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## Synthesis of Novel Heterocyclic N-Oxide Glycosides: Glycosylation of Myxin Analogs

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SYNTHESIS OF NOVEL HETEROCYCLIC N-OXIDE GLYCOSIDES: GLYCOSYLATION  
OF MYXIN ANALOGS

by

JARIN JOYNER

Under the Direction of Peng George Wang

ABSTRACT

Phenazines and heterocyclic *N*-oxides have proven to be interesting classes of antitumoral as well as antibiotic agents . The natural product myxin (1-hydroxy-6-methoxyphenazine- *N*5, *N*10-dioxide) which belongs to both of these unique classes of molecules, has been found to cause bio-reductively activated, radical-mediated DNA strand cleavage via a de-oxygenative mechanism, making it a potential antitumoral as well as anti-bacterial candidate. In order to investigate as well as improve the bioactive properties of myxin, the following study was designed to synthesize glycosylated myxin analogs. A small catalog of these compounds were synthesized, some of them exhibiting comparable biological activity to that of myxin.

INDEX WORDS: Myxin, Phenazines, Heterocyclic *N*-oxides, Glycosylation, Carbohydrates, Bio-reductive

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OF MYXIN ANALOGS

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JARIN JOYNER

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Arts and Sciences

Georgia State University

2013

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OF MYXIN ANALOGS

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December 2013

## **DEDICATION**

I would like to dedicate this work to my parents, whose continued guidance has not only served as an inspiration to succeed, but to also make a lasting impression on society.

## **ACKNOWLEDGEMENTS**

I would like to acknowledge my research advisor Dr. Peng George Wang for his mentorship and supervision of my thesis. I would also like to acknowledge my lab mates for their assistance within my research project.

## TABLE OF CONTENTS

ACKNOWLEDGEMENTS .....	v
LIST OF TABLES .....	viii
LIST OF FIGURES .....	ix
1 INTRODUCTION.....	1
2 METHODOLOGY.....	6
2.1 Synthesis of 1,6-Dimethoxyphenazine .....	7
2.2 Synthesis of 1-Hydroxy-6-methoxyphenazine .....	8
2.3 Synthesis of Myxin (1-Hydroxy-6-methoxyphenazine-N5, N10-dioxide).....	9
2.4 Synthesis of 2,3,4,6-Tetra-O-acetyl- $\alpha$ -D-glucopyranosyl bromide .....	10
2.5 Synthesis of 2,3,4,6-Tetra-O-acetyl- $\alpha$ -D-galactopyranosyl bromide .....	10
2.6 Synthesis of 6-Methoxyphenazine-1- $\beta$ -D-tetraacetylgluco-pyranoside .....	11
2.7 Synthesis of 6-methoxyphenazine-N10-oxide-beta-D-tetraacetylgluco-pyranoside	12
2.8 Synthesis of 6-Methoxyphenazine-N5-oxide-1-beta-D-gluco-pyranoside .....	13
2.9 Synthesis of 6-Methoxyphenazine-1- $\beta$ -D-tetraacetylgalacto-pyranoside.....	14
2.10 Synthesis of 6-Methoxyphenazine-N5-oxide-beta-D-tetraacetylgalacto- pyranoside.....	15
2.11 GI <sub>50</sub> Assay .....	15
3 RESULTS AND DISCUSSION .....	16
4 CONCLUSION .....	21
REFERENCES.....	23
APPENDICES.....	24

**Appendix A** ..... **24**

**LIST OF TABLES**

Table 1 GI <sub>50</sub> Assay (μM).....	21
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**LIST OF FIGURES**

Figure 1.1 Phenazines: Pyocyanin (1), Iodinin (2), Myxin (3).....	1
Figure 1.2 Bio-reductive Mechanism of Tirapazamine (TPZ).....	3
Figure 1.3 Bio-reductive Mechanism of Myxin.....	4
Figure 1.4 Examples of Acetylated Glucoside Donors.....	5
Figure 2.1 Synthetic Scheme of Myxin .....	6
Figure 2.2 Synthesis of 6-Methoxyphenazine-1- $\beta$ -D- glycosides.....	7
Figure 2.3 Synthesis of 1,6-Dimethoxyphenazine .....	7
Figure 2.4 Synthesis of 1-Hydroxy-6-methoxyphenazine .....	8
Figure 2.5 Synthesis of Myxin.....	9
Figure 2.6 Synthesis of 2,3,4,6-Tetra-O-acetyl- $\alpha$ -D-glucopyranosyl bromide.....	10
Figure 2.7 Synthesis of 2,3,4,6-Tetra-O-acetyl- $\alpha$ -D-galactopyranosyl bromide .....	10
Figure 2.8 Synthesis of 6-Methoxyphenazine-1- $\beta$ -D-tetraacetylgluco-pyranoside .....	11
Figure 2.9 Synthesis of 6-methoxyphenazine-N5-oxide-beta-D-tetraacetylgluco-pyranoside.....	12
Figure 2.10 Synthesis of 6-Methoxyphenazine-N5-oxide-1-beta-D-gluco-pyranoside.....	13
Figure 2.11 6-Methoxyphenazine-1- $\beta$ -D-tetraacetylgalacto-pyranoside .....	14
Figure 2.12 6-Methoxyphenazine-N5-oxide-beta-D-tetraacetylgalacto-pyranoside .....	15
Figure 3.1 Attempted Glycosylation of <b>3b</b> , <b>9</b> , and <b>3</b> .....	18
Figure 3.2 Novel Glycosylated Compounds .....	19

## 1 INTRODUCTION

Phenazines are a class of naturally occurring compounds that are comprised of a nitrogen containing, heterocyclic moiety that vary in both their chemical and physical properties based on the type and position of substituents present. More than 100 different phenazine structural derivatives have been identified in nature, bacteria being the only source of these natural compounds, and over 6000 compounds that encompass phenazine as the central moiety have been synthesized.<sup>1</sup> Phenazine natural products are isolated as secondary metabolites from bacterial genera such as *Pseudomonas*, *Streptomyces*, and other genera from soil and marine habitats.<sup>2</sup> Both natural and synthetic phenazines have been of particular interest due to the fact that they have been known to exhibit an extensive range of biological activities including antitumoral, antibiotic, and anti-parasitic properties.<sup>2-3</sup> While the physiological function of naturally occurring phenazines have yet to be fully understood, their mode of action has been proposed to include biological targets such as polynucleotide interactions, inhibition of topoisomerase I and/or II, and free-radical scavenging.<sup>4</sup> The polynucleotide interacting phenomena exhibited by many phenazines such as pyocyanin(1), iodinin (2), and myxin (3) stems from their planar and aromatic structure which is comparable to known intercalators such as daunomycin, chromomycin, and ethidium bromide.<sup>5</sup>

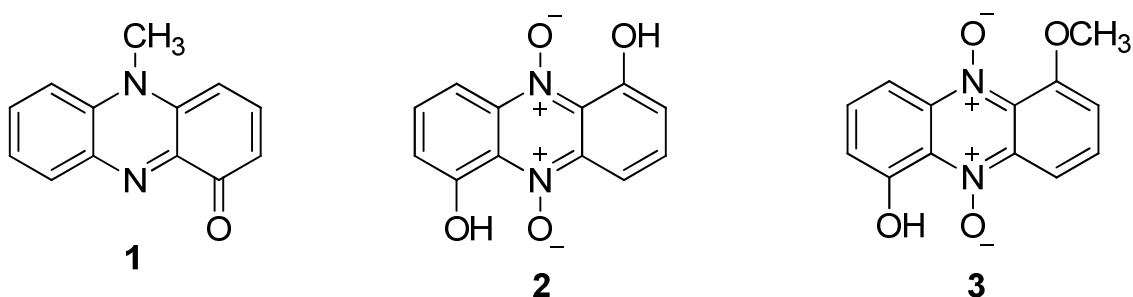


Figure 1.1 Phenazines: Pyocyanin (1), Iodinin (2), Myxin (3)

These respective structural characteristics allow for  $\pi$ - $\pi$  interactions between the phenazines and the base pairs of DNA, which in turn allows them to intercalate. In addition, phenazines 1,2 and 3 have

been shown to also bind to either base pairs in double stranded DNA, RNA polymerase, and/or to ribonucleoside 5'-triphosphate.<sup>2</sup> Although, there has yet to be any naturally occurring phenazine that inhibits topoisomerase to date, many synthetic phenazine analogs have been synthesized that have shown inhibition of topoisomerase I/II.<sup>6</sup> This particular strategy consisting of the target of the topoisomerase enzymes stems from the fact that proliferating cells, like cancer cells contain large concentrations of topoisomerases.<sup>2</sup> Phenazines have also displayed antibiotic activity through radical scavenging phenomena. Certain phenazines such as **1** have been shown to generate radicals that when inside cells, can accept electrons and thereby interrupt electron transport and respiratory flow in the cell.<sup>2</sup>

One particular phenazine that has reemerged as a compound of interest due to its antitumoral properties, and for which the following study is based upon is that of **3** (1-hydroxy-6-methoxyphenazine-N5, N10-dioxide, or myxin). Myxin, a natural product, was first isolated from *Sorangium* sp. By Peterson et al. in 1966.<sup>7</sup> The uniqueness of myxin is of interest due to the fact that it not only belongs to the class of phenazine compounds, but also to a class known as heterocyclic N-oxides. Heterocyclic N-oxides have been known to be an attractive class of compounds that exhibit antitumor and antibacterial agency through their bio-reductively activated, hypoxia-selective DNA-damaging properties.<sup>8</sup> Many studies that have investigated the bio-reductive properties of heterocyclic N-oxides have been based off of a lead compound known as tirapizamine (TPZ, **4**, *Figure 1.2*), which has been investigated in phase I, II, and III of clinical trials for the treatment of various cancers.<sup>9</sup> The hypoxia-selective cytotoxicity of TPZ and other compounds in the respective class of compounds lies in its ability to undergo an intracellular one-electron reduction mechanism (**5**, *Figure 1.2*) followed by a deoxygenative mechanism to form a key DNA damaging intermediate (**7**, *Figure 1.2*) and other metabolite byproducts (**6**, *Figure 1.2*).<sup>10</sup> In the presence of molecular oxygen, the radical intermediate is back oxidized to the parent molecule and does not proceed through the DNA-damaging intermediate (*Figure 1.2*).

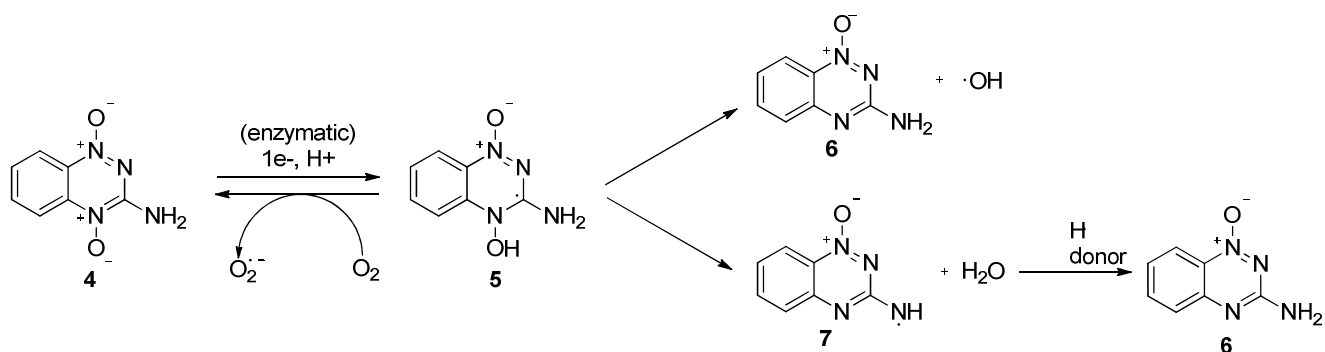


Figure 1.2 Bio-reductive Mechanism of Tirapazamine (TPZ)

The use of TPZ in a clinical setting has been limited in the fact that it has exhibited poor extra-vascular support and thus has lead researchers to investigate similar heterocyclic N-oxides with improved activity.<sup>11</sup> Myxin (**3**) however, causes bio-reductively activated, radical-mediated DNA strand cleavage under both aerobic and anaerobic conditions via a deoxygenative mechanism analogous to that characterized for tirapazamine under anaerobic conditions (*Figure 1.3*).<sup>10</sup> This observation has led to biological activity comparisons drawn between myxin and TPZ that have shown that myxin has comparable activity to that of TPZ in anaerobic conditions and improved activity in aerobic conditions.<sup>10</sup> The fact that myxin is active under both of these conditions exhibits its potential antibacterial as well as anti-tumoral potential. It has been suggested that myxin's aerobically active properties lie within the fact that the radical anion (**8**) possesses phenoxy radical characteristics. This intermediate, which is generated following the enzymatic one electron reduction of myxin (**3**) has been hypothesized to be more stable and thus less sensitive to oxygen, which in turn allows the intermediate to proceed through the reductive mechanism and generate DNA damaging radicals.<sup>10</sup> Therefore, myxin's double agency in the phenazine class as well as the heterocyclic N-oxide class has rendered it as an interesting compound of interest in the fields anticancer and antibacterial research.

In order to investigate further improvements of the biological activity of myxin along with its other analogs, the synthesis of glycoside conjugates of myxin analogs was proposed. Carbohydrates car-

rying an aromatic aglycon have proven to be very important compounds with examples such as antibiotics such as vancomycin and chromomycin.<sup>12</sup> An increase in the understanding of the important roles that glycoconjugates play in biological processes has led to a demand for these respective compounds in medicinal and pharmacological studies.<sup>13</sup> In light of these observations, the synthesis of glycosylated myxin analogs was carried out in order to investigate the effect that conjugated glycosides had on the bioactivity of myxin analogs. In addition to the glycosylation of **3**, the glycosylation of compound **9** was also of interest due to the fact that previous studies observed that it had significantly lower bioactivity than its precursor, **10** under both aerobic and anaerobic conditions.<sup>10</sup> Chowdury et al. suggest that **9** may engage in redox cycling similar to that of **4**, but that the radical anion is incapable of being further reduced allowing for the release of its own cytotoxic species i.e. the hydroxyl radical.<sup>10</sup> However, this “ease of reduction” of compound **9** may be improved by the addition of acetylated glycosides, which in turn increases the electrophilic nature of the aromatic system and thus stabilizes the radical anion.<sup>14</sup>

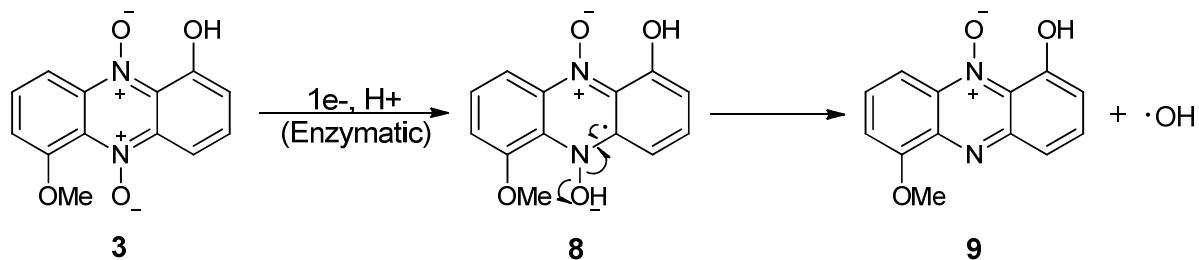


Figure 1.3 Bio-reductive Mechanism of Myxin

The chemical synthesis of glycosides typically involves the transformation of a particular sugar into a fully protected glycosyl donor with a leaving group at its anomeric center.<sup>13</sup> These leaving groups can encompass a list of potential functional groups such as halides, acetates, trifluoroacetates, and trichloroacetimidates (Figure 1.4).<sup>12</sup>

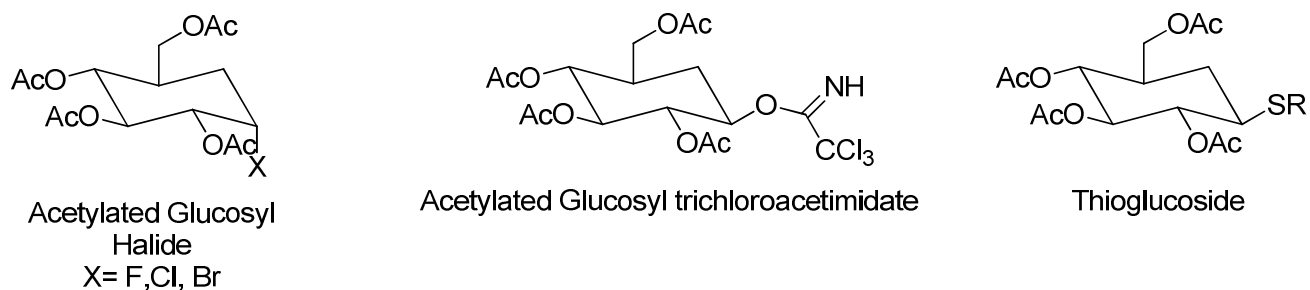


Figure 1.4 Examples of Acetylated Glucoside Donors

Glycosylation of a suitably protected glycosyl acceptor, which generally contains a free hydroxyl group, then is carried out. The glycosyl donor can be viewed as “transferring” the glycosyl moiety (which is generally an electrophile) to the glycosyl acceptor (which is generally a nucleophile). Therefore, the leaving group as well as the protecting groups are the main fundamental parameters with respect to the yield and anomeric selectivity of glycosylation reactions.<sup>13</sup> However, there are many problems associated with the glycosylation of phenol groups such as that in compounds such as **3** and **9**. Some of these difficulties include the fact that phenols are considered weak nucleophiles, and sensitivity to pH.<sup>12</sup> Due to the stereo-selectivity, ease of use, and strong electrophilic characteristics of glycosyl halides, glycoside bromide was selected as the “donor” and compounds **3** and **9** along with other myxin analogs were selected as the “acceptors.” This Koenigs- Knorr method encompassed the use of various phase transfer catalysts under basic conditions, which were utilized in these glycosylation reactions in order to obtain an optimal yield. Due to the potential improved solubility as well as more positive reduction potential of **9**, it was hypothesized that the myxin analogs such as **9** would exhibit improved bioactivity following their conjugation with different acetylated glycosides.

## 2 METHODOLOGY

In the process of chemically synthesizing the central phenazine moiety found in compound **3**, many modifications to the synthetic routes reported by Chowdury et al. were made.<sup>10</sup> All synthetic routes were scaled up to yield larger quantities of the respective product. All materials were purchased at the highest possible purity from Sigma Aldrich Chemical Company. Of the various synthetic routes that could have been carried out in the generation of **3** and its precursors, only the route that included the cleavage of a single methoxy group in the 1,6-dimethoxy phenazine compound prior to its oxidation was carried out habitually (*Figure 2.1*). The glycosylation of **9** was carried out using modifications to glycosylation techniques described by Wu et al (*Figure 2.2*).<sup>15</sup> In the synthesis of 6-methoxyphenazine-1- $\beta$ -D- glycosides, only the glucose analog of the unacetlyated compound was synthesized. All <sup>1</sup>H NMR, <sup>13</sup>C NMR, and ESI mass spectra are provided in the appendix.

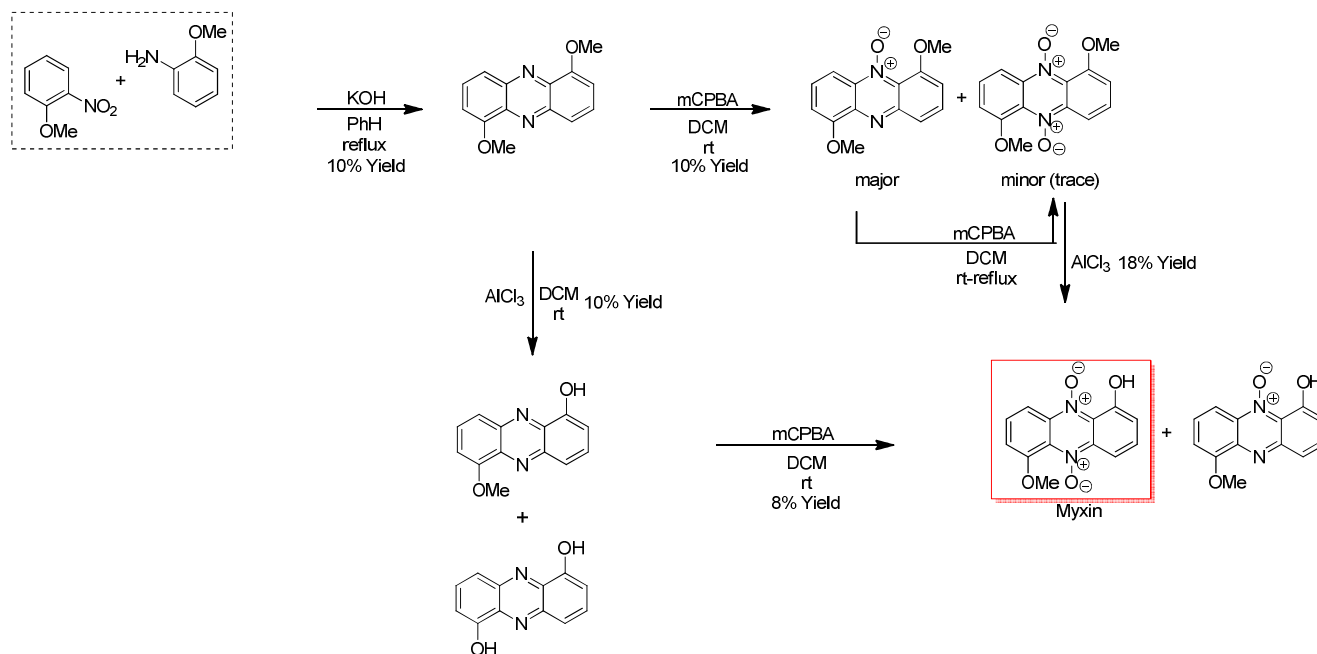


Figure 2.1 Synthetic Scheme of Myxin

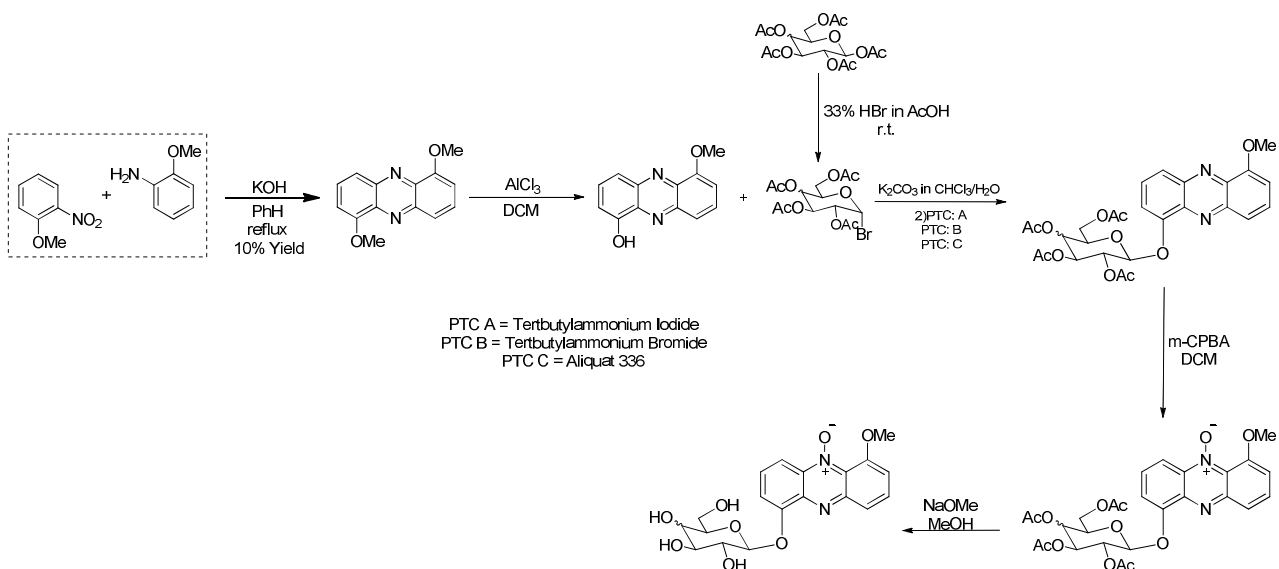


Figure 2.2 Synthesis of 6-Methoxyphenazine-1-β-D- glycosides

### 2.1 Synthesis of 1,6-Dimethoxyphenazine

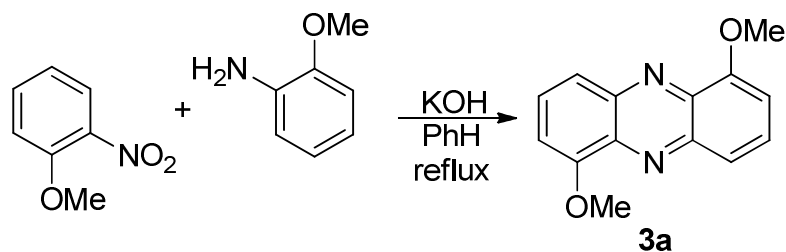


Figure 2.3 Synthesis of 1,6-Dimethoxyphenazine

1,6- Dimethoxyphenazine was synthesized by first adding 0.80 mol (90.22 mL) of o-anisidine to 500 mL of anhydrous benzene. 1.44 mol (176 mL) of o-nitroanisole along with 4 mol (224.42 g) of powdered potassium hydroxide was then added to the solution. The solution was heated at reflux for 8 hours with vigorous stirring. Upon cooling overnight, the benzene solution was decanted off and cold water was added to the reaction flask in order to break up the solid and extract any remaining potassium hydroxide. The solid was then collected via vacuum filtration and washed twice with ethanol. After filtration, the solid was recrystallized using hot ethanol and dried with an oil pump to yield 14% of product.  $R_f = 0.50$  (100%

ethyl acetate) *Spectra 1*.  $^{13}\text{C}$  NMR *Spectra 2*. Mass spectra (ESI) was also collected in order to verify the structure *Spectra 3*.

## 2.2 Synthesis of 1-Hydroxy-6-methoxyphenazine

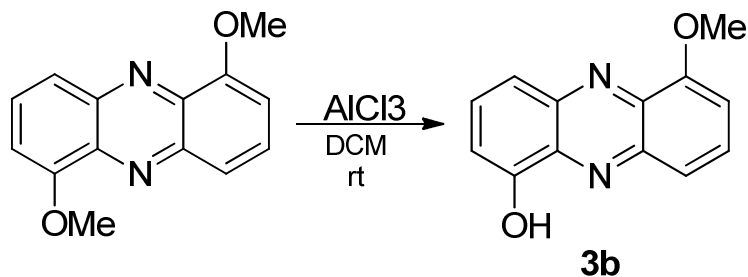


Figure 2.4 Synthesis of 1-Hydroxy-6-methoxyphenazine

1-Hydroxy-6-methoxyphenazine was made by adding 0.064 mol (16.21 g) of 1,6-dimethoxyphenazine to 300 mL of dichloromethane in a 500 mL round bottom flask. Then 0.351 mol (43.46 g) of aluminum chloride was added to the solution under 0°C condition and allowed to stir for 20 minutes. The progression of the reaction was verified through TLC analysis using 5:1 ethyl acetate to hexane,  $R_f = 0.63$ . The mixture was then worked up by adding solution to 0.5 N HCl in order to quench the mixture, followed by extraction with dichloromethane. Product was then further purified using silica gel column chromatography (1:1 ethyl acetate to hexane). Percent yield = 17%.  $^1\text{H}$ NMR *Spectra 4*.  $^{13}\text{C}$ NMR *Spectra 5*. MS (ESI) *Spectra 6*.

### 2.3 Synthesis of Myxin (1-Hydroxy-6-methoxyphenazine-N5, N10-dioxide)

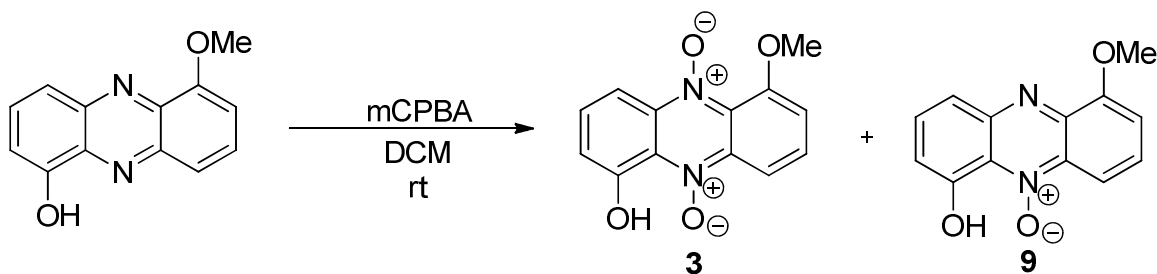


Figure 2.5 Synthesis of Myxin

To a solution of 0.0044 mol (1.0 g) of 1-Hydroxy-6-methoxyphenazine in 50 mL of dichloromethane was added 3.0 g of m-CPBA. The mixture was stirred at room temperature for 12 hours and evaluated by TLC using 2:1 hexane to ethyl acetate  $R_f = 0.63$  for compound **3** and 0.45 for compound **9**. Mixture was then worked up by diluting with another 100 mL of DCM and washed with 5%  $\text{Na}_2\text{SO}_3$ ,  $\text{NaHCO}_3$  and brine washes were then followed by drying with  $\text{Na}_2\text{SO}_4$ . Products were separated via silica column using 1/3/1 ethyl acetate/hexane/DCM yielding 75% of compound **9** and 5.31% of compound **3**. For compound **3**,  $^1\text{H}$  NMR *Spectra 7*.  $^{13}\text{C}$ NMR *Spectra 8*. Product was also evaluated using mass spectrometry (ESI) *Spectra 9*. For compound **9**,  $^1\text{H}$  NMR *Spectra 7a*.

#### 2.4 Synthesis of 2,3,4,6-Tetra-O-acetyl- $\alpha$ -D-glucopyranosyl bromide

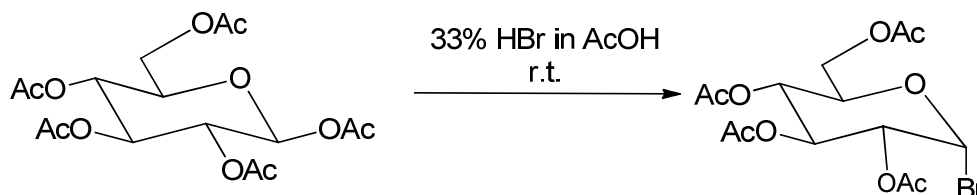


Figure 2.6 Synthesis of 2,3,4,6-Tetra-O-acetyl- $\alpha$ -D-glucopyranosyl bromide

The synthesis of 2,3,4,6-Tetra-O-acetyl- $\alpha$ -D-glucopyranosyl bromide was carried out by adding 8 mL of 33% HBr in acetic acid to 1 g ( $2.60 \times 10^{-3}$  mol) of  $\beta$ -D-glucose pentaacetate under nitrogen at 0°C. After 5 minutes, the solution was allowed to stir to completion at room temperature ( $R_f = 0.35$  1:1, 1 hexane: 1 ethyl acetate). Upon completion of the reaction, the solution was diluted with 60 mL of dichloromethane and 30 mL of cold H<sub>2</sub>O. The organic layer was collected and washed with NaHCO<sub>3</sub> until the aqueous layer was at a pH of 7. 90% of product was yielded. <sup>1</sup>HNMR Spectra 10. <sup>13</sup>CNMR Spectra 11. MS (ESI) Spectra 12.

#### 2.5 Synthesis of 2,3,4,6-Tetra-O-acetyl- $\alpha$ -D-galactopyranosyl bromide

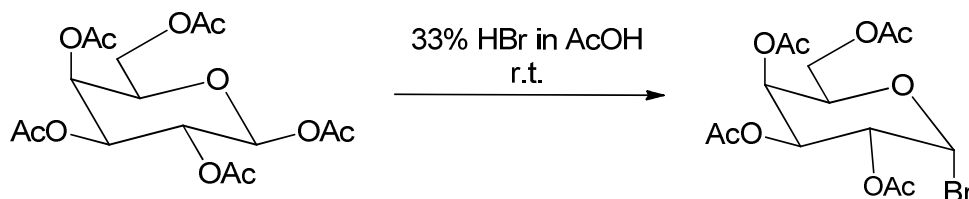


Figure 2.7 Synthesis of 2,3,4,6-Tetra-O-acetyl- $\alpha$ -D-galactopyranosyl bromide

The synthesis of 2,3,4,6-Tetra-O-acetyl- $\alpha$ -D-galactopyranosyl bromide was carried out by adding 8 mL of 33% HBr in acetic acid to 1 g ( $2.60 \times 10^{-3}$  mol) of  $\beta$ -D-galactose pentaacetate under nitrogen at 0°C. After 5 minutes, the solution was allowed to stir to completion at room temperature ( $R_f = 0.35$  1:1, 1 hexane: 1 ethyl acetate). Upon completion of the reaction, the solution was diluted with 60 mL of dichloromethane and 30 mL of cold H<sub>2</sub>O. The organic layer was collected and washed with NaHCO<sub>3</sub> until the aqueous layer was at a pH of 7. 87% of product was yielded. <sup>1</sup>HNMR Spectra 13. <sup>13</sup>CNMR Spectra 14. MS (ESI) Spectra 15.

### 2.6 Synthesis of 6-Methoxyphenazine-1- $\beta$ -D-tetraacetylgluco-pyranoside

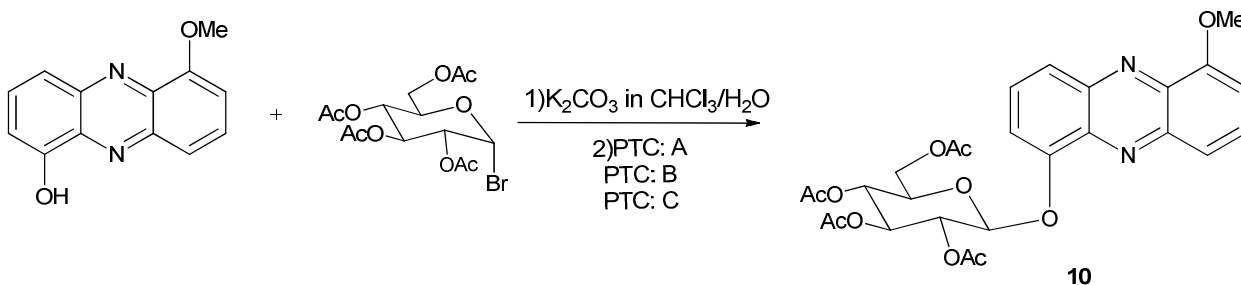


Figure 2.8 Synthesis of 6-Methoxyphenazine-1- $\beta$ -D-tetraacetylgluco-pyranoside

113 mg ( $5.00 \times 10^{-4}$  mol) of 1-hydroxy-6-methoxyphenazine along with 410 mg of 2,3,4,6-Tetra-O-acetyl- $\alpha$ -D-galactopyranosyl bromide ( $1.05 \times 10^{-3}$  mol) was dissolved in 8 mL of CHCl<sub>3</sub>. 8 mL of H<sub>2</sub>O was then added along with 138 mg ( $1.00 \times 10^{-3}$  mol) of K<sub>2</sub>CO<sub>3</sub> to the mixture. A catalytic amount of tertbutylammonium iodide (PTC A)/tertbutylammonium bromide (PTC B)/aliquat 336 (PTC C) was added to the mixture. The reaction was allowed to stir at 50.0° C for 24 hours. The reaction temperature was then decreased to room temperature and the reaction was allowed to stir for an additional 12 hours. After completion of the reaction, the reaction mixture was worked up by adding 0.1 M HCl and extracting the organic layer. The organic layer was further washed with brine and

once again extracted. The final compound was purified via column with 1:1, hexane: ethyl acetate.  $R_f = 0.21$  using 1:1, hexane: ethyl acetate. Percent yield with PTC A and PTC B was only trace amounts. Percent yield with PTC C was 75%. <sup>1</sup>HNMR *Spectra 16*. <sup>13</sup>CNMR *Spectra 17*. MS (ESI) *Spectra 18*.

## 2.7 Synthesis of 6-methoxyphenazine-N10-oxide-beta-D-tetraacetylgluco-pyranoside

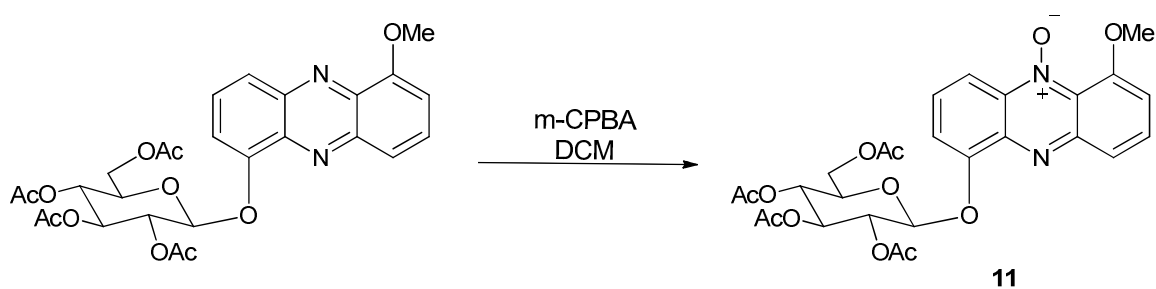


Figure 2.9 Synthesis of 6-methoxyphenazine-N5-oxide-beta-D-tetraacetylgluco-pyranoside

The synthesis of 6-methoxyphenazine-N5-oxide-beta-D-tetraacetylgluco-pyranoside was carried out by dissolving 50 mg of 6-methoxyphenazine-N5-oxide-beta-D-tetraacetylgluco-pyranoside ( $8.73 \times 10^{-5}$  mol) in dichloromethane. 0.106 g of m-CPBA ( $6.14 \times 10^{-4}$  mol) was then added to the solution and the solution was allowed to stir for 24 hours under argon at room temperature. Over the course of the reaction, the solution gradually turned from yellow to orange. After completion of the reaction, the organic layer was diluted with dichloromethane and washed with  $\text{NaHCO}_3$  followed by brine. The product was purified by column using 1:1, hexane: ethyl acetate.  $R_f = 0.67$  using 100% ethyl acetate. Yielded 90% of product. <sup>1</sup>HNMR *Spectra 19*. <sup>13</sup>CNMR *Spectra 20*. MS (ESI) *Spectra 21*.

## 2.8 Synthesis of 6-Methoxyphenazine-N5-oxide-1-beta-D-gluco-pyranoside

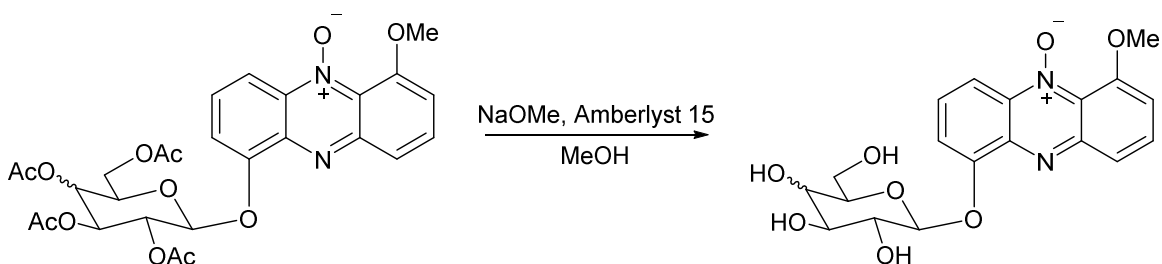


Figure 2.10 Synthesis of 6-Methoxyphenazine-N5-oxide-1-beta-D-gluco-pyranoside

10 mg ( $2.47 \times 10^{-5}$  mol) of 6-Methoxyphenazine-N5-oxide-1-beta-D-gluco-pyranoside was dissolved in a mixture of 6 mL of anhydrous methanol and 1 mL dichloromethane. A small amount sodium methoxide was then added, and the solution was allowed to stir at room temperature for one hour. After completion of the reaction, Amberlyst 15H form was added to the solution carefully, until the solution reached a pH of 7. Solution was diluted further with methanol and evaporated.  $R_f = 0.25$  using 10: 1, dichloromethane: methanol. Yielded 99% of product. <sup>1</sup>HNMR *Spectra 22*. <sup>13</sup>CNMR *Spectra 23*. MS (ESI) *Spectra 24*.

## 2.9 Synthesis of 6-Methoxyphenazine-1- $\beta$ -D-tetraacetylgalacto-pyranoside

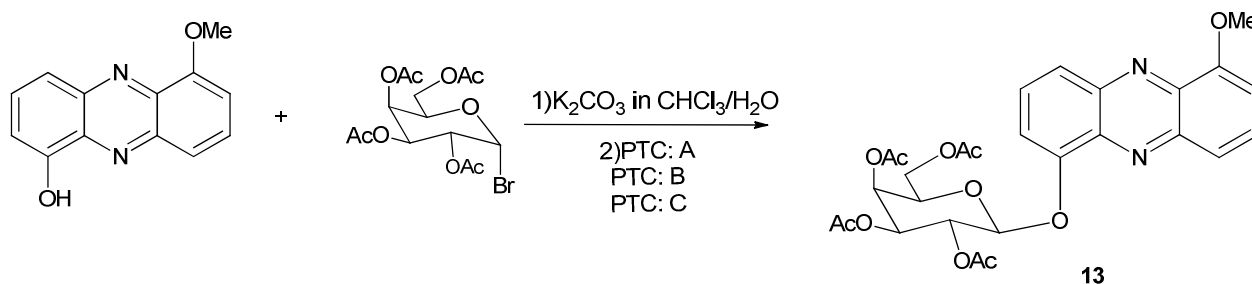


Figure 2.11 6-Methoxyphenazine-1- $\beta$ -D-tetraacetylgalacto-pyranoside

200 mg ( $8.84 \times 10^{-4}$  mol) of 1-hydroxy-6-methoxy phenazine was dissolved in a 8 mL  $CHCl_3/8$  mL  $H_2O$  mixture. 800 mg ( $2.05 \times 10^{-3}$  mol) of 2,3,4,6-Tetra-O-acetyl- $\alpha$ -D-galactopyranosyl bromide was then added to the solution. 276 mg of  $K_2CO_3$  was then added to the solution followed by a catalytic amount of either PTC A (tetrabutylammonium iodide), PTC B (tetrabutylammonium bromide), or PTC C (aliquat 336). The solution was allowed to stir under nitrogen for 24 hours at  $50^\circ C$ . After completion of the reaction, the solution was diluted with 30 mL of dichloromethane. Solution was then worked up by first adding 30 mL of 0.1 M HCl and then extracting the organic layer. Product was purified by column using 2:1, hexane: ethyl acetate.  $R_f = 0.25$  using 1:1, hexane: ethyl acetate. When using both PTC's A and B, the percent yield was only in trace amounts. PTC C yielded 80%.  $^1H$ NMR Spectra 25.  $^{13}C$ NMR Spectra 26. MS (ESI) Spectra 27.

## 2.10 Synthesis of 6-Methoxyphenazine-N5-oxide-beta-D-tetraacetylgalacto-pyranoside

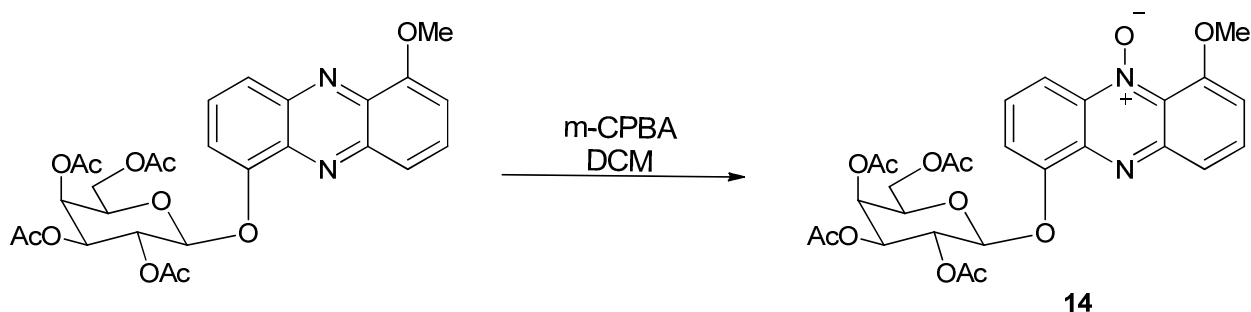


Figure 2.12 6-Methoxyphenazine-N5-oxide-beta-D-tetraacetylgalacto-pyranoside

Dissolved 50 mg ( $9.00 \times 10^{-5}$  mol) of 6-methoxyphenazine-1-β-D-tetraacetylgalacto-pyranoside in 10 mL of dichloromethane. Added 100 mg ( $5.80 \times 10^{-4}$ ) of m-CPBA to the reaction mixture and allowed to stir at room temperature for 24 hours. Over the course of the reaction, the solution turned from yellow to orange. After completion of the reaction, the organic layer was diluted with dichloromethane and washed with  $\text{NaHCO}_3$  followed by brine. The product was purified by column using 1:1, hexane: ethyl acetate.  $R_f = 0.67$  using 100% ethyl acetate. Yielded 90% of product. <sup>1</sup>HNMR Spectra 28. <sup>13</sup>CNMR Spectra 29. MS (ESI) Spectra 30.

## 2.11 GI<sub>50</sub> Assay

Cancer cell lines HCT-8 (colon cancer cells), HeLa (immortalized cervical cancer cells), AGS (gastric cancer cells), MCF-7 (breast cancer cells), and HepG2 (liver cancer cells) were kindly provided by Dr. Jin-Xiong She of Georgia Regents University. The cancer cell line Molt-4 (acute lymphoblastic leukemia) was kindly provided by Dr. Binghe Wang of Georgia State University. HCT-8, HeLa, AGS and Molt-4 cancer cells were cultured in RPMI 1640

medium with 10% Fetal Bovine Serum and 1% of the respective drug target. MCF-7 and HepG2 cancer cells were cultured in DMEM medium with 10% Fetal Bovine Serum and 1% respective drug target. All cells were incubated in 5% CO<sub>2</sub> at 37°C and were given medium and plate changes as needed. The evaluation of 50% growth inhibition (GI<sub>50</sub>) by proliferation assay was carried out by diluting HCT-8, HeLa, and AGS cells to a concentration of  $3.5 \times 10^4$  cells/mL and MCF-7, Molt-4, and HepG2 cells to a concentration of  $4 \times 10^5$  cells/ml. 100  $\mu$ L of the cell suspension was seeded in 96 well plates. The plates were incubated in 5% CO<sub>2</sub> at 37°C overnight, giving ample time for adherent cell lines to adhere to the plates. The following day, 1  $\mu$ L of the prepared compound dilutions (3-fold dilutions) was added to each well, so that the highest final concentration was 50  $\mu$ M with 8 successive dilutions. Cells were then incubated for 72 hours. A Dojindo CCK-8 kit was used to estimate the number of viable cells in each well according to the manufacturer's instructions. After 4h incubation, OD values were measured at 450nm using the multifunctional microplate reader. The data was then analyzed to construct a GI<sub>50</sub> curve and calculate the GI<sub>50</sub> value using Sigmaplot software. Growth inhibition was calculated using the following formula:  $GI = (\text{control OD} - \text{experimental OD}) / \text{control OD}$ .

### 3 RESULTS AND DISCUSSION

The initial synthesis of compound **3** and its phenazine precursors (**3a** and **3b**) proved successful. However, synthesizing desirable yields of these respective compounds proved challenging. This can be attributed to the fact that in each step of this synthesis, the desired compound was a minor product. An example can be considered compound **3b**, which forms 1,6-dihydroxy phenazine as the major product. The glycosylation of compound **3b** proved successful as well. These glycosylation reactions were carried out using the Koenigs- Knorr method, using K<sub>2</sub>CO<sub>3</sub> as the weak base and various phase

transfer catalysts to improve the yield of the glycosylation product. In this process, it was determined that commercially available phase transfer catalyst PTC C gave an optimal yield of 75% of both the galactoside and glucoside products, while PTC A and PTC B gave minimal yields. Because acetyl glycoside bromides were utilized as the donor in the glycosylation reactions, only a  $\beta$ - linkage of the phenazine acceptor was formed, hence the selective capabilities of glycosyl bromides. Oxidation of the subsequent glycoside phenazines (compounds **10** and compound **13**) only yielded mono-oxide products (compounds **11** and **14**). This formation of exclusively mono-oxide products can be attributed to the fact that the presence of the sugar “blocks” the oxidation of the *N10* nitrogen by a steric effect. Chowdury et al describes an effect similar to this in previous experiments where the free hydroxyl group of the 1-hydroxy-6-methoxy phenazine compound was protected with a TBDPS group. In this study, it was found that the TBDPS group did indeed sterically hinder the oxidation of the *N10* nitrogen and yielded the mono-*N5*-oxide as a major product.<sup>10</sup> In the case where there is no sugar present, like in the formation of compound **3**, previous studies suggest that the hydroxy group of the 1-hydroxy-6-methoxy phenazine compound directs the oxidation of the *N10* nitrogen and thus yields compound **9** as a major product and compound **3** as a minor product. It also must be noted that the glycosylation of compound **3** using the Koenigs- Knorr method along with the various phase catalysts proved unsuccessful. One potential reason for this could be the fact that the strength of hydrogen bond between the phenolic proton and “negatively charged” oxygen is too strong to be cleaved by a weak base such as  $K_2CO_3$ . Another reason could be simply due to a “steric effect” that arises from the *N10*-oxide that disrupts the nucleophilic character of the phenol. The same result was seen when compound **9** served as the acceptor (which comprised the *N10* mono-oxide), in that glycosylation using the Koenigs- Knorr method was unsuccessful (*Figure 3.2*).

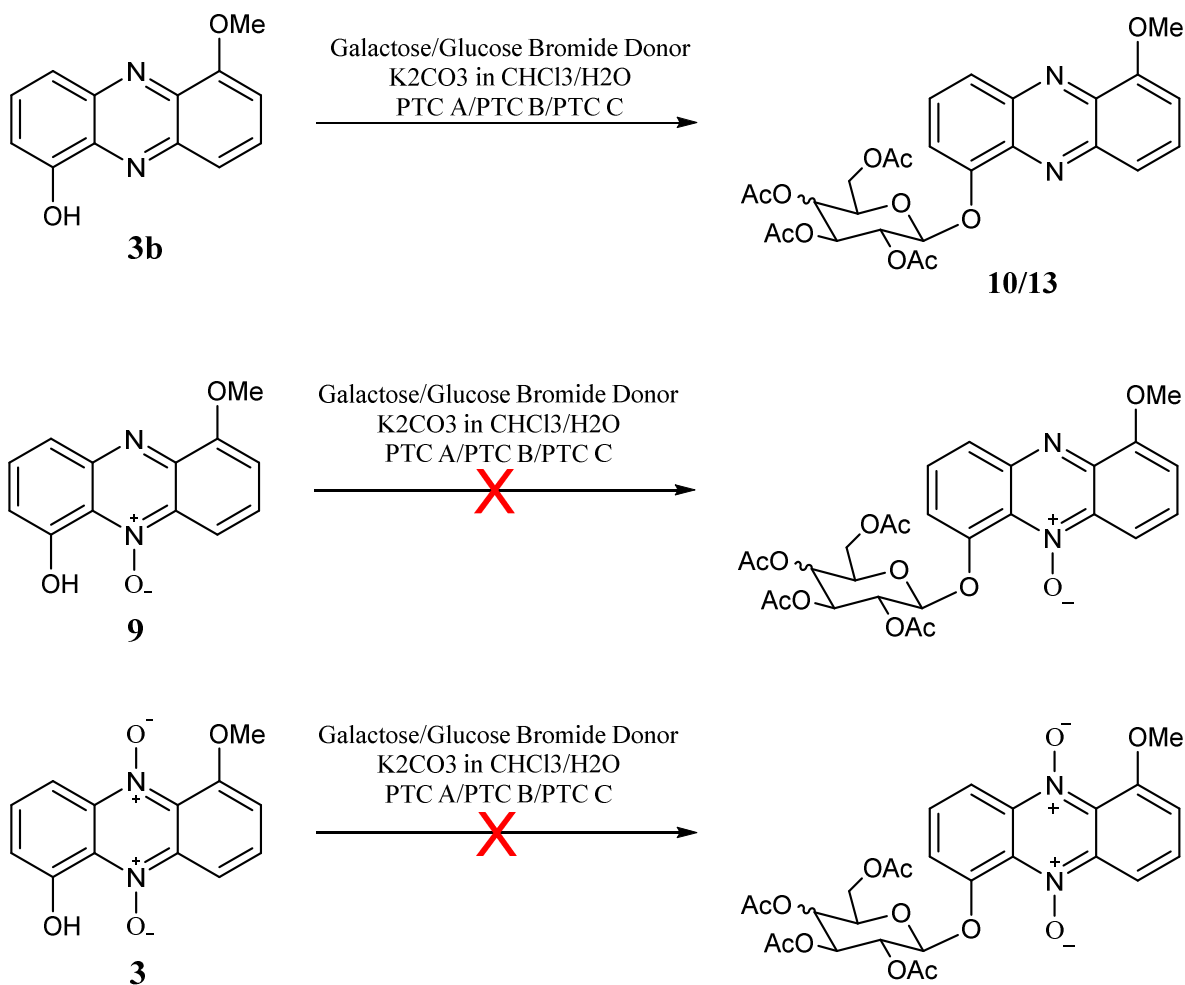


Figure 3.1 Attempted Glycosylation of **3b**, **9**, and **3**

Nonetheless, a small, initial catalog of novel glycosylated phenazines and heterocyclic N-oxides was created during these synthetic experiments (*Figure 3.2*). These compounds were then tested against various cancer cell lines including HeLa, Skov-3, HCT-8, HepG2, MCF-7, and Molt-4 and assayed in a GI<sub>50</sub> study.

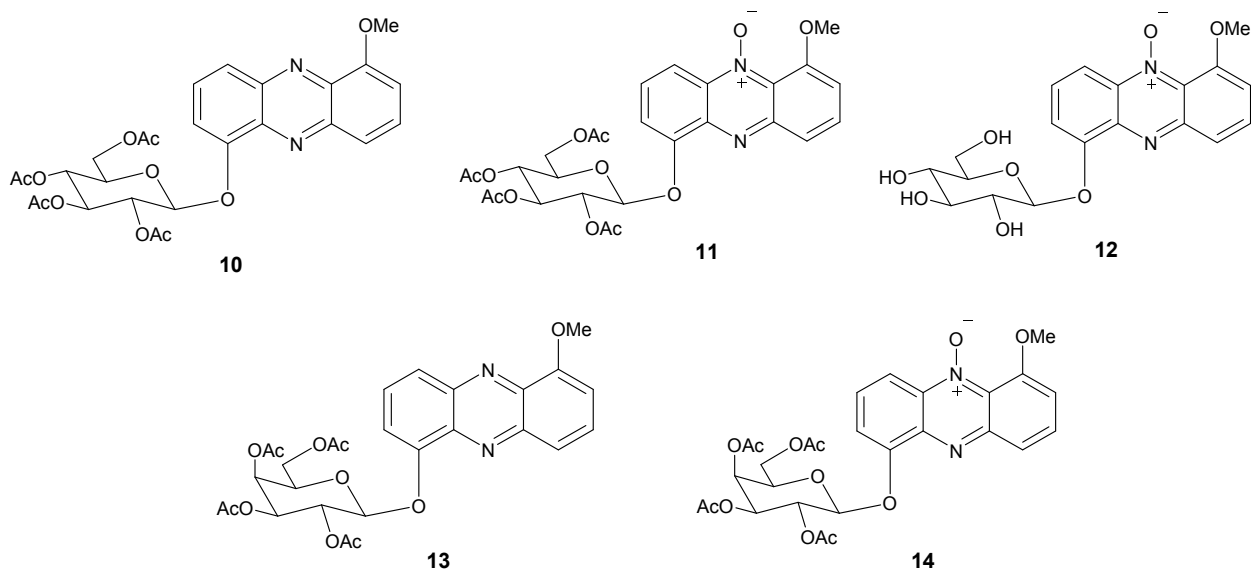


Figure 3.2 Novel Glycosylated Compounds

The  $GI_{50}$  study was carried out under a oxygenated environment meaning that the following compounds were assayed under aerobic conditions. All compounds in the assay were tested at a concentration of 50  $\mu$ L. For the sake of comparison of the biological activity of compounds **10-14**, doxorubicin (a commercial drug known for its high toxicity by intercalation), and compound **4** (tirapazamine, TPZ) were also included (*Table 3.1*). Due to the fact that the  $GI_{50}$  assay was carried out under aerobic conditions, it would be expected that compound **4** would not show any significant bioactivity based off the mechanism explained in *Figure 1.2*. Based off the  $GI_{50}$  values, compounds **11** and **14** exhibited improved bioactivity than compounds **4** and **9** and showed comparable bioactivity with compound **3** when tested with Hela and Skov-3 cell lines. When tested with cell lines HCT-8, MCF-7, and Molt-4, compounds **11** and **14** exhibited only improved bioactivity than compounds **4** and **9**. When the glycosylated compounds that were absent of the N-oxide moiety (compounds **10** and **13**) were tested in all cell lines, there was no sign of improved bioactivity in comparison to compounds **3**, **4**, and **9**. It also must be noted that compound **12**, which contained the unacetylated glucoside, N-oxide moiety exhibited no signs of improved bioactivity in comparison to compounds **3**, **4**, and **9**.

Possible explanations for the increased activity of compounds **11** and **14** in comparison to **4** and **9** can be attributed to the fact that the presence of the acetylated sugar increases the electrophilic nature of the overall molecular structure, which in turn increases its reduction potential, stabilizing the radical anion after its reduction. Another explanation of the improved bioactivity of these respective compounds can be due to the improved uptake of **11** and **14** into the cells due to improved solubility. However, it must be noted that compound **9** has the *N10* oxidized and compounds **11** and **14** have the *N5* oxidized, so direct comparisons cannot necessarily draw an exact conclusion to this difference in activity. Nonetheless, the comparison of compounds **9** against compounds **11** and **14** can still give clues into improving the activities of mono-N-oxide phenazines, which have shown significantly less bioactivity than myxin and tirapazamine overall.<sup>10</sup> The lack of improvement in bioactivity in compounds **10** and **13** against compounds **3**, **4**, and **9** most likely can be attributed to the lack of the N-oxide moiety and thus not allowing for any degree of reduction, which would in turn allow for production of DNA damaging radicals. This result therefore, is in agreement with early hypotheses. Finally, compound **12**'s lack of improved bioactivity vs. compound **11** can most likely be attributed to better uptake of an acetylated glucose as opposed to a free glucose in the structure. It can be predicted that glucose that is protected with acetyl groups can cross through the cell membranes more readily than unprotected glucose.

**Table 1 GI<sub>50</sub> Assay (μM)**

<b>Compound</b>	<b>Hela</b>	<b>Skov3</b>	<b>HCT-8</b>	<b>HepG2</b>	<b>MCF-7</b>	<b>Molt-4</b>
<b>4</b>	52.19	67.94	249.49	89.42	237.72	90.41
<b>Doxorubicin</b>	<0.22	<0.22	3.50	<0.22	0.01	<0.22
<b>3</b>	17.2	12.0	53.83	19.42	31.84	5.08
<b>9</b>	469	125.27	180.63	98.71	308.76	29.16
<b>11</b>	24.19	120.89	>100	>100	195.07	17.79
<b>14</b>	18.79	17.00	96.25	141.85	122.97	17.40
<b>10</b>	>100	>100	>100	>100	>100	17.40
<b>13</b>	>100	>100	>100	>100	>100	21.67
<b>12</b>	>100	>100	>100	>100	>100	33.63
<b>3a</b>	>100	>100	>100	>100	>100	>100

#### 4 CONCLUSION

Although the glycosylation of compounds **3** and **9** were deemed unsuccessful using the Koenigs-Knorr method, successful synthesis of glycosylated non-oxidized phenazines using the same method were indeed successful (**10**, and **13**). Further studies within the glycosylation of heterocyclic-N-oxides will include investigating different glycosylation techniques utilizing other carbohydrate donors such as glycosyl trichloroacetimidates and thioglycosides. Due to the fact that  $\beta$ -linked glycosides were synthesized, future studies will also include the synthesis of  $\alpha$ -linked glycosides as well. In addition, in order to further study myxin's bioactive properties in the field of cancer and antibiotics, functionalization with various halogen and alkyl groups will also be carried out. The GI<sub>50</sub> results of compounds **11** and **14** point to potential retained biological activity and thus allow for these compounds' further study within the applicative field of phenazines and heterocyclic- N-oxides. Ongoing studies of these compounds include many aspects including specific mechanisms of action. In order to study these mechanisms of action, ad-

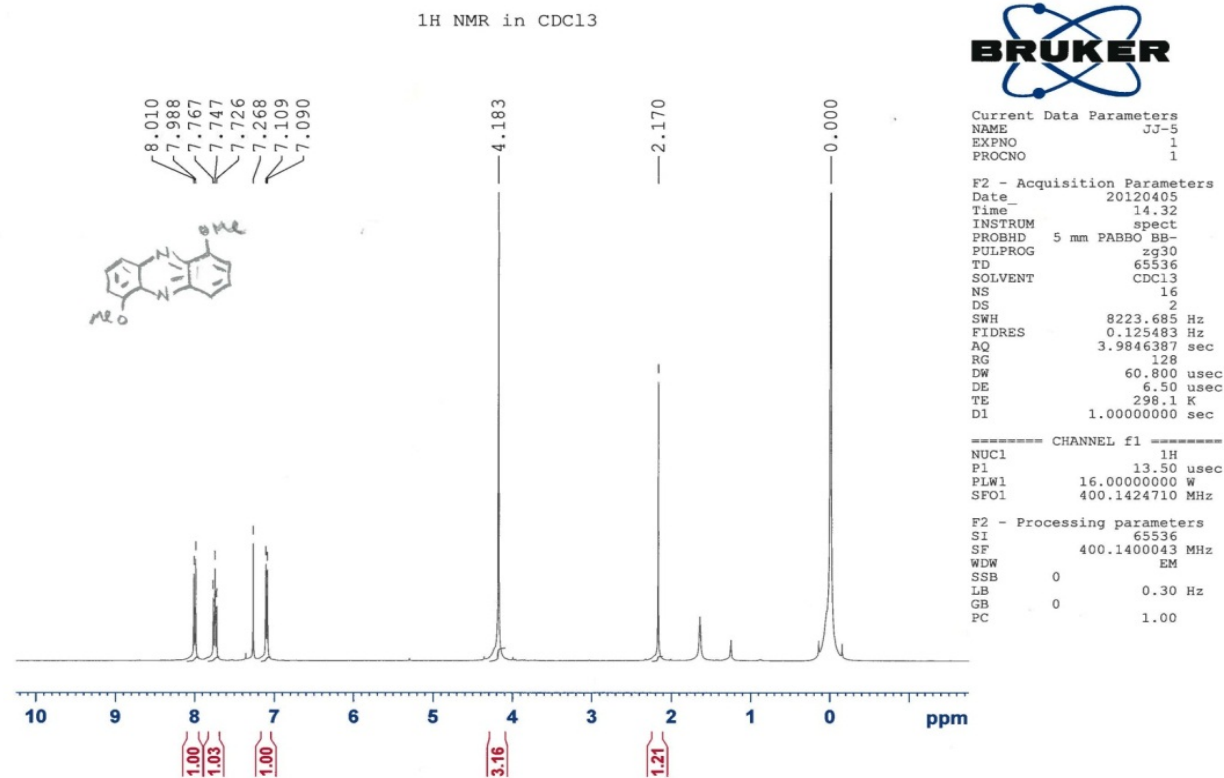
ditional assays including DNA cleavage tests (which would give further clues into whether these compounds indeed intercalate, or produce DNA damaging radicals) and assays that would investigate specific reduction potentials for all synthesized compounds.

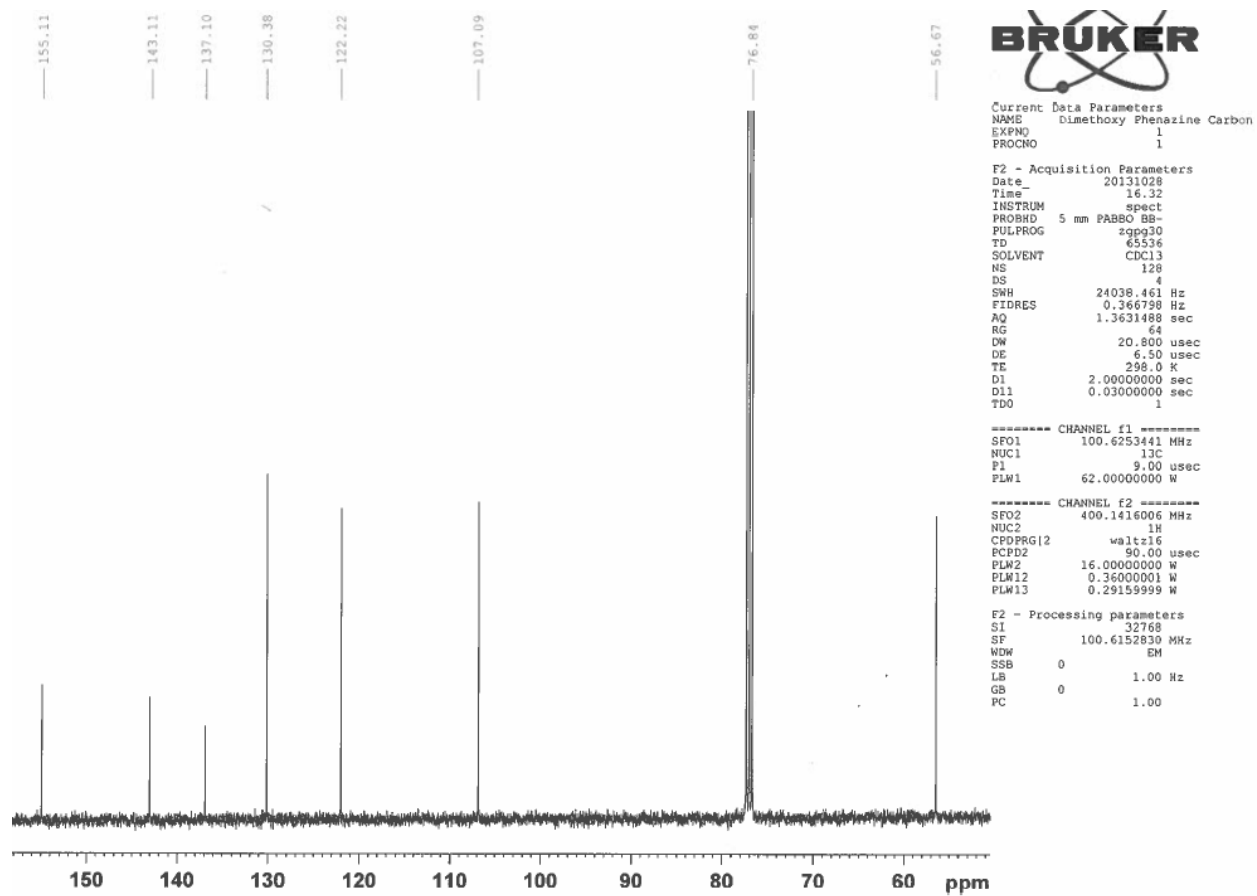
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## APPENDICES

## Appendix A

Spectra 1 <sup>1</sup>H NMR 1,6-Dimethoxyphenazine

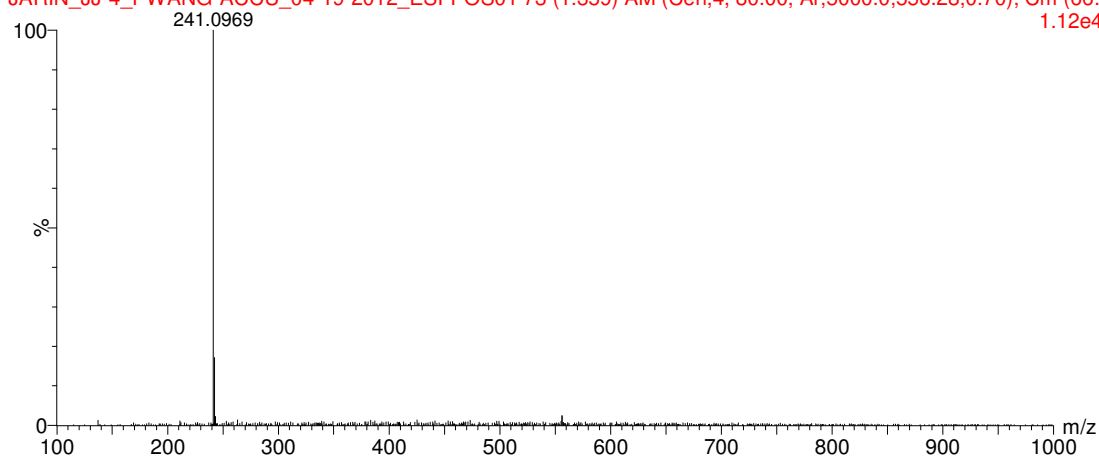


Spectra 2  $^{13}\text{C}$  NMR 1,6-Dimethoxyphenazine

100%MeOH+0.1%HCOOH

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JARIN\_JJ-4\_PWANG-ACCU\_04-19-2012\_ESI-POS01 73 (1.359) AM (Gen,4, 80.00, Ar,5000.0,556.28,0.70); Cm (66:7  
1.12e4



Elemental Composition Report

Single Mass Analysis

Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

230 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

Elements Used:

C: 1-100 H: 1-100 N: 1-15 O: 1-20

Minimum:

-1.5

Maximum:

5.0

5.0

50.0

Mass

Calc. Mass

mDa

PPM

DBE

i-FIT

Formula

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241.0977

-0.8

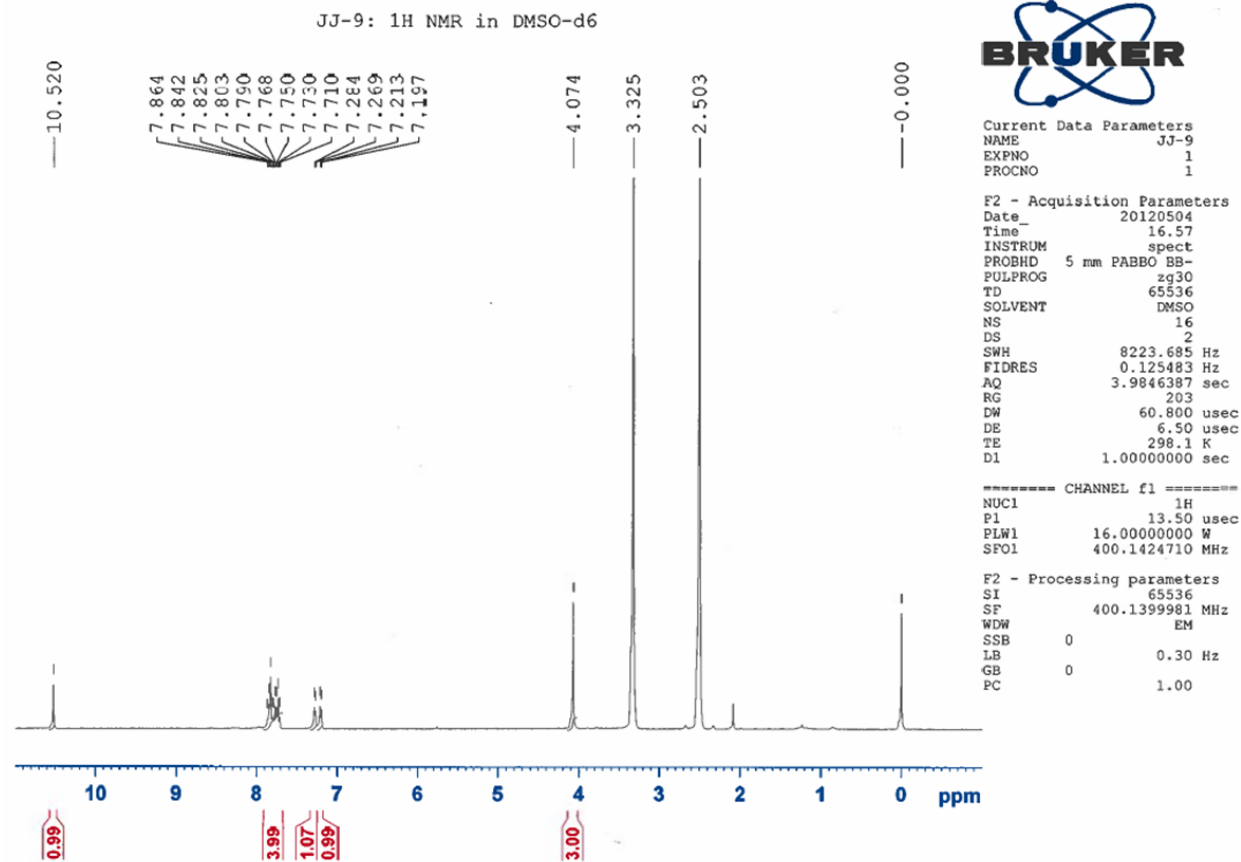
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9.5

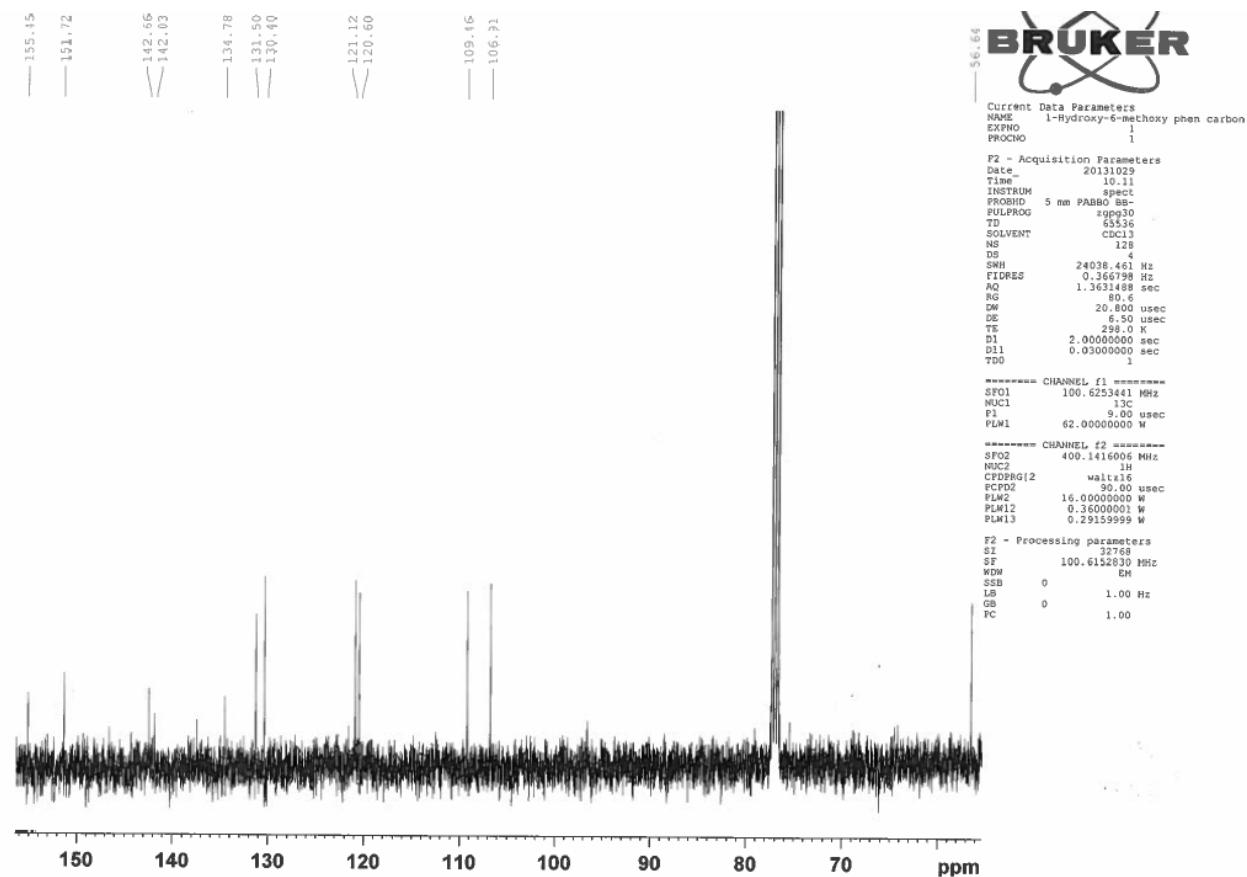
7.9

C14 H13 N2 O2

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*Spectra 4<sup>1</sup>H NMR 1-Hydroxy-6-methoxyphenazine*



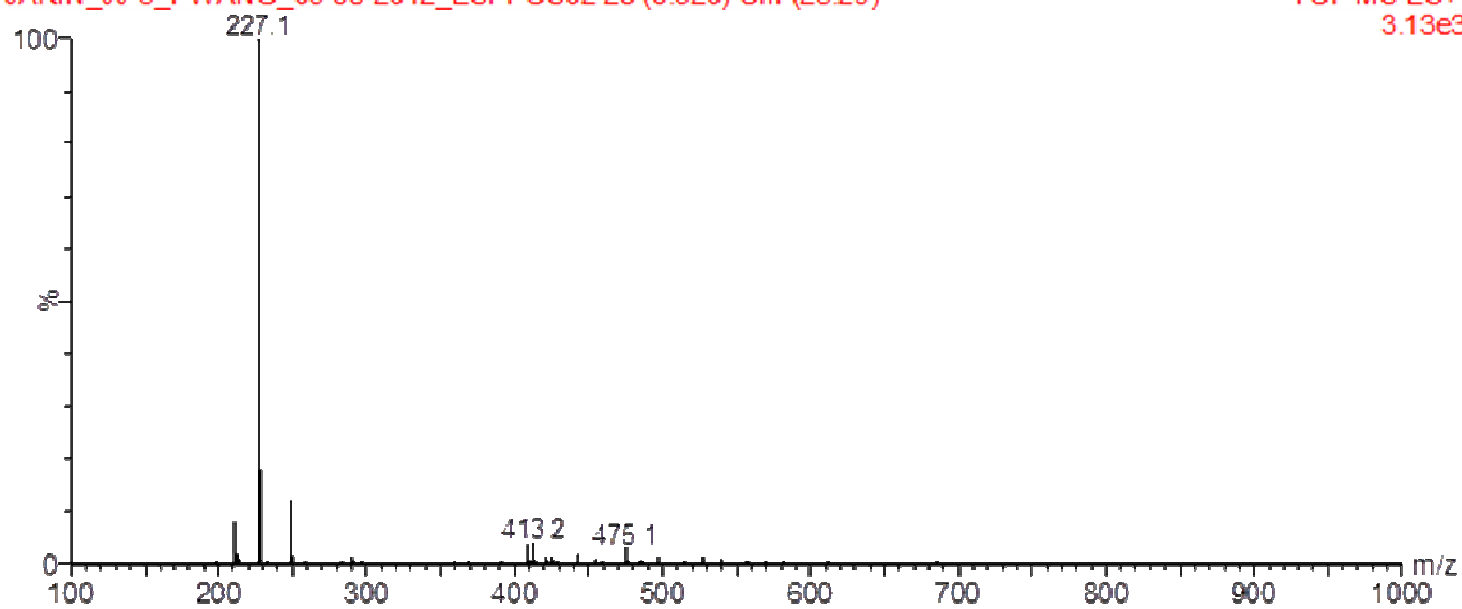
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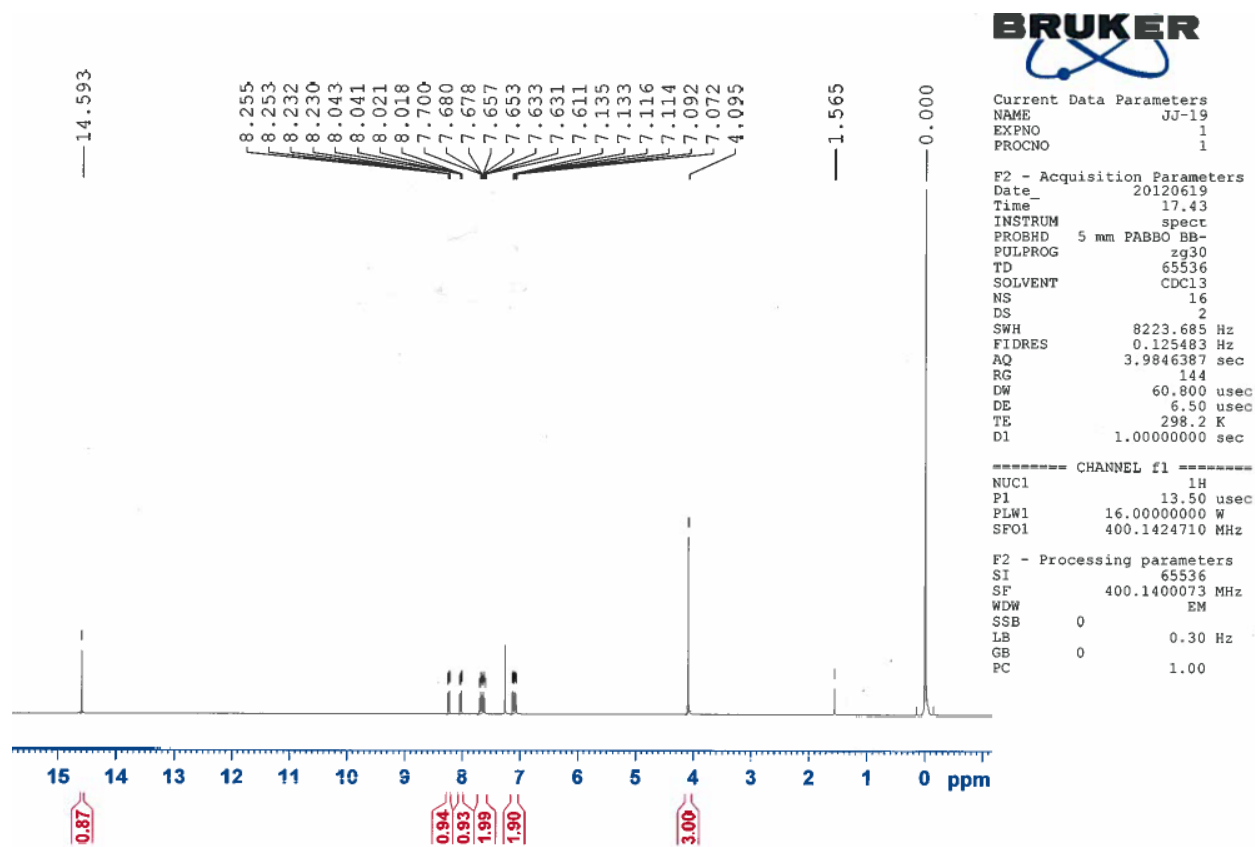
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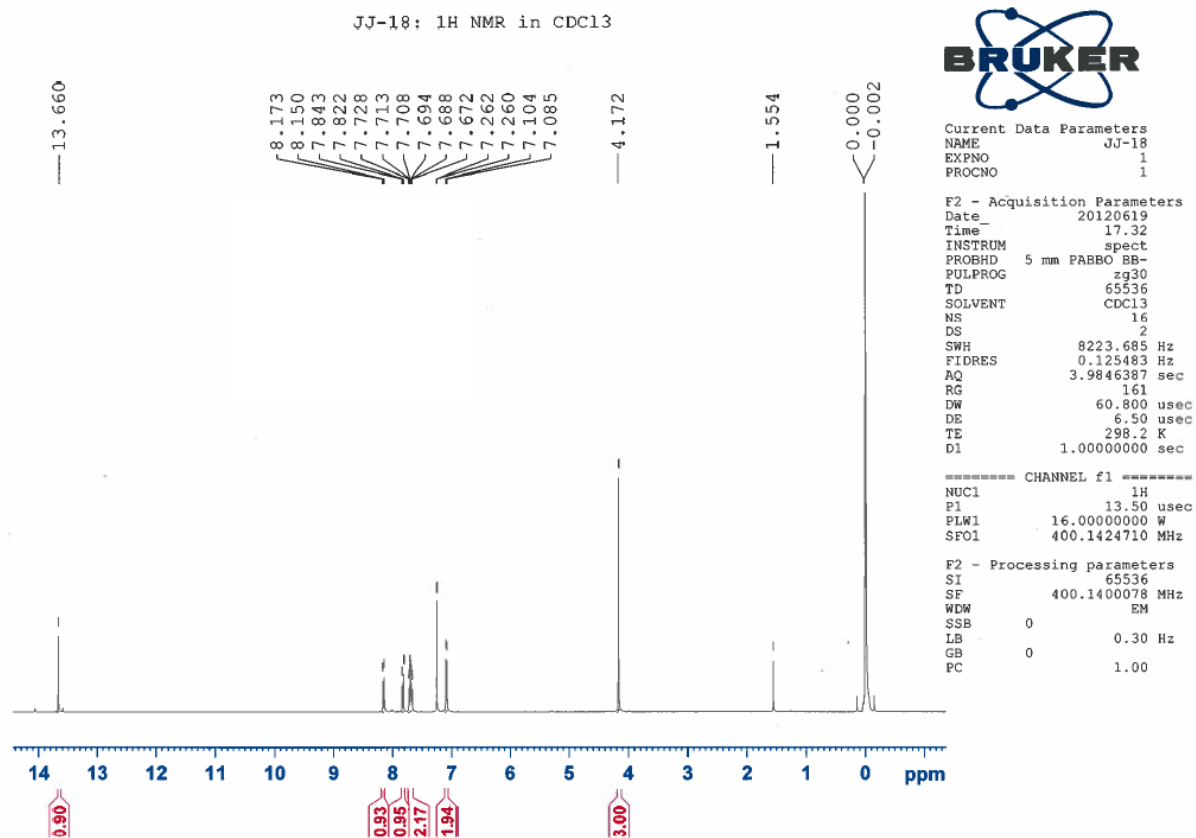
13:50:36 08-May-2012

TOF MS ES+  
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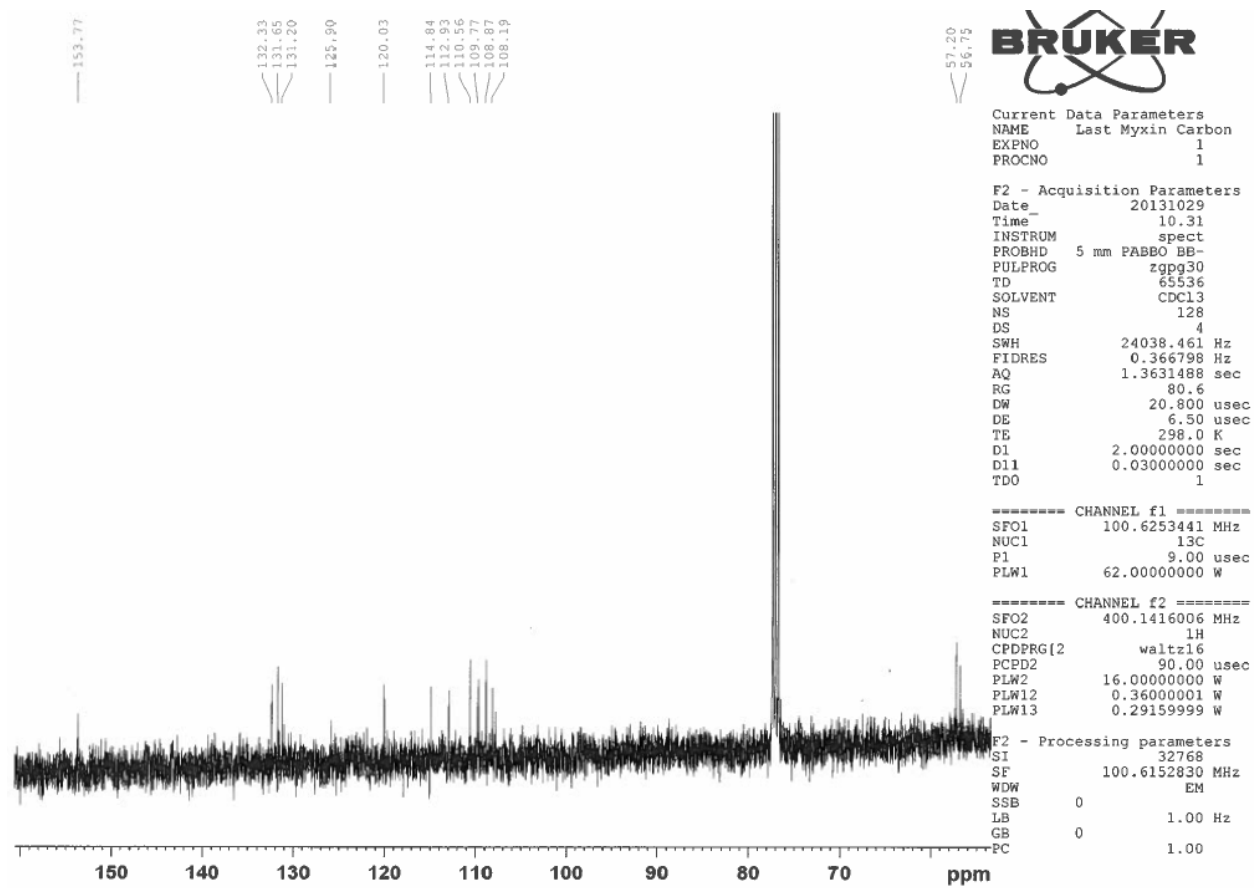


Spectra 6 Mass Spec (ESI) 1-Hydroxy-6-methoxyphenazine

Spectra 7 <sup>1</sup>H NMR Myxin



Spectra 7a  $^1\text{H}$  NMR 1-Hydroxy-6-Methoxy-N10-Oxide

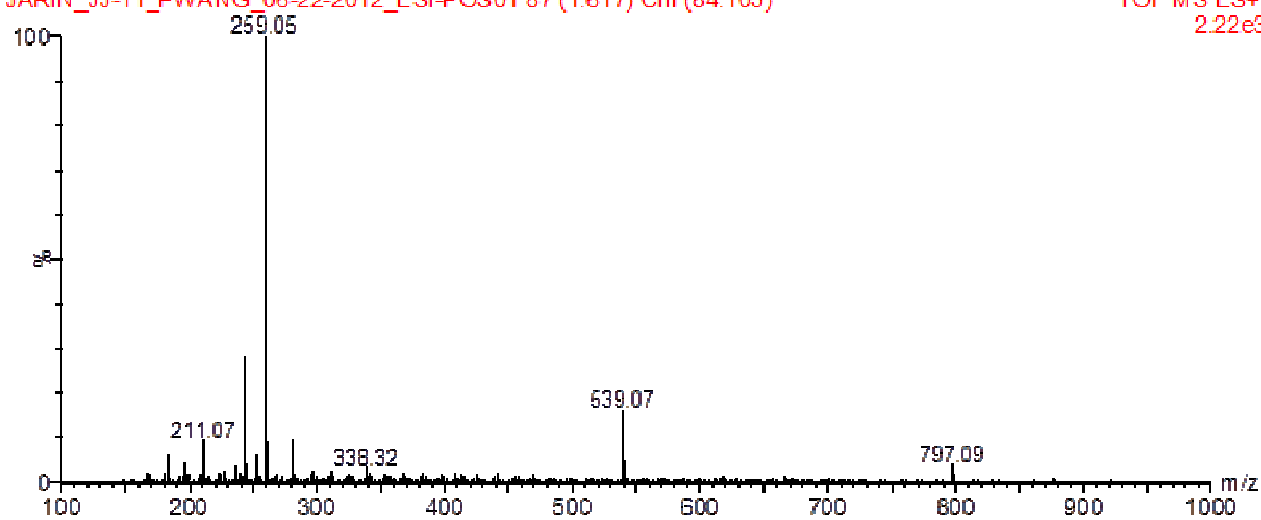
Spectra 8  $^{13}\text{C}$  NMR Myxin

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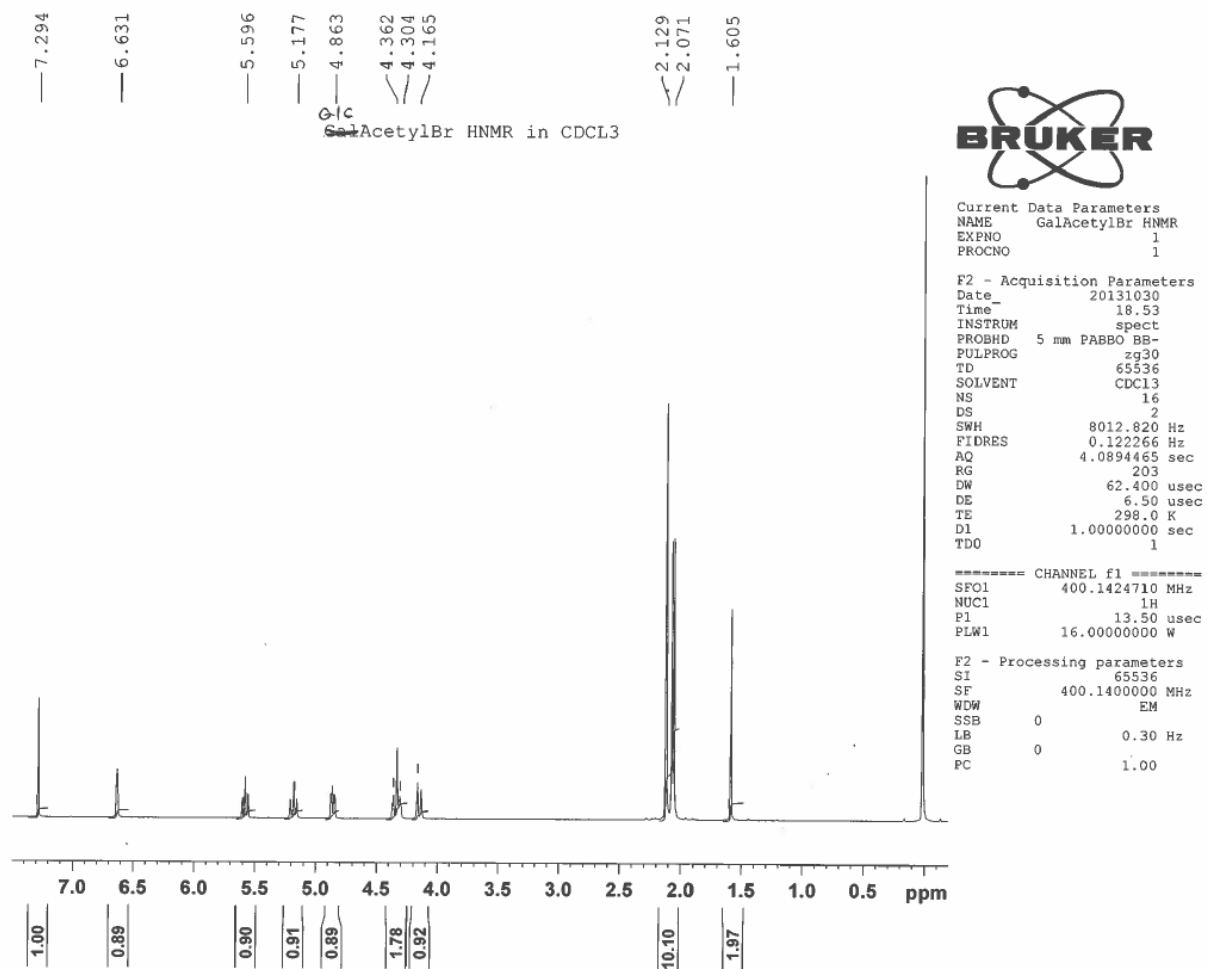
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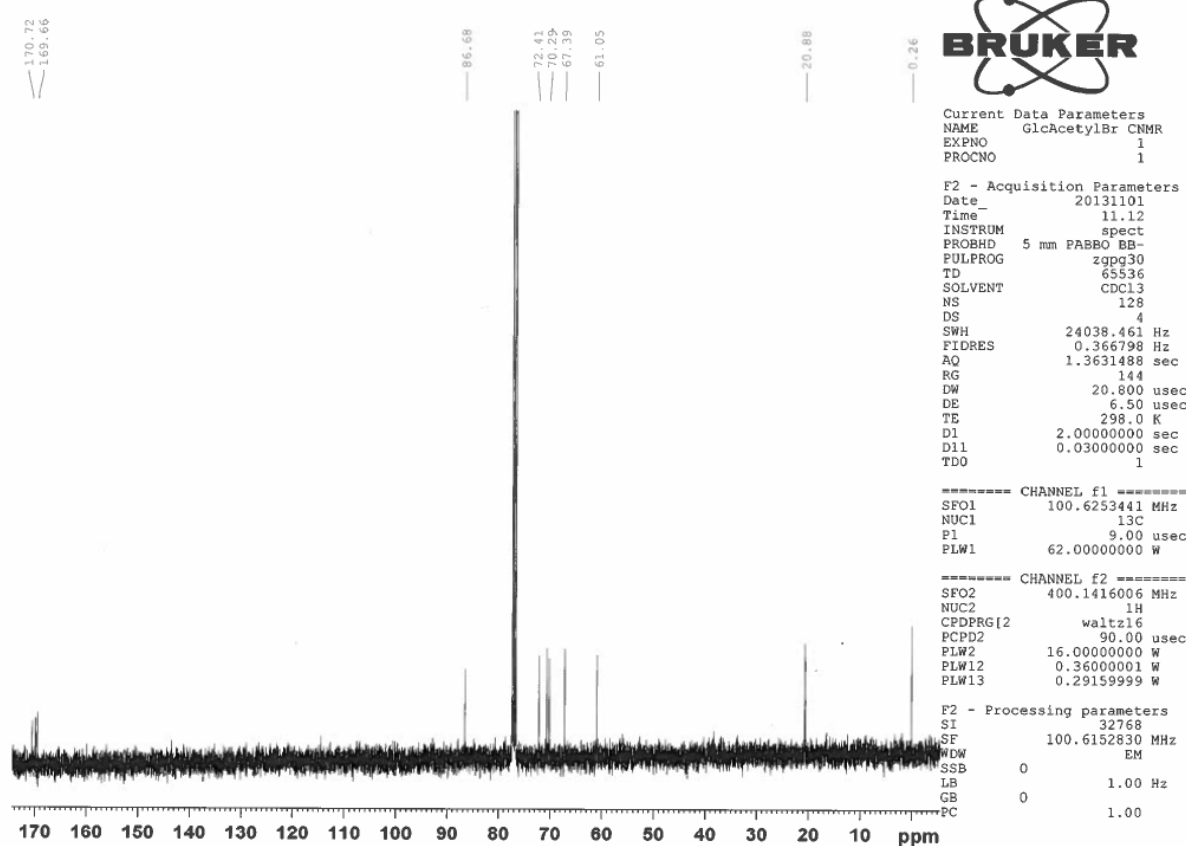
TOF MS ES+  
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*Spectra 9 Mass Spec (ESI) Myxin*

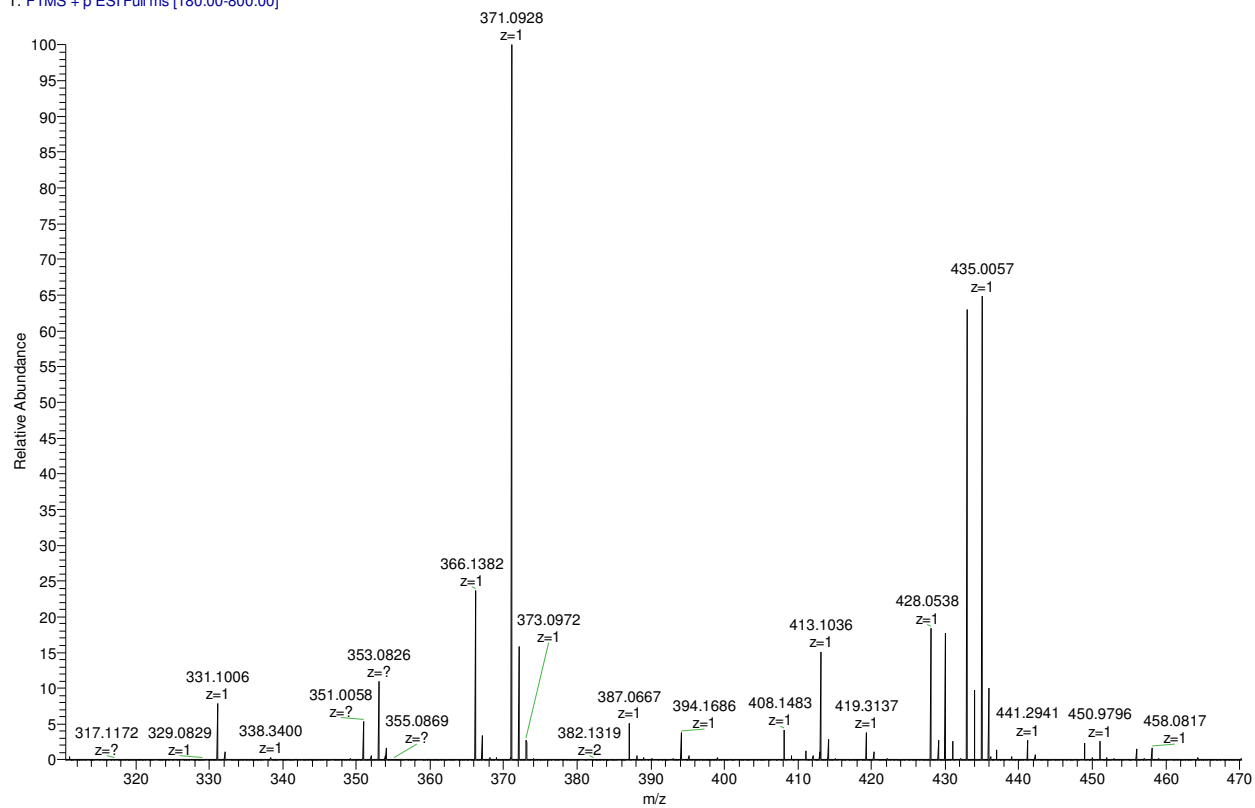


*Spectra 10* <sup>1</sup>H NMR 2,3,4,6-Tetra-O-acetyl- $\alpha$ -D-glucopyranosyl bromide

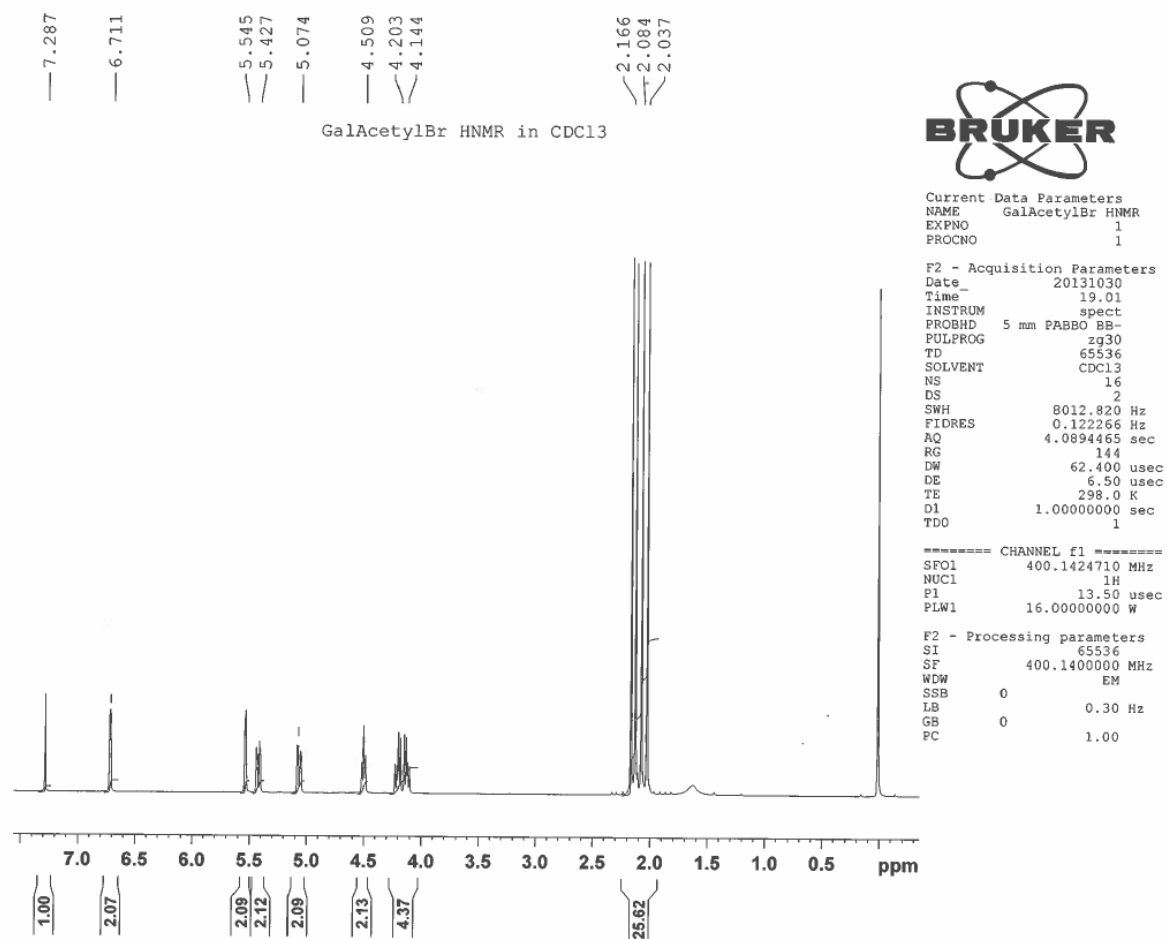


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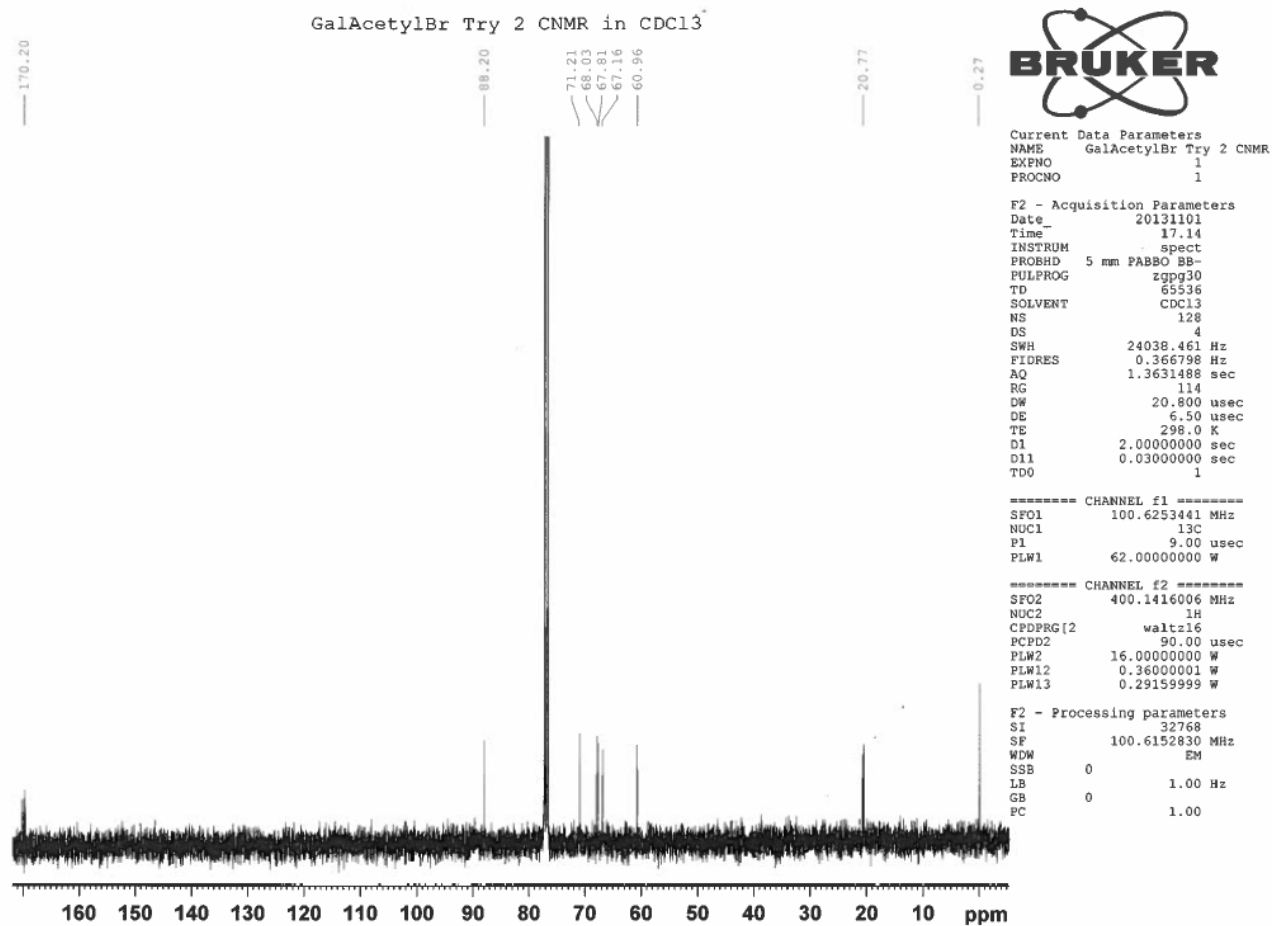
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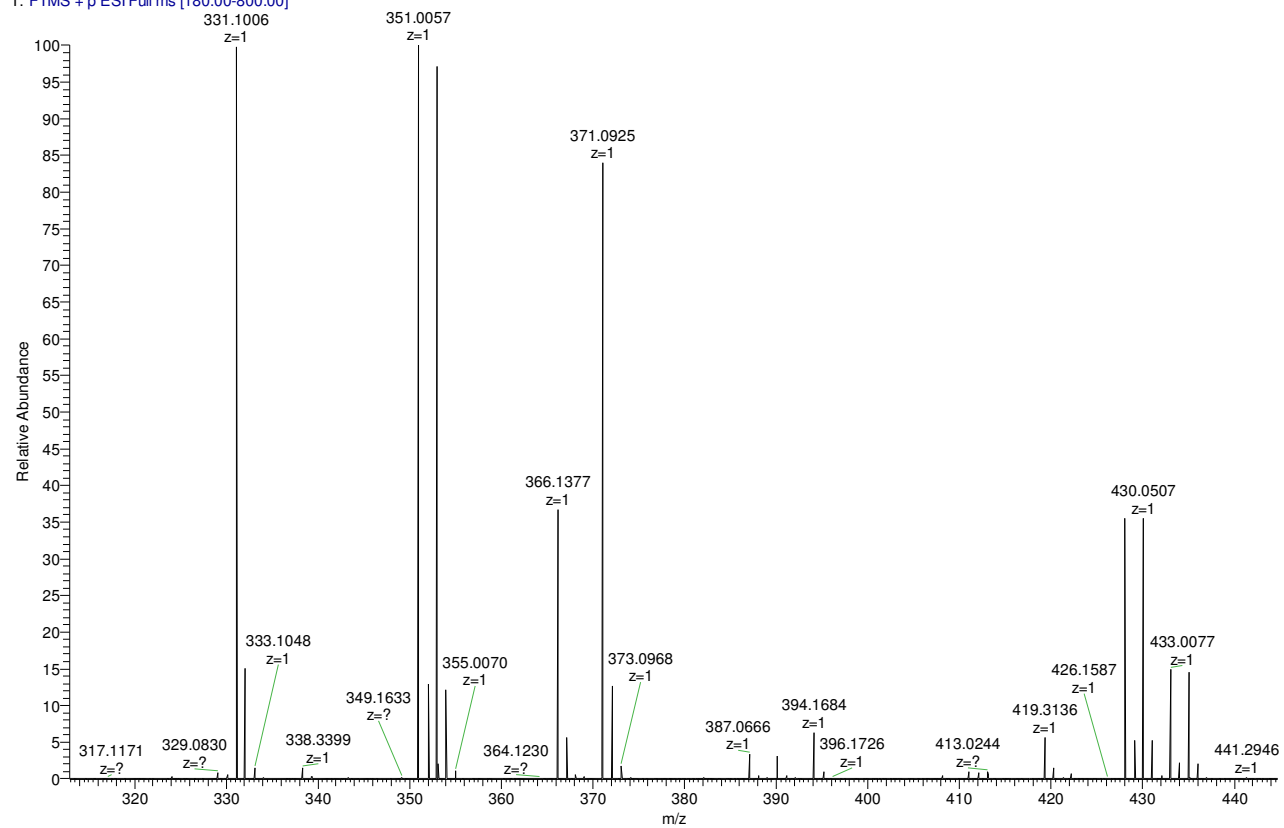


*Spectra 13 <sup>1</sup>H NMR 2,3,4,6-Tetra-O-acetyl- $\alpha$ -D-galactopyranosyl bromide*

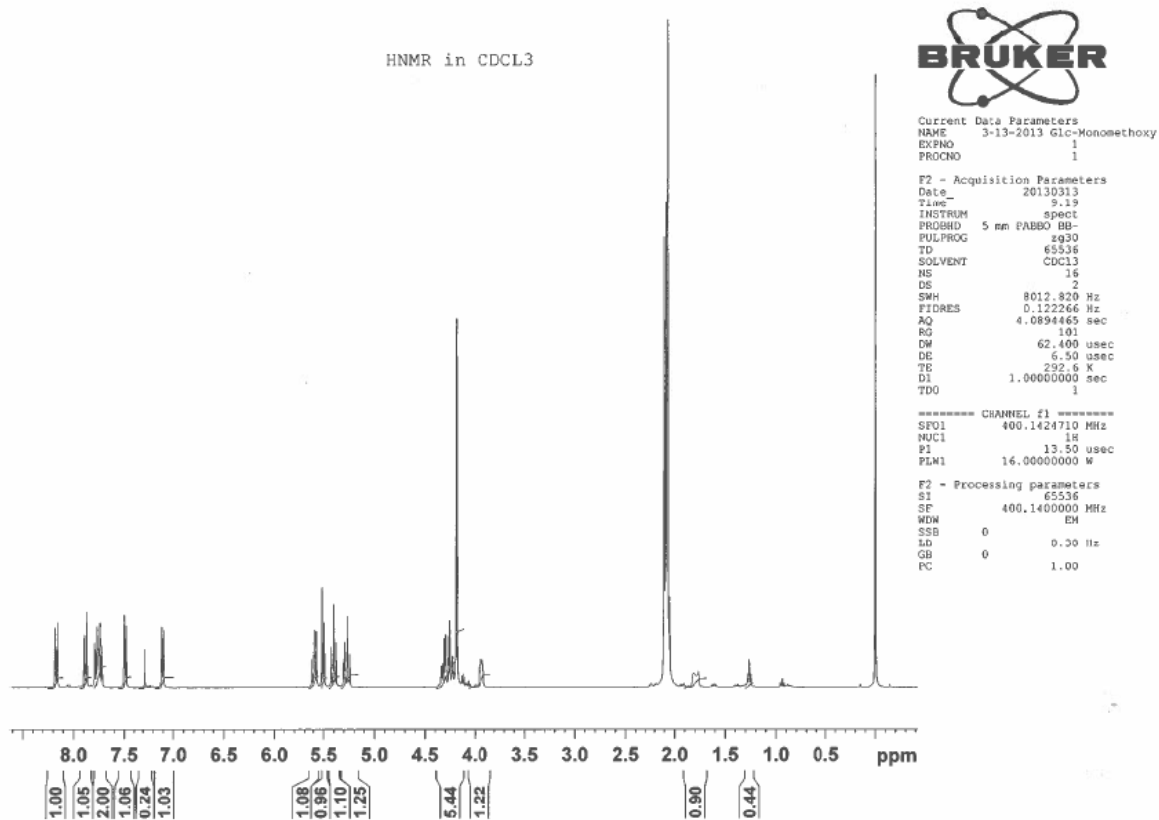


*Spectra 14* <sup>13</sup>C NMR 2,3,4,6-Tetra-O-acetyl- $\alpha$ -D-galactopyranosyl bromide

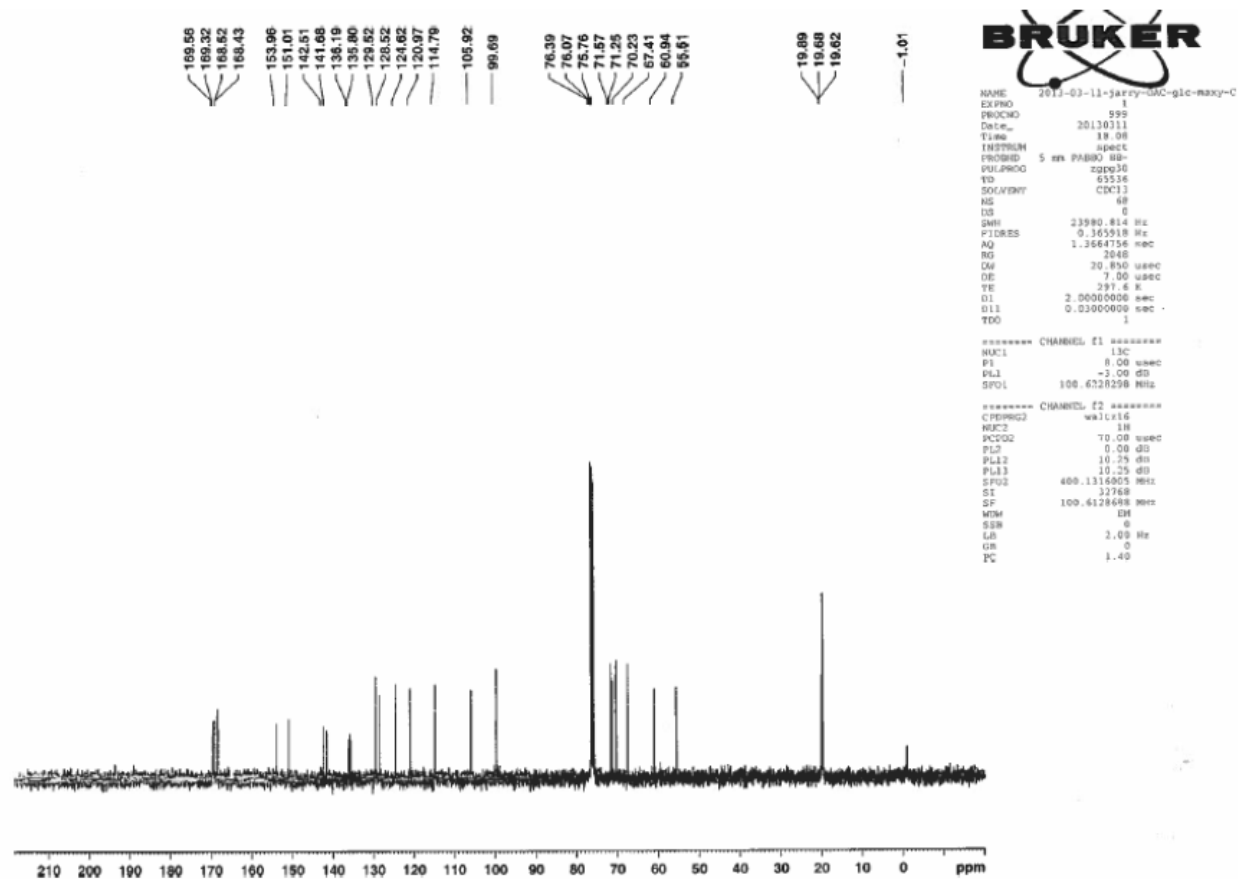
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*Spectra 15 Mass Spectra (ESI) 2,3,4,6-Tetra-O-acetyl- $\alpha$ -D-galactopyranosyl bromide*



*Spectra 16* <sup>1</sup>H NMR 6-Methoxyphenazine-1-β-D-tetraacetylgluco-pyranoside



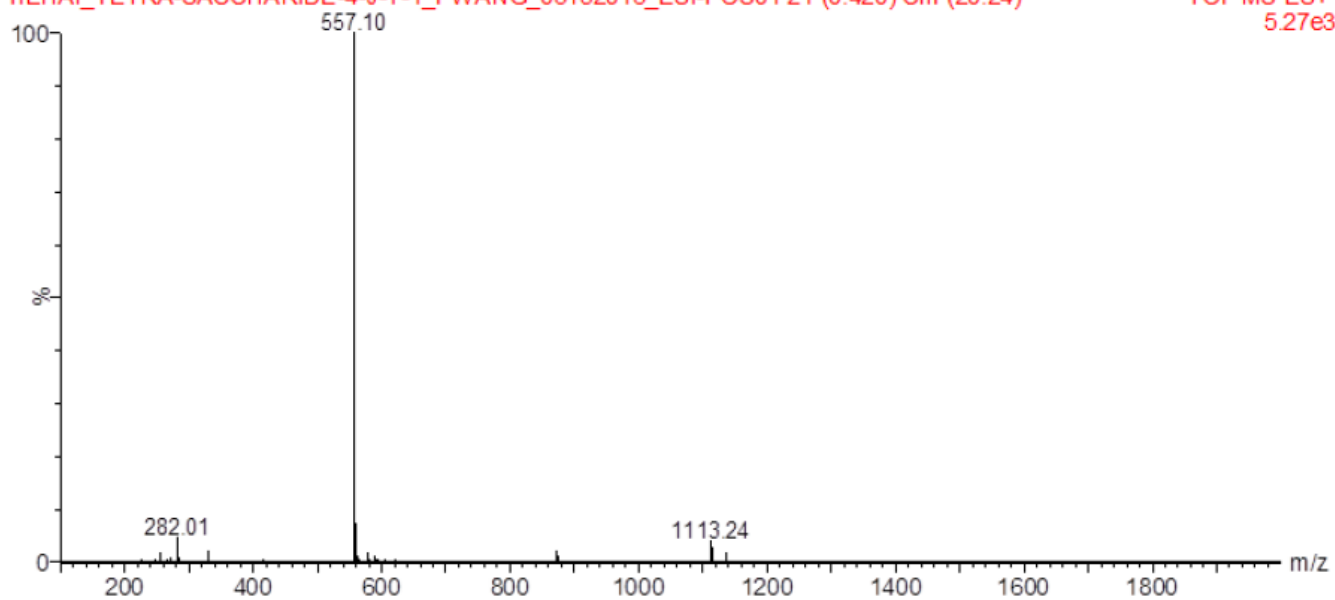
Spectra 17  $^{13}\text{C}$  NMR 6-Methoxyphenazine-1- $\beta$ -D-tetraacetylgluco-pyranoside

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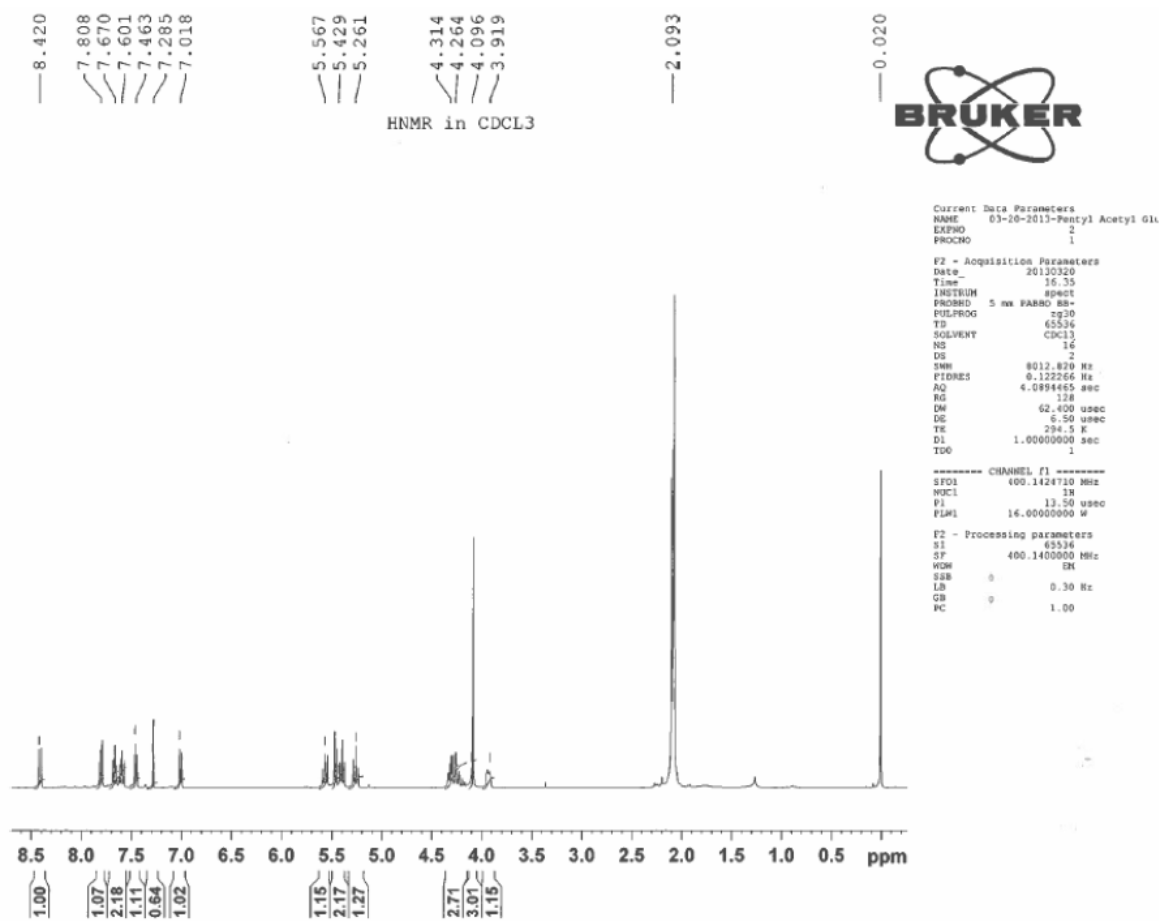
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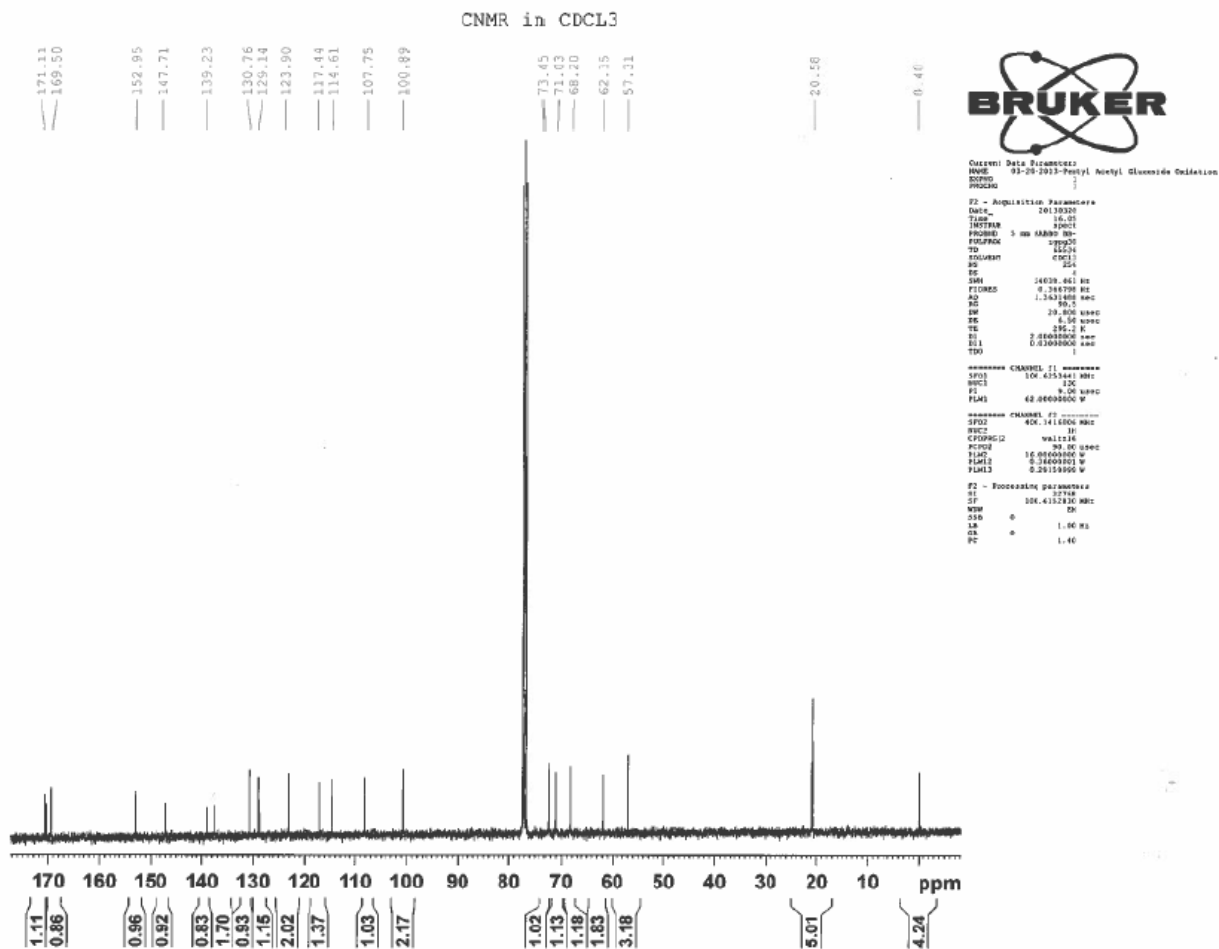
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*Spectra 18 Mass Spectra (ESI) 6-Methoxyphenazine-1-β-D-tetraacetylgluco-pyranoside*



Spectra 19 <sup>1</sup>H NMR 6-Methoxyphenazine-N5-oxide-1-beta-D-tetraacetylgluco-pyranoside



Spectra 20 <sup>13</sup>C NMR 6-Methoxyphenazine-N5-oxide-1-beta-D-tetraacetylgluco-pyranoside

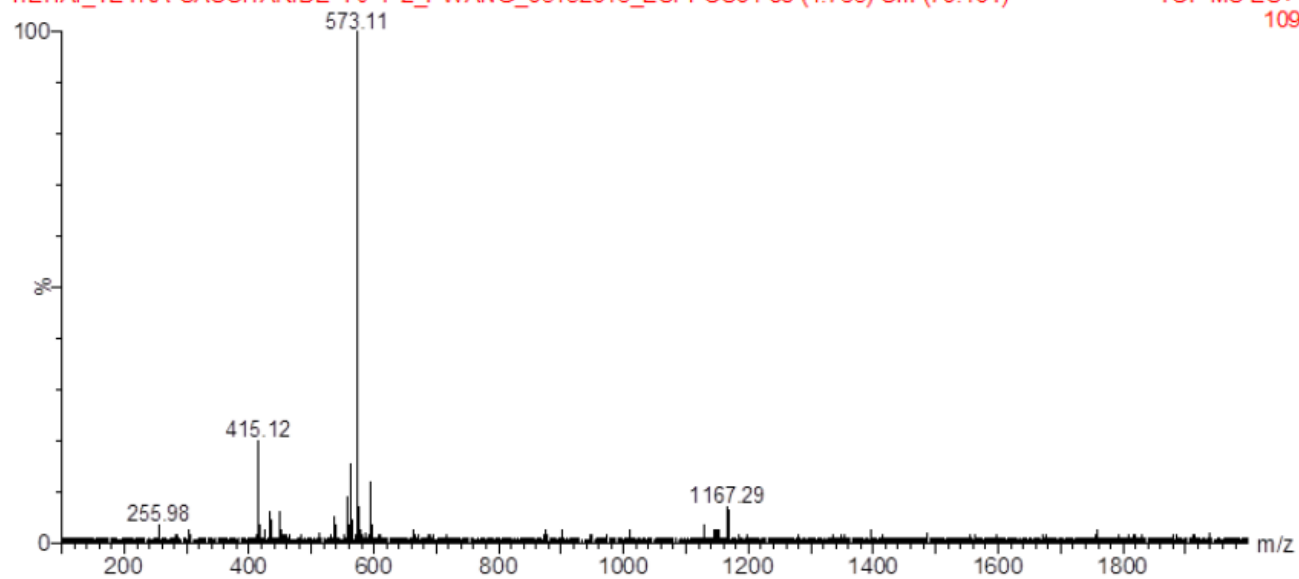
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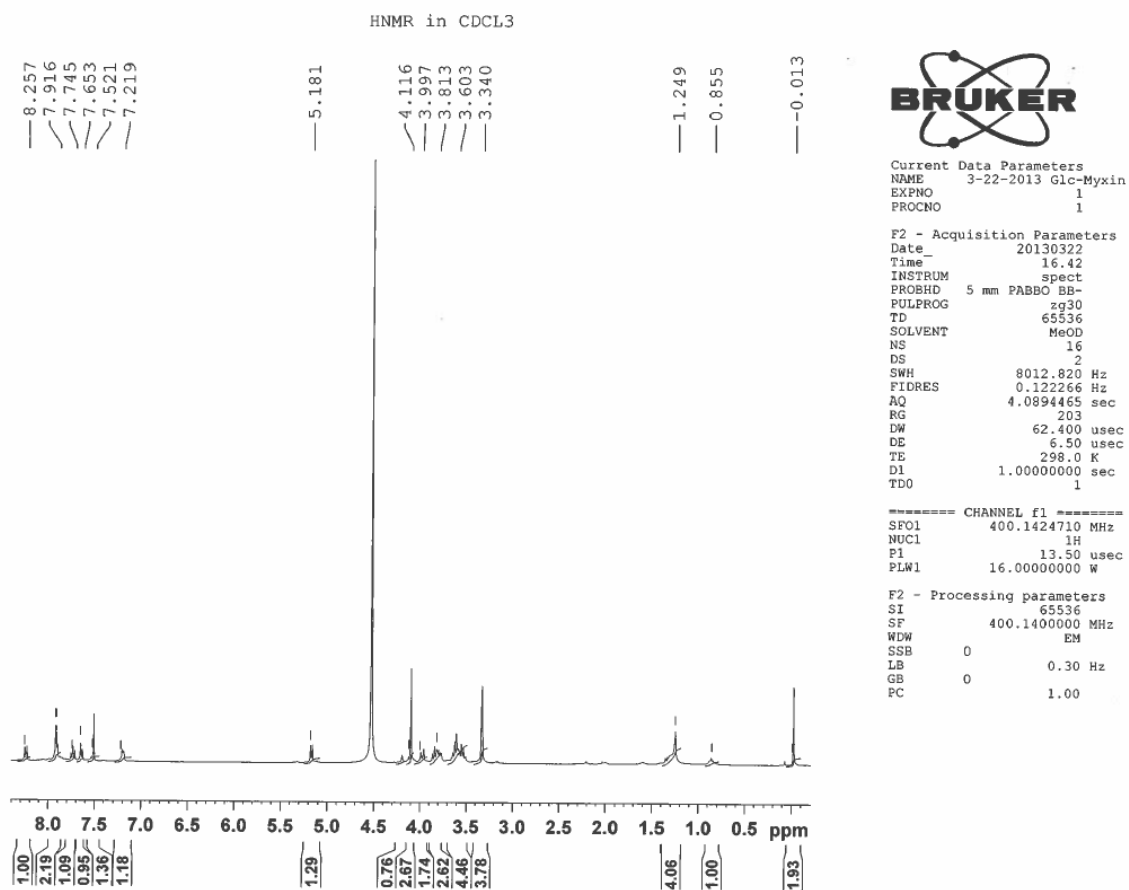
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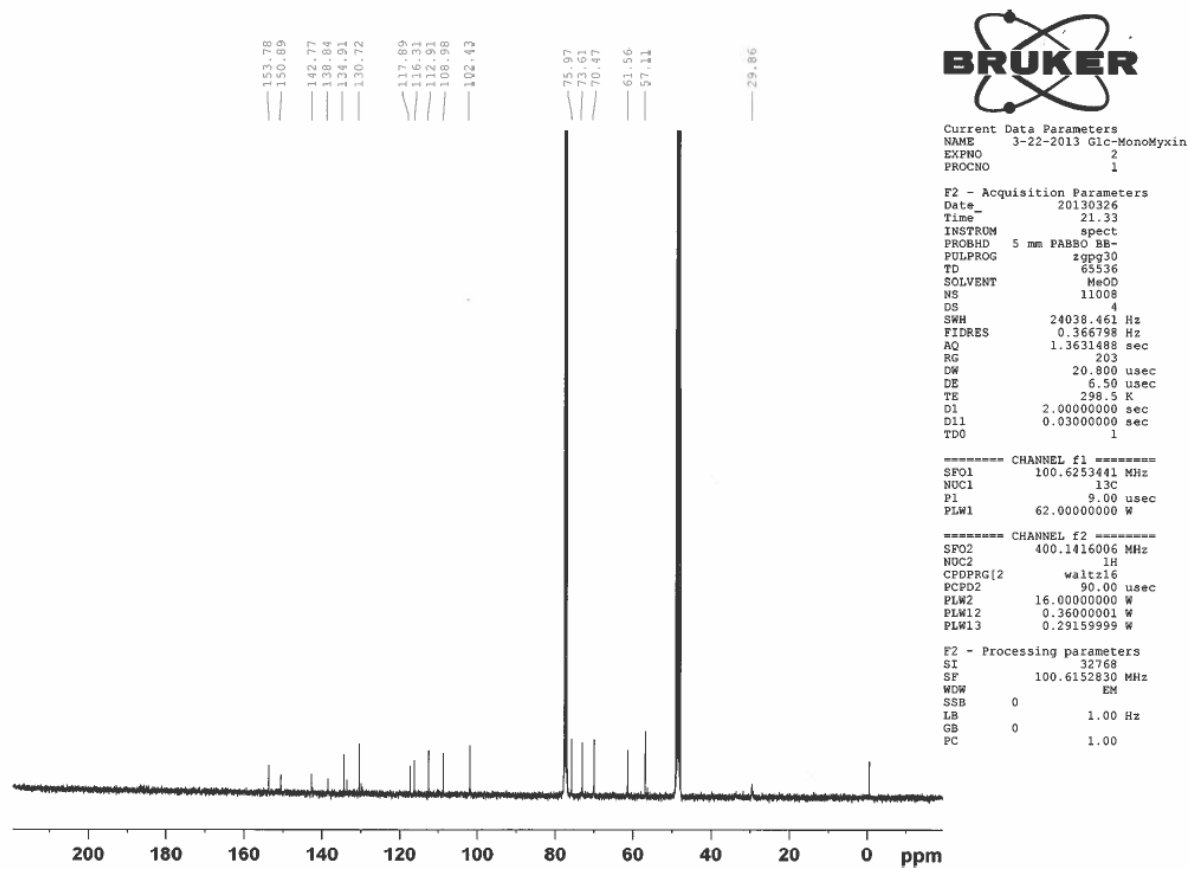
109



*Spectra 21 Mass Spec (ESI) 6-Methoxyphenazine-N5-oxide-1-beta-D-tetraacetylgluco-pyranoside*



*Spectra 22* <sup>1</sup>H NMR 6-Methoxyphenazine-N5-oxide-1-beta-D-gluco-pyranoside



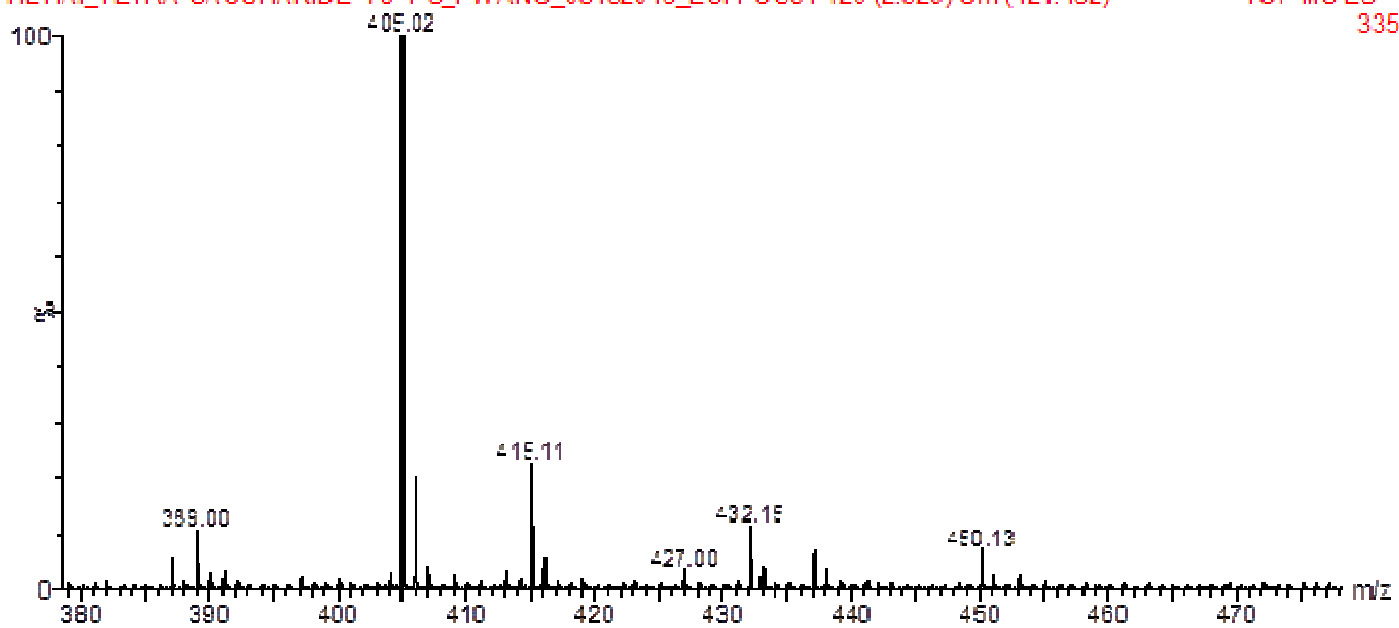
Spectra 23 <sup>13</sup>C NMR 6-Methoxyphenazine-N5-oxide-1-beta-D-gluco-pyranoside

diluted in 50% ACN

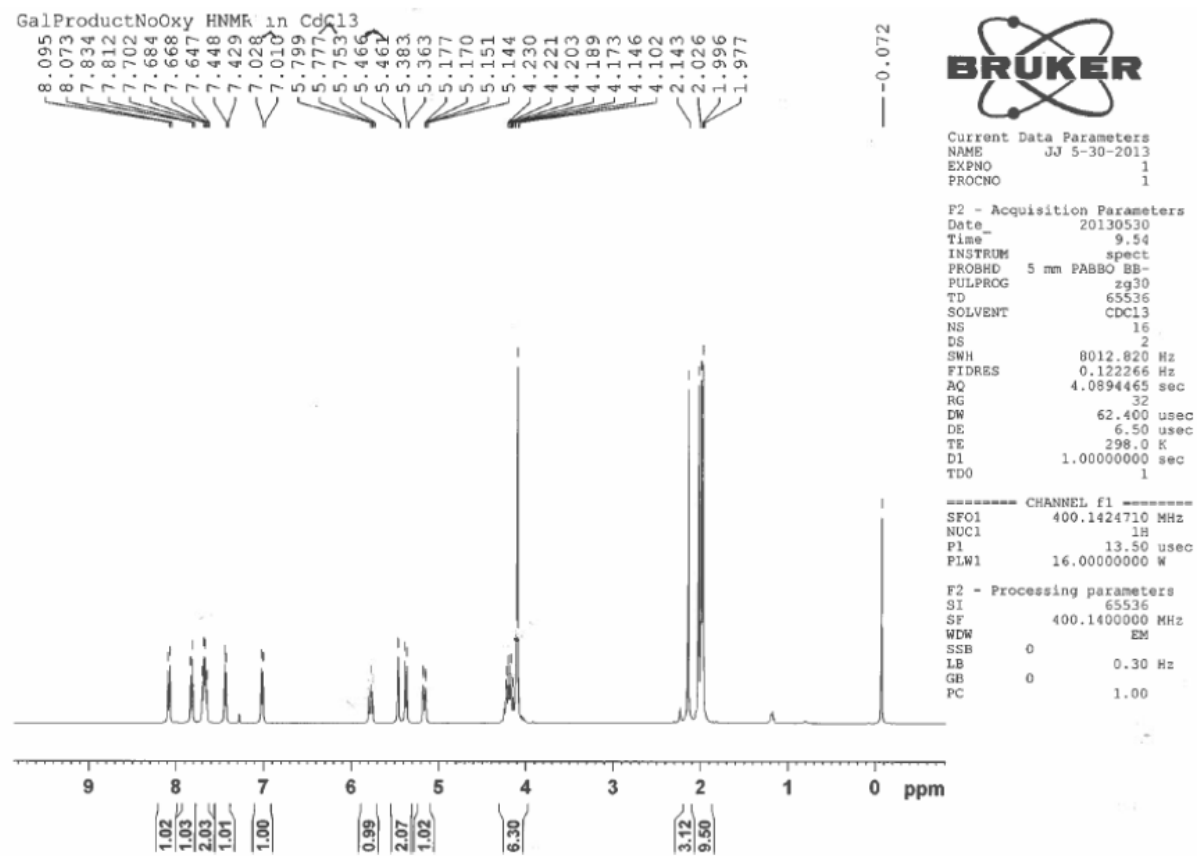
17:35:44 25-Mar-2013

TIE HAI\_TETRA-SACCHARIDE-4-J-Y-3\_PWANG\_03152013\_ESI-POS01 126 (2.520) Cm (121:132)

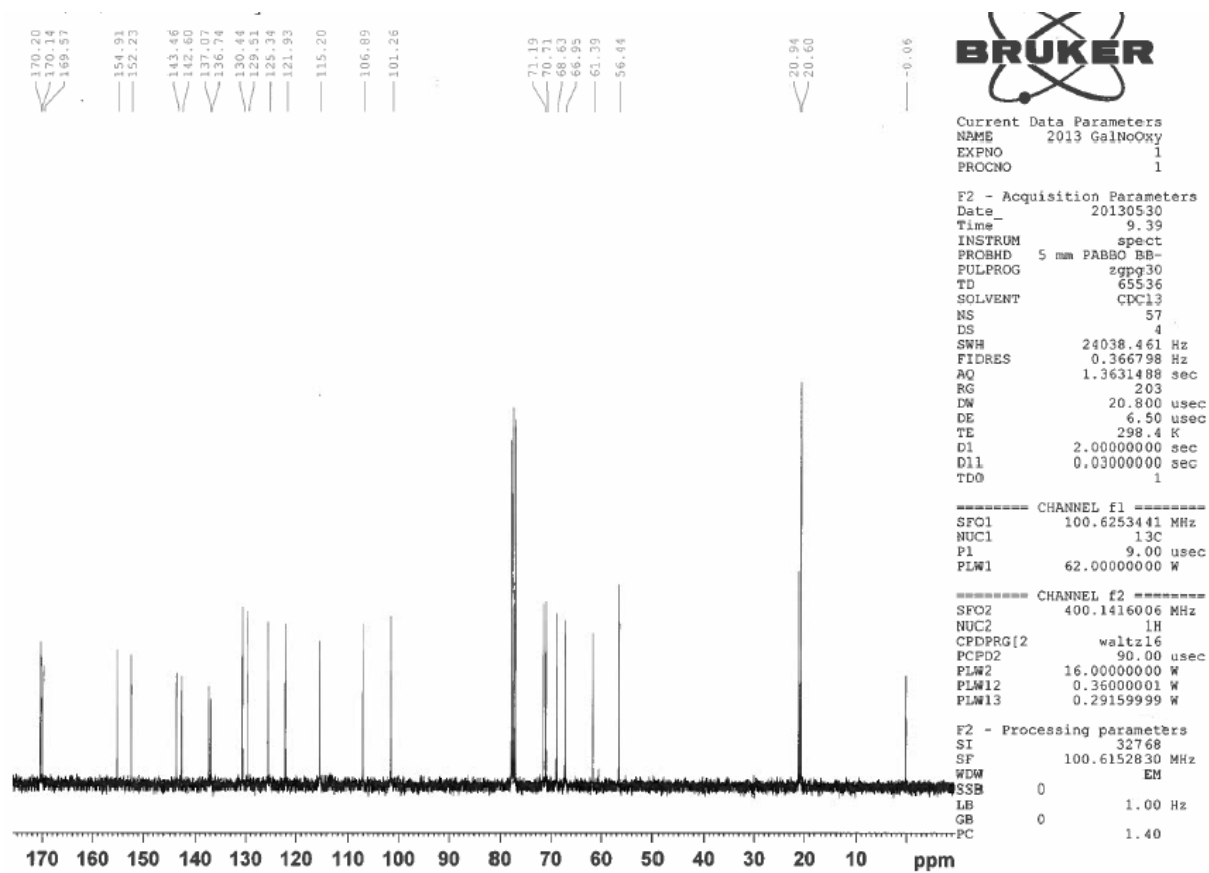
TOF MS ES+  
335



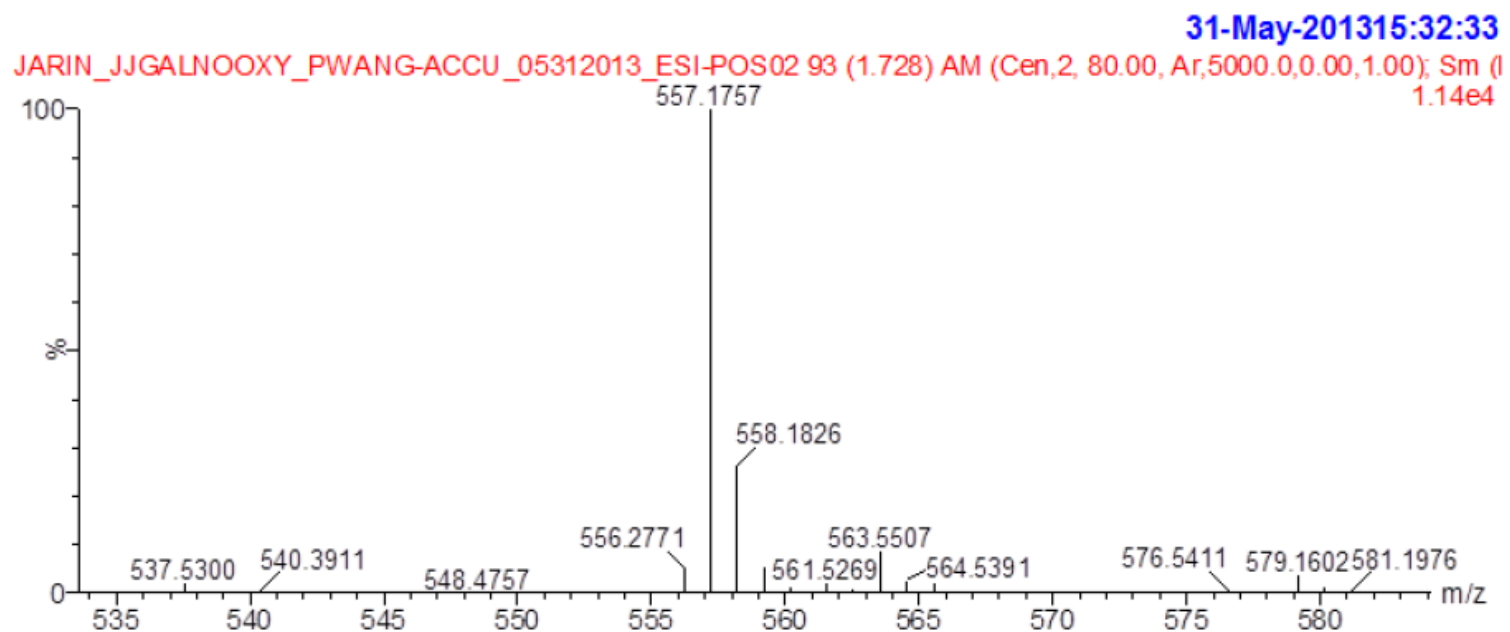
Spectra 24 Mass Spectra (ESI) 6-Methoxyphenazine-N5-oxide-1-beta-D-gluco-pyranoside



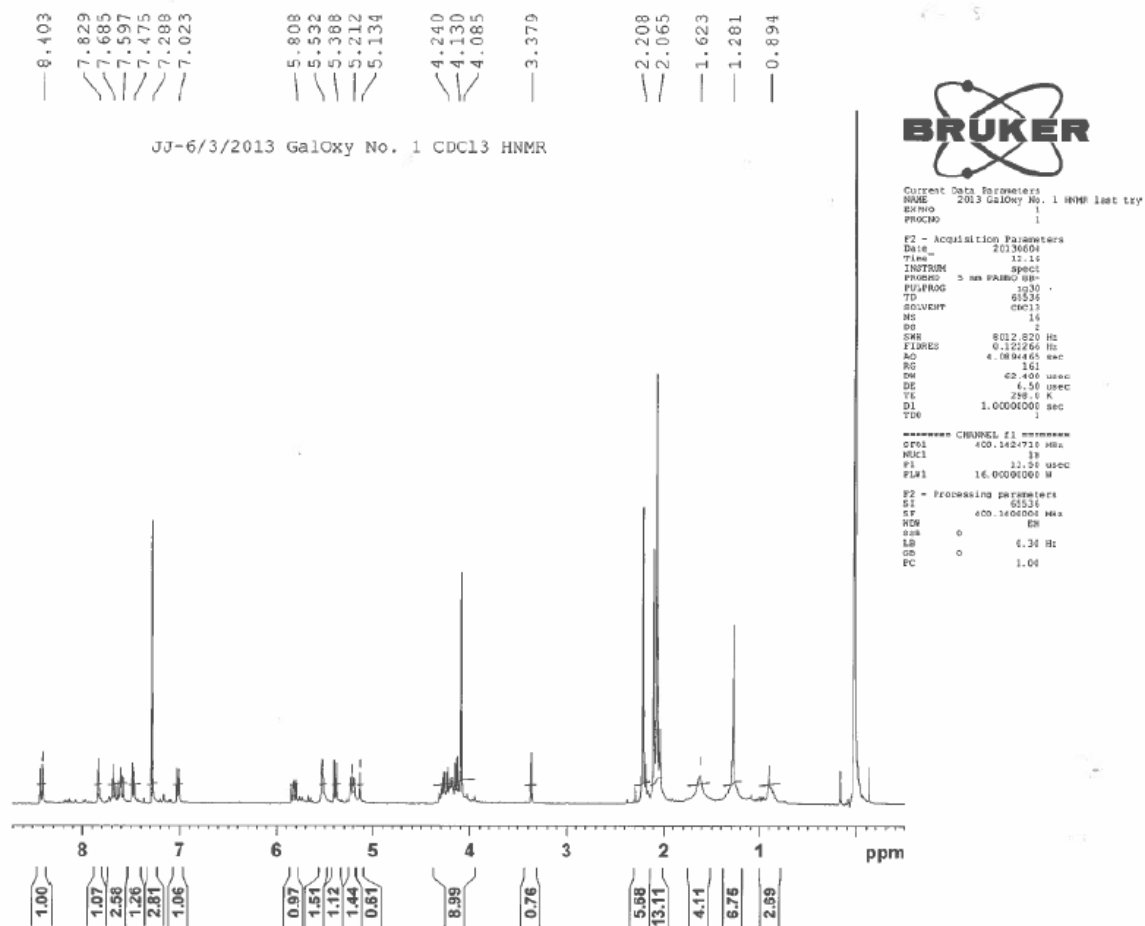
Spectra 25 <sup>1</sup>H NMR 6-Methoxyphenazine-1-β-D-tetraacetylgalacto-pyranoside



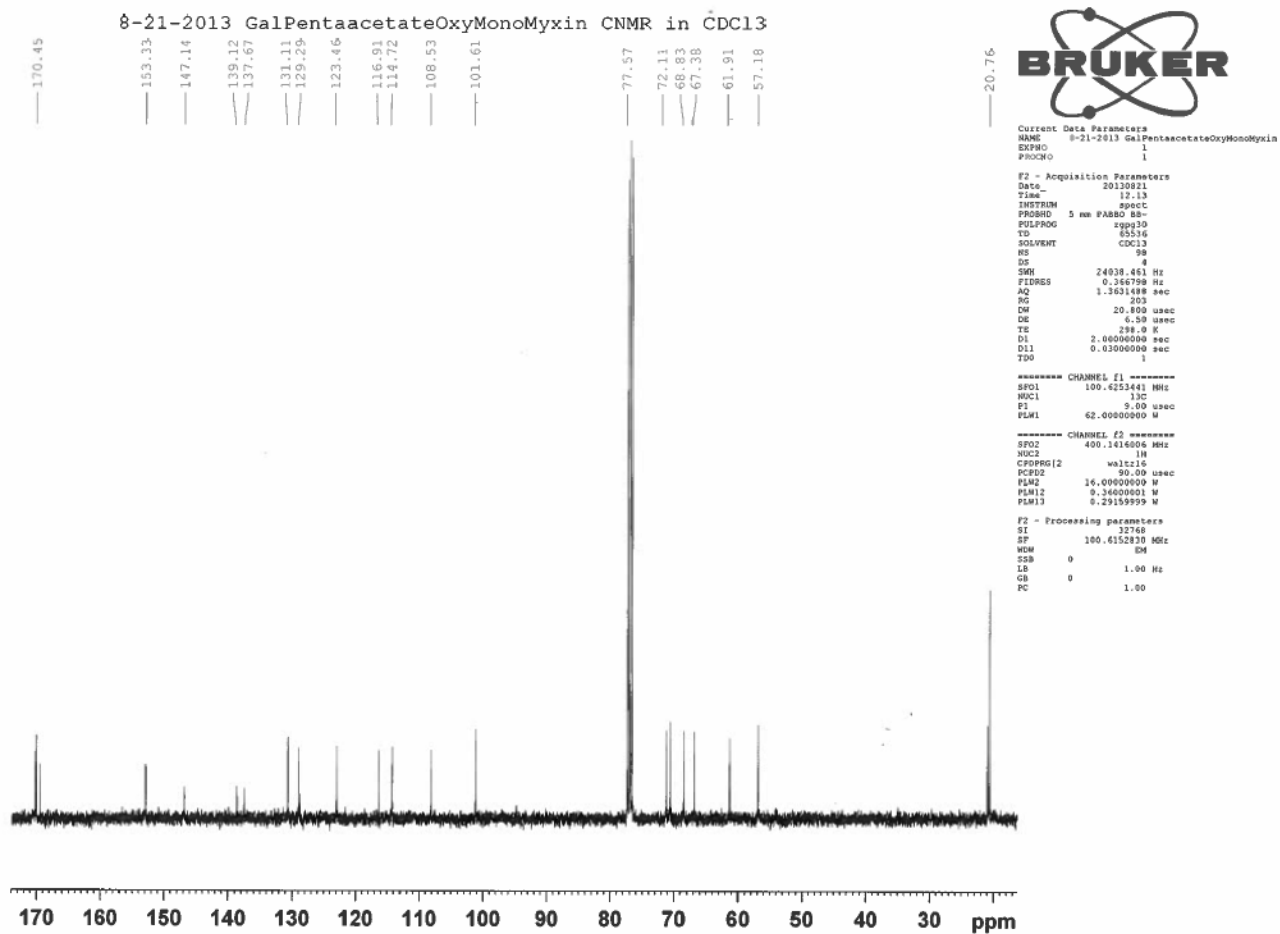
Spectra 26  $^{13}\text{C}$  NMR 6-Methoxyphenazine-1- $\beta$ -D-tetraacetylgalacto-pyranoside



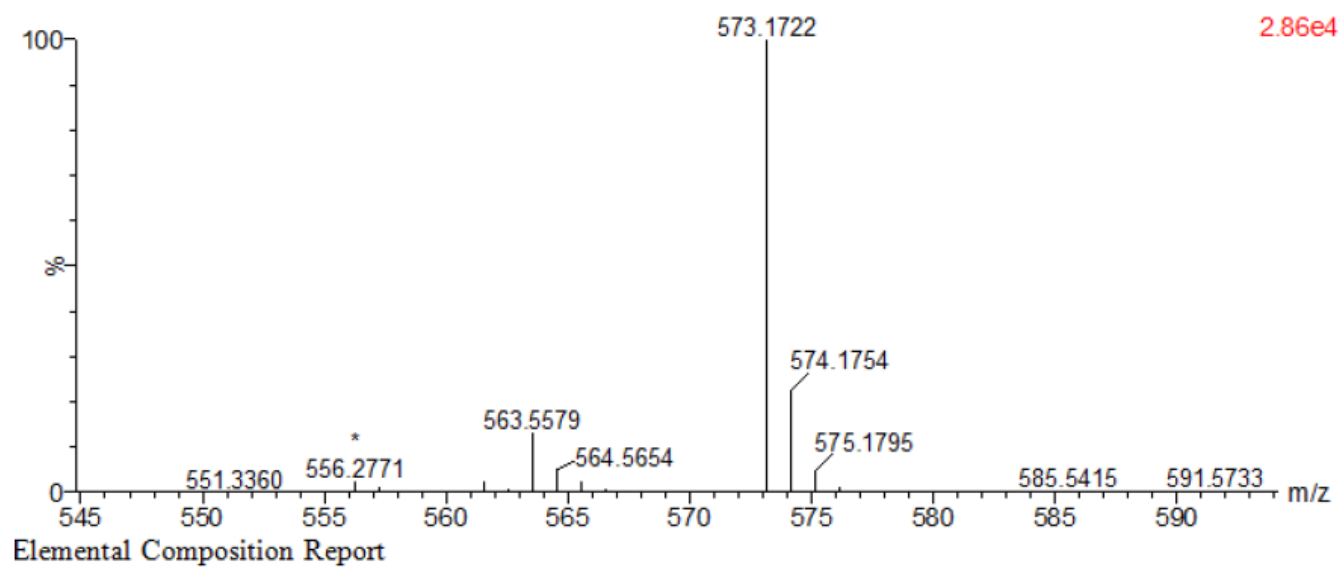
*Spectra 27 Mass Spectra (ESI) 6-Methoxyphenazine-1-β-D-tetraacetylgalacto-pyranoside*



Spectra 28 <sup>1</sup>H NMR 6-Methoxyphenazine-N5-oxide-beta-D-tetraacetylgalacto-pyranoside



Spectra 29 <sup>13</sup>C NMR 6-Methoxyphenazine-N5-oxide-beta-D-tetraacetylgalacto-pyranoside



*Spectra 30 Mass Spec (ESI) 6-Methoxyphenazine-N5-oxide-beta-D-tetraacetylgalacto-pyranoside*