Characterization of a Novel Cellulose Biosynthesis Inhibitor, CBI28, in *Gluconacetobacter xylinus*

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Abstract

To study the underlying mechanisms for microbial cellulose biosynthesis, a novel compound, CBI28, was used as an inhibitor along with classical genetics and EMS mutagenesis. An EZ-Link Biotin Hydrazide Kit was used to create a CBI28-Biotin conjugate for further studies. *Gluconacetobacter xylinus* cells were exposed to 10 µM CBI28 to induce cellulose biosynthesis inhibition, lysed and small hydrophobic molecules were extracted using methanol and Waters Oasis HLB SPE-Paks. Samples were separated and detected using the Ultra Performance Liquid Chromatograph-Mass Spectrometer/Photo Diode Array. Putative mutants were isolated but did not survive for further study. An ion with the expected mass of a CBI28-Biotin conjugate (552 m/z) was detected but not in sufficiently high concentrations for characterization. Metabolite studies revealed putative metabolites derived from the HLB SPE and methanol extractions with no significant difference in extraction methods. Potential metabolites with masses of ~281.77 m/z and ~79 m/z were detected in CBI28 exposed cells. Further analysis needs to be performed to determine if CBI28 metabolites prevent cellulose production.

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List of Abbreviations

acs – Acetobacter Cellulose Synthase

ATCC – American Type Cell Culture

CBI28 – Cellulose Biosynthesis Inhibitor 28

c-di-GMP – Cyclic Diaguanylic Acid (Cyclic Diguanylate)

cdg – Cyclic Diguanylate

CE – Capillary Electrophoresis

CE/LC/MSD – Capillary Electrophoresis/Liquid Chromatography/ Mass Spectrometry Detection

dgc – Diguanylate Cyclase

DMSO - Dimethyl Sulfoxide

DNA – Deoxyribonucleic Acid

EMS - Ethyl Methanesulfonate

ESI – Electrospray Ionization

HLB – Hydrophilic-Lipophilic Balance

IUPAC – International Union of Pure and Applied Chemisty

m/z – Mass to Charge Ratio

Min A – Minimal A Buffer

MS – Mass Spectrometer

MS/MS – Tandem Mass Spectroscopy

nm - Nanometres

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PDA – Photo Diode Array

pde – Phosphodiesterase

PEG – Polyethylene Glycol

pGpG – Diguanosine Diphosphate

pppGTP – Guanosine Phosphate

SIR – Single Ion Reaction

SPE – Solid Phase Extraction

TIC – Total Ion Count

TLC – Thin Layer Chromatography

UDP-glucose – Uridine Diphosphate-glucose

UPLC – Ultra Performance/Pressure Liquid Chromatography

UV – Ultraviolet

Chapter 1: Cellulose Biosynthesis in *Gluconacetobacter xylinus*

1.1 Cellulose Biosynthesis in *Gluconacetobacter xylinus*

Cellulose, β-1, 4-glucan, is among one of the most abundant molecules on earth, primarily because it is a major component of the cell walls of vascular plants. The molecule is not unique to plants, however, and appears on various evolutionary branches including several genera of bacteria, most algae, oomycetes, some slime moulds like *Dictyostelium*, and even one group of animals - tunicates (Brown, 1982) . In all cases, cellulose is produced as an extracellular polysaccharide that is either an integral element of the cell wall, such as in plants, algae, and oomycetes, or is used to filter feed and as structural support in tunicates (Brown Jr. & Saxena, 2007). It is therefore not surprising that cellulose is potentially required for survival in bacteria and is required for the proper functioning of plant and algal cells (Hisert *et al.*, 2005, Brown Jr. & Saxena, 2007). The best studied of the bacterial cellulose producers, *Gluconacetobacter xylinus* (previously *Acetobacter xylinum*), is a Gram-negative, aerobic, rod-shaped organism. It is a member of the family Acetobacteraceae (Boone, Castenholz & Garrity, 2001; Colvin, Sowden & Leppard, 1977). A distinguishing feature of *Gluconacetobacter xylinus (G. xylinus)*, as well as other members of the cellulose producing *Acetobacter* genus, is the production of a cellulose pellicle when the organism is cultured statically (Gromet-Elhanan & Hestrin, 1963). This feature has made *G xylinus* an important model system for the study of cellulose biosynthesis because it produces copious amounts of cellulose and it is easily cultured in laboratory studies.

G. xylinus extrudes cellulose from large complexes located on the longitudinal axis of the bacteria (Zaar, 1979). The cellulose is extruded as ribbons which further crystallize, (ribbons hydrogen bond together to form sheets and then microfibrils, to form pellicles of cellulose) (Hestrin & Schramm, 1954). The large multienzyme complexes have been identified using freeze fracture microscopy as pits located in between the inner and outer membrane of the bacteria at 35 Ångstrom (Å) (Kimura, Chen, Saxena, Brown Jr. & Itoh, 2001). There are 50 multienzyme complexes, identified through freeze fracture microscopy and immunolabelling, synthesizing cellulose ribbons and 12 to 25 of these glucan ribbons will assemble along the longitudinal side of the bacteria to form larger cellulose chains (Kimura, Chen, Saxena, Brown Jr. & Itoh, 2001). There are six major enzymes involved in the multisubunit complex identified below.

At least six genes, four of which are in one operon (*acsA-D*), appear to be required for *in vivo* cellulose production (Saxena, Kudlicka, Okuda & Brown, 1994). The cellulose synthase protein encoded by the Acetobacter Cellulose Synthase A (*acsA)* gene is known as or *bcsA* (bacterial cellulose synthase A) and is localized in the cytoplasmic membrane with portions that traverse to the outer plasma membrane (Saxena, Lin & Brown, 1990). The *acsA* gene is considered the catalytic subunit because it binds the substrate uridine diphosphate –glucose (UDP-glucose) (Lin, Brown, Drake & Haley, 1990).

The second gene in the operon, *acsB*, encodes a protein whose function was determined only once the allosteric activator, cyclic diguanylic acid (c-di-GMP), was identified (Ross *et al.*, 1987). Fragments of the AcsB protein reversibly bind c-di-GMP and are localized alongside the AcsA protein embedded in the cytoplasmic membrane (Kimura, Chen, Saxena, Brown Jr. & Itoh, 2001). The gene product does not directly result in a protein that binds to c-di-GMP, but instead a protein with a mass of 67 kDa binds to c-di-GMP (Mayer *et al.*, 1991). In addition, Weinhouse *et al*. discovered a second protein that binds c-di-GMP that is distinct from AcsB (Weinhouse *et al.*, 1997). The protein may be degraded into the 67 kDa protein or the protein may function to direct c-di-GMP to the AcsB protein. This protein is loosely associated with cellulose synthase (AcsA) and binds tightly to c-di-GMP in the presence of K^+ ions (Weinhouse *et al.*, 1997). The potassium ions have a positive charge and may also cause a charge induced change in the protein causing it to bind tightly to c-di-GMP. The effect observed for K+ is specific for that ion. This is significant because the presence of K+ appears to reduce the availability of c-di-GMP for cellulose synthase activation since exogenous, unbound c-di-GMP is unaffected by K+. Potassium ions have been known to inhibit cellulose biosynthesis by dissipating the transmembrane potential (Delmer, Benziman & Padan, 1982). The change in potential caused by potassium ions may disrupt cellulose export out of the cell inhibiting cellulose biosynthesis.

The function of the a*csC* and *acsD* genes is not clear (Wong *et al.*, 1990). Saxena *et al*. suggests that *acsC* has homology with a pore forming protein that may

form pores to allow cellulose to be secreted (Saxena, Kudlicka, Okuda & Brown Jr., 1994). The *acsD* gene causes *G. xylinus* to make cellulose II instead of cellulose I when disrupted (Saxena, Kudlicka, Okuda & Brown Jr., 1994). Cellulose microfibrils have a unidirectional polarity where cellulose I is described as having a parallel arrangement of cellulose microfibrils and cellulose II has an anti-parallel arrangement of cellulose microfibrils (Haigler, 1985).

There are two other genes upstream of the operon *acsA-D* which affects cellulose biosynthesis (Standal *et al.*, 1994). One gene encodes a cellulase and the other is a proline rich protein of unknown function. The latter protein is required for cellulose biosynthesis *in vivo*, but not *in vitro* (Standal *et al.*, 1994). The proposed function of the cellulase in cellulose biosynthesis is that the cellulase is used in chain termination or when the cellulose microfibrils deviate from the cellulose structure (Delmer, 1999). A disorganized chain would be a chain that does not conform to the highly structured organization of hydrogen bonded cellulose chains linked together into sheets and then microfibrils. It is hypothesized the cellulase functions essentially as an editor/terminator (Delmer, 1999).

An important feature of cellulose biosynthetic activity in *G. xylinus* is its allosteric activator, c-di-GMP, which was first identified by Ross *et al.* (1987). The cellulose synthase protein AcsA is allosterically activated by c-di-GMP (Ross *et al.*, 1987). In the cell 90% of the c-di-GMP is bound to the c-di-GMP binding protein AcsB (Kimura, Chen, Saxena, Brown Jr. & Itoh, 2001). The bound c-di-GMP is

released by the AcsB fragment to the cellulose synthase AcsA (Weinhouse *et al*., 1997). It is believed the close spatial arrangement of the AcsB protein and the cellulose synthase allows the release of c-di-GMP to the cellulose synthase (Weinhouse *et al.*, 1997). The proportion of bound and unbound c-di-GMP is modulated by the intracellular potassium concentration (Weinhouse *et al*., 1997). The cellulose synthase remains in an inactive or a low activity form until c-di-GMP activates the enzyme (Ross *et al.*, 1987). *In vitro*, the addition of submicromolar amounts causes cellulose production to increase by 200-fold achieving a cellulose biosynthesis rate that is half that of *in vivo* rates in *G. xylinus* (Ross *et al.*, 1985).

Production of c-di-GMP is regulated by two enzymes; diguanylate cyclase and a phosphodiesterase. Diguanylate cyclase is either a membrane bound protein or cytoplasmic protein which synthesizes the c-di-GMP from two molecules of pppGTP (Bochner & Ames, 1982). Phosphodiesterase A works to degrade c-di-GMP by cleaving c-di-GMP to pGpG (Ross *et al.*, 1987). Calcium cations inhibit the action of phosphodiesterase A, which suggests some sort of regulatory role through interaction with the cyclic dinucleotide (Ross *et al.*, 1987). Another phosphodiesterase, phosphodiesterase B, works to cleave pGpG to 5'GMP, which regenerates the pool of precursors to resynthesize c-di-GMP (Ross *et al.*, 1987).

Genes encoding the phosphodiesterase A (*pdeA*) and diguanylate cyclase gene (*dgc*) enzymes used to synthesize and degrade c-di-GMP, respectively, are located on three homologous operons named cyclic diguanylate (*cdg) (cdg1, cdg2, cdg3)* (Tal *et*

al., 1998). The structure of the *cdg* operon is described as having a *pdeA* gene upstream of the *dgc* gene (Tal *et al*., 1998). The operon also contains two flanking regions, *cdg1d*, which has no known function and *cdg1a*, which encodes a putative transcription factor that is regulated by oxygen (Tal *et al*., 1998). The N-terminus of the Dgc and PdeA proteins contained putative oxygen sensing domains (Tal *et al*., 1998). Both *dgc* and *pdeA* control the turnover of c-di-GMP which is an allosteric activator of cellulose biosynthesis (Ross *et al*., 1985).

1.2 Objectives

The use of chemical inhibitors to study complex processes is a widely used technique in biology. Screening of a 10,000-compound chemical library identified one compound (CBI28) that specifically affects cellulose synthesis, but not growth in *G. xylinus* (D. Bonetta, personal communication). It is unclear whether CBI28 action is against cellulose synthase itself or some other protein involved in cellulose synthesis. The primary objective of the research outlined in this thesis was therefore to gain a better understanding of the mode of action of CBI28 on cellulose biosynthesis. A number of different approaches were employed to achieve this goal. First, the genetic approach made use of forward genetics to screen for *G. xylinus* mutants that were resistant to CBI28. Since high levels of resistances often identify the target protein of an inhibitor, the hope was to identify the CBI28 target by isolating and characterizing CBI28-resistant mutations. A second approach was to tag CBI28 with biotin so that this version of the CBI28-Biotin conjugate could be used to affinity purify CBI28 target

proteins from *G. xylinus* extracts and precipitate them through affinity column precipitation. A third and final approach was aimed at characterizing if the active form of CBI28 needs to be metabolized by *G. xylinus* suggested by the experiment performed by Bonetta and Strap (Personal communication, 2008) which suggested that CBI28 may be metabolized to an active form (Bonetta & Strap, Personal communication, 2008). To find possible metabolites of CBI28, extracts of cell lysates were separated using chromatography techniques.

Figure 1.1. The Structure of CBI28 Molecule

CBI28 molecule is characterized by its high degree of conjugation, two benzene groups, a dioxin like portion and the carbonyl group. The carbon-carbon double bond is the major site for chemical reactions since benzene molecules are very stable. The compound was identified by screening 10 000 compounds for their ability to inhibit cellulose biosynthesis in G.xylinus using the Updegraff method.(Chembridge, San Diego, CA, 2008).

Chapter 2: Materials and Methods

2.1 Strains, Media and Growth Conditions

All reagents were purchased from Bioshop (Burlington, ON) unless otherwise specified.

The *Gluconacetobacter xylinus* strain used in this study is from the American Type Cell Culture collection (ATCC 53582). *G. xylinus* bacteria were cultured at 30^{o}C in a shaking incubator at 150 rpm in a Schramm-Hestrin (SH) medium which consists of glucose (20 g), peptone (5 g), yeast extract (5 g), Na_2HPO_4 (2.7 g), and citric acid (1.5 g) in one litre of H_2O (Hestrin & Schramm, 1954). The media was then sterilized in the autoclave for 30 minutes. No hydrated reagents were used.

The Minimal A Buffer (Min A) (10.5 g K_2HPO_4, 4.5 g of KH_2PO_4, 1 g of $(NH4)_2SO_4$ and 0.5 g of sodium citrate in 1 litre of distilled water) was made and autoclaved for 30 minute. This buffer was used to keep cells in an environment that was isotonically stable for Ethyl MethaneSulphonate (EMS) mutagenesis. No hydrated reagents were used.

Phosphate Buffered Saline Buffer (PBS) (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2) was used as the solvent for the biotin hydrazide conjugation.

2.2 Identification of CBI28 (Previously Performed Work)

The bacterial cellulose biosynthesis inhibitor (CBI28) was identified by D. Bonetta who screened a 10, 000-compounds chemical library from Chembridge (Chembridge Corporation, San Diego, CA). Bacterial cells were incubated at 28^{o}C for

five days until a cellulose pellicle was observed in the majority of wells (D. Bonetta, personal communication). Compounds that prevented pellicle formation but not cell growth were then retested (D. Bonetta, personal communication). Those compounds that inhibited cellulose synthesis were validated by measuring cellulose according to Updegraff (1969). The Updegraff method consists of combining cellulose fibers with acetic and nitric acid (Updegraff, 1969). The remaining cellulose is then allowed to react with anthrone and sulfuric acid and the resulting coloured compound is assayed spectrophotometrically at a wavelength of 635 nm (Updegraff, 1969). The major limitation of the Updegraff method is that is cannot be scaled up and the extensive use of high concentration sulfuric acid. As controls, *G. xylinus* cells were grown in liquid media containing 0.1% cellulase (Sigma, St.Louis, MO). Once the screening was performed individual chemicals that inhibit cellulose biosynthesis could be identified (D. Bonetta, personal communication). Identification numbers from Chembridge allowed identification of chemicals and their structures (D. Bonetta, personal communication). According to International Union of Pure and Applied Chemists (IUPAC) conventions CBI28 is (E)-3-phenyl-1-(2, 3, 4, 5-tetrahydrobenzo[*b*] [1, 4] dioxocin-8-yl) prop-2-en-1one.

2.3 Ethyl methanesulfonate Mutagenesis

In order to establish a mortality curve for ethyl methanesulfonate (EMS) mutagenesis, 50 mL of *G. xylinus* cells were cultured for five days at 30^{o} C shaking at 150 rpm to a density of 2.0×10^{8} cells/mL. Cells were counted using a

haemocytometer. Cellulose was then removed by adding 0.1 % cellulase (w/v) for 16 hours (Sigma: *Trichoderma Reesei,* St. Louis, MO). The cells were then centrifuged at 3700 rpm for 10 minutes. The supernatant was discarded and the cells then washed twice in sterile Min A buffer. The cells were resuspended in 2 mL of sterile Min A buffer and 30 µL of EMS (Sigma) was added (0.015%).

The cells were then incubated in EMS while shaking at 50 rpm at 30^{o}C including time equal to zero to construct the mortality curve. The time of incubation in EMS was optimized to determine at what time 50% of the population of bacteria died (time = 0, 5, 15, 30, 45, 60 min) since 50% cell mortality allows for sufficient mutagenesis and sufficient bacterial survival. Thus 50% mortality is an optimal ratio for mutagenesis and efficiency of screening. This is a mortality percentage that balances both mutagenesis and practical screening. Bacterial colonies were spread plated on SH agar plates for three days at 30^{o}C and then counted under a dissecting microscope using a clicker for each bacterial colony, to determine 50% population death. The cells were spread plated undiluted and at a 1:20 dilution. There were 12 plates where every time and dilution was represented on a plate. Once the colonies were counted from the plate, the numbers of colonies were tabulated and the mortality curve was constructed. The time that represented 50% population death was used as a guideline to perform the experimental screen for CBI28 resistant mutants. The time in the mortality curve was a guideline for incubation in EMS and the curve does not represent a lethal dose curve. There is no other data for statistical analysis because replicates were not performed. The mortality curve represented the amount of

colonies that survived after mutagenesis and handling on the SH Agar plates. It did not represent the original cell density of 2.0 x 10^8 cells/ mL that was started with since there was a processing time involved which contributed to further exposure to EMS and cell death. EMS was added at time zero to accommodate for the processing time which resulted in extended exposure to EMS for all samples.

To isolate mutants, cells were grown and cellulase was added as mentioned above. 50 mL of *G. xylinus* cells were cultured for five days at 30^{o}C shaking at 150 rpm to a density of 2.0 x 10^8 cells/ml. Cells were counted using a haemocytometer. Cellulose was then removed by adding 0.1 % cellulase (w/v) for 16 hours (Sigma: *Trichoderma Reesei,* St.Louis, MO). The cells were then centrifuged at 3700 rpm for 10 minutes. The supernatant was discarded and the cells then washed twice in sterile Min A buffer. The cells were resuspended in 2 mL of sterile Min A buffer and 30 µL of EMS (Sigma) was added. The cells were then incubated, shaking at 50 rpm at 30^{o}C for 41 minutes. These were centrifuged as before and washed twice in Min A buffer containing 1 M sodium thiosulfate to neutralize the EMS. All EMS waste was treated with 1 M NaOH to inactivate the EMS and sterilize the waste. The cells were then resuspended in 2 mL of Min A buffer and 100 µL of cells were spread plated at a 1:20 dilution and with no dilution on SH 2% (w/v) agar (Bioshop, Burlington) plates - which were made by adding agar (Bioshop, Burlington, ON) to SH liquid media. The plates were then incubated for three days at 30^{o}C.

G. xylinus cells that survived EMS mutagenesis and grew into colonies on the plate were individually inspected and counted. Cells that had been incubated in EMS to

50% survival were used to identify putative CBI28-resistant mutants through screening on SH plates supplemented with CBI28. CBI28 exhibits cellulose biosynthesis inhibition at 0.5 µM but 2.5 µM was used in screening to increase the chance of finding a resistant mutant (Bonetta, personal communication, 2007). In total 359,085 *G. xylinus* colonies were screened and visually inspected for cellulose production in the presence of 2.5 µM CBI28 on SH-agar plates. A dissecting microscope was used to visually screen for positive hits. Positive hits were defined as a round colony with cellulose fibrils emanating from its edges. Mutants were then patched onto varying concentrations of CBI28 (2.5, 5, 10 and 20 µM). These plates were also incubated for three days at 30^{o} C and were visually inspected.

2.4 CBI28 Biotin Conjugations Using the EZ-Link Biotin Hydrazide Kit

Biotin conjugation to CBI28 was performed using the Pierce EZ-Link Biotin Hydrazide Kit (Pierce, Thermo Scientific, Rockford, IL). The original protocol was changed because it was designed for labelling proteins. The original manufacturer's protocol for the EZ-Link Biotin Hydrazide Kit is described below.

The original protocol describes the addition of 1 mL of 20 mM, cold sodium meta-periodate solution to a cold protein mixture, mixing well and allowing oxidation to occur for 30 minutes at 4^{o} C. Glycerol is then added to a final concentration of 15 mM, mixed and incubated for five minutes on ice at 4^{o} C to stop the oxidation. The samples are then dialyzed overnight against a coupling buffer or a desalting column. This protocol was modified by removing periodate from the reaction because it may sheer the CBI28 molecule and eliminating all steps associated with periodate as well as

eliminating the dialysis for purifying proteins (K. Paal, personal communication). The biotin hydrazide solution is then added at a concentration of 5 mM and mixed for two hours at room temperature (21^{o}C) in the original protocol. The protocol was then modified to adding 100 µL 5 mM stock biotin hydrazide and 100 µL of 1 mM CBI28 in 800 µL of PBS buffer to make the final volume 1 mL (K. Paal, personal communication). The reaction was allowed to proceed at room temperature (21^{o}C) with vigorous shaking (K. Paal, personal communication). The reaction was also performed at 30^{o}C since increased temperature may cause increased reaction rate. In the original protocol, biotinylated molecules are then separated from non-reacted materials by dialysis or gel filtration. Since there were no proteins involved, once the reaction was complete, products were extracted from solvents and buffers to facilitate analysis by Mass Spectrometry (MS) and Thin Layer Chromatography (TLC) as follows.

All reaction mixtures were then extracted and purified using the Waters Oasis Solid Phase Extraction Cartridges (SPE Paks) using the manufacturer's protocols (Waters Corporation, Milford, MA). Briefly, 1 mL of methanol was applied to the SPE-Pak, followed by 1 mL of water, 1 mL of sample, 1 mL of 5% methanol, 1 mL of water and then the final elution with 1 mL methanol (Waters Corporation, 2008). The methanol was then evaporated in a fume hood overnight at room temperature and the remaining sample was then reconstituted in 600 µL of HPLC grade methanol. Several SPE Paks were used to optimize the method including: Hydrophilic-Lipophilic Balance (HLB), Cyanopropyl, C18, amino propyl, and diol. Samples in methanol were then separated with Capillary Electrophoresis-Mass Spectrometer (CE-MS) or Ultra

Pressure/Performance Liquid Chromatography-Mass Spectrometer (UPLC-MS) systems.

2.5 Elution of CBI28 Using the Capillary Electrophoresis-Mass Spectrometry/ Photo Diode Array

Capillary Electrophoresis separates electrically charged analytes in a conductive solution under the influence of an electric field by their charge, frictional forces and mass (Skoog, Holler & Crouch, 2007). The analytes are separated by the way they interact with the capillary and their electrophoretic mobility (Skoog, Holler & Crouch, 2007).

Capillary Electrophoresis analysis was performed on the reaction mixtures of the biotin hydrazide CBI28 conjugation reaction. The apparatus used was the Agilent 1200 Capillary Electrophoresis/ Liquid Chromatography /Mass Spectrometry Detection (CE/LC/MSD) system (Agilent, Mississauga, ON) equipped with an auto sampler, a Quadropole Mass Spectrometer (MS) (Agilent, Mississauga, ON) and a Photo Diode Array (PDA) (Agilent, Mississauga, ON) equipped with an electrospray ionization interface run at positive mode at 4.0 kV. The capillary used was a 125 cm fused-silica capillary (Part No. G1600-67311, Agilent Technologies, DE, Germany) 50 µm i.d. connected to the electrospray ionization interface of the MS (Agilent, Mississauga, ON).

The elution method was taken from Baldacci *et al.* (2004) as a method for oxycodone elution since oxycodone is similar in size to CBI28, oxycodone was available and there are no previous studies on CBI28 elution. Nitrogen gas was the

sheath gas and the sheath liquid consisted of 50:50 methanol: ammonium acetate buffer (v/v) with 1% formic acid to aid in positive ionization (Baldacci, Caslavska, Wey & Thormann, 2004). Samples were run using 25 mM ammonium acetate buffer pH 8 and electrokinetically injected for 20 s using a positive voltage of 10 kV (Baldacci, Caslavska, Wey & Thormann, 2004). Electrokinetic injection was performed by using an electric field to draw charged particles into the capillary (Agilent, 2001). Spectra were collected for the Total Ion Content (TIC) in the mass range of 100 m/z to 700 m/z. The range of the MS was sufficient to detect the mass to charge ratio of the reactants (CBI28 and biotin) and putative products of the reaction.

The Capillary Electrophoresis-Mass Spectrometry /Photo Diode Array (CE-MS/PDA) was used to elute and characterize CBI28, biotin and a possible chemical conjugate of the two. CBI28 and Biotin Hydrazide controls were eluted in the CE-MS/PDA. There was no solvent control peak because MS and PDA detectors were not turned on until three minutes had elapsed. The lapse in time between when the detector was turned on to detect analytes was so that the solvent can run through the capillary and not be detected because solvents peaks come off very early and are very large with respect to the analyte peaks resulting in the scaling of the detector being skewed and unable to detect the actual analytes. All compounds were eluted using a CE-MS method for biotin as described above. The capillary was first washed using 0.5 mL of 1M NaOH and then 0.5 mL of 0.1 M NaOH to remove any contaminants in the run while continuously monitoring the MS and PDA to ensure that analytes were not being lost in the washes (Baldacci, Caslavska, Wey & Thormann, 2004). Agilent

Chemstation was used to manage the method and for analysis of electropherograms.

2.6 Elution of CBI28 on the Ultra Performance Liquid Chromatography-Mass Spectrometry/Photo Diode Array

The Ultra Performance Liquid Chromatography (UPLC) system used in this study was a Waters Acquity UPLC with a PDA and Quadrupole MS (Waters Corporation, Milford, MA). The UPLC system was used because the CE-MS system had maintenance issues and a method for elution on the UPLC could be developed. The column used was a Waters Acquity BEH C18 column (Part No. 186004045). Caffeine was used as a standard to assess column integrity since caffeine is readily available, inexpensive and there is extensive literature available on caffeine elution (M. Allison, personal communication). Caffeine elution was performed by starting the Waters Acquity BEH C18 column at a flow rate of 0.4 mL/min and 12:88 acetonitrile: water at 1.20 min, increasing to 40:60 (v/v) acetonitrile: water at 1.60 min and holding at 40:60 (v/v) acetonitrile: water at 2.00 min and finally decreasing to 12:88 (v/v) acetonitrile: water at 4.00 min (M. Allison, personal communication).

Stock samples of 10 µM CBI28 were initially made in dimethylsulfoxide (DMSO). The DMSO was removed by evaporation using an evaporator and suspended in methanol. CBI28 was extracted using the HLB SPE-Pak as per the instructions provided by Waters (Waters Corporation, 2008). The HLB SPE-Pak was conditioned with 1 mL of methanol. Then 1 mL of water and 50 µL of 10 mM CBI28 were added. The sample was washed with 1 mL of 5% methanol and water and the final elution was performed by adding 1 mL of methanol (Waters Corporation, 2008). The methanol was evaporated overnight in a fumehood at room temperature. Samples were then

resuspended in 600 µL of HPLC grade methanol and eluted on the UPLC-MS. The solvent peaks usually elute very quickly and the detector is not activated for the first three minutes since solvent peaks skew scaling due to the size of the solvent peak resulting in analyte peaks that appear as noise because of the skewed scaling.

The elution method developed for CBI28 was done by starting the column at 10:90 (v/v) methanol: ammonium acetate buffer and increasing the gradient to 90:10 (v/v) methanol: ammonium acetate buffer over 10 min, holding 90:10 (v/v) methanol: ammonium acetate buffer for 5 min and then decreasing the gradient to 10:90 (v/v) methanol: ammonium acetate buffer for 5 min (M. Allison, personal communication). This method was developed to elute small hydrophobic compounds with run times and methanol proportions that are in excess since retention times and parameters for CBI28 were previously unknown. This elution method was used to test different mobile phases to provide the optimal conditions for CBI28 elution.

The elution of CBI28 was performed in various mobile phases such as acetonitrile/water, methanol/water, methanol/ammonium acetate pH 4, methanol/ammonium acetate pH 8. The same method was used for all mobile phase systems below. Methanol and 10 mM ammonium acetate pH 8 were used for subsequent metabolite analysis.

The mass spectrometer was optimized for CBI28 by infusing CBI28 at 10 ppm using the MassLynx software provided by Waters (Waters Corporation, Milford, MA). For the analysis of CBI28, the mass spectrometer was set to Single Ion Reaction (SIR) for increased sensitivity and specificity to the mass to charge ratio (m/z) of CBI28. For CBI28 analysis, the PDA was set to a wavelength of 255 nm for analyte detection.

2.7 Extraction of Metabolites from *Gluconacetobacter xylinus*

An extraction was performed to extract metabolites of CBI28 to determine if one of the metabolites is the active form of CBI28 which causes cellulose inhibition. Metabolites have to be extracted so the metabolites can be detected and further characterized. Extraction of CBI28-induced metabolites was performed by incubating *G. xylinus* in 50 mL of SH medium supplemented with 10 µM CBI28 for five days until the cells reached a density of ~ 1.0 x 10^9 cells/mL. Cells were counted using a haemocytometer and a 10 µM dose of CBI28 is not lethal to cells. There is no difference in growth rates of cells with exposure to CBI28 except that CBI28 causes cellulose inhibition. A control was set up that lacked CBI28 and was also incubated for five days using the same conditions mentioned in Section 2.1. Control cells were treated with 0.1% cellulase (w/v) or 60 µg/ml of cellulase to degrade cellulose and release cells (Setyawati, Chien & Lee , 2009). Both CBI28-treated and untreated samples were centrifuged at 3750 rpm for 10 min. The supernatant was decanted and the cell pellet retained and washed twice in sterile double deionized water with centrifugation done as before retaining the cell pellet after every wash. The cells were then resuspended in 10 ml of sterile double deionized water. The samples were then placed in a French Press to lyse the cells. Cells were passed through the French Press twice on medium pressure (~22 000 psi). The cell lysates were then collected, washed twice, and two extractions were performed. Either a methanol extraction or a HLB SPE-Pak extraction was performed on the samples to extract metabolites from cell lysates. The two extraction methods are described below.

The methanol extraction was performed by adding 10 mL of methanol and vortexing the samples to allow mixing. The mixture was allowed to incubate for 10 minutes and then centrifuged as before to remove cellular debris. The supernatant was decanted and retained. The supernatant was evaporated in a fume hood overnight at room temperature. The second extraction method made use of solid phase extraction with Waters Oasis HLB SPE-Paks as previously described.

All extracted samples were lypholized using a freeze dryer. Samples used for analysis were reconstituted in 600 µL of HPLC grade methanol.

In total, the samples generated were a 10 µM CBI28 with HLB extraction, a 10 µM CBI28 methanol extraction, a control HLB extraction in the absence of CBI28, a control methanol extraction with no CBI28 added. There were 4 equivalent replicates made for all samples listed. There was no solvent control due to the time delay for the detector to turn on and the range of the detector. The time delay was present to prevent solvent peaks from skewing scaling and obscuring analyte peaks since solvent peaks come off very quickly and are usually very large in relation to the analyte peaks.

2.8 Elution of CBI28-Induced Metabolites from *Gluconacetobacter xylinus*

CBI28 induced metabolites were eluted so metabolites could be separated from other cellular metabolites and characterized. The metabolites of CBI28 were eluted using the method that was previously developed for CBI28 (Section 2.6). This consisted of starting with a 10:90 (v/v) methanol/ammonium acetate mixture which was increased to 90:10 (v/v) methanol/ammonium acetate in 10 min (4mL column volume), held at 90:10 (v/v) methanol/ammonium acetate for 5 min (2 mL column volume) and

followed by decreasing to 10:90 (v/v) methanol/ammonium acetate for 5 min (2 mL column volume). The flow rate was 0.4 mL/min over all runs. Analysis of the samples was performed with the PDA set at 255 nm to detect analytes and another run was performed increasing the range of the PDA to 200-800 nm. The mass spectrometer had additional argon filters added to filter out noise from the carrier gas and the total ion count (TIC) was used to survey masses between 50-500 m/z. Replicates were performed by repeating the same method with other samples.

Chapter 3: Results

3.1 *Gluconacetobacter xylinus* Mutant Screen Using CBI28

It is often possible to identify the primary targets of chemical inhibitors by identifying mutations which confer a high level of resistance in the test organism. With this goal in mind, EMS mutagenesis was utilized to generate mutagenized *G. xylinus* cells that were screened for CBI28-resistance. To do this, it was first necessary to optimize the time of EMS incubation to ensure that sufficient and efficient mutagenesis had occurred. Typically, about 50% survival of bacteria is chosen. The time cells are incubated in EMS is determined empirically through a mortality curve. Fig 3.1 shows the mortality curve that was generated and the point at which 50% of the population died (41 minutes).

In total, 359 085 mutagenized *G. xylinus* colonies were screened and 19 putative mutants were identified that were capable of producing cellulose on 2.5 µM CBI28. The putative mutants were capable of producing cellulose on 2.5 µM CBI28 and appeared as round, dark colonies with cellulose microfibrils emanating from the centre. The mutants were retested on varying concentrations of CBI28 and 7 mutants maintained cellulose biosynthesis in the presence of 5 µM CBI28, 4 positive on 10 µM CBI28 and 1 retested positive on 20 µM CBI28. All the mutants retested positive on 2.5 µM CBI28. Putative mutants become unrecoverable throughout the screening process leading to discrepancies in reported numbers due to cell death at varying times.

The varying degree of resistance may be due to the nature of the mutations for instance, a mutation in a gene responsible for the metabolism of CBI28, mutations on

accessory proteins or mutations to the active site of the enzyme target. However, the mutants were not stored properly and so could not be further characterized.

3.2 CBI28 Conjugation to Biotin

Another method to identify CBI28 cellular targets is by affinity purification. Since affinity purification using biotin is well established, an attempt to conjugate CBI28 to biotin was made. If successful, the hybrid molecule would then be tested to ensure that it had retained activity *in vivo*. To do this, an EZ-Link Biotin Hydrazide Kit from Thermo Scientific was used. As mentioned in the materials and methods the conjugation protocol originally developed with the EZ-Link Biotin Hydrazide kit was extensively modified to accommodate the use of CBI28 instead of proteins. Reactants were characterized on the CE-MS initially and then the reaction mixture was characterized to determine if any products were formed.

The first phase of determining if a novel compound was synthesized is to characterize the individual reactants. The data on the biotin hydrazide was available in literature provided by the manufacturer, but that for CBI28 had to be determined. Both the CE-MS/PDA and the UPLC-MS/PDA were both used to elute the reactants of the experiment. Various mobile phases were tested as mentioned in Section 2.6. The most effective mobile phase for elution was methanol/ammonium acetate pH 8. The results consistently showed that Biotin Hydrazide was in excess of CBI28 (Fig 3.2). A soluble form of CBI28 as detected at a mass of 295.1 m/z (Fig. 3.2).Biotin consistently eluted on the CE-MS/PDA and UPLC-MS/PDA (Fig 3.2). The mass of 258.1 m/z was consistent with the mass of the biotin hydrazide and the retention time was consistent

Table 1. Testing CBI28 Resistance on Various Concentrations in Putative CBI28 Resistant Mutants. [1]

The table documents the retesting of putative mutants on CBI28 at 2.5, 5.0, 10.0 and 20.0 µM concentrations. There were originally 19 putative mutants but four perished before retesting occurred. Putative mutant number 1 was most promising and has the strongest resistance to CBI28 across all concentrations tested. Some putative mutants retested inconsistently but all putative mutants subsequently perished due to improper storage techniques or the mutations affected mechanisms related to cell viability.

Putative Mutant	2.5 µM CBI28	5.0 µM CBI28	10.0 µM CBI28	20.0 µM CBI28
1	+	+	+	+
2	+	-	-	-
3	+	-	+	-
4	+	-	+	-
5	+	-	-	-
6	+	+	-	-
7	+	+	-	-
8	+	+	+	-
9	+	-	-	-
10	+	+	-	-
11	+	?	-	-
12	+	?	?	-
13	+	+	-	-
14	+	-	-	-
15	+	+	-	-

[1]Positive (+) represents cellulose production where negative (-) represents no cellulose production. Boxes marked with question marks (?) have phenotypes that were not discernible due to contamination or no growth. There were originally 19 putative mutants but four subsequently were not recoverable before the retest . There were 15 putative mutants retested but 3 tests had no growth which caused aberrant results. All putative mutants perished after the experiments.

with values in literature (Klampfi, Buchberger, & Haddad, 2000). CBI28 was also successfully eluted. A soluble form of CBI28 consistently eluted at ~ 6.36 min on the CE-MS (Fig 3.3). The mass spectrometer detected CBI28 and the PDA characterized the ultraviolet absorption spectra of CBI28 (Fig. 3.4). The PDA used along with the MS on the CE-MS was also useful in obtaining a UV-Vis Spectra of CBI28 at 1.603 min (Fig.3.4). The UV-Vis spectrum of CBI28 has characteristic absorption peaks at 120, 275 and 330 nm (Fig 3.5). The total reaction mixture was injected into the CE-MS after purification in the Waters HLB SPE-Paks.

The mass of the CBI28-Bioton Conjugate was calculated by adding the mass of CBI28 (294.13 u) to the mass of the Biotin Hydrazide (258.1 u). A putative CBI28-Biotin Conjugate was detected at an expected mass of 552 m/z that shouldered with Biotin at 5.203 min (Fig 3.6 and Fig 3.7). The relative amounts were also determined. The ion of 552 m/z was present at 16.9% of the peak, however, when re-tested two months after using the same sample the relative amounts had decreased to 5.6 % the original (Fig 3.7).

3.3 Effects of CBI28 on *Gluconacetobacter xylinus* Metabolites

It is unknown if the active form of CBI28 is CBI28 itself or a metabolite of CBI28. In addition, it is not clear if CBI28 directly or indirectly affects cellulose biosynthesis. To explore these alternatives, CBI28-induced metabolites were measured using liquid chromatography.

The same method of eluting CBI28 on the CE-MS was used with the UPLC-MS. CBI28 was eluted on the UPLC-MS at 9.87 min at concentration of 10 ppm stock

solution (Fig.3.8). The peak at 9.36 min may have been an impurity, a resonance form or an isomer. The peak has an expected mass of ~ 294.96 m/z (Fig. 3.9).

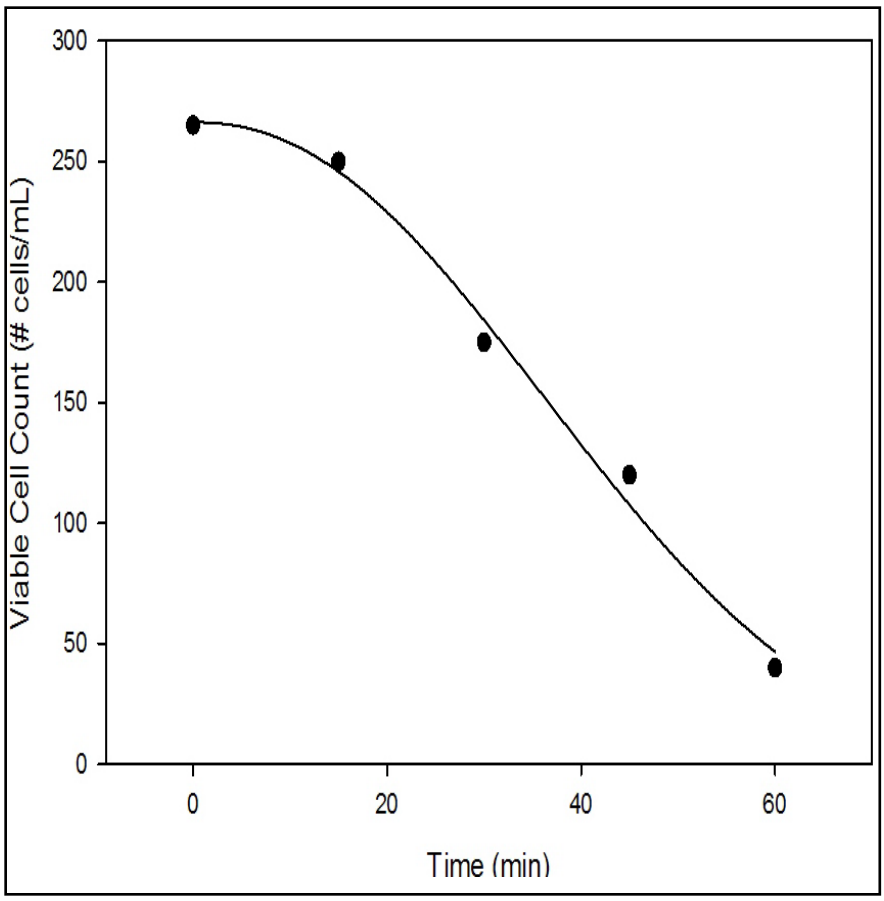


Figure 3.1. Mortality Curve of *G. xylinus* Incubated in EMS for Various Times to Optimize EMS Mutagenesis

The mortality curve represents the amount of *G. xylinus* colonies that survived the timed incubation in EMS. Several times were used to construct a curve which shows that 50% of the cells survived at ~41 min. One sample was completed per time point since it was used as a guideline to provide parameters to perform the EMS mutagenesis. The time of 41 min was used to screen for putative mutants on SM Agar plates supplemented with 2.5 µM CBI28. The graph does not represent the number of cells that were initially grown but the number of cells that survived the mutagenesis on the SM Agar plate. Cells perished due to processing time of samples.

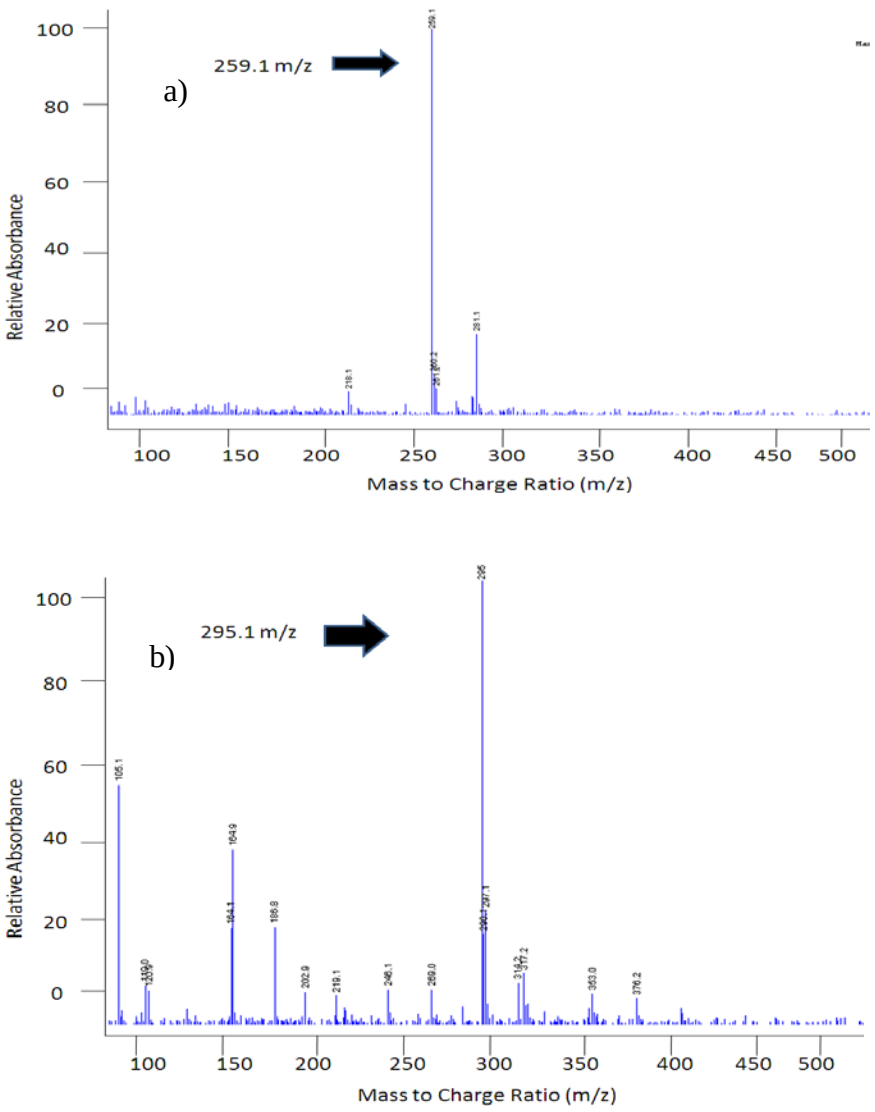


Figure 3.2. CBI28 and Biotin Hydrazide Standards Eluted on the CE-MS

Mass Spectrum of a peak which shows a) the mass for biotin hydrazide (258.1 m/z) at 7.042 min and b) the mass of the soluble form of CBI28 (295.1 m/z) at 9.056 min. This is due to acid hydrolysis of the dioxin like portion since the ether linkages are easily cleaved. Both CBI28 and Biotin were eluted using 25 mM ammonium acetate and methanol as per *Baldacci et al.* (2004). Biotin eluted consistently according to literature (Klampfi, Buchberger, & Haddad, 2000).

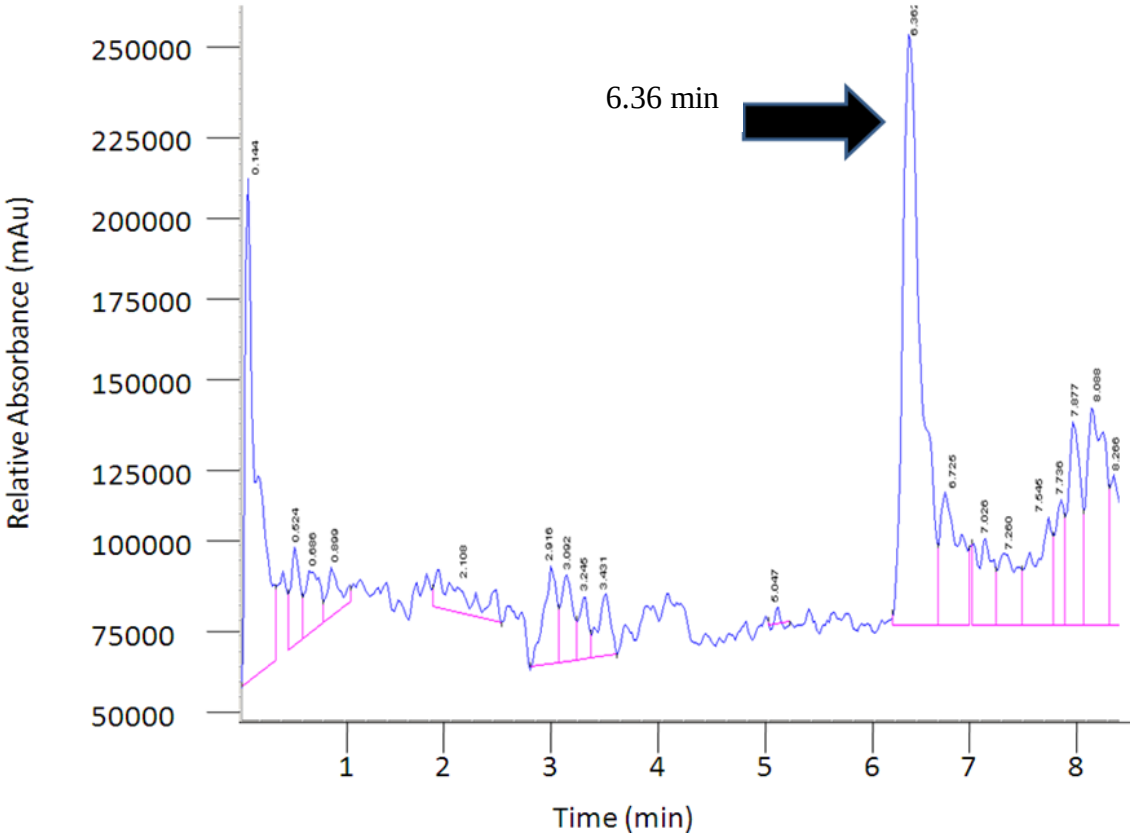


Figure 3.3. Detection of CBI28 on the CE-MS at 6.36 min

The figure represents the electropherogram of CBI28 on the CE-MS with peak at 6.36 min. The first peak is most likely a solvent peak since it comes off the capillary very quickly. Peaks after 6.36 min are due to background signals. CBI28 was eluted using 25 mM ammonium acetate and methanol as per *Baldacci et al.* (2004).

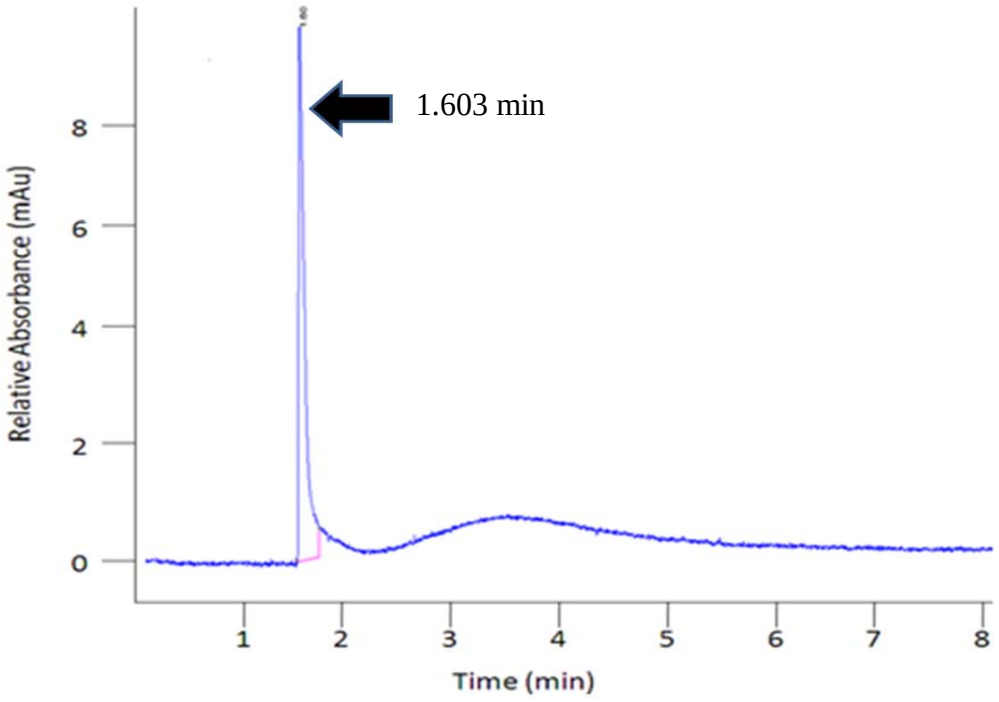


Figure 3.4. Detection of CBI28 on the Diode Array Detector with a Peak at 1.603 min

Detection of CBI28 using a Diode Array Detector - UV detection with peak at 1.603 min. The detection was performed using a detection of 260 nm. CBI28 is quickly detected by the PDA since it uses light to detect analytes and it does not have to travel to reach the detector. Elution was performed using 25 mM ammonium acetate and methanol as per *Baldacci et al.* (2004).

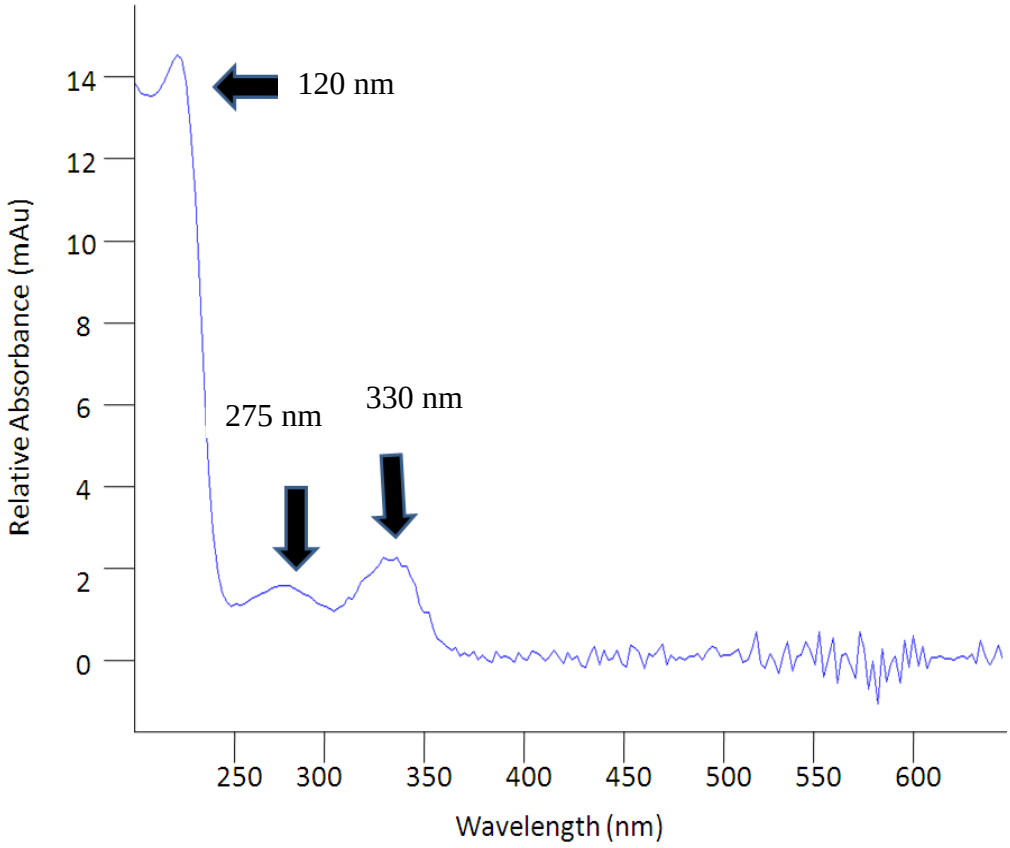


Figure 3.5. UV Spectrum of CBI28 between 200 nm and 800 nm Showing Characteristic Ultra Violet Profile

UV spectrum of CBI28 with characteristic peaks at 120 nm, 275 nm and 330 nm detected using the CE-MS/DAD. CBI28 was dissolved in methanol after extracting CBI28 from DMSO using a HLB SPE-Pak. The sample was then loaded onto the CE-MS/PDA and eluted using 25 mM ammonium acetate and Methanol as per *Baldacci et al.* (2004).

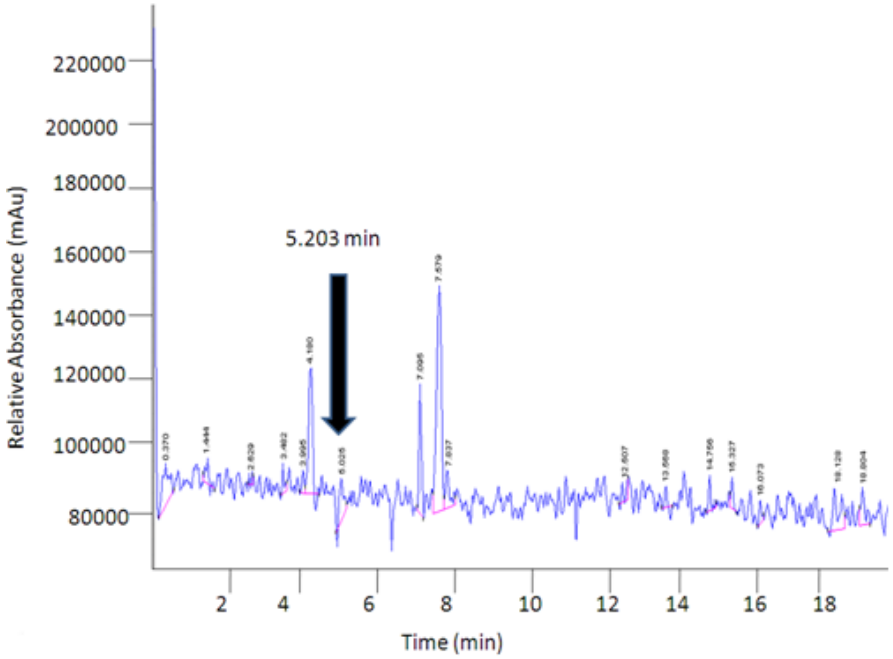


Figure 3.6. Electropherogram of CB28-Biotin Conjugate on the CE-MS Using a Total Ion Count (TIC)

Electropherogram of the CBI28-Biotin Conjugate on the CE-MS which shows a Total Ion Count (TIC) of the reaction mixture on the CE-MS before ion extraction for the mass of 552m/z at 5.203 min. The reaction mixture was eluted using 25 mM ammonium acetate and methanol as per *Baldacci et al.* (2004). The CE-MS revealed that the biotin-CBI28 conjugate was being eluted with biotin as the peaks were very close together resulting in the peaks merging into one peak.

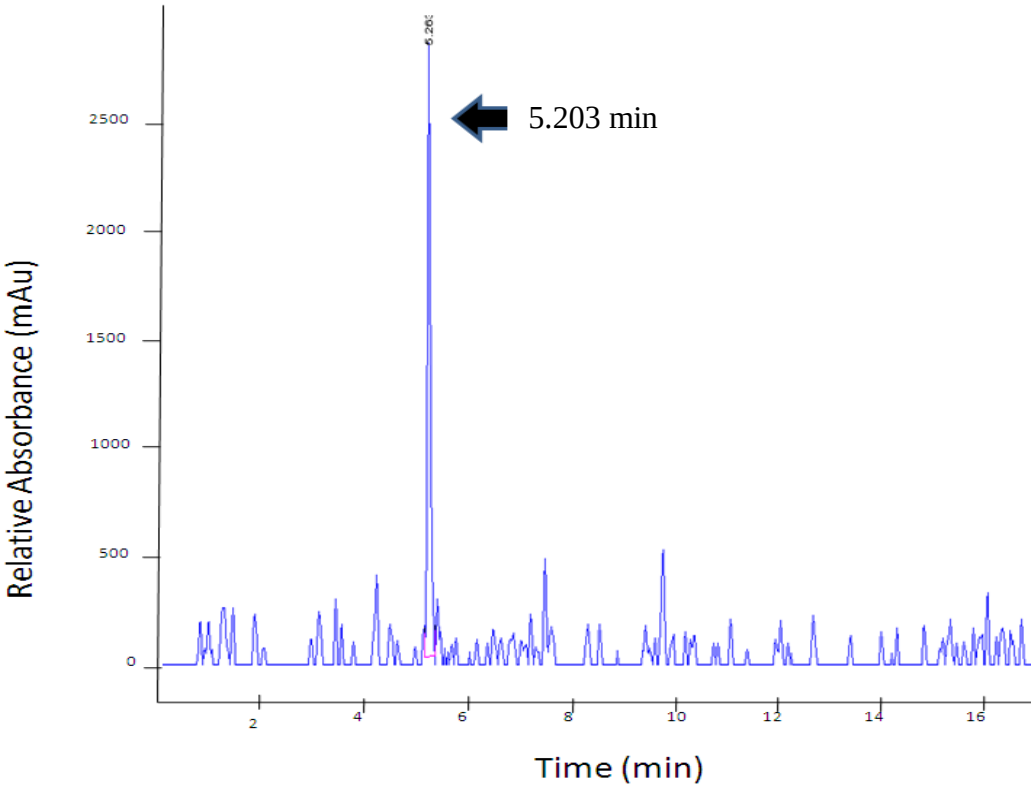


Figure 3.7. Ion Extraction for 552 m/z of CBI28-Biotin Conjugate from the Total Ion Content (TIC)

Ion extraction for 552 m/z (expected size of the CBI28-Biotin conjugate) at 5.203 min was performed using the Agilent CE-MS software. The software allowed for selection and extraction of the 552 m/z ion which allows for the detection of low levels ions. The 552 m/z ion was in low abundance in all replicates with the best replicate had an abundance of 17.2 % but then decreased to 5 % of the peak. The 552 m/z ion was degraded over time due to hydrolysis which makes the ion difficult to recover.

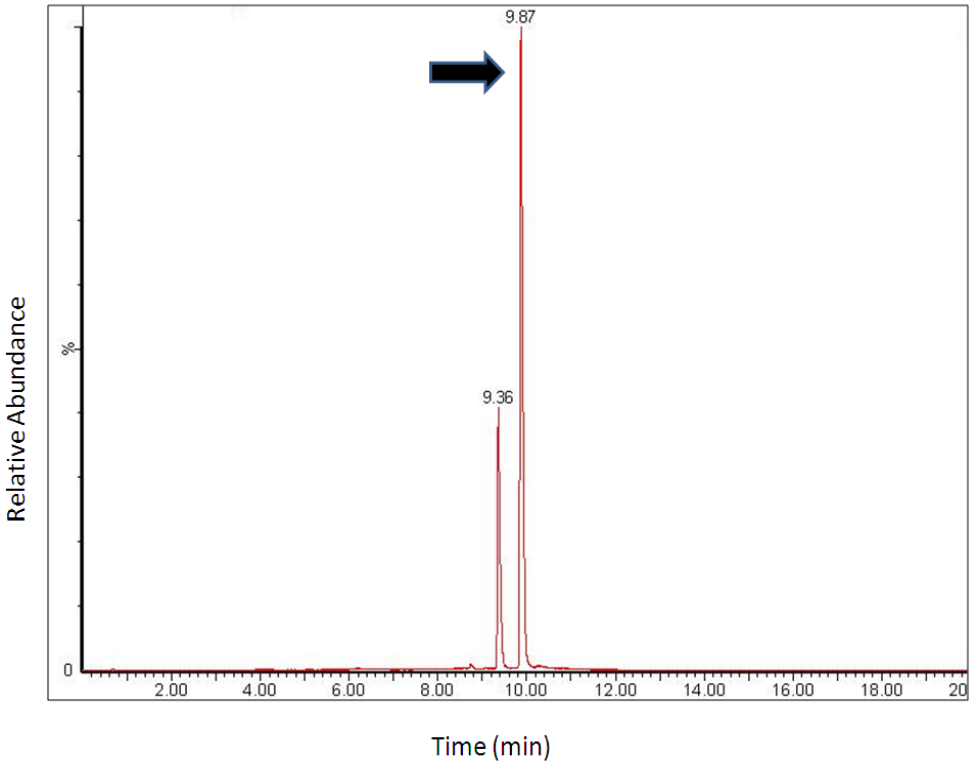


Figure 3.8. Chromatogram of CBI28 on the Ultra Performance Liquid Chromatography Mass Spectrometer with Retention Times of 9.87 min

The chromatogram illustrates the elution of CBI28 on the UPLC-MS. The figure shows the elution of CBI28 in methanol/water at a concentration of 10 ppm. The peak at 9.87 min was the peak that matched the mass of CBI28 (294.13). The other peak at 9.36 min has the same mass of ~ 294.16 m/z and may be a resonance form of CBI28 or an impurity in the sample. CBI28 was extracted using an HLB SPE-Pak and eluted on the UPLC-MS/PDA using an elution method developed to elute hydrophobic molecules with water and methanol. The elution was performed under the Selective Ion Reaction (SIR) setting which selected for the 294 m/z increasing sensitivity, reducing noise and streamlining detection.

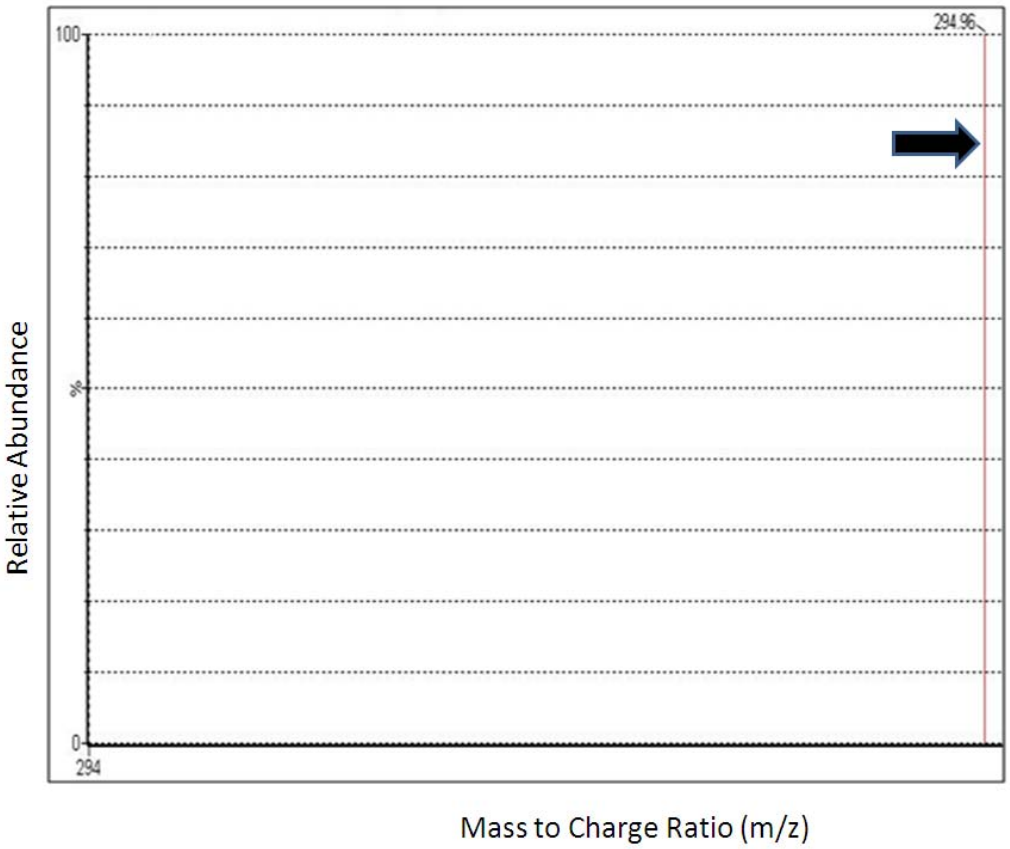


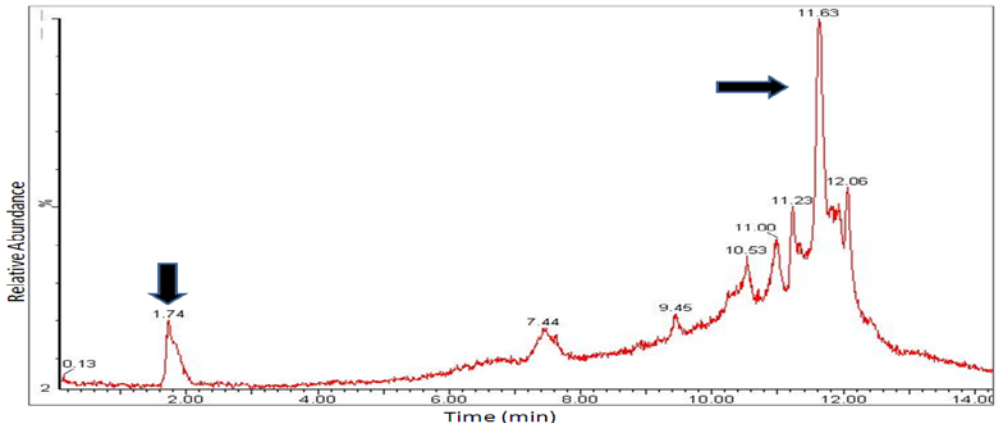
Figure 3.9. Mass Spectrum of Single Reaction Monitoring for 294 m/z which show the Characteristic Mass to Charge Ratio of the Soluble form of CBI28

This mass spectrum shows the mass to charge ratios of ions in the peak at 9.87 min. The mass spectrum clearly shows that there is a m/z = 294. The elution was performed using the Single Ion Reaction setting which only selects for 294 m/z increasing sensitivity, reducing noise and enhancing detection. The range of detection is between 294 m/z and 295 m/z. The ion of CBI28 detected was smaller in size because the soluble for of CBI28 has portions that are cleaved due to hydrolysis of the ether groups. CBI28 was extracted using an HLB SPE-Pak and eluted on the UPLC-MS/PDA using an elution method developed to elute hydrophobic molecules using methanol and water.

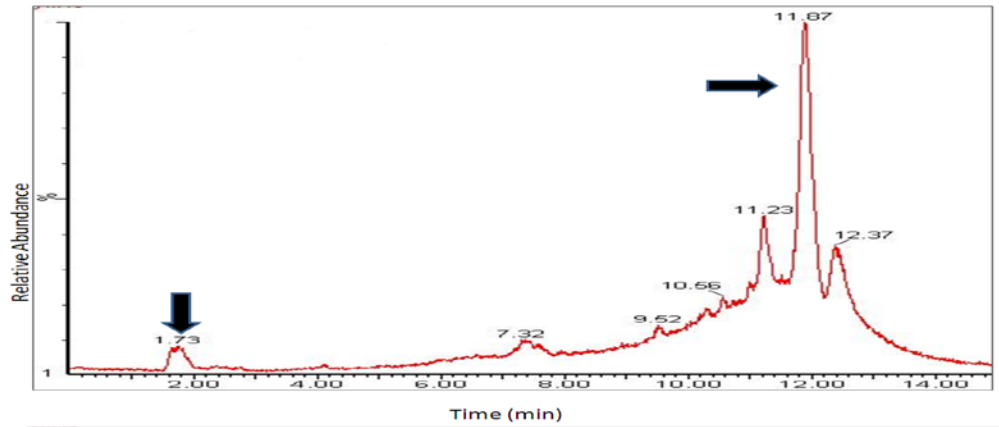
The next phase of the experiment was to determine if any metabolites were detectable. The samples were processed as mentioned above (Section 2.7) and the samples lacking CBI28 were compared to experimental samples that were supplemented with CBI28. There were several masses that occurred in the experimental samples supplemented with CBI28 that were not observed in the control samples lacking CBI28 (Fig 3.10, Appendix 2 - Appendix 4). There were also compositional shifts in some peaks which gave rise to novel masses as compared to control samples (Fig 3.11, 3.12, Appendix 5 – Appendix 9). The two methods of cell lysate extraction were also compared (Appendix 2, Appendix 3, Appendix 4).

The Waters Oasis HLB SPE-Pak and a simple methanol extraction were used to extract any hydrophobic metabolites or derivatives of the CBI28 compound. The chromatograms showed different peaks and background noise for the two different types of metabolite extraction methods but major ions were observed for both extractions methods (Fig. 3.10, Appendix 2, Appendix 3, Appendix 4).The methanol extraction and the HLB-SPE extraction methods yielded ions with a m/z =281.1 (11.791 min) (Fig. 3.11, Appendix 6, Appendix 7). There was a peak at ~1.775 min which was the time of elution of that peak as measured by the UPLC-MS (Fig. 3.10, Appendix 2, Appendix 3, Appendix 4) that was present in all the samples but the compositional masses changed between the controls and the experimental samples (Fig. 3.12, Appendix 8, Appendix 9). There was a mass of ~ 78 m/z which is consistent with the mass of substituted benzene at10.53 min (Fig 3.12, Appendix 8, and Appendix 9).

a



b



c

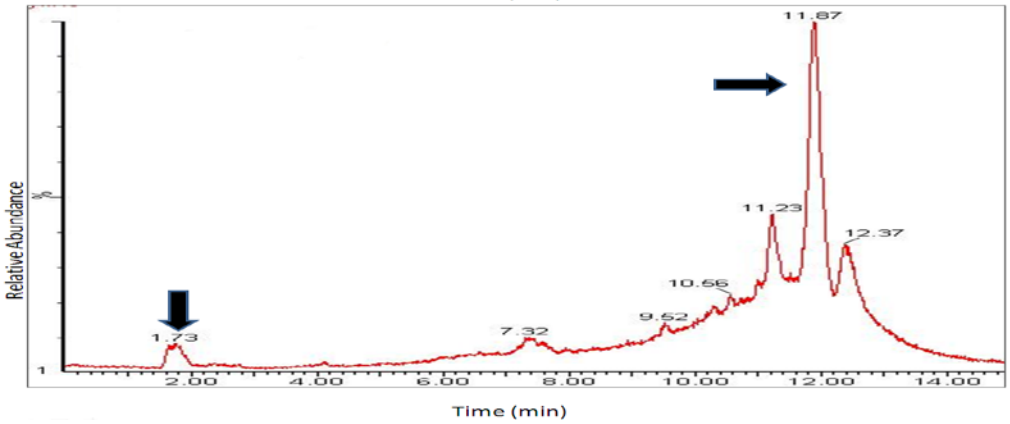


Figure 3.10. Chromatogram of Cell Extracts treated with 10 µM CBI28 using a HLB and Methanol Extraction and in the Absence of 10 µM CBI28

a) The sample in the absence of CBI28 analysis on the UPLC-MS as described in the materials and methods section. b) The chromatogram of the Waters Oasis HLB SPE-Pak Extraction. c) The chromatogram of the methanol extraction. The samples show major peak differences in the 10-13 minute range where numerous compounds eluted. Samples were eluted using the method developed for hydrophobic molecules using 10 mM ammonium acetate and methanol. The MS was set to scan between 50-500 m/z.

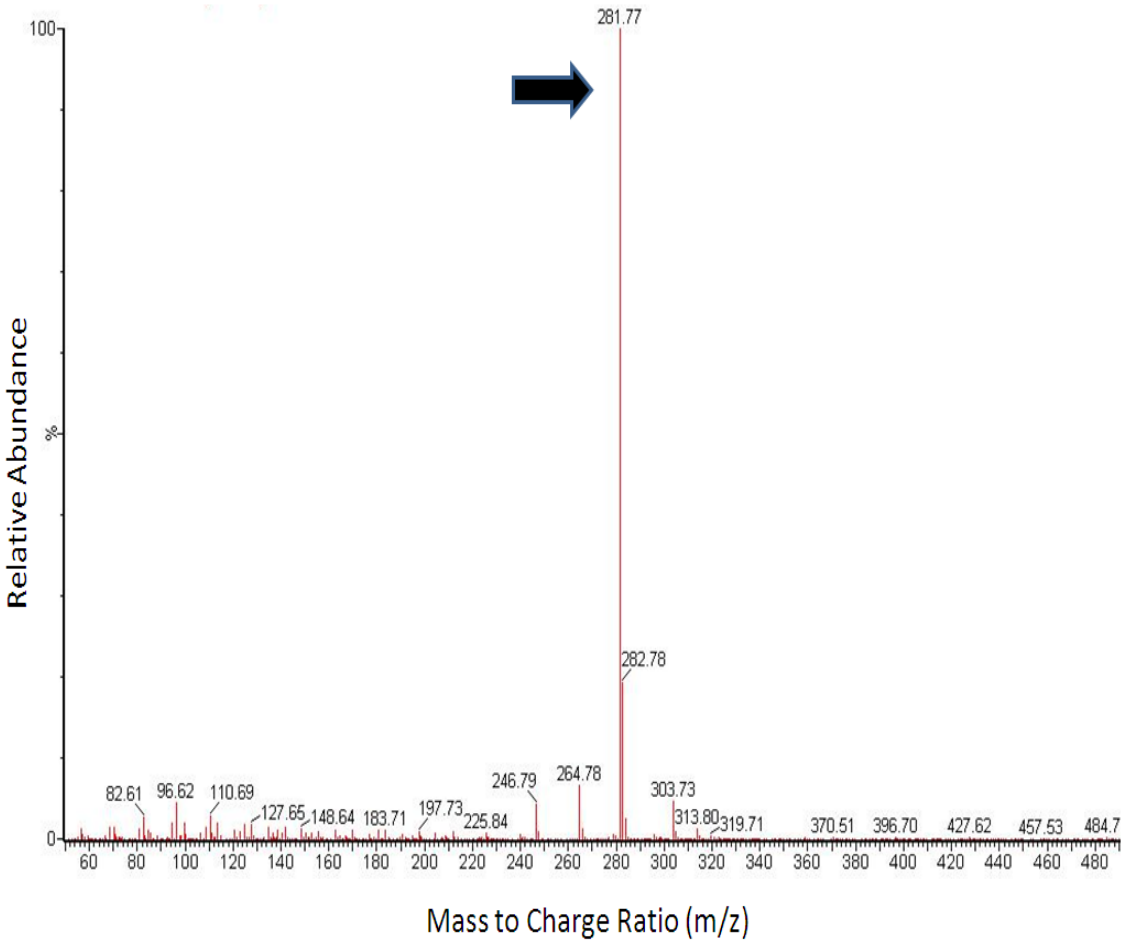


Figure 3.11. Mass Spectrum of an Ion Found with a Mass to Charge Ratio of ~281.77 m/z which is Apparent Only in Cells Treated with 10 µM CBI28 and May Be Putative Active form of CBI28

The mass spectrum shows a m/z of 281.77 which is a novel metabolite when compared to cell extracts in the absence of CBI28 at ~ 12.2 min (Appendix 4). The mass may represent a breakdown product of CBI28. The mass of m/z of 281.77 was present in both HLB and methanol extractions but did not occur in control samples (Appendix 4). The same elution was maintained through the metabolite analysis using 10 mM ammonium acetate and methanol. The elution method was specifically designed for hydrophobic molecules. It is assumed the metabolites would initially be similar in properties to CBI28 when cells processed CBI28 for excretion. The MS was set to scan for all masses between a range of 50 -500 m/z.

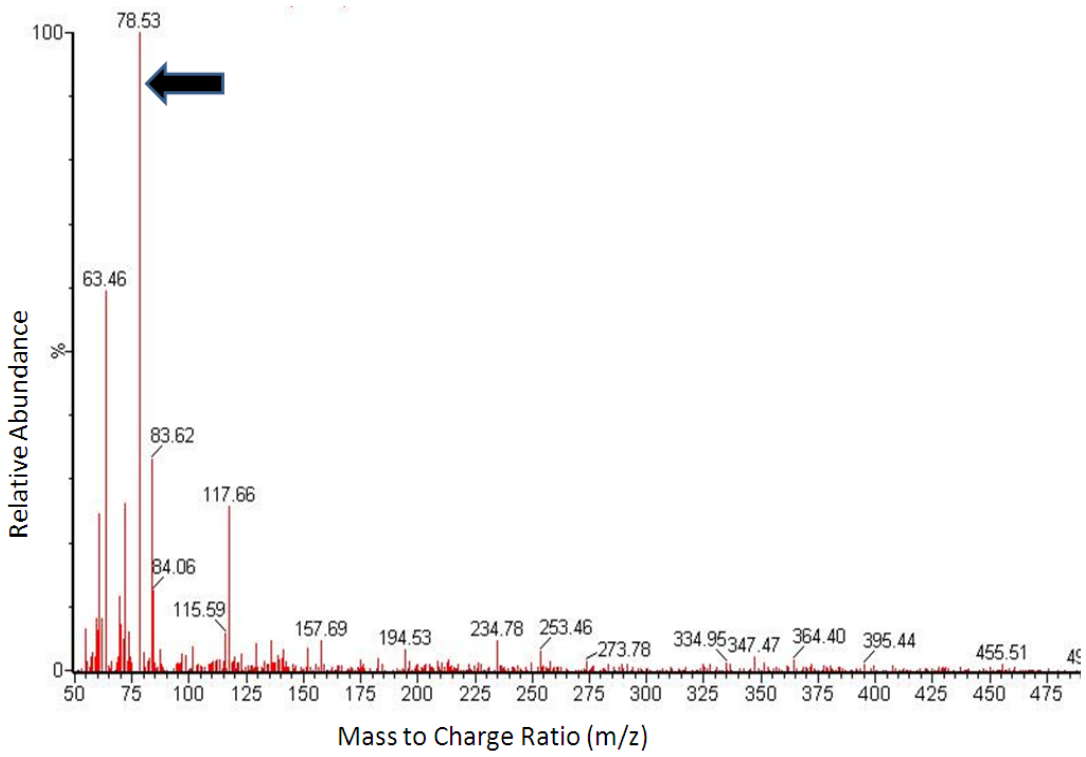


Figure 3.12. Mass of 78.53 m/z Identified in Samples Treated with 10 µM CBI28 Which May Represent a Fragment of a Metabolized Form of CBI28

The mass spectrum shows a peak at m/z= 78.53 at 1.775 min which may be an ion that is derived from the benzene like portions of CBI28. The ~78 m/z ion may represent an ion that comes from CBI28 or a metabolite derived from CBI28 that causes cellulose inhibition. The method for elution is the same as before using 10 mM ammonium acetate and methanol. The MS was set to scan for all masses between a range of 50 -500 m/z.

There were similar peaks in the methanol and HLB SPE-Pak extractions but the HLB SPE-Pak extraction had less background noise.

The range in which the PDA detected was increased to the entire range of the UV-Spectrum (200 nm – 800 nm). The control and experimental samples had no discernible differences (Fig. 3.13). All spectra coalesced into waves with no definitive peaks (Fig. 3.13). UV detection utilizing the PDA proved to be inadequate for detecting unknown compounds in a diverse mixture of compounds such as in cells. The MS provided additional information and since it detected masses, characteristic ions could be hypothesized.

A mass of approximately ~489 m/z was detected which might correspond to the glucuronidation of CBI28 (Fig. 3.14). However, when dealing with low intensity ions and unknown metabolites requires that column integrity and detection are optimal. An analysis of the column was performed to confirm column integrity. A caffeine standard of 1.2 ppm was run on the column and the results were consistent with the literature, in that caffeine had a retention time of 2.75 min and a predicted mass to charge ratio of ~194 m/z (Kele & Guiochon, 2000) (Appendix 1, Fig. 3.16). The caffeine sample showed that there were contaminants in the first run that were not present in the second run as seen in the reduced size of the peak (Appendix 1). The debris was evidenced in the differences in the size of the peaks on the PDA (relative abundance). The peaks showed there were other contaminants in the column from before. If debris was in the column it would cause erroneous peaks in the MS and may be indicative of column damage resulting in skewed peaks.

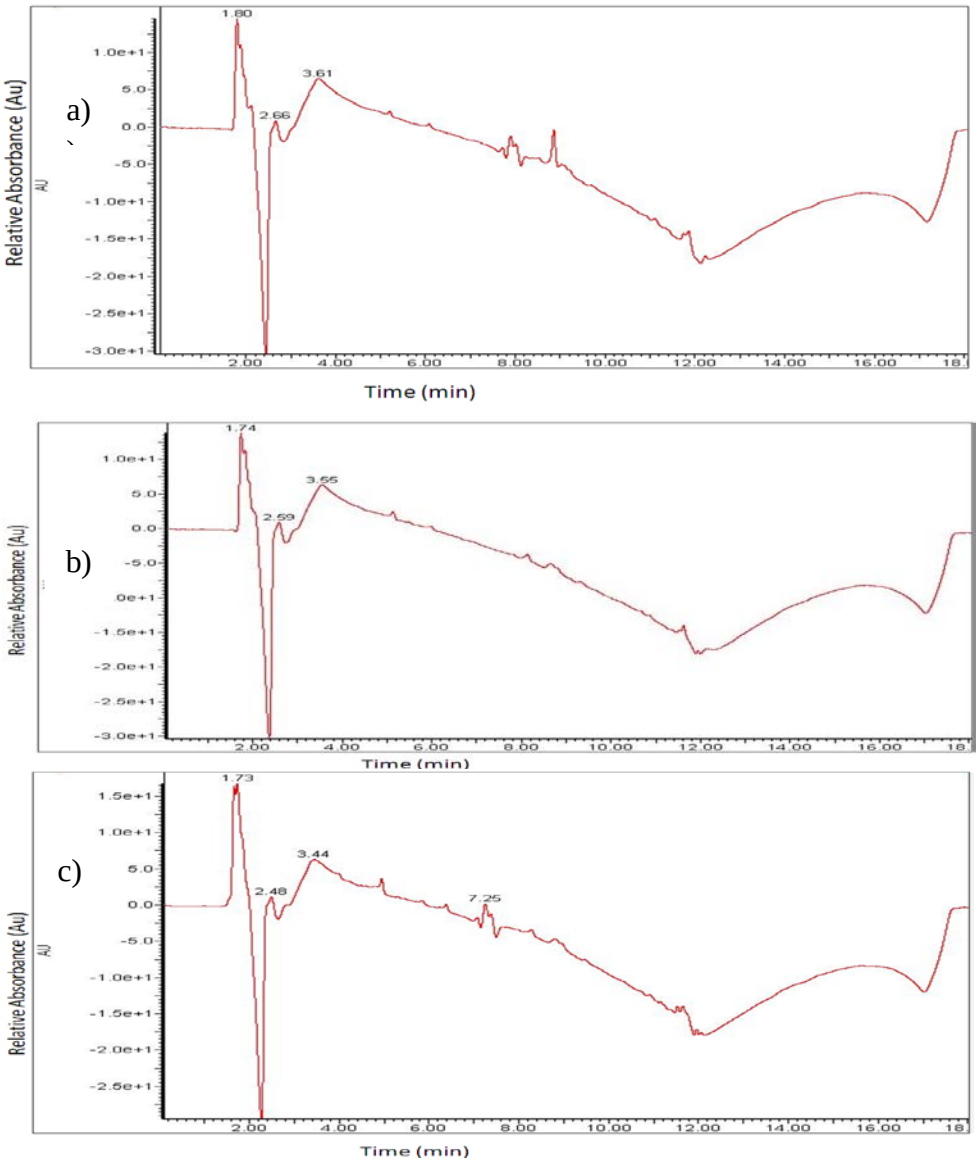


Figure 3.13. Comparison of Cell Extracts in the Absence of CBI28 and with HLB and Methanol Extractions Using the Ultra Performance Liquid Chromatography Photo Diode Detection Between 200 nm and 800 nm

a) Control – Absensce of CBI28 b) Experimental HLB SPE-Pak Extraction c) Experimental Methanol Extraction. PDA detection carried out on cell *G. xylinus* metabolites showed inconclusive results as there were no discernible peaks due to the broadness of the UV range scanned. The elution method was uniquely designed for hydrophobic compounds and the analytes were detected using the photo diode array. The photo diode array detected signals between 200 nm and 800 nm.

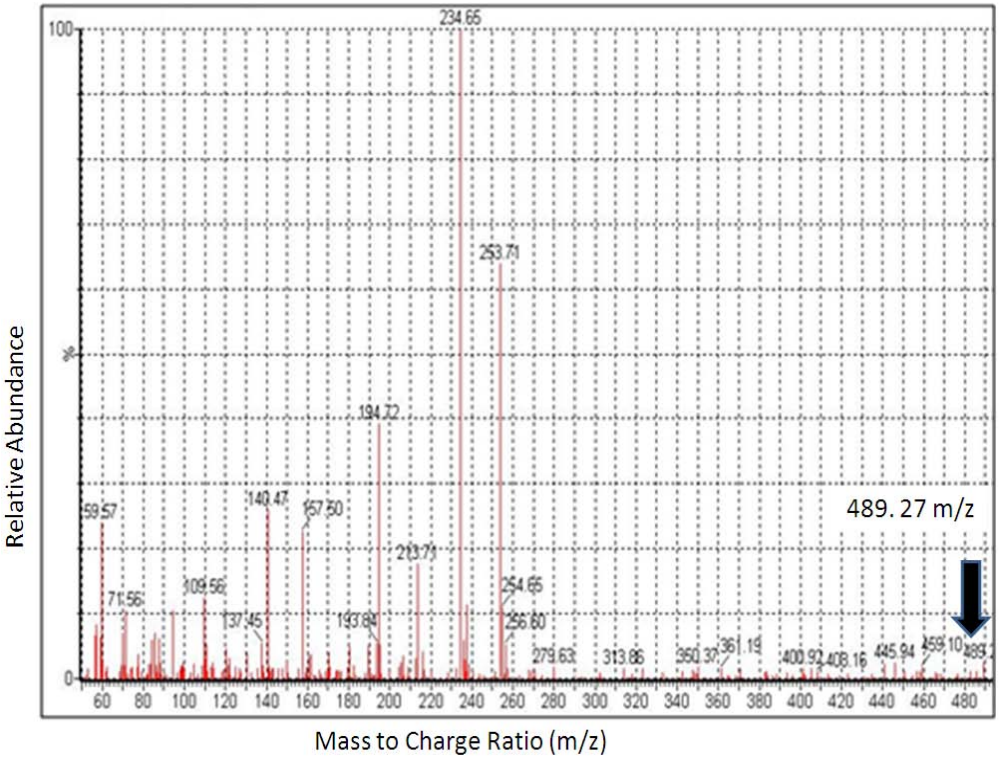


Figure 3.14 Mass Identified as 489 m/z which May be A Glucuronidated Form of CBI28 but in Very Low Abundance

The mass spectrum shows a peak at 1.775 min that has a m/z = 489.27 in low amounts that may be a glucuronidation event with CBI28. The peak may be noise due to the low amount detected or it may have been excreted out. Glucuronidation is a method for bacteria to excrete molecules by making them more hydrophobic. CBI28 may have been modified for glucuronidation but there is no strong evidence to prove glucuronidation since the abundance of expected ion is low. Elution was performed using a method designed for hydrophobic molecules using 10 mM ammonium acetate and methanol. The mass spectrometer scanned between 50 – 500 m/z.

The caffeine standard was run first before any analysis was done ensuring the integrity of the column before use. The caffeine standard was eluted consistently with replicates and a PDA detector was used to detect caffeine at a wavelength of 210 nm. The results were consistent and demonstrated that the column was functioning as expected. There was, however, some debris in the column.

Chapter 4: Discussion and Future Directions

4.1 *Gluconacetobacter xylinus* Mutant Screen Using CBI28

One of the most common drug resistance mechanisms in bacteria is mutation in the drug target site (Harbottle, Thakur, Zhao & White, 2006). Using this principle, it was reasoned that CBI28 targets could be identified by isolating resistant mutants. In total, 19 mutants were isolated and one mutant had resistance to 20 µM CBI28. However, these mutants were not maintained appropriately and were lost. It is hypothesized that the mutants might also have been unstable and lost resistance. Future experiments of this kind will warrant that cells with desirable phenotypes should be preserved either by lyophilisation or by freezing in glycerol (Malik, 1988; Swift, 1936). This would ensure that unstable mutants would be preserved for a later date and there would be a stock strain if the mutants lost their phenotype or were no longer viable.

Another reason that this experiment was unsuccessful is that an insufficient number of colonies were screened. Therefore, an alternative method of screening for resistant mutants might include screening for mutants in liquid media rather than on solid media. Screening could be done using microtiter plates, for example, (Kitaoka & Robyt, 1997) so that pellicle formation may be observed when resistance to CBI28 mutants is present. Once identified, the resistant mutants could then be isolated away from wild-type cells. This has the advantage that a greater number of mutants can be screened in a small format.

The instability of the putative mutants may be due to a survival mechanism. There has been evidence of bacterial survival mechanisms related to cellulose biosynthesis (Hisert *et al.*, 2005). The putative mutants may have been unstable because cellulose biosynthesis genes may be involved in bacterial survival. Mutating cellulose biosynthesis genes may therefore affect bacterial survival. Mutating cellulose biosynthesis genes may then detrimentally affect cell survival causing cell instability and subsequent cell death.

4.2 CBI28 Conjugation to Biotin

The most direct method to identify the cellular targets of CBI28 is by affinity purification. Since affinity purification using biotin is well established, the first step towards this goal was to create a CBI28 conjugated to biotin. Once created, the hybrid molecule could have been tested to ensure that it retained activity *in vivo*. CBI-biotin molecules that retained their activity would then be incubated with cellular extracts and target proteins could then be purified by biotin-streptavidin affinity chromatography. Upon purification, protein identity would have been determined by peptide mass fingerprinting.

The conjugation of biotin to CBI28 might have yielded three possible products that could be used for further studies (Fig. 4.1). The full biotin conjugate in Figure 4.1 c is the mass that was detected at 552 m/z ratio (Fig. 3.5). The mass of 552 m/z which is the expected mass of the CBI28-Biotin conjugate made up 17.2 % of the peak and this number dropped to ~5 % after two months due to hydrolysis by water in the buffer (Duffy & Leisten, 1956). The mass was calculated by adding the mass of CBI28 to the

mass of Biotin Hydrazide and the relative abundance was calculated using the MS. Even though samples were stored in the freezer, it seemed hydrolysis of the CBI28-Biotin Hydrazide conjugate occurred reducing the abundance of the ion over a period of time. Collecting fractions of putative conjugates was not possible due to the unreliability of the CE-MS. Fraction collection of putative CBI28-Biotin conjugates could be performed with the UPLC-MS through the waste line. However, collecting fractions may still be inadequate since the water seems to cause hydrolysis of the amide linkages (Duffy &Leisten, 1956).

The biotin kit that was used in this study was originally designed for proteins and several modifications had to be made to try and link the biotin to a small hydrophobic molecule like CBI28. The periodate ion is a very strong nucleophile designed to sheer proteins and leave carbonyl groups that would then act as a site for linkage with the biotin hydrazide (Pierce, Thermo Scientific, 2008). The periodate ion and all steps related to it was eliminated because there was no need to add a very strong nucleophile to the mixture which could possibly attack CBI28 and fragment the molecule into smaller components. In addition, an excess of the biotin hydrazide was added to the conjugation reactions. Due to the scale of the reactions, the product quantities were insufficient for analysis by NMR to determine structural properties. The temperature of the reaction was increased to 30^{o} C, which was expected to increase the rate of reaction. The temperature might have also increased the rate of hydrolysis as well resulting in hydrolysis of any conjugate formed making levels of conjugate below detection levels.

a)

b)

c)

Figure 4.1. Putative Forms of the CBI28-Biotin Conjugate that May Occur in a Reaction with Biotin Hydrazide where CBI28 is Degraded or Remains Intact

The two top molecules (a and b) represent CBI28 splitting at the double bond and binding to biotin. c) The whole molecule of CBI28 binds to biotin. The blue box represents CBI28 and the red box represents Biotin. These molecules represent potential molecules that may form through the biotin hydrazide reaction with CBI28. These can be used for subsequent study if isolated. Products from the reaction were not isolated due to hydrolysis resulting in no further analysis being performed to determine structure or functionality. Nuclear Magnetic Resonance can be used to predict structure but a more efficient and stable product must be formed for further use. The Michael Addition using various catalysts might prove useful and efficient to generate a stable compound in sufficient quantities for study.

4.3 Effects of CBI28 on *Gluconacetobacter xylinus* Metabolites

The application of CBI28 to membrane preparations of *G. xylinus* does not inhibit cellulose biosynthesis (D. Bonetta, personal communication). This result can be interpreted in a number of ways, but two obvious explanations are the active form of CBI28 is metabolically modified *in vivo* or that the cellulose synthase complex is not the direct target of CBI28 action. To explore these alternatives, CBI28-dependent metabolites from *G. xylinus* cells were qualitatively assessed using liquid chromatography and mass spectroscopy.

Analyzing the total chemical content of a cell is a challenging task (Welthagen *et al.*, 2005). Assessing the metabolites present in a cell is complex because of the sheer diversity of the compounds present, the labile nature of some metabolites and the varying concentrations of metabolites (Welthagen *et al.*, 2005). The metabolite analysis proved to be more difficult due to the diversity of species that were present in the cell lysates (Huang & Regnier, 2007). Based on the results, it is still unclear if CBI28 is metabolized in some way. The addition of a glucuronic acid molecule to CBI28 would result in a mass to charge ratio of 489.1. This might indicate a possible modification, such as glucuronidation (Fig. 3.14) (Soleim & Scheline, 2009). The levels of the putative glucuronidated ion were very low but this was due to the hydrophilic nature of the molecule and the fact that glucuronidated compounds are excreted from cells (Soleim & Scheline, 2009). An ion with a m/z= 388.1, which is the mass of CBI28 with the addition of a hydroxyl group was not detected in the samples (Nojiri & Omori, 2002). This is unexpected because traditionally bacteria will add hydroxyl groups to

benzene molecules to break the benzene ring and make them more soluble (Nojiri & Omori, 2002). The CBI28 molecule has two benzene moieties that may have hydroxyl groups added by *G. xylinus*. There were also ions such as the m/z=281.1 which were present in experimental samples but not in control samples (Appendix 5-Appendix 7). The mass to charge ratios of some ions were also similar to benzene which suggests both fragmentation and metabolism is in multistep processes (Fig. 3.6, Appendix 8 and Appendix 9). Glycosylation was more difficult to demonstrate due to the fact that the ion would be similar in size to the glucuronidated form of CBI28. The mass spectrometer was also set to monitor the range of 50-500 m/z and any additional glycosylated groups would be out of the range so no glycosylation events could be detected since it would be too large to detect with the pre-set range.

The PDA detector was utilized but the amount of information it provided was limited. The PDA was initially set to 255 nm and there were several peaks indicating that there were compounds present. A wavelength of 255 nm was chosen because the C-H bond is excited at that wavelength and was also used to assess the diversity of compounds present in the sample. The range increase caused all the peaks to level out and no peaks were discernible because the range was too large and too many signals were detected. The major downfall of the PDA is that it only detects molecules if they are UV active for that given wavelength. With a plethora of unknown metabolites present in a cell it is difficult to determine which molecule is UV active at a given wavelength. The PDA does not provide enough information to definitively identify a compound or metabolite with unknown UV properties. The UV spectra were obtained

for CBI28 but the metabolites it converts into or even fragments of CBI28 may have different UV activity. Thus molecules can have different excitation wavelengths making the PDA and ineffective method of detection in a complex mixture of compounds (de Pascual-Teresa, Treutter, Rivas-Gonzalo, & Santos-Buelga, 1998). Mass spectroscopy is more informative for determining chemical identity especially when tandem mass spectroscopy (MS/MS) is performed (Welthagen *et al.*, 2005). Characteristic fragmentation patterns can be determined and the MS can be used a filter to remove noise (Welthagen *et al.*, 2005). Tandem mass spectroscopy coupled with two-dimensional chromatography is useful for separating unknown metabolites.

It should be mentioned that the instrumentation used had a detection limit of 1 ppm. This is a poor detection limit in metabolomic studies but due to accessibility, the Waters Acquity System was the only system available to perform the experiment. A tandem mass spectrometer would have significantly improve the limit of detection.

4.4 Future Directions

4.5 CBI28-resistance in *Gluconacetobacter xylinus*

If CBI28 resistant mutants can be isolated in the future an exciting possibility for their characterization would include the use of an Illumina Genome Analyzer (Illumina Inc., 2009). The Illumina Genome Analyzer is a high-throughput sequencer. The DNA is immobilized on a chip and subsequently amplified using a polymerase chain (PCR) reaction (Illumina Inc., 2009). The strands are duplicated and sequenced-by-synthesis using four fluorescently labelled, reversible terminators that are imaged

through laser excitation (Illumina Inc., 2009). The image results are entered into the analysis pipeline (Illumina Inc., 2009). This makes the sequencer extremely robust and fast but very similar sequences are difficult to assemble since the genomic DNA is initially sheared (Illumina Inc., 2009).

The wild type *G. xylinus* genome will have to be sequenced and constructed using specialized software for comparison with mutant sequences. Once the sequencing has been performed, then mutant genomes can be compared to wild type genomes to determine where EMS mutagenesis altered genes related to CBI28 resistance. The two-dimensional protein gels could lead to proteins that are modified in mutant samples. These protein modifications could be utilized to determine how CBI28 affects cellulose biosynthesis. Cellulose production could be directly studied through putative mutants.

4.6 CBI28-Biotin Conjugation

Given the shortcomings of the biotin-CBI28 conjugation experienced here, the conjugation should be done using an alternative approach (K. Paal, personal communication). One of the most traditional methods for linking two conjugated molecules together in organic synthetic chemistry is the Michael Addition (Kamal, Reddy, & Rajender, 2005). The Michael Addition can be extremely efficient reaching 80-90% product (Kamal, Reddy, & Rajender, 2005). The reaction using CBI28 would make use of thiolamines to provide a synthetic linker to link CBI28 and biotin (Paal, Personal Communication, 2008). A thiolamine would be a good candidate for the link

because it provides a thiol group that would act as a nucleophile and attack carbanion linking to CBI28. The amino group would provide another less reactive nucleophile to attack a biotin-ester. The thiolamine linker will have a carbon chain between the thiol group and the amino group and the length of the group does not matter; however, the longer the linker chain the less likely it will be biologically active (Jin, Legros, Leclercq, Hardcastle, & Jarman, 1997). The amino group will then be used to perform a two-step Michael Addition linking both CBI28 and Biotin to a thiolamine linker. The amino group would also be a less competitive nucleophile than the thiol group and may also be blocked (Dixon & Perham, 1968). Traditional methods of Michael Addition are available but newer methods make use of catalysts.

To perform the actual Michael Addition there are newer methods involving catalysts that will make the reaction more efficient and proceed faster. The catalysts range from polymers and clays to ionic solutions. The ionic solution method is not desirable because it makes use of an expensive, non-readily available compound that acts as the catalyst (Ranu & Dey, 2004). Another method employs clays/minerals that provide a surface that will act as the catalyst enhancing the Michael Addition reaction rate (Sharma, Kumar, & Chakraborti, 2006; Zahouily, Abrouki, Rayadh, Sebti, Dhimane & David., 2003). These clays would have to be obtained commercially or synthesised in multiple steps (Zahouily, Abrouki, Rayadh, Sebti, Dhimane & David., 2003). The last method employs polyethylene glycol (PEG) as the catalyst which is more common, inexpensive and recyclable (Kamal, Reddy, & Rajender, 2005). The reaction using PEG also takes two hours with monitoring to reaction completion

(Kamal, Reddy, & Rajender, 2005).

Reactions could be monitored using TLC plates. The products of the Michael Addition could be extracted using ether and a silica column once the reaction is complete (Kamal, Reddy, & Rajender, 2005; Ranu & Dey, 2004; Sharma, Kumar, & Chakraborti, 2006; Zahouily, Abrouki, Rayadh, Sebti, Dhimane & David., 2003). Extracting enough product to test bioactivity was previously problematic. Using avidin to extract any products in the reaction mixture which contain biotin would not be suitable because disassociating avidin from biotin requires stringent conditions that may alter the conjugate before it is tested for biological activity (Kizke *et al.*, 2005).

The reactants would be subjected to UPLC-MS to determine if the reaction did occur and if a CBI28-Biotin conjugate was formed. If enough sample was extracted, it could be analyzed using Nuclear Magnetic Resonance (NMR) to determine its structure and whether it matches predictions of a CBI28-Biotin conjugate. Once the samples are extracted, they could be assayed for biological activity using wildtype *G. xylinus*. The assay will be performed either in liquid or on agar plates supplemented with the CBI28-Biotin conjugation at varying concentrations. The *G. xylinus* would be spread plated on the plates or inoculated in the liquid culture. The plates or microtiter plates would then be incubated to see if cellulose is still inhibited while making sure the cells are still viable. There would also be CBI28 (with corresponding concentrations to the CBI28-Biotin conjugate), DMSO control and in the absence of CBI28.

4.7 CBI28-dependent metabolite analysis

An improvement on the chromatographic techniques used in this study, would be to use two-dimensional chromatography to improve metabolite in conjunction with activity assays (Huang & Regnier, 2007). To do this, the metabolites could be fractioned, collected in microtiter plates and assayed for inhibition of cellulose production *G. xylinus.* Active fractions could then be analyzed using UPLC to further resolve the active compounds. These could then be characterized using tandem mass spectroscopy (Welthagen, Shellie, Spranger, Ristow, Zimmermann & Fieh., 2005).

Alternatively, isotopic labelling of CBI28 could be used to determine which metabolites are derived from CBI28. In this case, extracts from treated cells with mixtures of labelled and unlabelled CBI28 would be used (Huang & Regnier, 2007). The expectation would be that labelled CBI28 and its metabolites would have different retention times as well as a different m/z value (Huang & Regnier, 2007). The peaks would then be present in ratios that are similar to the ratios of isotopically labelled CBI28 and non-isotopically labelled CBI28 (Huang & Regnier, 2007). Isotopes of hydrogen would be used because it is easily accessible and more easily substituted onto molecules because of the fast rate of protonation and deprotonation. Isotopes of carbon can also be used but would be more difficult to supplement onto the molecules because carbon is used as a backbone for the CBI28 molecule. Thus the peaks reveal which ones are derived from CBI28. Ultimately, the information gained from tandem mass spectroscopy and the knowledge of the starting material, CBI28, could allow a hypothesis based on the structures and/or functional groups that could be used as a

probe to inhibit cellulose biosynthesis *G. xylinus*.

The novel cellulose biosynthesis inhibitor, CBI28, was used to elucidate the processes of cellulose biosynthesis. Attempts at generating mutants proved to be difficult to analyze because it seems that bacterial survival may be related to cellulose biosynthesis. Synthesizing biotin derivatives for analysis was also difficult due to recovery and the chemical synthesis of a stable product. Metabolomic analysis of CBI28 treated cells revealed putative metabolites that may contribute to inhibition of cellulose biosynthesis but further analysis needs to be performed. Several different approaches may be applied to these problems. For mutational analysis, screening in liquid media and better storage may allow putative mutants to be analyzed. If the cellulose biosynthesis pathways are related to bacterial survival then mutational analysis may prove difficult but this also reveals information about cellulose biosynthesis and CBI28. Synthesizing a biotin-CBI28 product may prove useful for biochemical studies and determining protein targets. It was discovered that both there is no difference between methanol and Waters Oasis HLB SPE-Pak extraction except in some cases methanol extractions caused more noise. Metabolites may also aid in tracking the life cycle of CBI28 as it passes through the cell especially if coupled to high performance techniques such as tandem mass spectroscopy, isotopic labelling and tandem chromatography. Tools need to be further developed to determine the mode of action of CBI28 and how it affects cellulose biosynthesis. The tools used to determine the mode of action of CBI28 may be guided by this research.

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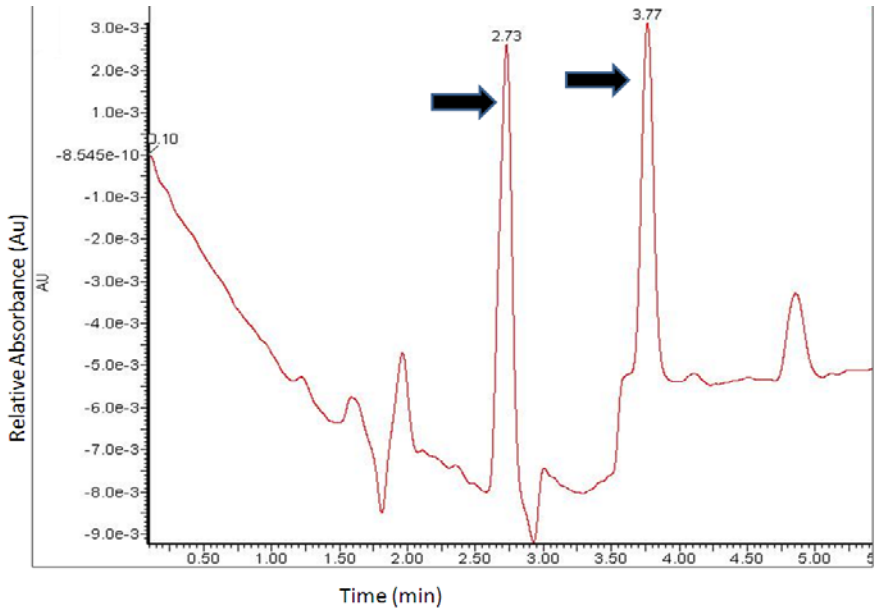
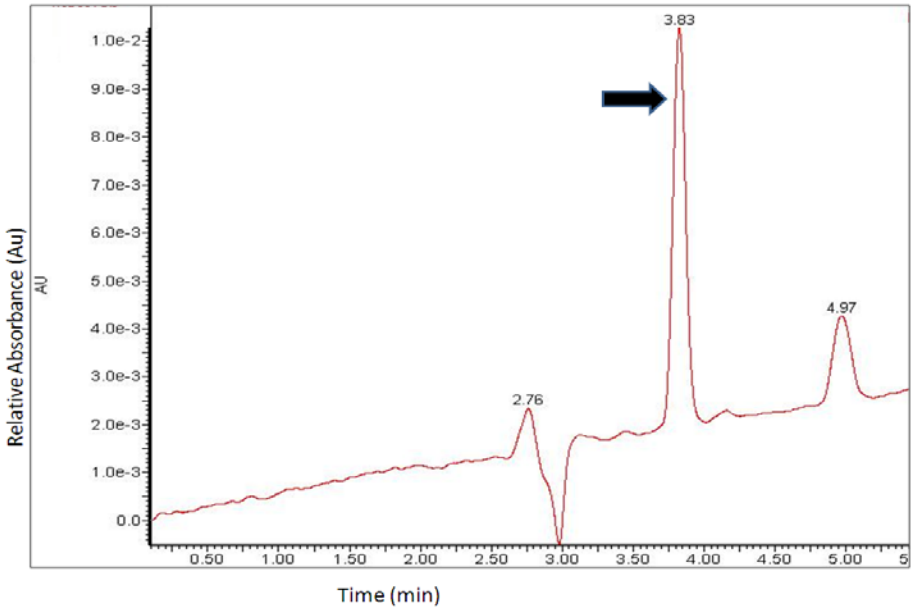
Appendix 1 – Caffeine Standards Used to Test Column Integrity



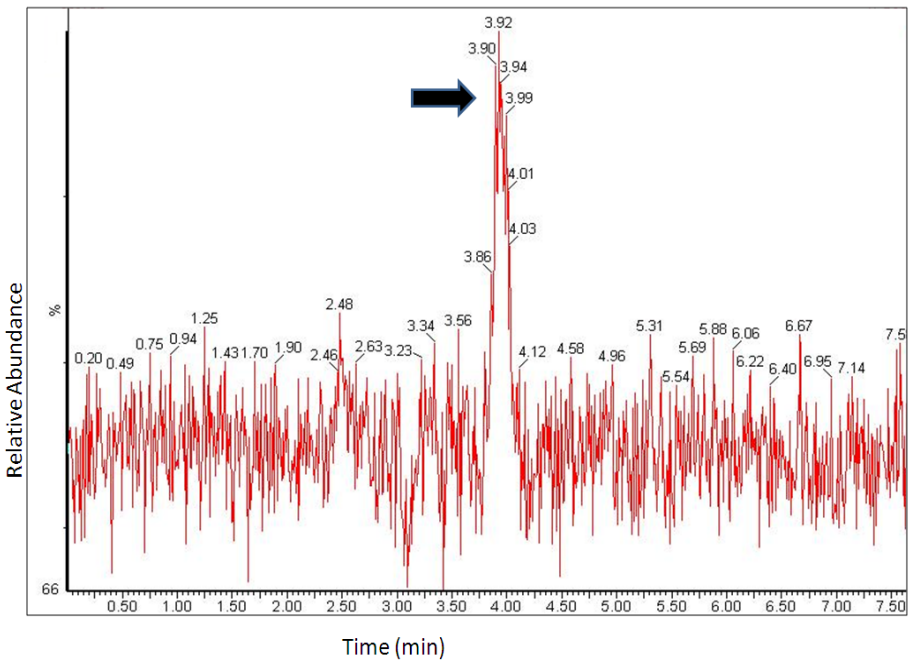
Appendix 1.1. – Caffeine Standard Used to Test Column Integrity Showing Debris

The chromatogram depicts the use of caffeine at a concentration of 1.2 ppm to test the integrity of the Waters C-18 column. The UPLC-MS/PDA was used to elute caffeine with a previously developed method (M.Allison, personal coomunication). Caffeine elution using UPLC is well documented in literature (Kele & Guiochon, 2000). The retention time of 3.77 min is expected for the retention time of caffeine (Kele & Guiochon, 2000). The large peaks as monitored by the PDA reveal column debris from previous use around 2.75 min, 5.00 min and 2.00 min. The retention time of caffeine may also have been skewed because of the column debris. Once the debris was found the column was washed to eliminate debris.



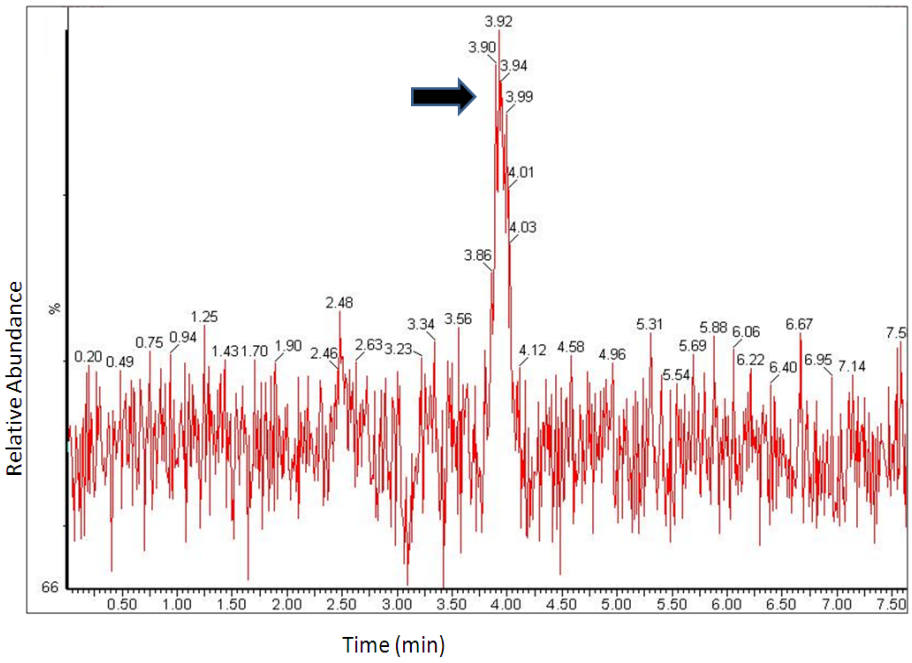
Appendix 1.2. – Caffeine Standard Used to Test Column Integrity Showing a Properly Functioning Column

The chromatogram depicts the use of caffeine at a concentration of 1.2 ppm to test the integrity of the Waters C-18 column. The UPLC-MS/PDA was used to elute caffeine with a previously developed method (M.Allison, personal communication). Caffeine elution using UPLC is well documented in literature (Kele & Guiochon, 2000). The retention time of 3.83 min is expected for the retention time of caffeine (Kele & Guiochon, 2000). The chromatogram of this caffeine run was performed after the initial run and shows no debris. This run indicates that the column has been successfully washed as seen by only one large peak detected by the PDA at 3.83 min. This retention time is closer to the accepted retention time of caffeine (Kele & Guiochon, 2000). The peaks at 2.76 min and 4.97 min are decreased in size also indicating that debris has been cleared from the column, the wash was successful and the integrity of the column is intact.



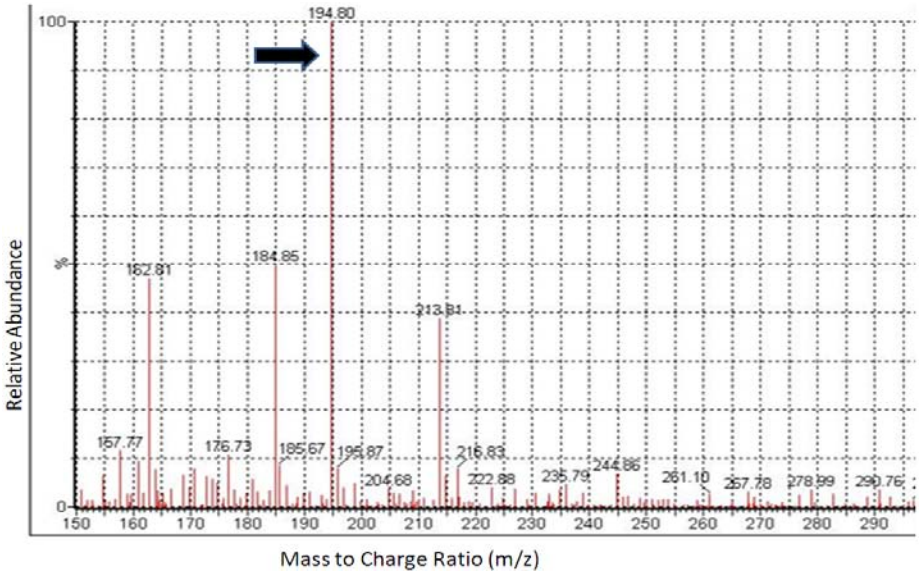
Appendix 1.3. – Mass Spectrum (TIC) of Caffeine Standard at 1.2 ppm

The mass spectrum shows caffeine at a concentration of 1.2 ppm using the Total Ion Count (TIC). TIC scans all masses between a given range which was 50 – 500 m/z. The mass of caffeine was detected at 3.92 min which is consistent with literature values (Kele & Guiochon, 2000). The mass of caffeine at a concentration of 1.2 ppm was detected even though the detection limit of the mass spectrometer was 1 ppm. The concentration of caffeine tested was close to the detection limit of the mass spectrometer which explains the signal to noise ratio and the high level of background noise. The mass spectrum was taken from the first caffeine run to determine column integrity where debris was found in the column. No signals for other debris molecules were found because they might not have been ionized. The UPLC-MS/PDA was used to elute caffeine with a previously developed method (M.Allison, personal coomunication). Caffeine elution using UPLC is well documented in literature (Kele & Guiochon, 2000)



Appendix 1.4. – Replicate Mass Spectrum (TIC) of Caffeine Standard at 1.2 ppm

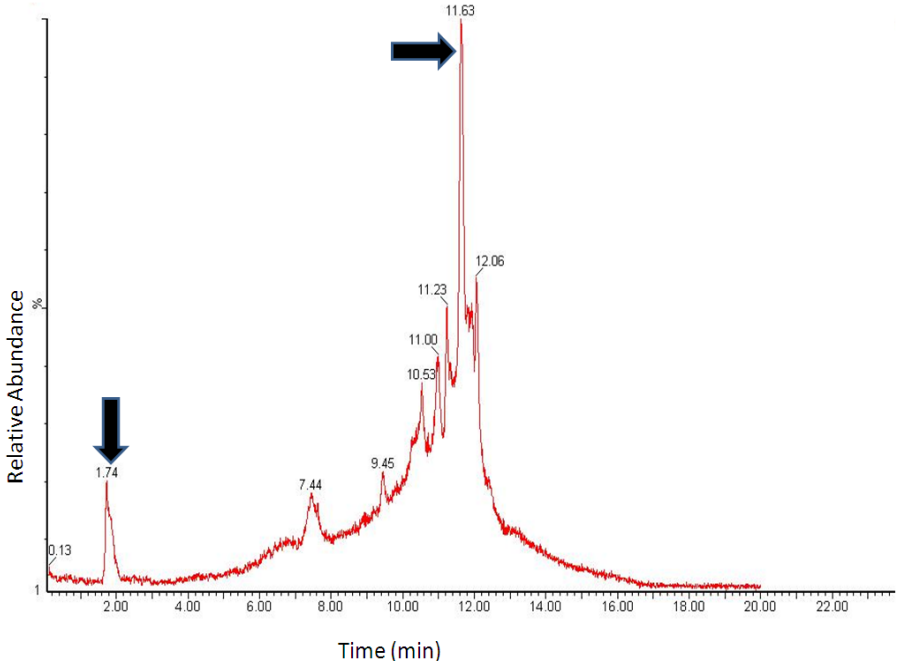
The mass spectrum shows caffeine at a concentration of 1.2 ppm using the Total Ion Count (TIC). TIC scans all masses between a given range which was 50 – 500 m/z. The mass of caffeine was detected at 3.92 min which is consistent with literature values (Kele & Guiochon, 2000). The mass of caffeine at a concentration of 1.2 ppm was detected even though the detection limit of the mass spectrometer was 1 ppm. The concentration of caffeine tested was close to the detection limit of the mass spectrometer which explains the signal to noise ratio and the high level of background noise. The above mass spectra was taken from the second run of caffeine to determine column integrity where there was far less to no debris in the column. The UPLC-MS/PDA was used to elute caffeine with a previously developed method (M.Allison, personal communication). Caffeine elution using UPLC is well documented in literature (Kele & Guiochon, 2000)



Appendix 1.5. – Mass Spectrum of Caffeine Peak at 194.80 m/z from Chromatogram of 1.2 ppm Caffeine Standard at 3.92 min

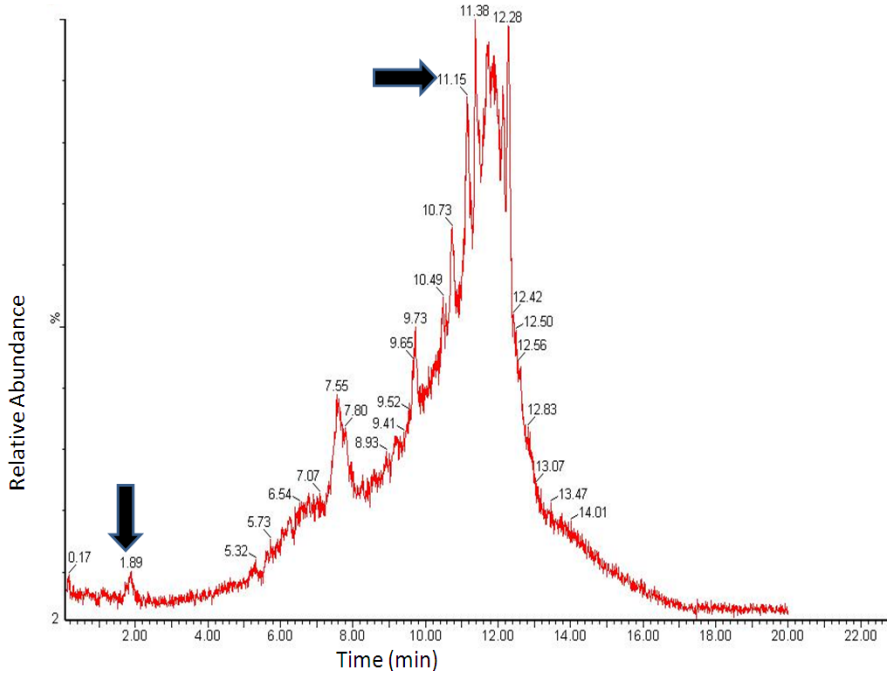
The mass spectrum shows the detection of the characteristic ion of caffeine with a mass of 194.80 m/z. The ion detected with a mass of 194.80 m/z is consistent with literature values caffeine and the retention time occurred at the appropriate time (Kele & Guiochon, 2000). There are also several other masses detected in lower abundance which may represent column debris or background noise due to the low concentrations (1.2 ppm) being detected. The low concentration of caffeine used may decrease the signal to noise ratio. The UPLC-MS/PDA was used to elute caffeine with a previously developed method (Allison, Personal Communication). Caffeine elution using UPLC is well documented in literature (Kele & Guiochon, 2000)

Appendix 2 – Chromatograms of *G. xylinus* Cell Extracts in the Absence of CBI28



Appendix 2.1. – Chromatogram of *G. xylinus* Cell Extracts in the Absence of CBI28

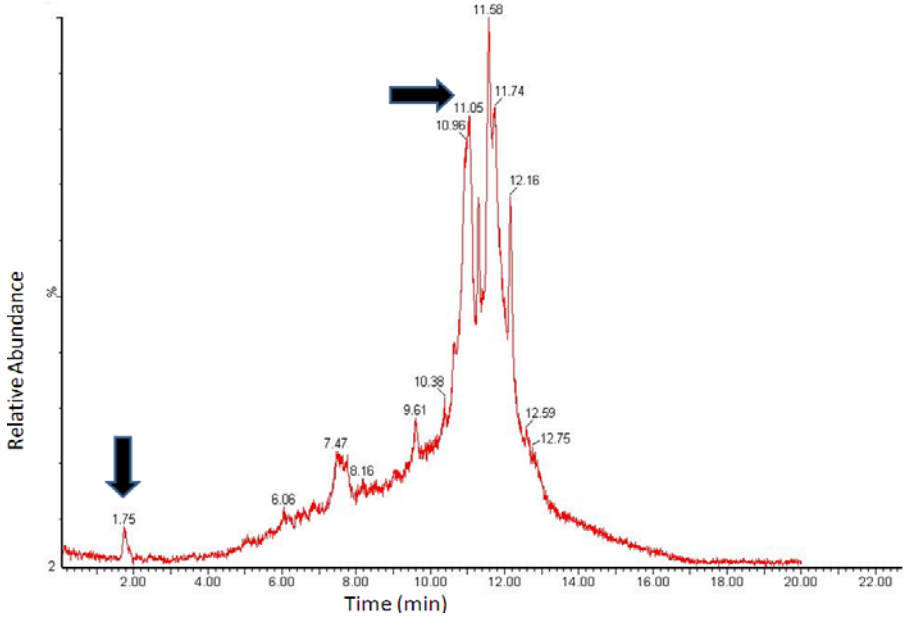
The chromatogram shows cell lysates in the absence of CBI28 that were eluted using a uniquely developed gradient elution method for this metabolomic analysis. The method uses 10 mM ammonium acetate and methanol. The extraction was performed with a Waters Oasis HLB SPE-Pak to extract any hydrophobic metabolites. The method for extraction was the standard method for extraction using the HLB SPE-Pak provided by Waters Corporation (2008). The chromatogram shows peaks at 1.74 min and between 10 -13 min. There are several peaks that have common ions for HLB-SPE Pak extractions and methanol extractions as well as being present in experimental samples. The cell lysates in the absence of CBI28 was also used as a comparison against CBI28 treated cells which revealed potentially novel metabolites related to CBI28.



Appendix 2.2. – Chromatogram of *G. xylinus* Cell Extracts in the Absence of CBI28

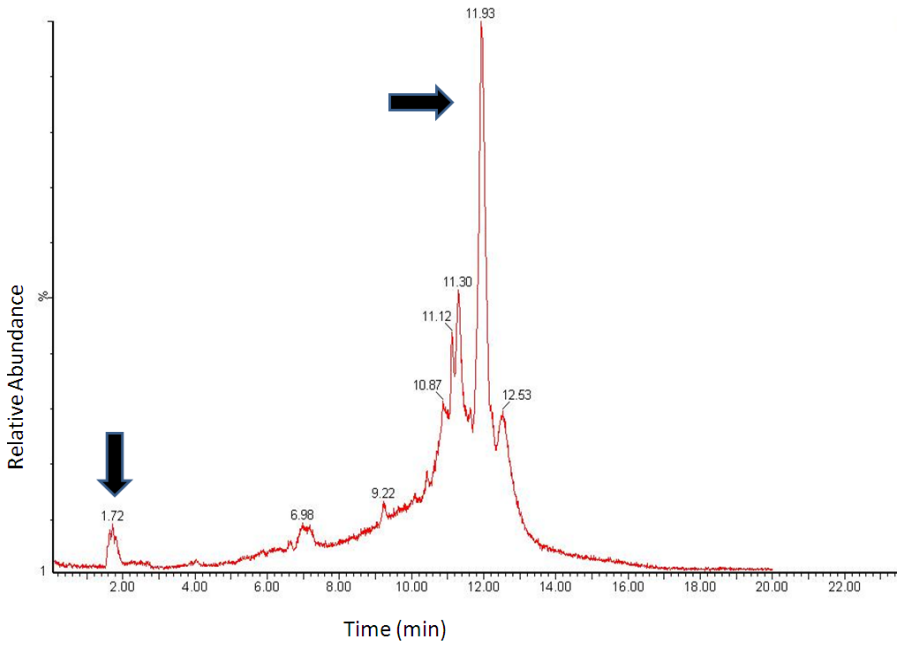
The chromatogram shows cell lysates in the absence of CBI28 that were eluted using a uniquely developed gradient elution method for this metabolomic analysis. The method uses 10 mM ammonium acetate and methanol. The extraction was performed using methanol to extract hydrophobic metabolites. The method for extraction was adding methanol to cell lysates and then extracting methanol and evaporating off excess methanol. The chromatogram shows characteristic peaks at 1.89 min, 8.00 min and 10.00 – 13.00 min. There are several peaks that have common ions for HLB-SPE Pak extractions and methanol extractions as well as being present in experimental samples. The cell lysates in the absence of CBI28 was also used as a comparison against CBI28 treated cells which revealed potentially novel metabolites related to CBI28. The profile of this chromatogram is different between 11.00 and 13.00 min where the peak is more pronounced than in the Waters Oasis HLB SPE-Pak extraction. There were no differences, however, in masses detected between the methanol and Waters Oasis HLB SPE-Pak extractions in the absence of CBI28.

Appendix 3 – Chromatograms of *G. xylinus* Cells Exposed to 10 µM CBI28 and Extracted Using the Waters Oasis HLB SPE-Pak



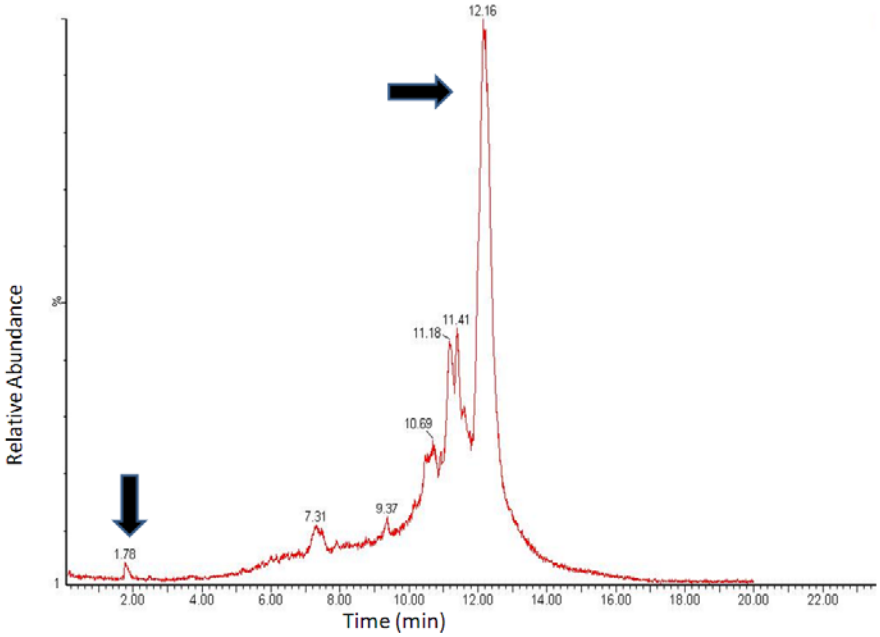
Appendix 3.1. – Chromatogram of *G. xylinus* cells Treated with 10 µM CBI28 and Extracted with and HLB SPE-Pak

The chromatogram shows *G. xylinus* cells exposed to 10 µM CBI28, lysed and then eluted using a uniquely developed gradient elution method for this metabolomic analysis. The method uses 10 mM ammonium acetate and methanol. The extraction was performed with a Waters Oasis HLB SPE-Pak to extract any hydrophobic metabolites. The method for extraction was the standard method for extraction using the HLB SPE-Pak provided by Waters Corporation (2008). The chromatogram shows peaks at 1.74 min, and between 10.00 – 13.00 min. Several peaks occurred in both methanol and Waters Oasis HLB SPE-Pak that may be putative metabolites.



Appendix 3.2. – Chromatogram of Replicate *G. xylinus* Cells Exposed to 10 µM CBI28 and Extracted using the HLB SPE-Pak

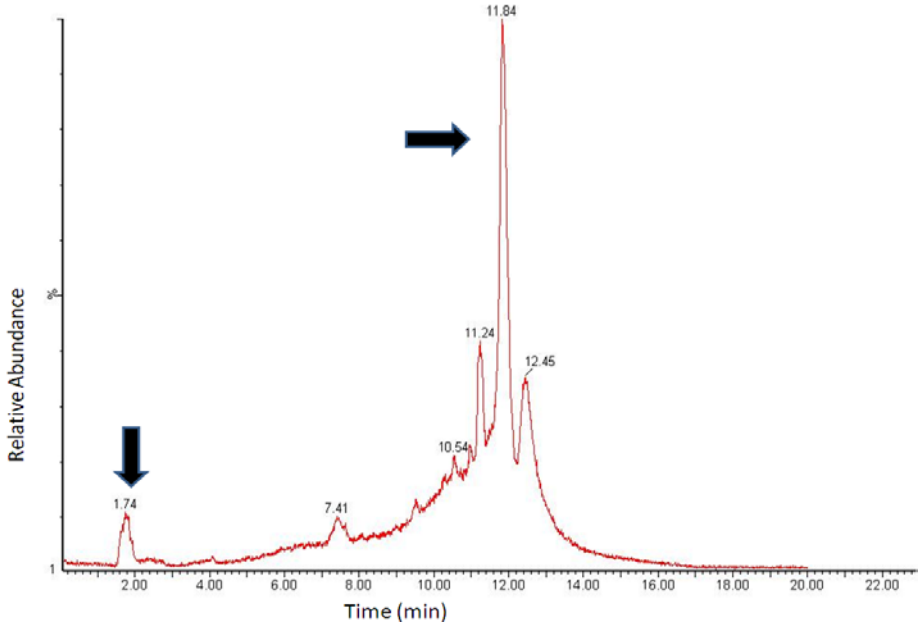
The chromatogram shows *G. xylinus* cells exposed to 10 µM CBI28, lysed and then eluted using a uniquely developed gradient elution method for this metabolomic analysis. The method uses 10 mM ammonium acetate and methanol. This chromatogram is also a replicate for cells extracted with the Waters Oasis SPE-Pak exposed to 10 µM CBI28. The extraction was performed with a Waters Oasis HLB SPE-Pak to extract any hydrophobic metabolites. The method for extraction was the standard method for extraction using the HLB SPE-Pak provided by Waters Corporation (2008). The chromatogram shows peaks at 1.72 min, and between 10.87 – 12.53 min. Several peaks occurred in both methanol and Waters Oasis HLB SPE-Pak that may be putative metabolites. There are some differences in the peaks between 10.00 and 13.00 min but there were no differences in the detected major ions.



Appendix 3.3. – Chromatogram of Replicate *G. xylinus* cells Treated with 10 µM CBI28 Extracted Using and HLB SPE-Pak

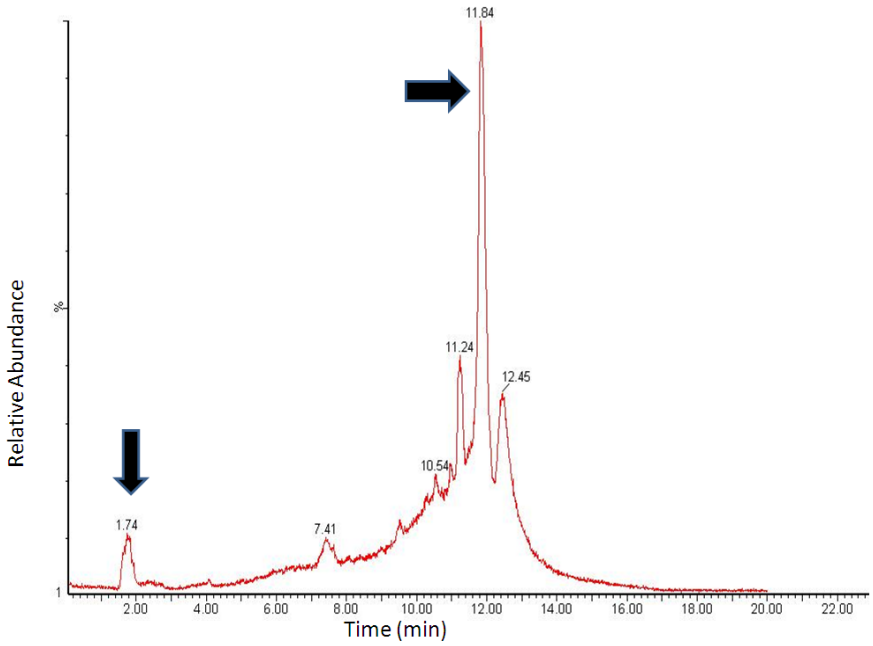
The chromatogram shows *G. xylinus* cells exposed to 10 µM CBI28, lysed and then eluted using a uniquely developed gradient elution method for this metabolomic analysis. The method uses 10 mM ammonium acetate and methanol. This chromatogram is also a replicate for cells extracted with the Waters Oasis SPE-Pak exposed to 10 µM CBI28. The extraction was performed with a Waters Oasis HLB SPE-Pak to extract any hydrophobic metabolites. The method for extraction was the standard method for extraction using the HLB SPE-Pak provided by Waters Corporation (2008). The chromatogram shows peaks at 1.78 min, and between 10.69 - 12.53 min. Several peaks occurred in both methanol and Waters Oasis HLB SPE-Pak that may be putative metabolites. There are some differences in the peaks between 10.00 and 13.00 min but there were no differences in the detected major ions.

Appendix 4 – Chromatograms of *G. xylinus* Cells Exposed to 10 µM CBI28 and Extracted Using Methanol



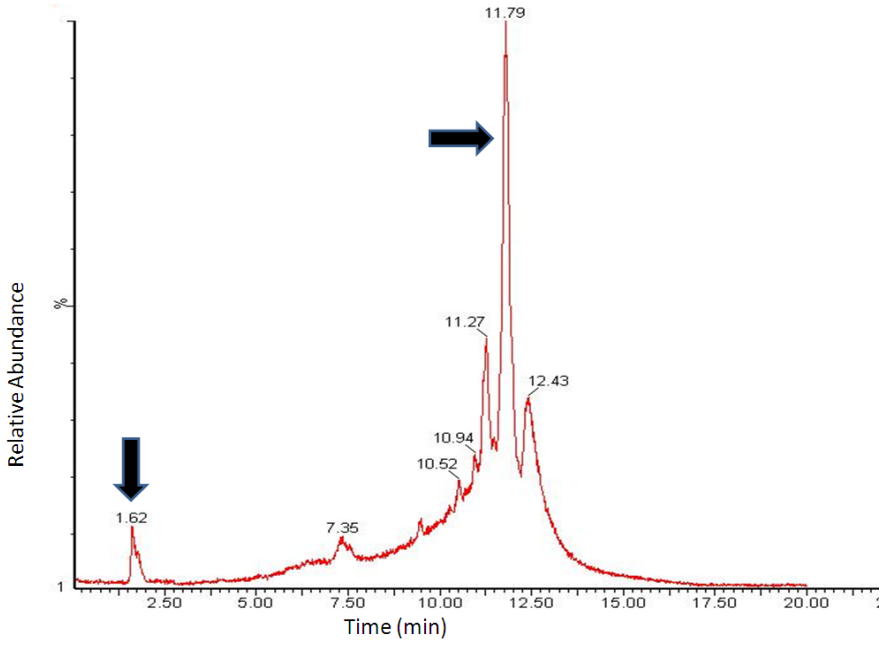
Appendix 4.1. – Chromatogram of *G. xylinus* cells Exposed to 10 µM CBI28 Extracted Using Methanol

The chromatogram shows *G. xylinus* cells exposed to 10 µM CBI28, lysed and then eluted using a uniquely developed gradient elution method for this metabolomic analysis. The method uses 10 mM ammonium acetate and methanol. The method for extraction was adding methanol to cell lysates and then extracting methanol and evaporating off excess methanol. The chromatogram shows characteristic peaks at 1.74 min and 10.54 – 12.45 min. Several peaks occurred in both methanol and Waters Oasis HLB SPE-Pak that may be putative metabolites. There are some differences in the peaks between 10.00 and 13.00 min but there were no differences in the detected major ions. There did not seem to be any major differences between extractions methods or even samples in the absence of CBI28 but the differences were in the detected ions from peaks.



Appendix 4.2. – Chromatogram of *G. xylinus* cells Exposed to 10 µM CBI28 Extracted Using Methanol

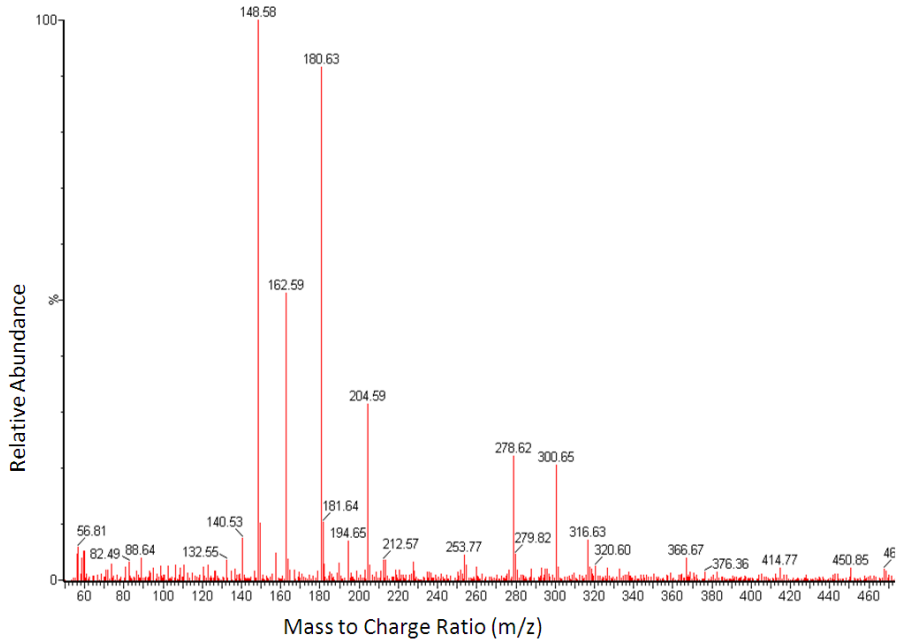
The chromatogram shows *G. xylinus* cells exposed to 10 µM CBI28, lysed and then eluted using a uniquely developed gradient elution method for this metabolomic analysis. The method uses 10 mM ammonium acetate and methanol. This chromatogram is also a replicate for cells extracted employing the methanol extraction exposed to 10 µM CBI28. The method for extraction was adding methanol to cell lysates and then extracting methanol and evaporating off excess methanol. The chromatogram shows characteristic peaks at 1.74 min and 10.54 – 12.45 min. Several peaks occurred in both methanol and Waters Oasis HLB SPE-Pak that may be putative metabolites. There are some differences in the peaks between 10.00 and 13.00 min but there were no differences in the detected major ions. There did not seem to be any major differences between extractions methods or even samples in the absence of CBI28 but the differences were in the detected ions from peaks. This chromatogram is very similar to the first elution run using methanol.



Appendix 4.3. – Chromatogram of *G. xylinus* cells Exposed to 10 µM CBI28 Extracted with Methanol

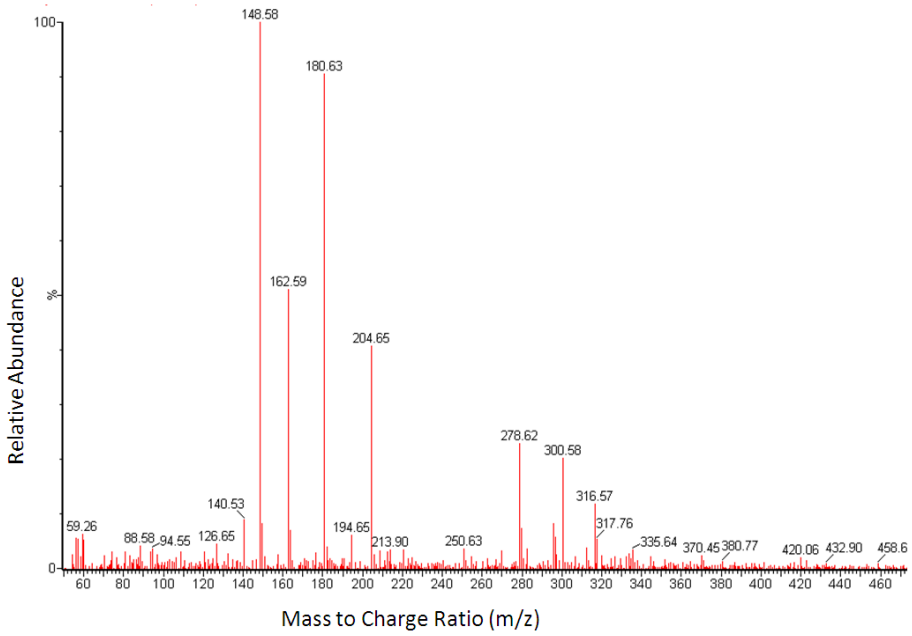
The chromatogram shows *G. xylinus* cells exposed to 10 µM CBI28, lysed and then eluted using a uniquely developed gradient elution method for this metabolomic analysis. The method uses 10 mM ammonium acetate and methanol. This chromatogram is also a replicate for cells extracted employing the methanol extraction exposed to 10 µM CBI28. The method for extraction was adding methanol to cell lysates and then extracting methanol and evaporating off excess methanol. The chromatogram shows characteristic peaks at 1.62 min and 10.52 – 12.43 min. Several peaks occurred in both methanol and Waters Oasis HLB SPE-Pak that may be putative metabolites. There are some differences in the peaks between 10.00 and 13.00 min but there were no differences in the detected major ions. There did not seem to be any major differences between extractions methods or even samples in the absence of CBI28 but the differences were in the detected ions from peaks.

Appendix 5 – Mass Spectra of *G. xylinus* Cells in the Absence of CBI28 Extracted Using the HLB SPE-Pak Lacking a Peak at 281 m/z at ~ 12.000 min



Appendix 5.1. – *G. xylinus* Cells in the Absence of CBI28 Extracted Using the HLB SPE-Pak Lacking a Peak at 281 m/z at 12.106 min

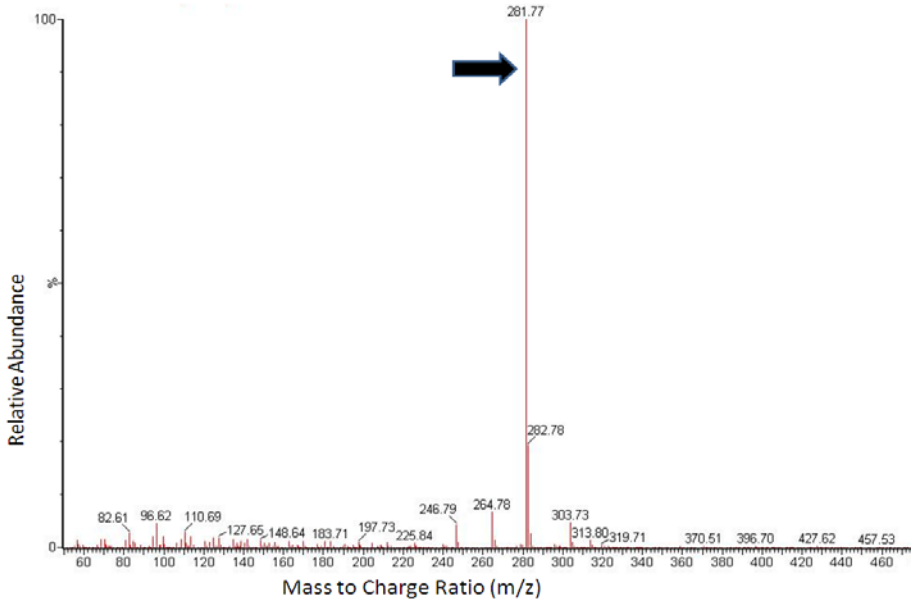
The mass spectrum shows the absence of a characteristic ion of 281 m/z at a time of 12.106 min. This run was used as a control to show the lack of the ion in *G. xylinus* cells in the absence of CBI28 when compared to experimental samples that were exposed to CBI28. There are other ions present in abundant concentrations that occurred only in this run or were common in all runs. These ions may come from the large amount of metabolites in cells which made analysis more difficult. The mass spectrum is derived from a chromatogram where *G. xylinus* cells in the absence of CBI28, lysed and then eluted using a uniquely developed gradient elution method for this metabolomic analysis. The method uses 10 mM ammonium acetate and methanol. The extraction was performed with a Waters Oasis HLB SPE-Pak to extract any hydrophobic metabolites. The method for extraction was the standard method for extraction using the HLB SPE-Pak provided by Waters Corporation (2008).



Appendix 5.2. – *G. xylinus* Cells in the Absence of CBI28 Extracted Using Methanol and Lacking Peak at 281 m/z at 12.255 min

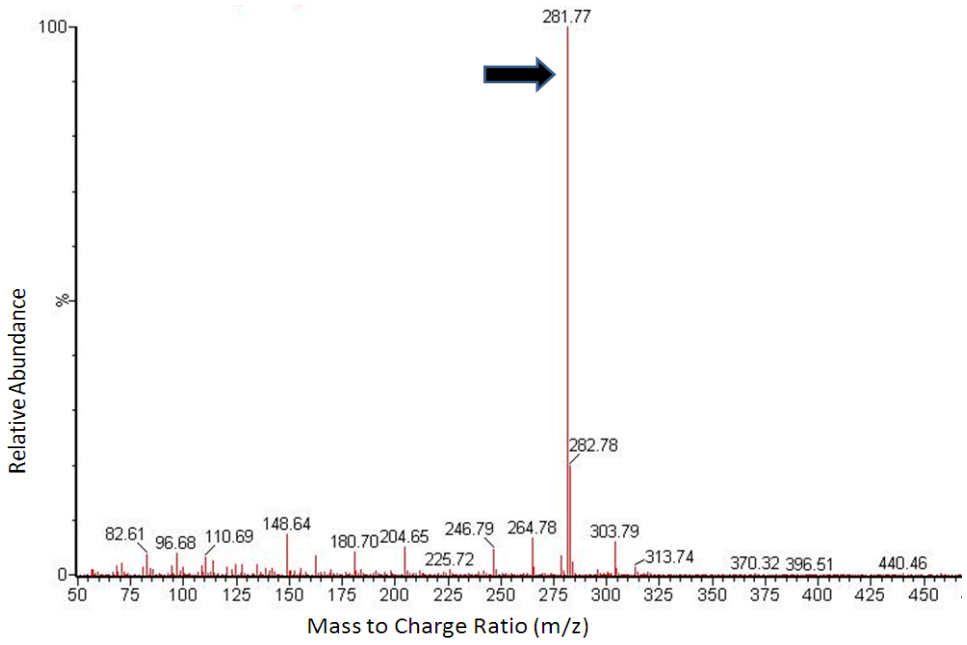
The mass spectrum shows the absence of a characteristic ion of 281 m/z at a time of 12.255 min. This run was used as a control to show the lack of the ion in *G. xylinus* cells in the absence of CBI28 when compared to experimental samples that were exposed to CBI28. There are other ions present in abundant concentrations that occurred only in this run or were common in all runs. These ions may come from the large amount of metabolites in cells which made analysis more difficult. The mass spectrum is derived from a chromatogram where *G. xylinus* cells in the absence of CBI28, lysed and then eluted using a uniquely developed gradient elution method for this metabolomic analysis. The method uses 10 mM ammonium acetate and methanol. The method for extraction was adding methanol to cell lysates and then extracting methanol and evaporating off excess methanol. The methanol extraction was used to extract hydrophobic metabolites. The mass spectrum also compares the two different extraction methods. There is no difference between methanol and Waters Oasis HLB SPE-Pak extraction.

Appendix 6 – Mass Spectra of Putative Metabolite with Peak at 281 m/z from Waters Oasis HLB SPE-Pak Extraction at ~ 12.000 min



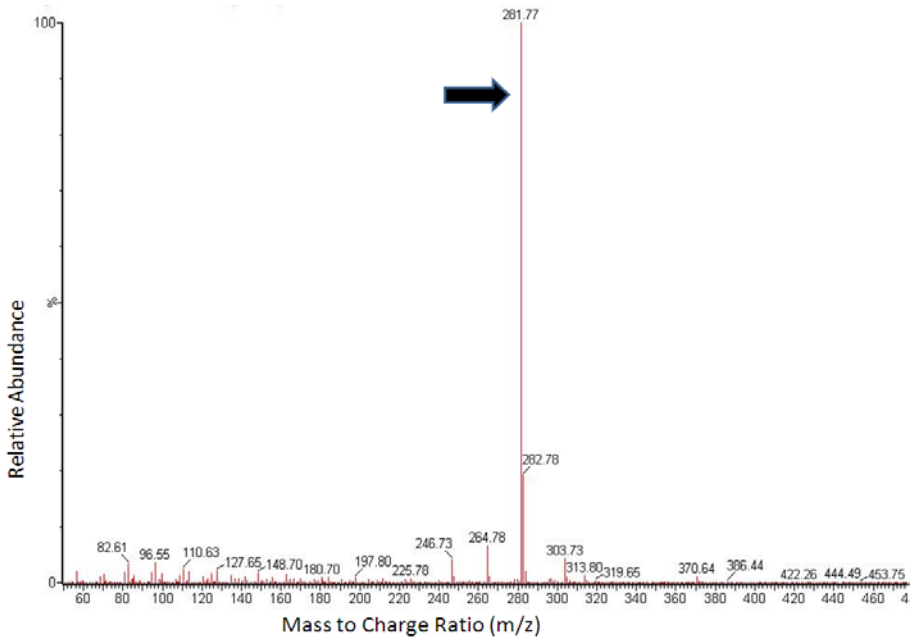
Appendix 6.1. – Putative Metabolite with Peak at 281 m/z from HLB Extraction at 12.193 min

The mass spectrum is derived from a chromatogram where *G. xylinus* cells were exposed to 10 µM of CBI28, lysed and then eluted using a uniquely developed gradient elution method for this metabolomic analysis. The method uses 10 mM ammonium acetate and methanol. The extraction was performed with a Waters Oasis HLB SPE-Pak to extract any hydrophobic metabolites. The method for extraction was the standard method for extraction using the HLB SPE-Pak provided by Waters Corporation (2008). The mass spectrum shows an ion of 281.77 m/z at a time of 12.193 min. The 281 m/z ion only occurs in samples that were exposed to CBI28 and not in samples in the absence of CBI28. This may suggest that this ion is a putative metabolite and may contribute to cellulose biosynthesis inhibition. The ion at 281 m/z is also present in methanol and Waters Oasis SPE-Pak extractions at approximately the same time. There are small time differences between samples which may be due to natural variation in the instrumentation and samples.



Appendix 6.2. – Replicate Putative Metabolite with Peak at 281 m/z from HLB Extraction at 11.984 min

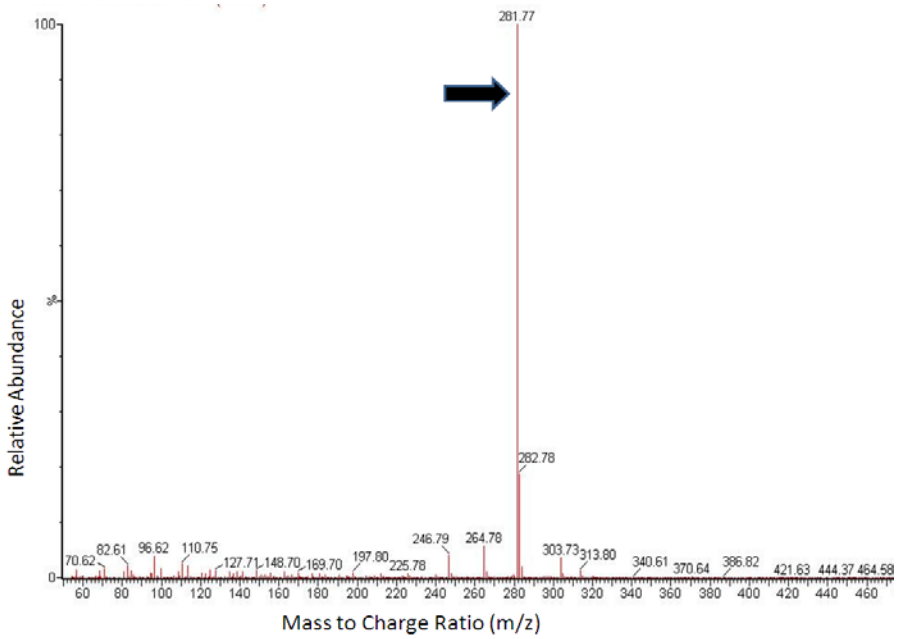
The mass spectrum is derived from a chromatogram where *G. xylinus* cells were exposed to 10 CBI28, lysed and then eluted using a uniquely developed gradient elution method for this metabolomic analysis The method uses 10 mM ammonium acetate and methanol. The mass spectrum is also a replicate for cells extracted with the Waters Oasis SPE-Pak exposed to 10 µM CBI28 giving rise to an ion of 281 m/z at approximately 12.00 min. The extraction was performed with a Waters Oasis HLB SPE-Pak to extract any hydrophobic metabolites. The method for extraction was the standard method for extraction using the HLB SPE-Pak provided by Waters Corporation (2008). The mass spectrum shows an ion of 281.77 m/z at a time of 11.984 min. The 281 m/z ion only occurs in samples that were exposed to CBI28 and not in samples in the absence of CBI28. This may suggest that this ion is a putative metabolite and may contribute to cellulose biosynthesis inhibition. The ion at 281 m/z is also present in methanol and Waters Oasis SPE-Pak extractions at approximately the same time. There are small time differences between samples which may be due to natural variation in the instrumentation and samples.



Appendix 6.3. – Replicate Putative Metabolite with Peak at 281 m/z from HLB Extraction 11.870 min

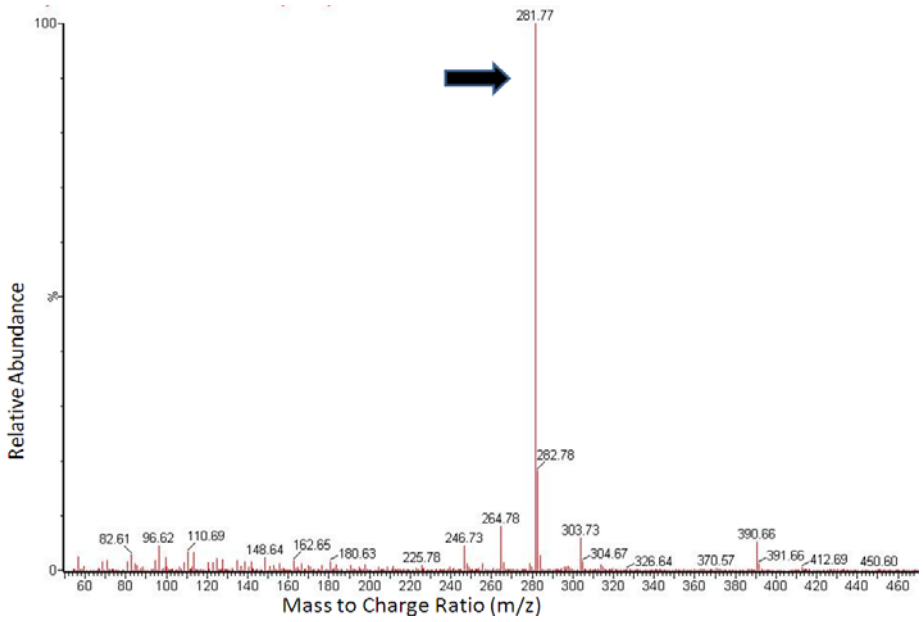
The mass spectrum is derived from a chromatogram where *G. xylinus* cells were exposed to 10 CBI28, lysed and then eluted using a uniquely developed gradient elution method for this metabolomic analysis. The method uses 10 mM ammonium acetate and methanol. The mass spectrum is also a replicate for cells extracted with the Waters Oasis SPE-Pak exposed to 10 µM CBI28 giving rise to an ion of 281 m/z at approximately 12.00 min. . The extraction was performed with a Waters Oasis HLB SPE-Pak to extract any hydrophobic metabolites. The method for extraction was the standard method for extraction using the HLB SPE-Pak provided by Waters Corporation (2008). The mass spectrum shows an ion of 281.77 m/z at a time of 11.870 min. The 281 m/z ion only occurs in samples that were exposed to CBI28 and not in samples in the absence of CBI28. This may suggest that this ion is a putative metabolite and may contribute to cellulose biosynthesis inhibition. This ion may be the active, soluble form of CBI28 that causes cellulose biosynthesis inhibition. The ion at 281 m/z is also present in methanol and Waters Oasis SPE-Pak extractions at approximately the same time. There are small time differences between samples which may be due to natural variation in the instrumentation and samples.

Appendix 7 – Mass Spectra of Putative Metabolite with Peak at 281 m/z at ~ 12.000 min from Methanol Extraction



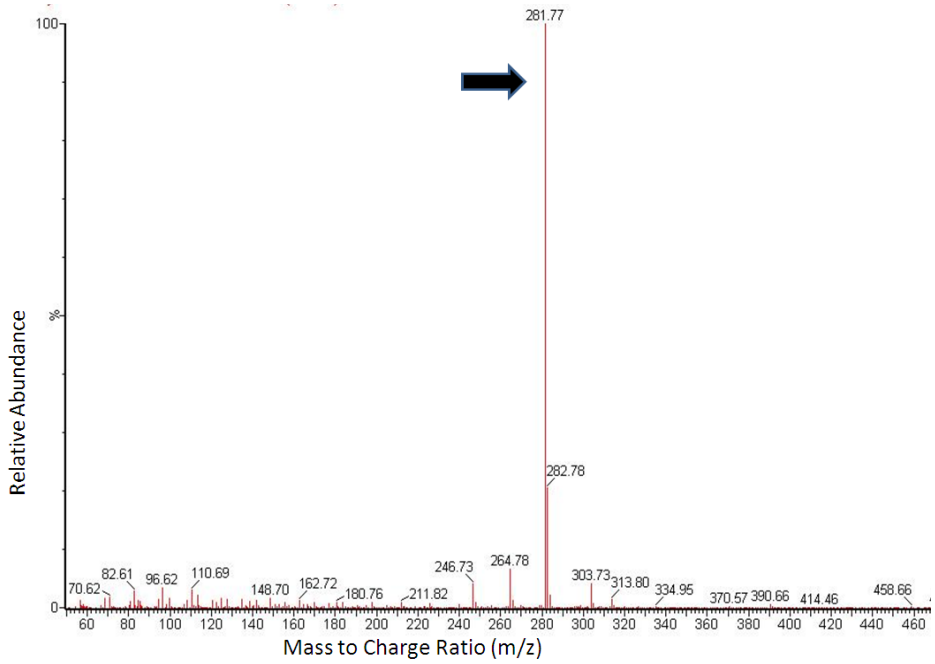
Appendix 7.1. – Putative Metabolite with Peak at 281 m/z at 11.835 min from Methanol Extraction

The mass spectrum is derived from a chromatogram where *G. xylinus* cells were exposed to 10 CBI28, lysed and then eluted using a uniquely developed gradient elution method for this metabolomic analysis. The method uses 10 mM ammonium acetate and methanol. The method for extraction was adding methanol to cell lysates and then extracting methanol and evaporating off excess methanol. The methanol extraction was used to extract hydrophobic metabolites. The mass spectrum also compares the two different extraction methods. There is no difference between methanol and Waters Oasis HLB SPE-Pak extraction. The mass spectrum shows a peak of 281.77 m/z at a time of 11.835 min. The ion only occurs in experimental samples that are exposed to CBI28 and not in samples in the absence of CBI28. The ion of 281.77 m/z may represent a putative metabolite that may cause cellulose inhibition. This ion may also be the soluble, active form of CBI28 that causes cellulose biosynthesis inhibition. The 281.77 m/z ion is present in both extraction methods at approximately the same time. Differences in time may be to inherent variance in instrumentation and samples but the mass of the ion remains the same and the time of detection is approximately 12.00 min.



Appendix 7.2. – Replicate Putative Metabolite with Peak at 281 m/z at 11.748 from Methanol Extraction

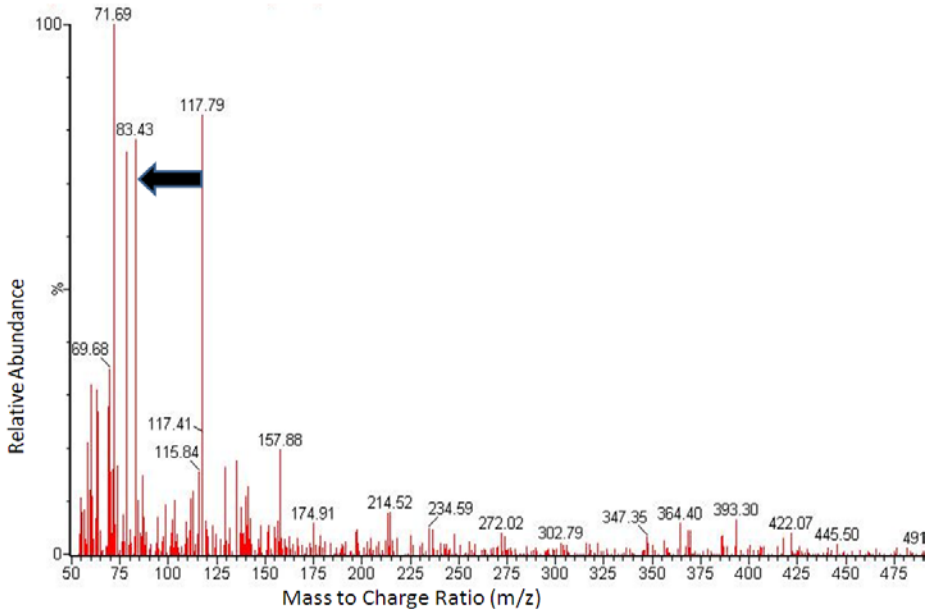
The mass spectrum is derived from a chromatogram where *G. xylinus* cells were exposed to 10 CBI28, lysed and then eluted using a uniquely developed gradient elution method for this metabolomic analysis. The method uses 10 mM ammonium acetate and methanol. The method for extraction was adding methanol to cell lysates and then extracting methanol and evaporating off excess methanol. The methanol extraction was used to extract hydrophobic metabolites. The mass spectrum also compares the two different extraction methods. There is no difference between methanol and Waters Oasis HLB SPE-Pak extraction. The mass spectrum shows a peak of 281.77 m/z at a time of 11.748 min. This mass spectrum is a replicate of the methanol extraction that yielded the 281.77 m/z ion. The ion only occurs in experimental samples that are exposed to CBI28 and not in samples in the absence of CBI28. The ion of 281.77 m/z may represent a putative metabolite that may cause cellulose inhibition. This ion may also be the soluble, active form of CBI28 that causes cellulose biosynthesis inhibition. The 281.77 m/z ion is present in both extraction methods at approximately the same time. Differences in time may be to inherent variance in instrumentation and samples but the mass of the ion remains the same and the time of detection is approximately 12.00 min.



Appendix 7.3. – Replicate Putative Metabolite with Peak at 281 m/z at 11.809 min from Methanol Extraction

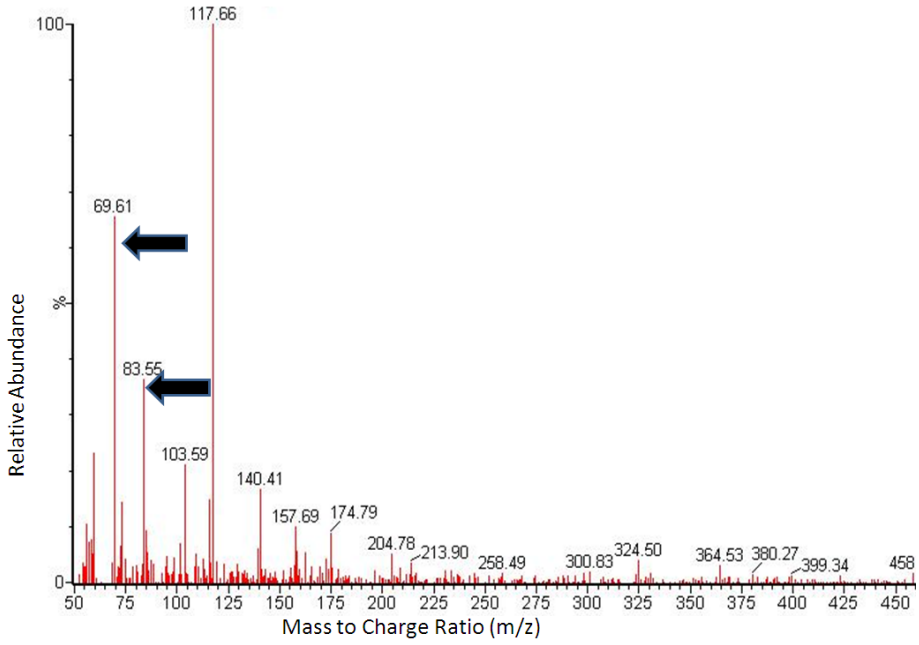
The mass spectrum is derived from a chromatogram where *G. xylinus* cells were exposed to 10 CBI28, lysed and then eluted using a uniquely developed gradient elution method for this metabolomic analysis. The method uses 10 mM ammonium acetate and methanol. The method for extraction was adding methanol to cell lysates and then extracting methanol and evaporating off excess methanol. The methanol extraction was used to extract hydrophobic metabolites. The mass spectrum also compares the two different extraction methods. There is no difference between methanol and Waters Oasis HLB SPE-Pak extraction. The mass spectrum shows a peak of 281.77 m/z at a time of 11.809 min. This mass spectrum is a replicate of the methanol extraction that yielded the 281.77 m/z ion. The ion only occurs in experimental samples that are exposed to CBI28 and not in samples in the absence of CBI28. The ion of 281.77 m/z may represent a putative metabolite that may cause cellulose inhibition. This ion may also be the soluble, active form of CBI28 that causes cellulose biosynthesis inhibition. The 281.77 m/z ion is present in both extraction methods at approximately the same time. Differences in time may be to inherent variance in instrumentation and samples but the mas of the ion remains the same and the time of detection is approximately 12.00 min

Appendix 8 – Mass Spectra of Putative Benzene Fragment at ~ 1.700 min with Peak at ~ 83 m/z from Waters Oasis HLB SPE-Pak Extractions



Appendix 8.1. – Putative Benzene Fragment at 1.775 min with Peak at 83 m/z from HLB Extraction

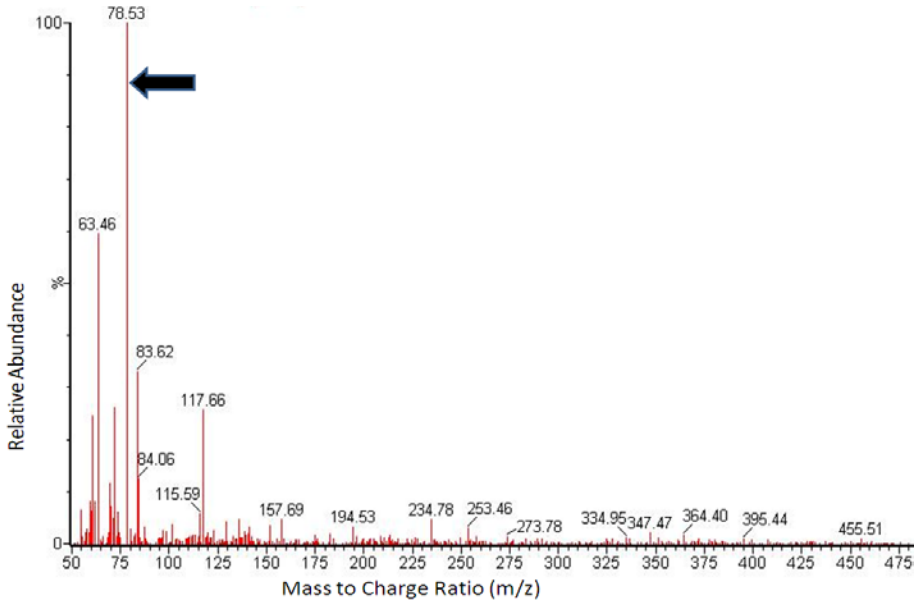
The mass spectrum is derived from a chromatogram where *G. xylinus* cells were exposed to 10 µM of CBI28, lysed and then eluted using a uniquely developed gradient elution method for this metabolomic analysis. The method uses 10 mM ammonium acetate and methanol. The extraction was performed with a Waters Oasis HLB SPE-Pak to extract any hydrophobic metabolites. The method for extraction was the standard method for extraction using the HLB SPE-Pak provided by Waters Corporation (2008). The mass spectrum shows an 83 m/z ion at a time of 1.775 min. The ion occurs in experimental samples exposed to CBI28 and not in samples in the absence of CBI28. The 83.43 m/z ion has a mass that is similar in size to benzene molecule. CBI28 has two benzene molecules in its structure which may represent a fragment of CBI28. The ion may also be a novel metabolite that may contribute to cellulose biosynthesis inhibition. There are several other ions in the sample that are close in mass to charge ratio. These ions are not consistent in other mass spectrum and may represent noise or the diverse set of metabolites in a cell. There is large variance with this ion. The ion with a mass of 117.79 m/z is present in higher levels in the samples extracted with the Waters Oasis HLB SPE-Pak as compared to the methanol extraction.



Appendix 8.2. – Replicate Putative Benzene Fragment at 1.662 min with Peak at 83 m/z and 69 m/z from HLB Extraction

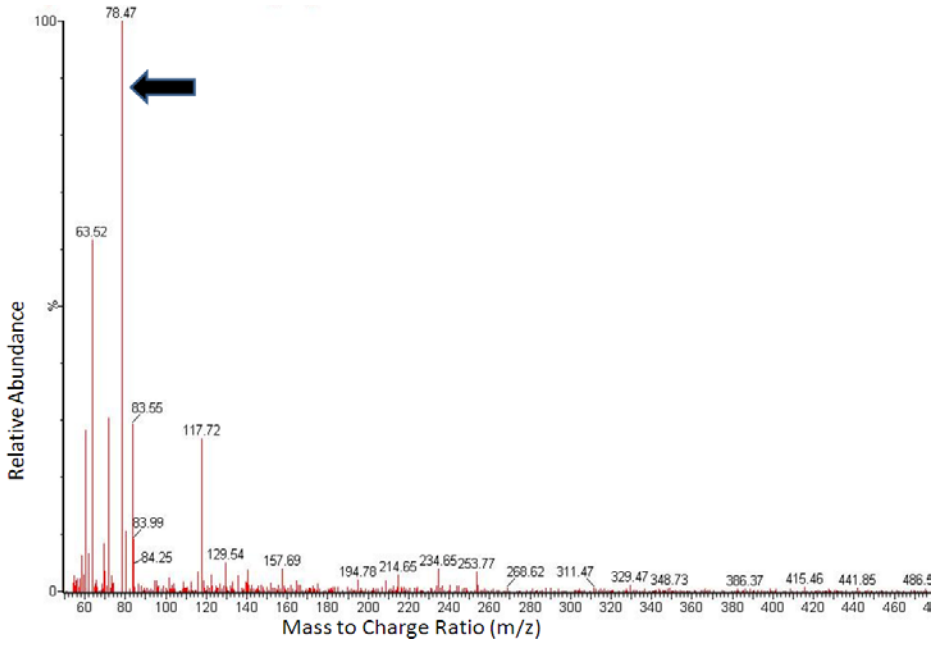
The mass spectrum is derived from a chromatogram where *G. xylinus* cells were exposed to 10 µM of CBI28, lysed and then eluted using a uniquely developed gradient elution method for this metabolomic analysis. The method uses 10 mM ammonium acetate and methanol. The extraction was performed with a Waters Oasis HLB SPE-Pak to extract any hydrophobic metabolites. The method for extraction was the standard method for extraction using the HLB SPE-Pak provided by Waters Corporation (2008). This mass spectrum is also a replicate for the Waters Oasis HLB SPE-Pak extraction that yielded the 83 m/z ion. The mass spectrum shows an 83 m/z ion at a time of 1.662 min. The ion occurs in experimental samples exposed to CBI28 and not in samples in the absence of CBI28. The 83.55 m/z and 69.61 m/z ion has a mass that is similar in size to benzene molecule. CBI28 has two benzene molecules in its structure which may represent a fragment of CBI28. The ion may also be a novel metabolite that may contribute to cellulose biosynthesis inhibition. There are several other ions in the sample that are close in mass to charge ratio. These ions are not consistent in other mass spectrum and may represent noise or the diverse set of metabolites in a cell. There is large variance with this ion. The 117.66 m/z is present in higher levels in samples extracted with the Waters Oasis HLB SPE-Pak as compared to the methanol extraction.

Appendix 9 – Mass Spectra of Putative Benzene Fragment at ~ 1.700 min with a Peak at ~ 78 m/z from the Methanol Extraction



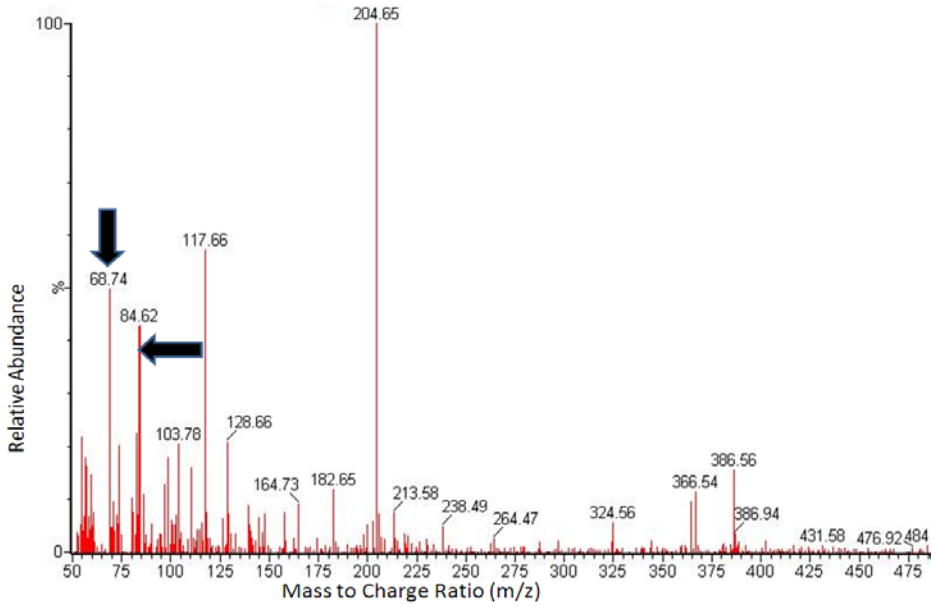
Appendix 9.1. – Putative Benzene Fragment at 1.779 min with a Peak at 78.53 m/z from the Methanol Extraction

The mass spectrum is derived from a chromatogram where *G. xylinus* cells were exposed to 10 CBI28, lysed and then eluted using a uniquely developed gradient elution method for this metabolomic analysis. The method uses 10 mM ammonium acetate and methanol. The method for extraction was adding methanol to cell lysates and then extracting methanol and evaporating off excess methanol. The methanol extraction was used to extract hydrophobic metabolites. The mass spectrum also compares the two different extraction methods. There was less noise in this mass spectrum which is not consistent for the design protocol for the Waters Oasis HLB SPE-Pak. The methanol extraction may have omitted some ions or did not achieve high enough concentrations to be detected. The mass spectrum also shows an ion with a 78.53 m/z at 1.779 min. CBI28 has two benzene molecules in its structure which may represent a fragment of CBI28. The ion may also be a novel metabolite that may contribute to cellulose biosynthesis inhibition. There are several other ions in the sample that are close in mass to charge ratio. These ions are not consistent in other mass spectrum and may represent noise or the diverse set of metabolites in a cell. There is little difference in the time the ions were detected but there is variance between the mass to charge ratio of the ions. The 117.66 m/z ion is in lower abundance than in samples extracted with the Waters Oasis HLB SPE-Pak.



Appendix 9.2. – Replicate Putative Benzene Fragment at 1.758 min with Peak at 78.47 m/z from the Methanol Extraction

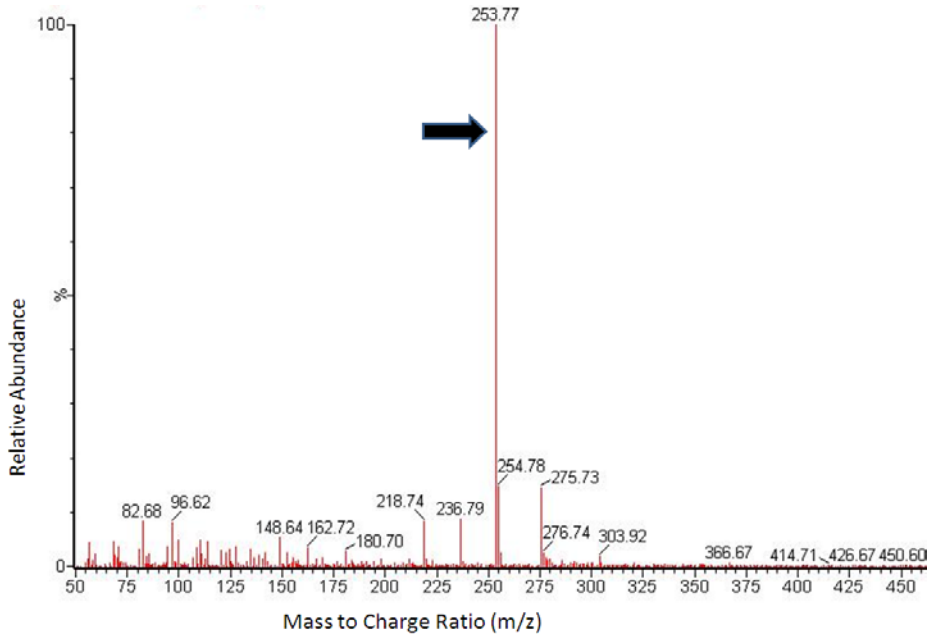
The mass spectrum is derived from a chromatogram where *G. xylinus* cells were exposed to 10 CBI28, lysed and then eluted using a uniquely developed gradient elution method for this metabolomic analysis. The method uses 10 mM ammonium acetate and methanol. The method for extraction was adding methanol to cell lysates and then extracting methanol and evaporating off excess methanol. The methanol extraction was used to extract hydrophobic metabolites. This mass spectrum is a replicate of the run and mass spectrum that yielded a ~78 m/z using the methanol extraction. The mass spectrum also compares the two different extraction methods. There was less noise in this mass spectrum which is not consistent for the design protocol for the Waters Oasis HLB SPE-Pak. The methanol extraction may have omitted some ions or did not achieve high enough concentrations to be detected. The mass spectrum also shows an ion with a 78.47 m/z at 1.758 min. CBI28 has two benzene molecules in its structure which may represent a fragment of CBI28. The ion may also be a novel metabolite that may contribute to cellulose biosynthesis inhibition. There are several other ions in the sample that are close in mass to charge ratio. These ions are not consistent in other mass spectrum and may represent noise or the diverse set of metabolites in a cell. There is little difference in the time the ions were detected but there is variance between the mass to charge ratio of the ions. The 117.72 m/z ion is in lower abundance than in samples that were extracted using the Waters Oasis HLB SPE-Pak.



Appendix 9.3. – Putative Benzene Fragment at 1.627 min with Peak at 84.62 m/z and 68.74 m/z from Methanol Extraction

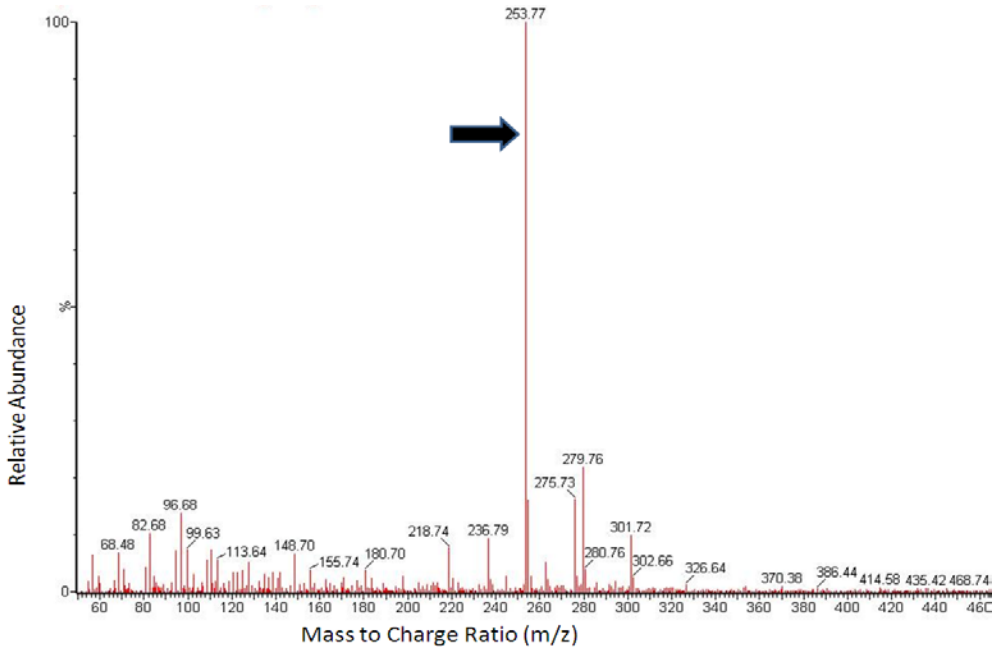
The mass spectrum is derived from a chromatogram where *G. xylinus* cells were exposed to 10 CBI28, lysed and then eluted using a uniquely developed gradient elution method for this metabolomic analysis. The method uses 10 mM ammonium acetate and methanol. The method for extraction was adding methanol to cell lysates and then extracting methanol and evaporating off excess methanol. The methanol extraction was used to extract hydrophobic metabolites. This mass spectrum is a replicate of the run and mass spectrum that yielded a ~78 m/z using the methanol extraction. The mass spectrum also compares the two different extraction methods. There was less noise in this mass spectrum which is not consistent for the design protocol for the Waters Oasis HLB SPE-Pak. The methanol extraction may have omitted some ions or did not achieve high enough concentrations to be detected. The mass spectrum also shows an ion with a 84.62 m/z and a 68.74 m/z at 1.627 min. CBI28 has two benzene molecules in its structure which may represent a fragment of CBI28. The ion may also be a novel metabolite that may contribute to cellulose biosynthesis inhibition. There are several other ions in the sample that are close in mass to charge ratio. These ions are not consistent in other mass spectrum and may represent noise or the diverse set of metabolites in a cell. There is little difference in the time the ions were detected but there is variance between the mass to charge ratios of the ions. There is a 204.85 m/z ion in high abundance that does not appear in other samples as well as a 117.66 m/z ion in higher abundance than in samples extracted with the Waters Oasis HLB SPE-Pak.

Appendix 10 – Representative Mass Spectra of a Common 253 m/z Ion in the Absence of CBI28 and in *G.xylinus* Cells Exposed to 10 µM CBI28 and Extracted Using Methanol and the Waters Oasis HLB SPE-Pak



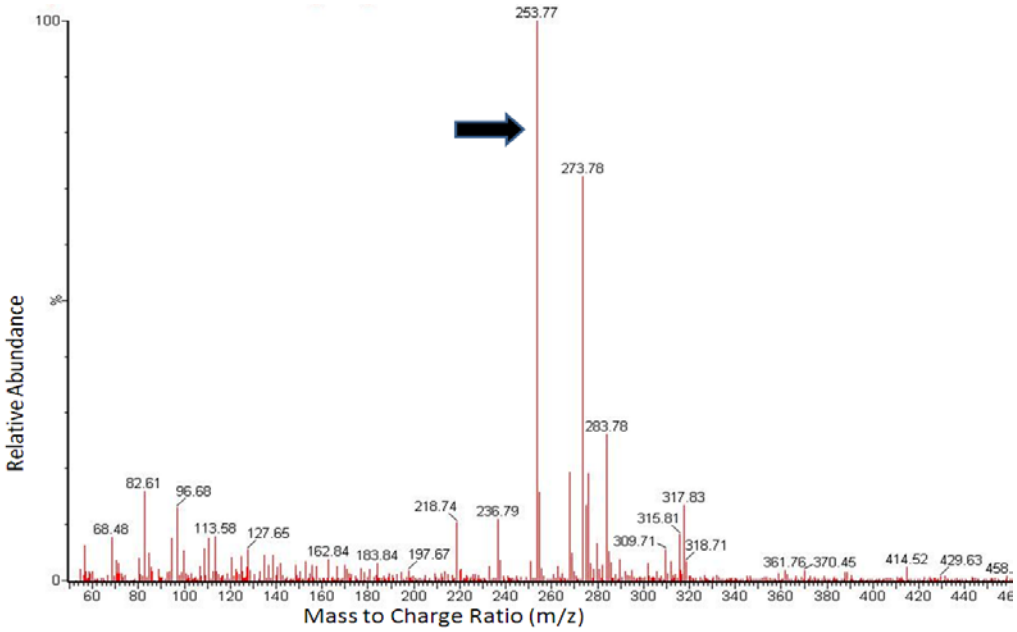
Appendix 10.1. – Representative Samples in the Absence of CBI28 Showing Peak at 253.77 m//z at 11.616 min Common in All Samples

The mass spectrum represents common masses that occur in all samples. The 253.77 m/z ion occurs at 11.616 min. This sample was extracted and processed in the absence of CBI28 by both methanol and the Waters Oasis HLB SPE-Pak. Both extraction methods yielded the same ion of ~253 m/z at approximately 11.600 min. There is also no difference between samples exposed to CBI28 or not. The ~ 253.77 m/z ion appears to be in all samples and is mostly likely present because it is a metabolite derived from *G. xylinus* cell lysates. Since it is present in all samples it is probably a metabolite from the cells that are being processed. The 253.77 m/z ion is clearly present in a much higher abundance than other ions that are seen inconsistently in other sample replicates.



Appendix 10.2. – Representative HLB Extracted Sample Exposed to 10 µM CBI28 Showing Common Peak at 253 m/z at 11.555 min

The mass spectrum represents common masses that occur in all samples. The 253.77 m/z ion occurs at 11.555 min. The *G. xylinus* cells were exposed to 10 µM CBI28 then lysed and extracted by the Waters Oasis HLB SPE-Pak. The samples were then eluted using a uniquely developed gradient elution method for this metabolomic analysis. The method uses 10 mM ammonium acetate and methanol. The extraction was performed with a Waters Oasis HLB SPE-Pak to extract any hydrophobic metabolites. The method for extraction was the standard method for extraction using the HLB SPE-Pak provided by Waters Corporation (2008). Both extraction methods yielded the same ion of ~253 m/z at approximately 11.600 min. There is also no difference between samples exposed to CBI28 or not. The ~ 253.77 m/z ion appears to be in all samples and is mostly likely present because it is a metabolite derived from *G. xylinus* cell lysates. Since it is present in all samples it is probably a metabolite from the cells that are being processed. There was also little noise in these samples as compared to the methanol extraction which demonstrates the superior efficiency and specificity of the Waters Oasis HLB SPE-Pak. The 253.77 m/z ion is clearly present in a much higher abundance than other ions that are seen inconsistently in other sample replicates.



Appendix 10.3. – Representative Methanol Extracted Sample Exposed to 10 µM CBI28 with Peak at 253 m/z at 11.293 min Common in All Samples

The mass spectrum represents common masses that occur in all samples. The 253.77 m/z ion occurs at 11.293 min. *G. xylinus* cells were exposed to 10 CBI28 lysed and then eluted using a uniquely developed gradient elution method for this metabolomic analysis. The method uses 10 mM ammonium acetate and methanol. The method for extraction was adding methanol to cell lysates and then extracting methanol and evaporating off excess methanol. The methanol extraction was used to extract hydrophobic metabolites. Both extraction methods yielded the same ion of ~253 m/z at approximately 11.600 min. There is also no difference between samples exposed to CBI28 or not. The ~ 253.77 m/z ion appears to be in all samples and is mostly likely present because it is a metabolite derived from *G. xylinus* cell lysates. Since it is present in all samples it is probably a metabolite from the cells that are being processed. There was more noise in the methanol extracted samples which may be due to other metabolites and the superior efficiency and specificity of the Waters Oasis HLB SPE-Pak. The 253.77 m/z ion is clearly present in a much higher abundance than other ions that are seen inconsistently in other sample replicates.