# Novel chemical approaches in CHO sialic acid analog glycoengineering

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# Abstract

Biophamaceuticals promise a wide variety of benefits to modern medicine. The ability to produce highly effective, low-cost treatments for genetic diseases and other illnesses places a high degree of importance on biopharmaceutical techniques. Acting in concert or as an analogous replacement, biopharmaceutical proteins are a bold, promising technology poised to have major impacts on human health. Glycoproteins are a particularly well-researched biotherapeutics class that display high specificity and efficacy in treating numerous diseases.

Bulk production of protein therapeutics requires careful monitoring of many factors. Cell type, media quantification, and glycosylation patterning are all relevant points of analysis. Glycosylation in particular, how sugar structures are assembled on amino acid residues, is an extremely important regulatory mechanism. Chinese hamster ovary cells are often used in order to provide a viable production platform with human-similar glycosylation.

Within glycosylation, it has been found that terminal sialic acid (N-acetylneuraminic acid, NeuAc, NeuGc) content on the glycan correlates with many positive in vivo effects, including increased half-life, reduced clearance, and more direct site recognition. Because of the importance of terminal sialic acid, many techniques exploit differing aspects of cell biology in order to increase the volume of terminal sialic acid. We study the effects of chemical biology and the utilization of analogs of ManNAc, a sialic acid precursor commonly used to increase available cellular sialic acid as a biochemical approach to promote sialic acid availability.


Advisor: Dr. Michael Betenbaugh

Reader: Dr. Kevin Yarema

## Acknowledgements

As with any work of this magnitude, it would have been impossible to get as far as I have without the generous help of my peers and supervisors.

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Ruby, Abby, and Adam, I'd like to thank for being excellent support when it came to the logistics of managing my experience. Thank you for eagerly and willingly providing extra cells and keeping the machine running when I've been occupied with other tasks. I expect great things from all of you!

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## Table of Contents

## List of Figures

## List of Tables

# Chapter 1: Introduction

Biopharmaceuticals, drugs of origin from a living organism, showed $100 billion in 2010 earnings, a trend which shows no inclination towards slowing down. [47] Broadly, biopharmaceuticals consist of the classes of proteins, hormones, and other complex macromolecules that are difficult to synthesize without the specialized biological equipment inherent in living cells. [16, 19]

In contrast to traditional, lab-synthesized chemical pharmaceuticals, biopharmaceutical provide several useful advantages, which include known activity, controlled side effects, cost effectiveness, as well as high specificity and effectiveness. [40] Drugs such as Remicade and Rituxan provide relief in ways that dramatically improve the quality of life in their recipients. [39, 53, 58] In fact, many engineered proteins are designed to improve upon native traits inherent to their standard function. "Biosuperiors," are hoped to act in place of their original incarnation, and achieve even better results. [33, 38]

These benefits are not without drawbacks. Biopharmaceuticals are often cleared very quickly from serum, and can suffer from low activity. [21, 24] However, a common feature these drugs share lies in their post-translational modification. Over two-thirds of biopharmaceutical proteins are heavily glycosylated. Glycosylation refers to sugar structures built up from protein residues, and the uniqueness of these outer shells are responsible for many aspects of solubility, activity, serum half-life, and structural stability. [12, 18]

In order to conserve the glycan structures and thereby valued aspects of the produced proteins, it is important to utilize a platform which can produce the protein with high fidelity, volume, and speed. Due to the importance of a protein's glycosylation, a common optimization

goal in biopharmaceutical production is the act of "humanizing" a protein by modifying labeling structures called glycans. [21, 63]

Chinese hamster ovary cells are a valued platform used to produce upwards of 80% of on-market human protein therapeutics. The reasons for the use of these particular cells, opposed to humans, are due to their low maintenance, efficient growth rate, and highly conserved mechanisms that produce human-like glycosylation patterns. [7, 52, 54]

## *1.1 Cell Culture*

In order to produce a broad volume of a desired therapeutic, it is important to utilize a platform which expresses human-mimetic proteins, and produces them at a high rate. Cell culture has proven to be a reliable way to produce bulk scale proteins. [61]

Broadly, cell culture refers to the process of growing cells within a liquid medium, rather than harvesting them from a treated animal. The medium is then supplemented with nutritional items or other tools used to parameterize various aspects of cellular metabolism or protein throughput. [1, 42] Cells in the liquid medium are then maintained under a variety of conditions of agitation, temperature, $O_2$ and $CO_2$ feeds. [50] Mammalian cells are typically grown in media supplemented with essential amino acids which cannot be synthesized by the cells, as well as numerous buffers and drugs. [40, 44]

Chinese hamster ovary (CHO) is a commonly used platform for expressing human proteins. This is due to multiple factors. The primary factor is low maintenance. CHO are robust and can be used for multiple conditional examinations. [2, 10] A second is the relatively low complexity of the CHO genome, allowing for clarity of genetic analysis and well-documented

knowledge regarding gene inserts and substitutions commonly used in genetic engineering for protein expression. [33]

Of interest to this work is the similarity of glycosylation machinery conserved between human subjects and CHO. [4]

## *1.2 Glycosylation*

### *1.2.1 Glycosylation Overview*

Glycans arise as a result of post-translational modification in the golgi apparatus in a process known as glycosylation. Many important protein characteristics arise by covalently attaching sugar residues in glycosidic linkages extending from an amino acid residue. [11] Glycosylation can broadly be described in two forms, contingent on the linkage between the protein's core structure and the initializing carbohydrate. N-glycosylation arises on sites with a residue sequence of Asn-X-Ser/Thr, where X can be any non-proline amino acid. [6] By contrast, O-glycosylation occurs when the initial sugar attaches to an oxygen present on a serine or threonine residue. [26]

Glycosylation regulates many important factors of a fully complete glycoprotein, including solubility, resistance to degradation, immune response, cellular signaling, adhesion, as well as protein/protein interactions and the proper structure of a protein itself. [4, 10] Many of these important characteristics are particularly controlled by the terminal (most distant from the protein) sugars on the glycan structure. Sialic acid (N-acetylneuraminic acid) is of particular note, and is the only functional carbohydrate with a negative charge. [5, 6] Sialylation refers to the addition of sialic acid to a forming glycan, and is of particular interest to bioengineers, and will be addressed in detail in further sections.

Although glycoproteins can vary considerably both in their protein structure as well as the final form of their completed glycans, all glycans share a common "core" structure of carbohydrate moieties. In particular, N-acetylglucosamine and mannose (GlcNAc and Man, respectively) consist of a vital set of linkages upon which variants in glycans form. Glycan labels are described by listing the sugar in question, and the order of carbons on the central ring structure that are interlinked. For example, the preserved core structure shows the following series of bonds and moieties: Man(α)1,3(Manα1,6) Manβ-1,4GlcNAc β-1,4GlcNAc-Asn. The descriptor Man(α)1,3(Manα1,6) refers to the linkages of the initial mannose's first carbon to the three carbon on the second mannose, as shown below in Figure 1. [11, 20]

From this core structure, regulation via additional processing and severed residues diversify glycan structures within the Golgi apparatus to the wide variety of shapes which can be used to characterize glycoproteins.

*1.2.2 Glycan Variation*

Glycan structures are characterized into complex, high mannose, and hybrid types. A schematic representation is shown of these types, as well as the core structure, below in Figure 1.

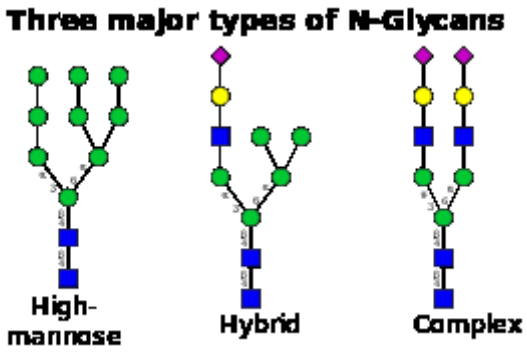



**Figure 1 Common glycoforms.** The high mannose type displays a preponderance of mannose, complex by contrast shows a wider variation of sugars. The hybrid type is mixed, and shows a mannose branch as well as an alternating, complex branch. Table 1 refers to the various labels and sugars commonly associated with glycan maps as this one. [65]

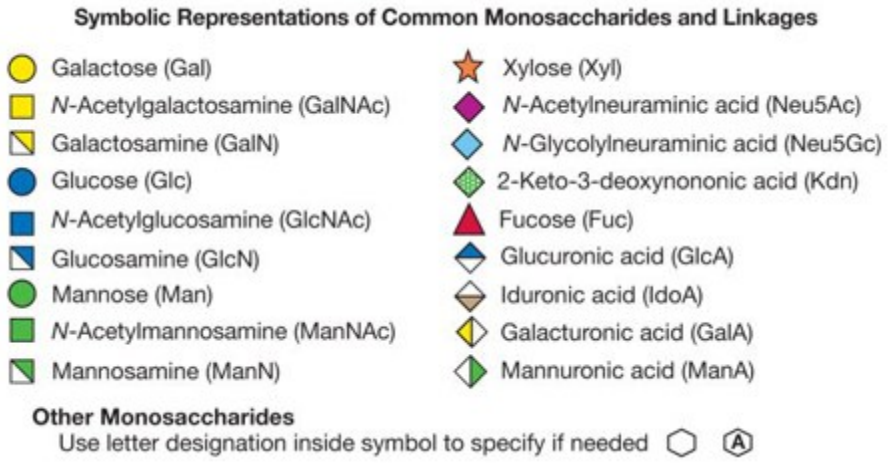
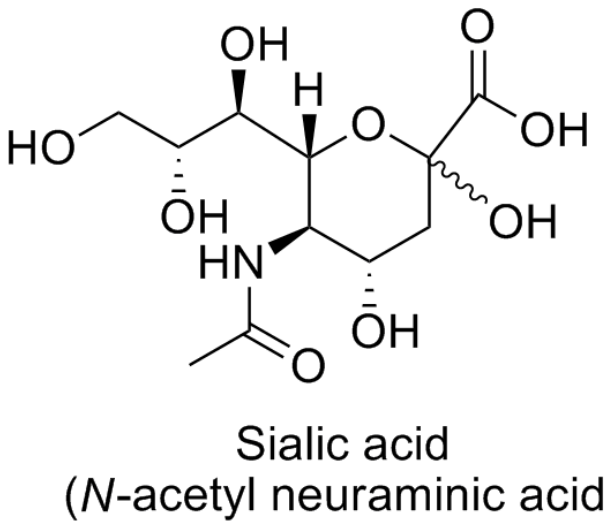



**Table 1 Schematic representation of common sugars in glycan forms.** While some sequences in glycans are relatively predictable, the table displays common sugars and their linkages within glycans. [65]

Between two and six connected mannose residues comprise high mannose glycans extending from the core structure. Hybrid type continues this overall trend, by displaying a characteristic GlcNAc β1,4 linkage to a β1,4 mannose in the pentasaccharide core. [60]

By contrast, complex types include a galactose (Gal) β1,4 bond to a β1,2-GlcNAc, and end in a terminal sialic acid (Sia) group. Structures of this type and this terminal group are produced similarly in CHO-K1 cells. [63]




Sialic acid
(*N*-acetyl neuraminic acid)

**Figure 2 N-acetyl neuraminic acid.** The most commonly occurring sialic acid (in addition to Neu5Ac and Neu5Gc, among others), *N*-acetyl neuraminic acid is responsible for many important biotherapeutics effects. [59]

### *1.2.3 Sialyation and Sialic Acid*

Sialyation is the process by which a sialic acid is linked to a terminal galactose on a glycoprotein's glycan. Sialic acids consist of a broad family of related, negatively charged, nine carbon polyhydroxylate α-keto acids, the most common of which is NeuNAc, N-acetylneuraminic acid. Sialyation correlates strongly to several very important effects on proteins of interest: increased serum halflife, immune evasion, and proper secretion of many valuable biopharmaceutical products of interest, such as erythropoietin (EPO). [56, 58]

Although sialic acid as a class promotes these positive benefits as the terminal glycan, other important things to note are that NeuNAc is not the only sialic acid incorporated into human terminal groups. Many cellular processes regulate terminal sugars, and these alternates compete with the desirable SA throughput. Sialic acid synthesis is described in Figure 3. [37, 38, 39]

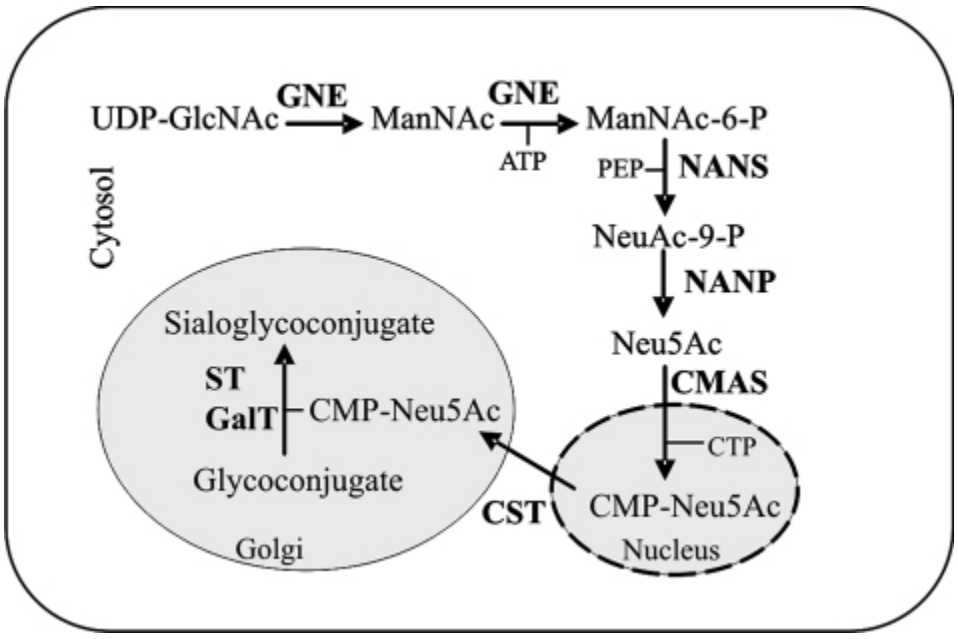



**Figure 3 Sialic Acid Pathway.** UDP-GlcNAc, ManNAc, et al are shuttled through various enzymatic processes in order to finally transform the molecules into CMP-Neu5Ac or other such SA variants, before finally being assigned to a glycan within the Golgi. [12]

An important factor in glycan formation is a cell's available pool of sialic acid. NeuNAc generation can occur from a wide variety of precursor molecules, such as ManNAc, UDP-GlcNAc, and other precursors. [6, 64] Evidence has shown that a cell's pool of a given terminal sugar correlates strongly to the degree by which that terminal sugar is displayed. [61, 64] Therefore, a viable engineering and optimization avenue is to increase the volume of available cytosolic sialic acid, and its precursors, which in turn can be translated into SA. [53, 55] Many avenues have been described to approach this end, including upregulating enzymes responsible for precursor turnover, resulting in increased flux through the pathway, and other efforts to humanize biotechnology platforms to create more human-mimetic sialic acid patterns. [42, 43, 56]

In addition to sialic acid, the patterns of branching which describe human proteins are highly specific. Not only is the actual sialic acid itself important, but the pattern as well. Engineering enzymes either to upregulate the host's throughput to desired varieties of SA, or to

mimic non-native human patterns, has proven to be a challenging, if rewarding, method of improvement. [28, 30, 37]

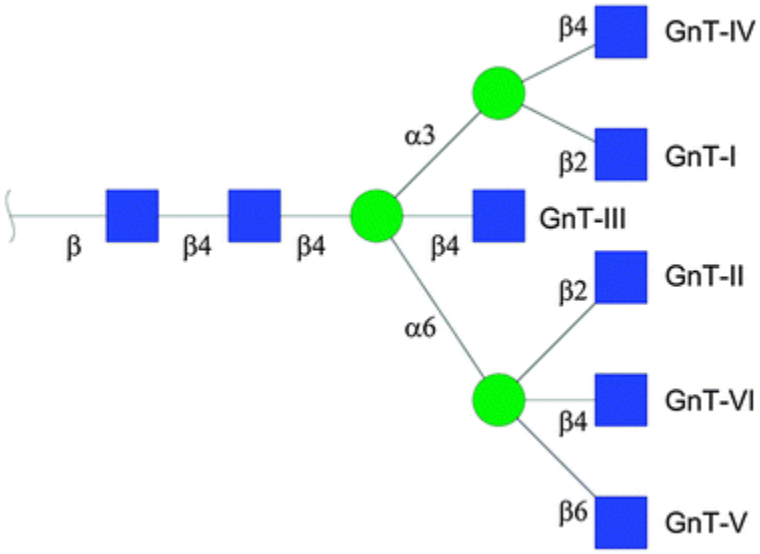



**Figure 4 Branching patterns of glycosyltransferase class enzymes (GnT).** As shown, the ability of a glycan to split into its branched linkages is contingent largely on the GnTs present in the platform. While these enzymes are native to most human cells, CHO are missing α2,6-driving GnT. [12, 33]

However, genetic engineering yields several drawbacks. The effort necessary to create a cell line with improved glycosylation mechanisms is substantial, often involving expensive reagents, in conjunction with long-term studies to confirm the stable incorporation of the gene into the cell line. [38, 48] Additionally, when it becomes necessary to repeatedly engineer enzymes related to glycosylation pathways, given an enzyme's highly specific function, the necessary time and monetary input increases substantially. [3, 12, 25]

One effective, broad-scale alternative to increase sialic acid content is through chemical means. In particular, through the molecule of interest and sialic precursor, ManNAc. [3, 9]

# Chapter Two: ManNAc Analogs

## *2.1 N-Acetyl Mannosamine monohydrate (ManNAc)*

While increasing cellularly available SA can be accomplished through many means, the direct addition of SA is inarguably the simplest in concept. In practice, mere addition of SA requires more finesse than simply adding it to a culture's media. Sialic acids as a group are ineffective at crossing the cell membrane. [3, 9, 25] However, the same is not true of their numerous precursors, such as ManNAc (N-acetyl Mannosamine monohydrate). As described above, ManNAc feeds into the SA synthesis pathway via the enzyme GNE, which converts ManNAc to ManNAc-6-Phosphate, which is in turn converted to NeuNAc-9-P by NANS, after which it follows the diagram described in Figure 3. [65]

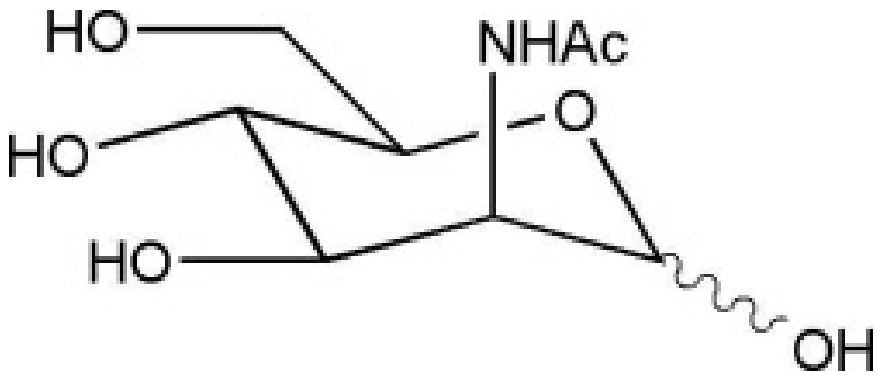



**Figure 5 N-acetylmannosamine.** ManNAc is a key precursor to sialic acid, and is a useful tool in increasing readily available SA content in a culture's cytoplasmic space. It is important because it, unlike the desired SA, is capable of crossing the plasma membrane and penetrating the cell. [37]

Like all chemical compounds, engineers have sought to improve upon ManNAc in the form of ManNAc analogs, which take the core ManNAc structure and augment them with various side chains and modified groups. [2]

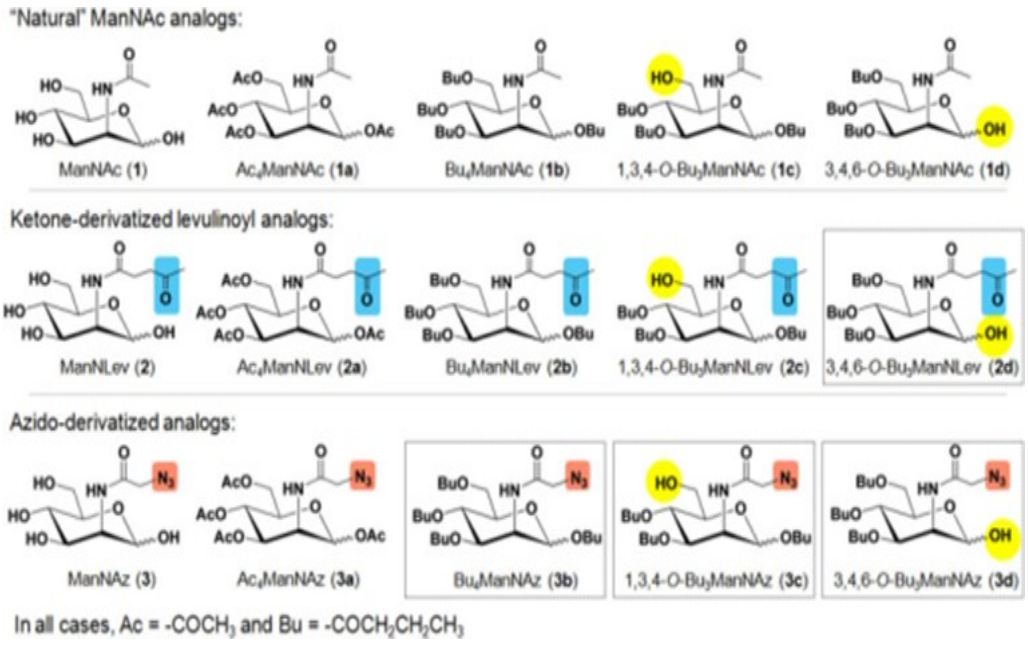



**Figure 6 Derived ManNAc analogs.** As shown, a wide variety of chemical modifications to the core ring structure as well as side groups is possible for ManNAc. These molecules are all similar enough to "standard" ManNAc that they are uptaken into the SA metabolic pathway, though they yield different effects when installed as terminal glycans. [2]

## *2.2 ManNAc Analogs*

There exist a wide variety of synthesized ManNAc analogs, all of which show varied levels of effectiveness in increasing protein SA content, promoting cell survivability, or other utility such as available azide groups for labeling or separation purposes. A variety of these analogs are displayed in Figure 6.

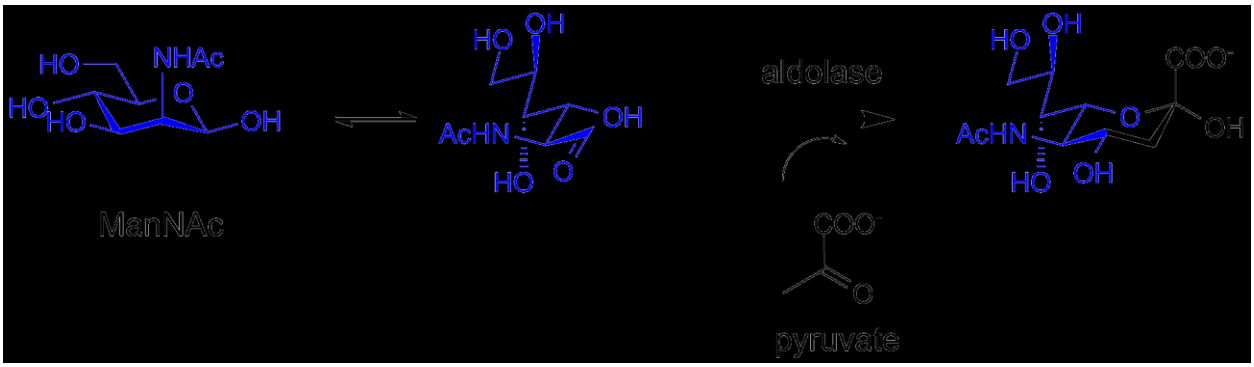



**Figure 7 Simple ManNAc Pathway.** ManNAc is capable of undergoing isomeric shifts, but the addition of pyruvate drives it into its useable stage as SA. [2]

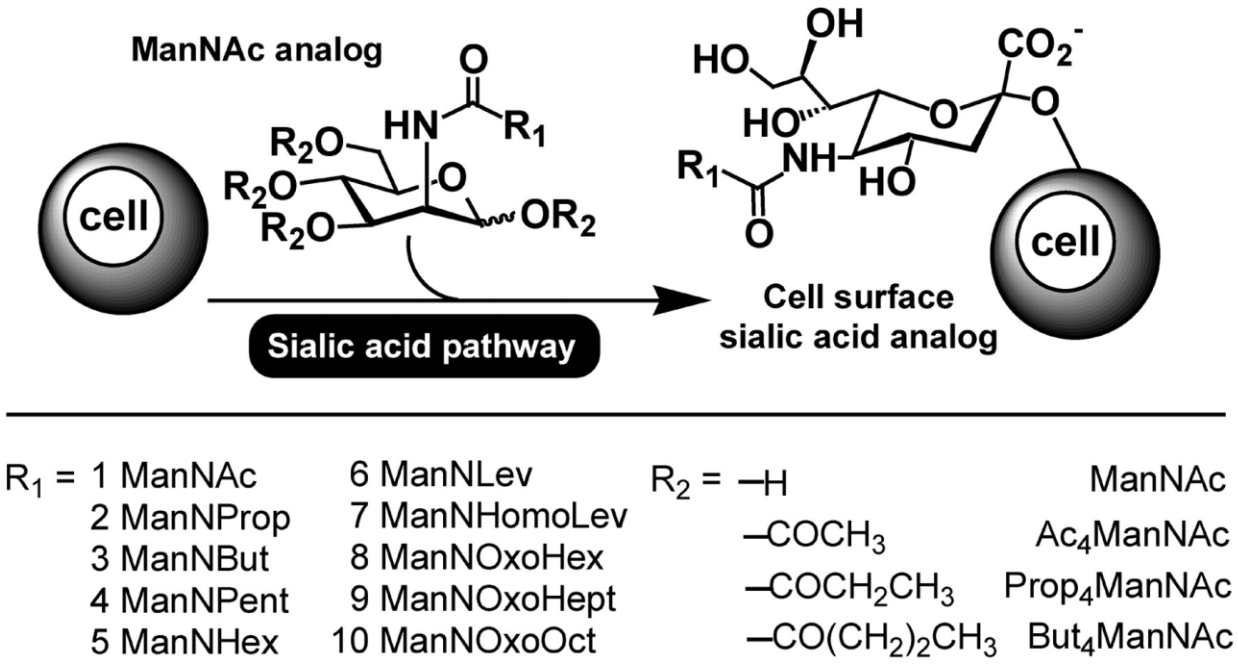



**Figure 8 Sialic acid pathway versatility.** The SA pathway is relatively non-specific provided the feed molecules follow certain constraints. As demonstrated above, many ring variants as well as side groups are all incorporated into the glycan. [54]

*2.2.1 $Ac_4$ManNAc*

$Ac_4$ManNAc has seen use in cultures in attempts to sialic acid content on glycoproteins of interest. Prior work shows that the $Ac_4$ analog accomplishes this well, but at a cost. [3] $Ac_4$ has been shown to have numerous cytotoxic effects, and its low dosing requirements correspond to a minimal effectiveness in the increase in SA content. Relative to ManNAc, it is a viable alternative particularly in that it can be synthesized without a great deal of additional difficulty and can be used at far lower volumes to achieve corresponding benefits. [24, 25]

*2.2.2 Butyrated Analogs*

Butyrate is commonly used to functionalize membrane penetrants. As a hydrophobic group, it easily crosses the cell membrane. Compared to the acetyl group, which is often associated with poisonous metabolic products, butyrate's toxicity threshold tends to be much higher. For these reasons, we examined the utility of Bu groups as additions to the ManNAc

analogs and whether or not they would be able to provide useful function over $Ac_4$ManNAc. [51]

In this study, we examined the ability of Ac4 and two proposed competitor molecules: 1,3,4-O-$Bu_3$ManNAc and $Bu_4$ManNAc.

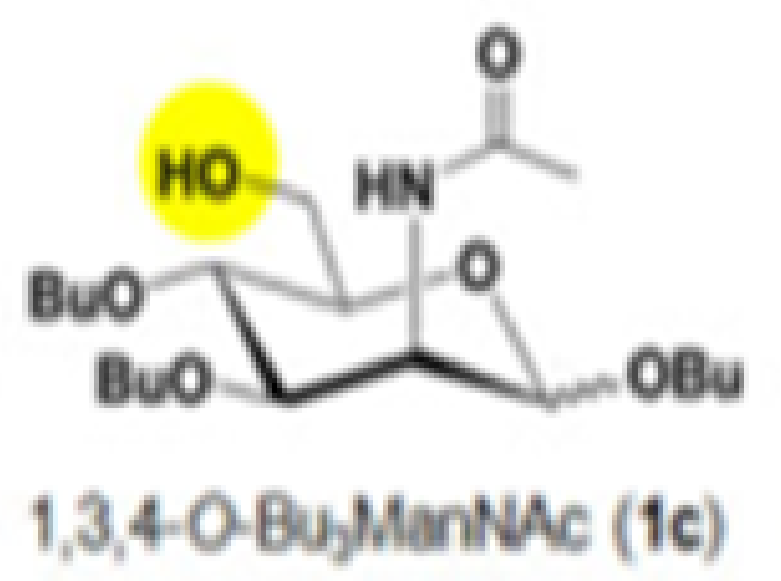



**Figure 9 1,3,4-O-$Bu_3$ManNAc.** The molecule of interest in this work. Not the positions and modifications from standard ManNAc of the Bu groups at the 1, 3, and 4 carbons. [2]

## *2.3 Materials and Methods*

### *2.3.1* Cell Culture and Treatment

Adherent CHO-K1 cells (Sigma-Aldrich, St. Louis, MO) were cultured in variable well plates utilizing a staged sequence of one day growth under F-12K Medium (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific, Waltham, MA) for one day in order to promote initial cellular growth and plate adherence. Cells were grown in conditions of 5% CO2 and 37C$^o$ for 24 hours in a Series 8000 Direct Heat and Water Jacket $CO_2$ incubator (Thermo Scientific, Waltham, MA). After this initial growth period, media was removed and cells were washed with Dulbecco's phosphate buffered saline (PBS) (Life

Technologies, Carlsbad, CA), which was then aspirated off. Cells were then fed a uniform volume of OptiMEM Serum-Free media mixed with varied concentrations of ManNAc, 1,3,4-O-$Bu_3$ManNAc, $Bu_4$ManNAc, $Ac_4$ManNAc variants. ManNAc and analogs used were solubilized in ethanol or a water/ethanol mix. The volume of ethanol used to solubilize and mix the chemicals into the media was found to constitute at maximum .04% of the media volume for the highest concentration (.68μM). [55, 66] Per previous investigation, this concentration shows minimal effect on culture viability until an ethanol composition of media reaches 1mM over 24 hours. [55, 66] These analogs and base ManNAc were generously provided by the Kevin Yarema lab, Department of Biomedical Engineering, Johns Hopkins University. In total, each concentration condition per set was tested in triplicate per set, three for growth and three for lysate collection. Each condition was tested this way a minimum of three times, with conditions of interest repeated upto eight.

*2.3.2 Collection*

After a further 24 hour period after the secondary incubation period, a selection cultures had media and cell waste aspirated off, and were then washed with PBS. One volume of cells was removed from the plate using .25% -Trypsin EDTA (Life Technologies, Carlsbad, CA), and a second volume of identical treatment was lysed directly by the addition of RIPA buffer and consolidated via a rubber policeman. Each sample was separately moved to a 1ml centrifuge tube (Sigma-Aldrich, St. Louis, MO) and labeled.

The whole cell lysate samples were then sonicated at 20 amps for a pulse of five seconds to disrupt remaining cell debris. The samples were then centrifuged at 10,000 RPM in a centrifuge for 10 minutes. (Eppendorf, Hamburg, Germany).The supernatant, containing the protein samples, was then collected for further analysis.

*2.3.3 Cell Density Counts*

Living cell counts were determined via a Tryphan blue (Sigma-Aldrich, St. Louis, MO) assay at a 1:1 dilution using a hemocytometer (Thermo Fisher, Waltham, MA). Counts per sample were done in triplicate, per individual well sampled. These values were then scaled up to an approximate number of cells per standard recommendations from Fisher at the chosen dilution factor.

$$Cells * \frac{Dilution\ Factor}{\#\ of\ Squares} * 10{,}000 \frac{cells}{ml} = Approx\ Cells/Ml$$

Due to the large volume necessary to glean data from the broad number of parameters tested, wells of varied size were utilized to conduct multiple studies in parallel. In order to have a uniform distribution (due to variable available grow space and the necessity of media volume), cell counts were then scaled to a per $mm^2$ basis for density.

*2.3.4 Enzyme Linked Immunosorbent Assay (ELISA)*

A BCA assay (Thermo Scientific, Waltham, MA). was performed on whole cell lysate samples to determine total protein volume collected from the cell lysate and evaluated using a spectrophotometer at the 562nm wavelength (BIO-RAD, Hercules, CA; Sigma-Aldrich, St. Lous, MO). These values were compared to uniform standards on a 96-well basis, allowing for the testing of 38 duplicates per plate. Individual assay equations were tabulated in order to establish standard deviation within the protein volumes collected. Protein volumes collected were used to determine concentrations within the volume of lysate remaining, which was then diluted to a standard value equivalent to the lowest concentration found. For instance, with two

protein samples of 30µg/µl and 10µg/µl, to have parity, both samples would need to be fed at 10µg/µl into the electrophoretic analysis.

*2.3.5 SDS-Page*

After uniform scaling per the BCA assay evaluation, samples were stained in 1x Laemelli blue buffer with β-mercaptoethanol (BIO-RAD, Hercules, CA; Sigma-Aldrich, St. Lous, MO) and denatured by boiling at 90C$^{o}$ for 5 minutes.

Samples of interest were loaded into 10% polyacrylamide gels with uniform concentration, as evaluated per the Pierce ® BCA protein assay kit (Thermo Scientific, Waltham, MA). Equal volumes at the selected concentration were loaded into the gel, with each position carefully monitored upon initial loading relative to the ladder.

Denatured samples were separated by running at 120V for 80 minutes at room temperature. Protein was then transferred from the gel to an Immuno-Blot ® polyvinylidenedifluoride (PVDF) membrane (BIO-RAD, Hercules, CA) at 200V for 75 minutes at room temperature under mild agitation.

*2.3.6 Western/Lectin Blot*

A western blot was performed using lectins as the primary and secondary antibody to evaluate the content of terminal sialic acid in the whole cell lysate. Lectins are proteins which bind to specific sugars, rather than conjugate sites per standard antibodies used in the evaluation of specific protein content. Using the PVDF membrane developed by the SDS-PAGE method, it was then incubated in 5% milk dissolved in PBST (phosphate buffered saline , with with 0.1% Tween-20 (Life Technologies, Carlsbad, CA; Sigma-Aldrich, St. Louis, MO) added) for 60

minutes. Following preblocking, the membrane was treated with PBST containing a 1:500 dilution of biotinylated Ig-like Lectin I (Life Technologies, Carlsbad, CA), as a primary antibody to sialic acid. After incubation for 2 hours, the membrane was washed for 5 minutes in PBST 3 times. The 15 minute wash period was followed by a treatment in 5ml of 1:2000 dilution of streptavidin (Life Technologies, Carlsbad, CA), in PBST. The study was terminated with three further 5 minute PBST washes.

The lectin blots were resolved using a Western Blot Chemiluminescence SuperSignal (Life Technologies, Carlsbad, CA) detection kit to visualize light response to biotinylation, a 1:1 mixture of reagents in the kit were used to develop the blots.

## *2.4 Results*

### *2.4.1 Effect of Analogs on Cell Growth*

*2.4.1.a* Operating Frame and Concentration Ranges

Figure 10 shows initial studies into growth rates over the course of one week using various concentrations of $Ac_4$ManNAc and 1,3,4-O-$Bu_3$ManNAc. As the compounds of interest, these were used to generate a kill curve and operating range of concentrations for each compound. In the case of both compounds, higher concentrations have shown a dramatic decrease in cellular growth over time. The $Ac_4$ManNAc variant is known to be used primarily within the range of 50-400μM. [3, 4, 5, 25]

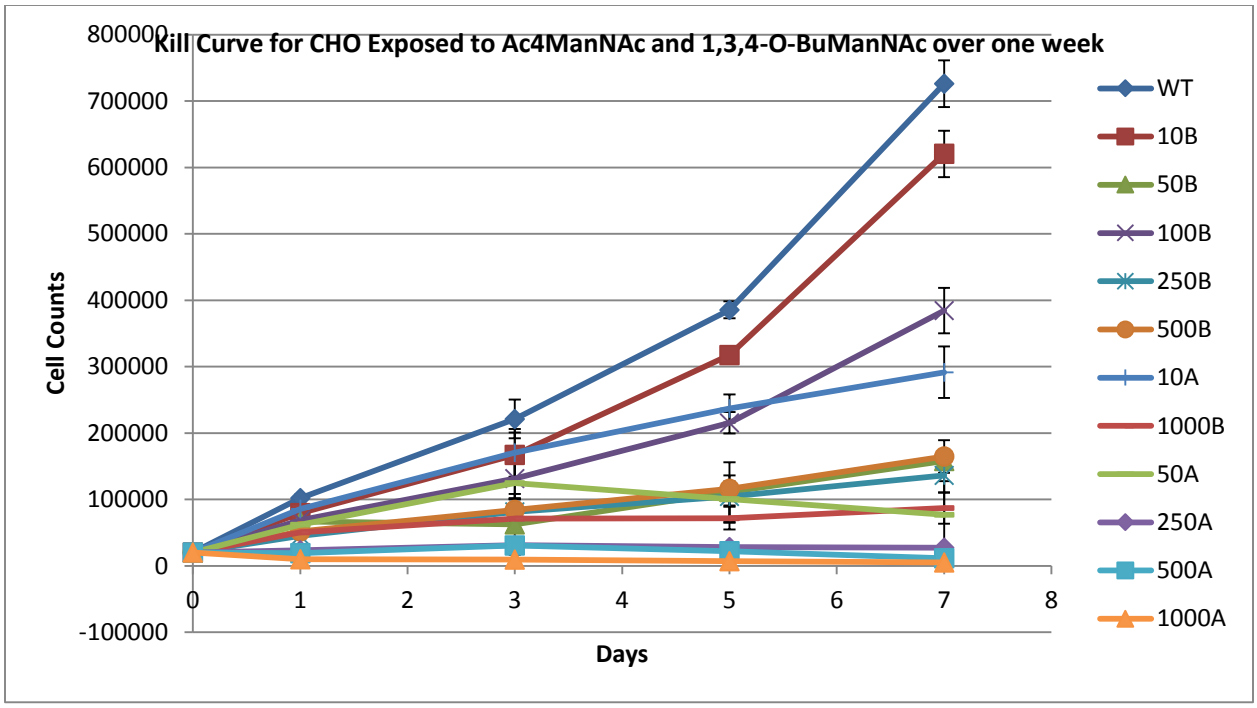



**Figure 10 Kill Curve.** 1,3,4-O-Bu$_3$ManNAc and Ac$_4$ManNAc were tested in parallel to examine the relative toxicity and determine an optimal range of cell function and treatment. As shown, the 1000µM concentration-treated cells fared the worst, with diminishing negative effects on cell growth as the concentration decreases. Additionally, Bu$_3$ appears to be overall less toxic, as the 1000µM concentration still outgrows all but the two lowest (10 and 50µM) Ac$_4$ treatments.

By contrast, 1,3,4-O-Bu$_3$ManNAc showed negative effects on growth, but these effects were dramatically attenuated compared to the sister Ac$_4$ compound. In fact, when the results were normalized (Figure 11), cells under 1,3,4-O-Bu$_3$ManNAc treatment outgrew the Ac$_4$-treated cells by an appreciable margin. After the initial kill curve assay, it was determined that based on Ac$_4$'s established industrial ranges and an observable shift from lag phase into exponential phase within some of the treatments and WT at day 5, as compared to a significant decrease in growth or even death, that five days of observation would prove to be a useful range to observe and reproduce results.

To adequately compare competition at uniform concentrations, treatments were performed at the same concentration to determine that concentration's effect on growth, protein yield, and overall SA content.

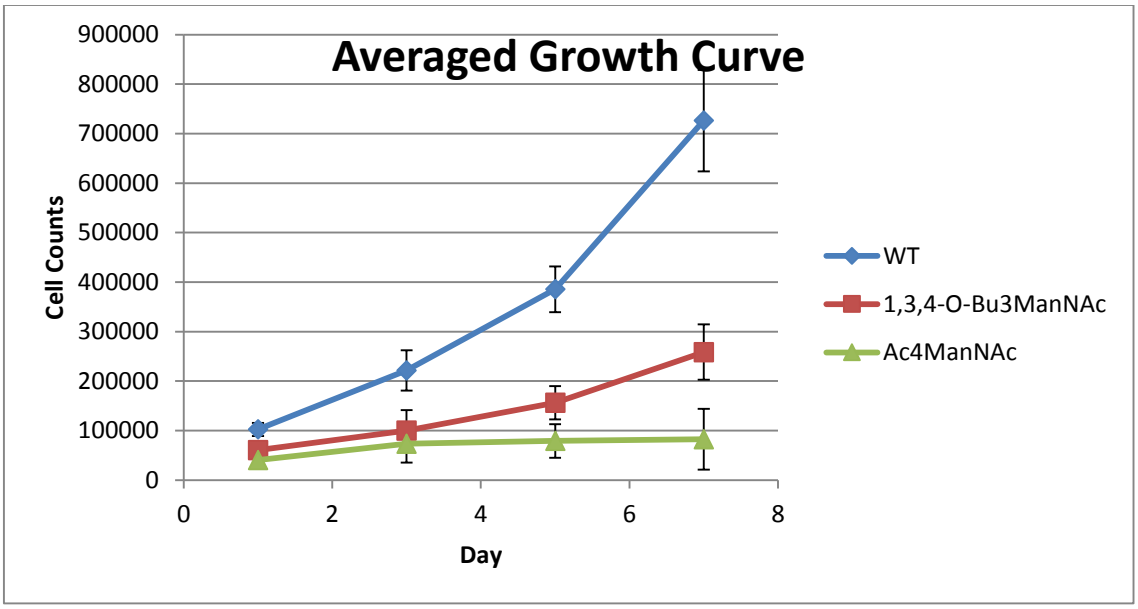



**Figure 11 Averaged Kill Curve.** This takes the data from above and averages the growth for $Ac_4$ManNAc and 1,3,4-O-$Bu_3$ManNAc treatments, forming a roughly linear curve over the days. While WT reaches an exponential phase, the relative weighting of the low yield of cell mass higher concentrations lower the total cell volume for the aggregate, giving the shape presented. The important thing to note is that even when averaging across concentration (which are fed at identical µM between the two chemicals), $Bu_3$ yields a higher total number of cells across the samples tested.

*2.4.1.b Growth Conditions*

While the cells were treated uniformly for environmental conditions, different concentrations of the various chemicals used displayed profound effects on culture growth, particularly as the length of the experiment continued. Figures 12, 13, and 14 show the collected average of cell volume for days 1, 3, and 5 respectively. It is important to note that each figure is the aggregate of multiple data points sampled within a uniform time frame rather than a bulk collection of data. Due to the volume of labor necessary to obtain these results, it would be prohibitive to gather the entirety of this data during the course of a single week.

For example, one experiment consisted of treatments 1-11, (or other such combinations) which were sampled in triplicate at 24 hours after initial feeding, 72 hours after initial feeding, and 120 hours after initial feeding, while a second experiment conducted the following week

consisted of treatments 1, 3, 12, 13, and 14 (or other such combinations). The volume of data collected per treatment type is factored in to the standard deviations displayed in the figures, and efforts were put forth to describe each treatment with a large volume of samples irrespective of the relative importance of those samples.

| Chemical | Concentration | Label |
|---|---|---|
| None | Null | 1 |
| ManNAc | 100µM | 2 |
| ManNAc | 250µM | 3 |
| ManNAc | 1000µM | 4 |
| $Bu_4$ManNAc | 50µM | 5 |
| $Bu_4$ManNAc | 250µM | 6 |
| $Bu_4$ManNAc | 500µM | 7 |
| $Ac_4$ManNAc | 50µM | 8 |
| $Ac_4$ManNAc | 100µM | 9 |
| $Ac_4$ManNAc | 250µM | 10 |
| $Ac_4$ManNAc | 500µM | 11 |
| 1,3,4-O-$Bu_3$ManNAc | 20µM | 12 |
| 1,3,4-O-$Bu_3$ManNAc | 30µM | 13 |
| 1,3,4-O-$Bu_3$ManNAc | 50µM | 14 |
| 1,3,4-O-$Bu_3$ManNAc | 75µM | 15 |
| 1,3,4-O-$Bu_3$ManNAc | 100µM | 16 |
| 1,3,4-O-$Bu_3$ManNAc | 125µM | 17 |
| 1,3,4-O-$Bu_3$ManNAc | 150µM | 18 |
| 1,3,4-O-$Bu_3$ManNAc | 250µM | 19 |
| 1,3,4-O-$Bu_3$ManNAc | 500µM | 20 |

Table 2 Key for Growth Studies

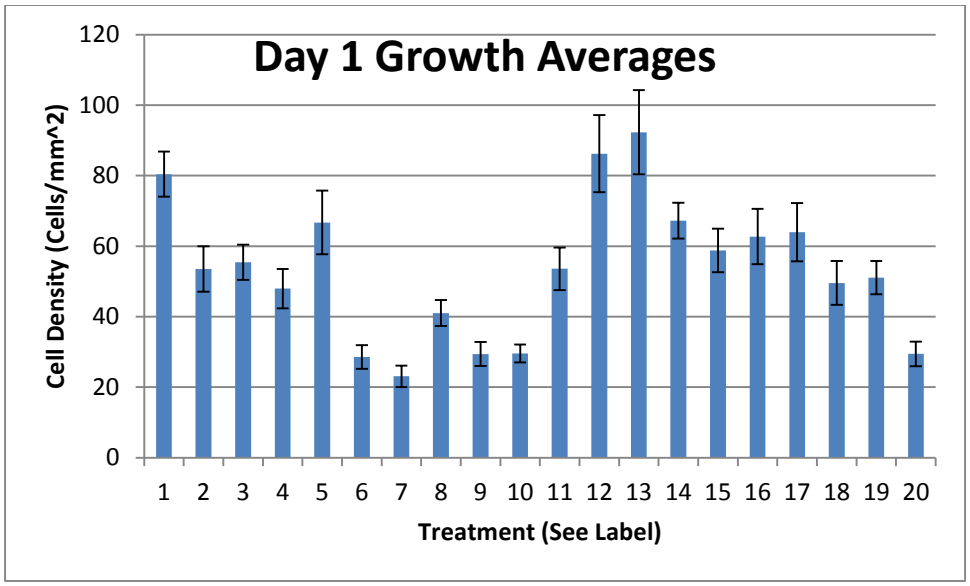



**Figure 12 Averaged Growth, Day 1.** Over numerous concentrations tested and days utilized, an average cell density per $mm^2$ was determined for 24 hours (1 day) of growth after initial chemical feed. The corresponding chemical and concentration of the treatment are shown above in Table 2. Relative to WT, the 1,3,4-O-$Bu_3$ManNAc performs the best in terms of non-negative effects on cell growth. $Bu_4$ was most toxic, with ManNAc and $Ac_4$ displaying worse outcomes than $Bu_3$ but better than $Bu_4$.

Figure 12 describes several profound results. Comparatively, concentrations of 1,3,4-O-BuManNAc (samples 11-20) tended to show relatively similar growth to WT (Sample 1) at lower concentrations (sub-125µM). Although treatments 12 and 13 (respectively 20 and 30 µM) appear to show an increased growth relative to WT, this lies easily within the range of standard deviation for WT and the samples themselves. This is still a positive results, and shows that CHO cells treated at those concentrations appear to suffer minimal drawbacks compared to higher 1,3,4-O-$Bu_3$ManNAc concentrations and similar concentrations of $Bu_4$ and $Ac_4$ treatment.

$Bu_4$ particularly threatened cell growth, and was carefully monitored the following days, while $Ac_4$ lay between the extremes of 1,3,4-O-$Bu_3$ManNAc and $Bu_4$ManNAc itself showed observable, but lesser effects on cell growth on the first day of study.

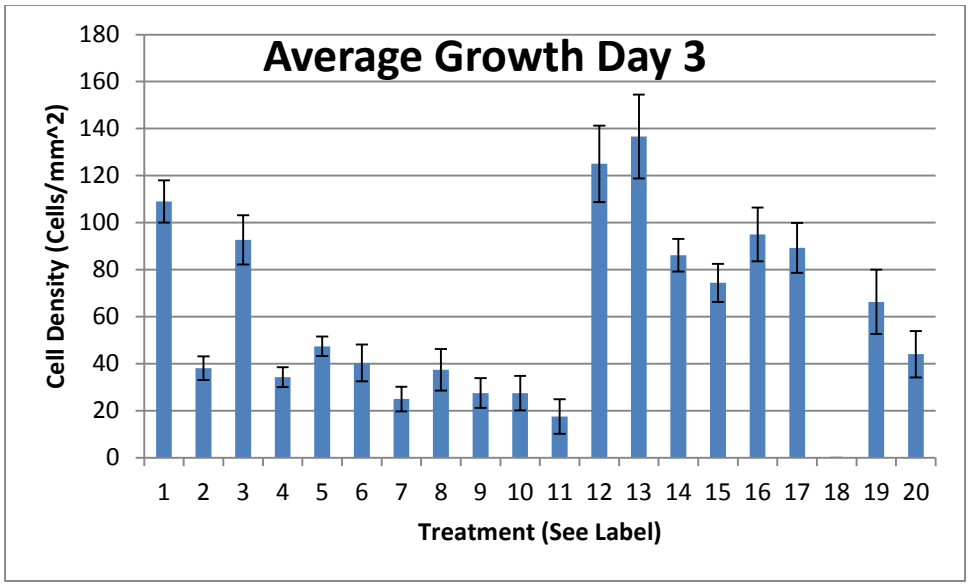



**Figure 13 Averaged Growth, Day 3.** An average cell density per $mm^2$ was determined for 72 hours (3 days) of growth after initial chemical feed. The corresponding chemical and concentration of the treatment are shown above in Table 2. Relative to WT, the 1,3,4-O-$Bu_3$ManNAc performs the best in terms of non-negative effects on cell growth. $Bu_4$ was most toxic, with ManNAc and $Ac_4$ displaying worse outcomes than $Bu_3$ but better than $Bu_4$.This is exacerbated even further from Day 1. Sample 18 (150 **µM**) was removed due to incomplete data.

Day 3 shows a more distinct stratification and diversity of growth volume. While all cells except for treatment 11 displayed growth during this time frame, WT, 11, and 12 showed dramatic outgrowth of the other treatments, though 3, 16, and 17 showed similar results as well, forming a secondary tier below the first three in terms of overall cell growth. Bu4 continued to show the most negative effects on growth, and Ac4 was solidly beaten by the Bu3 variant. Given that minimization of growth inhibition is one facet of the study in its entirety, 3, 16, and 17 could remain competitive, or even be more desirable than WT, 11, and 12 if the overall yield of SA/Protein/Growth was found to be favorable.

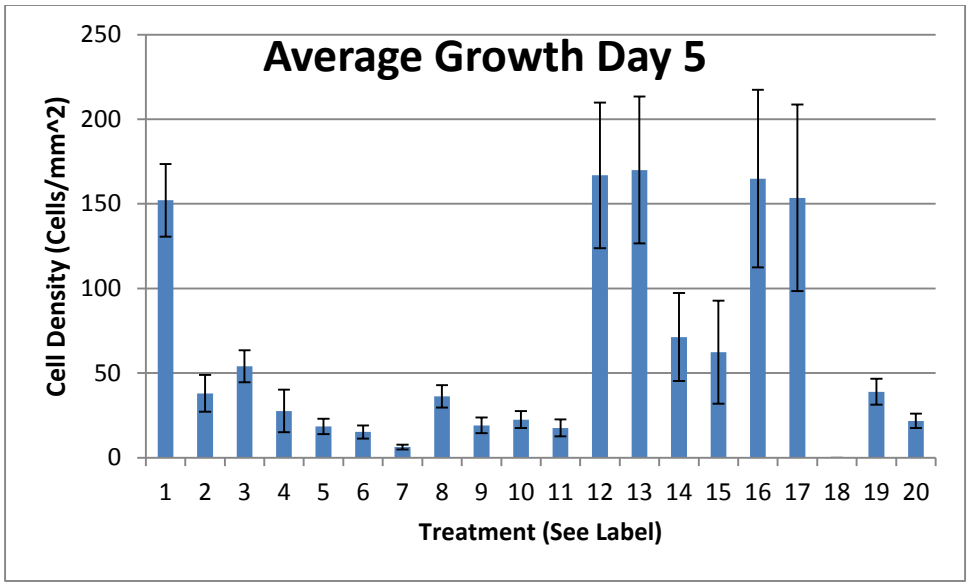



**Figure 14 Averaged Growth, Day 5.** An average cell density per $mm^2$ was determined for 120 hours (5 days) of growth after initial chemical feed. The corresponding chemical and concentration of the treatment are shown above in Table 2. Trends from Days 1 and 3 are improved further, with the aberrant results of 14-17 departing from the expected trend of a downward or constant decrease from sample 12.

By Day 5, the growth experiment has more or less shown the expected trend. Base ManNAc, Bu4, and the current Ac4 standard have all dramatically fallen off as compared to WT and all but the highest two Bu4 concentrations (19, 20). 14 and 15 prove to be aberrations as compared to the growth of 12, 13, 16, and 17. Being between 16 and 13, it could be inferred that they would show slight decreases from 13, or comparable values to 16, at the very least. Yet this was not the case. Possible errors in formulation, or outliers in the creation of the graph could lead to this strange results, but "Goldilocks" effects on metabolism have been noted in other cellular treatments as well. The standard deviations calculated show a range at which the two could overlap, but the lack of accuracy is a cause for further investigation.

Figure 15 shows the comprehensive effects of the overall growth from day to day, simply reaffirming that the results on Day 5 follow an observable trend from Day 1 to completion.

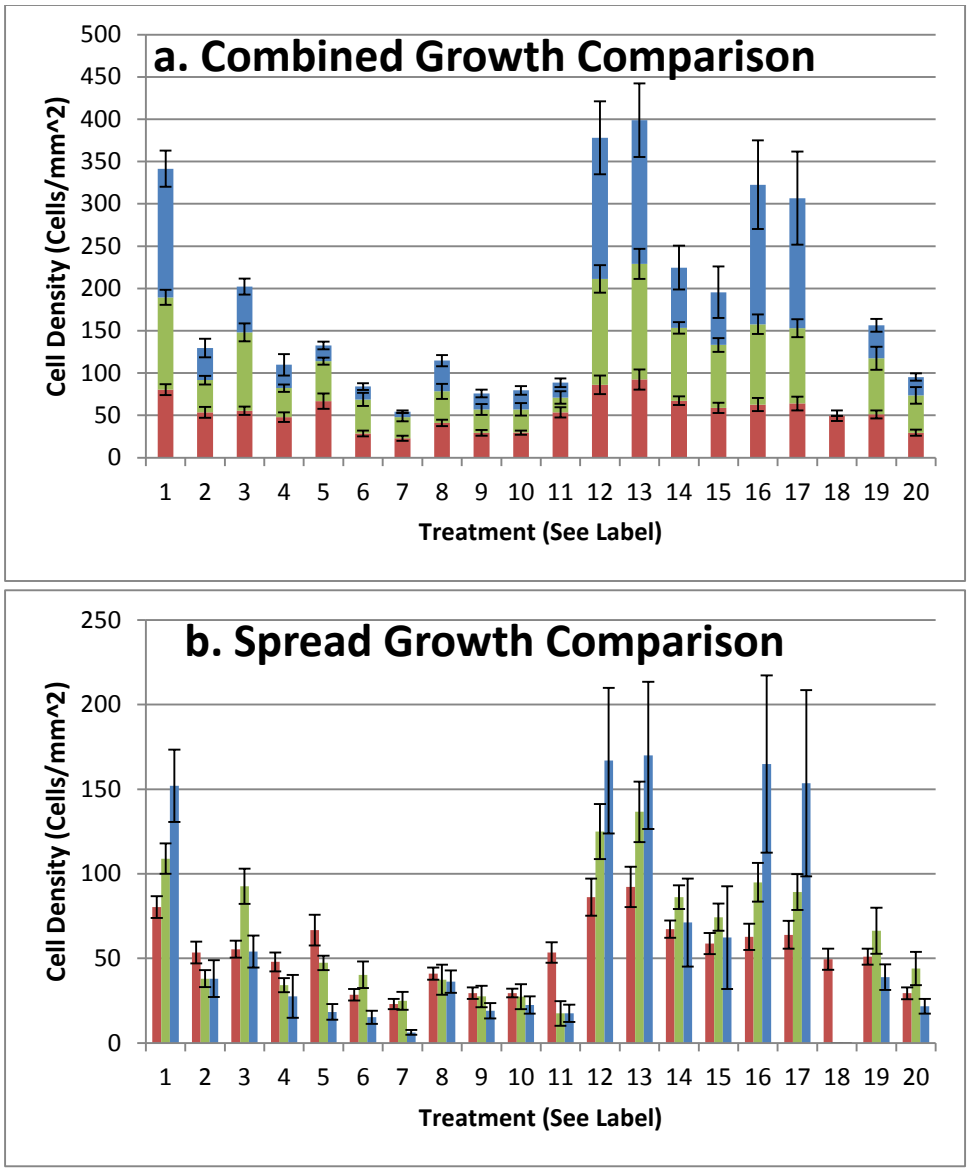



**Figure 15 Aggregate Growth. A)** This figure displays the compiled data from previous, showing net magnitude of growth. The 1,3,4-O-$Bu_3$ManNAc cluster (12-20) readily outperforms the other clusters, save for wild type. Provided the chemical yields similar protein per cell mass yield and SA content, it could prove a boon for large scale cultures. **B)** Comparatively shows the spread and progression of growth over time.

Clear competitors for SA yield optimization have begun to emerge based on the growth study, namely WT, 3, and the broad collection of $Bu_3$'s from 12-17. However, to avoid missing out on a potential high-yield of sialyated protein per cell mass, $Ac_4$ and $Bu_4$ were to be used at select concentrations in further evaluations.

Explanations for these effects are numerous. While analog behavior has been theorized previously, it bears reiterating that Bu4 is likely too hydrophobic to properly cross the cell membrane, and likely merely contributes to intercellular toxicity than upregulated SA yield. $Ac_4$ManNAc's toxic effects are noted prior in the background.

### *2.4.2 Cross-Analog Comparison of Protein Yield*

Protein yield was determined from a uniform volume of cell lysate distributed in duplicate in a 96 well plate, and evaluated on a per plate basis using 9 BCA standards to establish fluorescence to µg/µl ratio. Samples of interest were narrowed down to WT, and µM concentrations of: 1000 ManNAc; 50 $Bu_4$ManNAc; 50, 100, 250 $Ac_4$ManNAc; and 30, 50, 100, and 250 1,3,4-O-$Bu_3$ManNAc, based on parameters described in the growth curve. Some were selected for their cell growth yield, while others were chosen merely to retain a useful comparison. Figure 16 on the following page shows the results of the first day's protein yield.

| Chemical | Concentration | Label |
|---|---|---|
| None | Null | 1 |
| ManNAc | 1000µM | 2 |
| $Bu_4$ManNAc | 50µM | 3 |
| $Ac_4$ManNAc | 50µM | 4 |
| $Ac_4$ManNAc | 100µM | 5 |
| $Ac_4$ManNAc | 250µM | 6 |
| 1,3,4-O-$Bu_3$ManNAc | 30µM | 7 |
| 1,3,4-O-$Bu_3$ManNAc | 50µM | 8 |
| 1,3,4-O-$Bu_3$ManNAc | 100µM | 9 |
| 1,3,4-O-$Bu_3$ManNAc | 250µM | 10 |

Table 3 Key for Protein Yield Studies

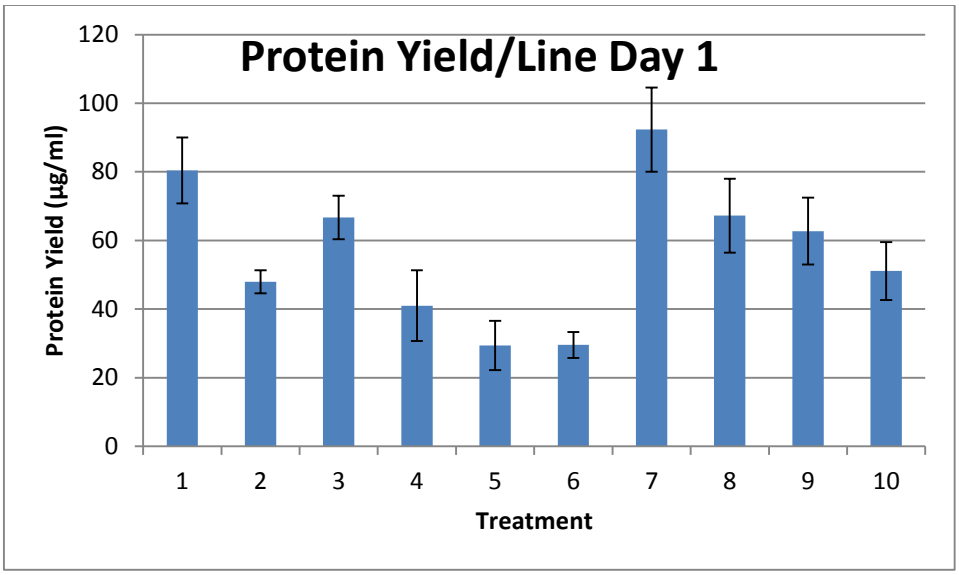



**Figure 16 Protein Yield for Select Samples, Day 1.** From the range of growth effects in figures 12-15, select concentrations were used to determine a protein yield, shown above. Corresponding labels and chemical/concentration data are defined in Table 3. As shown, the lower concentrations of chemicals in general lead to higher protein yield, and the $Bu_3$ cluster demonstrates superior performance.

While the goal of these experiments remains to maximize SA yield, paying adequate attention to protein output from a treatment method is also vital to note for the purposes of bulk-scale production of biotherapeutics. Even in the event of highly sialyated proteins, the expense necessary to cultivate a cell culture with low cellular or protein yield could prove to be a failed investment.

Day 1 demonstrates that $Ac_4$ManNAc and $Bu_4$ManNAc appear to express lower cellular protein relative to wild type, while low concentrations of 1,3,4-O-$Bu_3$ManNAc appear to increase it, or at the very least not encourage lower protein expression. A possible explanation for this is that chemicals such as sodium butyrate are known to influence increased protein expression [51]. In the process of glycomic processing, the admission of Bu groups into the cell may be facilitating this mechanism.

Of course, there are other, simpler issues which could confound this result inherent in the small volumes used and in the relative inaccuracy of the BCA assay, as the sample curves which

arise per 96-well plate can be extremely variable. Another issue may simply be lower cell volume per lysate, leading to a correlational decrease in overall protein availability.

To rectify this simple confound, Figure 17 was constructed. Figure 17 displays the protein yield from Figure 16, above, scaled on a per cell density basis assessed in the growth experiments. Similar trends as far as the relative magnitude of the various cell line's protein/cell yield indicate that the later samples of 1,3,4-O-$Bu_3$ManNAc tend towards better protein yield than the other analogs. Overall, the protein to cell count remain relatively similar, and lie within standard deviations of each other, with the exception of samples 4 and 5. This result is reasonable, as apoptotic processes or metabolism diverted towards cell survival may decrease protein yield as well as growth in the case of the Bu4 analog. The overall expression being similar for the other treatments is reasonable given that SA content, rather than protein production, is the target point of optimization. Any benefits in expression, rather than mere negation of negatives, are tangential, though merit further study.

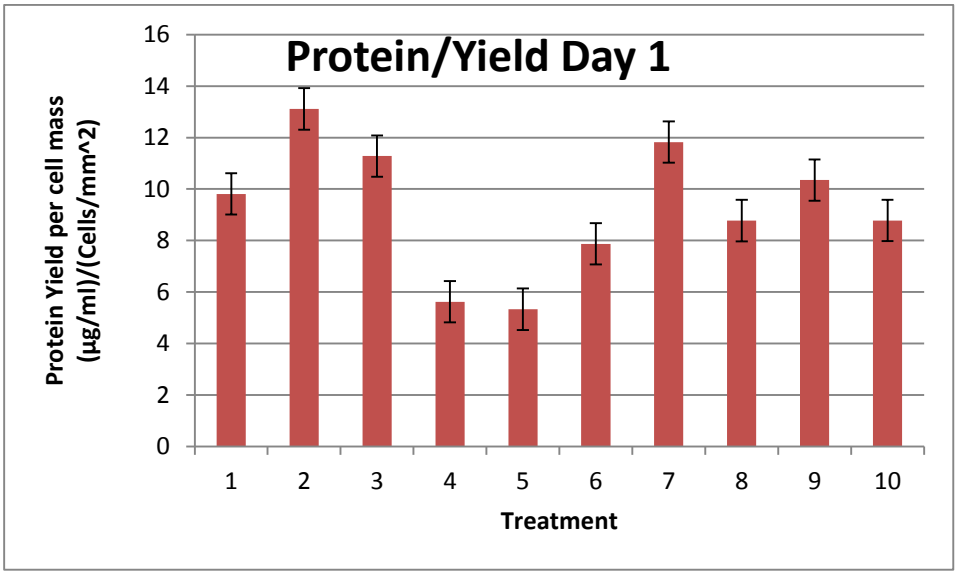



**Figure 17 Protein Yield per Cell Density for Select Samples, Day 1.** This graph frames the protein data on a per cell mass basis, allowing for quantitative comparison and removing issues of high cell volume corresponding to high protein values. While less apparent than previous figures, this figure nonetheless continues the dominance of 1,3,4-O-$Bu_3$ManNAc, though Samples 2 and 3 also do a fair showing of their own, particularly sample 2.

Figures 18 and 19 continue the analysis of protein and protein/cell data.

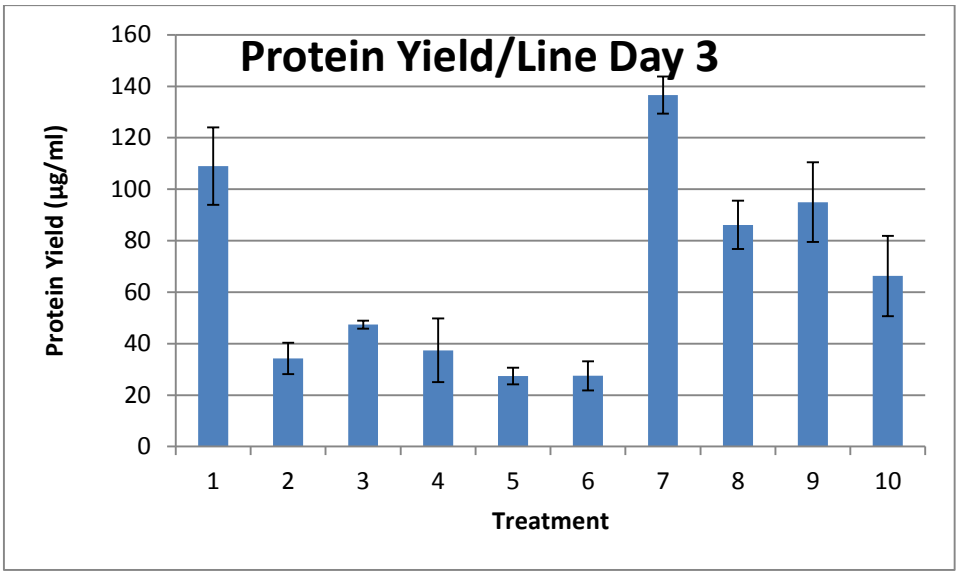



**Figure 18 Protein Yield for Select Samples, Day 3.** The gap between WT and $Bu_3$ samples widens. As could be inferred, the lower concentrations of all chemical show a greater preponderance towards higher protein yield than the higher concentrations.

Compared to Day 1, the overall protein yield drops off in the same fashion as the cell growth does. General protein yield goes up, confirming that the overall protein content increases with the volume of cells, which did continue to grow per Figure 15. WT and 1,3,4-O-BuManNAc continue to be top performers, with lower concentrations of $Bu_3$ yielding the most WT-similar results. The lowest concentration, 30 µmol 1,3,4-O-$Bu_3$ManNAc even appears to increase protein yield, though this could simply be an aberration rather than a key experimental result; all that matters is that $Bu_3$'s performance relative to WT and the other analogs remains consistently better. This is particularly true between differing analogs at the same concentrations. (3, 4, 8; 5, 9; 6, 10).

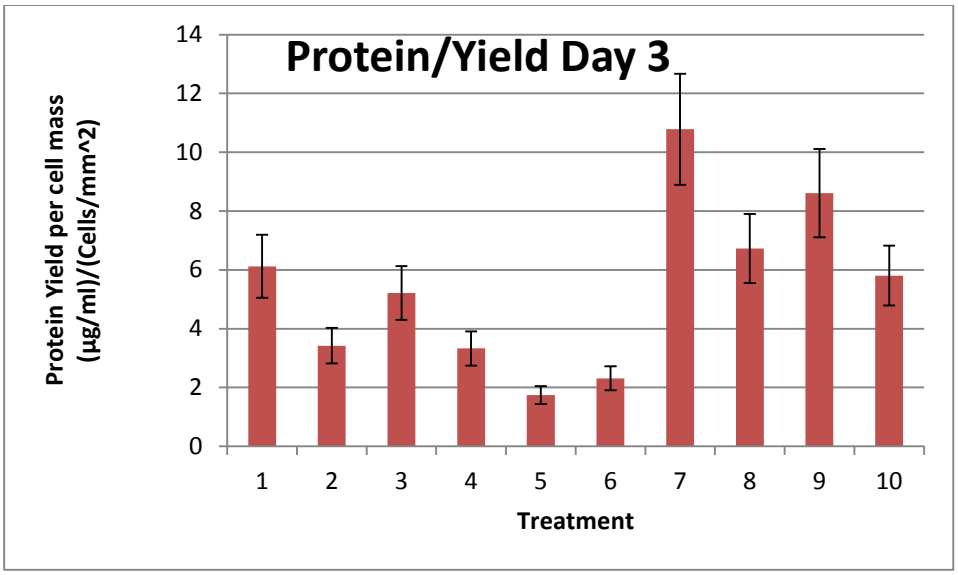



**Figure 19 Scaled Protein Yield for Select Samples, Day 3.** As with the raw protein data, the $Bu_3$ samples show a greater degree of protein yield. The $Ac_4$ however, is the more interesting data cluster, as the chemical not only appears to diminish growth in a far more pronounced way, but it also seems to reduce protein yield/cell in ways that other analogs seem not to do.

When scaled again to the growth values, the trend of similarity continues, though the overall protein yield for the Bu3 analogs does remain consistent. A possible avenue of inquiry between Day 1 and 3 is that $Ac_4$ and ManNAc may be metabolized at a faster rate than 1,3,4-O-$Bu_3$ManNAc. A factor that is interesting to note is that while the yield/cell decreases across the board from Day 1, the decrease is most pronounced for ones which were previously high performing (2, 3, 6).

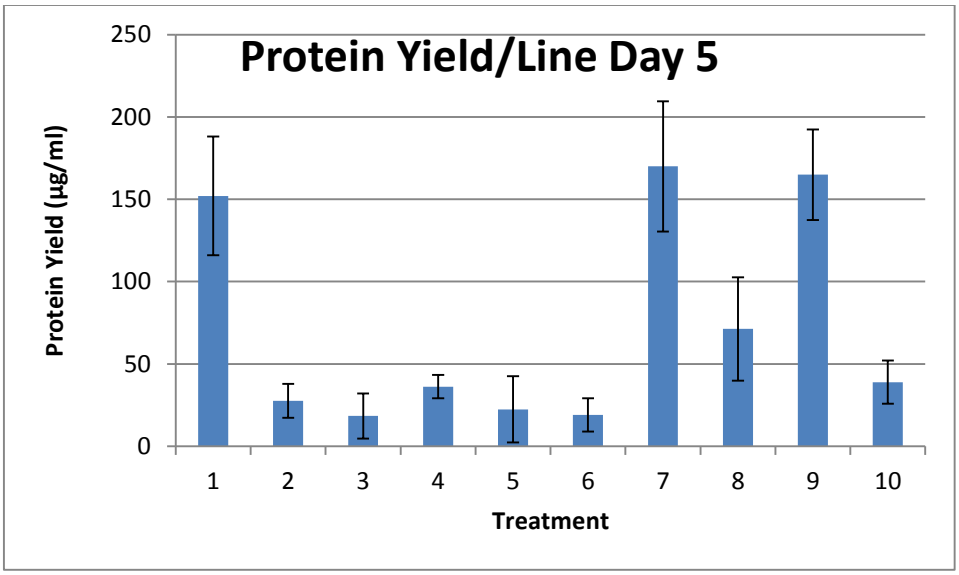



**Figure 20 Protein Yield for Select Samples, Day 5.** Sample 8 (50µM 1,3,4-O-$Bu_3$ManNAc) continues its strange dip relative to its neighboring concentrations. Other than this, $Bu_3$ has a far easier time of achieving desired protein yields proportional to cell mass than its competitors.

Compared to Day 3, which correlated to an overall increase in protein and likely cell mass, the protein found on Day 5 was diminished dramatically in all but treatments 1, 7, and 9. The fact that 8 doesn't follow this trend, or that 7 rather than 8 does, is unusual and bears further investigation, though the overall trend and investigation remain as expected. Day 5's experimental results reduce the need to conduct additional days of study, as divergence between WT and the $Bu_3$ treatments and the ManNAc, $Bu_4$, and $Ac_4$ treatments is dramatic.

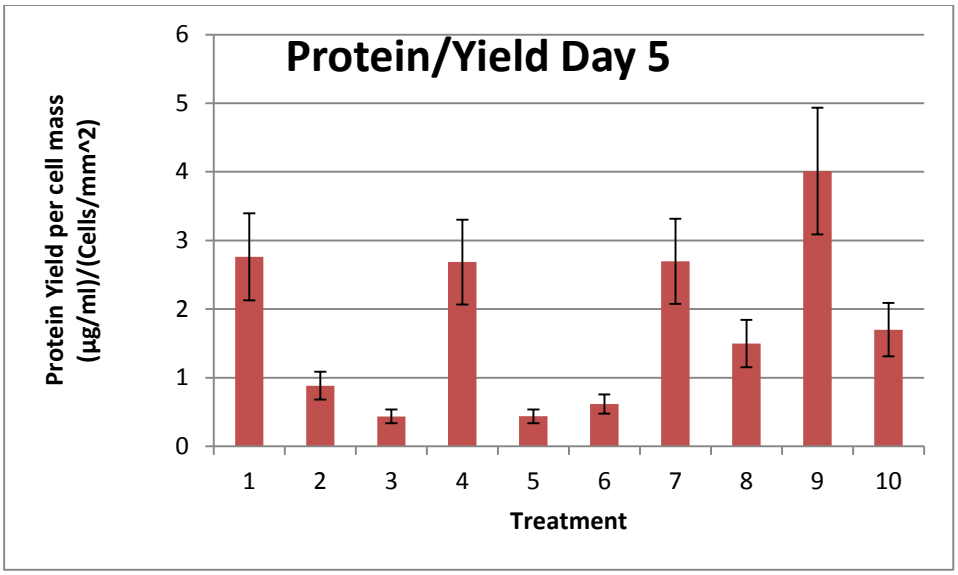



**Figure 21 Scaled Protein Yield for Select Samples, Day 5.** The overall trend here narrows, with a pronounced spike in protein/cell mass of sample 4 (50µM $Ac_4$). While it could be expected, as a whole, that when scaled to their cell masses, the average protein yield would be similar across the board, non-$Bu_3$ variants appear nonetheless diminished in their ability to produce expected protein, perhaps due to increased emphasis on cell survival.

The protein per cell yield for Day 5 raises many interesting questions. While the relationship between the magnitude of the columns is similar in shape to Figure 20, treatment 4 shows a dramatic departure from the general shape of Figure 20. Another point of interest is the large, continued drop in ratio. Where treatment 1 (Wild Type) showed a decrease from 10 µg/ml/(cell density) to 6 µg/ml/(cell density) from Day 1 to Day 3 in Figures 17 and 19, the change of about 6 to under 3 marks a similar, higher decrease. Overall protein yield per cell volume seems to be decreasing over time, even as cell volume increases, irrespective of treatment method. This could arise from cell toxicity from long term exposure, or could be from a shift in cellular metabolism from lag to exponential phase in the case of wild type.

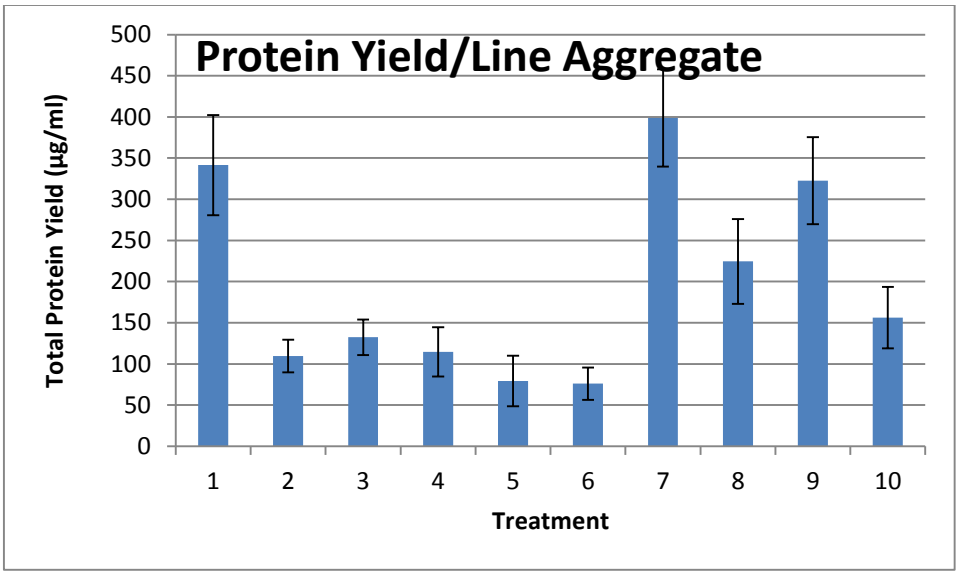



**Figure 22 Aggregate Protein Yield.** When taken collectively, the overall yield of protein swings heavily in favor of the $Bu_3$ cluster (7-10), while the $Ac_4$, $Bu_4$, and even ManNAc show dramatically lower results.

Figure 22 (above) shows the aggregate of protein collected over the course of the study, while Figure 23 (below) displays the aggregate of protein scaled to cell density.

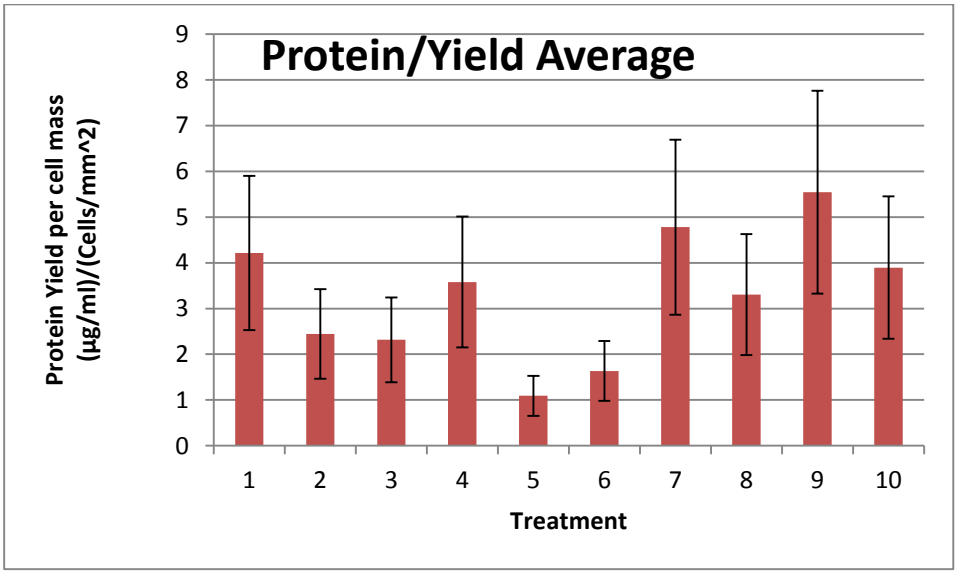



**Figure 23 Scaled Average Protein Yield.** As compared to Figure 21, this figure provides a far more uniform distribution of protein per cell mass, as might be expected. The $Bu_3$ outliers are still within WT's standard deviation, and though they do give a strong performance, this graph merely exposes $Ac_4$'s negative effect on protein yield rather than essentially improving the content within $Bu_3$.

In summary, $Bu_3$ continues to compete on-par with WT for overall protein yield and cell growth, and demonstrably outperforms the other analogs. $Ac_4$ and $Bu_4$ in particularly show

severe drawbacks for the purpose of developing increased SA yield. Comparatively, the $Bu_3$ performs at a minimum of slightly over 3 (µg/ml)/(cell/mm$^2$) at its lowest value, while the only competitor is the 50 µM $Ac_4$ feed. Provided $Bu_3$ can show similar sialic acid content, the improved protein yield should encourage usage of the 1,3,4-O-$Bu_3$ManNAc analog over the $Ac_4$ for large scale processes.

### *2.4.3 1,3,4-O-$Bu_3$ManNAc Protein Yield*

The previous section aims to examine the effects of the analogs in comparison to each other. This section is a brief investigation into trends within concentrations of 1,3,4-O-$Bu_3$ManNAc.

| **Concentration (1,3,4-O-$Bu_3$ManNAc** | **Label** |
|---|---|
| o µM/Wild Type | 1 |
| 20 µM | 2 |
| 30 µM | 3 |
| 50 µM | 4 |
| 75 µM | 5 |
| 100 µM | 6 |
| 125 µM | 7 |
| 250 µM | 8 |
| 500 µM | 9 |

**Table 4 Concentrations Used for 1,3,4-O-$Bu_3$ManNAc determination**

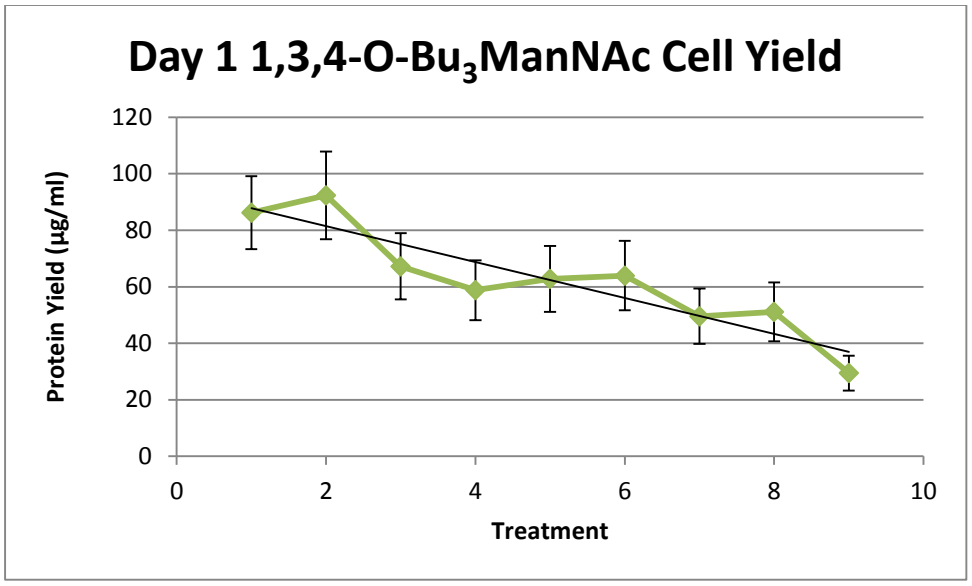



**Figure 24 Day 1 Protein Yield for 1,3,4-O-Bu$_3$ManNAc.** Unlike the previous figures, which were designed to showcase a comparison across chemical types and concentrations, this protein yield narrows its view to only concentrations of Bu$_3$. This shows a downward trend, as might be expected.

Figures 24, 25, and 26 show the yields of concentrations of 1,3,4-O-Bu$_3$ManNAc in order of increasing concentration. Between Days 1 and 3, Bu3 proves consistent, showing and overall and generally proportional increase in protein yield across the concentrations, and also shows a downward trend of protein yield as concentration increases.

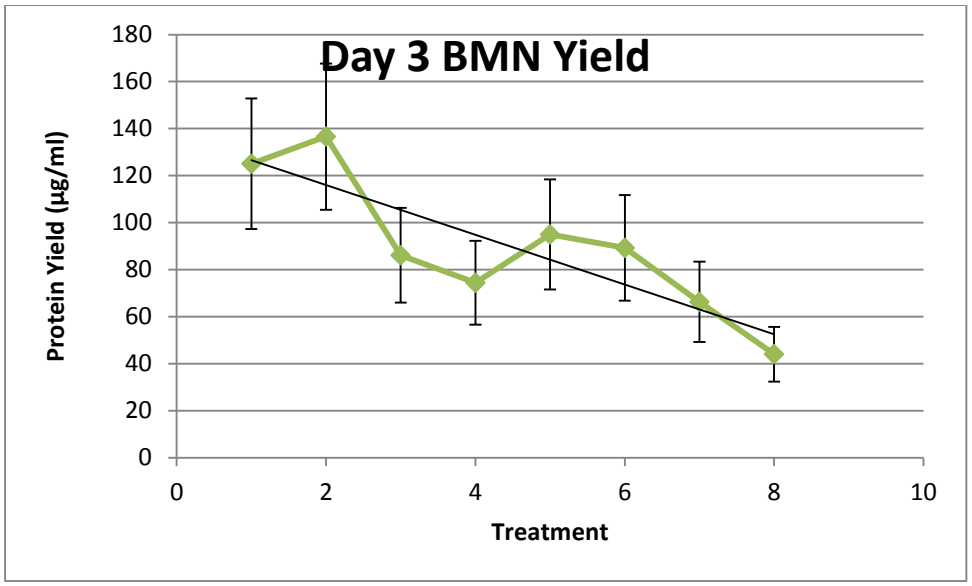



**Figure 25 Day 3 Protein Yield for 1,3,4-O-Bu$_3$ManNAc.** As with figure 24, the downward trend with increasing concentration is simply noted.

Between Day 3 and 5, this downward trend remains semi-apparent, but owing to the unexpected result of the 100 and 125 µM concentrations of 1,3,4-O-Bu$_3$ManNAc (previously described to show lower than expected cell growth and protein yield), the overall trend is distorted.

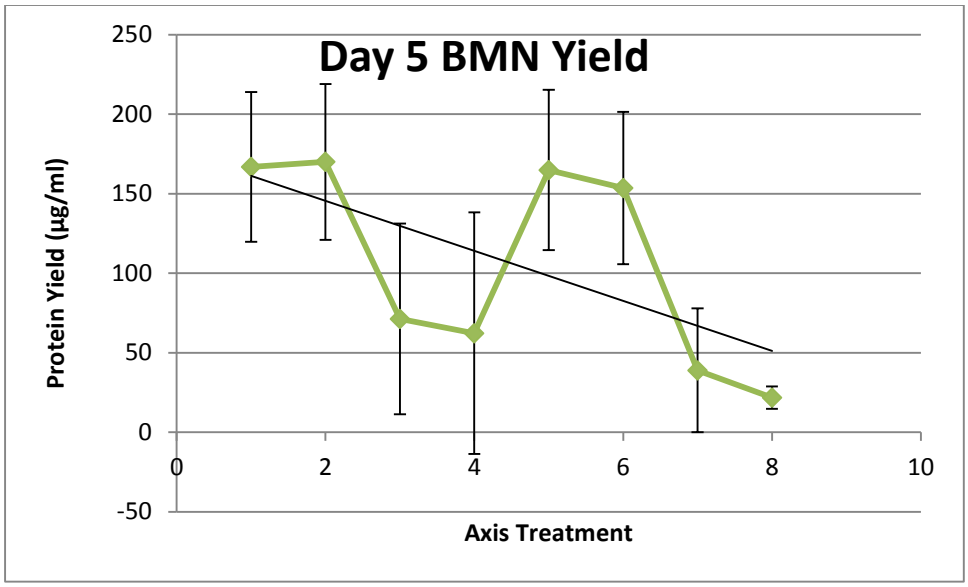



**Figure 26 Day 5 Protein Yield for 1,3,4-O-Bu$_3$ManNAc.** While a downward trend is relatively obvious from the first and last data points, the middle create an interesting conundrum, addressed as the odd results of the concentrations of treatment 6 and 7 listed in the growth curve of Figures 12-15 as samples 15 and 16.

While a simple result, it is not trivial to acknowledge that increasing concentrations of 1,3,4-O-Bu$_3$ManNAc does incur several risks associated with the other analogues.

*2.4.4 Lectin Blot*

Using the BCA technique described previously, uniform protein volumes were seeded into an SDS-Page gel, separated, and transferred into a PVDF membrane. They were then preblocked, treated with a primary antibody (Lectin I) for SA, and a secondary antibody for the primary (Streptradivin). The results of these lectin blots, which display relative SA content for a uniform volume of protein, are shown below. Relative darkness indicates higher presence of sialic acid.

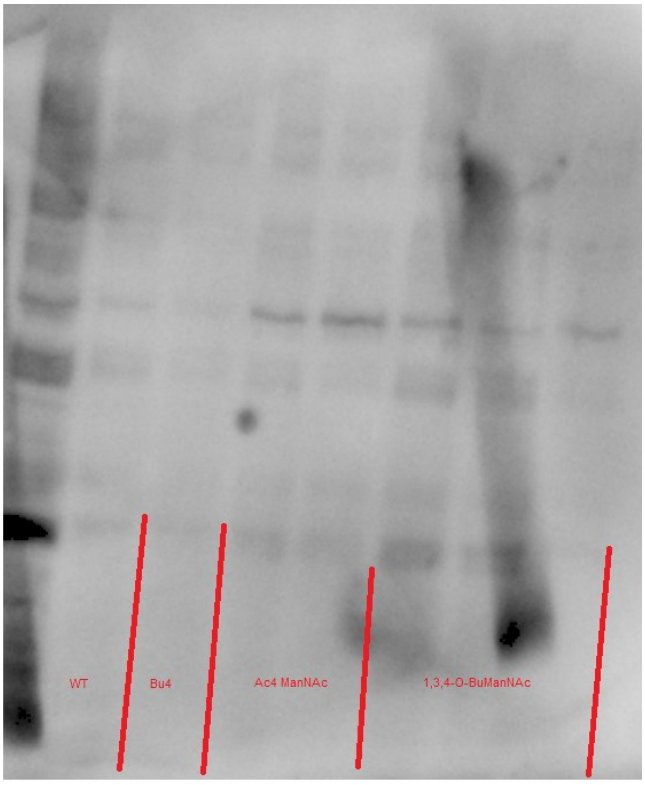



**Figure 27 Lectin Blot, Day 1a.** As shown above, the darker bands represent sialic acid content. This in turn can be compared to the WT band. Because this is simply a cursory, rather than quantitative look, only the clusters are labeled. Within the rightmost (1,3,4-O-$Bu_3$ManNAc) band, concentration of the analog fed decreases, showing lighter bands at lower concentrations. As a whole, $Ac_4$ and $Bu_3$ showed significant increases on SA content.

Figure 27 shows an initial lectin blot performed on the first day's samples. As theorized, the analogs used increase overall sialic acid content, relative to WT, except for Bu4. Although each band indicates a different concentration of analog used, this blot simply establishes a positive effect on SA content compared to wild type when using either 1,3,4-O-BuManNAc or Ac4ManNAc. The band to the far left, which is darkest, is a ladder rather than sample. As this assessment was performed on whole cell lysate rather than a specific protein, kDa labels are not included.

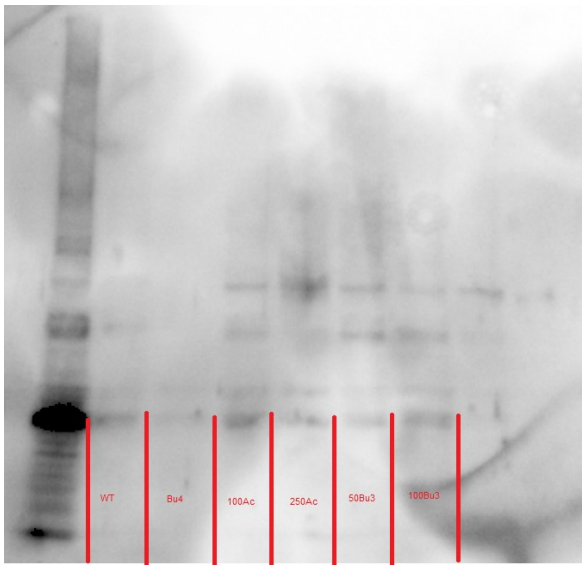



**Figure 28 Lectin Blot, Day 1b.** Here bands are labeled according to the analog fed. Although somewhat blurry at the lower end of the band, all non $Bu_4$ bands show significantly higher SA content than WT, particularly the 250µM and $Bu_3$ bands.

Figure 28 displays a similar trend to the blot above, and is also a sample of Day 1. The far right lane is difficult to visualize, and has not been labeled due to a lack of useful data from the distortion. However, in the remaining lanes, 50µmol and 100µmol 1,3,4-O-$Bu_3$ManNAc show similar darkness to the prevailing 100 and 250µmol $Ac_4$ManNAc. Bu4 continues to underperform, while WT is not significantly darker than the analogs.

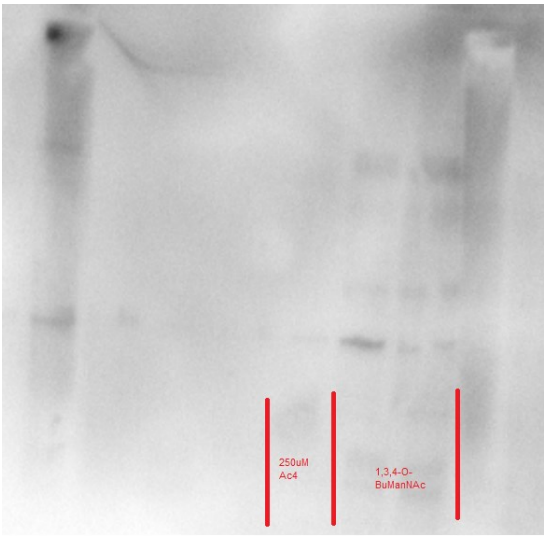



**Figure 29 Lectin Blot, Day 3** The labeled bands, though obscure, show the difference in $Bu_3$, high-feed $Ac_4$ and other analogs added in over Day 3. Where Day 1 (Figures 27 and 28) are relatively similar across the various chemicals and concentrations, the difference becomes more stratified as time goes on.

Day 3 marks the transition point in both protein yield and protein yield per cell growth. This blot is included to show that lectin blot assays were done on the SA content for the Day 3 samples. However, due to numerous logistical issues, Day 3 consistently failed to provide any other useful information other than SA was present in various bands. Notably, the bands marked for 1,3,4-O-$Bu_3$ManNAc showed the darkest overall presence relative to the illusiveness of the other bands.

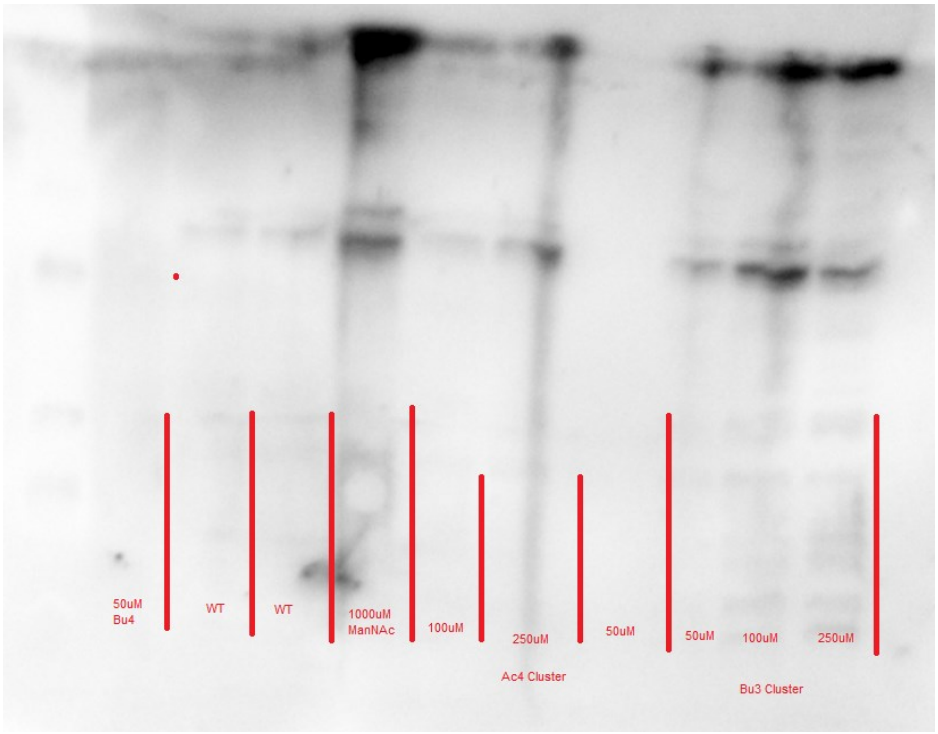



**Figure 30, Day 5a.** This blot shows an analysis of Day 5, and shows particularly dark bands at the maximum (250μM) feed level. Other bands show modest increases in darkness relative to the high feed. $Bu_3$'s cluster is consistently dark, while $Ac_4$ shows mixed results, with very little display in 50μM feed volumes. This blot also shows a 1000μM feed, and while very dark, is not substantially more competitive than 250μM $Ac_4$ or 100/250μM $Bu_3$.

Figure 30's blot shows the results of a Day 5 investigation. Relative to the similar concentrations of $Ac_4$, $Bu_3$ shows darker bands and a higher presence of SA throughout the lanes, particularly compared to baseline ManNAc and untreated WT protein.

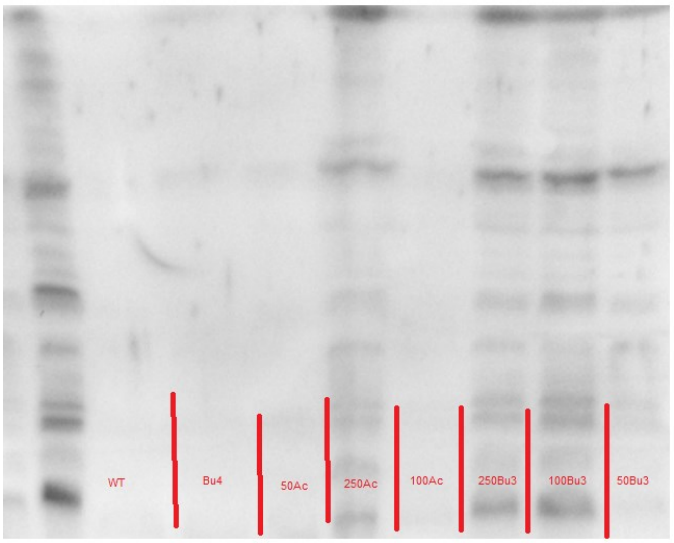



**Figure 31 Lectin Blot, Day 5b.** This blot is the most characterized and most distinctly shows the difference between WT, $Bu_4$, and $Ac_4$ and $Bu_4$ variants. $Bu_3$ shows a uniformly high SA content, and shows a competitive comparison of the 100Bu3 and 250$Ac_4$ bands, indicating a higher SA per concentration value when feeding $Bu_3$.

Compared to the previous Day 5 sample, this blot evaluates each lane independently. Lanes were chosen to maximize contrast and capitalize on previous lectin blots in hopes of creating a blot which more clearly represents the qualitative data cleaned from previous investigative studies. In the blow, $Bu_4$ and WT show mild differences in SA content, while 50Ac has a darker band, virtually indistinguishable, however, from the 100Ac band. By contrast, 250$Ac_4$ is very dark, showing a notable increase in SA content. But the comparison with the 1,3,4-O-$Bu_3$ManNAc samples is illuminating.

While the 250 and 100µmol bands of the $Bu_3$ studies are virtually indistinguishable, they show a marked increase in SA content over the 50; furthermore, they show comparable increase in SA to the 250$Ac_4$ band, with the 50 and 100 $Bu_3$ bands obviously outperforming the 50 and 100 $Ac_4$ bands. The 50$Bu_3$ band outperforms, quite visibly, all bands except for the 250Ac4 band. The increased concentrations of 100 and 250$Bu_3$ also visibly show darker bands than the 250$Ac_4$.

Taken as a whole, $Bu_3$ seems to give greater SA content/protein than $Ac_4$. When taken into consideration with the previous means in which Bu3 had better protein and cell mass yields, 1,3,4-O-$Bu_3$ManNAc is a clear winner in the comparison between the analogs.

A more direct comparison using an excision from Figure 29 was developed to provide a greater contrast, showing that SA increased between the 50, 100, and 250 µmol samples, shown in Figure 32 below.

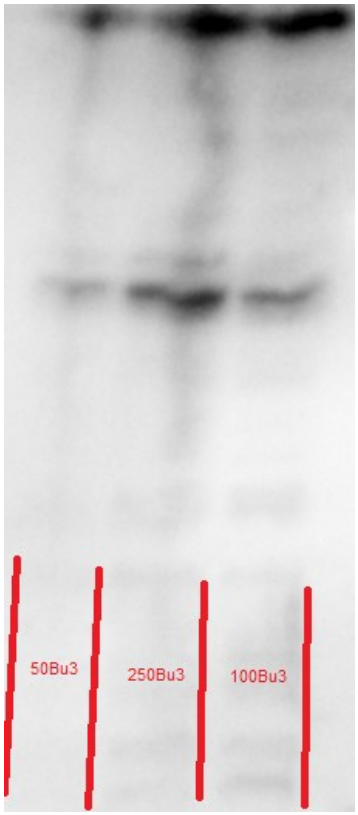



**Figure 32 Lectin Blot Day 3 1,3,4-O-$Bu_3$ManNAc direct comparison.** Removal of the other lanes allows for precise visualization of the increasing SA content characteristic of increased concentration of $Bu_4$. As shown, the darker bands correlate to higher concentrations.

# Chapter 3: Conclusions

All analogs, with the dubious exception of the $Bu_4$ analog, were shown to increase SA content relative to wild type. Comparatively, 1,3,4-O-$Bu_3$ManNAc was less toxic on the effects of cell growth over the course of the days studied, particular compared to the current $Ac_4$ standard. This trend continued to the overall protein yield. While there were some oddities in the values of 75 and 100 μmol 1,3,4-O-$Bu_3$ManNAc. For uniform protein concentrations, lectin-blotted 1,3,4-O-$Bu_3$ManNAc showed a higher increase in SA content than comparable $Ac_4$, and showed a steady increase In SA content with increasing 1,3,4-O-$Bu_3$ManNAc concentration. Furthermore, it showed a *greater* degree of increase in SA content when compared to higher concentrations of $Ac_4$ManNAc, in particular the comparison of 100(u)mol 1,3,4-O-$Bu_3$ManNAc as compared to the 250 μmol $Ac_4$ManNAc.

Overall, 1,3,4-O-$Bu_3$ManNAc has qualitatively shown to be an equivalent, if not superior, alternative to increasing sialic acid content through a chemical rather than genetically engineered method.

# Chapter 4: Further Research

## *4.1 HPLC*

High performance liquid chromatography (HPLC) can be used in order to separate a mixture based on numerous characteristics. Reverse phase HPLC allows for high specificity of analysis.

### *4.1.1 Nucleotide Sugars*

Nucleotide sugars are metabolites that often form intermediates in the formation of glycan structures (a total of twelve for uridine, cytosine, guanine, and adenine, each with a mono-, di-, and tri- phosphate). Specific focus on CMP-Neu5Ac could be beneficial for its membrane permeability and SA throughput.

Methods have been detailed for various solvents and methods for the extraction and quantification of nucleotide sugars. Further development of metabolic models could benefit from the determination of the effects on various metabolic pathways and the change in NT sugar composition on a per-analog exposure basis.

By monitoring these effects, it would help in the development of further *in silico* modeling of sialic acid flux pathways.

### *4.1.2 Glycan Depletion*

PNGase F (New England BioLabs, Ipswich, MA) is a common enzyme used to cleave N-glycans from proteins in order to study numerous traits of de-glycosylated proteins. One other benefit involves the separation of the actual glycans.

After cleaving the glycans and separating them out from the whole cell lysate, using HPLC to determine SA peaks could come in handy for quantitative analysis of overall SA yield within the lysate content.

## *4.2 Specific Proteins*

Using the metabolic model proposed above, further investigation into cell line parameters could be further evaluated for particular proteins of interest. Although this study encompassed broadly the overall effects of SA regulation via a whole-cell lysate analysis, the ultimate goal of most cell culture work is to increase industrial ability to produce high-quality glycoproteins. Applying these methods to a specific therapeutic could yield quite useful data.

## *4.3 Other Analogs*

Although only ManNAc and three other analogs ($Ac_4$, $Bu_4$, and 1,3,4-O-$Bu_3$ManNAc) were tested, there exist a huge variety of chemical modifications which have shown interesting properties. The 3,4,6-O-$Bu_3$ManNAc variant shows useful apoptotic properties, and could be cultured for in situ or in vitro cancer models and metabolic effectors.

Additionally, the usage of azide groups in the core ring structure of the analog could show many useful diagnostic traits due to the inherent Az-Az conjugation and the growing popularity of click chemistry methods.

## *4.4 Combined Approach of Analogs and Genetic Engineering*

A viable avenue of inquiry could involve the combined approach of the chemical analogs co-used with genetically engineered lines already showing increases in terminal sialic acid

content. It would be interesting to find out whether or not bioengineered platforms optimized for throughput could be further augmented by chemical means.

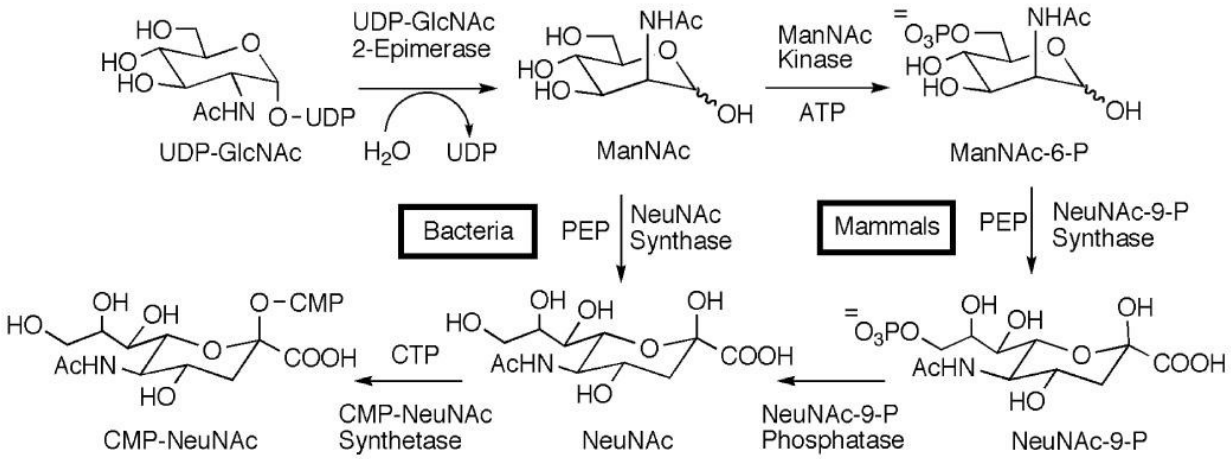



**Figure 33 Variant ManNAc Synthesis Pathway.** Bacteria are relatively less complex in their synthesis pathway. Transfection of the gene into CHO to examine metabolic throughput might be a viable means of investigation. [22]

Various organisms utilize variants of the sialic acid synthesis pathway. Examining these variants and testing for throughput or cell line minimization may prove to be of interest.

## *4.5 Treatment Phase*

Cells in this study were treated in the initial growth phase, and examination was stopped before exponential phase due to cell death or parity. Testing the effects of the analogs at different stages of cellular life cycles to account for yield may achieve industrial-tier results for mass-scale production.

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# Curriculum Vitae

## Mark C. Stuczynski II

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## Education

**Johns Hopkins University** Baltimore, Maryland

Master of Science in Engineering (M.S.E.) in Chemical and Biomolecular Engineering, June 2015

- Thesis: *Metabolic glycoengineering optimization in mammalian cell cultures*
- Cumulative GPA: 3.77
- Relevant Coursework: Metabolic Systems Biotechnology, Nanomedicine and Supramolecular Materials, Bioengineering in Regenerative Medicine, Drug Development and Discovery, Advanced Reaction Engineering

Bachelor of Science in Chemical and Biomolecular Engineering, May 2014

- School Ranking: #1 in the US for Bioengineering
- Concentration in Molecular and Cellular Bioengineering
- Minor in Psychological and Brain Sciences

## Research Experience

**Dr. Michael J. Betenbaugh Lab,** December 2010-Present Baltimore, Maryland

*Research Assistant,* Department of Chemical and Biomolecular Engineering Johns Hopkins University

*Site-directed evolution of human paraoxonase-1*

- Summer 2012
- Objective: Use ePCR to create high-efficiency mutants of HuPON-1 to treat organophosphate poisoning
- Used serial dilution of recombinant CHO-S cells expressing HuPON, use ePCR to generate mutants
- Collected with FACS, screen for best mutants, repeated process until HuPON-1 expression delivered a high-efficiency strain to aid in organophosphate treatments

*Bioengineering of recombinant glycosylated erythropoietin/IgG*

- Fall 2013-Present
- Objective: Create cell constructs for recombinant erythropoietin expression for large-scale culture production and modeling purposes
- Designed vectors containing six variants of hybridized and/or modified versions of erythropoietin and various regulatory protein sequences
- Screened cultures post-transfection for monoclones for preservation and scaleup, included a glycan pattern analysis via HPLC/LCMS
- Evaluated novel glycoforms for the recombinant models via western and lectin blot analysis

*In-Vitro Glycosylation Bolstering via ManNAc analogs*

- *Project Lead*
- Summer 2013-Present
- Objective: Improve sialic acid content in various desirable protein therapeutics to create more human-mimetic drugs
- Fed cells various sialic acid precursor analogs ranging from novel organic compounds and industry standards
- Evaluated apoptosis-induced utility of some compounds in cancer cell lines, with considerations towards targeting methods and cell safety

## Laboratory and Design Experience

**Chemical and Biomolecular Engineering Lab** Johns Hopkins University
*540.313 Chemical and Biomolecular Engineering Lab* Senior Capstone Evaluation

*Modeling and Quantification of Biocatalytic Activity for Unknown Enzymes*

- Objective: Examine catalytic activity of two enzymes for production of a specified bioproduct.
- Compared two unknown enzymes genetically engineered into *E. coli* for conversion rate and volume of product for a specialized biopharmaceutical using multiple spectroscopy methods
- Created a MATLAB model which factors in temperature, suspension, and volumetric flux data

*Membrane Separation of Rhodamine B from Potential Anti-hemorrhagic Compound*

- Objective: Separate contaminant Rhodamine B reactant from solution containing a desired compound while minimizing losses
- Developed a process model which separates Rhodamine B to leave behind 1ppm of contaminant while minimizing product loss to below 20%

## Extracurricular and Leadership Activities

**Graduate Student Liaison Committee,** August 2014-June 2015 Baltimore, Maryland

- *Master's Student Chair,* September 2014-Present
- Mentored underprivileged students in STEM fields once a week until the summer

**Johns Hopkins Newsletter,** June 2013-September 2014 Baltimore, Maryland

- *Staff Writer, Science and Technology*
- Wrote weekly on a wide variety of science topics in astronomy, genetic engineering, and communications.

**Johns Hopkins Pen and Paper Gaming,** August 2010-June 2015 Baltimore, Maryland

- *President,* April 2012-May 2014
    - Raised membership and club participation by 400% during fall semester of 2012
    - Raised club funding by 200% by fall semester 2013, and a further 50% in 2014

**Johns Hopkins Cycling Team,** September 2010-May 2015 Baltimore, Maryland

- Interacted and lead a wide variety of teammates from various disciplines and backgrounds
- Practiced both team strategy and individual perseverance

## Professional Experience

**Johns Hopkins University,** January 2015 Baltimore, Maryland
*Course Instructor,* 540.269 Here Be Dragons: A Systems Biology Approach to Fantastic Creatures

- Organized a course syllabus for a three week course as a graduate instructor.
- Successfully integrated topics into an engaging, informative, and novel course.
- Covered topics of cell metabolism, biochemistry, biomechanics of flight, chemical kinetics and reaction schema, genetics, differential equations, ecology, fluid mechanics, MATLAB programming, sociology, and history.

**Johns Hopkins University,** August 2014-June 2015 Baltimore, Maryland
*Teaching Assistant,* 660.461 Engineering Business and Management

- Evaluated students on subjective criteria regarding causal analysis in complex business and integration scenarios.
- Hired for a second semester, and additional course under same instructor for upcoming semester.

## Skills

**Lab:** Transformation and transfection, fluorescence microscopy, SDS-PAGE and other gel electrophoresis techniques, plasmid design, western blot, lectin blot, flow cytometry, ZFN, TALEN, HPLC, PCR, e PCR, HEK, CHO, INS-1, *e.coli*, cell culture, metabolic flux analysis, LCMS
**Computer:** Aspen, LaTeX, MATLAB, Simulink
**Foreign and Linguistic Experience:** Lived and studied abroad in Islamabad, Pakistan; Kuala Lumpur, Malaysia; and Muscat, Oman, for a net period of ten years. Working proficiency in American Sign Language and Spanish.