Whole cell biosynthesis of N-acetylneuraminic acid derivatives

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Abstract

Sialic acids are a family of N- or O-substituted derivatives of neuraminic acid (a 9-carbon monosaccharide), with roles in cell-cell interaction and immunology. Research on the biology of sialic acids is limited by access to these compounds, as effective synthesis routes for derivatives are limited. Herein, a system designed to produce various sialic acids by whole-cell biosynthesis is presented, using the native ManNAc-6-phosphate epimerase NanE of *Escherichia coli* and the sialic acid synthase NeuB from *Neisseria meningitidis*. Over-expression of this pathway in *E. coli* lacking native catabolic pathways demonstrated successful conversion several N-Acetylglucosamine (GlcNAc) derivatives to corresponding sialic acids, including N-acetylneuraminic acid (Neu5Ac), N-propanoylneuraminic acid (Neu5Pr), and N-butanoylneuraminic acid (Neu5Bu). Initial yields were ≈ 30 mg L^{-1} of culture broth for Nue5Ac and Neu5Pr, and 4 mg L^{-1} for Neu5Bu. Production of both N-isobutanoylneuraminic (Neu5iBu) and N-benzanoylneuraminic acids (Neu5Br) was not observed, indicating that this biosynthetic pathway may be limited by the steric size of substitutions. Further work is required to fully characterize the observed products.

Keywords: sialic acid, metabolic engineering, biosynthesis

Acknowledgements

I am grateful to Dr. Christopher Boddy for the opportunity to work with him and his group in pursuing this research project. Dr. Boddy's encouragement, insight, and knowledge has been inspirational.

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Table of Contents

Abstract i

Acknowledgements ii

Table of Contents iii

List of Figures v

List of Tables viii

Introduction 1

Background 1

Project Goals 3

Sialic acid quantification 6

Results 7

Production of Neu5Ac 7

Production of Neu5Bz and Neu5Pr 9

Purification of Neu5Pr 9

Production of Neu5Bu and Neu5iBu 9

Further characterization of Neu5Pr and Neu5Bu 11

Characterization of GlcNPr substrate 13

Growth experiment with azidoglucose substrate 13

Discussion 14

Conclusion 16

Experimental 17

Construction of sialic acid biosynthetic plasmid pDS3 17

Sialic acid production in *E. coli* in shake flask culture 18

HPLC detection and quantification by DMB hybridization 19

Western blotting to detect His-tagged proteins 20

Neu5Ac standard curve 21

Work Cited 22

Appendix 26

NMR and mass spectra for GlcNPr feedstock 26

Supplementary HPLC-UV chromatograms 27

List of Figures

Figure 1. General structure of sialic acids (left) and of N-acetylneuraminic acid (Neu5Ac, right). Adapted from [17]. 2

Figure 2. Schematic for production of Neu5R sialic derivative from GlcNR feedstock, where R is the functional group corresponding to the derivative (e.g. R = Ac for Neu5Ac production from GlcNAc). Modified from [24]. 4

Figure 3. Alternative metabolic pathways for GlcNR substrate in E. coli. The box represents the cell wall, and enzymes or transporters are indicated beside arrows. Modified from Horsman et al., 2016. 6

Figure 4. Western blot shows lysates of BL21, BRL02, and BRL04 cultures at 24 and 48 h, in which expression of His-tagged neuB and nanE had been induced. Strong bands detected for both proteins, at both time points and in all cultures. 8

Figure 5. HPLC-UV chromatogram (λ = 350 nm) for DMB-derivatized sample from growth experiment with GlcNAc feedstock with BRL-04 cells at t = 48 h. Peak with magenta underline: RT = 4.05 min, [Neu5Ac] = 29 mg / L 8

Figure 6. HPLC-UV chromatograms (λ = 350 nm) for DMB-derivatized sample from growth experiment with GlcNPr feedstock with both BRL-04 and BL21 cells at t = 72 h, as well as derivatized Neu5Ac standard, and blank (water). Triplicate BRL04 cultures showed peak at 7.2 minutes putatively corresponding to Neu5Pr, concentration 30 mg L^{-1}. No corresponding peaks were observed in BRL02 or BL21 cultures. 10

Figure 7. HPLC-UV chromatograms (λ = 350 nm) for DMB-derivatized sample from second growth experiment with GlcNPr feedstock with BRL-04 cells at t = 48 h, as

well as derivatized Neu5Ac standard, assay blank, and instrument blank. Two peaks are observed in the growth experiment sample, at 3.43 and 6.82 minutes. 12

Figure 8. Plasmid map of pDS3, containing C-terminal His-tagged nanE and neuB genes under the control of an inducible T7 promoter. 18

Figure 9. Standard curve for DMB-derivatized Neu5Ac, from 10 – 160 mg / L. 21

Figure 10. NMR and mass spectra (EI+ TOF) for diluted GlcNPr feedstock sample. MS peak at 258 corresponds to GlcNPr + Na^+. 26

Figure 11. HPLC-UV chromatograms (λ = 350 nm) for DMB-derivatized sample from growth experiment with azidoglucose feedstock with BRL-04 cells at t = 48 h, with derivatized Neu5Ac standard, and assay blank (DMB solution). Integral of peak observed at 3.5 minutes was inconsistent between replicates, and also appeared in BRL02 cultures (not shown). 27

Figure 12. HPLC-UV chromatograms (λ = 350 nm) for DMB-derivatized sample from first growth experiment with GlcNBu feedstock with BRL-04, BRL02, and BL21 cells at t = 72 h. Peaks at t = 7 minutes in BRL04 cultures putatively correspond to Neu5Bu product. Note that BL21 trace was taken before instrument was fully equilibrated. 28

Figure 13. HPLC-UV chromatograms (λ = 350 nm) for DMB-derivatized sample from second growth experiment with GlcNBu feedstock with BRL-04 cells at t = 48 and 144 h. Peaks at t = 7 minutes in BRL04 cultures putatively correspond to Neu5Bu product, but peaks at 3.5 minutes were also observed and could correspond to production of Neu5Ac. 29

Figure 14. HPLC-UV chromatograms (λ = 350 nm) for DMB-derivatized sample from growth experiment with GlcNiBu feedstock with BRL-04 cells at t = 72 h, with derivatized Neu5Ac standard, and assay blank (DMB solution). Very small potential peaks are indicated at t = 3.5 minutes but were below the sensitivity of the assay and could not be confirmed. 30

Figure 15. HPLC-UV chromatograms (λ = 350 nm) for DMB-derivatized sample from growth experiment with GlcNiBu feedstock with BRL-04 and BL21 cells at t = 72 h, with derivatized Neu5Ac standard, and assay blank (DMB solution). BL21 cells showed strong double peak in this experiment which could not be further identified. 31

Figure 16. HPLC-UV chromatograms (λ = 350 nm) for DMB-derivatized sample from growth experiment with GlcNiBu feedstock with BRL-04 cells at t = 72 h, with derivatized Neu5Ac standard, and assay blank (DMB solution). One replicate of BRL04 cells showed at stronger peak at t = 3.5 minutes (bottom left)..... 32

List of Tables

Table 1. Summary of growth experiments with BL21, BRL02, and BRL04 cells transformed with sialic acid biosynthetic plasmid pDS3. Not detected indicated by dash; Averages presented, n = 3. 11

Introduction

Background

Metabolic engineering is the discipline of modifying metabolic pathways to optimize the production of a given product or phenotype [1] [2]. Techniques from molecular biology, systems biology, and fundamental biochemistry are applied to engineer desired outcomes [3]. Advances in the field of metabolic engineering are increasingly achieved through the application of new methods in computational biology, protein engineering, and synthetic biology [4]. The application of high-throughput techniques to metabolic engineering is also accelerating progress in the field [5].

N-acetylneuraminic acid (Neu5Ac) is the most common member of N- or O-substituted derivatives of neuraminic acid (a 9-carbon monosaccharide) [6], and access to these derivates (sialic acids) is important to the study of their biology, especially their roles in cell-cell interactions and immunology [7] [8] [9]. Sialic acids have been found on the surface of most vertebrate cells [10]. Physiologically, sialic acids in humans have been shown to be essential to kidney function [11], to affect neuronal plasticity [12], and to mediate inflammatory responses [13]. Sialic acids are also produced by many pathological bacteria and aid in evading host immune response [14]. Sialic acids have potential roles as therapeutics, with a sialic acid derivative, zanamivir, being a common anti-influenza drug [15]. However, research and development in sialic acids is limited by availability to compounds due to difficult and expensive available syntheses [16].

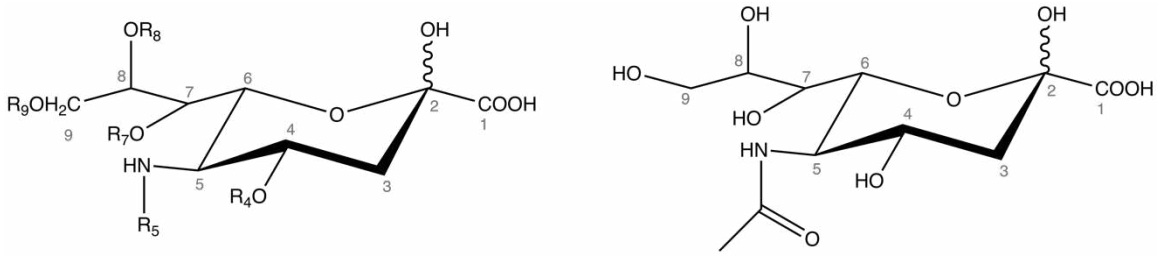

Figure 1. General structure of sialic acids (left) and of N-acetylneuraminic acid (Neu5Ac, right). Adapted from [17].

Chemical synthesis of Neu5Ac and derivatives is difficult due to the number of stereocenters as well as the need to protect many similar functional groups simultaneously [18]. While chemical synthesis routes exist, they involve many steps and low yields [19] [20].

Chemo-enzymatic synthesis routes include one-step conversion of N-acetylmannosamine (ManNAc) and pyruvate to Neu5Ac [8], and two-step conversion of N-Acetylglucosamine (GlcNAc) to ManNAc, followed by conversion to Neu5Ac [21]. Chemo-enzymatic synthesis requires purified enzymes and potentially expensive substrates (ManNAc) and cofactors (ATP) [21], and is a high-cost process unsuitable for large-scale production of sialic acids.

Whole cell biosynthesis of sialic acids is a promising technology as intermediates and cofactors can be provided by host cells. Initial work in this domain by Boddy *et al* [16] and others [22] developed biosynthetic fermentation pathways using inexpensive feedstocks such as glucose or N-Acetylglucosamine (GlcNAc). These methods have used genetically modified *E. coli* with sialic acid synthases from other organisms such as *Campylobacter jejuni* and *Neisseria meningitidis*. Initial methods have been optimized to

produce Neu5Ac at titres of up to 26 g L^{-1} [23], and improved by knockout of genes involved in sialic acid catalysis [24].

However, the fermentation pathway previously described cannot produce derivatives of Neu5Ac. Synthesis of derivatives typically involves design of a novel scheme for each compound [25]. The family of sialic acids comprises over 50 naturally occurring members, and access to these compounds is required to perform fundamental research on their biology [26]. A versatile biosynthesis capable of producing several Neu5Ac derivatives could permit increased access to these compounds, enabling further research as well as development of new therapeutics.

Project Goals

The present work proposes a biosynthetic route for producing a variety of sialic acid derivatives using a single pathway (shown in Figure 2), using GlcNAc-derivative feedstocks to produce corresponding Neu5Ac derivatives. This pathway was designed by PhD candidate Luis Villegas and the purpose of this work was to construct it with His-tagged genes and to validate it with a variety of substrates. The pathway is designed to generate sialic acid derivatives with modifications at the fifth Carbon (see Figure 1), specifically, by modification of the acetyl group.

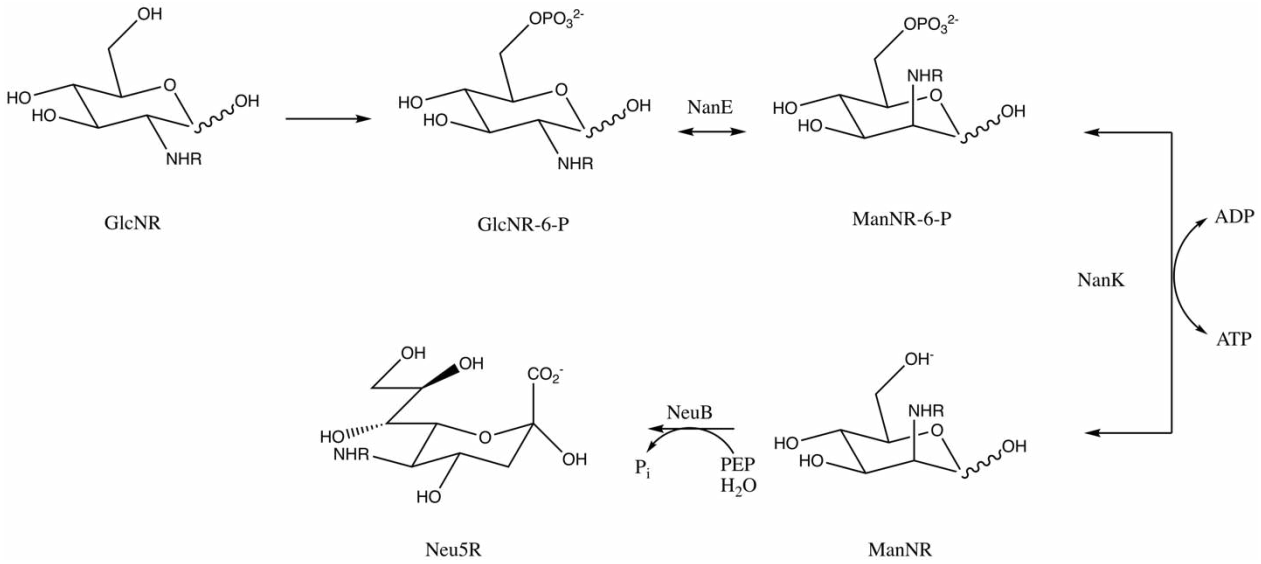

Figure 2. Schematic for production of Neu5R sialic derivative from GlcNR feedstock, where R is the functional group corresponding to the derivative (e.g. R = Ac for Neu5Ac production from GlcNAc). Modified from [24].

The pathway design is shown in Figure 2. A GlcNAc-derivative (hereafter GlcNR, where R corresponds to the functional group replacing the acyl group of GlcNAc) is imported into the cell and phosphorylated upon import [27]. The phosphorylated GlcNR (GlcNR-6-P) is epimerized by ManNAc-6-phosphate epimerase (NanE) [28] to the corresponding mannose-based derivative (ManNR-6-P). ManNR-6-P is dephosphorylated by ManNAc kinase (NanK) (*Ibid.*). These equilibrium reactions generate a small pool of ManNR, the dephosphorylated mannose-based derivative. NeuB, a sialic acid synthase from *Neisseria meningitidis* (other enzymes described are native to *E. coli*), irreversibly converts ManNR with consumption of phosphoenolpyruvic acid (PEP) to the corresponding sialic acid derivative, Neu5R [29].

It must be noted that the favoured direction for NanK is toward the phosphorylation of ManNAc to produce ManNAc-6-P [28]. However, as is common in enzymes, some leaky reverse activity is possible. This system depends on any ManNR

produced being converted irreversibly (due to consumption of PEP by NeuB) to Neu5R. Thus, consumption of ManNR will drive equilibrium toward regeneration of the pool and continued production of Neu5R.

Since biosynthesis is here carried out in whole cells, competing metabolic pathways exist which could reduce or eliminate yields. Some key alternative pathways are summarized in Figure 3. GlcNR feedstock may be directed toward central metabolism by the N-Acetylglucosamine 6-phosphate deacetylase NagA [30]. Neu5R may be reconverted to ManNR by the N-acetylneuraminate lyase NanA [31]. Neu5R product is exported from cells by an unknown mechanism but may be re-imported by sialic acid transporter NanT (*Ibid.*).

Previous work by the Boddy group had produced two strains of *E. coli* lacking genes encoding the catabolism and transport genes shown in Figure 3: BRL02 is a $nanT^-$ $nanA^-$ strain [16], and BRL04 is a $nanT^-$ $nanA^-$ $nagA^-$ strain [24]. While sialic acid biosynthesis has been successful in both strains, higher titres have been obtained in BRL04 due to a more limited sialic acid catabolism.

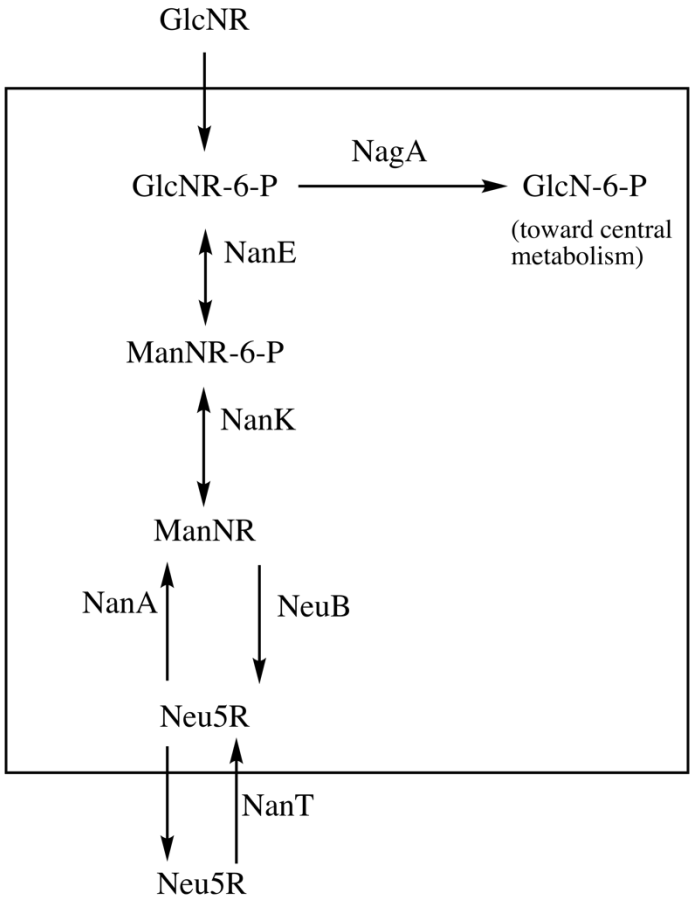

Figure 3. Alternative metabolic pathways for GlcNR substrate in E. coli. The box represents the cell wall, and enzymes or transporters are indicated beside arrows. Modified from Horsman et al., 2016.

Sialic acid quantification

Determination of sialic acid in this study was performed by derivatization with 1,2-diamino-4,5-methylene dioxybenzene (DMB) [32] [33], followed by detection through HPLC-UV (λ = 350 nm) [25]. A standard curve was generated using known concentrations of Neu5Ac, and it was assumed that since DMB binds in a 1:1 ratio with high specificity for nine-carbon sugars [34] that this standard curve would apply to the various Neu5Ac derivatives produced.

Results

Production of Neu5Ac

To initially validate the pathway, an experiment to produce Neu5Ac from GlcNAc was designed. *E. coli* BRL02 and BRL04 strains were selected for production experiments because of their limited sialic acid catabolism, as well as *E. coli* BL21(DE3) since it is commonly used for protein production and is the parent strain for BRL02 and BRL04. Cells were grown for 48 h in minimal media and given one feeding 0.5% GlcNAc upon induction with IPTG [35] [25].

Western blotting of cell lysates using Anti-His antibodies showed that both NanE and NeuB were highly expressed (see Figure 4). Sialic acid was detected by HPLC-UV (see representative chromatogram in Figure 5) in the BRL04 production culture at 29 mg / L, but was not detected in the BRL02 or the BL21 cultures. A summary of all HPLC-UV results from experiments with GlcNAc derivatives is presented in Table 1 (page 11).

Figure 4. Western blot shows lysates of BL21, BRL02, and BRL04 cultures at 24 and 48 h, in which expression of His-tagged neuB and nanE had been induced. Strong bands detected for both proteins, at both time points and in all cultures.

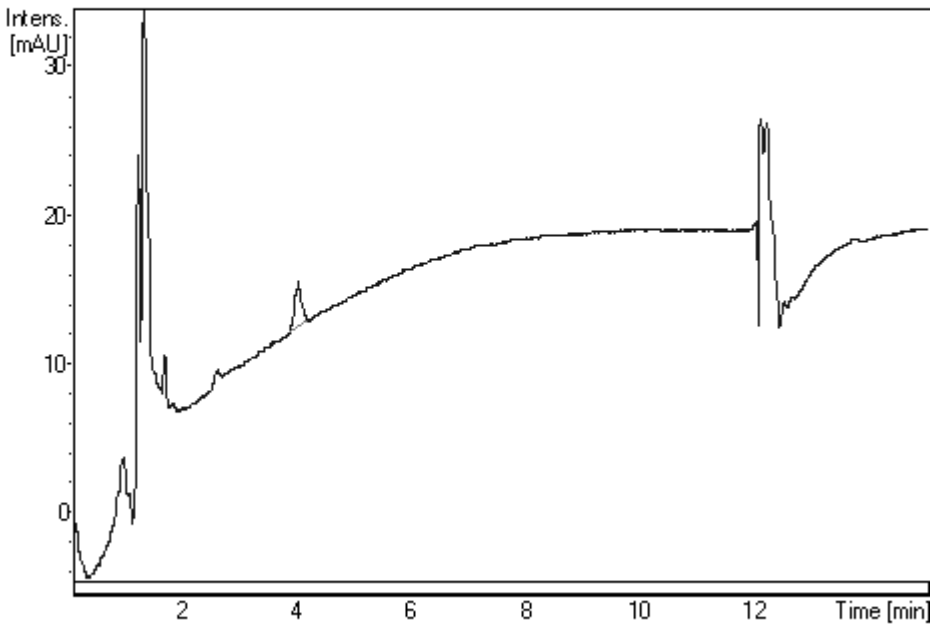

Figure 5. HPLC-UV chromatogram (λ = 350 nm) for DMB-derivatized sample from growth experiment with GlcNAc feedstock with BRL-04 cells at t = 48 h. Peak with magenta underline: RT = 4.05 min, [Neu5Ac] = 29 mg / L.

Production of Neu5Bz and Neu5Pr

Growth experiments were performed using both GlcNBz and GlcNPr substrates to attempt to characterize the performance of the system with both small and sterically bulky modifications. HPLC-UV showed production of Neu5Pr at 30 mg / L in BRL04 cultures (similar production level as for Neu5Ac, see Figure 6 and Table 1) but production of Neu5Bz was not detected.

Purification of Neu5Pr

An attempt was made to purify Neu5Pr product by lyophilizing cell broth, followed by filtration and methanol trituration. While this procedure for similar compounds (e.g. legionaminic acid) visibly crashed out salts upon addition of methanol, a homogeneous mixture was formed with the lyophilized broth that could not be further separated.

Production of Neu5Bu and Neu5iBu

Since production of Neu5Pr was successful, substrates with increasingly bulky modifications were tested. Growth experiments were performed using GlcNBu and GlcNiBu substrates. Neu5Bu was produced at 4 mg / L in BRL04 cultures (see Figure 12 in Appendix); however, Neu5iBu product was not detected (see Figure 14 – Figure 16 in Appendix). Again, a summary of all growth experiments is presented in Table 1.

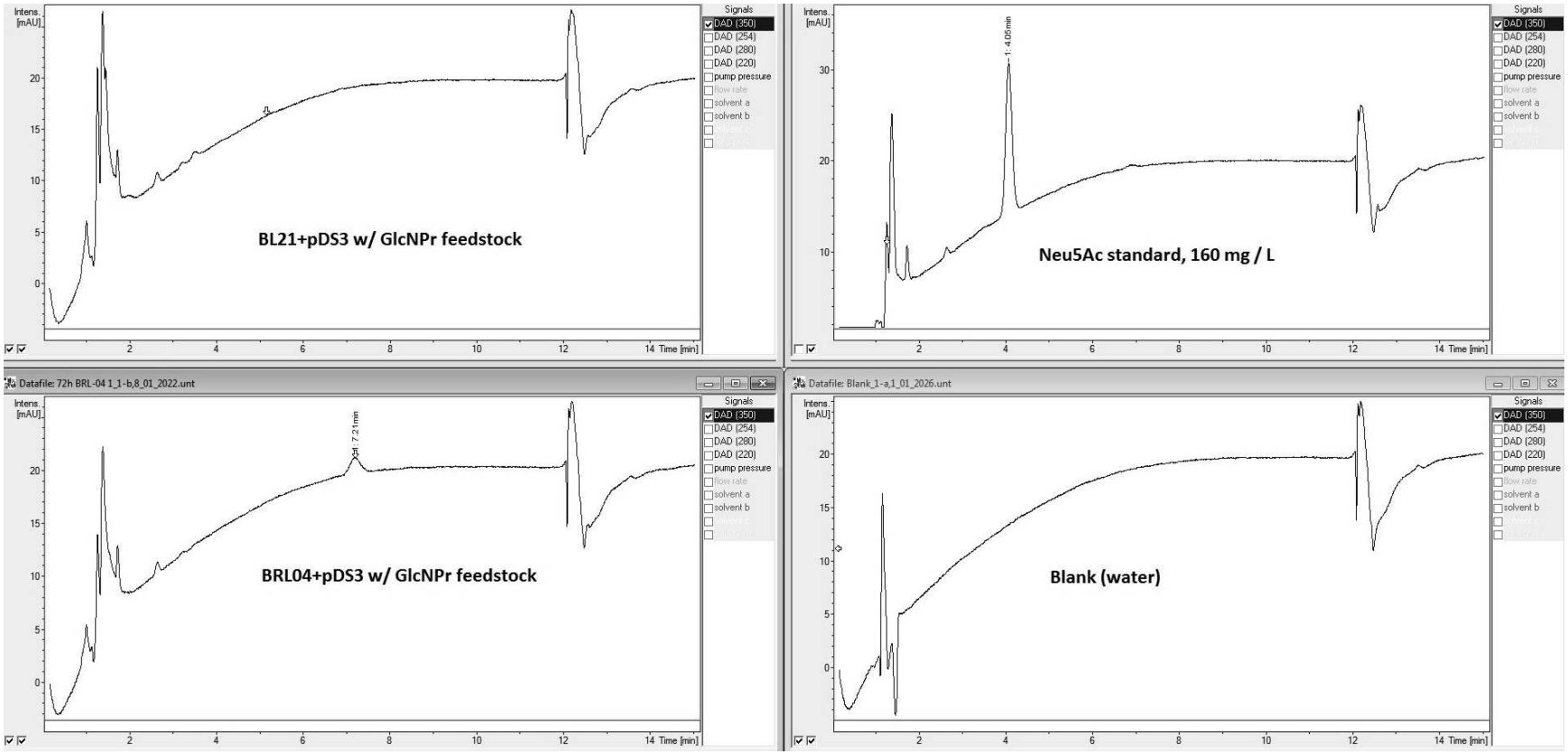

Figure 6. HPLC-UV chromatograms (λ = 350 nm) for DMB-derivatized sample from growth experiment with GlcNPr feedstock with both BRL-04 and BL21 cells at t = 72 h, as well as derivatized Neu5Ac standard, and blank (water). Triplicate BRL04 cultures showed peak at 7.2 minutes putatively corresponding to Neu5Pr, concentration 30 mg L^{-1}. No corresponding peaks were observed in BRL02 or BL21 cultures.

Table 1. Summary of growth experiments with BL21, BRL02, and BRL04 cells transformed with sialic acid biosynthetic plasmid pDS3. Not detected indicated by dash; Averages presented, n = 3.

Feedstock	Culture	RT (min)	Integral (mAU)	[Sialic acid] (mg L^{-1})
GlcNAc	BL21	–	–	–
	BRL02	–	–	–
	BRL04	4.05	121.93	29
GlcNPr	BL21	–	–	–
	BRL02	–	–	–
	BRL04	7.02	123.84	30
GlcNBu	BL21	–	–	–
	BRL02	–	–	–
	BRL04	7.09	31.33	4
GlcNiBu	BL21	–	–	–
	BRL02	–	–	–
	BRL04	–	–	–
GlcNBz	BL21	–	–	–
	BRL02	–	–	–
	BRL04	–	–	–

Further characterization of Neu5Pr and Neu5Bu

To more fully characterize Neu5Pr and Neu5Bu products, additional growth experiments were performed with the goal of increasing titres. HPLC-UV analysis suggested that the desired derivatives were produced, but that Neu5Ac was produced as well due to peaks at both the expected retention times for Neu5Ac and Neu5Pr/Bu (see Figure 7, and Figure 14 in Appendix).

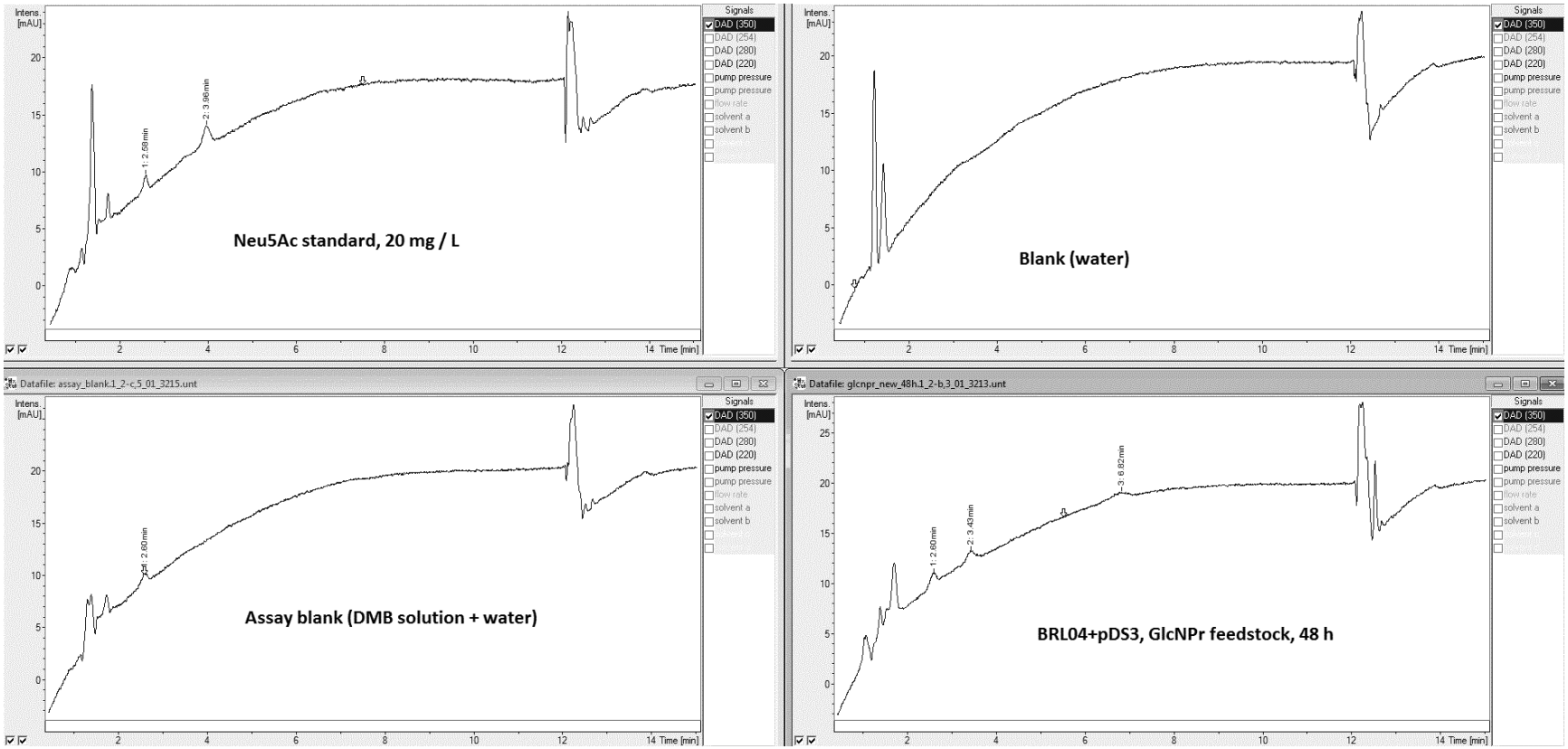

Figure 7. HPLC-UV chromatograms (λ = 350 nm) for DMB-derivatized sample from second growth experiment with GlcNPr feedstock with BRL-04 cells at t = 48 h, as well as derivatized Neu5Ac standard, assay blank, and instrument blank. Two peaks are observed in the growth experiment sample, at 3.43 and 6.82 minutes.

Characterization of GlcNPr substrate

Mass and ^{1}H NMR spectra were obtained for GlcNPr substrate to investigate whether glucosamine or other impurities were present in the feedstock. Mass spectra suggested a high purity of GlcNPr, as any signal corresponding to glucosamine was indistinguishable from background signal (see Figure 10).

Growth experiment with azidoglucose substrate

A growth experiment was performed with azidoglucose, with the hypothesis that intracellularly it may be converted to N-azido glucosamine and further converted to N-azido neuraminic acid. Variations between HPLC-UV spectra of replicates precluded strong interpretation of results (see Figure 11 in Appendix).

Discussion

Given the dependence on unfavoured reverse conversion of ManNAc to ManNAc-6-P by NanK in the biosynthetic pathway, production of Neu5Ac and Neu5Pr is a success despite low initial yields of about 0.6%. Further optimization of growing conditions and feeding regimens will be required to increase titres.

Some work was done to attempt to purify products by lyophilizing cell broth followed by methanol trituration. While this method was not successful, future work may attempt purification using an ion exchange column to bind hydrophilic products, followed by elution with formic acid.

Neu5Bz product was not detected in cell broth by HPLC-UV analysis. However, since the sensitivity of the DMB conjugation assay was determined to be about 5 mg / L, it is possible that Neu5Bz was produced in lower titres that could not be detected. The use of HPLC-MS/MS could make detection possible if this product was generated. This applies also to the growth experiment designed to produce Neu5iBu.

The production of both Neu5Ac and Neu5Pr from GlcNPr substrate, when previous experiments had produced only Neu5Pr, suggested that the substrate was not pure; glucosamine or other impurities in the feedstock could allow for preferential production of sialic acid. Given the low conversion of GlcNPr to Neu5Pr, it is possible that small impurities would be efficiently converted to Neu5Ac in similar yields. An experiment to validate this hypothesis would be a growth experiment using glucosamine as a substrate. Note however that mass spectra for the GlcNPr substrate indicated that the

purity was high, with any glucosamine impurities being indistinguishable from the background signal.

While sialic acid determination was performed in this work by HPLC-UV analysis with DMB-derivatization, it was only possible to determine the presence of sialic acids without confirmation of their specific identity. Analysis of culture broths should be performed by HPLC-MS/MS in order to determine which derivatives are being produced. This would unambiguously identify the products without an immediate need for the higher titres that would permit less technically challenging purification.

Conclusion

Biosynthesis of sialic acid derivatives with modifications on the fifth Carbon was demonstrated to be possible through the NanE, NeuB pathway herein presented. Production of Neu5Ac was successful as a positive control, and production of Neu5Pr was demonstrated at similar titres. Production of Neu5Bu was achieved at lower yield. While production of Neu5iBu, Nue5Bz, and Neu5Az was attempted, results were inconclusive and further analysis is necessary.

One of the limitations of this study was the reliance on HPLC-UV to detect products. Positive identification of products would require purification, and higher-confidence results should be initially obtained through the use of HPLC-MS/MS. HPLC-MS/MS would permit clearer interpretation of the more ambiguous results that we obtained.

With further optimization it may become possible to product these sialic acid derivatives in higher titres, making possible a more economical synthesis than is currently available. Increased availability of these compounds will permit more rapid advances in our understanding of their biology and potential applications.

Experimental

Construction of sialic acid biosynthetic plasmid pDS3

E. coli XL1-Blue Competent Cells were used for cloning, following supplier's protocols for transformations. Briefly, competent cells were thawed on ice and 0.5 – 1 μL vector was added, with incubation on ice for 30 minutes. Heat shock was performed at 42 ºC for 45 seconds, then incubation on ice for 2 minutes. 1 mL preheated LB medium was added, and solution incubated for 1 hour at 37 ºC, 225 – 250 rpm. 100 μL were plated on appropriate selection plates and incubated overnight.

Source plasmids containing both nanE and neuB were selected from prior work in the group: pBRL21 contained neuB, and pBRL06 contained nanE, with both plasmids having NdeI and EcoRI sites flanking the respective genes. Both genes were excised by digestion with NdeI and EcoRI, and cloned into pMGXAmpHis: a low-copy Amp^R vector under control of an inducible T7 promoter, with a hexa-histidine tag following the EcoRI site [25]. In this way, pDS1 (nueB) and pDS2 (nanE) were produced. To form a bicistronic synthetic inducible operon containing both nanE and neuB, nanE was excised from pDS2 by XbaI and AvrII digestion, and inserted into pDS1 at the XbaI site to form pDS3 (see plasmid map in Figure 8).

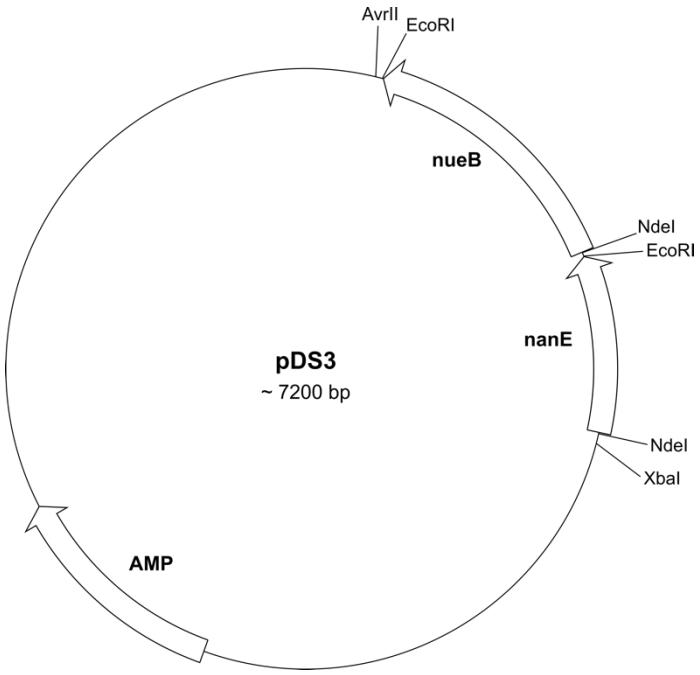

Figure 8. Plasmid map of pDS3, containing C-terminal His-tagged nanE and neuB genes under the control of an inducible T7 promoter.

Each plasmid was validated by restriction digests to validate successful insert ligation. Plating was performed with appropriate antibiotics for selection of successful ligation products. *E. coli* BL21(DES) cells were transformed with vectors and cell lysates assayed by Western blotting to validate protein expression.

Sialic acid production in *E. coli* in shake flask culture

BL21, BRL02 and BRL04 cells were transformed with pDS3 vector. Seed cultures from single colonies were grown in LB with appropriate antibiotics at 37 ºC, 200 rpm overnight (18 h). Production cultures in F2 media (consisting of per litre, 12.24 g K_2HPO_4, 6.0 g KH_2PO_4, 4.0 g $(NH_4)_2SO_4$ and 175.5 mg $MgSO_4$) with appropriate antibiotics were inoculated with 0.5% seed culture, 0.25% casitone, and 0.5% (v/v) glycerol, then grown at 37 ºC, 200 rpm until an OD_{600} of 0.4 – 0.6 was achieved (2 – 4 h).

At this point (t = 0), cultures were inoculated with 0.5% of the appropriate GlcNAc derivative, and protein expression was induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Production cultures were incubated at 30 ºC, 200 rpm. At t = 24 h, sample aliquots were collected, and antibiotics were again supplemented to the cultures. At t = 48 h sample aliquots were collected, and the growth experiment was stopped. Aliquots were centrifuged at 13,000 rpm for 2 min, and supernatant was removed for analysis. Triplicate cultures of all strains were used as possible depending on the quantity of substrate available.

Some variations of this procedure were attempted, splitting the feeding of 0.5% GlcNAc derivative to two feedings at t = 0 h and t = 24 h, as well as extending growth experiments to 72 or 144 h. Aliquots were taken at t = 48 h and no significant differences in titre were found for longer experimental durations.

HPLC detection and quantification by DMB hybridization

Collected cell culture supernatants were mixed with a DMB solution (8 mM DMB (Sigma-Aldrich), 1.5 M acetic acid, 14 mM sodium hydrosulfite, 0.8 M β-mercaptoethanol) in a 1:1 volume mixture. Samples were placed in a heat block at 60 ºC for two hours, shielded from light due to ready photodegradation of DMB.

Derivatized samples were analysed using a 1260 Infinity High Performance Liquid Chromatography system (Agilent Technologies) using a Prontosil C18, 5 µm, 125 mm x 4 mm column. Flow rate: 1.00 mL min^{-1}, λ= 350 nm. Mobile phase: 84% water: 9% acetonitrile: 7% methanol constant for 12 minutes, followed by a linear gradient of acetonitrile from 9% to 100% over 1 minute until completion. A standard curve was

generated using known concentrations of an authentic standard of Neu5Ac from 10 – 160 mg L^{-1} (R^2 = 0.9996). The area under the curve of the peak corresponding to derivatized sialic acid was used for quantification.

Western blotting to detect His-tagged proteins

Collected cell pellets generated by centrifugation of 1.5 mL cell broth as previously described were resuspended in 300 µL B-PER Bacterial Protein Extraction Reagent (Thermo Scientific) and mixed for at least one minute. Crude lysates were run on SDS-PAGE Mini-Protean TGX Stain Free gels (BioRad) following protocol provided by the supplier, and imaged under UV

Proteins were transferred to a PVDF membrane, and blocking, transfer, and antibody dilution buffers prepared according to instructions provided with the antibody kit (HRP-conjugated Anti-His monoclonal antibody (Genescript)). Immobilon Western Chemiluminescent Horse Radish Peroxidase substrate was used for visualization according to supplier's protocol (ThermoFisher Scientific).

Neu5Ac standard curve

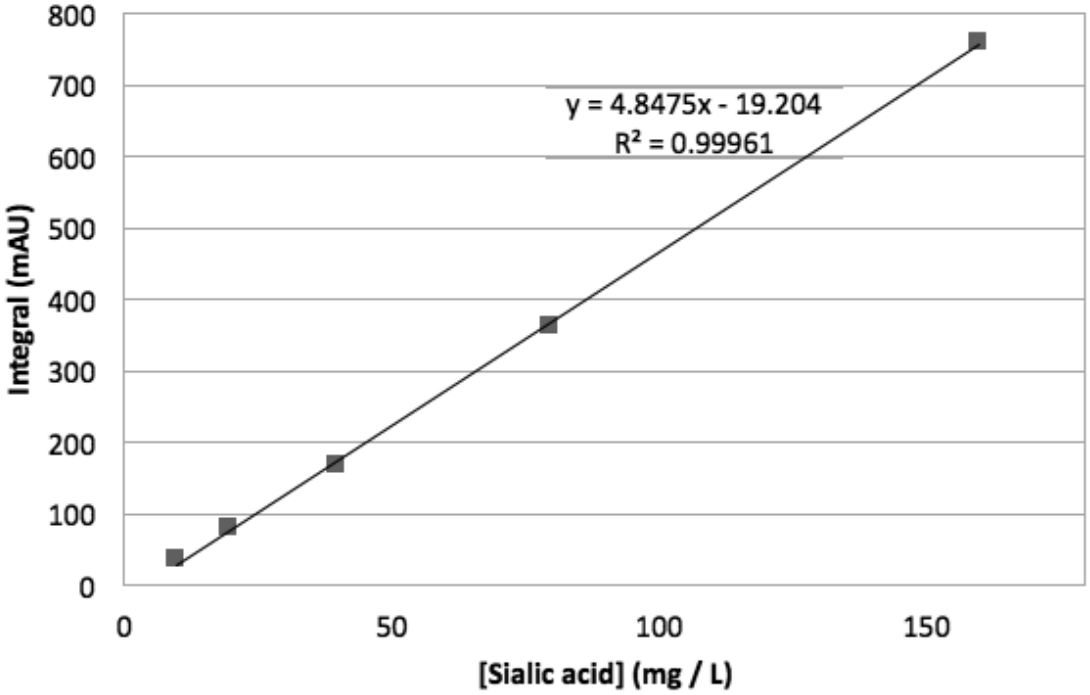

Figure 9. Standard curve for DMB-derivatized Neu5Ac, from 10 – 160 mg / L.

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Appendix

NMR and mass spectra for GlcNPr feedstock

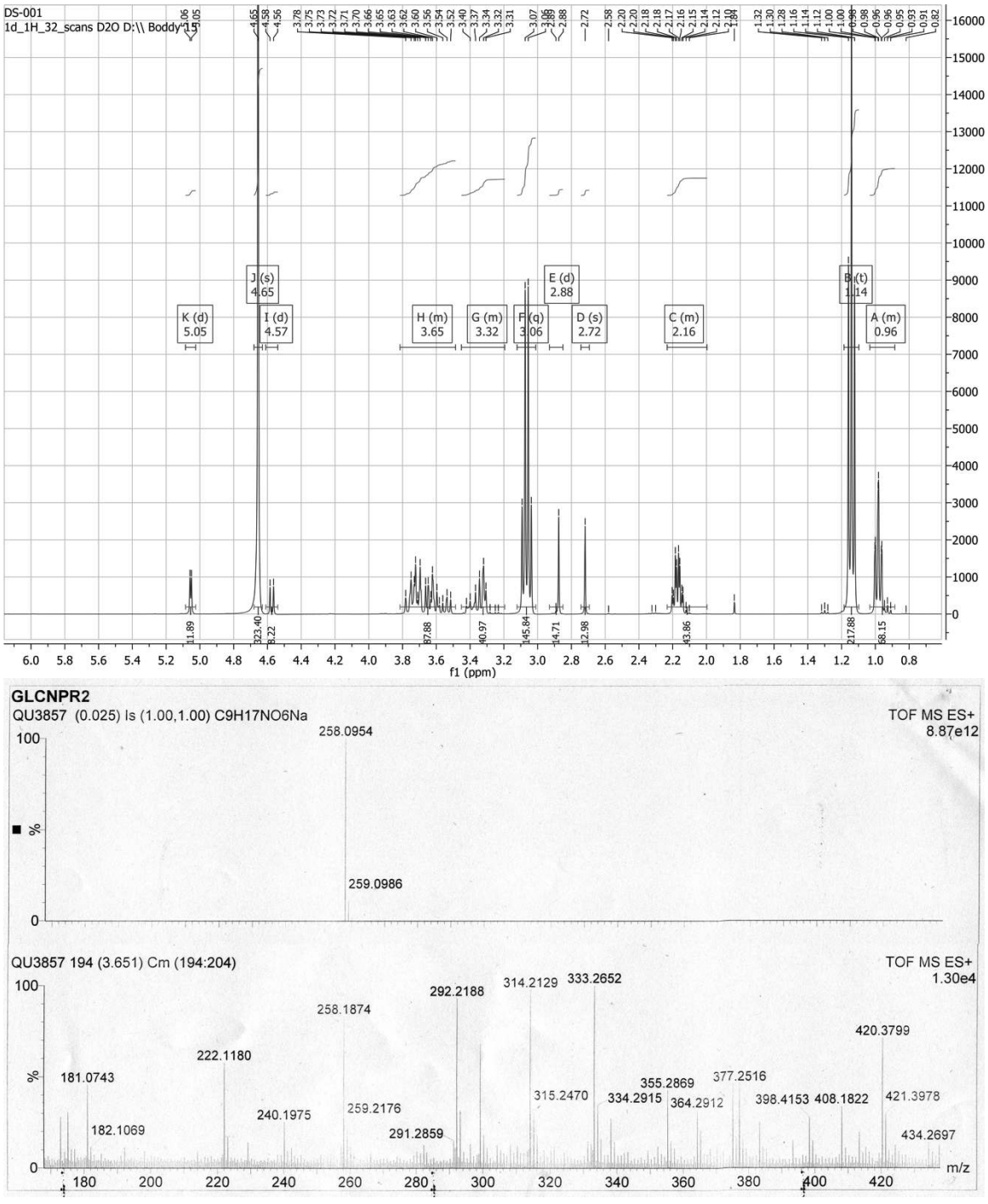

Figure 10. NMR and mass spectra (EI+ TOF) for diluted GlcNPr feedstock sample. MS peak at 258 corresponds to GlcNPr + Na^+.

Supplementary HPLC-UV chromatograms

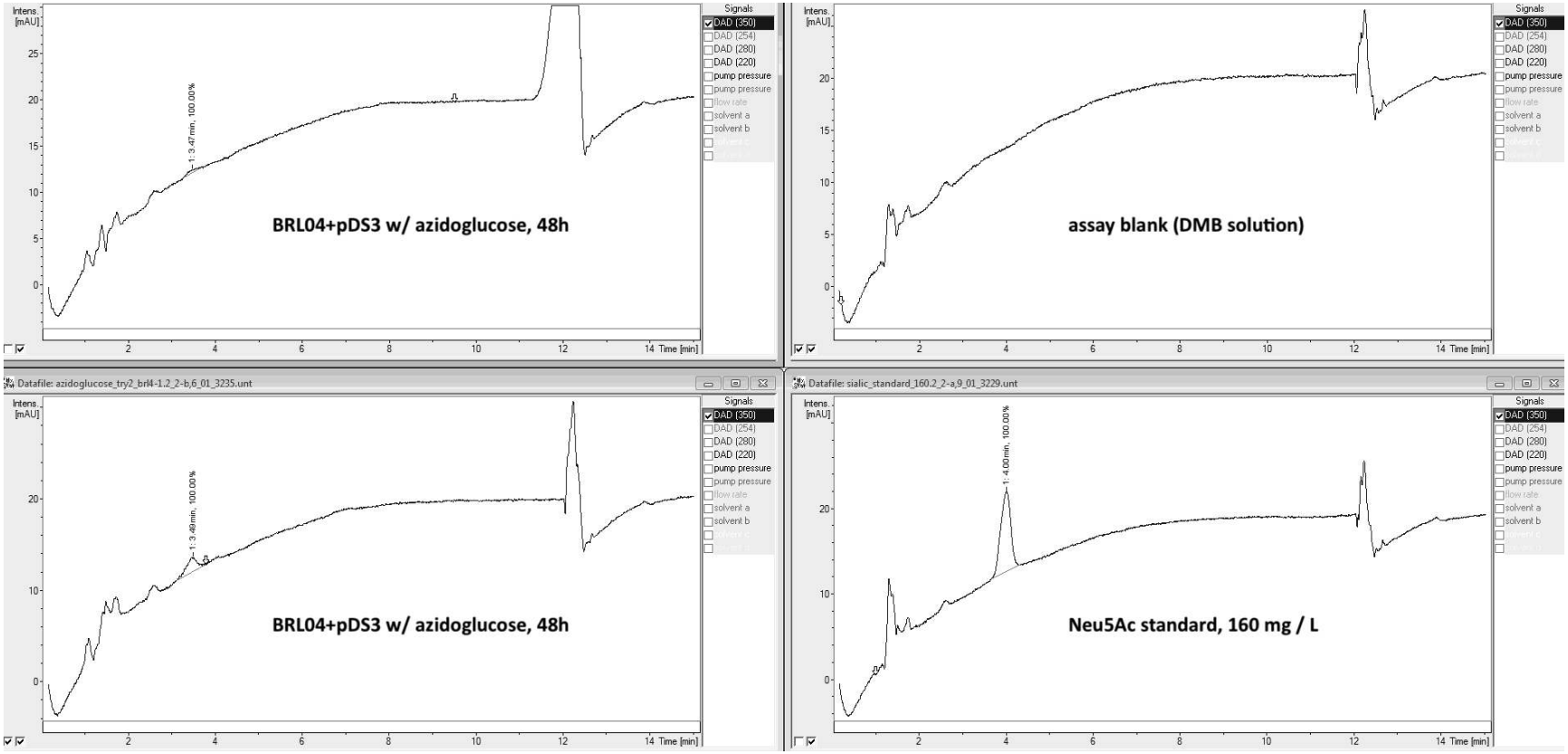

Figure 11. HPLC-UV chromatograms (λ = 350 nm) for DMB-derivatized sample from growth experiment with azidoglucose feedstock with BRL-04 cells at t = 48 h, with derivatized Neu5Ac standard, and assay blank (DMB solution). Integral of peak observed at 3.5 minutes was inconsistent between replicates, and also appeared in BRL02 cultures (not shown).

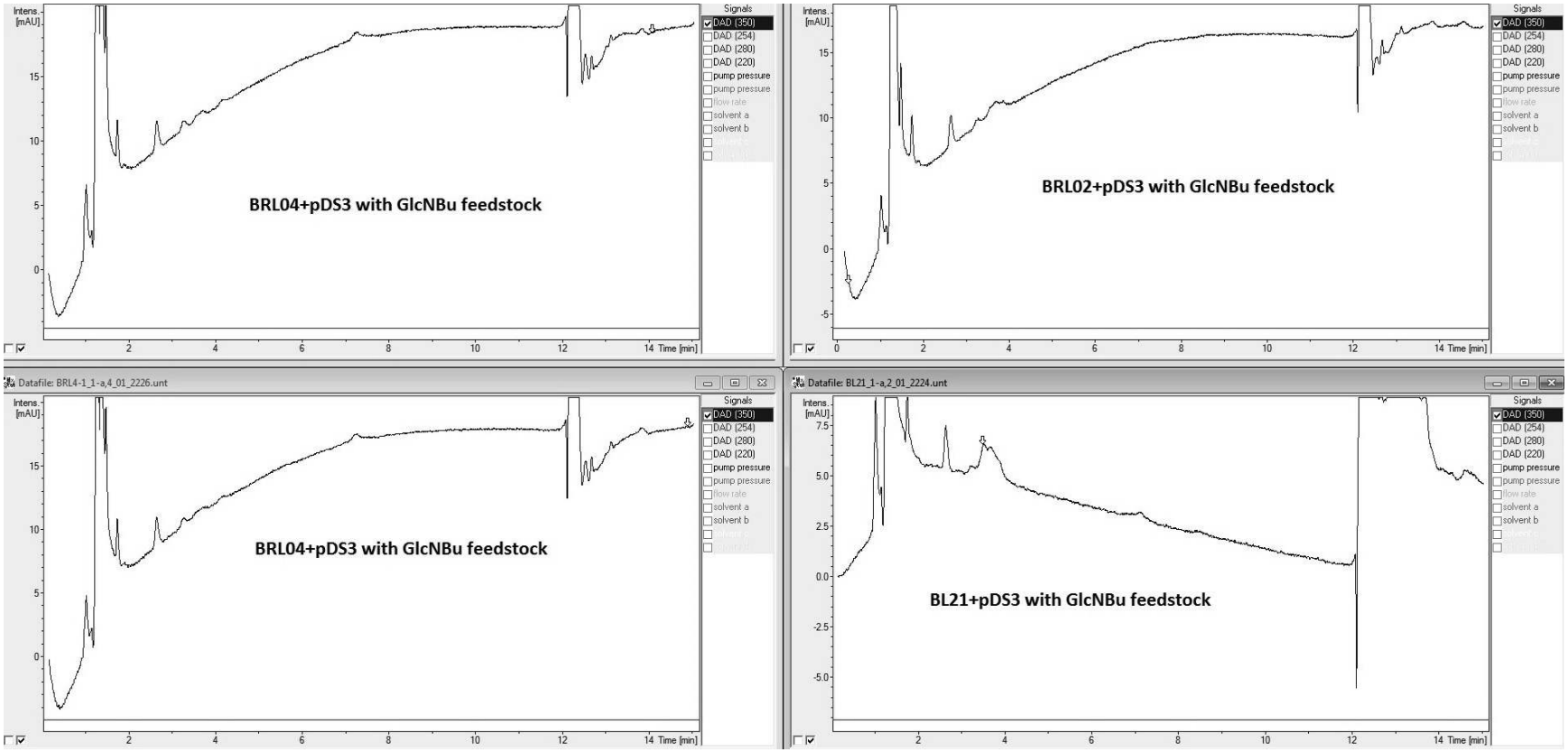

Figure 12. HPLC-UV chromatograms (λ = 350 nm) for DMB-derivatized sample from first growth experiment with GlcNBu feedstock with BRL-04, BRL02, and BL21 cells at t = 72 h. Peaks at t = 7 minutes in BRL04 cultures putatively correspond to Neu5Bu product. Note that BL21 trace was taken before instrument was fully equilibrated.

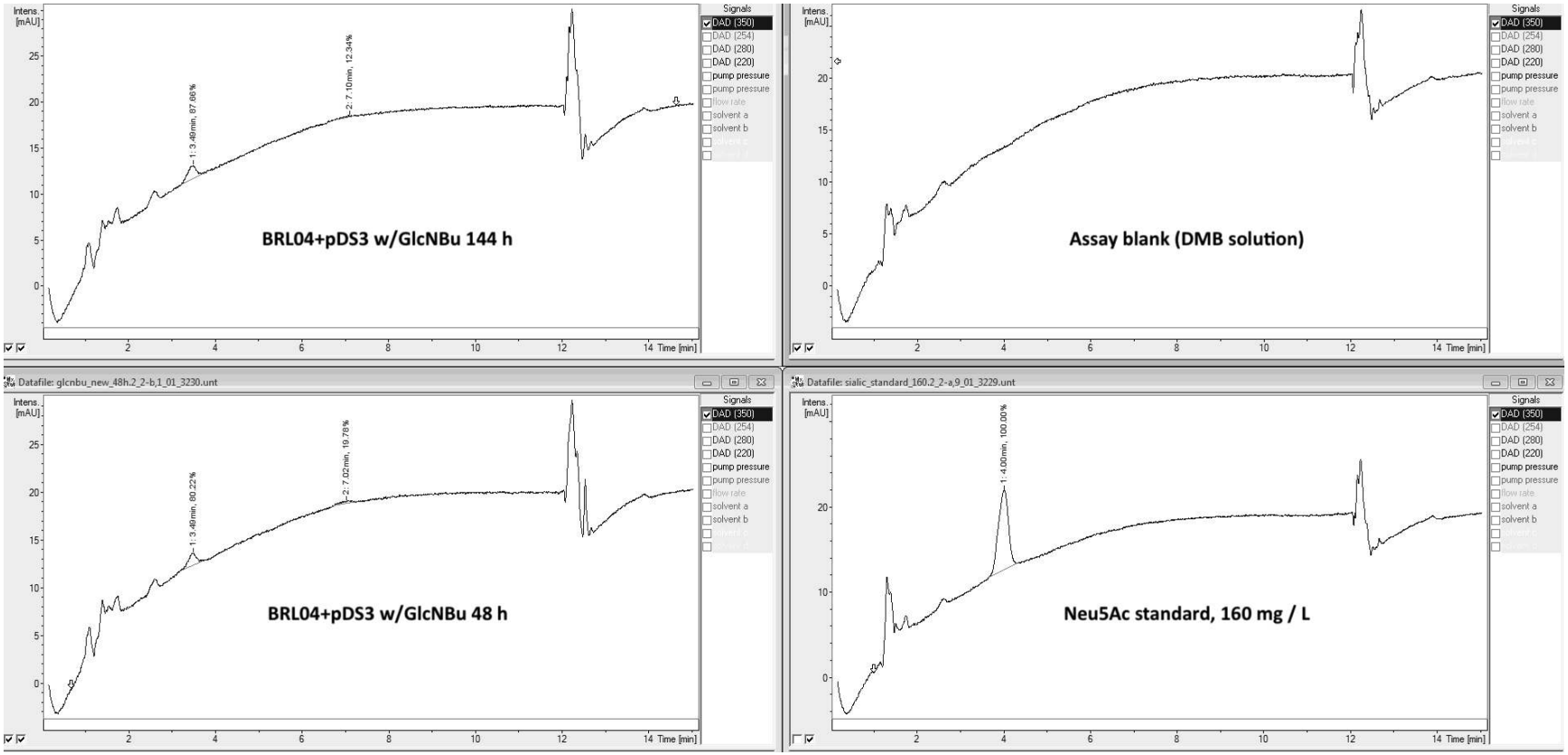

Figure 13. HPLC-UV chromatograms (λ = 350 nm) for DMB-derivatized sample from second growth experiment with GlcNBu feedstock with BRL-04 cells at t = 48 and 144 h. Peaks at t = 7 minutes in BRL04 cultures putatively correspond to Neu5Bu product, but peaks at 3.5 minutes were also observed and could correspond to production of Neu5Ac.

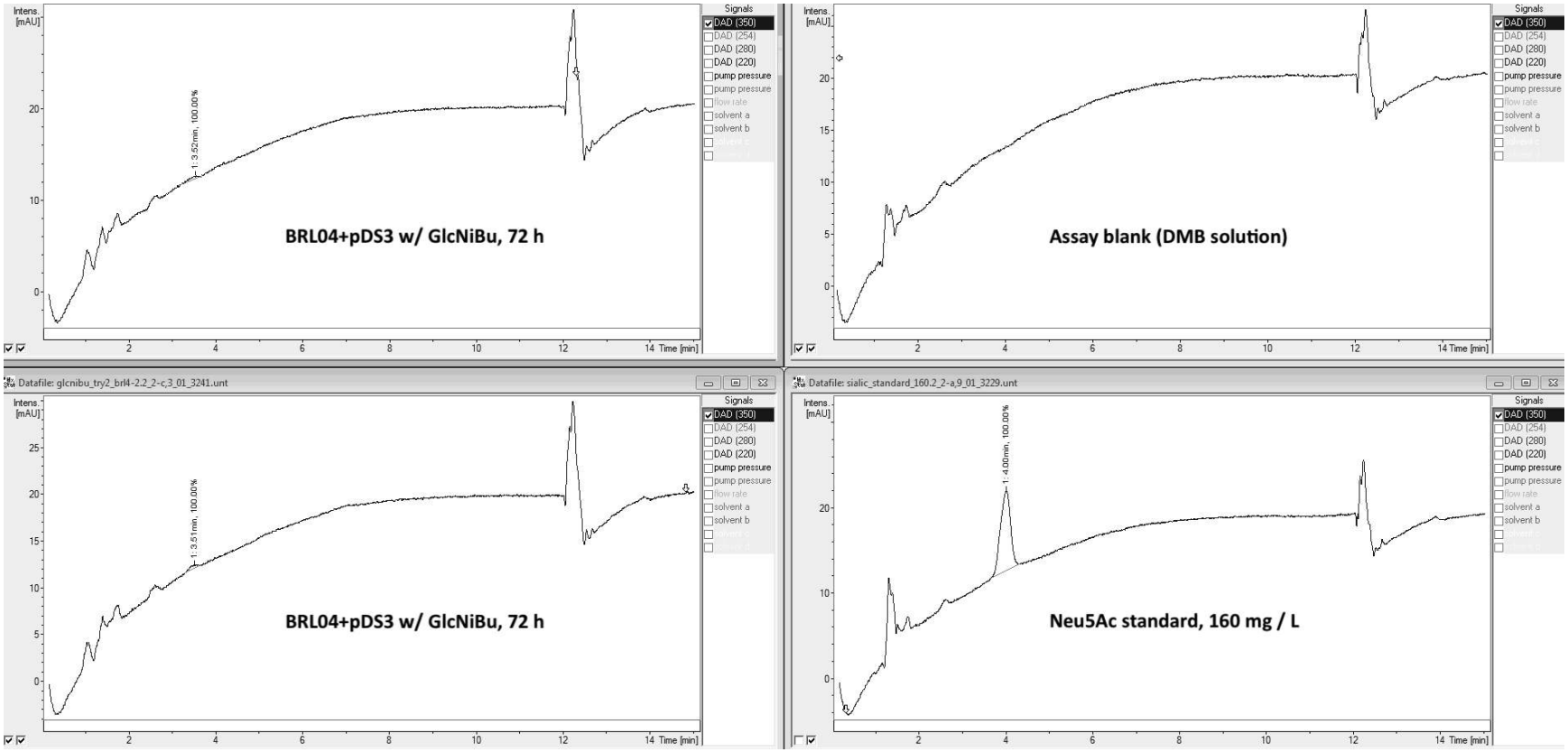

Figure 14. HPLC-UV chromatograms (λ = 350 nm) for DMB-derivatized sample from growth experiment with GlcNiBu feedstock with BRL-04 cells at t = 72 h, with derivatized Neu5Ac standard, and assay blank (DMB solution). Very small potential peaks are indicated at t = 3.5 minutes but were below the sensitivity of the assay and could not be confirmed.

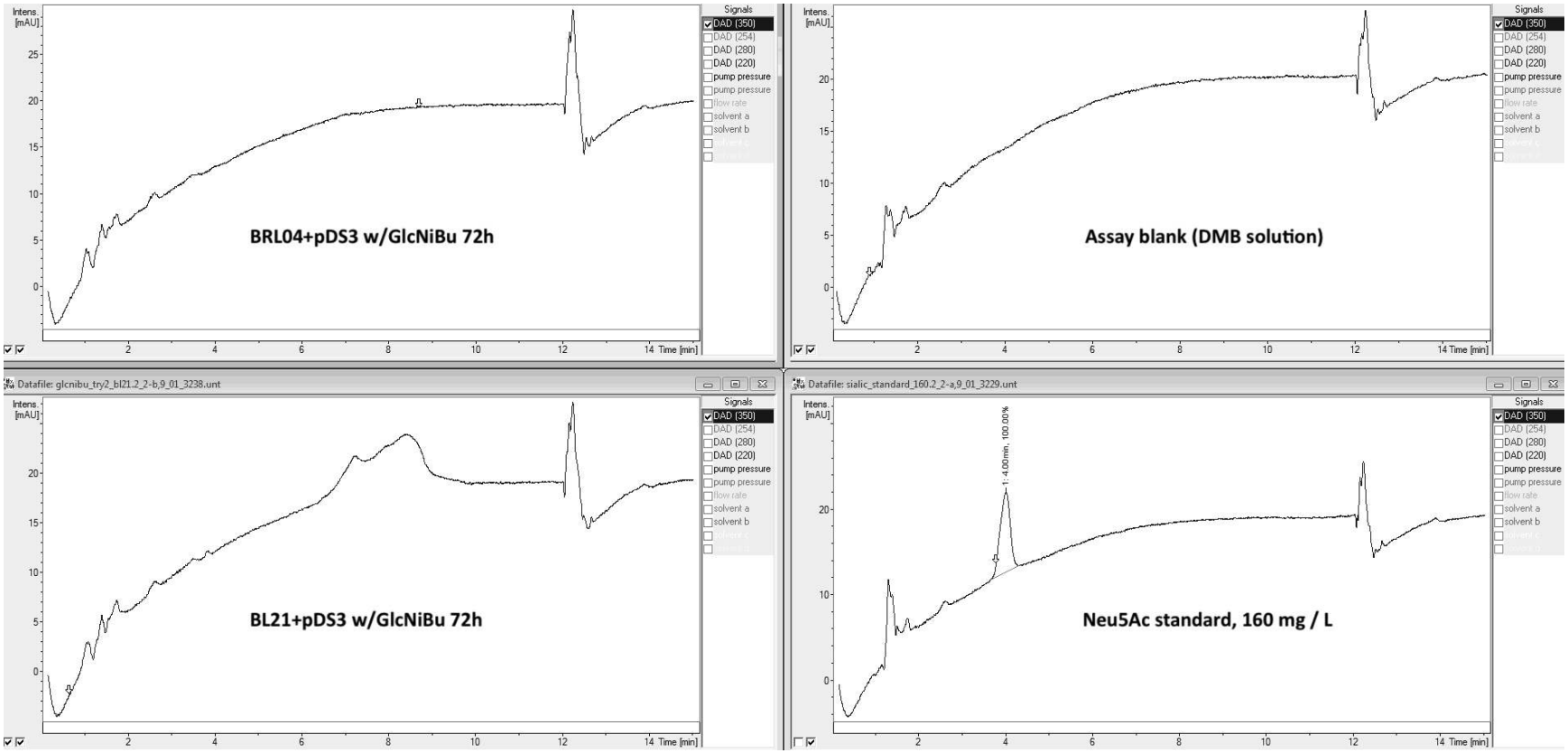

Figure 15. HPLC-UV chromatograms (λ = 350 nm) for DMB-derivatized sample from growth experiment with GlcNiBu feedstock with BRL-04 and BL21 cells at t = 72 h, with derivatized Neu5Ac standard, and assay blank (DMB solution). BL21 cells showed strong double peak in this experiment which could not be further identified.

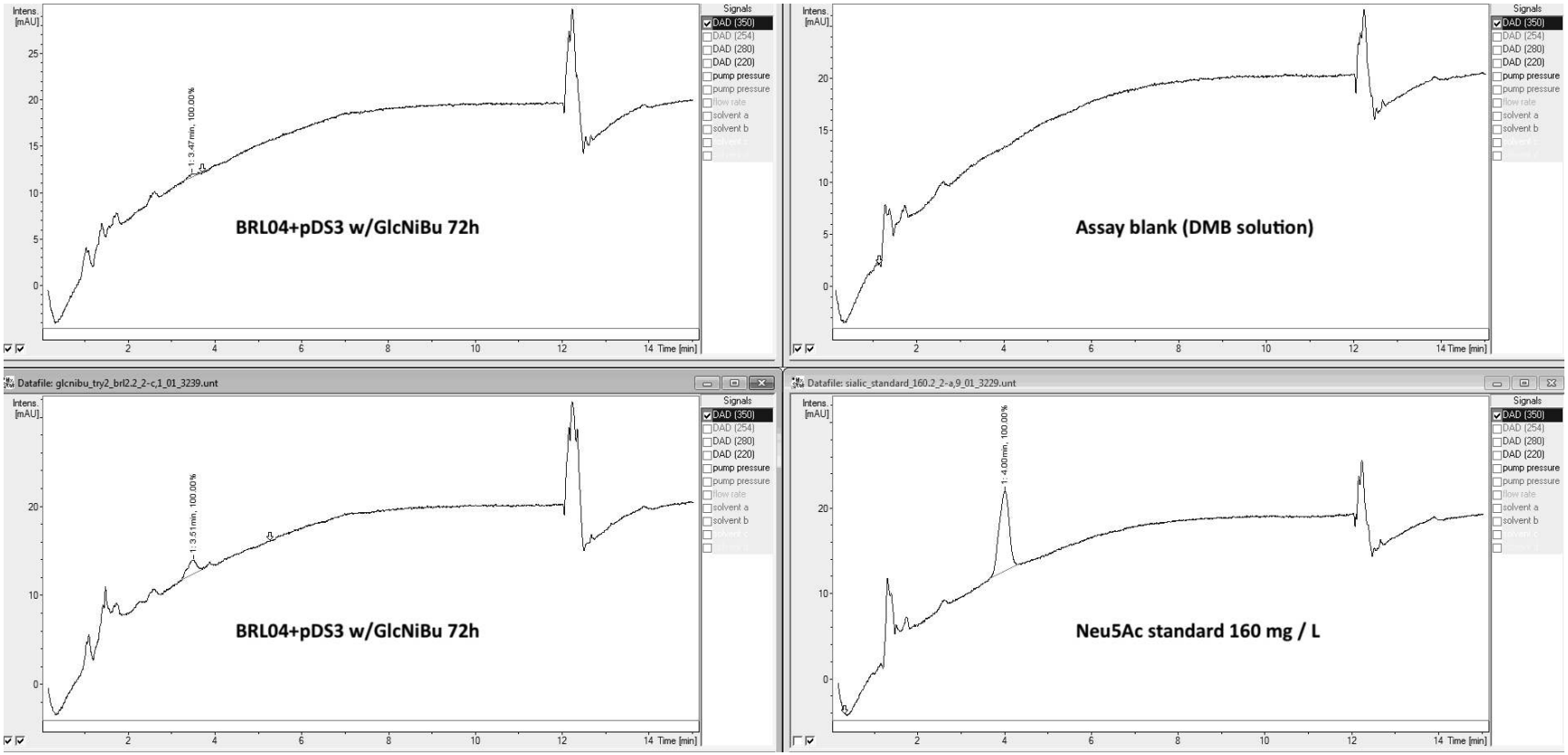

Figure 16. HPLC-UV chromatograms (λ = 350 nm) for DMB-derivatized sample from growth experiment with GlcNiBu feedstock with BRL-04 cells at t = 72 h, with derivatized Neu5Ac standard, and assay blank (DMB solution). One replicate of BRL04 cells showed at stronger peak at t = 3.5 minutes (bottom left).