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Development of a nucleotide sugar purification method using a mixed mode column & mass spectrometry detection

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ABSTRACT

Analysis of nucleotide sugars, nucleoside di- and triphosphates and sugar-phosphates is an essential step in the process of understanding enzymatic pathways. A facile and rapid separation method was developed to analyze these compounds present in an enzymatic reaction mixture utilized to produce nucleotide sugars. The Primesep SB column explored in this study utilizes hydrophobic interactions as well as electrostatic interactions with the phosphoric portion of the nucleotide sugars. Ammonium formate buffer was selected due to its compatibility with mass spectrometry. Negative ion mode mass spectrometry was adopted for detection of the sugar phosphate (fucose-1-phophate), as the compound is not amenable to UV detection. Various mobile phase conditions such as pH, buffer concentration and organic modifier were explored. The semi-preparative separation method was developed to prepare 30 mg of the nucleotide sugar. ^{19}F NMR was utilized to determine purity of the purified fluorinated nucleotide sugar. The collected nucleotide sugar was found to be 99% pure.

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1. Introduction

Nucleotide sugars contain sugar or sugar derivatives connected through the glycosidic hydroxyl group to the 2nd phosphate of a nucleoside 5′-pyrophosphate. The biosynthesis of protein and lipid-linked oligosaccharides utilizes nucleotide sugars for glycosylation [1,2]. Therefore, analysis of nucleotide sugars is an important step in understanding the glycosylation mechanism in cells.

Inhibition of fucosylation, the process of adding fucose sugar units to a molecule, is of interest in the field of antibody-dependent cellular cytotoxicity (ADCC). The antibody binds to the target antigen and engages CD16 on the effector cell. CD16 is a low affinity IgG receptor Fc III (FcγRIII) found on the surface of natural killer cells, such as monocytes and macrophages. Binding of the antibody causes the release of pore-forming proteins and proteases, which lyse the target cell. Absence of fucose on the antibody improves binding to the effector cell which, in turn, enhances cell lysis [3]. Fucosylation may be reduced through the use of a small molecule inhibitor of the fucosylation pathway.

Various chromatographic methods have been reported for the separation of nucleotides, nucleotide-sugars and sugar-phosphates. These include reversed-phase ion-pairing (RP-IP), porous graphite carbon (PGC), hydrophilic interaction liquid chromatography (HILIC) and mixed mode chromatography [1,2,4–10]. Guanosine diphosphate (GDP) sugars and their metabolites were separated by ion-pairing chromatography utilizing ODS-columns (C-18) and ion-pairing agents such as tributylamine with acetic acid [4].

Tetrabutylammonium hydrogen sulfate has been shown to be successful at separating these analytes as well. However, the ion-pairing agent was not compatible with the mass spectrometer [5]. In addition, to these studies, separation of various nucleotide sugars, such as GDP-mannose, GDP-fucose and GDP-rhamnose were successfully performed using a C-18 reversed-phase column and triethylammonium acetate buffer as an ion-pairing reagent for increasing retention [1]. Unfortunately, this buffer has low volatility and would be difficult to remove from collected purified samples. Also, nucleoside triphosphates were not explored in the study.

Columns with porous graphite carbon (PGC) were shown to separate nucleotides according to the base and the sugar moiety rather than the ionic phosphate groups, unlike ion-pairing reversed-phase chromatography. However, PGC columns required complex cleaning methods, which included boiling in TFA and rinsing with sulfite,

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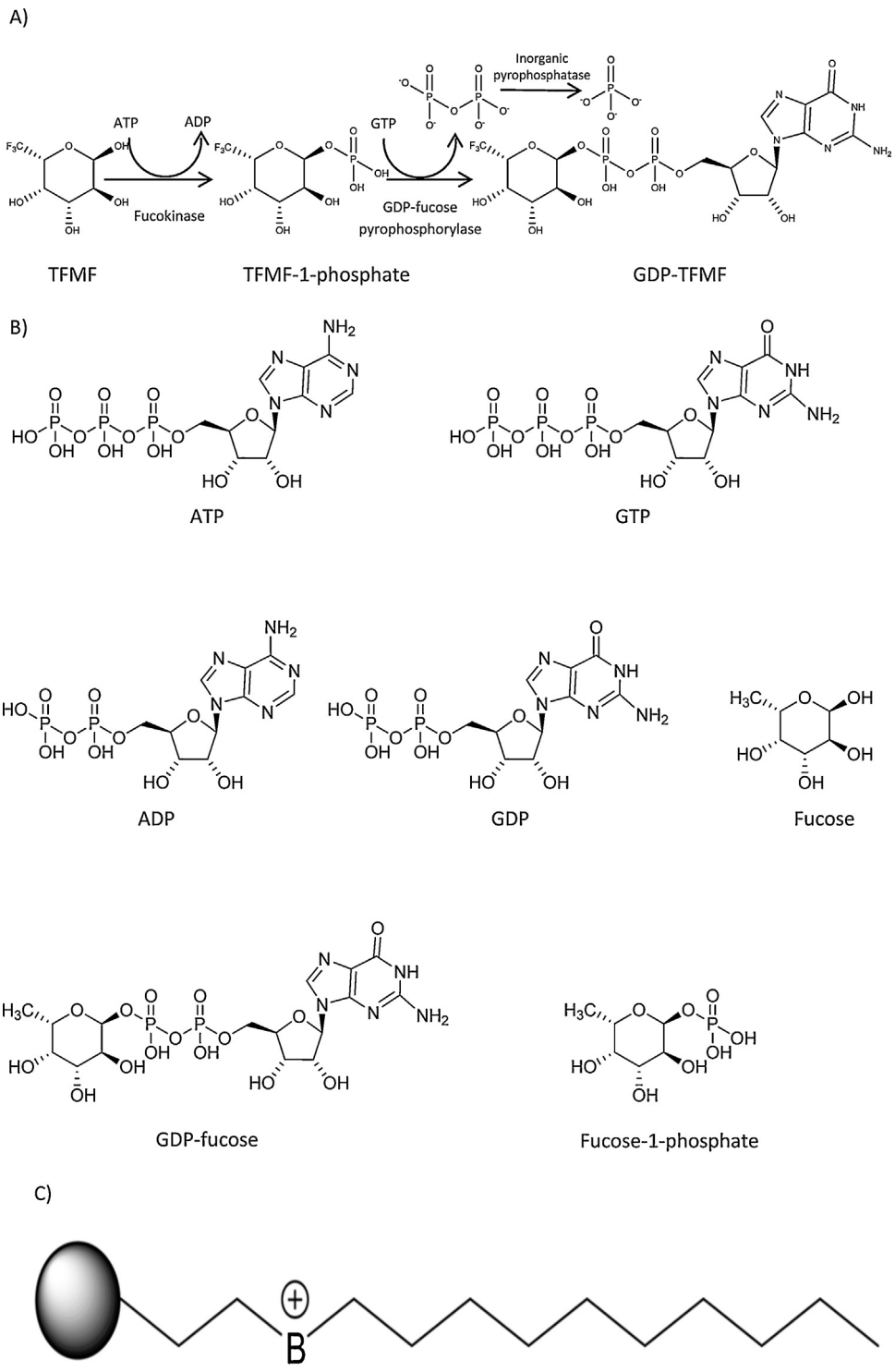

Fig. 1. (a) Chemoenzymatic synthesis of GDP-TFMF from TFMF-fucose and the intermediate TFMF-1-phosphate. (b) Structures of ATP, GDP, ATP, ADP, GDP-fucose, fucose-1-P and fucose. (c) The schematic depiction of the Primesep SB stationary phase with strong embedded basic ion-pairing groups bound to silica.

as well as an acid/base treatment to achieve separation [2]. Changes in the column could lead to long equilibration time and reproducibility issues. One possible explanation for this was a slow change on the PGC surface from oxidation [6].

HILIC has been useful at separating hydrophilic analytes and has been successful for the separation of nucleotides, sugar phosphates and nucleotide sugars [7–9]. Recently, separation of nucleotides was achieved with a cyclofructan based column using ammonium acetate buffer in both gradient and isocratic methods [7]. Unlike other HILIC stationary phases, cyclofructan offers dual retention mechanisms. Traditional hydrogen bonding/dipolar interactions can be supplemented by dynamic ion interaction effects for anionic analytes. This is due to cyclofructan binding certain buffer cations to form a positively charged cavity in the stationary phase which will bind the negatively charged phosphates of the nucleotides. While this mode is useful for nucleotide separations, it did not address separating nucleotide sugars or sugar phosphates that could potentially be present.