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Sugar nucleotides analogues as chemical tools for *in vitro* glycoproteins functionalization

1. Introduction

Glycoproteins are a large class of biomolecules essential for numerous cellular activities. They are constituted of a protein backbone to which sugar chains are covalently attached. The glycosylation of proteins represents one of the most important post translational modification and requires enzymes called glycosyltransferases. Sugar nucleotides are substrates of these enzymes, which make them crucial building blocks for the glycosylation process. The most naturally occurring sugar nucleotides are nucleoside diphosphate-sugars (NDP-sugars) which are structurally composed of a monosaccharide and a nucleoside, connected to each other by a pyrophosphate bond [1]. In this article, we will briefly discuss the chemical synthesis of such important molecules. Then, we will present how the glycosylation machinery can be exploited *in vitro* to functionalize glycoproteins for labelling and/or therapeutic purposes.

2. General approaches for NDP-sugars chemical synthesis

As for enzymatic syntheses, no general chemical method for the efficient preparation of 'all' NDP-sugars has yet emerged [1]. The existing approaches mainly focus on the construction of the pyrophosphate bond through a coupling reaction between the sugar and the nucleoside building blocks (Scheme 1- Strategy A). Noteworthy, another general strategy for synthesizing NDP-sugars involves the anomeric linkage between an electrophilic glycoside and a nucleoside-5'-diphosphate (Scheme 1- Strategy B). Nevertheless, the latter procedure remains less attractive because it requires to find the suitable electrophile and has the inconvenient of lacking anomeric diasteroselectivity [1].

The first strategy might be preferred for the synthesis of NDP-sugars, but it relies on a challenging P-O-P connection that requires the activation of one of the two building blocks [2]. Indeed, the difficulty in such bond construction, arises from slow kinetics and possible side reactions which highly complicate the purification step, usually performed by HPLC and/or size exclusion chromatography. The chemical synthesis of this diphosphate bond has been first accessed via a condensation reaction between two pentavalent phosphate carried by both the sugar and the nucleotide precursor [3]. Many advances have been published concerning this P(V)-P(V) approach, in order to increase both the kinetic and the yield of the reaction. They mainly rely on improving the activation of the nucleoside precursor through the selection of different attached leaving groups (LG). In

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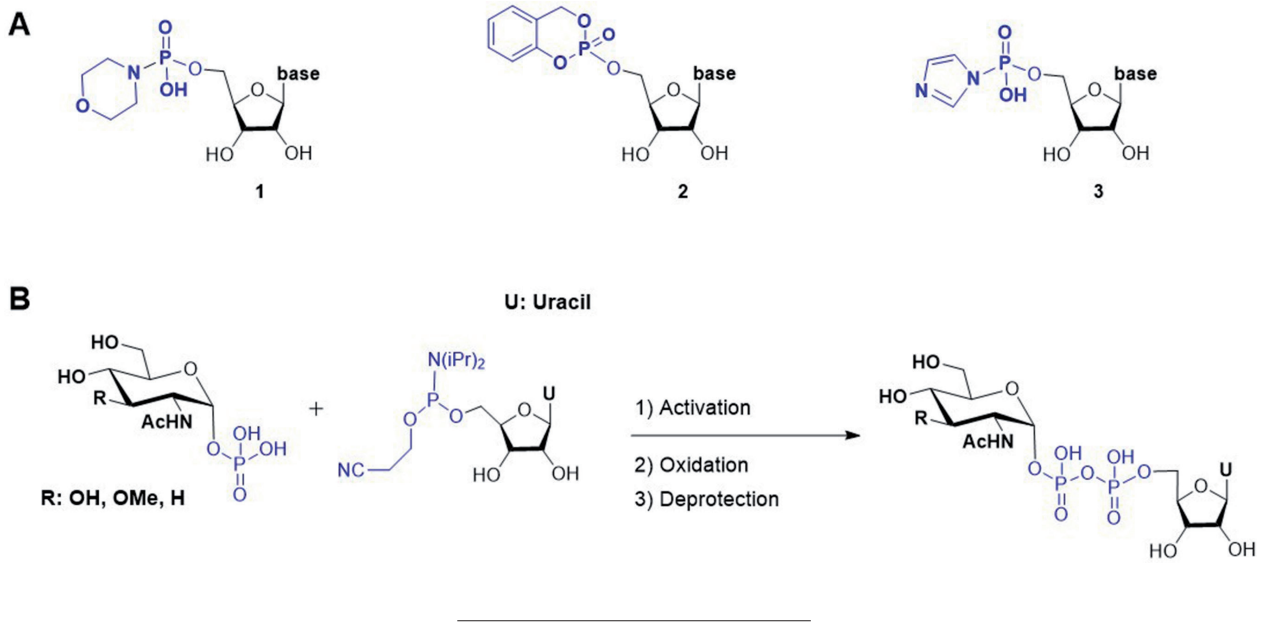
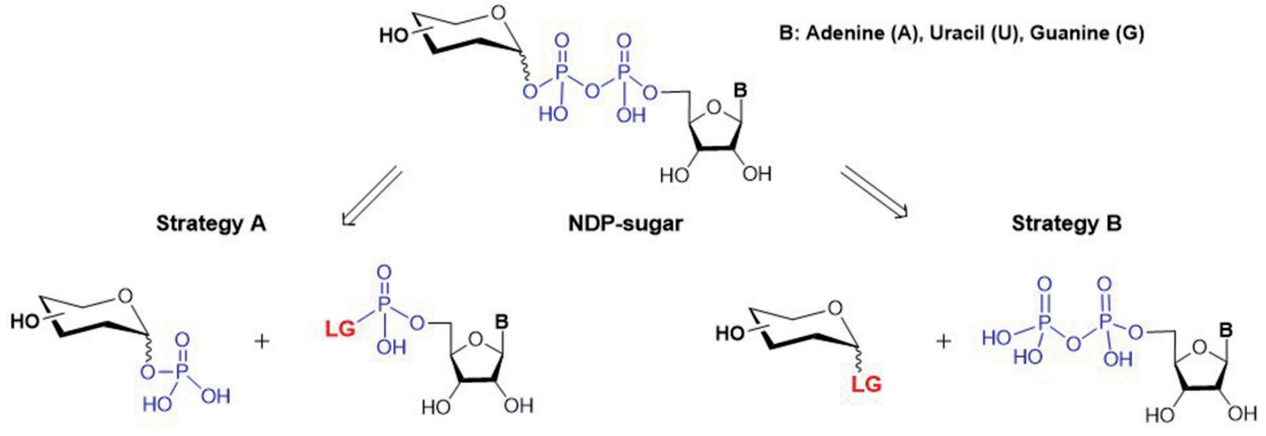


Scheme 1. General strategies for NDP-sugar synthesis.

this case, various activated nucleosides have been employed (Scheme 2.A): starting from the classical nucleoside phosphomorpholidate **1** [4], to newer activated intermediates such as cyclosal nucleotide **2** [5], and nucleoside phosphorimidazolide **3** [6]. In addition to those P(V)-P(V) connections, other approaches have emerged in order to enhance the effectiveness of the coupling reaction. For instance, the Van der Marel's group developed a P(V)-P(III) strategy [7] in which the sugar phosphate was coupled with a nucleoside phosphoramidite, followed by the oxidation of the phosphate-phosphite intermediate in one pot, which led to the formation of UDP-*N*-acetylglucosamine (UDP-GlcNAc) and two of its unnatural analogues (Scheme 2-B). In fact, while leading to the formation of natural NDP-sugars, the development of all these chemical approaches particularly served the synthesis of unnatural analogues, designed either as enzyme inhibitors [8-10], or alternative substrates of glycosyltransferases [11].



Scheme 2. A) Examples of activated nucleoside precursors for the synthesis of NDP-sugars. B) P(V)-P(III) approach for the synthesis of UDP-GlcNAc and analogues.

3. From sugar nucleotides analogues to functionalized glycoproteins

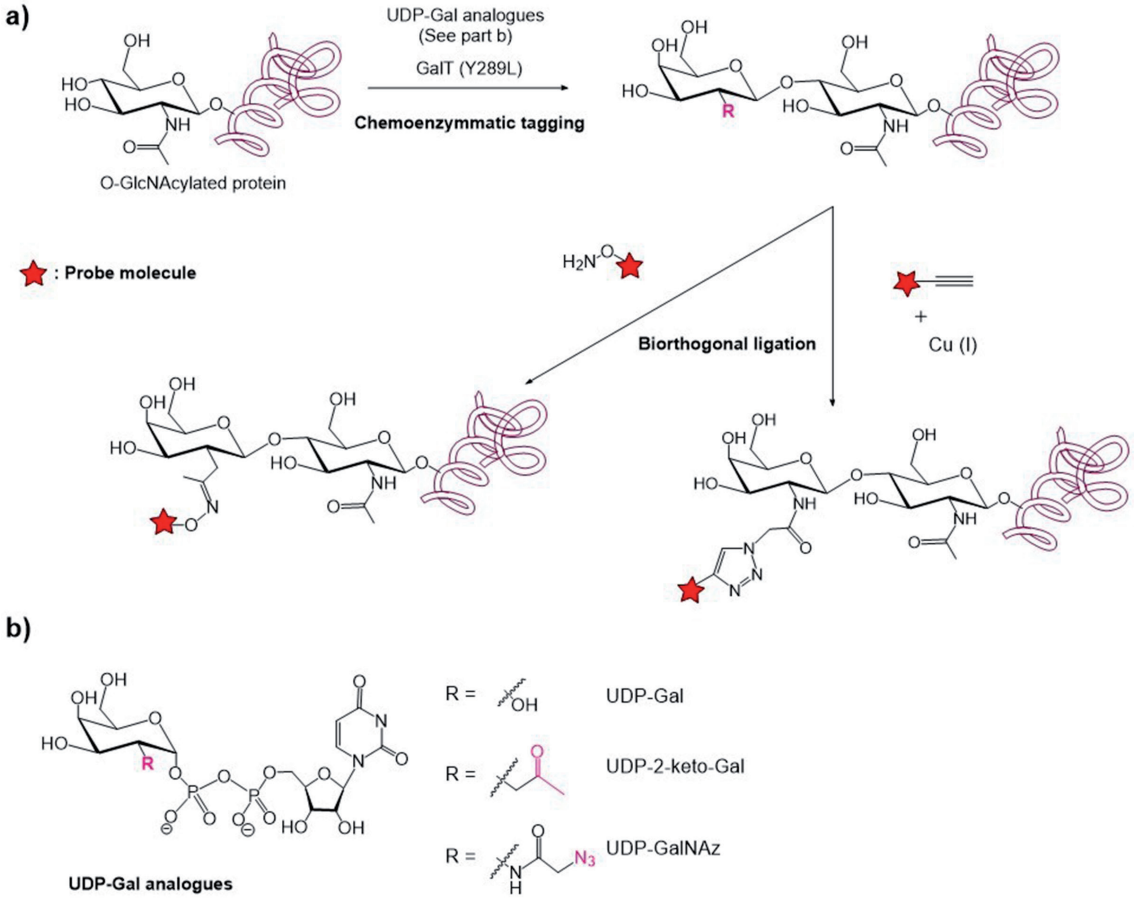
Protein glycosylation can take place at the carboxamide side chain of asparagine or glutamines (*N*-glycosylation) or at the hydroxyl group of serine/threonine side chains (*O*-glycosylation) [12]. Among the enzymes involved in the two processes, glycosyltransferases act by transferring stereo- and regio-selectively appropriate monosaccharides from their activated forms, the sugar nucleotides, to the nascent oligosaccharide chain.

With the aim of developing glycoengineered proteins with new properties, the use of the glycosylation machinery *in vitro* emerged, encouraged by the improvements made in both glycosyltransferases accessibility and sugar nucleotides synthesis [13]. Besides the naturally occurring sugar nucleotides such as uridine diphosphate galactose (UDP-Gal) and guanosine diphosphate mannose (GDP-Man), unnatural analogues could also be used in this approach. The aim is to introduce into the protein's glycan chains, a modified monosaccharide bearing an orthogonal function, with a unique reactivity. The latter allows to functionalize chemoselectively the glycoprotein with a payload that bears the other complementary orthogonal function. In this case, the challenge lies mainly in choosing and/or designing appropriate glycosyltransferases that can accept those unnatural sugar nucleotides together with the synthesis of the sugar nucleotides analogues themselves [14].

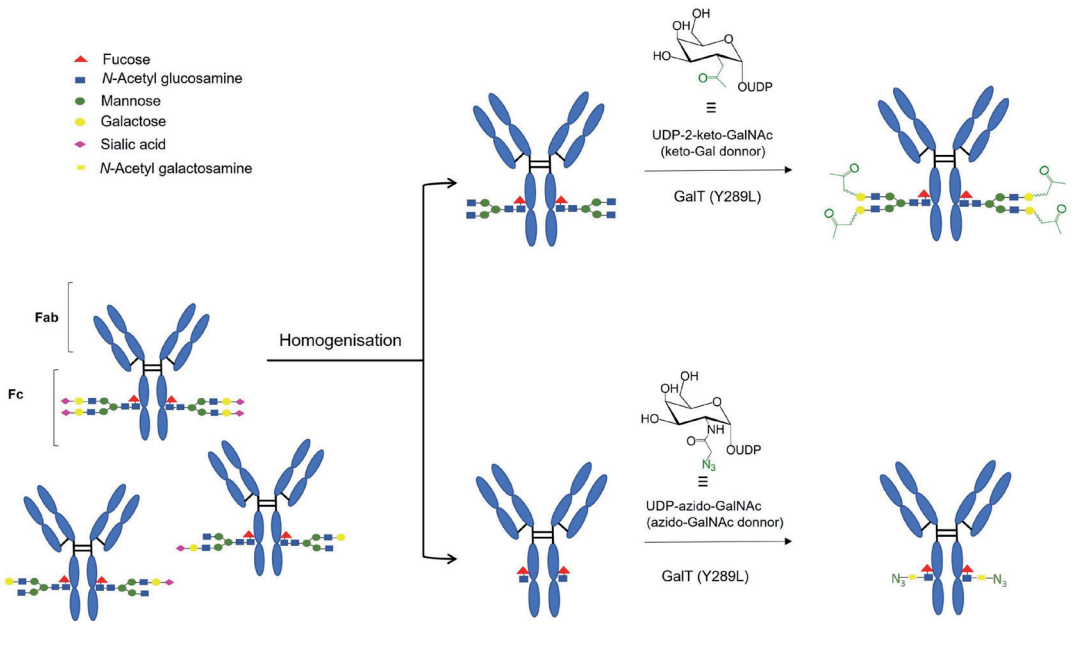
For instance, using a modified UDP-Gal derivative, Qasba *et al.* managed to study the O-GlcNAc glycosylation of proteins [15], which is the covalent modification of serine and threonine residues by *N*-acetylglucosamine (GlcNAc). For that purpose, they exploited the fact that galactosyltransferases (GalT) are enzymes that are capable of selectively transferring a galactose from UDP-Gal precisely to the terminal GlcNAc (targeted for detection). Inspired by this natural machinery, they developed a mutant of GalT (Y289L) capable of transferring a 2-keto galactose derivative from its corresponding UDP-activated form (UDP-2-keto-Gal) to the GlcNAc acceptors. After that, they used the introduced ketone function as a handle to link, via oxime ligation, a fluorescent probe for detection (Scheme 3).

Alternatively, Hsieh-Wilson and co-workers used the azide-modified UDP-Gal analogue (UDP-GalNAz) in order to label O-GlcNAc proteins with a 2-*N*-acetyl-azido-galactose [16], exploiting the same GalT mutant. The subsequent click reaction between the azido reporter and the alkyne function of the fluorescent probe, permitted the rapid detection of those O-GlcNAc proteins (Scheme 3).

Moreover, for therapeutical purposes, such chemoenzymatic approaches also served to introduce drugs in the glycan chains of proteins such as antibodies. Indeed, Immunoglobulin G, the most abundant antibody isotype in human serum, bears two *N*-linked glycans attached precisely at asparagine (Asn)- 297 in the crystallizable fragment (Fc). Each IgG *N*-glycan, which is always biantennary, is composed of the following sequence: two *N*-acetylglucosamine (GlcNAc) - mannose (Man) - mannose antennae - *N*-acetylglucosamine (GlcNAc). This forms the common core glycan that can be enriched by additional carbohydrates [17]. As for labelling O-GlcNAc glycosylation, Qasba *et al.* exploited the same chemoenzymatic approach to insert the 2-keto galactose into the core glycans of IgG (Scheme 4), which was further conjugated using aminooxy containing auristatin F, leading to an antibody-drug conjugate that exhibited specific binding and killing of HER2-overexpressing cancer cells [18]. Another glycan-mediated antibody functionalization, known as the GlycoConnect technology, was also developed by the van Delft's group [19]. In this case, UDP-GalNAz and the GalT (Y289L) mutant were used to introduce the 2-*N*-acetyl-azido-galactose into the core glycan of the antibody (Scheme 4). Then, the subsequent click reaction permitted to conjugate different cytotoxic payloads especially effective in cancer therapy.



Scheme 3. a) An overview of glycoengineering approaches for O-GlcNAc proteins labelling. b) Substrates of GalT (Y289L). Adapted from [14].



Scheme 4. Example of antibody's glycoengineering.

4. Discussion

The glycan-mediated functionalization of proteins presented herein is governed by the selectivity of the chosen glycosyltransferase towards the sugar nucleotide donor and the glycan acceptor. It is hence site selective and allows for homogeneity. Besides, in the case of antibodies functionalization, targeting the only two N-297 glycans of IgGs permits to control the drug to antibody ratio (DAR) while avoiding modifications of the antibody's Fab subunit, which is necessary for the specific recognition and binding to the antigen expressed in cells to which the drug is directed. For all these reasons, the use of a glycoengineering method, especially to functionalize antibodies, is becoming more and more attractive.

In the "Bio-Organic Chemistry laboratory", we are interested in exploring and developing a novel glycoengineering method using a modified mannose, which could be exploited to functionalize antibodies. In a more fundamental aspect, we focus also on studying the tolerance of mannosyltransferases (ManTs) regarding mannose's modifications. In fact, ManTs use guanosine diphosphate-mannose (GDP-Man) as a donor substrate to transfer selectively a mannose into the suitable acceptor. Such an outcome has to be verified when using modified GDP-Man analogues, bearing for example an azide or an alkyne, for subsequent click reactions (Scheme 5).

By pursuing this project, we managed to chemically obtain one clickable GDP-mannose analogue, while currently working on the synthesis of others. Besides expanding the existing library of unnatural sugar nucleotides analogues, the prepared molecules could constitute potent tools to initiate the use of a mannosylation machinery as a new approach for *in vitro* glycoproteins functionalization.

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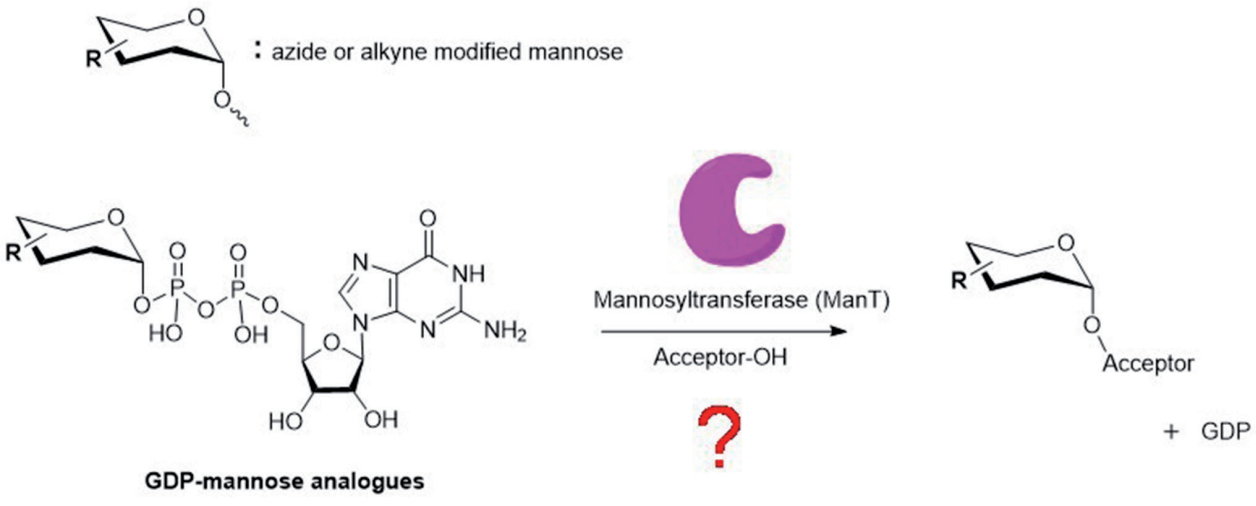

Scheme 5. ManT catalysed mannosylation of an acceptor.

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