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The Synthesis of Carbasugars via Ring-closing Metathesis Applied to the Inhibition of M. Tuberculosis Cell Wall Biosynthesis

Abstract
Because Galactofuranose (D-Galf) constitutes an essential part of the cell wall of M. tuberculosis, the inhibition of its biosynthesis pathway has become a therapeutic target. Various molecules containing a structural unit mimicking the galactofuranose monosaccharide have been synthesized as enzyme inhibitors.

Our goal is to generate carbasugars derived from cyclohexene in which the ring oxygen is replaced by a methylene group. These pseudosugars are designed to mimic transition states or intermediates involved in the mechanism of UDP-galactopyranose mutase (UGM), an essential enzyme for the viability of M. tuberculosis. Thanks to the construction of this carbocycle by ring-closing metathesis, a set of molecules have been synthesized and their inhibition profile has been evaluated against UGM.

Keywords
Mycobacteria, tuberculosis, carbasugars, ring-closing metathesis, phosphonates, inhibitors.

1. Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*. Among the most important contagious diseases, TB constitutes a major health problem threatening the world population. In 2011, the bacterium produced 8.7 million new infections and caused 1.4 million deaths.[1]

The recent appearance of extremely drug resistant strains forces the development of new therapeutic strategies to cure tuberculosis. Among the various pharmacological approaches, the biosynthesis of the cell wall is a validated target for the discovery of anti-tubercular agents. Indeed, isoniazid, pyrazinamide and ethambutol, three of the first line drugs to treat tuberculosis, are inhibitors of this biochemical pathway. Therefore, new enzymes of the cell wall biosynthesis can be considered as interesting targets.

The glycoconjugates present in the cell wall of mycobacteria (lipoarabinomannan LAM and mycolylarabinogalactan mAG) are essential for the virulence and the survival of this microorganism. The galactofuran, component of mAG, is biosynthesized by three enzymes: UDP-Galactopyranose Mutase (UGM) and two galactofuranosyl transferases ($Glf T_1$ and $Glf T_2$). Compounds inhibiting these three enzymes might thus constitute a new class of antitubercular agents.

2. Occurrence of galactofuranose in nature

Galactose is a carbohydrate that can be present as an open chain in equilibrium between two cyclic forms: a pyranose **1** (D-Gal*p*, six-membered ring) and a furanose **2** (D-Gal*f*, five-membered ring) (Figure 1).

In mammals, galactose is exclusively found in the pyranose form as a component of many glycoconjugates such as the ABO blood group antigens, the glycosaminoglycan keratan sulfate and many glycolipids.[2,3] Interestingly, the furanose form is abundant in protozoa, fungi, bacteria,[3-7] lichens and marine organisms, but totally absent in mammals.

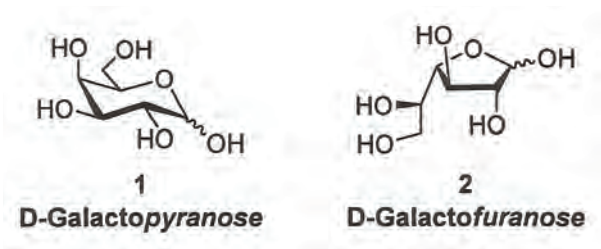

Figure 1: The cyclic forms of D-galactose.

In bacteria, β-D-Gal*f* is a component of the glycoconjugates present in Gram positive and Gram-negative bacteria. It exists as

a repetitive unit or can form a linkage with other sugars. Some of these bacteria are very pathogenic; in fact, Gal*f* often plays a crucial role in the viability and the virulence of the contagious agent.

This sugar has been identified in mycobacteria such as *M. tuberculosis* or *M. leprae*. It constitutes the major part of its cell wall, known as mycolyl-arabinogalactan complex (Figures 2 and 3).[8] The galactan is formed by up to 30 residues of alternating (1→6) and (1→5) linked β-D-galactofuranosides.

3. Structure of the mycobacterial cell wall

As illustrated in Figures 2 and 3, the cell wall of *M. tuberculosis* is composed by three major parts: the peptidoglycan (PG), the glycolipid core (LAM, mAGP, LM) and the mycolic acids.

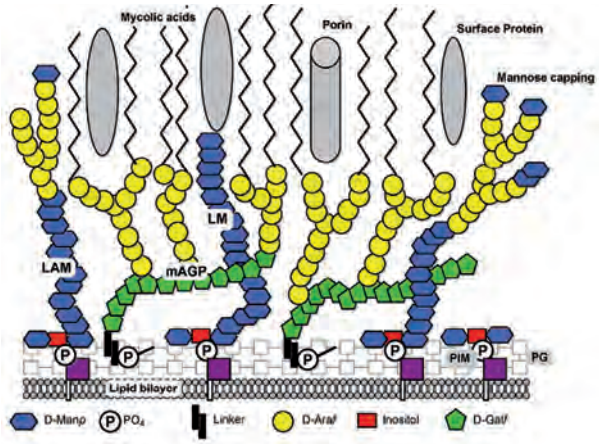

Figure 2: Mycobacterium cell wall and their components (D-Manp = D-mannopyranose, D-Araf = D-arabinofuranose, LM = lipomannan, LAM = LipoArabinoMannan, PG = peptidoglycan, mAGP = mycolylArabinoGalactan).

3.1. Biosynthesis of the mycolylArabinoGalactan mAG, a key component of the cell wall.

The galactan (represented in green in Figures 2 and 3) is a small polymer linking the outer layer and the inner layer of the cell wall. This polysaccharide is connected to the peptidoglycan by a disaccharide linker and to the mycolic acids by the arabinan. It is composed by a linear chain of 30 residues of alternating β-(1→5), β-(1→6)-D-Gal*f*. In the O-5 of each galactofuranose, three D-Ara*f* are attached via α-(1→5) bond. The main arabinofuranosidic chain contains 31 linear α-(1→5)-D-Ara*f* structures (the arabinan represented in yellow in Figure 2 and in blue in Figure 3), branched in position 2 and 3 via α-(1→2)-D-Ara*f* and α-(1→3)-D-Ara*f* connection.[9-11] After the formation of arabinogalactan, an esterification occurs between the terminal Ara*f* moiety and the mycolic acids to afford the whole mAG.[12]

3.2. Galactofuranose biosynthesis

The biosynthesis of the galactan is catalyzed by three enzymes (Figure 4): UDP-galactopyranose mutase (UGM) and two galactofuranosyltransferases (Gl*f*T1 and Gl*f*T2). UDP-Gal*f* **5** is the biosynthetic precursor of Gal*f*. It is generated from UDP-galactopyranose **4** by UDP-Galactopyranose Mutase (UGM). Once produced, the two enzymes Gl*f*Ts transfer the Gal*f* residues from UDP-Gal*f* to the growing oligosaccharide. These enzymes are attractive targets to develop new therapeutic strategies to combat TB.

4. UDP-Galactopyranose Mutase (UGM)

UDP-Galactopyranose Mutase was cloned and identified for the first time in 1996 in *E. coli*. A radiometric assay confirmed the activity of UGM as being involved in the interconversion of UDP-Gal*p* into UDP-Gal*f*.[13]

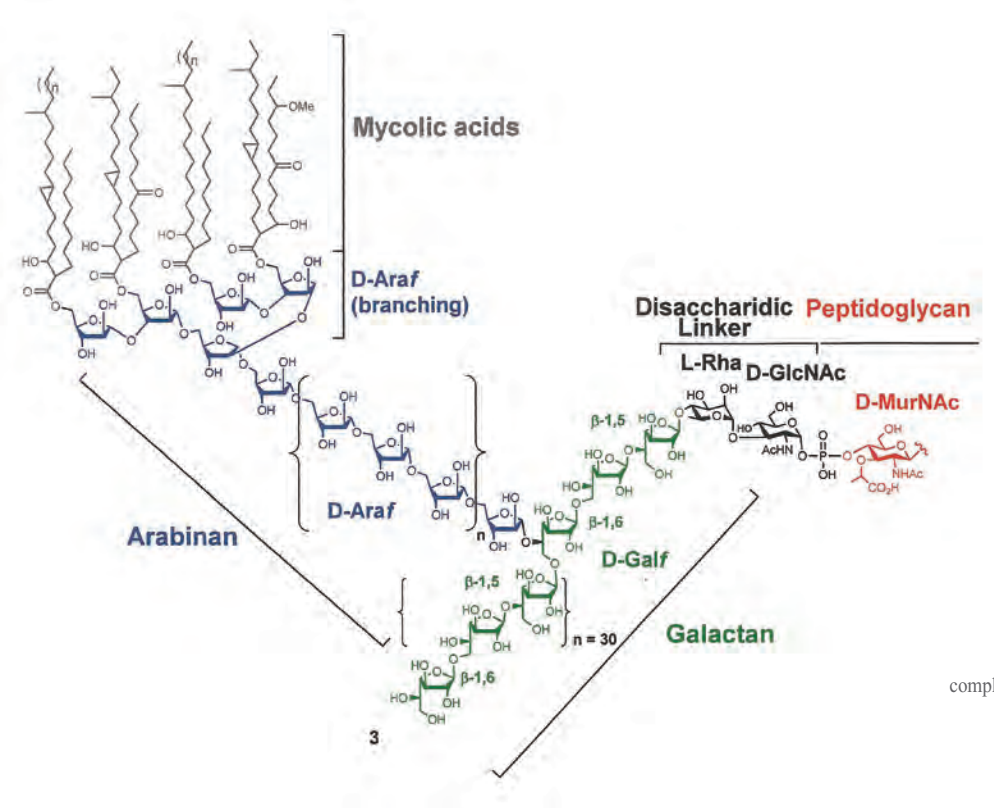

Figure 3: Structure of mAGP complex incorporated in the cell wall of mycobacteria.

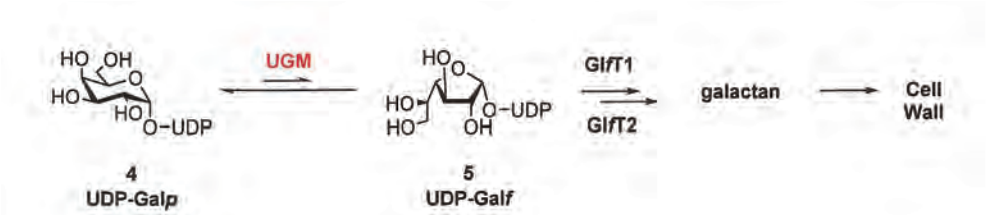

Figure 4: Biosynthetic pathway of galactofuran.

This enzyme, found in many eukaryotic and prokaryotic microorganisms, is crucial for the survival and the virulence of these species.[14] Indeed, the deletion of the gene coding for UGM in eukaryotes revealed morphological changes in the cell surface of *Aspergillus nidulans*, *Aspergillus niger*, *Leishmania major* and *Aspergillus fumigatus* because of the loss of Gal*f*.[15-18] In addition, an attenuation of the virulence of *L. major* and *A. fumigatus* has been evidenced as a consequence of UGM absence.[17,19] On the other hand, UGM has been also identified in prokaryotes such as *Escherichia coli*, *Klebsiella pneumoniae*, *Mycobacterium tuberculosis* and *Deinococcus radiodurans*[13,20-22] as a dimer protein.[23]

4.1. Mechanism of UGM

UGM is a flavoenzyme (an enzyme that binds a FAD cofactor) whose mechanism appears to be totally different from the other FAD binding proteins. This mechanism has thus been the subject of intense investigations, sometimes controversial. Figure 5 describes the mechanistic steps that are usually well-admitted in the literature.

The enzymatic reaction begins by the release of UDP, from UDP-Gal*p* **4**, to provide the oxycarbenium **6**. The nucleophilic addition of the FAD on the anomeric position produces a galactose-flavin adduct **7**. After ring opening of the cycle, an acyclic intermediate **8** is formed followed by a ring contraction to generate the furanose-flavin species **9**. Then, the release of FAD generates an oxycarbenium **10** which, after a nucleophilic attack of UDP, provides UDP-Gal*f* **5** (Figure 5).

The compounds that present an analogy with UDP-Gal*f* **5** or mimicking the intermediates **10** and **8** constitute an interesting tool to study the mechanism of this essential enzyme.

4.2. Design of UGM transition state analogues

Few years ago, Caravano et *al.* designed some UDP-exoglycals as mimics of the oxycarbenium intermediate **10** (Figure 6). These sugars behaved as time-dependent inactivators of UGM under non-reducing conditions. In comparison to **11a**, the molecules **11b** and **11c** displayed much slower inactivation rates due to the presence of the fluorine atom. It was thus concluded that the mechanism of UGM inactivation proceeds via two-electron processes.[25]

More recently, our laboratory published the synthesis of a novel series of UDP-galactitol as analogues of the acyclic intermediate **8**. The four molecules **12**, **13**, **14**, **15** have in common a UDP and a galactitol subunits that are attached together with different linkers (Figure 7). The binding affinity of these sugars for UGM increase as follows: **15** < **14** < **13** < **12**. Compound **12** is the best inhibitor of this series: it binds UGM tightly in a reversible manner which was surprising given the electrophilic nature of the epoxide functionality. It could be concluded that the variation in the orientation of the UDP and the galactitol dramatically affects the binding properties.[26]

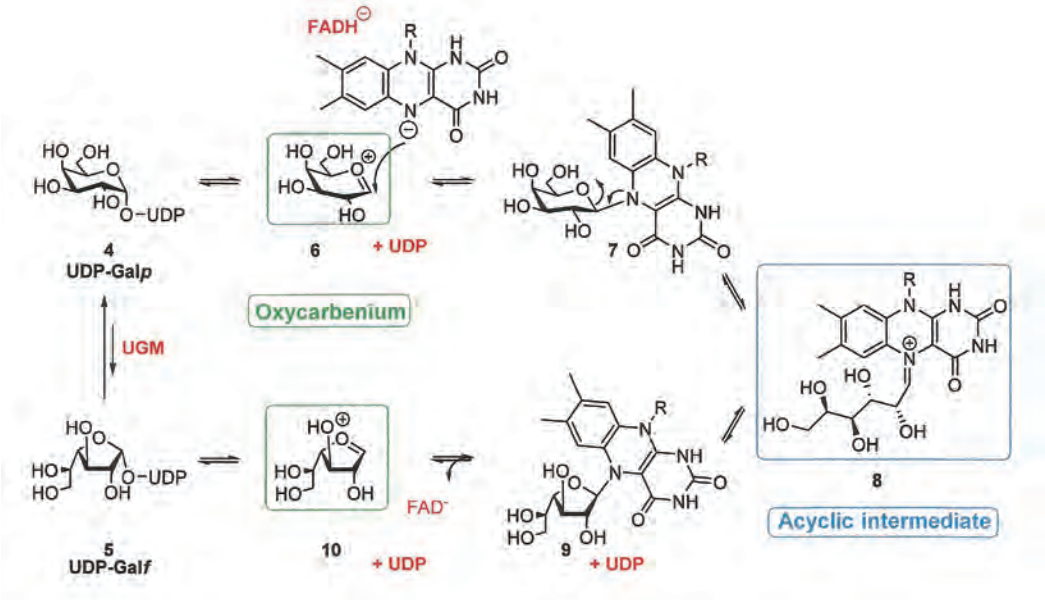

Figure 5: Possible intermediates involved in the mechanism of UGM.[24]

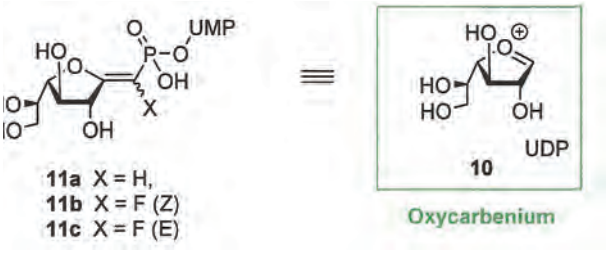

Figure 6: Exofluorinated glycals transition state analogues of UGM.[25]

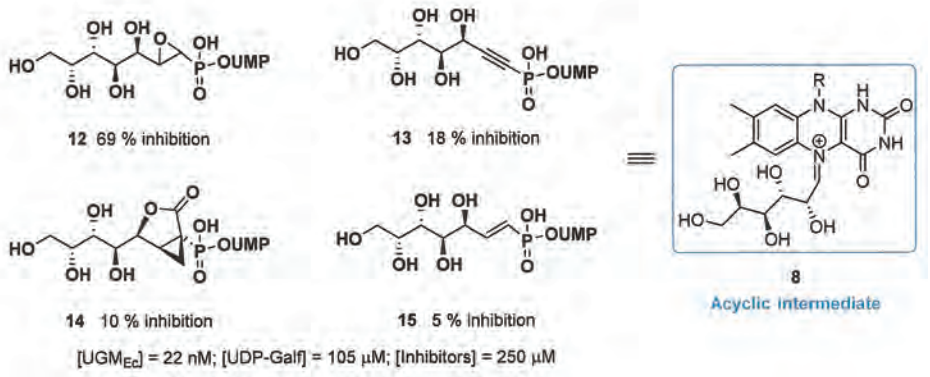

Figure 7: Synthesis of a series of UDP-galactitols with different electrophilic substituents.[26]

5. Carbasugars analogues of UGM mechanistic intermediates

The carbocyclic structures that we envision to synthesize have been designed to be mimics of UDP-Gal*f* and could also be analogues of the two intermediates **8** and **10** involved in the mechanism of UGM (Figure 8). In one hand, they have the same stereochemical outcome (polyol) than their analogue **5** with the only difference that the ring oxygen of UDP-Gal*f* is replaced by a methylene group which confers some stability for the carbocycle. On the other hand, the cyclohexene with its sp^2 character mimics the oxycarbenium **10** and by its extended cycle may also mimic the acyclic galactitol-iminium form **8**.

According to the previous work of our laboratory, the cyclohexene **16** that we would like to synthesize displays a galactitol chain, a UDP moiety and an extended arm which can increase the flexibility of the cycle (Figure 9). These three elements are necessary for optimizing the binding affinity of the analogues with UGM.

Thanks to the carbasugar moiety, the target molecules could be defined as mechanistic intermediate analogues while maintaining UDP as a key subunit of the substrate.

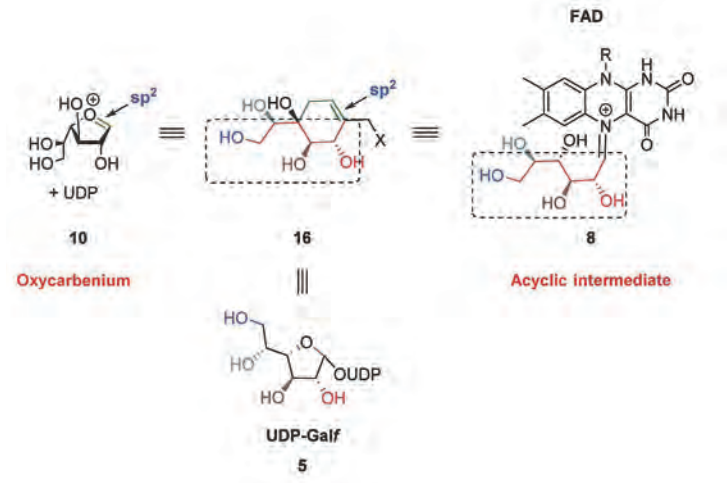

Figure 8: Polyhydroxylated cyclohexene **16** as mimics of the mechanistic intermediates **8** and **10**.

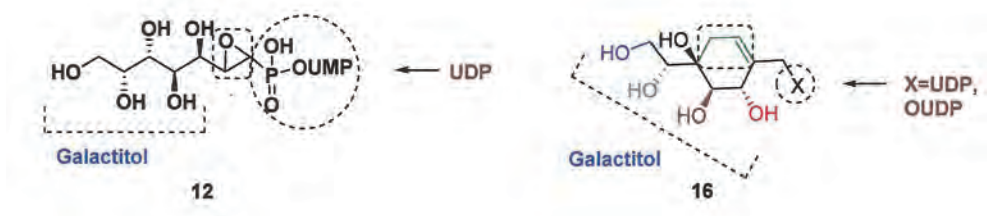

Figure 9: Structural analogy between UDP-cyclohexene **16** and UDP-galactitol derivative **12**.

6. Synthesis of carbasugars via RCM

In 1966, McCasland's group reported the synthesis of "*carbohydrate mimetic*"[27-32] known as carbasugars[33]. The substitution of the ring oxygen by a methylene group[34-36] improves the chemical and enzymatic stability of these derivatives towards degradative enzymes. Indeed, the methylene group, being bioisoster of oxygen, may improve the properties of these carbasugars and make them important synthetic target with a wide range of biological applications. Some carbasugars show antiviral and antitumor properties therefore, they may find applications in the treatment of some diseases such as AIDS and cancer.[37-43]

Among other methods, ring-closing-metathesis (RCM) has become an important way to prepare carbocycles in the recent literature[44-46]. This synthetic method allows the transformation of the acyclic sugar into a carbasugar ring, a cyclopentene or cyclohexene derivatives. By using this key transformation, the synthesis can begin with a cheap sugar bearing all the stereochemical information necessary to obtain the final molecule.
The cyclohexene **19** can be generated by ring closing metathesis between the two olefins (present in position **1** and **4**) that can be introduced further to successive steps starting from the D-galactonolactone **17** bearing the same stereochemistry than galactofuranose (Figure 10).

In our original design, we also anticipated that the alkene functionality of the carbasugar subunit could be exploited for the generation of other inhibitors. Strategically, the idea was to use the same synthetic advanced precursors in order to synthesize novel UDP-Gal*f* analogues. Conceptually, we wished to explore further the interactions of UGM with UDP-Gal*f* analogues modified at the anomeric position, a region of the substrate where all the catalytic process occurs.
First, we generated two categories of molecules bearing phosphonate and phosphate (R = CH_2P and CH_2OP, represented in blue in Figure 11) as two functional groups.

The UDP-phosphonate carbasugars can be analogues of UDP-Gal*f*, due to the presence of the UDP moiety and the analogy with the sugar part. The presence of a non-scissible C-C bond in the anomeric position gives certain stability towards degradative enzymes.

In comparison with UDP-phosphonate pseudosugars, the only difference between the compounds **21** and **22** (Figure 12) is the presence of an oxygen atom between the anomeric position of the carbocycle and the phosphorus atom. By insertion of oxygen, we can extend the bond length and the bond angles which can give flexibility to the system within the UGM active site and then may favor the interactions with the uridine moiety. Furthermore, electrostatic interactions can occur between the phosphate carbasugars and the guanidinium moiety of Arginine (Figure 12). Since phosphonates and phosphates have significantly different pKa's, these modifications can also influence the binding properties of the inhibitors.

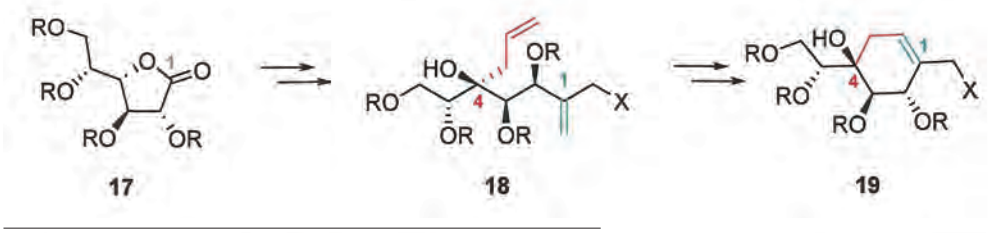

Figure 10: Synthetic pathway to generate the cyclohexene **19** from D-galactonolactone **17**.

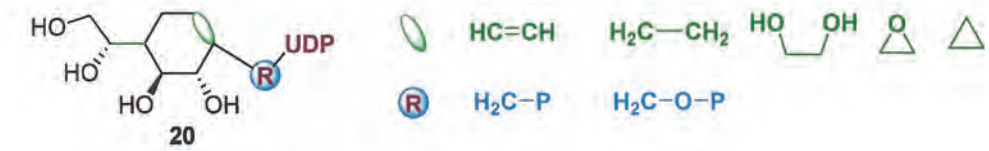

Figure 11: Generation of a novel series of UDP-carbasugar by standard and chemoselective derivatization of the alkene.

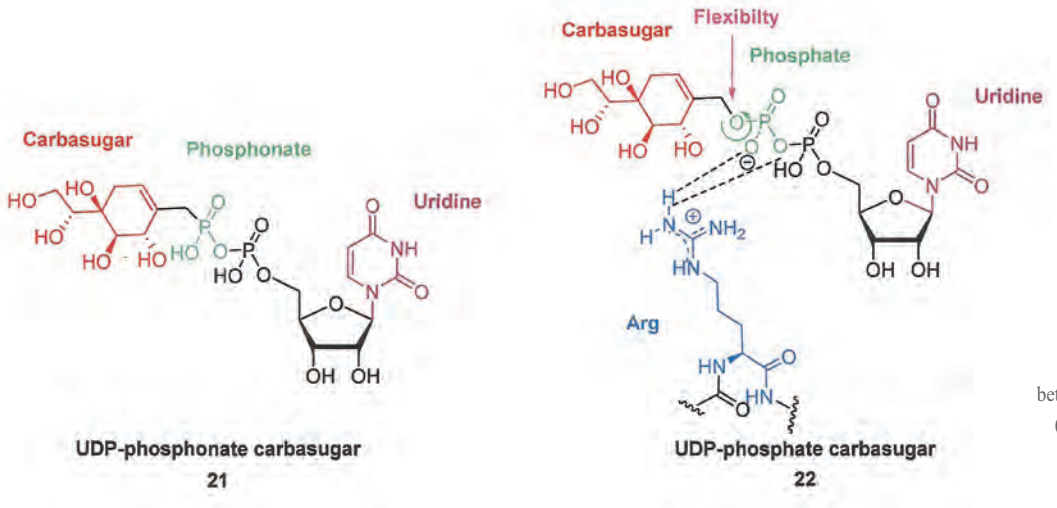

Figure 12: Difference between UDP-phosphonate (**21**) and UDP-phosphate (**22**) carbasugars in interaction with UGM.

7. Generation of UDP-galactose analogues as potential UGM inhibitors

As illustrated in Figure 13, the alkene functionality can be transformed in one step into a large variety of polyhydroxylated cyclohexanes. The double bond of the cyclohexene **20** can be transformed into various functional groups in one step by hydrogenation, dihydroxylation, epoxidation and cyclopropanation to afford a novel family of UDP-carbasugars.

The cyclohexene by its sp^2 character will mimic the oxycarbenium intermediate involved in the UGM mechanism. Then, the cyclohexane will give information about the importance of the double bond on the conformation of the UDP-Gal*f* analogues. The replacement of C=C by the diol functionality will allow to favors hydrogen bond interaction with the amino acids or the cofactor within the active site and then increase the affinity with the enzyme. However, the epoxide may give a strained character to the UDP-carbasugar and then may enhance the binding affinity with the enzyme. Finally, the cyclopropane can be an analogue of the epoxide and may give rise to a strong conformational restriction to the carbocycle.

8. Conclusion and outlook

To summarize, the aim of this project is to synthesize a family of UDP-carbasugars (cyclohexene) analogues of UDP-Gal*f* in order to study the mechanism and the binding properties of UGM. Two different categories have been synthesized: the molecules with a phosphonate moiety and others with phosphate group. On these two scaffolds, a divergent synthesis has been carried out by functionalizing the double bond of the carbocycle. These products have then be coupled with uridine monophosphate to provide the different UDP-carbasugars.
The inhibition properties of all these molecules are underway in our laboratories.

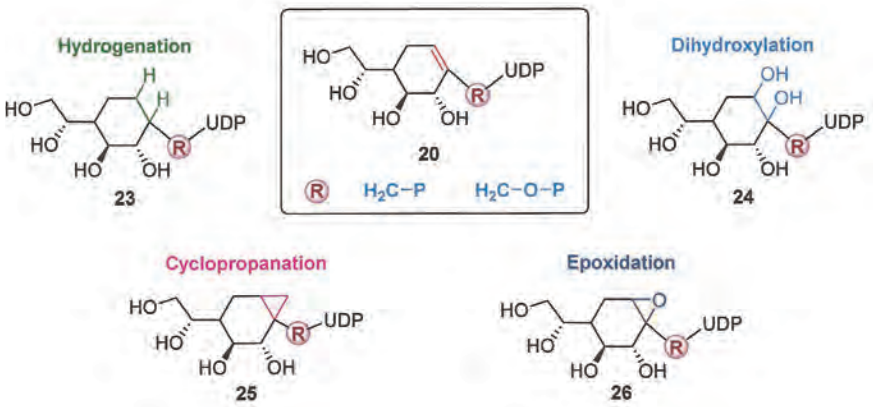

Figure 13: Synthesis of a family of UDP-carbasugars as UGM competitive inhibitors.

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