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Synthesis of GPI anchor analogues to support the discovery of new molecular targets of *Trypanosoma cruzi*

Síntese de análogos de âncora de GPI: uma contribuição para a descoberta de novos alvos moleculares de *Trypanosoma cruzi*

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ABSTRACT

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Glycosylphosphatidylinositol (GPI) anchors are essential molecules to attach glycoconjugates and proteins in protozoan's cell surface. *Trypanosoma cruzi* produces a range of unique GPI structures that anchor mucins and *trans*-sialidases which participate in important processes involved in the interaction between parasite and host. As an effort to study *T. cruzi* GPI anchor biosynthesis and possibly use it as a potential target for an antichagasic drug, this work aims to synthesize GPI anchor analogs (labelled or not) and analyze the potential of these molecules as substrates in the GPI biosynthetic pathway. In this context, a pseudo-disaccharide **31** was synthesized by *O*-glycosylation reaction between azide glycosyl donors (**32 or 33a-d**) and *myo*-inositol acceptor (**34**), prepared from glucosamine (**35**) hydrochloride and methyl α-D-glucopyranoside (**36**), respectively, using orthogonal protection/ deprotection. Five different glycosyl donors (**32** and **33a-d**) were prepared to investigate the influence of their protective groups on the stereoselectivity of the *O*-glycosylation reaction in the presence of different solvents to afford the required GPI α-linkage**.** In addition, the synthesis of the *myo*-inositol acceptor **34** was achieved using several protection/deprotection steps, besides the Ferrier rearrangement, to form a functionalized cyclitol derivative that enables the regioselective introduction of the azide glycoside unit and phospholipid moiety on its C-1 and C-6 positions, respectively**.** Then, *O*-glycosylation of acceptor **34** with donor **33c** was accomplished in diethyl ether, using TMSOTf as promoter to give exclusively α-anomer **31c** in high yield. After deallylation of **31c**, the phosphodiester moiety bearing an octyl chain (**87**), prepared by the H-phosphonate approach, was appended to the pseudo-disaccharide to yield, after deprotection, target compounds **30a**. The same synthetic strategy was applied to the preparation of **30c**, even though in the protective form, compound **91** bearing an alkyl-naphthyl side chain (**90**). Currently, compound **30a** is being tested as substrates of GPI anchor biosynthesis in *Euglena gracilis* cell membranes, a non-pathogenic unicellular algae, which may potentially be used as a model for phylogenetically related human parasites. After incubation of the potential

GPI substrate **30a** with *E. gracilis* microsomal membranes for generation of metabolites, the analysis by LC-MS and, eventually, isolation of the products will be performed for further characterization. Products that show any substrate or inhibitory activities will be also assayed in *T. cruzi* microsomal membrane.

Keywords: GPI anchors, orthogonal protection/deprotection; glucosamine; *myo*-inositol; phosphodiester.

RESUMO

MOROTTI, A. L. M. Síntese de análogos de âncora de GPI: uma contribuição para a descoberta de novos alvos moleculares de *Trypanosoma cruzi*. 2018. 178p. Tese (Doutorado). Faculdade de Ciências Farmacêuticas de Ribeirãi Preto-Universidade de São Paulo, Ribeirão Preto, 2018.

Âncoras de glicosilfosfatidilinositol (GPI) são estruturas essenciais para a ancoragem de glicoconjugados e proteínas na superfície celular de protozoários. *Trypanosoma cruzi* produz uma gama de estruturas únicas de GPI, as quais ancoram mucinas e *trans*-sialidases, que participam de processos envolvidos na interação entre parasita e hospedeiro. Afim de estudar a biossíntese de âncora de GPI de *T. cruzi* e possivelmente utilizá-la como um potencial alvo anti-*T.cruzi*, este trabalho visa sintetizar análogos de âncoras de GPI e analisar o potencial destas moléculas como substratos da via biossintética de GPIs. Neste contexto, um pseudo-dissacarídeo **31** foi sintetizado através de *O*-glicosilação entre os doadores derivados de azido-glicopiranosídeo (**32** ou **33a-d**) e o acceptor de *mio*-inositol (**34**), preparados a partir de cloridrato de glucosamina (**35**) e metil-α-D-glucopiranósido (**36**), respectivamente, usando proteção/desproteção ortogonais. Cinco diferentes dadores de glicosídicos (**32** e **33a-d**) foram preparados para investigar a influcia dos seus grupos protetores na estereoselectividade da reações de *O*-glicosilação na presença de diferentes solventes para estudar o favorecimento da configuração α, presente em GPIs. Ademais, a síntese do aceptor de *mio*-inositol **34** foi realizada em 12 etapas pela estratégia do rearranjo Ferrier para formar um derivado de ciclitol, além de diversas proteções/desproteções, funcionalizado que permite a introdução regiosselectiva da unidade de azido glicose (**32-33a-d**) e uma porção de fosfolípido no seu C-1 e posições C-6, respectivamente. Assim, *O*-glicosilação entre doador **33c** e o acceptor **34**, foi realizada utilizando TMSOTf como promotor para originar o composto **31c** com boa estereoseletividade para α, com elevado rendimento (~70%). Após a dealilação de **31c**, a porção fosfodiéster contendo uma cadeia C-8 (**87**), preparada pela abordagem do H-fosfonato, foi anexada ao pseudo-dissacarídeo para gerar, após desprotecção global, o composto alvo **30a**. A mesma estratégia sintética foi aplicada ao preparo do composto **91** contendo uma cadeia lateral alquil-naftil (**90**) que está em últmas etapas de desproteção para gerar o composto final **30c**.

Atualmente, o composto **30a** está sendo testado como substrato da biossíntese de âncoras de GPI em membranas microssomais de *Euglena gracilis*, uma alga unicelular não patogênica, que pode potencialmente ser utilizada como modelo para parasitas humanos filogeneticamente relacionados. Após a incubação do potencial substrato de GPI **30a** com membranas microssomais de *E. gracilis* para geração de metabólitos, será realizada análise do extrato por LC-MS e, eventualmente, isolamento dos produtos formados para posterior caracterização. Os produtos que apresentarem atividade como substrato ou como inibidores da biossíntese de GPI em *E. gracilis* serão também ensaiados na membrana microsomal do *T. cruzi.*

Palavras-chave: âncoras de GPI, proteção/desproteção ortogonal, glicosamina, *mio*-inositol, fosfodiéster.

1. INTRODUCTION

1.1. Chagas Disease and *Trypanosoma cruzi*

Chagas disease affects around 7 millions of people worldwide, being a substantial cause of morbidity and mortality, particularly in developing countries, being the source of about 7500 deaths annually. Low financial returns and reduced income of affected individuals do not arouse interest in research and development of new treatments by the pharmaceutical industry (WHO, 2018). Chagas disease was a concern limited to Latin American territory. However, due to an increase in immigration in the last decades, the disease has started to spread around other continents (DNDi, 2018).

The causative agent of Chagas disease is the hemiflagellate protozoan *Trypanosoma cruzi*, which belongs to the family of *Trypanosamatidae* and order *Kinetoplastida*. The parasite was discovered by Carlos Chagas in 1909, who first identified *T. cruzi* in the blood of a domestic cat and later in a two years old girl. The main transmitter of *T. cruzi* is a vector bug, *Triatoma infestans*, which infects humans (and other mammals) through their contaminated feces while feeding with the individual blood. Other forms of infection also occur through the ocular or oral mucosa, blood transfusion, congenital and laboratory accidents (WHO, 2018). The protozoan has a heteroxenic life cycle, which consists in one phase in the invertebrate vector and another phase in vertebrates (mammals) (NOIREAU; DIOSQUE; JANSEN, 2009; NEVES, 2000). *T. cruzi* undergoes intracellular multiplication phases in the vertebrate host being found as trypomastigotes (blood) and amastigotes (tissues). In the insect, the protozoan is present as epimastigotes. Chagas' disease presents three clinical phases: the acute one, generally asymptomatic or with unspecific symptoms, which the parasite is in abundance in the individual’s bloodstream; the intermediate phase, in which the host is asymptomatic until manifestation of the third phase, the chronic one, characterized by irreversible lesions in different organ tissues. Among individuals in the chronic phase, 30% of them develop cardiopathies while 10% suffer from damage to the esophagus, colon, nervous system or more than one organ, remaining with the disease until the end of their lives (WHO, 2018). In Brazil, cardiac complications related to Chagas disease are significant cause of implantation of pacemakers and heart transplantations, as

well as an important cause of death in adults between 30 and 60 years old (BRAZIL, 2013).

1.2. Therapy against Chagas Disease

Currently, there is no vaccine against Chagas Disease, being prevention and elimination of the vector bug the most effective means of protection against the illness. The scarce therapeutic arsenal for the treatment of affected individuals consists of two drugs: benznidazole and nifurtimox (Figure 1) (SALES-JUNIOR et al., 2017). Both compounds act as pro-drugs that require nitro-reduction for further formation of free radicals and metabolites which bind to the parasite's nuclear and mitochondrial DNAs, causing damage to these structures (SALES-JUNIOR et al., 2017; RAJAO et al., 2014). On the other hand, these nitroaromatic compounds are also responsible for significant side effects, lack of selectivity, and are only effective in the early stage of the infection. Patients in the acute phase of the disease, which are those who could successfully respond to treatment with benznidazole or nifurtimox represent less than 1% of infected ones. Moreover, nifurtimox was discontinued in Brazil, because some *T. cruzi* strains have shown to be resistant to this drug. In summary, all these facts call attention to the urgent search for more selective and specific treatments against Chagas disease (SUETH-SANTIAGO et al., 2017; ANDRADE et al., 1999).

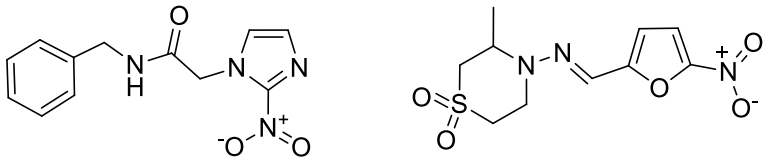


Figure 1: Currently approved drugs for the treatment of Chagas Disease

1.2.1. Drug discovery and the search for specific targets in T. cruzi

Studies towards drug discovery for Chagas Disease continues to be a challenge, even after more than 100 years of the discovery of *T. cruzi* (WHO, 2009). The search for novel molecules that are selectively injurious to the parasite is the primary quest in the exploration for new drugs. Antiparasitic chemotherapy is usually

based on two classes of targets: the ones that are specific to *T. cruzi*, and those which are common to parasite and host. In the last case, there must be some selectivity by the bioactive compound for the protozoan's receptors or enzymes, aiming a more significant effect on the parasite and fewer impacts on the host (SUETH-SANTIAGO et al., 2017). In a study performed by Sueth-Santiago and collaborators (2017), *T. cruzi's* specific targets such as *trans*-sialidases, trypanothione reductase, and cruzipain have been gaining significant interest in the search for molecules against the parasite in the last two decades. In this context, our research group has shown interesting results by putting efforts on synthesizing new structures and by exploring higher selectivity on analogues of existing drugs against *T. cruzi* (MARCHIORI et al., 2017; ANDRADE et al., 2015; MARTINS-TEIXEIRA et al., 2013). Examples of compounds synthesised by our group against *T. cruzi* are shown in Figure 2. Andrade and co-workers (2015) synthesised a series of 27 analogues of benznidazole which some presented very good *in vitro* activities against trypomastigote and amastigote forms of two different strains of *T. cruzi.* Among them, compound **1** (Figure 2) showed IC_{50}= 7,0 µM and low toxicity in spleen cells isolated from C57BL/6 mice, leading to high selectivity for the parasite's cell.

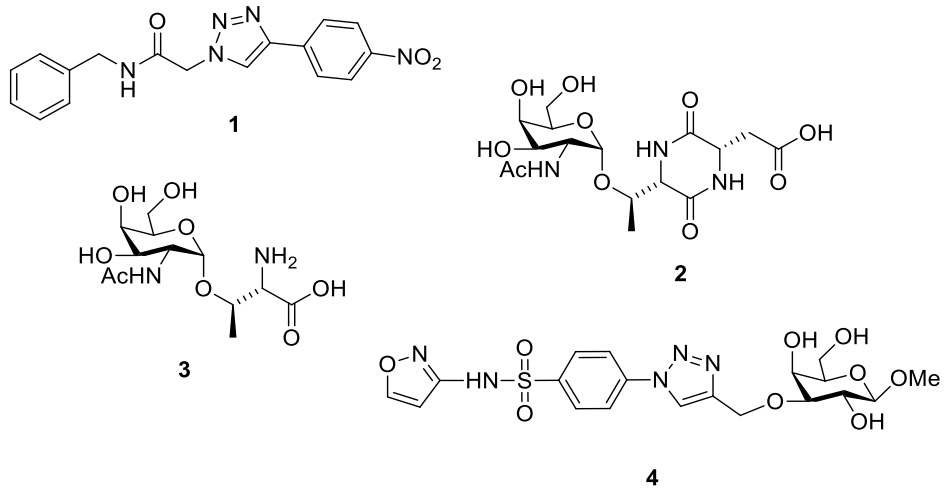


Figure 2*:* Compounds synthesised by our research group aiming for trypanocidal activity.

Diketopiperazine analogue **2** and precursor **3** (Figure 2) were proposed as a *trans*-sialidase inhibitor. Compound **3** showed the best activity towards the TcTS (79% of inhibition at 1.0 mM and IC_{50} value of 0.32 mM), whereas compound **2** appeared to have higher anti-trypanosomal activity against *T. cruzi* Tulahuen strain, presenting an IC_{50} value of 124 µM (benznidazole was used as control with value of 0.03 mM for IC_{50}). Moreover, compound **2** did not show cytotoxicity against mouse spleen cells.

Finally, compound **4** (Figure 2) was evaluated in its ability to avoid *T. cruzi*'s tripomastigostes invasion in the host's cells. Compound **4** blocked the parasite invasion by a galectin-3 binding-related mechanism, revealing that galectin-3 may be an essential host target for the design of anti-trypanosomal agents.

1.3. Glycosylphosphatidylinositol Anchors

Glycosylphosphatidylinositol (GPI) anchors are glycophospholipid-based structures which are found in the membrane leaflet of many eukaryotic cells. These molecules can be found attached to complex phosphosaccharides, glycoproteins and as surface glycolipids in protozoans, being predominantly abundant in these organisms (McCONVILLE; FERGUSON, 1993; FERGUSON, HART, KINOSHITA, 2017). More specifically in *T. cruzi*, GPI anchors are responsible for anchoring essential mucins and enzymes, such as *trans*-sialidase on the parasite's cell surface. Several studies revealed that GPI anchors are important for invasion and survival of parasites in host cells. The high density of GPI structures at all life-cycle stages of protozoan parasites suggests that the GPI biosynthetic pathway might be an interesting object of study towards the development of anti-parasitic drugs/vaccines. (MENDONCA-PREVIATO, et al. 2008; McCONVILLE; FERGUSON, 1993).

GPIs comprise a glycan core with a phosphoethanolamine linker and a phospholipid chain (Figure 3, part A). Most GPI anchors so far elucidated have a conserved scaffold: **$H_2N(CH_2)_2OPO_3H6Man\alpha1\rightarrow2Man\alpha1\rightarrow6Man\alpha1\rightarrow4GlcN\alpha1\rightarrow6$*myo*-Ino1-$OPO_3H$-Lipid,** except for *Entamoeba histolytica* (FERGUSON, 1997; MCCONVILLE; FERGUSON, 1993).

Given that all eukaryotes produce GPI anchors, it is essential to understand the differences regarding structural features (Figure 3, Parts A and B). The different GPI structures according to each organism are highlighted in Table 1. For instance, in mammalians, a fourth mannose residue is linked to Man III as well as a second phosphorylation in Man II (Figure 3, Part A). Lipid is usually alkylacylglycerol, or diacylglycerol derivatives and C-2 of inositol moiety present a palmitate lipid moiety (Table 1, Line 6). (TSAI et al., 2012).

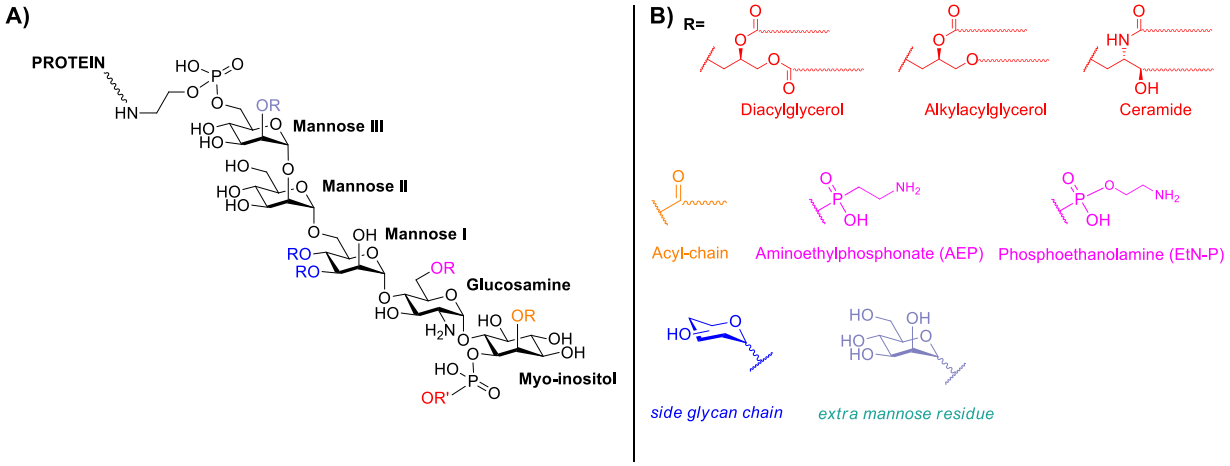


Figure 3: *A: Common structure of GPI anchors. B: Main components linked to the glycan core in specific colours, which correlates to the positions of same colours in the glycan core in part A.*

Regarding *T. brucei*, the variant surface glycoprotein (VSG) GPI anchor contains a saccharide chain branch at the C-3 of Man I. Dimyristoylglycerol, and *lysO*-1-*O*-stearoylglycerol are the main lipids composing *T. brucei*'s VSG and Procyclic Acidic Repetitive Protein (PARP) anchors. In addition, procyclic forms of the parasite present acylated inositol moiety in their GPIs and a residue of ethanolaminophosphate is only found in Man III (Table 1, Line 1). (MCCONVILLE; FERGUSON, 1993; FANKHAUSER et al., 1993; CONZELMANN et al., 1992).

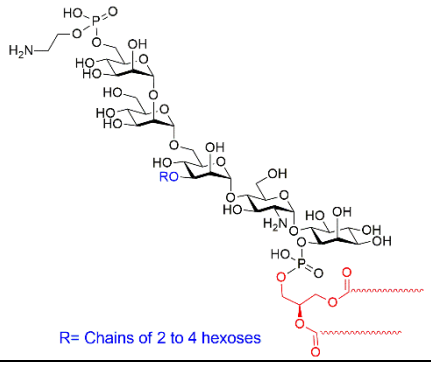
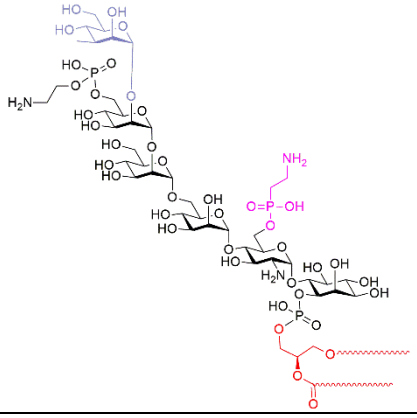
T. cruzi GPI´s glycan core comprises four mannose residues besides a 2-aminoethylphosphonate (AEP) group linked at the C-6 position of the GlcN. (Table 1, Line 2). The lipid linked to the PI moiety varies along the parasite's developmental state: Epimastigotes GPIs contain mainly 1-*O*-($C_{16:0}$)-alkyl-2-*O*-($C_{16:0}$)-acylglycerol-3-PI; metacyclic trypomastigotes present GPI anchors predominantly composed by different types of phosphoceramide-inositol, containing dihydrosphingosine ($C_{18:0}$), lignoceric ($C_{24:0}$) or palmitic acid ($C_{16:0}$) (Figure 1) (CARDOSO et al., 2013; SERRANO et al., 1995; MCCONVILLE; FERGUSON, 1993).

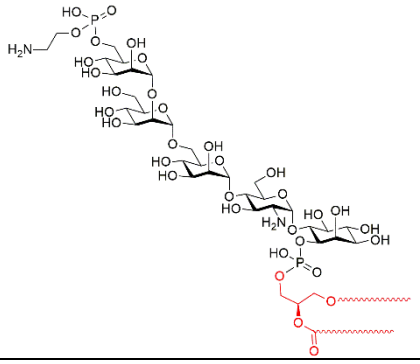
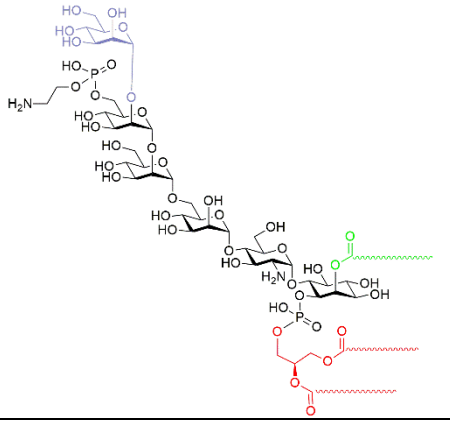
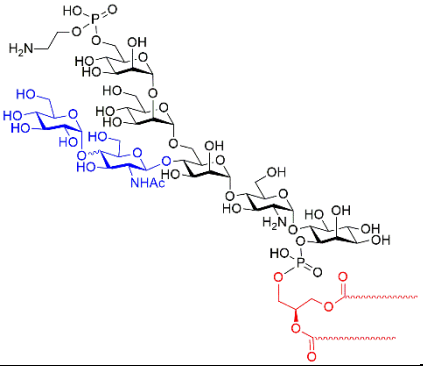
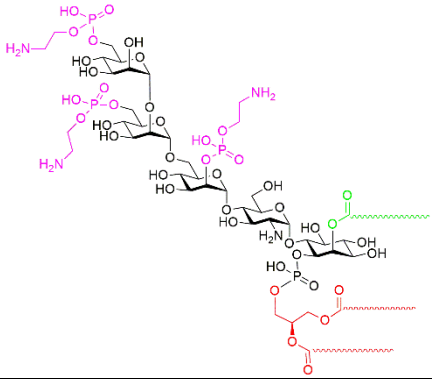
Leishmania spp. lipophosphoglycans (LPGs) are made of 1-*O*-alkyl-2-*lyso* phosphatidylinositol with one saturated C_{24} or C_{26} alkyl chain, which supports a negatively charged repeating polysaccharide, richly expressed by promastigote forms (up to 5×10^6 per cell). Glycoinositol phospholipids (GIPLs), related to 1-*O*-alkyl-2-*O*-acyl- or *lysO*-1-*O*-acylglycerol, and varied external glycans, are found in large quantities (approximately 10^7 molecules per cell) in all developmental stages (Table 1, Line 3, for *L. major*). (MCCONVILLE; FERGUSON, 1993).

Plasmodium spp. parasites present four mannose moieties at their GPI glycan core. The PI part of intraerythrocytic *P. falciparum* GPIs is varied on fatty acyl substituents, with palmitate or myristic acid frequently linked to C-2 of inositol. *Plasmodium spp*. GPIs do not contain any additional phosphoethanolamine substitution in their glycan core structures (Table 1, Line 4, for *P. falciparum*) (SUKHAREVA-BUELL, 2003; NAIK; DAVIDSON; GOWDA, 2000).

Finally, *Toxoplasma gondii* GPI anchors are found as free structures or anchoring proteins, and these structures mainly differ among themselves in carbohydrate composition connected to the glycan core. Type A GPIs contain a residue of GalNAc-linkedβ1→4 to Man I, whereas type B presents Glcα1→4GalNAc linked to the same mannose residue in the main core. Fatty acids are typically ($C_{18:1}$)-diacylglycerol, but the length varies depending on the life stage and virulence of *T. gondii* strain (Table 1, Line 5). (ECKERT; GEROLD; SCHWARZ, 2002; MCCONVILLE; FERGUSON, 1993).

Table 1. Structures of some protozoan parasites and mammalian GPIs. Table adapted from Morotti, Martins-Teixeira and Carvalho (2017).

Species	GPI structure
1. Trypanosoma brucei	
2. Trypanosoma cruzi	

Species	GPI structure
3. Leishmania major	
4.Plasmodium falciparum	
5. Toxoplasma gondii	
6. Mammalian	

1.3.1. Biosynthesis of GPI anchors

The first GPI structure elucidated was *T. brucei*'s Variant Surface Glycoprotein (VSG), and this parasite has been extensively used as an experimental model for

studies involving GPI anchor pathways. Since *T. brucei* GPI structures and pathways have been characterised, much of what is known about this class of molecules is related to this parasite, and the order of the events in a GPI biosynthesis is also based on these existing studies (MCCONVILLE; FERGUSON, 1993). What is identified about *T. cruzi* GPI anchors have been recently reported by Cardoso et al. (2013) although the order of each step may follow *T. brucei*'s GPI biosynthesis, as standard. On the other hand, structural variances between GPI anchors lead to the fact that biosynthesis of these molecules in different organisms is also diverse. Herein, what is acknowledged about the biosynthesis of *T. cruzi* is described with the assistance of Figure 4, and some examples of divergent biosynthesis from other eukaryotes will be discussed.

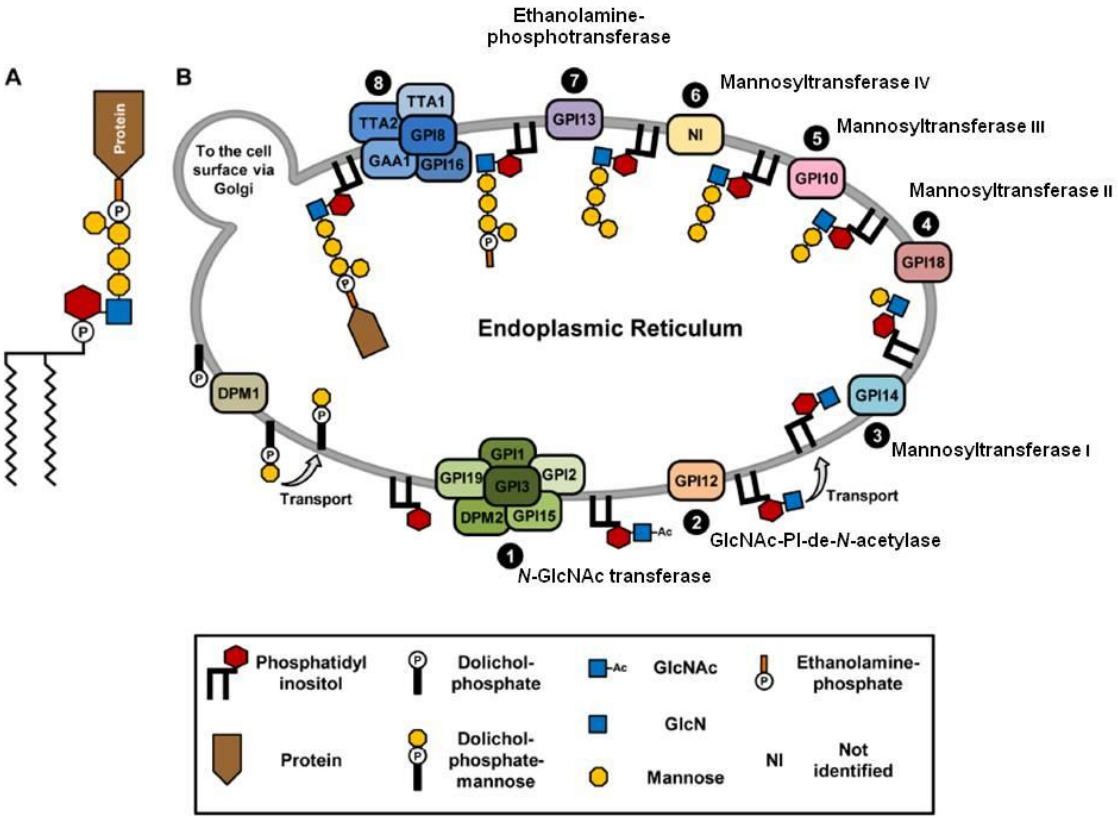


Figure 4: Biosynthesis of *T. cruzi* GPI anchors according to Cardoso and co-workers (2013). Coloured squares bring the names of genes Identified so far for the enzymes involved in the pathway. Abbreviations: **Glc*N*Ac**: *N*-acetylgucosamine; **Glc*N***: guclosamine; **Glc*N*Ac-PI-de-*N*- acetylase**: *N*-acetylglucosamine-phosphatidylinositol-de-*N*-acetylase.

The first step is the attachment of *N*-acetyl glucosamine by a *N*-GlcNAc transferase to the phosphatidylinositol (PI) moiety (Step 1, Figure 4). De-*N*-acetylase of glucosamine moiety allows the entrance of the molecule in the endoplasmic

reticulum (Step 2, Figure 4). Four different mannosyltransferases link, respectively, mannoses I, II, III, and IV to the GlcN-PI block; UDP-Mannose is the donor of all mannose residues in the GPI pathway (Steps 3 to 6, Figure 4). An ethanolamine phosphate moiety is linked to Man III of the central core by an ethanolamine-phosphotransferase for further linkage of a protein (Step 7 and 8, Figure 4). The ready GPI anchor leaves the endoplasmic reticulum in a vesicle towards the Golgi apparatus for later attachment to the cell membrane.

Some differences in the biosynthetic pathway of other organisms are emphasised: In *T. gondii*, conversion of Glc*N*Ac to Glc*N* also occurs outside the ER- when compared to *T. cruzi* biosynthesis, which contrast with common biosynthetic routes, that require GlcNAc to occur inside ER (SMITH et al., 2007). PI-acylation occurs in mammalian GPI biosynthesis and *T. gondii* at a particular step, which is after de-*N*-acetylation of PI-GlcNAc and before the addition of any mannose residue. In mammalian GPIs, the addition of the first mannose residue is followed by linkage of an extra ethanolamine phosphate in C-6 of this moiety (NIEHUS et al., 2014; PEKARI et al., 2001). Deacylation occurs after addition of all mannose residues, and failure in this process leads to inhibition of later steps (SMITH et al., 2007). In contrast, biosynthesis of *T. brucei'*s bloodstream form GPI counts on several acylations/deacylations along the pathway. Conversely, in *L. mexicana*, an additional ethanolaminephosphate is unusually linked to the core GlcN of some hybrid type GIPL anchor (MCCONVILLE; FERGUSON, 1993). *T. cruzi* and *P. falciparum* biosynthetic pathways appear to be similar to *T. brucei*'s, although there's no evidence of fatty acid remodelling before attachment of a protein to the ethanolaminephosphate residue (NAIK; KRISHNEGOWDA; GOWDA, 2003). Finally, *T. cruzi* GPIs undergo a change of PI lipids from alkylacylglycerol derivative to ceramide by an inositolphosphorylceramide (IPC) synthase during epimastigote to metacyclic trypomastigote stages. However, this stage is not considered part of the parasite’s GPI biosynthesis (RALTON; MCCONVILLE, 1998).

1.3.2.GPI Anchors as a target against protozoan parasites

Biosynthetic and structural differences between organisms are some of the reasons why GPI anchor can be useful as drug targets against parasites. Moreover, normal levels of GPI synthesis are not essential, in some instances, for mammalian

cell survival (SMITH et al., 2001; FERGUSON, 1999). Although eukaryotes have similar GPI pathways, differences in the timing of specific biosynthetic steps and substrate specificities of the enzymes have been reported. In the last two decades, various research groups have been investigating the possibility of validating proteins involved in GPI biosynthesis as drug targets against parasites such as protozoans and fungus (MOROTTI; MARTINS-TEIXEIRA; CARVALHO, 2017, SMITH et al., 2004). In this context, it is important to cite some examples of studies towards inhibition of GPI pathway.

GPI intermediates from *T. brucei* and *Leishmania* spp. undergo myristic acid remodelling, at some point of the biosynthesis. Myristate analogues containing oxygen substituting one methylene group were used as inhibitors of myristic acid remodelling process. The *O*-11 (Compound **5**; Figure 5) analogue killed almost all the parasites in culture within few hours at a concentration of 10 µM. The compound was able to cause alterations in the parasites' morphology, with impaired motility, loss of integrity shape and vacuole inclusions. Moreover, the analogue **5** showed no toxicity for human T lymphoid cells (DOERING et al., 1991).

Some molecules showed GPI inhibition although it was not possible to identify the specific biosynthetic step or particular target molecule involved. Synthetic cell-permeable analogues of a GPI intermediate, (Compounds **6** and **7;** Figure 5) were recognised, incorporated and metabolised by the *T. brucei* GPI pathway, but not by the HeLa cells representing the human pathway. This finding served as a chemical validation of the GPI biosynthetic pathway as a drug target against African sleeping sickness (SMITH et al., 2004).

The specificity of mannosyltransferases was studied in *T. brucei* with a series of synthetic analogues. In summary, a simple C_{18} alkyl chain could substitute the natural diacylglycerol lipid moiety, and the substrate recognition by MTIII required hydroxyl groups at positions C-2 and C-3 of the terminal mannose. On the other hand, the amine in glucosamine residue was not essential for the recognition. This examination led to the identification of **8** (Figure 5) as an inhibitor of Mannosyltransferase III with an IC_{50} of 1.7 µM, leading to the accumulation of Man_2GlcN-PI, (URBANIAK, et al., 2008). Most straightforward structures such as **9** (Figure 5) also inhibited the GPI pathway, possibly by competing with endogenous GPI intermediates for mannosyltransferase(s) (BROWN et al., 1997).

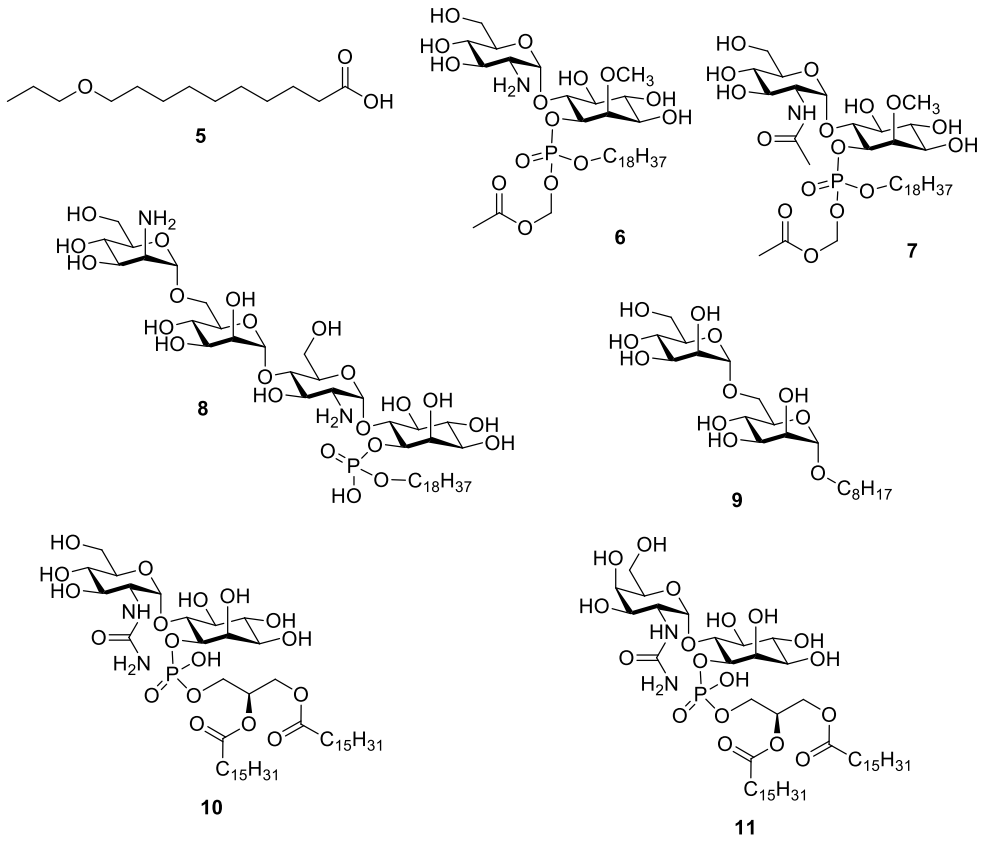


Figure 5: Compounds designed for Inhibition of GPI anchors of various organisms.

Smith and co-workers (2002) evaluated the substrate specificities of enzymes from *Plasmodium* GPI biosynthesis using analogues of D-GlcNR1→6-D-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol. Similarities were found comparing *Plasmodium* and mammalian (HeLa cells) enzymes, such as: (i) the 2-acetamido/amino and 3-OH groups in GlcN moiety are essential for the de-*N*-acetylase, inositol acyltransferase, and further mannosyltransferase I recognition; (ii) the 6-OH group in GlcN is not critical for substrate recognition by de-*N*-acetylase, inositol acyltransferase, the four mannosyltransferases, and the ethanolamine phosphate transferase; (iii) the 4-OH group of GlcN is required for recognition of the inositol acyltransferase and mannosyltransferase I. Conversely, differences between both pathways were also revealed: (i) Inhibition of *Plasmodium* inositol acyltransferase was observed with GlcN-[L]-PI, while GlcN-(2-*O*-alkyl) PI weakly and competitively inhibited the mannosyltransferase I; (ii) the *Plasmodium* de-*N*-acetylase is able to recognise analogues containing *N*-benzoyl, *α*-D-GlcNAc or *α*-D-GalNAc, whereas the human enzyme cannot. Two analogues, (Compounds **10** and **11**; Figure 5) were designed and found to be potent *Plasmodium*-specific de-*N*-acetylase suicide inhibitors (IC_{50}

~0.2 µM), being potential lead compounds for the development of antimalarial drugs (SMITH et al., 2002; SMITH et al., 2001).

1.3.3. Synthetic approaches for GPI anchors

According to examples cited above, several compounds, most of them being carbohydrate/lipid based, have shown to be successful in inhibiting enzymes involved in GPI anchor biosynthesis of various parasites when assayed *in vitro*. However, using these molecules *in vivo* is still a challenge regarding pharmacokinetic and pharmacodynamic aspects, considering that many parasites are intracellular (MITRAGOTRI; BURKE; LANGER, 2014). On the other hand, various strategies can be used to enable the entrance of carbohydrate-like molecules inside cells. Most procedures consist in proper functionalization of the carbohydrate moiety or formulation of carriers to circumvent problems such as lack of pharmacokinetic properties, poor bioavailability, fast serum clearance, and rapid degradation (MITRAGOTRI; BURKE; LANGER, 2014; AICH et al., 2010). Moreover, convincing evidences have shown that this class of molecules is a versatile platform for drug discovery (IVANOVA et al., 2017; HATAKEYAMA et al., 2011; AICH et al., 2010; MONTOYA-PELEAZ et al., 2005; BROWN et al., 2001).

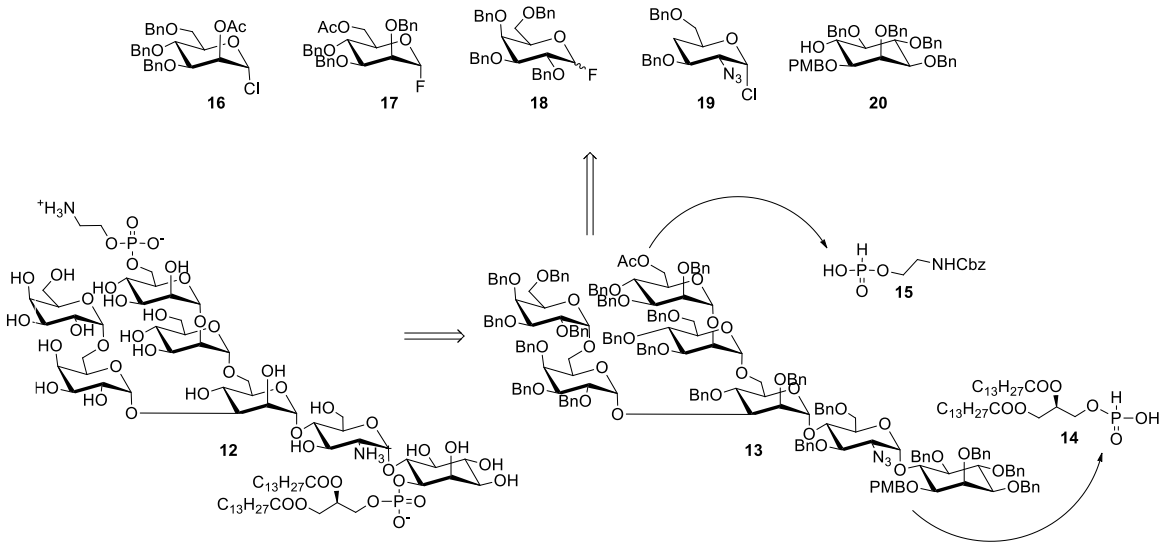
Natural GPIs are structurally diverse. Furthermore, GPI-anchored proteins are amphiphilic and associated with the cell membrane, making their isolation from nature difficult (LU; GAO; GUO, 2015). Studies of isolated GPIs from *P. falciparum* showed that parasitic GPIs can trigger an immune response of mammalian hosts. However, other groups could not find the same reported results. The discrepancies between these results might be due to the impurities associated with the isolation process of the parasitic GPI. To circumvent this problem, a series of synthetic fragments structurally related to P. falciparum GPI were synthesized and evaluated. This strategy allowed to observe that there were higher levels of IgG in sera which were exposed to the synthesized structures, compared to those not exposed to these synthesized molecules (BOUTLIS et al., 2002, BOUTLIS et al., 2003; HUDSON et al., 2003; SUGUITAN (2006). The progress in the chemical synthesis enabled the preparation of several GPIs and analogues of *P. falciparum* and *T. brucei* that allowed the study of these molecules against the immune response of the host. These findings, together with the fact that all protozoan parasites express large

amounts of GPIs, in comparison with mammals, suggest that these molecules offer a good alternative for the development of vaccines (TSAI et al., 2012).

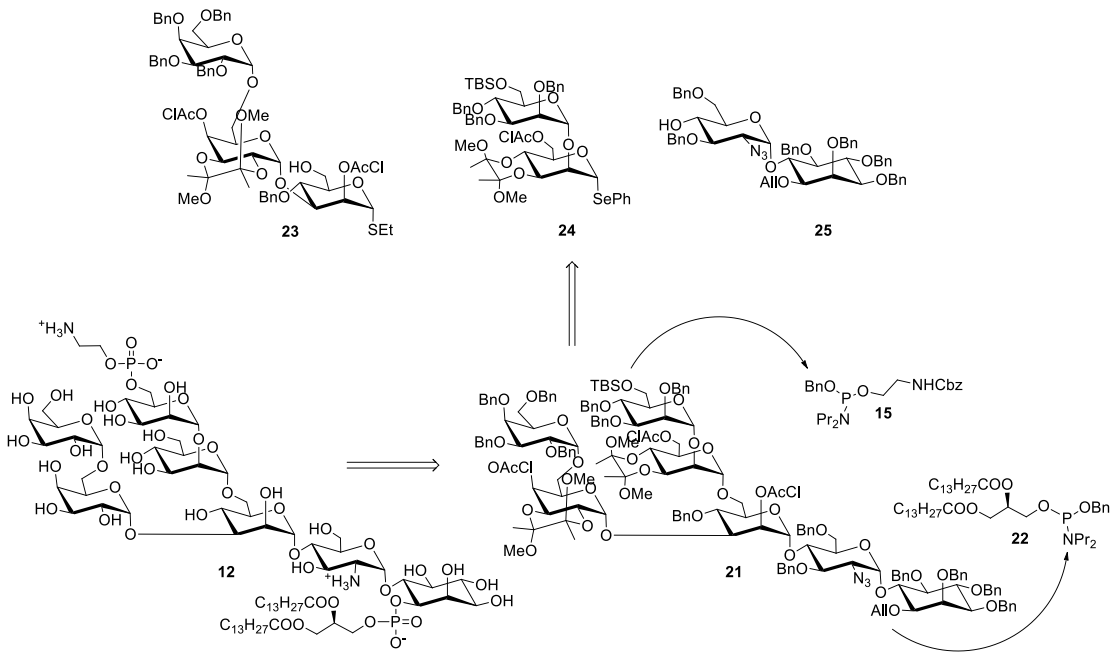
Numerous GPI anchor structures of various organisms have been synthesised. Synthesis of the *O*-glycan core usually starts with a suitably protected D-glucosamine to an optically pure, also protected *myo*-inositol derivative. From this block, there are basically two ways to synthesize the oligosaccharide chain present in GPIs: the linear and the convergent forms. The linear form is derived from monosaccharide donors which are individually added one by one to form an oligosaccharide (NIKOLAEV; MAHARIK, 2011). The first GPI anchor structure was synthesized by Murakata et al. (1991-1992), who proposed a linear synthesis. Glycosyl donors containing fluorine and chlorine were used in this attempt (Compounds **16-19**, Scheme 1), as well as a protected *myo*-inositol moiety as acceptor (Compound **20**, Scheme 1). The benzyl groups were used as permanent protective groups along the entire synthesis. The H-phosphonate chemistry allowed the insertion of two different phosphodiester units (Compounds **14-15**, Scheme 1). Overall deprotection was carried out by hydrogenolysis, in the presence of $Pd(OH)_2/C$ (Compound **12**, Scheme 1). This first synthetic strategy gave the VSG GPI of *T. brucei* (11) with 23% yield (Scheme 1) (MURAKATA; OGAWA, 1991; MURAKATA; OGAWA, 1992).

The convergent approach occurs with ready and more complex blocks, which are prepared in parallel for later union to form one single molecule resulting in less management of the protecting groups of the oligosaccharide chain. The portions related to ethanolamine phosphate and phospholipids are added before the overall deprotection of the GPI structure.

Several authors have reported GPI anchor synthesis using different strategies in a convergent way (CAMPBELL; FRASER-REID, 1994; CAMPBELL; FRASER-REID, 1995; BAESCHLIN et al., 1998; MAYER, SCHMIDT, 1999; KWON et al., 2005; LIU; STOCKER; SEEBERGER, 2006). As an example of convergent synthesis, Ley and collaborators (1998) performed the VSG GPI of *T. brucei*, which was adaptable for the synthesis of other GPIs (Scheme 2). Briefly, the strategy was based on three building blocks prepared in parallel, using butane-2,3-diacetal and chloroacetate as protecting groups (Compounds **23** and **24**, Scheme 2). (BAESCHLIN et al., 1998).



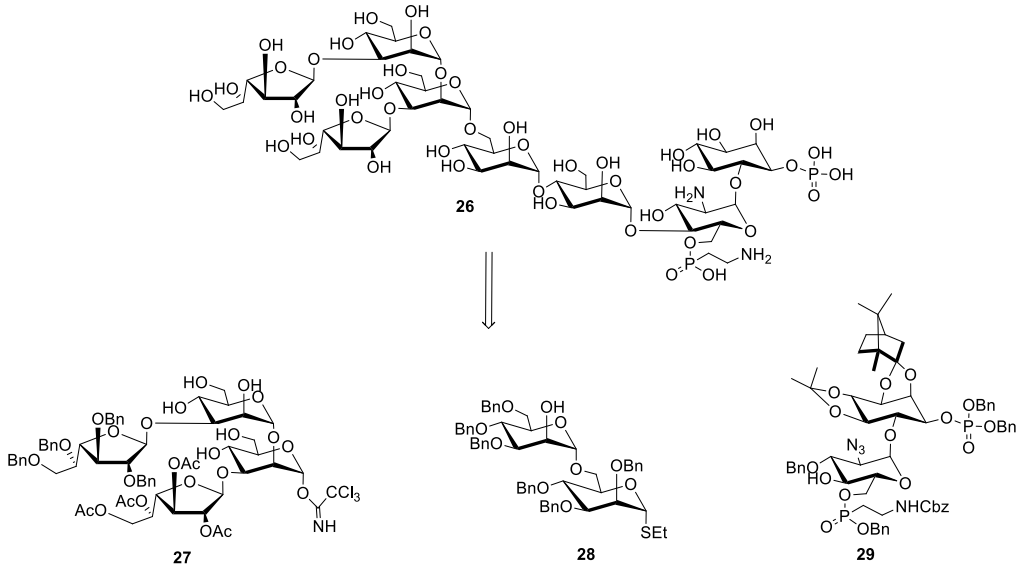
Scheme 1: Retrosynthesis of *T. brucei* GPI anchor performed by Ogawa and co-workers (1991/1992). Adapted from Nikolaev and Maharik, 2011.



Scheme 2: Retrosynthesis of *T. brucei* GPI anchor performed by Ley and co-workers (1998). Adapted from Nikolaev and Maharik, 2011.

Another example of convergent GPI synthesis was performed by Konradsson and Hederos groups (2005) for the preparation of *P. falciparum* phosphorylated *myo*-nositol heptassacaride (Scheme 3). The proposed synthetic route came from three building blocks (Compounds **27**, **28** and **29**). Donors derived

from galactofuranose, containing *O*-acetyl group, were coupled to mannose residues to obtain block **27** (Scheme 3), by *O*-glycosylation promoted by AgOTf. Stereochemistry (β) was assisted by the *O*-acetyl groups. Block **28** was obtained by *O*-glycosylation of two mannose residues, followed by 2'-*O*-debenzylation. Block **29** was obtained in six steps.



Scheme 3: Retrosynthesis of the heptasaccharide derivative of *P. falciparum* GPI anchor performed by Konradson and co-workers (2005). Adapted from Nikolaev and Maharik, 2011.

After the addition of all the blocks, the overall deprotection of the heptassacaride was first performed with deacetylation, under Zemplen conditions followed by *O*-debenzylation using sodium in ammonium solution. Finally, the acetal groups were removed by acid hydrolysis, generating the final product **26** (Scheme 3).

1.3.4. Labelled GPI Anchors as a strategy to study GPI pathways

Labelled GPI anchors have been used as part of efforts to elucidate molecular details of GPI biosynthesis and structural features. Many studies on the structure and components of the GPI anchor have relied on the incorporation of labelled lipids and sugars into GPIs anchor (FERGUSON; HALDAR; CROSS, 1985). Structural characterization of intermediates led to the initial elucidation of GPI anchors biosynthetic pathways (SHARMA et al., 1999; VARMA; HENDRICKSON, 2010). Currently, different chemical approaches have been applied to understand GPI-anchoring and its significance.

Lili Lu and co-workers (2015) developed a strategy for metabolic engineering of cell surface GPIs and GPI-anchored proteins using inositol derivatives carrying an azido group. The inositol containing azido group was exposed to cancer cell lines, MCF-7, Hela, K562, and SKM28, and could be incorporated to the GPI pathway. The cells were then tagged with biotin via click chemistry forming a fluorescent coat of GPIs with the modified inositol. This approach was suggested to be used to label GPI-anchored proteins with various tags for biological studies (LU; GAO; GUO, 2015). Another application of labelled GPI anchor was developed by the same research group which synthesised the first biotin-labeled GPI core glycans. These GPI conjugates were useful tool by their use to the scrutiny of pore-forming bacterial toxin-GPI interaction, revealing that the phosphate group at the C-1 position of GPI inositol had a significant impact on GPI-toxin binding (GAO et al., 2017).

GPI glycan moieties labelled with 2-aminobenzamide were also useful to compare the structure of phylamentous fungi *Aspergillus fumigatus* and yeasts *Saccharomyces cerevisiae* GPI anchors. The study showed that the nature of the inositol-linked phospholipids of *A. fumigatus* is phosphoceramide containing mainly phytosphingosine and monohydroxylated $C_{24:0}$ fatty acid. In contrast, *S. cerevisiae* presented diacylglycerol phospholipid moieties instead of a ceramide derivative (FONTAINE et al. 2003).

Imhof and c*o*-workers (2004) have reported that the ethanolamine phosphate side chain added to the first mannose residue is a prerequisite for the addition of the third mannose to its GPI precursor in *Saccharomyces cerevisiae (yeast)*. These findings were performed after incorporation of labelled *myo*-[2-^{3}H] inositol precursor to the growing cells of the yeast.

1.4. *Euglena gracilis*

Euglena gracilis is a free-living microalga, unicellular and flagellated, which inhabits fresh water. However, due to its high adaptability, it can survive to conditions such as very low pH (around 0.9), high salinity and high energetic ionising radiation (BUETOW, 1962; 2011). *Euglena* presents both plant and animal characteristics: it can live in the light, producing food by photosynthesis, or it can exist in the dark and using environmental sources, storing them as a *paramylon* (starch-like β-1,3-glycan).

Euglena is composed of all organelles of the eukaryotic cell and it does not have a cell wall like plant. Instead, it is composed of repetitive membrane domains

know as ridges and groves (Figure 6) (AHMADINEJAD; DAGAN; MARTIN, 2007; SOMMER, 1965). *Euglena* belongs to the phylum *Euglenozoa*, (NAKANO et al., 1987); which also includes Trypanosomatid parasites, such as *T. cruzi, T. brucei* and *Plasmodium.*

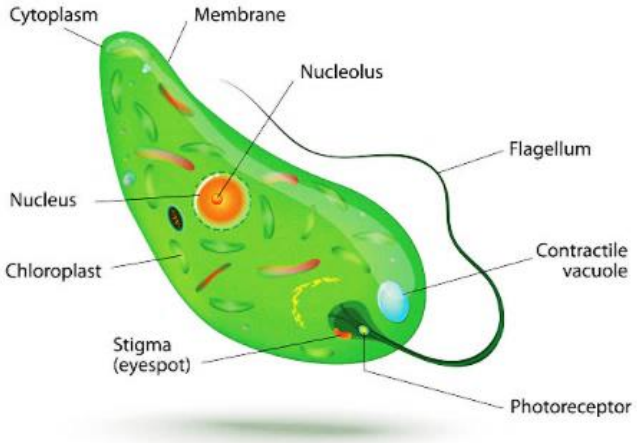


Figure 6: *Euglena gracilis* and cellular components

1.4.1. E. gracilis as a safe protozoan model

There is very limited data regarding research to elucidate the structural composition of glycoproteins in *E. gracilis* (IVANOVA, 2015). In a study conduced by Delacanal and Parodi (1985), *E. gracilis* entire cells were incubated with UDP-[^{14}C]Glc and radiolabelled lipid-linked *N*-glycans. Thus, *N*-linked glycoproteins were extracted at different time of incubation. The lipid-linked *N*-glycans were hydrolysed in mild acidic conditions to release monosaccharides, whereas *N*-linked glycoproteins were treated with endo-β-*N*-acetylglucosaminidase to cleave the bond between two *N*-acetylglucosamine moities to release *N*-linked oligosaccharides. The free monosaccharides and *N*-linked oligosaccharides were then compared to standards using paper chromatography. The results disclosed that dolichol-P-P-bound oligosaccharide migrated together with $Glc_3Man_9GlcNAc_2$ standard and appeared to contain glucose, mannose and *N*-acetylglucosamine residues. Based on these results, the authors suggested that the mechanism of protein *N*-glycosylation in *E. gracilis* is similar to higher eukaryotic cells phenomena.

A study performed by Ivanova and co-workers (2017) showed that fluorescent labelling could be successfully applied in glycosyl derivatives to track membrane-bound glycosyltransferases involved in the biosynthesis of various glycoconjugates in

Euglena gracilis. This methodology can be powerfully useful to probe the biosynthesis of other glycoconjugates, such as GPIs in *E. gracilis* and related organisms as *T cruzi.*

The investigation towards GPI anchor enzymes on microsomal membranes can also be extended to *T. cruzi*. Previato and co-workers (1998) conducted a characterization of the activity of a GlcNAc-transferase from Trypanosoma cruzi's microsomes. The authors were able to analyse the optimal environment for the enzyme's activity by incorporating *N*-[^{3}H] acetylglucosamine to a synthetic peptide acceptor (KPPTTTTTTTTKPP) and proved that the best conditions are related to pH 7.5-8.5 and the requirement of Mn^{2+}. The enzyme also proved to be unaffected by the natural products tunicamycin and amphomycin and is powerfully inhibited by UDP (PREVIATO et al., 1998).

The importance of GPI anchors on the cell surface of parasites and position of *Euglena* in the same phylum (*Euglenozoa*) of several protozoan parasites, as well as the lack of knowledge in this area of research, both in *E. gracilis* or in trypanosomatids, prompted the professor Rob Field's research group to investigate biosynthesis of these glycoconjugates in *E. gracilis*, with the possibility that *Euglena* could be used as an alternative model system for parasites. Given these findings, this work proposes that carbohydrate moieties to mimic GPI anchor precursors and serve as probe for GPI pathways in *E. gracilis* as an alternative and safe method to support drug discovery for Chagas disease.

The approach of using *E. gracilis* to uncover glycan pathways is recentlyand exclusively being standardized by the Rob Field's laboratory at John Innes Centre, in which *this* fresh-water algae have been used as source of several enzymatic and biochemical studies. Some advantages of this approach can be pointed out, such as the fact that no biosafety structure or training are necessary to manipulate *E. gracilis,* since it not a pathogen, making this algae a safe organism to standardize methodologies that can be applied to other organisms. *It also gives the opportunity* to obtain new compounds by enzymatic approach and to uncover the own *E. gracilis*' GPI pathways, as well as *T. cruzi*'s pathway. On the other hand, disadvantages can be related to the lack of validate methodologies in *E. gracilis* since GPI anchor precursors that may serve as controls are unknown, leading to the uncertainty that perhaps the GPI *E. gracilis* biosynthesis can be very different from *T. cruzi*'s.

The complexity and broad spectrum of mechanisms in which anchor GPIs may be involved in the parasite survival and infectivity arouse interest from a chemical and biological point of view. The synthesis of parts of GPI anchor becomes an interesting tool for the deepening of studies concerning the biological functions and interactions performed by this class of molecules for the search of new molecular targets, allowing the planning and development of new effective drugs. In addition, it may assist in the identification and development of potential structures to be used as antigen or adjuvant vaccines, capable of mimicking GPI anchor carbohydrates (BOONYARATTANAKALIN et al., 2008; SWARTS; GUO, 2010; TSAI et al., 2012).

5. CONCLUSIONS

Glycosyl donors were prepared from α-D-Glucosamine hydrochloride (**35**) in good yields, being the donors **33c** and **33d** not previously described in the literature. Synthesis of *myo*-inositol acceptor unit was successful achieved using *Ferrier* rearrangement strategy, affording acceptor **34** in moderate yields. Donors and acceptor were all tested in small scale in *O*-glycosylation reactions to check conditions and select the best donor in terms of good α anomer ratio, yield and reproducibility. From these experiments, donor **33c** was selected for large scale *O*-glycosylations.

O-glycosylation α/β ratio proved to be more depend on donor chemical properties than on solvents. Despite most of reactions had a higher generation of α anomer, yields and effectiveness of reactions were different. For instance, 1,2-dichloroethane seemed to promote extended time for reaction completion, which led to decomposition of some donors to hemiacetals. Large scale of *O*-glycosylation of acceptor **34** with donor **33c** was accomplished in diethyl ether, using TMSOTf as promoter. A key step involving the deallylation of compound **31c** was successfully performed with $PdCl_2$ and NaOAc in acetic acid giving a novel pseudo-disaccharide **77** in good yields. All synthesised pseudo-disaccharides **31a-d** are unkown in the literature.

Phosphodiester linkage between octanoyl H-phosphonate **87** and **77** was conducted according to Crossman and co-workers (2002), giving compound **90** with low yield, after partial deprotection and successful purification by semi-preparative HPLC. Product **90** was then subjected to overall deprotection giving the target product **30a,** which was sent to John Innes Centre for further *E. gracilis* microsomes assays. Moreover, injections of compound **30a** on LC-MS allowed the establishment of appropriate conditions for further analysis of products to be obtained after incubation of **30a** with microsomal membranes. For this purpose, *E. gracilis* was grown in dark conditions for 13 days for later isolation of microsomal membranes,

Furthermore, synthesis of anthracenyl (**84**) and naphthyl (**85**) lipid chains were successfully synthesised according to Riley and co-workers (2010) in few steps and moderate yields. Compound **85** underwent H-phosphonate approach and further phosphodiester linkage to the deallylated pseudo-disaccharide **77**, giving compound **91** in low yield. Overall deprotection of **91** is ongoing and last steps of the synthetic route are being optimized to achieve better yields and purity. The anthracenyl

derivative **84** will be submitted to the same procedures of phosphorylation once the route is optimized.

As future perspectives, the synthesis of compounds **30b-c** will be performed and optimization of the synthetic route aiming to achieve the final products in better quantities will be performed. In addition, standardization and further validation of the assays *in E. gracilis* microsomes will be performed with the scope that it must be prepared to give information whether the synthesised compounds **30a-c** would be either an GPI pathway inhibitor or substrate. Once assays are set in *E. gracilis*, results will be analysed, and the same methods will be appliedto *T. cruzi* microsomal membranes.

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