
REVIEW

Synthetic Neoglycoconjugates of Cell-Surface Phosphoglycans of *Leishmania* as Potential Anti-parasite Carbohydrate Vaccines*

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Abstract—*Leishmania* are a genus of sandfly-transmitted protozoan parasites that cause a spectrum of debilitating and often fatal diseases in humans throughout the tropics and subtropics. During the parasite life cycle, *Leishmania* survive and proliferate in highly hostile environments. Their survival strategies involve the formation of an elaborate and dense cell-surface glycocalyx composed of diverse stage-specific glycoconjugates that form a protective barrier. Phosphoglycans constitute the variable structural and functional domain of major cell-surface lipophosphoglycan and secreted proteophosphoglycans. In this paper, we discuss structural aspects of various phosphoglycans from *Leishmania* with the major emphasis on the chemical preparation of neoglycoconjugates (neoglycoproteins and neoglycolipids) based on *Leishmania* lipophosphoglycan structures as well as the immunological evaluation for some of them as potential anti-leishmaniasis vaccines.

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Leishmania are a genus of sandfly-transmitted protozoan parasites that cause a spectrum of debilitating and often fatal diseases in humans throughout the tropics and subtropics. The disease (termed leishmaniasis) torments over 12 million people worldwide, making 2.4 million people disabled and causing about 60,000 deaths annually. The WHO estimations show that 350 million people are thought to be at risk from the disease [1-3]. Although leishmaniasis is most common in tropical regions, it has also been diagnosed in overseas travelers and U.S. Gulf War veterans [4] and has emerged as an opportunistic infection of HIV patients [5]. The geographical distribution and pathology of leishmaniasis varies according to the species of the parasite. For instance, *Leishmania donovani* causes “visceral leishmaniasis” (also known as kala azar), characterized by an enlarged liver and spleen, that is often fatal, whereas *L. major* and *L. tropica* (in the Old World) and *L. mexicana* species complex, consisting of *L. mexicana*, *L. amazonensis*, and *L. venezuelensis*

mostly (in the New World) cause “cutaneous leishmaniasis” (known as oriental sore and characterized by self-limiting skin lesions) [1]. This form is the most common leishmanial disease, and is often fairly mild in comparison to the others. Widespread, chronic, non-ulcerative skin lesions resembling leprosy are indicative of “diffuse cutaneous leishmaniasis” [6, 7] that is commonly an effect of *L. aethiopica* (Old World) and *L. mexicana* species complex (New World) infection. “Mucocutaneous leishmaniasis” (or espundia) is due to *L. (V.) braziliensis* (*Viannia* subgenus) infection and most prevalent in Brazil, Bolivia, and Peru. It causes lesion in the mucous membranes, leading to devastation of tissues and extreme facial disfigurement [1, 8]. Secondary bacterial infections are common. *Leishmania (L.) mexicana* causes “Chiclero’s ulcer”, which can involve almost total destruction of the external ear.

The mechanism of infection [9] poses a serious challenge for therapeutic approaches. The vector for *Leishmania* is a sandfly of *Phlebotomus* (Old World) or *Lutzomyia* (New World) female species that inject the parasite when taking a blood meal. To initiate and propagate the infection, parasites then actively invade and reside within macrophages, the very part of the immune

Abbreviations: Hib, *Haemophilus influenzae* b; KLH, keyhole limpet hemocyanin; LPG, lipophosphoglycan.

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system that is designed to destroy them. The majority of the current anti-leishmanial drugs, such as Sb(V) based medications Pentostam and Glucantime or non-antimonial Amphotericin B, Paromomycin, and Miltefosine, seem to be inadequate due to factors such as cost, resistance, and toxic side effects [10-14]. Thus, a vaccine that would protect the human host by annihilating the parasites upon transfer from the insect vector would be welcome. Several different designs have been recently explored [15], but no generally applicable vaccine for leishmaniasis has yet come to light.

In principle, any unique feature *Leishmania* exposed on its cell surface could be investigated and exploited for the development of an anti-leishmaniasis vaccine [16]. *Leishmania* are digenetic organisms that alternate between the insect vector and a mammalian host. The most abundant macromolecule on the surface of the insect infectious stage of all *Leishmania* species is a complex glycoconjugate called lipophosphoglycan (LPG, see section "Cell-Surface and Secreted Phosphoglycans of *Leishmania*"). Although killed or attenuated parasites did not result a valid vaccine [15, 16], the LPG itself has been suggested to be a perspective vaccine candidate as the protective effect of the purified LPG fraction on mice challenged with *L. major* was demonstrated [17-19]. Here we discuss the preparation of synthetic neoglycoconjugates (neoglycoproteins and neoglycolipids) based on various elements of the LPG structure as potential anti-leishmaniasis vaccines as well as the immunological evaluation for some of them.

GLYCOCONJUGATE SYNTHETIC VACCINES

The carbohydrate vaccine technology is an area developing rapidly because of modern bioconjugation techniques [20-22], which allow an effective formation of carbohydrate-protein and carbohydrate-lipid conjugates. Traditional polysaccharide vaccines, i.e. highly purified immunogenic bacterial polysaccharides (usually capsular polysaccharides) [23, 24], have been associated with the short duration of the induced immunity and proved to be poorly immunogenic in infants and young children [25, 26]. That happens because the polysaccharide antigens are T-cell independent: they induce an immune response without the involvement of T-cells. The response lacks several important features that characterize the T-cell-dependent immune response, such as immunological memory, an antibodies class switch from IgM to IgG, and affinity maturation [23]. Conjugation of the bacterial polysaccharide to an immunogenic protein carrier (detoxified versions of strongly immunogenic proteins like diphtheria and tetanus toxins are often used) converts it to a T-cell-dependent antigen with enhanced immunogenicity. Thus, the new generation glycoconjugate vaccines against bacterial infections are immuno-

genic in infants and induce long lasting immunological memory resulting in a boostable response [27]. In 1990s, four anti-meningitis glycoconjugate vaccines against *Haemophilus influenzae* b (Hib) were introduced with great result: the Hib-induced meningitis has virtually been eradicated in countries with high immunization coverage [28]. Similarly, a tetravalent meningococcal glycoconjugate vaccine against *Neisseria meningitidis* serogroups A, C, Y, and W135 has recently been developed by Sanofi-Pasteur and licensed in the USA [29].

The glycan parts of modern glycoconjugate vaccines are, normally, native or functionalized bacterial capsular polysaccharides. These naturally derived carbohydrate polymers are heterogeneous mixtures that may include impurities and contaminants. The use of synthetic carbohydrate structures, which can be chemically produced as single compounds in a controlled manner without batch-to-batch variability, can eliminate these problems. Recently, a fully synthetic hexasaccharide conjugated to keyhole limpet hemocyanin (KLH) was shown to be an effective vaccine against prostate cancer [30]. The methodology has been used by Verez-Bencomo et al. [31] for the preparation of the first commercial synthetic anti-meningitis glycoconjugate vaccine against Hib. The results of clinical testing in the target population demonstrated that the glycovaccine containing fully synthetic phosphoglycans (which are fragments of capsular polysaccharides) is as effective as its natural counterpart. The vaccine was registered in Cuba in 2003 and is now part of the national immunization program. Similarly, a synthetic vaccine against *Shigella dysenteriae* type 1 based on a synthetic oligosaccharide conjugated to a protein was found to be more immunogenic than a glycoconjugate prepared from the native polysaccharide [32]. Synthetic preparation of the glycan part of the glycoconjugate vaccines also allows for chemical modifications of the structure that may not be possible to perform on the native material.

CELL-SURFACE AND SECRETED PHOSPHOGLYCANS OF *Leishmania*

During the parasite life cycle, which involves a promastigote stage(s) in the midgut of the insect vector and an amastigote stage in the phagolysosomes of the mammalian macrophage, *Leishmania* survive and proliferate in highly hostile environments. Their survival strategies involve the formation of an elaborate and dense cell-surface glycocalyx composed of diverse stage-specific glycoconjugates that form a protective barrier [33, 34]. Some of these macromolecules (including various phosphoglycans) were shown to be essential for virulence of the parasite [35-38].

LPG is a predominant cell-surface glycoconjugate of *Leishmania* promastigotes. Structurally, this is a poly(glycosyl phosphate) consisting of the β -Gal-(1 \rightarrow 4)- α -Man-1 \rightarrow OPO₃ repeating units (Fig. 1), where the nature of the

X and Y substituents varies according to the species. *Leishmania donovani* [34, 39] and *L. infantum* synthesize a linear phosphoglycan, whereas those of *L. mexicana* [40, 41], *L. major* [42-44], and *L. aethiopica* [45] have branched structures bearing non-stoichiometric mono- and/or disaccharide components (X) linked at O3 of the β -D-Galp residue. In *L. major*, the composition of the side chains varies and depends on the developmental stage of the parasite. In the LPG produced by *L. aethiopica* there are also some additional α -D-Manp residues (Y) linked to D-mannose residues in the main chain. All LPG molecules are capped at the non-reducing terminus with an oligosaccharide phosphate unit (basically, GalMan₃-phosphate, where some residues are non-stoichiometric; Fig. 1) and contain a glycan core (not shown) linked to an inositol-phospholipid anchor [33, 38] at the reducing end of the chain. The cap oligosaccharide structures appeared to be species- and stage-specific: GalMan₃ and GalMan₂ caps have been found in *L. donovani* and *L. mexicana* LPG [33, 40, 47], whereas for *L. major* LPG in promastigotes the cap was a Man₂ disaccharide [34, 42] and in amastigotes a GalMan disaccharide [34].

In addition to cell-surface LPG, *Leishmania* promastigotes produce a number of secreted glycoproteins,

where a peptide core is modified (glycosylated) with LPG-type phosphoglycans [47, 48]. *Leishmania donovani* secretes an acid phosphatase (sAP) in which the peptide chains are heavily glycosylated on C-terminal serine/threonine domains. The glycans (which are, in fact, phosphoglycans) consist of the same disaccharide phosphate repeating units found in the corresponding LPG (Fig. 1) and are phosphodiester-linked to select serine residues [34]. All species of *Leishmania* promastigotes synthesize secreted filamentous proteophosphoglycan, which forms a highly viscous gel surrounding the parasite cells. Compositionally this proteophosphoglycan consists of 95% phosphoglycans in a species-specific manner (Fig. 1). The poly(glycosyl phosphate) chains are attached to serine residues of the peptide component via phosphodiester groups.

Hydrophilic phosphoglycans have been found in culture supernatants of *Leishmania* promastigotes and have been structurally characterized for *L. major* [49], *L. mexicana* [50], and *L. donovani* [51]. They are essentially the corresponding LPG molecules without the glycan core—inositol-phospholipid region.

Leishmania mexicana amastigotes synthesize large amounts of a macromolecular amastigote-specific pro-

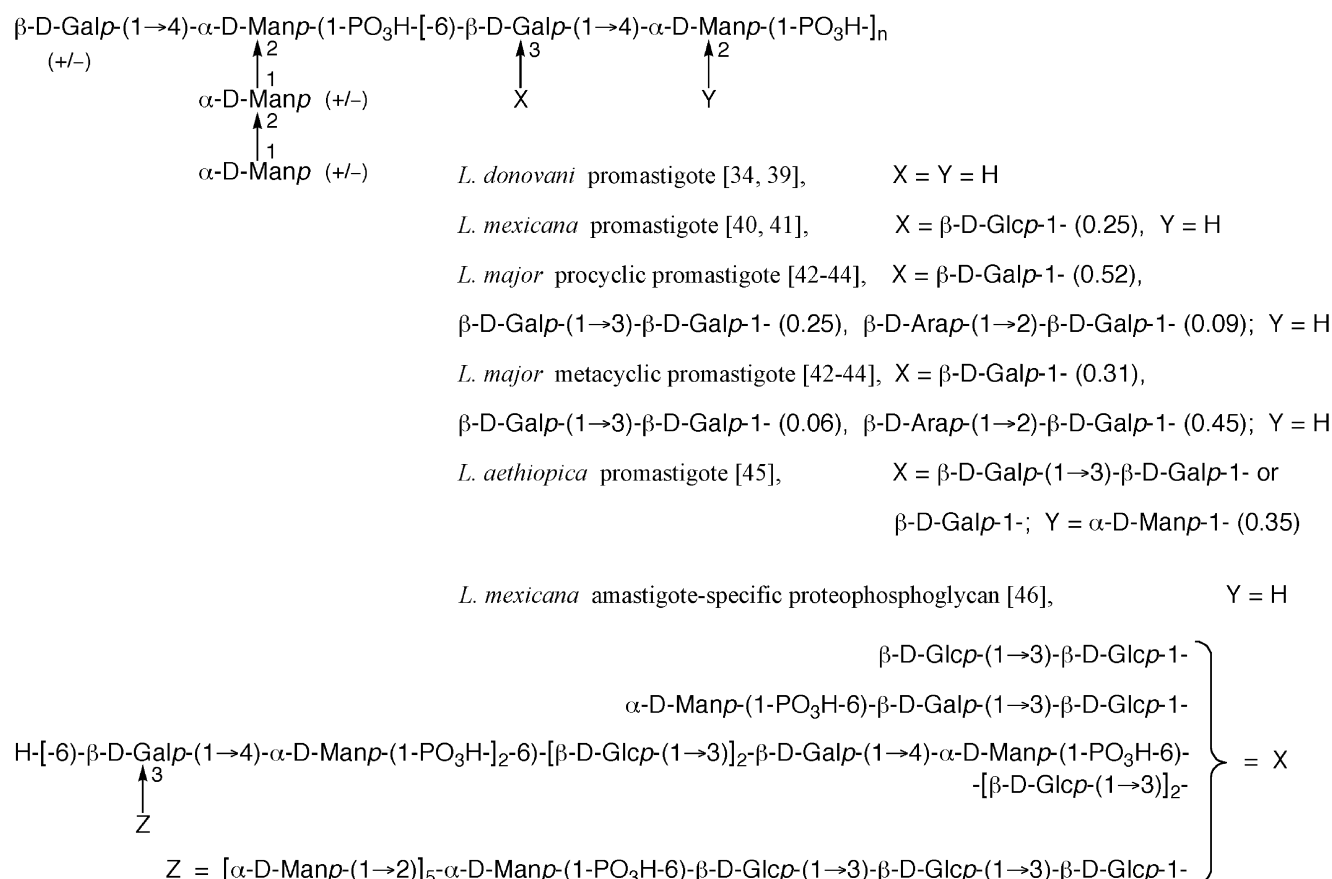


Fig. 1. Phosphoglycan region of lipophosphoglycans and proteophosphoglycans of *Leishmania*.

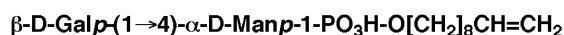
teophosphoglycan and secrete it (in mg/ml concentrations!) into the phagolysosome of the mammalian macrophage [46]. Some phosphoglycan chains are similar to those found in *L. mexicana* LPG, but the majority represent novel stage-specific highly branched structures (Fig. 1), including (1→3)-linked glucobiose and glucotriose moieties and long phosphorylated side chains. The phosphoglycans are attached to serine residues of the protein backbone, most likely through the phosphate groups. The amastigote-specific proteophosphoglycan is believed to activate the complement system via the mannose-binding pathway. It may also contribute to the binding of *Leishmania* to host cells and play a role in modulation of the biology of the infected macrophage [34].

CHEMICAL SYNTHESIS OF PHOSPHOGLYCANS OF *Leishmania* DESIGNED FOR FURTHER BIOCONJUGATION

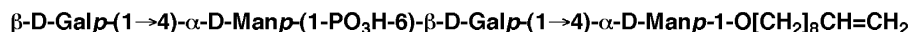
The most distinctive part of *Leishmania* LPG is their variable phosphoglycan domain, made of β -Gal-(1→4)- α -Man-1→OPO₃ phosphodisaccharide repeats linked to

each other through a phosphodiester group between the anomeric hydroxyl of D-mannose of one repeat and the 6-OH group of D-galactose of the adjoining repeat (see section "Cell-Surface and Secreted Phosphoglycans of *Leishmania*"). The preparation of neoglycoconjugates based on phosphosaccharide repeats upholds the need to develop routes for the chemical synthesis of *Leishmania* phosphoglycans. The development of synthetic approaches to phosphoglycans is challenging, because the phosphoglycans are hydrolytically labile due to anomeric phosphodiester linkage present between the repeating units. Synthesis of anomeric phosphodiesters is complicated, as both the correct stereochemistry at C1 and the lability of anomeric phosphodiester linkages must be taken into consideration [52, 53].

The first successful syntheses of *Leishmania* phosphoglycans were achieved by the Dundee University group [54-56] by exploiting the H-phosphonate chemistry. The glycosyl H-phosphonate route was proven to be the method of choice for the efficient and reliable assembly of various phosphodiester linkages characteristic of natural phosphoglycans [53]. The *L. donovani* phosphoglycans **1-5** (linear chains, built up from disaccharide



1



2



3



4



5

Fig. 2. *Leishmania donovani* phosphoglycan structures prepared by stepwise (1-4) and blockwise (5) chain elongation strategy.

phosphate repeating units in (1→6)-phosphodiester linkage and varying in length and by the presence of the Man₂-phosphate cap in **4** and **5**; Fig. 2) were constructed in a stepwise (**1-4**) [54, 55] and a blockwise (**5**) [56] chain elongation manner.

All the prepared phosphoglycans contain a dec-9-enyl aglycone moiety as they were purposely designed [55] for (a) studying *Leishmania* biosynthetic enzymes and (b) further preparation of neoglycoconjugates, which could be tested as potential anti-leishmaniasis vaccines. The hydrophobicity of the dec-9-enyl chain has been exploited for assaying, first, the mannosylphosphate transferases [57, 58] and then the galactosyltransferases [59, 60] involved in the LPG biosynthesis in *Leishmania*. As for the bioconjugation, the high lability of the anomeric phosphodiester bridges in phosphoglycans was given the top priority while selecting techniques for the neoglycoconjugate preparation. It was decided to avoid the use of any condensing reagents (which could easily interfere with the phosphate groups and facilitate the cleavage of the glycosyl phosphate bonds) and profit from the ozonolysis of the double bond in the dec-9-enyl aglycone linker instead, thus transforming it into an aldehyde functionality, which could be coupled to a protein or a lipid carrier by reductive amination (see section "The Preparation of Neoglycoconjugates Based on Phosphosaccharide Repeats Using Ozonolysis/Reductive Amination Technique").

The branched heptaglycosyl triphosphate phosphoglycan **6** (Fig. 3) from *L. mexicana* (containing β-D-glucose moiety in the side chain) was assembled via stepwise chain elongation [61]. A matching strategy was employed in the synthesis of the branched phosphoglycans **7-9** [62, 63] and **10-12** [64, 65] and Yashunsky, D. V., and Nikolaev, A. V., unpublished data) from *L. major* containing β-D-Gal and/or β-D-Gal-(1→2)-β-D-Ara side chains (Fig. 3).

Polymer-supported synthetic methodologies [66, 67] that employed glycosyl H-phosphonates for consecutive chain elongation have been also successfully developed for the preparation of *Leishmania* phosphoglycans as well as the H-phosphonate polycondensation strategy [67, 68].

THE PREPARATION OF NEOGLYCOCONJUGATES BASED ON PHOSPHOSACCHARIDE REPEATS USING OZONOLYSIS/REDUCTIVE AMINATION TECHNIQUE

As mentioned above, all the phosphoglycans designed and synthesized in Dundee were equipped with a dec-9-enyl aglycone linker to enable their future incorporation into neoglycoconjugates via oxidation of the terminal double bond. Ozonolysis of the phosphoglycans in methanolic solution afforded the corresponding 8-car-

bonyloctyl glycosides (or phosphosaccharide 8-carbonyloctyl diesters; Fig. 4), which were immediately used for coupling with a protein (or a lipid) carrier by reductive amination with NaCNBH₃ [69, 70] and Sizova, O. V., Nikolaev, A. V., and Ferguson, M. A. J., unpublished data). The conversion of the phosphoglycans to their 8-carbonyloctyl derivatives was shown to be complete and proceeds without destruction of the labile anomeric phosphodiester [69]. Ozonolysed compounds were mixed with NaCNBH₃ and a protein in a phosphate buffer solution to obtain a molar ratio of phosphosaccharide/protein NH₂ group/NaCNBH₃ of 1 : 3 (if another one is not specified) : 50. The prepared neoglycoconjugates were purified by gel filtration on a column of Superdex 200 HR (Amersham Biosciences, USA). Fractions containing the product were concentrated using ultrafiltration technique and Amicon centrifugal filters (Millipore, USA) (see the experimental procedures below). Following SDS-PAGE analysis showed that no starting protein was observed [69, 70].

To develop the optimal conjugation conditions, first [69], the *L. donovani* phosphoglycan **4** (Fig. 2) was ozonolysed and coupled to ribonuclease A (RNase A; M = 13,690 Da, 10 amino groups) using a molar ratio of phosphosaccharide/protein amino group of 1 : 5. That gave a glycoconjugate containing 1.1 phosphosaccharide (hapten) chains per RNase A molecule (i.e. one chain of a hapten per 12,440 Da of protein). The ozonolysed disaccharide phosphate **1** (Fig. 2) was coupled to BSA as a carrier (M = 66,382 Da, 59 amino groups) using a molar ratio of phosphosaccharide/protein amino group of 1 : 1, thus producing a glycoconjugate containing eight hapten chains per molecule of BSA (i.e. one chain of a hapten per 8,300 Da of protein). Although this was around three-fold lower than that reported for oligosaccharide-BSA conjugates prepared using similar reductive amination techniques [71, 72], in the latter cases a large molar excess of oligosaccharide over BSA amino groups (about 200 : 1) was used. Since (a) the synthetic phosphoglycans are rather precious and (b) a relatively low hapten/protein ratio (like one chain of a hapten per 10 kDa of protein) may be desirable from an antigen-presentation point of view [69], the developed conditions were used for the preparation ([70] and Sizova, O. V., Nikolaev, A. V., and Ferguson, M. A. J., unpublished data) of *Leishmania* phosphoglycan-tetanus toxin fragment C conjugates **13-19** (Fig. 4).

The engineered tetanus toxin fragment C (TetC) [73] does not retain any toxicity, but it retains immunogenicity [74]. It was already tested on the vaccine market [75] and has the advantage of inducing a Th-1 response that is necessary for the killing *Leishmania* parasites [76, 77]. The synthetic phosphoglycans were coupled to TetC using a molar ratio of phosphosaccharide/protein amino group of 1 : 3 [69, 70]. Thus, three *L. donovani*-TetC glycoconjugates **13-15** were prepared from three different linear phosphoglycans **2-4**, correspondingly. Ozonolysis of the

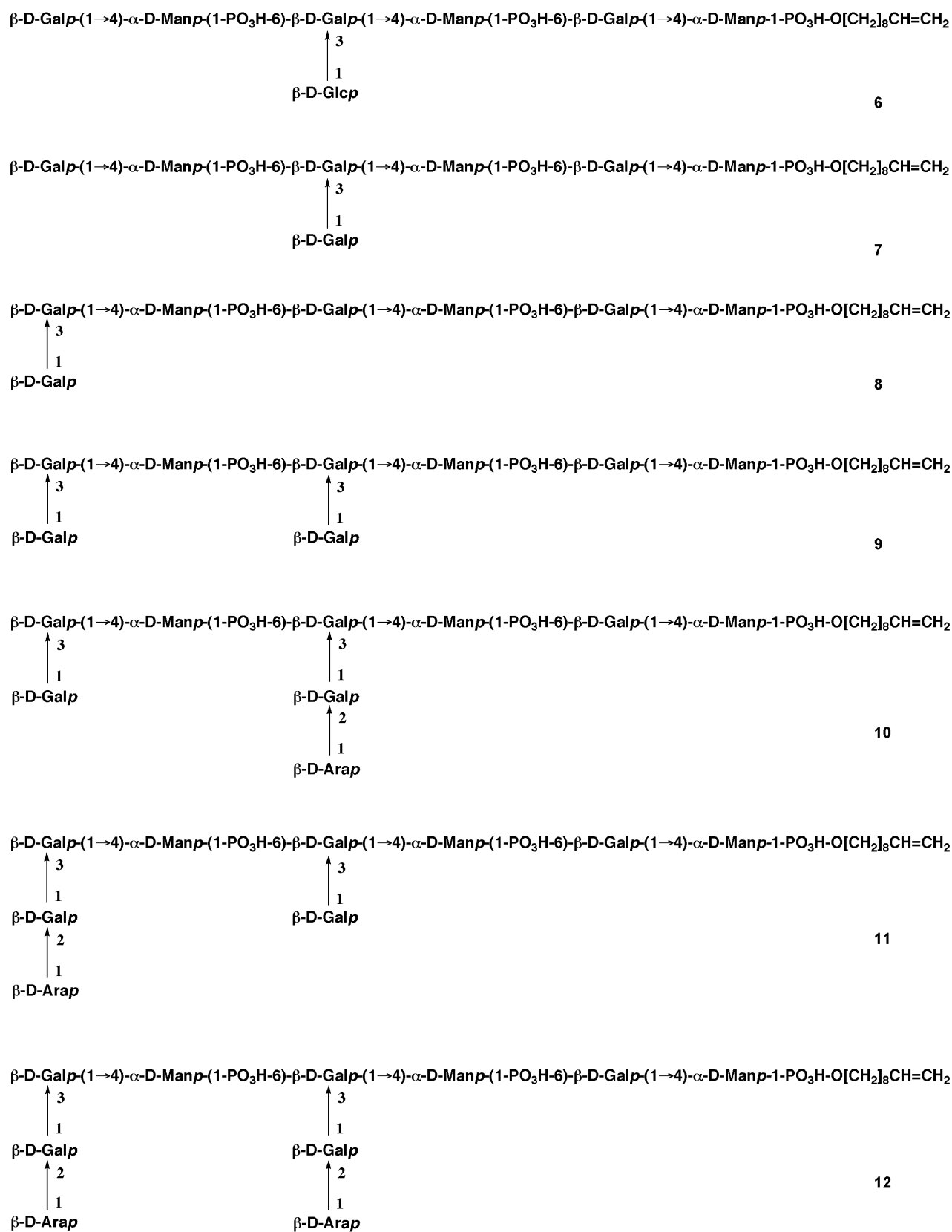
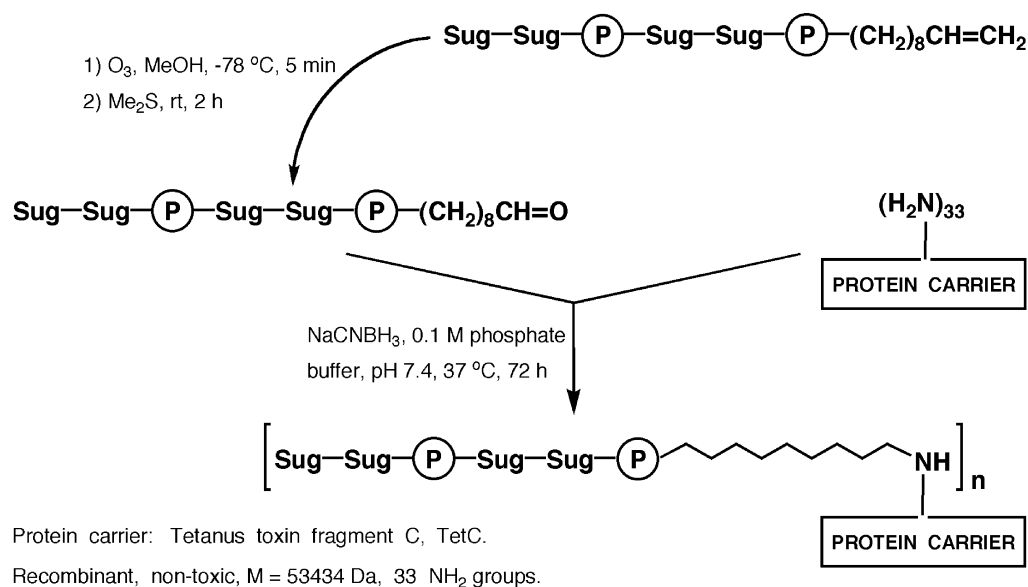


Fig. 3. *Leishmania mexicana* (6) and *L. major* (7-12) phosphoglycans prepared by the Dundee University group.



	Hapten/Protein mole/mole (n)	Number of NH ₂ groups per one hapten chain	Protein weight per one hapten chain
13 [Gal-Man-OPO ₂ H-Gal-Man-O(CH ₂) ₉] _n -TetC	3.82	8.6	14000 Da
14 [Gal-Man-OPO ₂ H-Gal-Man-OPO ₂ H-Gal-Man-O(CH ₂) ₉] _n -TetC	4.85	6.8	11000 Da
15 [Man-Man-OPO ₂ H-Gal-Man-OPO ₂ H-Gal-Man-OPO ₂ H-Gal-Man-O(CH ₂) ₉] _n -TetC	4.30	7.7	12420 Da
16 [Gal-Man-OPO ₂ H-(R ¹ →3)-Gal-Man-OPO ₂ H-Gal-Man-OPO ₂ H-O(CH ₂) ₉] _n -TetC R ¹ = Glcβ1-	4.15	8.0	12880 Da
17 [(R ² →3)-Gal-Man-OPO ₂ H-(R ² →3)-Gal-Man-OPO ₂ H-Gal-Man-OPO ₂ H-O(CH ₂) ₉] _n -TetC R ² = Galβ1-	3.37	9.8	15850 Da
18 [(R ³ →3)-Gal-Man-OPO ₂ H-(R ³ →3)-Gal-Man-OPO ₂ H-Gal-Man-OPO ₂ H-O(CH ₂) ₉] _n -TetC R ³ = Araβ1→2Galβ1-	3.80	8.7	14060 Da
19a [Gal-Man-CH ₂ PO ₂ H-Gal-Man-CH ₂ PO ₂ H-O(CH ₂) ₉] _n -TetC	2.00	16.5	26700 Da
19b [Gal-Man-CH ₂ PO ₂ H-Gal-Man-CH ₂ PO ₂ H-O(CH ₂) ₉] _n -TetC	9.70	3.4	5500 Da
20 Man-Man-PO ₃ NH ₄ -Gal-Man-PO ₃ NH ₄ -Gal-Man-PO ₃ NH ₄ -Gal-Man-O(CH ₂) ₉ -NH(CH ₂) ₂ -PO ₃ NH ₄ -O C ₁₆ H ₃₃ O C ₁₆ H ₃₃ O			

Fig. 4. Preparation and some characteristics of synthetic neoglycoconjugates based on *Leishmania* LPG phosphosaccharide repeats.

branched *L. mexicana* phosphoglycan **6** (Fig. 3), followed by protein coupling produced the *L. mexicana*–TetC glycoconjugate **16**, while the *L. major*–TetC glycoconjugates **17** and **18** were generated from the *L. major* phosphoglycans **9** and **12** (Fig. 3), respectively. The prepared conjugates were carrying from 3.37 to 4.85 phosphosaccharide haptens per molecule with, on average, one chain of a hapten per 13,300 Da of protein. The SDS-PAGE analysis of the prepared neoglycoproteins showed that no starting TetC protein was observed (Fig. 5).

The modified *L. donovani*–TetC glycoconjugates **19a** and **19b** (Fig. 4) were prepared from the synthetic C-glycosyl phosphono-analogue of the LPG fragment with a structure of β -Gal-(1 \rightarrow 4)- α -Man-(1 \rightarrow CH₂PO₂H-6)- β -Gal-(1 \rightarrow 4)- α -Man-1 \rightarrow CH₂PO₂H-O(CH₂)₈CH=CH₂ **21** [78]. Replacing the anomeric O1 in D-mannose residues with CH₂ groups in **21** does not, probably, change much conformation of the molecule, but makes the C-glycosyl phosphono analogues significantly more stable chemically than parent phosphoglycans and resists the action of phosphatases as well. All of those make the phosphono analogues of the parasitic and bacteria phosphoglycans strong candidates for synthetic glycoconjugate vaccines [79, 80]. Ozonolysis of compound **21**, followed by coupling to TetC with a molar ratio of phosphonosaccharide/protein amino group of 1 : 3 furnished the glycoconjugate **19a** containing just two hapten chains per molecule, while the higher loaded conjugate **19b** (with 9.7 hapten chains per molecule; Fig. 5) was prepared using a

higher molar ratio of the phosphonosaccharide over TetC amino groups of 2 : 3.

Finally, the neoglycolipid **20** (Fig. 4) was synthesized [69] by coupling the ozonolysed phosphoglycan **4** and 1,2-di-O-hexadecyl-*sn*-glycero-3-phosphatidylethanolamine in a mixture of chloroform–methanol–water using a molar ratio of phosphosaccharide/amine/NaCNBH₃ of 1 : 20 : 50. After purification on an Octyl-Sepharose column followed by Kieselgel 60, the glycoconjugate was isolated in 36% yield. For immunological studies, the neoglycolipids could be incorporated into liposomes that act as a carrier and as an adjuvant [81].

Experimental procedures for the preparation of neoglycoproteins 13–19 ([70] and Sizova, O. V., Nikolaev, A. V., and Ferguson, M. A. J., unpublished data). *Ozonolysis of phosphosaccharides*. Synthetic phosphosaccharides **2**, **4**, **6**, **9**, **12**, or **21** (as their ammonium or triethylammonium salts; 0.3–0.6 mg of a phosphosaccharide in one experiment) were dissolved in 1 ml of methanol or a mixture of methanol–water (5 : 1). The solution was cooled to –78°C and ozone (from ozone generator OZ06; Peak Scientific Ltd., Scotland) was bubbled (~5 min) into it via a sintered-glass bubbling tube until a pale blue colour persisted. Oxygen was then bubbled (~10 min) through the solution until it became colorless to eliminate excess O₃. Dimethylsulfide (0.01 ml) was added to reduce the ozonide, and the solution was allowed to warm to room temperature and left for 2 h. The products (corresponding 8-carbonyloctyl phosphosaccharides) were dried by evap-

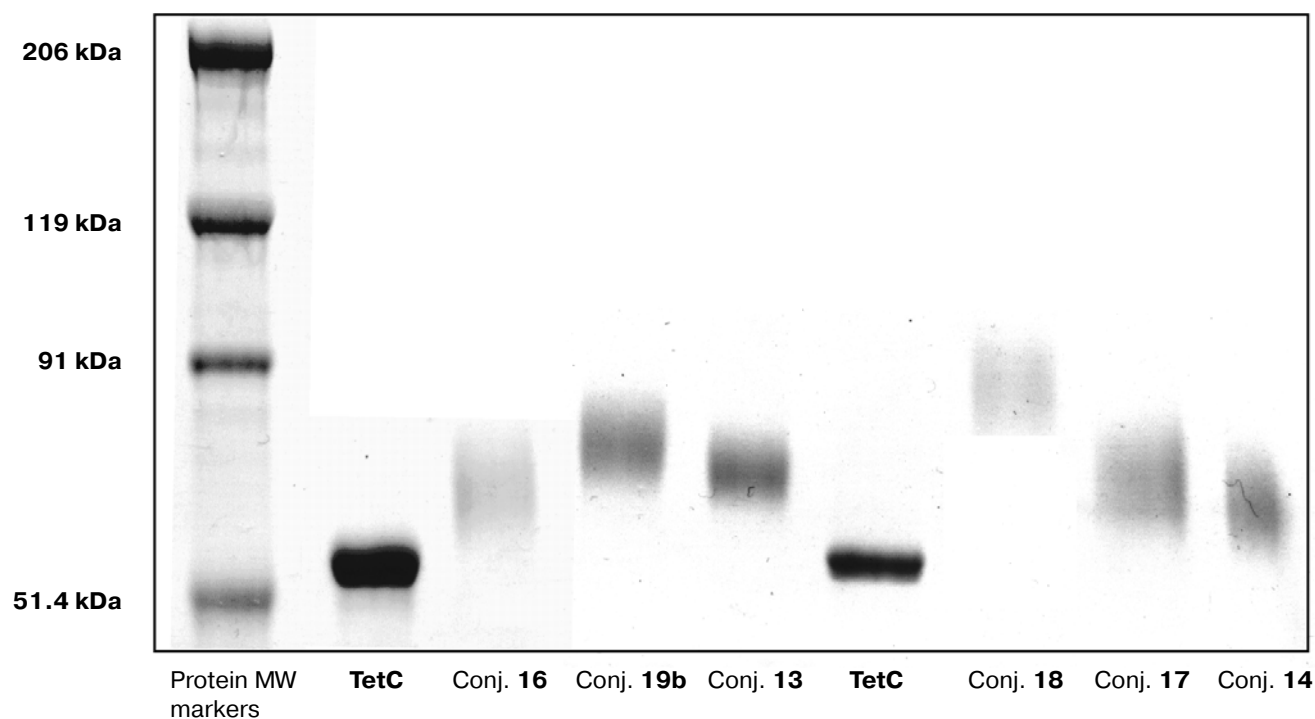


Fig. 5. SDS-PAGE analysis of TetC before and after its transformation into synthetic neoglycoproteins based on *Leishmania* LPG phosphosaccharide repeats.

oration of methanol therefrom (twice) and used immediately for coupling with the protein.

Coupling of phosphosaccharides to TetC and rapid purification of neoglycoconjugates. The prepared 8-carbonyloctyl phosphosaccharides and NaCNBH₃ were dissolved in 0.1 M phosphate buffer (0.54–0.70 ml, pH 7.4) and added to a TetC solution (0.06–0.15 ml) in the same buffer to obtain a molar ratio of phosphosaccharide/protein amino group/NaCNBH₃ of 1 : 3 : 50 (for the preparation of the glycoconjugate **19b** only, the ratio was taken as 2 : 3 : 50.) The mixture was incubated at 37°C for 72 h and then centrifuged at 13,000–14,000 rpm (30 min, 4°C). The prepared neoglycoproteins stayed in the supernatant solution and were purified by gel filtration on a column of Superdex 200 HR (void volume 8.5 ml) using 0.1 M phosphate buffer, pH 7.2, as eluant at a flow rate of 0.5 ml/min. Neoglycoproteins were detected with a UV detector at 280 nm; eluate of 1 min was collected into each tube. For each coupling experiment, the elution profile appeared to be as a single or one major peak, eluting in the area between 25 and 33 min. The appropriate fractions were combined (the total volume of the peak was about 2–3 ml). The glycoconjugate solutions were concentrated by ultrafiltration (5,000g, 30 min, 4°C) using an Amicon Ultra-0.5 Centrifugal Filter (Millipore) with an Ultracel-30 Membrane (30 kDa nominal molecular weight limit) and an Avanti centrifuge (Beckman, USA), thus providing samples of neoglycoproteins **13–19** in phosphate buffered saline (volume of each sample 0.30–0.89 ml; protein concentration 1.13–4.28 mg/ml). The following SDS-PAGE analysis of the samples showed that no starting TetC protein was observed (Fig. 5).

Oligosaccharide concentrations were determined by phenol-sulfuric acid assay [82] performed in triplicate. Protein concentrations were determined by the bicinchoninic acid assay (Pierce, USA) according to the manufacturer's instructions. SDS-PAGE was performed for native and coupled (to phosphosaccharides) TetC using 8% gel and the discontinuous buffer system of Laemmli [83]. Gels were stained with Coomassie blue to detect protein bands.

IMMUNOLOGICAL STUDIES OF *Leishmania* SYNTHETIC NEOGLYCOCONJUGATES

The glycoconjugates **14** (*L. donovani*–TetC), **16** (*L. mexicana*–TetC), **17** (*L. major*–TetC with D-Gal side chains), and **18** (*L. major*–TetC with D-Ara→D-Gal side chains) were used by Bates and Rogers (University of Liverpool) as glycovaccines (without any adjuvants) to immunize BALB/c mice, which were then challenged by the bite of *L. mexicana* infected *Lutzomyia longipalpis* sand flies [84]. Intriguingly, only the *L. mexicana*–TetC glycovaccine **16** showed significant protection compared with the control TetC immunization, resulting in a 50%

reduction in lesion size and a 94% (!) reduction in parasite burden. The other glycovaccines **14**, **17**, and **18**, neither the *L. mexicana* phosphoglycan **6** (Fig. 3) alone, were unable to provide protection against infected fly bite challenge. Thus, that was the first (to the best of the authors [84] knowledge) demonstration that a synthetic glycoconjugate vaccine can have a direct antiparasite effect in leishmaniasis or any other parasitic disease. It seems that these glycovaccines may induce species- and glycan structure-specific protection, although the ability of the *L. mexicana*–TetC glycovaccine **16** to protect against other species of *Leishmania* has not been tested yet.

THE PREPARATION OF NEOGLYCOCONJUGATES BASED ON THE TETRASACCHARIDE CAP STRUCTURE

The Cambridge (Massachusetts, USA) group [16, 85] has designed and synthesized two potential *Leishmania* vaccine constructs based on a structure of the tetrasaccharide phosphate cap (GalMan₃-phosphate) of LPG, while the phosphate group being omitted thus simplifying strongly the conjugation technique (see section “Chemical Synthesis of Phosphoglycans of *Leishmania* Designed for Further Bioconjugation”). Chemically prepared tetrasaccharide **22** (Fig. 6) was equipped with a 6-aminohexyl aglycone linker to facilitate further bioconjugation. The GalMan₃ tetrasaccharide itself has previously been a subject of two synthetic approaches [86, 87].

Two neoglycoconjugates **25** and **28** (Fig. 6) were prepared by joining the tetrasaccharide antigen **22** with protein carrier KLH or with lipid immunostimulator tripalmitoyl-S-glycerylcysteine **27**, respectively. The KLH proteins are known to be highly heterogeneous and have enormous molecular masses (4,500–13,000 kDa) as well as ill-defined structures, but their immunogenic capabilities can be superior [88]. Prior to the conjugation, the protein itself was reacted with N-succinimidyl bromoacetate to form the N-(bromoacetyl)-modified carrier **26**, while the 6-aminohexyl tetrasaccharide **22** was coupled with pentafluorophenyl (acetylsulfanyl)acetate **23** to give the tetrasaccharide **24** with masked SH functionality in the linker. Further coupling of **24** with the modified protein **26** in the presence of hydroxylamine was followed by capping of any remaining bromoacetyl groups with 2-aminoethanethiol to furnish the neoglycoprotein **25**, which was purified by gel filtration on a Pd-10 column (Sephadex G-25). The reported conjugation ratio of the GalMan₃ hapten chains to each KLH molecule was 55 : 1 (which is about one chain of the tetrasaccharide hapten per 82,000–236,000 Da of protein).

The lipopeptide N-palmitoyl-S-[(2*R*,2*S*)-2,3-di(palmitoyloxy)propyl]-L-cysteine **27** (tripalmitoyl-S-glycerylcysteine, Pam₃Cys) has been already reported as a part of a fully synthetic vaccine [89]. It can work both as

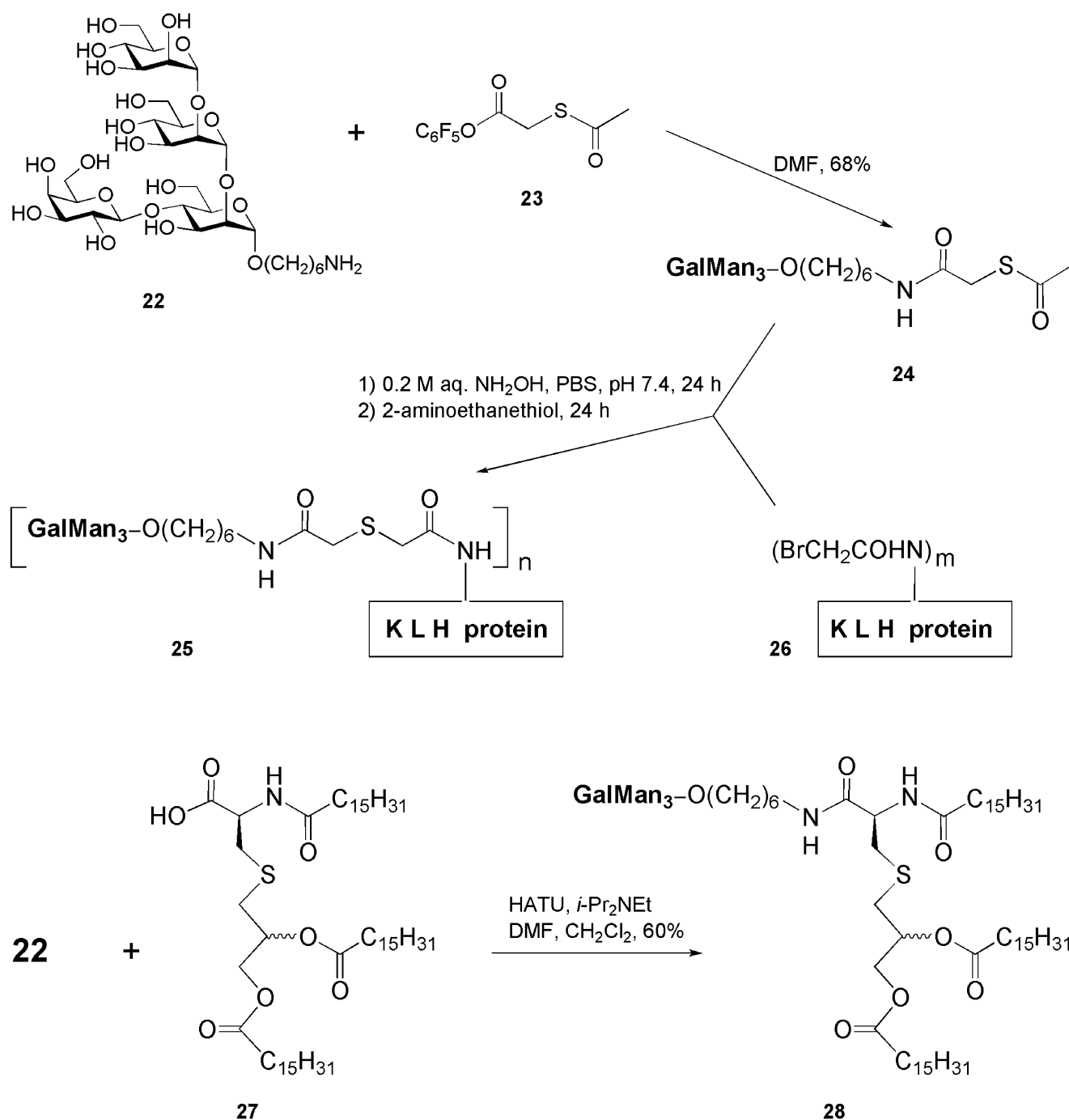


Fig. 6. Synthetic neoglycoconjugates based on the tetrasaccharide cap structure from *Leishmania* LPG.

a carrier and an adjuvant [90] and is readily available by synthesis [91]. Coupling of lipopeptide **27** with the 6-aminohexyl tetrasaccharide **22** was performed in the presence of HATU and diisopropylethylamine and afforded the neoglycolipid **28** (60%) after purification on a silica column. Both synthetic glycovaccines have been tested for their immunological evaluation in BALB/c mice, but the results have not been reported yet.

FUTURE ADVANCES

Despite appreciable progress in the preparation of neoglycoconjugates based on synthetic cell-surface structures of *Leishmania*, there are still several unresolved issues that prevent the neoglycoconjugates from being routinely synthesized to furnish further immunological testing. Part of them is dealing with rapid and effective

preparation of synthetic LPG fragments and, therefore, novel methods towards anomeric phosphodiester are in demand. This may also include a search for novel activating (for the H-phosphonate condensation) and oxidizing reagents. The oxidation step appeared to be a problem for the preparation of some sterically hindered glycosyl phosphosaccharides [52, 53] as the intermediate H-phosphonic diester, instead of its smooth transformation to the phosphodiester, may undergo hydrolysis of the glycosidic linkage as a concurrent reaction.

We have already cited in the introduction to this review that any unique feature *Leishmania* expose on its cell surface could be investigated and exploited for the development of an anti-leishmaniasis vaccine [16]. As all LPG molecules contain at the reducing end of the chain a glycan core of the α -Gal-(1 \rightarrow 6)- α -Gal-(1 \rightarrow 3)- β -Gal-(1 \rightarrow 3)-[α -Glc-1 \rightarrow PO₃H-6]- α -Man-(1 \rightarrow 3)- α -Man-(1 \rightarrow 4)- α -GlcNH₂ structure, which, in turn, is (1 \rightarrow 6)-linked to a 1-O-alkyl(C₂₄₋₂₆ chain)-2-*lyso*-phosphatidyl-*myo*-inositol lipid anchor [33, 38], it seems valuable to prepare neoglycoconjugates based on the above structure. The preparation of the glycan core-*myo*-inositol conjugate has been recently reported [92, 93].

LPG molecules are expressed on the promastigote (i.e. insect's midgut stage) membrane of all species of *Leishmania* [33, 40, 94], while their expression in amastigote parasites (existing in human phagolysosomes) significantly decreases, with an exception of *L. major* amastigotes [95]. At this stage, small molecules called glycoinositolphospholipids, which structures are, in some extent, similar to the above heptasaccharide glycan core (or part of it) linked to an inositolphospholipid anchor [33, 38, 44], remain the dominant surface structures. Thus, it looks worthy to prepare neoglycoconjugates based on the glycoinositolphospholipid structures and test them as potential anti-leishmaniasis vaccines.

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