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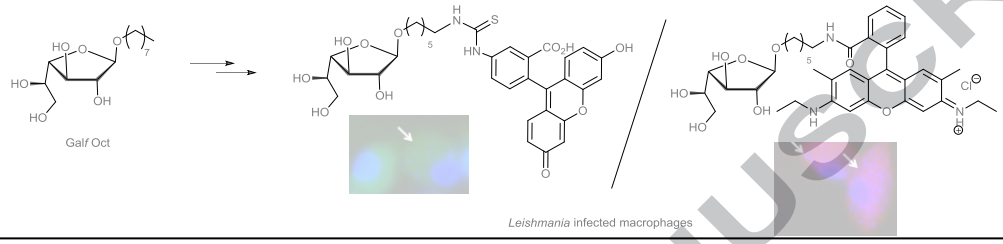

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Synthesis and biological properties of galactofuranosyl-containing fluorescent dyes

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ABSTRACT

Two fluorescent galactofuranosides were synthesized and their biological activities evaluated on non-infected and *Leishmania* infected macrophages. Both tagged scaffolds were able to penetrate macrophages. Compared to the activity of the parent octyl galactofuranoside used as a reference, the fluorescein-conjugate showed altered biological properties while the rhodamine 6G one synergistically acted with the lipid chain to significantly increase antiparasitic activity.

The presence of hexoses with striking five-membered ring was highlighted only in non-mammal living kingdom. As a consequence, these natural compounds, and the related biological pathways, are targets of choice for the development of some molecular tools useful to increase basic knowledge and/or to fight some related diseases. These diseases include especially tuberculosis and leishmanial infections. The first one is caused by *Mycobacterium tuberculosis* which infects millions of people each year throughout the world.[1] On the other hand, the leishmaniasis disease is mainly endemic in Eastern Africa, Brazil, India, Middle East and Mediterranean countries. Signs of infection by *Leishmania* species can vary and range from skin disorders to deadly visceral forms.[2] In both cases, WHO calls on governments, scientists, civil society and private companies to unite to stop the constant growth of these diseases.

Several teams have shown that furanosyl-containing conjugates may impact both *M. tuberculosis* and *L. donovani* lifecycle.[3] For instance, it was established that non-natural nucleotide-furanoses can be recognized and transferred by galactofuranosyl transferases on growing galactan.[4,5] Such nucleotides act at an early stage of this biological process and they significantly disturb the biosynthesis of this key galactan. Several UDP-analogues were also produced in order to inhibit this transferase and also the key UDP-galactopyranose mutase.[6-12] Considering recent knowledges related to the glycoconjugates part of *Mycobacteria* and *Leishmania* cell walls, some studies have focused on the biological impact of furanosyl-containing glycolipids.[2,13-19] Until now, the best results were obtained using a bicatenar thiofuranoside as a potent biocide agent against *M. tuberculosis*.[15] Nevertheless, the corresponding mode of action was not elucidated.

Octyl galactofuranoside (Gal*f*Oct) is a very simple glycolipid, whose structure however differs from the one of standard alkyl glycosides by the presence of the carbohydrate polar head in a furanose form. Leveraging its amphiphilic properties[20] has recently led us to investigate the impact of this compound on microorganisms that are able to biosynthesize and to metabolize furanosyl-containing conjugates.[21] A more detailed study was performed on *Leishmania donovani* using a small library of Gal*f*Oct derivatives.[18] The furanose form clearly contributes to a significant decrease of membrane fluidity leading to profound alterations in the leishmanial promastigote membrane and organelles. It was also shown that Gal*f*Oct was able to decrease intramacrophagic amastigote multiplication. This result led us to propose herein furanosyl-containing fluorescent dyes to characterize the intracellular distribution of Gal*f*Oct and to help

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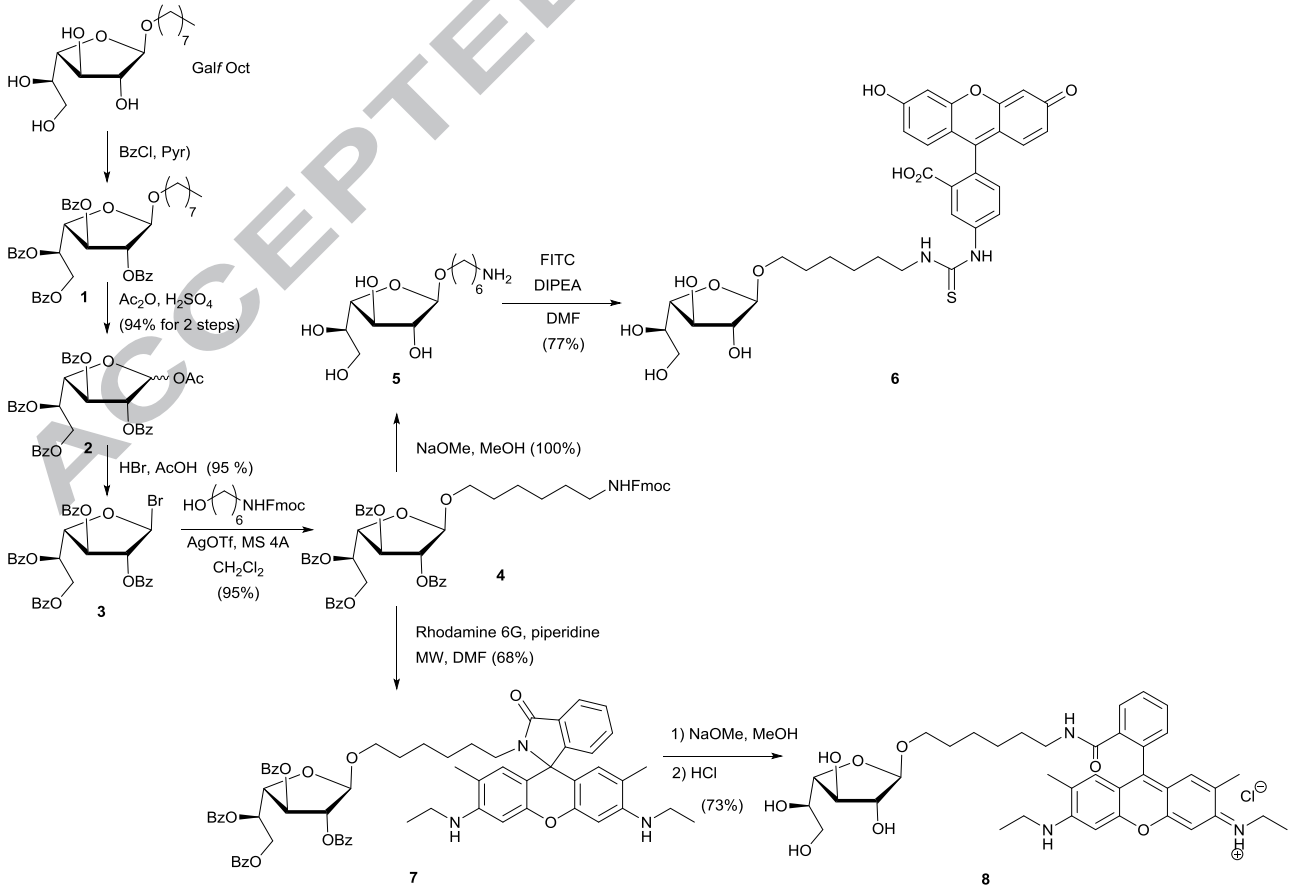
understanding of the mechanism of interaction of furanosides with the intracellular parasites.

Considering the respective size of the furanolipid and the most common fluorescent dyes, significant alterations of physicochemical and biological properties cannot be ruled out. Nevertheless, we were encouraged by recent studies in which a lipid chain was partly substitute by a fluorescent tag.[22-24] Therefore, we focused our attention on the conjugation of our furanoside with xanthene derivatives like (i) fluorescein, known for its low cellular permeability in assays involving living cells, or (ii) the red-shifted fluorescent rhodamine 6G because its positive charge strengthens interactions with negatively charged membranes.[25] Interestingly, these two related structures show a maximum of fluorescence in different pH range and would thus allow to monitor the trafficking of Gal*f*Oct in presence of infected macrophages, whatever the nature of the microenvironment. Moreover, the latter probe overcomes *in vivo* auto-fluorescent issues and ensures good diffusion through organic tissues.

The synthesis of the targeted fluorophores was based on a common intermediate **4** bearing a masked terminal amino function on the lipid part. Its synthesis started from Gal*f*Oct that was obtained in one step according to a modified Fischer glycosylation of octanol.[20] Subsequent benzoylation, acetolysis and bromination under standard conditions afforded the furanosyl donor **3** in a good 85% overall yield. The desired building block **4** was then obtained with 95 % yield by glycosylation of the commercially available *N*-Fmoc-protected 6-amino-1-hexanol using silver(I) triflate as the promoter. Further full removal of basic-sensitive esters and carbamate groups was efficiently performed under Zemplen conditions. Finally selective thiocarbamoylation in a dimethylformamide (DMF) solution in the presence of diisopropylethylamine (DIPEA) using fluorescein isothiocyanate (FITC) afforded the desired fluorescein conjugate **6** in 77% yield. Its structure was corroborated by 1D and 2D NMR spectroscopy: signal at δ 4.86 ppm correlated with a $^{3}J_{H1,H2}$ 1.9 Hz coupling constant, and signal at δ 109.4 ppm for C-1 highlighted the five-membered ring and the desired 1,2-*trans* β-configuration. Maximum excitation and emission wavelengths were measured in phosphate buffered saline (PBS) solution (pH 7) at 468 nm and 520 nm, respectively.

The rhodamine conjugate was prepared slightly differently, following a procedure described by Adamczyk and Grote.[26] Transacylation of the starting ethyl ester rhodamine 6G in DMF was thus performed in the presence of piperidine. This base allowed selective deprotection of the terminal amino function that could subsequently add to the carbonyl atom ethyl ester of rhodamine 6G to give, after elimination of ethanol, the desired benzoylated derivative **7**. The reaction was performed under microwave activation (100 W) at 90 °C and allowed to access the target conjugate **7** in only 30 minutes in 68% yield. The presence of a ^{13}C NMR signal at δ 65 ppm characteristic of the sp^3-hybridized carbon attach to a nitrogen indicated the major formation of the closed spirolactam form. Final debenzoylation was carried out as described above. The resulting water soluble rhodamine 2'-amide **8** was obtained as the open cationic specie following acidification with hydrochloric acid. This was corroborated by the disappearance of the ^{13}C NMR signal at δ 65 ppm. The measurement of fluorescent properties afforded λ_{ex}=528 nm and λ_{em}=552 nm in PBS.

The synthetic furanosyl-containing fluorescent probes were then investigated using macrophages infected by leishmanial parasites. Two effects were monitored: the trafficking of Gal*f*Oct labelled with fluorescein or rhodamine, and the possible alteration of the biological properties following the labelling. On one hand, *L. donovani* promastigotes grown at stationary phase



Scheme 1. Synthesis of the galactofuranosyl-containing fluorophores **6** and **8**.

(>5 days of culture) were used to infect human macrophages overnight (doi 10:1). After 3 washes, infected macrophages were treated for 6 or 18 hours with 80 µM of conjugate **6** or **8,** or with 80 µM of fluorescein, rhodamine 6G or of Gal*f*Oct used as controls (Figures 1A-1D). After three washes and fixation with 4% formalin, nuclei were labeled with Hoechst 33342 at 0.001 µM for 30 minutes. Then pictures of each condition were taken using epifluorescence microscopy (Nie, Nikon®) with a 50x oil immersion lens. All conditions were done in triplicate and repeated three times. Conjugate **8** was also tested once (in triplicate) using confocal microscopy on uninfected cells (Figure 1E).

On another hand, amastigotes growth inhibition rate was calculated on the infected cells incubated for 48 hours with 80 µM of either compounds **6** or **8**, fluorescein, rhodamine 6G or Gal*f*Oct as treated control or medium alone as untreated control. Then slides were washed three times and colored with May Grünwald Giemsa stain. The enumeration of infected cells and intraparasitic multiplication was determined *via* microscopic examination using a 100x oil immersion lens. A minimum of 200 macrophages were counted on each well for every condition. Amastigotes multiplication rate was computed taken into account infected cells harboring two or more amastigotes in their cytoplasm. All conditions were done in quadruplicate and repeated twice. Statistical analysis was performed using Mann-Whitney test and GraphPad Prism 5® software. Results were considered significant for $p < 0.05$.

On one hand, no trace of fluorescein was found either in the membrane or inside the macrophage (Figure 1D). On the other hand, compounds **6**, **8** and rhodamine 6G were shown to enter in all macrophages after 6 hour incubation (Figures 1A to 1C) and to stay in cytoplasm for at least 18 hours (data not shown). Intracytoplasmic distribution was uniform throughout the cytoplasm and no co-labelling with Hoechst was observed in nuclei (Figure 1). No accumulation dyes was observed around amastigote nuclei as well. Regarding antiparasitic effect, compound **6** and fluorescein alone presented no significant effect, whereas compound **8** significantly reduced macrophage infection ($p < 0.05$) and amastigote multiplication ($p < 0.05$) after 48 hours treatment, even without accumulation around amastigote nuclei (Figure 2). Surprisingly, there was a significantly higher efficacy of the compound **8** compared to Gal*f*Oct ($p < 0.05$) (Figure 2). In addition, we observed phenotypic surface alterations on MGG-stained slides after treatment with rhodamine 6G alone (data not shown).

Thus the effect of Gal*f*Oct-rhodamine 6G **8** could be favored by a cytotoxic effect on macrophages of rhodamine 6G itself even if there was no significant effect of rhodamine 6G on macrophagic infection (Figure 2). In addition modification of the global charge or the hydrophilic properties induced by the presence of covalently linked rhodamine 6G could explain the better antiparasitc effect of compound **8** compared to Gal*f*Oct. These properties were described elsewhere as responsible for the cytotoxicity of rhodamine 6G compared to other rhodamine on other cell lines.[27,28] It could have also participated to the stiffening of the membrane induced first by the alkyl furanoside.[18] Taken together, these observations suggest a synergistic effect of rhodamine 6G and Gal*f*Oct. Remarkably, and despite the same site of anchorage, the fluorescein ring inhibited the antiparasitic effect of Gal*f*Oct. The lack of effect of compound **6** was not due to an inhibition of intracellular diffusion of compound **6**, as fluorescence was observed inside cells (Figure 1), but most likely to a modification of Gal*f*Oct properties due to its linkage to fluorescein. As fluorescein itself has low cellular permeability, its detection inside cells suggests that the labeled compound gained entry through the Gal*f*Oct moiety.

In conclusion, this paper reports for the first time the synthesis of two fluorescent labeled galactofuranosyl-containing conjugates, one green fluorescein-based probe and one red rhodamine 6G derivative. In addition to the determination of their physicochemical properties, we also evaluated their impact in an *in vitro* model. This study highlighted the difficulty to foresee the impact of a grafted tag onto a very small molecule on the resulting biological properties. Herein, the Gal*f* derivative bearing a fluorescein dye showed low toxicity on macrophages while the presence of rhodamine 6G at the end of the lipid chain of Gal*f*Oct significantly improved antileishmanial activity.

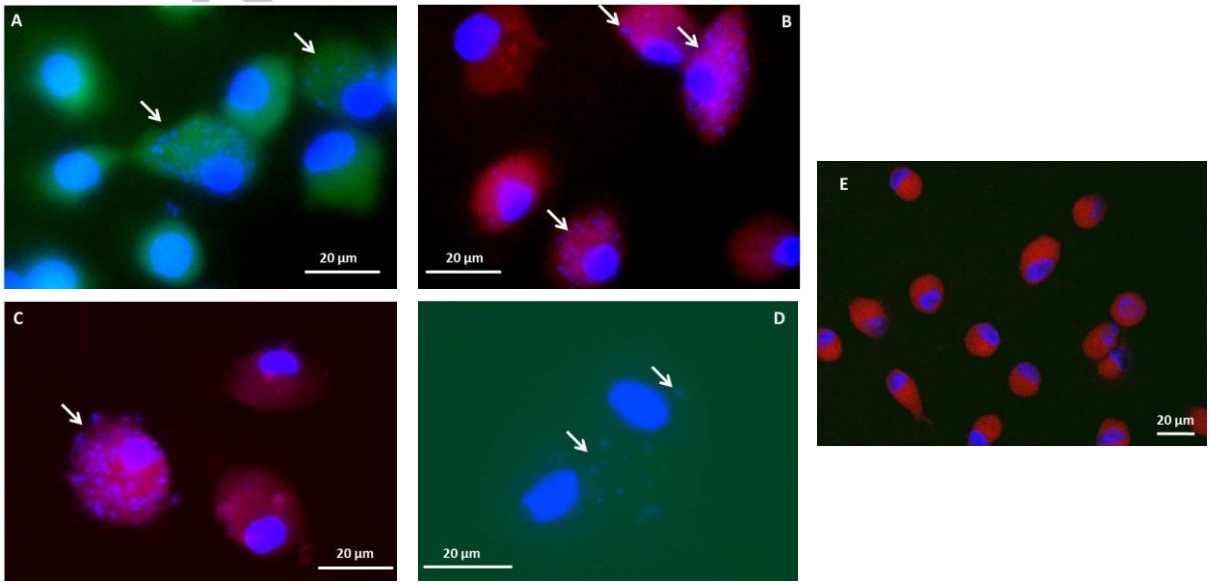


Figure 1. Cell imaging after labeling with fluorescent compounds. Epifluorescence pictures (**A**, **B**, **C** and **D**) of *Leishmania* infected macrophages (arrow) treated for 6 hours with compound **6** (**A**), compound **8** (**B**), rhodamine 6G (**C**) or fluoresceine (**D**) (50 x oil immersion lens). Confocal picture (**E**) of uninfected macrophages treated with compound **8** for 6 hours (20 x lens)

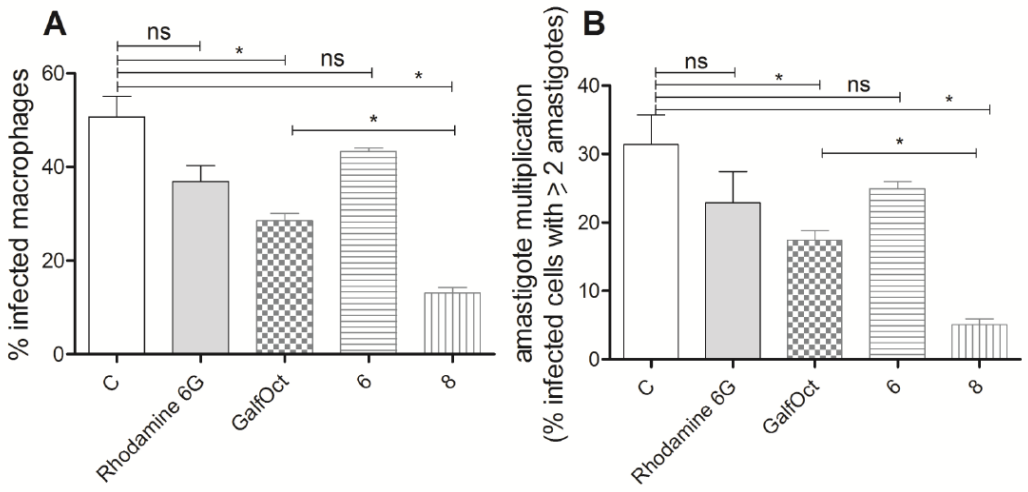


Figure 2. Effect of labeled or native Gal*f*Oct on cell invasion and *Leishmania* parasite growth. Rate of infected macrophages (**A**) or intracytoplasmic amastigote multiplication (**B**) after 48 h treatment with compound **6** or **8**, compared to untreated macrophages (C) or macrophages treated with the reference furanose (Gal*f*Oct) or with rhodamine 6G. All compounds were used at a concentration of 80 µM.

Further investigations on the action mechanism and in particular the impact on the membrane of the parasite are under way. Therefore, one should be careful when drawing conclusions on cellular interactions using labeled compounds, or before their use in *in vivo* models.

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Supplementary Material

Experimental details, NMR data and description of biological tests are available in Supplementary material.

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