Cover Page

Universiteit Leiden

Chapter 7

Summary and future prospects

Glycoconjugates are involved in numerous fundamental biological processes and are the subject of many studies. Glycoconjugates can bind on the basis of their specific carbohydrate structures to lectin receptors. Lectin receptors can be either membrane bound or soluble and play a key role in a wide variety of cellular recognition and communication processes. Many of the lectins, in particular those found on cells of the immune system are members of the C-type lectin family and these include the mannose receptor (MR), Dectin-1 and 2 as well as DC-SIGN. These carbohydrate-binding receptors have been exploited in immunological studies and targeting strategies. Significant attention has been directed to the design and synthesis of artificial glycoconjugates for binding to specific lectins. The research in this thesis is mainly focussed on glycoconjugates that carry oligomannosides and artificial mannose clusters that can be recognized by the mannose receptor (MR) or other mannose binding lectins.

In **Chapter 1** a selection of different mannose binding lectin-targeting strategies is described. Although carbohydrate protein interactions vary depending on the lectin of interest, the majority is considered to be multivalent. The synthesis of selected examples of linear and dendritic

multivalent mannosyl conjugates is discussed. In addition examples of the synthesis of high mannose *N*-glycan conjugates are described.

Chapter 2 describes the design, synthesis and evaluation of three conjugates comprising the irreversible cathepsin inhibitor DCG-04, a BODIPY dye and a mono-, tri- or heptamannoside, featuring natural *O*-glycosidic linkages (**1**, **2**, **3**, Figure 1). In a modular synthesis approach the oligomannosides were introduced via a Cu(I)-catalyzed "click" reaction in these conjugates. It was shown that the heptamannoside conjugate showed the least effective cathepsin labelling in cell lysates, whereas the uptake of the tri- and heptamannoside conjugates outcompeted the monomannoside conjugate in living cells. Also in live cells the trisaccharide proved to be a more effective inhibitor than its heptasaccharide counterpart. This outcome could be related to the resistance of the conjugates to the endo/lysosomal removal of the mannose residues by mannosidases and further research is needed to clarify this finding. For example the naturally occurring nonamannoside conjugate **4** can be probed. On the basis of the existence mannose-6-phosphate receptors and the targeting study of Hoogendoorn *et al.*[1] it is proposed to synthesize and evaluate structurally related conjugates of which the 6-hydroxyl groups in the oligomannosides is provided with a phosphate monoesters (**5**, Figure 1) The potential hydrolysis of the phosphate functions by phosphatases can be prevented by replacement of the 6-phosphate groups by 6-*C*-phosphonates (**6**, Figure 1).
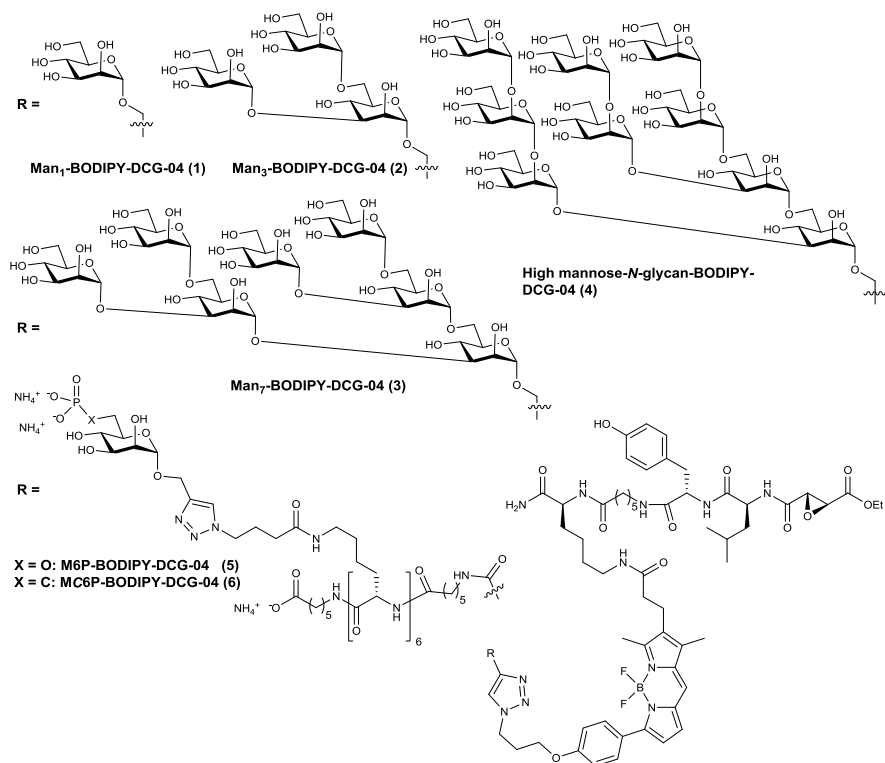
Figure 1: The structures of the conjugates **1**, **2** and **3** presented in Chapter 2 and the structures of the proposed high mannose-*N*-glycan- (**4**), mannose 6-*O*-phosphate- (**5**) and mannose-6-*C*-phosphonate-DCG-04 (**6**) conjugates.

In **Chapter 3** the synthesis and evaluation of Man_1-BODIPY-cyclophellitol **7** and Man_3-BODIPY-cyclophellitol **8** as "caged" activity based probes for glucocerebrosidase, the key enzyme in Gaucher disease, is described (Figure 2). The mannosides in these probes should function as homing device to target the fluorescent cyclophellitol inhibitor via the mannose receptor to Gaucher cells. The self-immolative linker system was installed between the mannoside and the cyclophellitol to allow liberation of the probe in the lysosome through the action of lysosomal mannosidases.
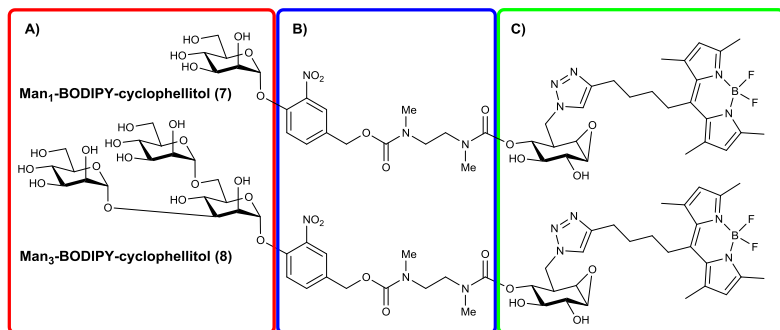
Figure 2: The structures of the conjugates presented in Chapter 3.

In a convergent approach the mono- and trimannosides were prepared and the self-immolative linker was introduced at the reducing end of the mannosides by nucleophilic aromatic substitution. An alternative and high yielding route of synthesis to a partially protected azido-cyclophellitol was developed in which the stereoselective introduction of the epoxide was followed by the introduction of the azide. Having the mannosylated linker systems and the partially protected azido-cyclophellitol and BODIPY-alkyne in hand the assembly of the probes went uneventful. Biological evaluation of the probes showed that they were significantly less effective inhibititors of the target enzyme than their BODIPY-cyclophellitol counterparts without the targeting device. This indicates ineffective cleavage of the mannosyl residues from the probes by intracellular mannosidases.

Guided by the successful uptake of the probes with the aid of the (oligo)mannosides as targeting device it is proposed to apply a disulfide based sulfhydryl-assisted self- immodulative linker.[2] As depicted in Figure 3, after internalization of the probe by Gaucher cells, cyclophellitol can be liberated via reduction of the disulfide bond by cellular glutathione,[3] thioredoxin,[4,5] or γ-interferon-inducible lysosomal thiol reductase and ensuing intramolecular ring formation.[6]
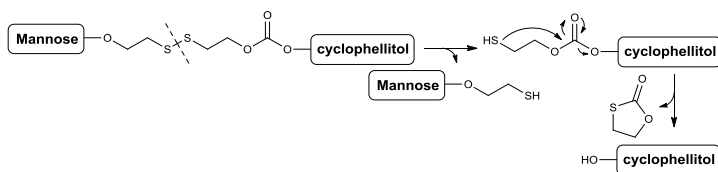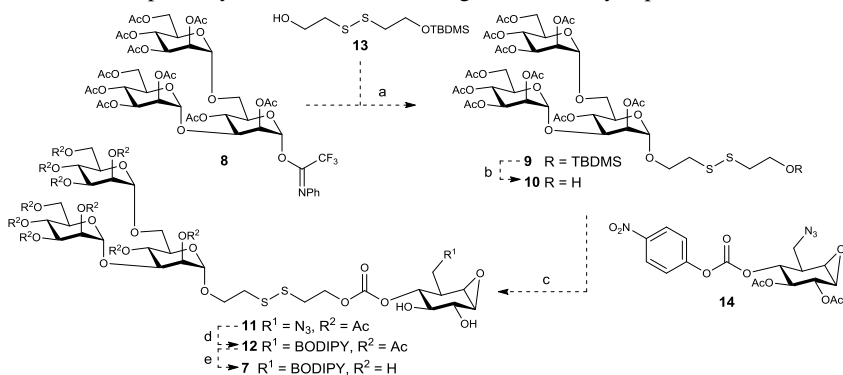
Figure 3: Proposed mechanism of a disulfide based sulfhydryl-assisted self immodulative linker.

The first biodegradable disulfide based conjugate, which is proposed, contains the same oligomannose homing device as described in Chapter 3 (Scheme 1).

**Scheme 1:** Proposed synthesis of disulfide bridged mannose-cyclophellitol construct **7**.

Reagents and conditions: (a) Disulfide **13**, TfOH, DCM; (b) TBAF, DCM/MeOH; (c) **14**, TEA, DMF, rt.; d) NaOMe/MeOH; e) Alkyne-BODIPY, CuSO$_4$, sodium ascorbate DMF/H$_2$O.

Based on the work of Bhuniya *et al.*[2] the partially protected TBDMS-2-hydroxyethyl disulphide linker **13**[7] is condensed with trimannoside (*N*-phenyl)trifluoroimidate **8** to give disulfide trimer **9**. Removal of the TBDMS group in **9** allows coupling with 4-nitrophenyloxycarbonate azidocyclophellitol derivative **14** to give compound **11**. Further processing by global deacetylation[8] and conjugation of alkyne-BODIPY with Cu(I)-cat. "click" chemistry leads to the proposed conjugate **7**.

In the same vein other mannose based targeting devices can be used. For instance, it is proposed to combine the self-immolative disulfide linker

reported by Chen *et al.*[9] with the synthetic mannose cluster, capable for lysosomal targeting of a cathepsin inhibitor, as developed by Hillaert *et al.*[10] A potential route of synthesis to this conjugate **15** is presented in Scheme 2. Azido-cyclophellitol derivative **18** can be coupled with TIPS-protected disulfide linker **17**[11]. Protective group manipulations followed by conversion of the carboxylic acid into the OSu-ester **21** allows the installation of mannose cluster **16**[10]. In the final event the BODIPY tag is introduced by "click" chemistry affording the proposed conjugate **15**.

Scheme 2: Proposed synthesis of disulfide bridged mannose cluster-cyclophellitol construct **15**.
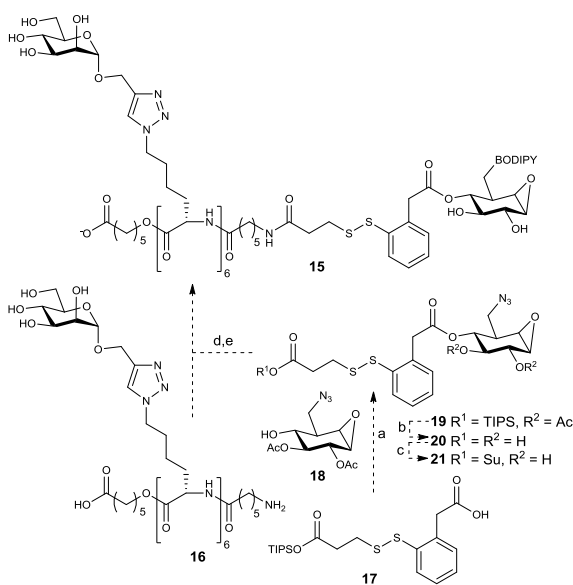
Reagents and conditions: (a) **18**, DIC, DMAP, THF, 0 °C to rt.; (b) *i.* HF/pyr, pyridine/MeCN, 0 °C to rt; *ii.* NaOMe/MeOH; (c) DIC, HOSu, 0 °C to rt.; (d) **8**, DMSO, rt.; (e) Alkyne-BODIPY, CuSO$_4$, sodium ascorbate DMF/H$_2$O.

Chapter 4 describes the synthesis and preliminary immunological evaluation of twenty mannosylated peptide conjugates. One, two, three and six copies of a mono-mannoside, three different dimannosides and a trimannoside, were conjugated to a peptide containing the MHC-class-I epitope SIINFEKL. The respective target peptides were provided with the

required number of azidolysines, while all (oligo)mannosides were equipped with an alkyne allowing an efficient Cu(I) catalyzed "click" mediated synthesis of the twenty mannosylated peptide conjugates. Worth noting is the increase in overall yield of the conjugates by the use of CupriSorb® resin to remove copper from the reaction mixture after the Cu(I) catalyzed "click" reaction. Preliminary immunological evaluation showed an increase in T cell response of almost all mannosylated conjugates in comparison with the non-mannosylated parent peptide. In addition, the uptake of the conjugates was likely mannose receptor mediated. Surprisingly, the monomannoside conjugates with one or two monomannosides showed the highest T cell response compared to the other constructs and no clear correlation between the type and number of (oligo)mannosides in the conjugates and the T cell response was apparent. A possible explanation for this finding is the reduced proteolytic susceptibility and processability of the mannosylated epitope conjugates. It is also unclear at present which lectins are involved in binding and or transporting the conjugates. It is proposed to introduce biodegradable linkers between the (oligo)mannose clusters and the epitope using the same chemistry as proposed for the modified cyclophellitol conjugates (*vide supra*, Scheme **1** and **2**) to influence the processing the conjugates.

As discussed above, mannose-6-phosphate conjugates are promising devices for receptor-mediated uptake. To study the relation between mannose-6-phosphate-receptor mediated uptake and antigen presentation, multivalent mannose-6-phosphate/phosphonate epitope conjugates are proposed (Figure **4**).
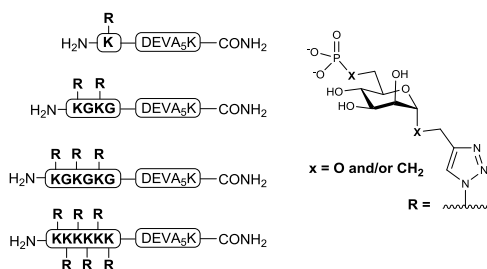

Figure 4: Schematic representation of mannose-6-phosphate/phosponate *O/C*-mannoside conjugates.

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Chapter 5 describes the synthesis of mannose configured α- and β-cyclophellitol **22**, and their aziridine counterparts **23**. The chiral cyclohexene, the common intermediate for both epoxides and aziridines, was obtained by adaptation of the procedure of the group of Madsen for the synthesis of cylcophellitol.[12] Using the procedure of Li *et al.*[13] for the conversion of the α-epoxide to the β-aziridine, the *manno*-configured β-aziridine cyclophellitol was generated. The synthesis of the α-aziridine started with the β-epoxide, the minor product of the cyclohexene epoxidation reaction. Opening of the epoxide with sodium azide and mesylation of the formed hydroxyl was followed by reduction of the azide with LiAlH₄ to produce the α-aziridine cyclophellitol. The absolute configurations of the α- and β benzyl-protected epoxides compounds were elucidated by NMR spectroscopy. Both the α- and β-epoxide adopt a $^4H_3$ conformation as corroborated by DFT calculations.

Both aziridine α-**23** and β-**23** can be converted into two-step labelling activity based probes **24** and **26** by introduction of an azide ligation handle via either acylation or alkylation of the aziridine nitrogen (Scheme 3).[14] These two-step probes can be further converted into direct activity based probes **25** and **27** by introduction of a BODIPY reporter group, using a Cu(I) catalyzed "click" reaction.

Scheme 3: Proposed functionalization of mannose configured aziridine cyclophellitol **23**.
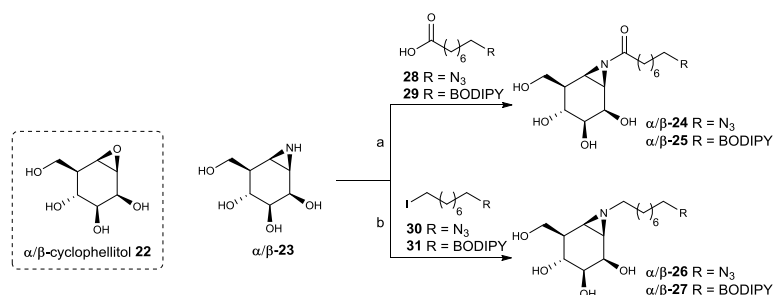
Reagents and conditions: a) acid **28** or **29**, EEDQ, DMF, 0 °C; b) iodide **30** or **31**, K₂CO₃, DMF, 90 °C.

Chapter 6 deals with the synthetic accessibility of differently configured 2-deoxy-2-fluoro glycosides with various (substituted) *N*-phenyltrifluoroacetimidate leaving groups at the anomeric centre as potential glycosidase inhibitors. The research in this chapter is based on the work of Walvoort *et al.*[15] who showed that 2-deoxy-2-fluoro-β-glucose bearing an anomeric *N*-phenyltrifluoroacetimidate has an improved inhibitory profile towards the enzyme glucocerebrosidase in comparison with commonly used inhibitors featuring other leaving groups such as fluoride, 2,4-dinitrophenyl and diphenylphosphate. Various naturally occurring glycosidases are known that hydrolyze different epimeric glycans using the Koshland double-replacement mechanism and therefore glucose- mannose- and galactose configured probes were targeted in this chapter. Unfortunately it turned out that all the 2-deoxy-2-fluoroglycosyl imidates were highly unstable and degraded partly or completely during HPLC purification. Only α-2-deoxy-2-fluoro-mannosyl *N*-phenyltrifluoroacetimidate and a few α- and β-2-deoxy-2-fluoroglucosyl N-phenyltrifluoroacetimidate derivatives could be prepared in low yields. All 2-fluoro-galactose configured imidates were too instable and could not be isolated.

It is proposed to use thioimidates as more stable leaving groups in the 2-deoxy-2-fluoro glycosides probes. Several possible routes of synthesis to 2-deoxy-2-fluoro-glucosyl thioimidate probes can be envisaged (Scheme 4).

Scheme 4: Proposed synthesis of thioimidate glycosidase probes **32-37**.
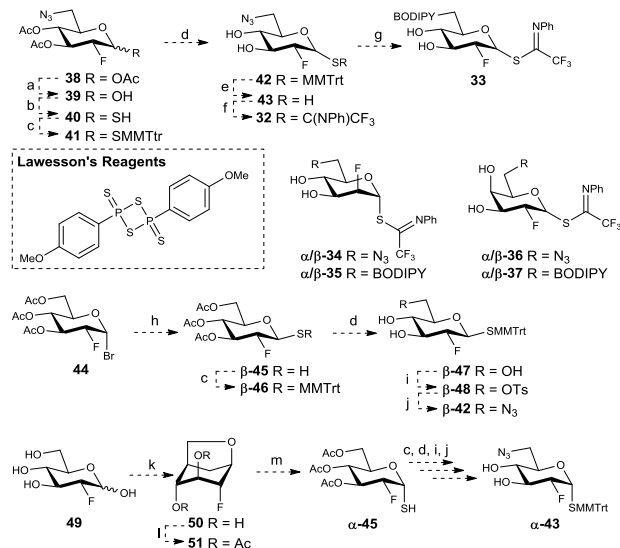
Reagents and conditions: (a) Piperidine/DMF; (b) Dioxane, Lawesson's reagent, 80 °C; (c) MMTrtCl, pyridine; (d) NaOMe/MeOH; (e) TFA, TES-H, DCM; (f) ClC(=NPh)CF$_3$, K$_2$CO$_3$, acetone; (g) Alkyn-BODIPY, CuSO$_4$, sodium ascorbate, DMF/H$_2$O; (h) *i.* thiourea; *ii.*Na$_2$S$_2$O$_5$, DCM/H$_2$O; (i) TsCl, pyridine; (j) NaN$_3$, DMF; (k) *i.* TsCl, pyridine; *ii.* 3N NaOH (aq.); (l) Ac$_2$O, pyridine; (m) *i.* (TMS)$_2$S, DCM, TMSOTf; *ii.* Ac$_2$O, pyridine *iii* KSAc/MeOH.

In the first route 2,6-dideoxy-2-fluoro-6-azido-glucose derivative **38** is converted to the corresponding thiol **40** with the aid of the Lawesson's reagents.[16] Protection of the thiol function with an monomethoxytrityl (MMTrt) group can give the individual α- and β-anomers **α-41** and **β-41**.[17] Deacetylation and deprotection of the MMTrt-group is followed by reaction with *N*-phenyl trifluoroacetimidoyl chloride under basic conditions[18] to give the individual α- and β-anomers of the potential thio-imidate probe **32**. A BODIPY fluorophore can be introduced at the azide of compound **32** with alkyn-BODIPY using "click" chemistry giving probe **33**.

In the second approach 2-deoxy-2-fluoro-glucose derivatives **44** and **49** are used as starting compounds, thereby postponing the introduction of the azide to a later stage of the synthesis (Scheme 4). Treatment of anomeric bromide **44** with potassium thioacetate followed by selective deacetylation of the

thioester gives **β-45**. Ensuing, protection of the thiol with a MMTrt group and deacetylation of the remaining acetyl groups sets the stage for the successive introduction of the azide at the 6-position and the thio-imidate at the anomeric centre to furnish β-probe **43**. The corresponding α-probe is accessible by opening of known 1,6-anhydro-glucose derivative **51**[23] with (TMS)$_2$S[19] followed by global acetylation and selective deacetylation of the anomeric thioester to give **α-45**. Using the procedure described above, **α-45** can be transformed into the α-thio-imidate probe **32**.

The proposed 2-deoxy-2-fluoro-glucosyl thioimidate inhibitors can be evaluated as pharmacological chaperones of the enzyme GBA for the treatment of the Gaucher's disease.[20] It is also proposed to evaluate lipophilic thio-imidate **53**, **54** and **55** as a closer mimic of the natural substrate glucosylceramide (**52**, Figure 5).
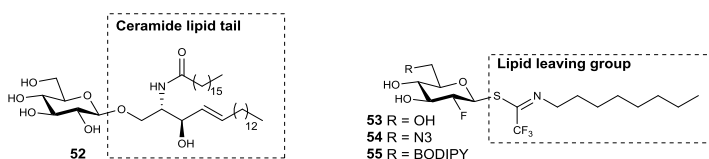

Figure 5: Structure of glucosylceramide (**52**) and its imidate based mimic inhibitor **53**-**55**.

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