Synthesis and application of glycans unique to S. mansoni

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Introduction and outline

General

Schistosomiasis is an acute and chronic disease caused by blood dwelling parasitic trematodes of the genus *Schistosoma*.[1] The World Health Organization (WHO) classified it as the second most socioeconomically devastating parasitic disease, second only to malaria. The number of infections is estimated at 249 million people per year, of which 200.000 end in loss of life.[2]–[5] Schistosomiasis mainly occurs in developing countries especially in areas without access to clean drinking water and inadequate sanitation (Figure 1).[6] The disease is contracted by exposure to schistosome infected water.

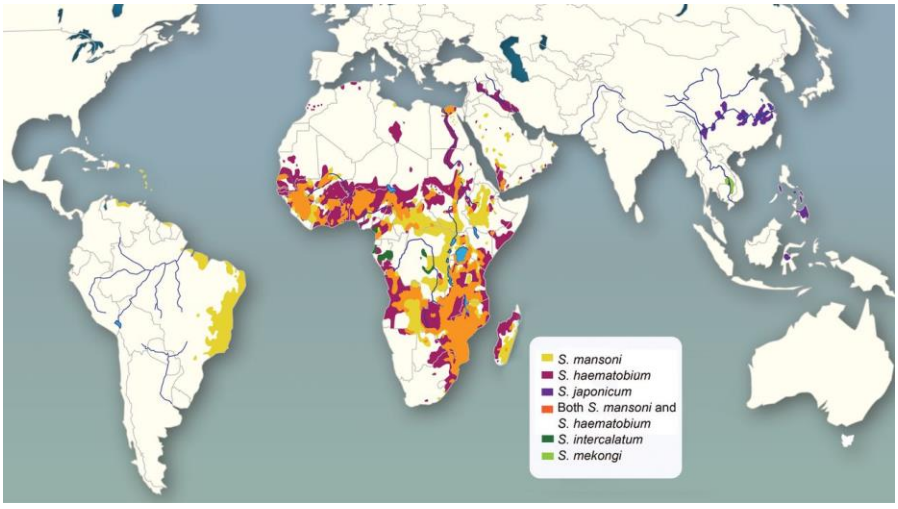


Figure 1: Global distribution of schistosomiasis[7]

Life cycle of schistosoma

The life cycle of *S. mansoni*, depicted in Figure 2, consists of two main stages: the water stage (1-5) and the host stage (6-10). Eggs are secreted by the host and hatch when they come into contact with fresh water.[8] The eggs release miracidia, which in turn infect fresh water snails. The miracidia reproduce asexually forming sporocysts in the snail. When these sporocysts burst they release cerceriae in the water.[3] These cerceriae are able to penetrate the skin of a host by releasing secretion vessels from their acetabular glands. These vessels contain a variety of proteins such as serpins, elastases, paramyosin and glutathione-S-transferases.[9]–[11] While entering the host, the cerceriae undergo a variety of changes. They lose their tail and become schistosomulae, that are able to circulate in the bloodstream and migrate to the lung capillaries.[12] In particular at this stage they shed their thick carbohydrate based layer (glycocalyx), which seems to be crucial for the evasion of the hosts' immune system.[9] After nine days of incubation in the lung capillaries, the schistosomulae migrate to the hepatic portal system, where they mature into schistosomes, while feeding on red blood cells. After they have fully matured a male and female schistosome permanently pair up and migrate to the mesenteric venules of the bowels. The paired schistosomes release approximately 200 eggs per day, most of which are excreted via the stool, some however, get trapped into various host organs causing inflammation, granuloma and fibrosis.[13]

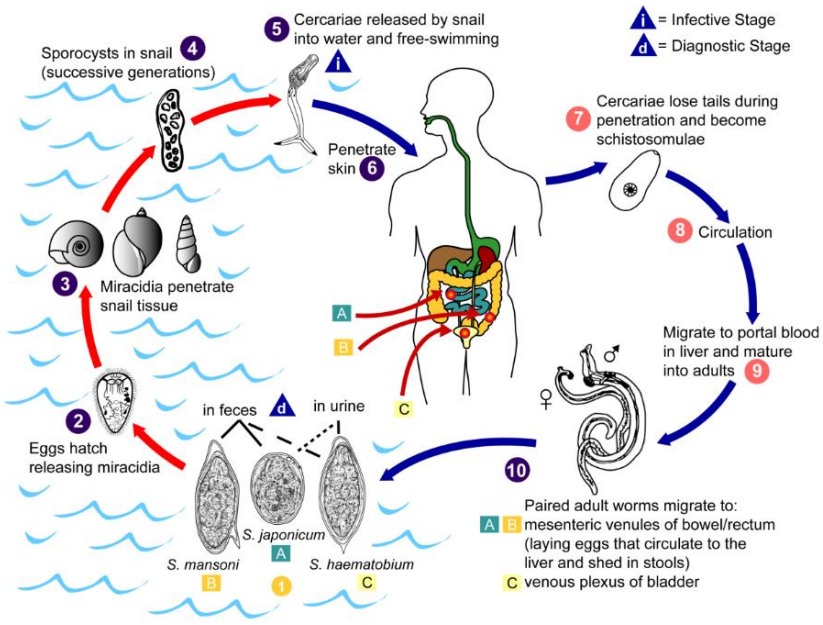


Figure 2: The life cycle of S. Mansoni.[14]

Schistosomiasis

Schistosomiasis is divided into two major classes: acute and chronic schistosomiasis. Acute schistosomiasis causes Katayama fever and occurs between six and eight weeks after the initial infection. When infected for the first time symptoms include fever, headache, abdominal pain and eosinophilia, while for people from endemic areas this stage can be asymptomatic.[15], [16] This phenomenon is explained by prior exposure *in utero* in schistosoma infected mothers. [17], [18]

Chronic schistosomiasis is caused by granulomatous inflammation against schistosome eggs that have become lodged in various organs and tissues. This inflammatory response can then cause tissue lesions in those organs. The symptoms of this stage of the disease are ulceration, abscess formation, chronical diarrhea and per rectal bleeding.[19]

Present treatment

The common treatment for schistosomiasis is Praziquantel (PZQ), which has been in use since the 1980's. It is sold in tablet form as a racemic mixture of which only the R enantiomer (see Figure 3-A) is active against schistosomiasis. The S enantiomer has little to no anti helminth activity, but instead is the cause of many of the side effects of PZQ.[20]

Although it has been used for over thirty years its precise mode of action is still not fully understood. It has been observed that PZQ causes a rapid intake of Ca^{2+}, which in turn causes paralytic muscular contraction, which dislodges the schistosome from the host.[21] Angelucci *et al.* proposed that PZQ acts as an adenosine analogue (see Figure 3-B and C), since they observed that PZQ blocked the uptake of purines, which in some vertebrates modulate the uptake of Ca^{2+} ions.[22] Besides these Ca^{2+} induced contractions, the outer protective layer of the worm, the tegument, is disrupted as well by PZQ. As a result of this the nematode is no longer able to resist digestion by the host.[23], [24] Notably, despite being used for well over thirty years very few PZQ-resistant strains have emerged.[25], [26]

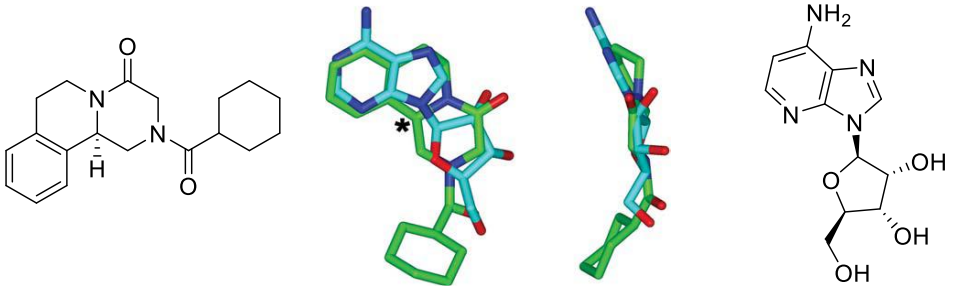


Figure 3: A: Structure of R-praziquantel, B: superimposition of praziquantel and adenosine, the asterisk marks the chiral centre of PZQ, C; structure of adenosine[22]

Despite its effectiveness, the use of PZQ is not without problems, as the S-enantiomer causes side effects such as diarrhea, vomiting, dizziness, vertigo, and headaches. The severity of these symptoms depends on the severity of the infection.[27] Unfortunately, there is no cost effective synthesis for enantiomerically pure PZQ at this time. Besides these negative side effects the drug has two more drawbacks. The first being that PZQ only targets adult worms, so often a second treatment is needed.[28] The second drawback is that PZQ does not offer immunity to protect against a future infection, making vaccination an attractive option. At the time of writing two possible vaccine candidates are being tested in clinical trials, however no results have been made available about these trials.[29], [30]

Diagnosis

There are various tools used for the diagnosis of schistosomiasis. The most frequently used and easiest to perform test is the WHO recommended Kato-Katz (KK) thick stool smear technique, which is based on the egg count in a stool sample.[31] Although the KK test is easy to perform it lacks in its accuracy. The accuracy of the KK test is good when the worm load in the host is high, but in case of a low worm load the accuracy drops as the egg secretion is not as high.[32] Since the schistosome egg excretion varies on a day

to day basis, the sensitivity of the KK test drops even further.[33], [34] The low accuracy at low worm load makes it difficult to assess if another PZQ treatment is required.[35] Another problem is that the eggs tend to clump together and therefore may not be present in the sample used for the KK test.

While the previous method describes a direct parasitological approach, much research has been conducted to develop an immunological diagnosis. Schistosomes, like all organisms, express a large range of complex glycans, some of which are unique to these species and these can therefore be used as an analytical marker and possibly as a target for a cure. Some examples of glycans of the glycocalyx of *S. mansoni* that so far appear unique to the *Schistosoma* genus are shown in Figure 4.[36]

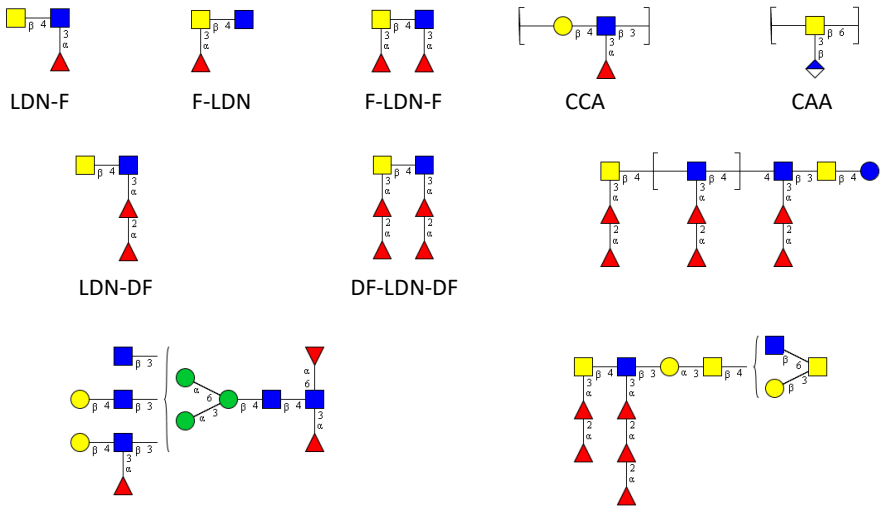


Figure 4: biological representation of glycans present in the glycocalys of S. Mansoni. Yellow square: GalNAc, yellow circle: Gal, blue square: GlcNAc, blue circle: Glc, green circle: Man, red triangle: Fuc, blue and white diamond: GlcA. LDN: LacDiNAc, F: fucosyl, DF: di-fucosyl, CCA: circulating cathodic antigen, CAA: circulating anodic antigen[37], [38]

The two most important glycans that are used for diagnosing schistosomiasis are the circulating anodic antigen (CAA), a polymer of 6-[GlcA-β-(1-3)-GalNAc-β-(1-] and the circulating cathodic antigen (CCA), a polymer of Lewis X.[39], [40] Another unique structural element are the L-fucose α-(1→2)-L-fucose chains present in many antigens. These fucose chains are present in every developmental stage of *S. mansoni*, unlike CAA and CCA, which are only present in adult worms. Several antibodies against multi-fucosylated glycans have been discovered, but the specific target of most of them is as of yet unknown. [41]

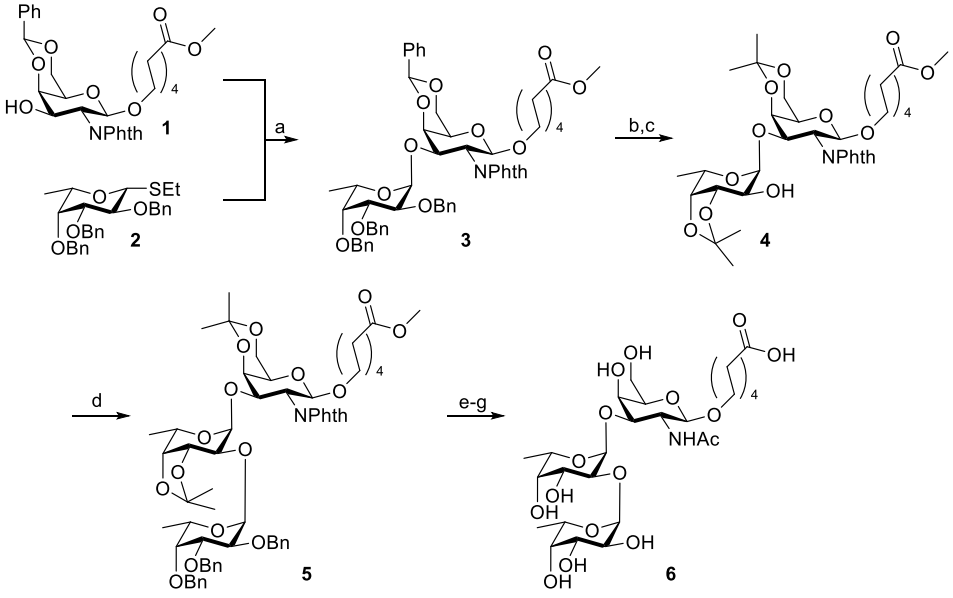
Diagnostic tests have been designed to detect anti-schistosomal antibodies or circulating schistosomal antigens in plasma, urine, serum or sputum.[7] Generally the antigens used in antibody detection assays are cercarial, egg or worm extracts containing multiple components, resulting in a lower specificity. To generate more specific diagnostic tools pure antigen could be used.[42], [43] As most of the biosynthesis enzymes that make these specific antigens are as of yet unknown, synthetic chemistry can provide the right tools to achieve this. Below, the syntheses of schistosome antigens reported to date are described.

Chemically synthesized antigens of *S. mansoni*

Several sugars of the *S. mansoni* glycocalyx have been synthesized by different groups. Most of them focused on the introduction of one or more of the α-(1-2)-fucosyl moieties. Short multi-fucosylated sugars have been synthesized by Hindsgaul and coworkers and Van Roon *et al*.[44], [45] Their syntheses are described in Schemes 1-3. Hindsgaul and co-workers generated a di-fucosyl galactosamine trisaccharide, while van Roon *et al.* assembled oligofucose chains appended to a glucosamine moiety. Larger multi-fucosylated glycans have been synthesized by Kanaya *et al*.[46] These glycans have a GalNAc-β-(1→4)-GlcNAc moiety (LacdiNAc or LDN) as the backbone. Vliegenthart and coworkers focused on a set of CAA fragments instead of the α-(1-2)-fucosides and synthesized up to the pentamer level of CAA (Scheme 6).[47]–[49] The key elements of these syntheses are discussed below in Schemes 1-6.

Hindsgaul and coworkers synthesized GalNAc-Fuc-Fuc trisaccharide **6** from appropriately protected building blocks **1** and **2** (Scheme 1).[44] Galactosamine **1** was fucosylated with donor **2** in presence of dimethyl(methylthio) sulfonium triflate (DMTST) and 2,6-di-*tert*-butyl-4-methylpyridine (DTBMP) giving disaccharide **3** in 80% yield.[50], [51] Disaccharide **3** was then subjected to palladium catalyzed hydrogenation to remove all benzyl ethers as well as the benzylidene acetal. The generated pentol was then protected with two *iso*-propylidene groups, leaving the fucosyl C2 hydroxyl unprotected (**4**). The characteristic α-(1-2)-fucose was introduced by reacting donor **2** with disaccharide **4** using AgOTf/bromine as the activator system.[52] This furnished trisaccharide **5** in 61% yield. Trisaccharide **5** was deprotected by first removing the *iso*-propylidene groups and hydrolysis of the methyl ester by acid catalyzed hydrolysis using 60% aqueous acetic acid at 50°C for 30 minutes. Prolonged exposure to these conditions led to decomposition of the formed trisaccharide. Next, the amine was liberated by treatment with hydrazine, after which the amine was selectively acetylated with Ac_2O in methanol. Finally, the benzyls were removed by catalytic hydrogenation giving **6** in 42% yield over three steps. The linker was equipped with a carboxylate functionality to enable conjugation to carrier proteins, by using peptide coupling reagents.

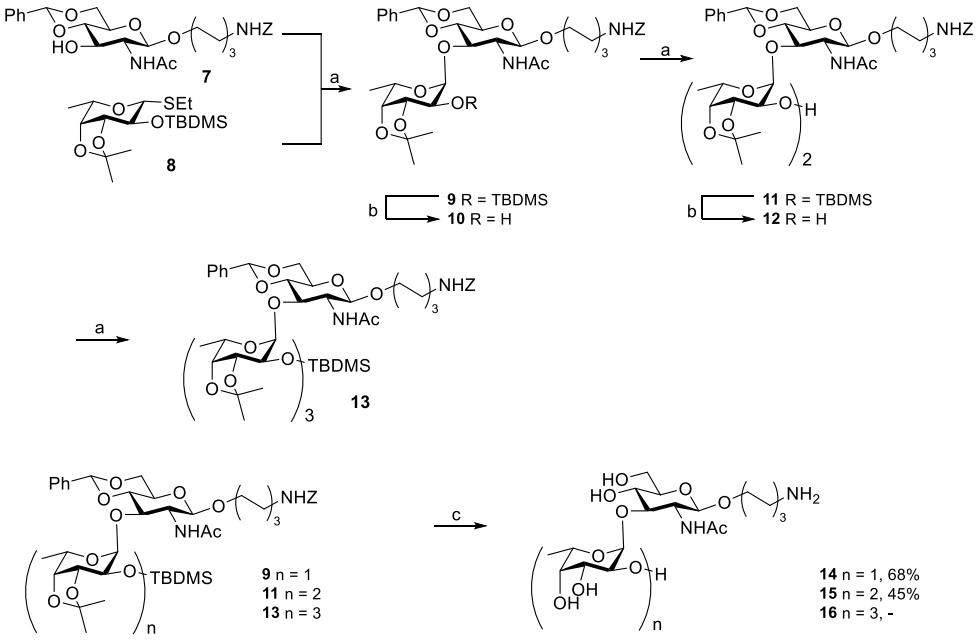
*Scheme 1: Synthesis of GalNAc-Fuc-Fuc **6** antigen by Hindsgaul and coworkers.[44]*



Reagents and conditions: **a**: DMTST, DTBMP, MS (4Å), DCM, 80%, **b**: Pd/C, H_2, EtOH, **c**: *p*-TsOH (cat.), DMP, ACN/DMF, **d**: AgOTf, Br_2, DTBMP, MS (4Å), DCM, 61%, **e**: 60% AcOH, 50˚C, **f**: i) N_2H_4 ·AcOH, MeOH, 65˚C, ii) Ac_2O, MeOH, **g**: Pd/C, H_2, MeOH, 42%.

Van Roon *et al.* synthesized a small set of fucosylated GlcNAc oligosaccharides (Scheme 2).[45] In these syntheses building block **8** was used as their fucosyl donor, which was orthogonally protected at the C2-O with a *tert*-butyldimethylsilyl (TBDMS) ether. In order to obtain disaccharide **9**, fucosyl donor **8** and glucosamine acceptor **7** were condensed using $CuBr_2$ with tetrabutylammonium bromide (TBABr) in a 1:1 mixture of DCM/DMF to obtain α-selectivity (α/β = 12/1).[53]. Disaccharide **9** was obtained in an excellent yield of 85% in a fully stereoselective manner. The TBDMS group was removed selectively by tetrabutylammonium fluoride (TBAF) in THF, which gave rise to disaccharide acceptor **10**. The fucosyl chain was elongated using the same coupling procedure as before resulting in trisaccharide **11** in 52% yield. The TBDMS ether on **11** was removed and the resulting acceptor **12** was extended in a similar manner as for **7** and **10**, giving tetrasaccharide **13** in 17% yield. The substantial difference in yield was attributed to steric hindrance of the bulky *iso*-propylidene groups on the acceptor. Besides the yield, the stereoselectivity dropped as well (α/β = 5/2). The protected glycans **9**, **11** and **13** were deprotected by treatment with 60% aqueous acetic acid, removing all the acetals, followed by catalytic hydrogenation. The di- and trisaccharide **14** and **15** were obtained in 68% and 45%, respectively. Unfortunately, tetrasaccharide **16** could not be obtained, as the terminal fucose was cleaved during hydrolysis of the *iso*-propylidene and benzylidene acetals.

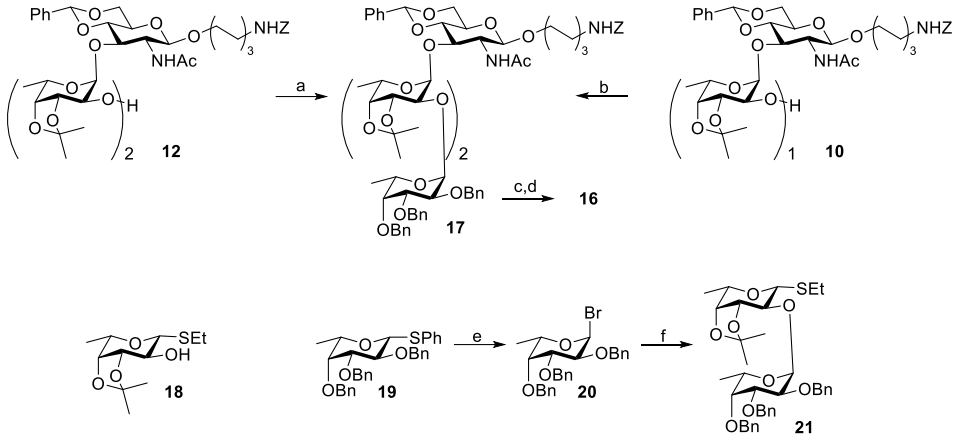
*Scheme 2: Synthesis of GlcNAc-Fuc-Fuc antigen **15** by van Roon et al.[45]*



Reagents and conditions: **a**: $CuBr_2$, TBABr, DMF:DCM (1:1), **9** 85%, **11** 52% (α/β, 12:1), **13** 17% (α/β, 5:2), **b**: TBAF, THF, 88-93%, **c**: i) 60% AcOH, 50 °C, ii) H_2, Pd/C, EtOH, H_2O, HCl, **14** 68%, **15** 45%, **15** 0%.

To prevent decomposition of the tetrasaccharide, Van Roon *et al.* turned to benzylated fucosyl **19** as a donor (Scheme 3). Donor **19** was successfully condensed with trisaccharide acceptor **12** using the same protocol as used for the synthesis of **9**, **11** and **13**, resulting in tetrasaccharide **17** in 17% yield. Although this time tetrasaccharide **17** could be obtained after acidic hydrolysis and catalytic hydrogenation (40%, over two steps), the yield of the glycosylation reaction was still poor. In order to increase the yield, they resorted to a [2 + 2] block coupling. To this end donor **19** was converted to the anomeric bromide **20** using bromine in DCM and then condensed with acceptor **18**, to give disaccharide donor **21** in 68% yield. Acceptor **10** and donor **21** were coupled under the activation of $CuBr_2$ as described above, which gave tetrasaccharide **17** in 36% yield. Notably, the stereoselectivity of this glycosylation dropped significantly, as 16% of undesired β-linked product was isolated as well.

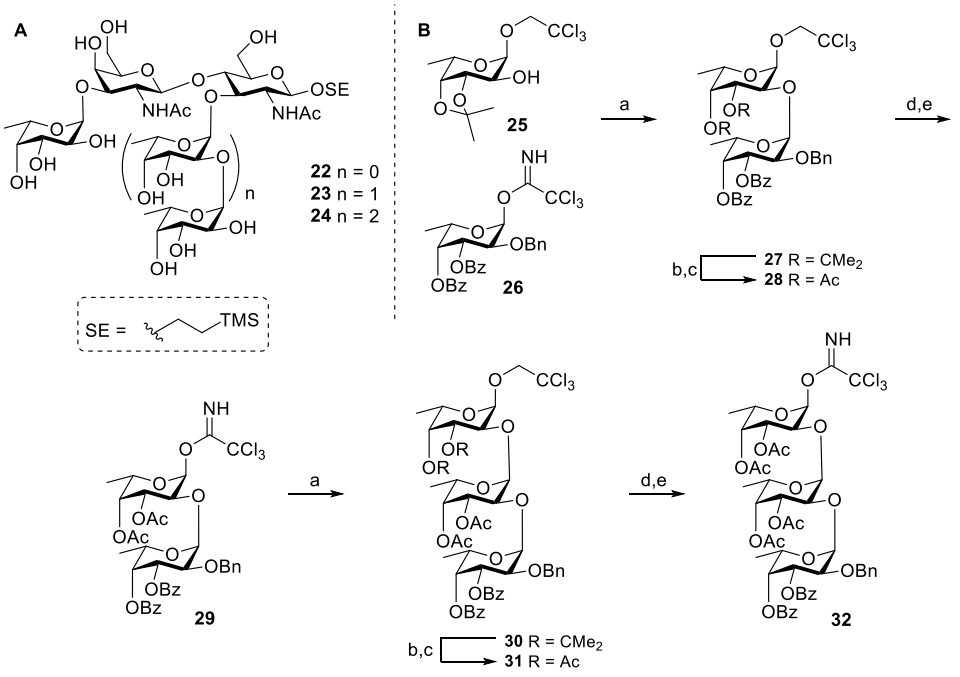
*Scheme 3: Alternative synthesis of GlcNAc-Fuc-Fuc-Fuc antigen **16** by van Roon et al.*[45]



Reagents and conditions: **a**: **19**, $CuBr_2$, TBABr, DMF:DCM (1:1), 16%, **b**: **21**,$CuBr_2$, TBABr, DMF:DCM (1:1), 36% (16% of the β-isomer), **c**: 60% AcOH, 50 °C, 57% **d**: H_2, Pd/C, EtOH, H_2O, HCl, 70% **e**: Br_2, DCM, 0°C, **f**: **18,** TBABr, DMF:DCM (1:1), 68%.

Kanaya *et al*. have synthesized several fucosylated *N*-acetylgalactosamine-β-(1-4)-*N*-acetylglucosamine (LDN) disaccharide structures, examples of which are highlighted in Scheme 4Scheme 5.[46] The α-(1-2)-fucosyl chains were introduced *en block* to the LDN backbone using donors **26**, **29** or **32**, which were synthesized from the non-reducing end (Scheme 4B). Fucosyl acceptor **25** was orthogonally protected with a trichloroethyl (TCE) group on the anomeric center and an *iso*-propylidene on the C3- and C4-hydroxyls. [54] Imidate donor **26** was chosen over thiophenyl donor **19** as that was prone to form a fucose-succinimide complex upon activation with NIS/TfOH.[55] Imidate donor **26** was condensed with *iso*-propylidene bearing fucosyl acceptor **25** to give disaccharide **27** in 75% yield. The *iso*-propylidene group was removed selectively by hydrolysis in 80% acetic acid, followed by acetylation of the obtained diol with Ac_2O and pyridine, which gave disaccharide **28** in 86% yield. The TCE group was then removed selectively with Zinc in AcOH, and the resulting hemi-acetal was treated with a catalytic amount of DBU and trichloroacetonitrile in DCM to give α-imidate disaccharide **29** in 77% yield over two steps. This disaccharide donor was in turn condensed with acceptor **25** giving trisaccharide **30** in 80% yield. The *iso*-propylidene in **30** was removed and exchanged for two acetyl esters as described above giving **31**. Trisaccharide **31** was transformed into imidate donor **32** by the same procedure as described for the conversion of **28** into donor **29**.

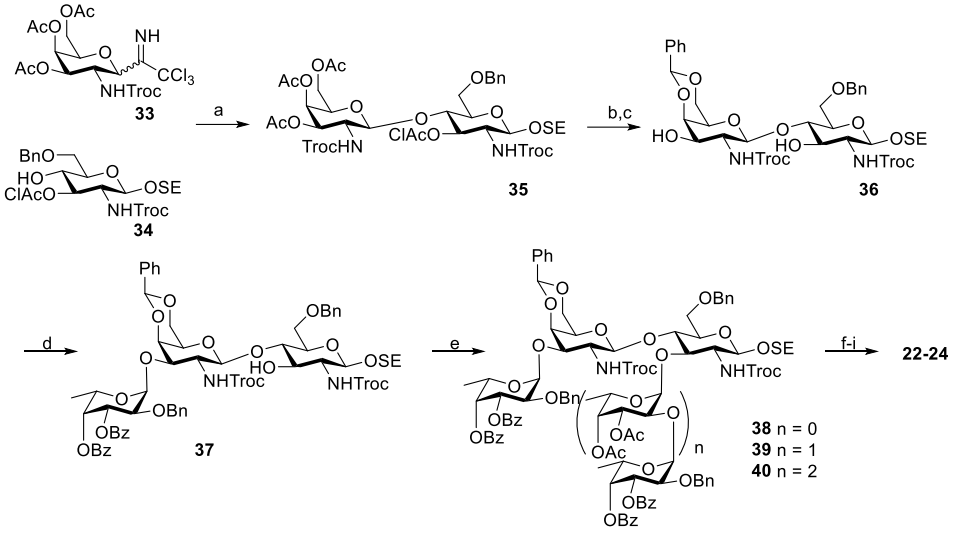
*Scheme 4: **A**: Synthesized structures, **B**: Synthesis of fucosyl chain donors **29** and **32**.*[46]



Reagents and conditions: **a**: TMSOTf (cat.), MS (4Å), DCM, -40˚C, **27** 75%, **30** 80%, **b**: 80% AcOH, 50˚C, **c**: Ac_2O, pyr., **28** 86%, **31** 63%, **d**: Zn, AcOH, 55 ˚C, **e**: DBU, CCl_3CN, DCM, -20 ˚C, **29** 77%, **32** 78%.

The LDN backbone was synthesized by condensing galactosamine imidate donor **33** with glucosamine acceptor **34**, which gave disaccharide **35** in 79% (*Scheme **5***). The acetyl and chloroacetyl groups were hydrolyzed by guanidinium nitrate and sodium methoxide in a mixture of methanol and chloroform. Subsequently, the C'6- and C'4-OH were selectively protected with a benzylidene group to give **36** with both the C3- and C'3-hydroxyls free.[56] The C'3-OH was selectively fucosylated using donor **26**, which gave rise to trisaccharide **37**. Next, the α-(1-2)-fucosyl chain on the C3-OH was introduced by condensing **37** with either **26**, **29** or **32**, which resulted in the isolation of **38**, **39** and **40** in yields of 78%, 67% and 43%, respectively. The LDN fragments **38**-**40** were deprotected in three steps. First the NHTroc group was changed into an acetamide by liberating the amines with zinc, followed by acetylation of the freed amines. Next, the benzylidene acetal and benzyl ethers were removed by catalytic hydrogenation. For ease of purification the glycans were fully acetylated, purified and de-acetylated using NaOMe in methanol, giving **22**, **23** and **24** in a yield of 23%, 32% and 35%, respectively.

*Scheme 5: Synthesis of fucosylated fragments **22**-**24**.[46]*

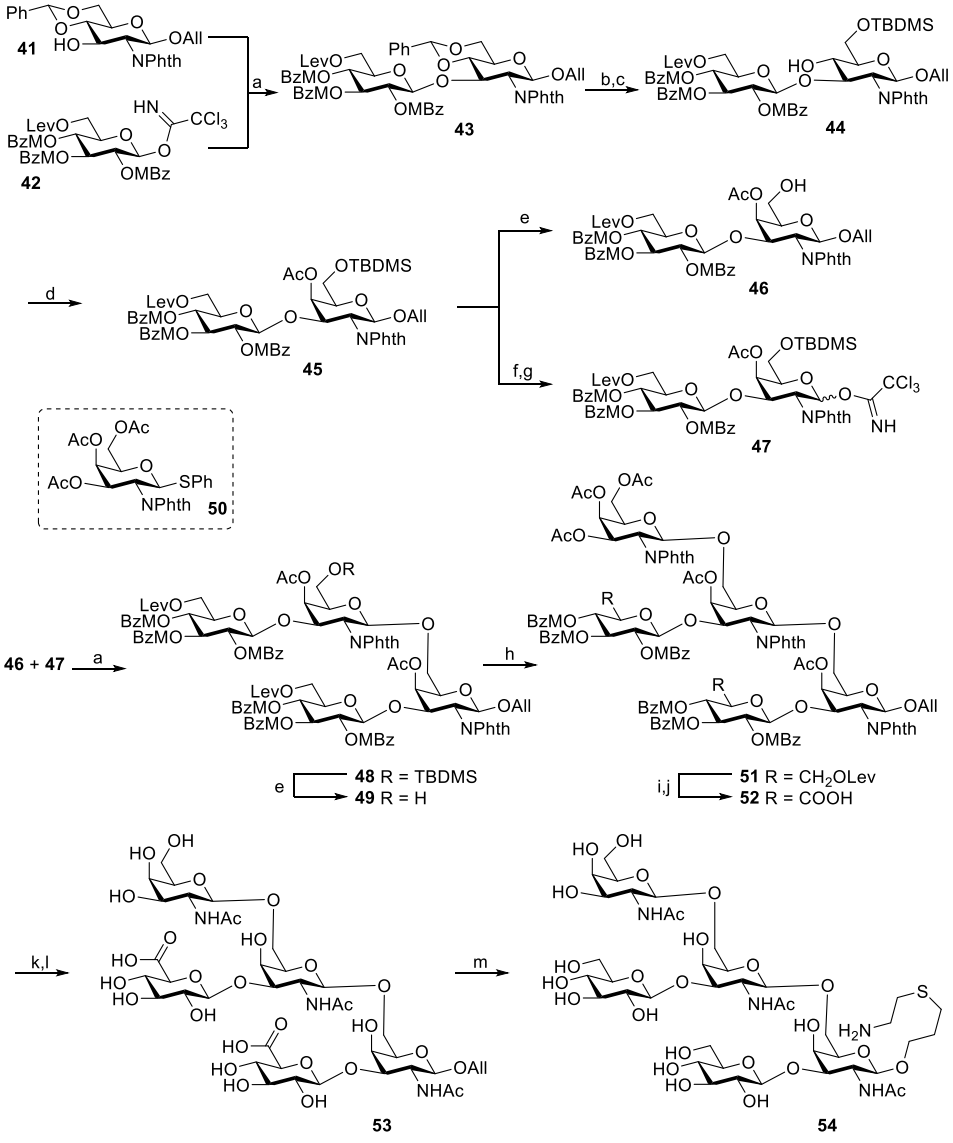


Reagents and conditions: **a**: TMSOTf (cat.), MS (4Å), DCM, 79%, **b**: guanidinium nitrate, NaOMe, MeOH/$CHCl_3$, **c**: benzaldehyde dimethyl acetal, $NaHSO_4 \cdot SiO_2$, ACN, 58% (2 steps), **d**: **37**, TMSOTf (cat.), MS (4Å), DCM, -40 ˚C, 66%, **e**: TMSOTf (cat.), MS (4Å), DCM, -40 ˚C , **38** 78%, **39** 67%, **40** 43%, **f** Zn, Ac_2O, AcOH, **g**: Pd/C, H_2, MeOH, **h**: Ac_2O, pyr., **i**: NaOMe, MeOH, **22** 23%, **23** 32%, **24** 35%.

The structure of CAA was first determined in 1994 by Vliegenthart and coworkers. Using NMR spectroscopy they proved that CAA consisted of a polymer composed of (→6)-[glcA-β-(1-3)]galNAc-(1→) repeats.[39] The same group synthesized a small set of CAA fragments.[47]–[49] These CAA molecules were conjugated to bovine serum albumin (BSA) in order to discover the optimal antigen. Their studies consisting of ELISA's and SPR's showed that the di- and tetrasaccharide CAA fragments were recognized best by several anti CAA monoclonal antibodies. There were, however, a lot of CAA antibodies that did bind with the native polymer but did not bind to any of the synthesized fragments, indicating that synthesis of bigger fragments was required.[57]–[59]

A brief overview of the synthesis of the CAA fragments by Vliegenthart and coworkers is shown in Scheme 6. They started from D-glucose and D-glucosamine, to synthesize both acceptor **41** and donor **42**, respectively. D-glucosamine was chosen over D-galactosamine as the starting material as it was significantly cheaper. The glucosamine moiety could be inverted to the corresponding galactosamine at the disaccharide stage. Their synthesis uses a late state oxidation, as a glucuronic acid donor was considered to be too unreactive for usage in glycosylation reactions.[60]

*Scheme 6: Synthesis of CAA pentamer **53** by Vliegenthart and co-workers.[47]–[49]*



Reagents and conditions: **a**: TMSOTf (cat.), MS (4Å), DCM, **43** 89%, **52** 73% **b**:TFA, DCM/H_2O, 98%, **c**: TBDMSCl, pyr, 96%, **d**: i) Tf_2O, pyr, DCM, 0 ˚C, ii) TBAA, DMF, 78%, **e**:*p*-TsOH (cat.), ACN, H_2O, **46** 91%, **49** 82% **f**:i) $(PPH_3)_4RhCl$, EtOH, ii) NIS, DCM, H_2O, **g**: CCl_3CN, DBU, DCM, 71% (two steps), **h**: **50**, NIS, AgOTf, MS (4Å), tol, 66%, **i**: N_2H_4·OAc, EtOH, Tol, 92%, **j**: PDC, MS (4Å), DCM, 73%, **k**: $MeNH_2$, EtOH, **l**: Ac_2O, MeOH, 0 ˚C, 65%, **m**: cysteamine hydrochloride, H_2O, UV, 80%.

Disaccharide **43** was synthesized by condensing donor **42** and acceptor **41** under influence of a catalytic amount of TMSOTf, which afforded disaccharide **43** in an excellent 89% yield. The benzylidene acetal in **43** was hydrolyzed under acidic conditions, followed

by selective protection of the released primary hydroxyl group with a TBDMS group, giving **44** in 95% yield. The free hydroxyl on the C4 position of glucosamine was inverted by triflation, followed by treatment with tetrabutylammonium acetate (TBAA) to give compound **45** in a 78% yield.[61], [62] Disaccharide **45** was then turned into either acceptor **46** or donor **47**. Acceptor **46** was obtained by selective removal of the TBDMS under mild acidic conditions to prevent any migration of the C4-acetyl to the C6-OH position. Donor **47** was synthesized by removal of the anomeric allyl group and subsequent imidoylation of the anomeric alcohol.[63]–[65] With both donor **47** and acceptor **46** in hand, tetrasaccharide **48** could be synthesized by utilizing the same coupling conditions as described for the formation of disaccharide **43**, which gave **48** in 73% yield. The TBDMS ether of **48** was removed under the same mild acidic conditions, as described for the transformation of **45** into **46**, resulting in tetrasaccharide **49**. Acceptor **49** and galactosamine donor **50** were condensed with the NIS/AgOTf activator couple to give pentasaccharide **51** in 66% yield. The primary hydroxyls on the glucose moieties were orthogonally deprotected by removing the levulinoyl (Lev) groups using hydrazine acetate, thus liberating them for oxidation. Initially, a Swern oxidation was performed followed by further oxidation by $NaClO_2$.[66] Unfortunately, this method did not give full conversion to the two carboxylic acids even after 96 hours of stirring. A different oxidation method using pyridinium dichromate (PDC) was explored next.[67], [68] PDC was able to oxidize both primary alcohols to the carboxylic acids in 4.5 hours in 73% yield. After successful oxidation **52** was deprotected with methylamine and subsequent acetylation of the free amines giving **53**.[69] The allyl was then functionalized by treatment with cysteamine hydrochloride in water under UV-light to obtain amine spacer containing pentasaccharide **54**.[70]

Outline of this thesis

The research in this Thesis describes the development of synthetic routes towards several glycans that are unique to *S. mansoni* and their use in diagnostic tools. **Chapter 2** describes the synthesis of Fuc-α-(1-2)-Fuc-α-(1-3)-GlcNAc and Fuc-α-(1-2)-Fuc-α-(1-3)-GalNAc, the shortest unique repeating units present in many glycans of the glycocalix of *S. mansoni*. A novel approach to the installment of the Fuc-α-(1-2)-Fuc was explored, together with an in depth NMR analysis and conformational analysis of the fully protected trisaccharides. The synthesized glycans were tested for biological activity by ELISA against monoclonal antibodies and sera of (un)-infected people. **Chapter 3** dives deeper into the synthesis of the α-(1-2)-Fuc linked oligofucosides, which are not anchored to any backbone. Two synthetic approaches toward these oligofucosides, comprising either elongation from the reducing end or from the non-reducing end, were explored and compared. Valuable insights on the applicability of the protective groups on the C3- and C4-hydroxyls of the fucosides, in terms of size and rigidity, were

discovered. Oligofucosides up to the tetramer were prepared and all glycans obtained were screened against monoclonal antibodies and sera of (un)-infected people. The synthesis of (di)-fucosylated GalNAc-β-(1-4)-GlcNAc disaccharides (GalNAc-β-(1-4)-GlcNAc, LacdiNAc or LDN), is described in **chapter 4**. A wide variety of orthogonal protective groups for both acceptor and donor glycosides were screened to attain the most favorable glycosylation reactions. Although F-LDN, LDN-F and F-LDN-F oligosaccharides were assembled successfully, the subsequent introduction of di-fucosyl side chains could not be achieved. **Chapter 5** describes the synthesis of CAA fragments. Two synthetic routes have been tested, one based on the use of monosaccharide glucose and galactose synthons, the other on isolating the desired disaccharide from Chondroitin A, a cheap and readily available biopolymer. A summary of this thesis and several future prospects are outlined in **chapter 6**.

References

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