Abstract

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This thesis describes chemical synthesis of carbohydrates and glycopeptides useful in studies of biologically interesting systems. The following topics are addressed: [Papers I-IV and supporting information]:

[I] The non-reducing end di- and trisaccharide structures of an erythrocyte glycolipid responsible for the rare NOR polyagglutination were chemically synthesized. The syntheses were based on a α-D-Gal*p*-(1→4)-β-D-Gal*p*NAc-(1→) structural element found only recently in Nature, and derivatives thereof have not been synthesized before. Both the synthesized oligosaccharides specifically inhibited human anti-NOR antibodies, with the trisaccharide being 300 times more active than the disaccharide.

[II] Derivatives of lactose with the galactose ring substituents replaced by 2´, 3´, 4´ and 6´ deoxy and 3´ acylamino functions were prepared. The lactosyl derivatives were tested as acceptors for the *Neisseria menigitidis N*-acetylglucosaminyltransferase catalyzed β-(1→3) glycosylation reaction, using UDP-GlcNAc as donor. The 6´-deoxy compound showed nearly a threefold increase in activity compared with the reference substance phenyl β-lactoside, whereas the 2´ and 4´-deoxy derivatives were less active. The 3´-deoxy and 3´-acylamino derivatives will be used in studies of the inhibitory capacity.

[III] In order to develop the non-viral Bioplex vector system for non-viral gene delivery to hepatocytes, biotinylated ligands were synthesised to study the structure-function relationship of specific binding and uptake to the asialoglycoprotein receptor ASGPr. Cluster glycosides containing two, three and six β-D-Gal*p*NAc residues were synthesized and tested for binding and uptake to liver cells. The derivative displaying six GalNAc units showed the highest uptake efficacy. However, the number of GalNAc units above three seems only to have a minor contribution to the overall affinity, while using longer spacer between the GalNAc ligands markedly influenced the uptake efficacy.

[IV] An analgetically active glycopeptide from the cone snail *Conus geographus*, contulakin G, has recently been analyzed and synthesized. Contulakin-G has been found to be a neurotensin agonist and have entered pre-clinical trials for short-term management of post-operative pain. The glycan part of contulakin-G has been found to be important for the biological *in vivo* activity. In order to further investigate the importance of the glycan part, three analogues of contulakin-G have been synthesized, were the α/β conformation of the anomeric centers as well as the glycosidic bond pattern of the disaccharide have been altered.

In addition to the contulakin G analogues, a heavily posttranslational modified glycopeptide from *Conus textile*, tx5a, has been synthesized.

Keywords: Carbohydrate, NOR-antigen, glycosyltransferase, asialoglycoprotein receptor, biotinylated ligands, glycopeptide, contulakin-G, tx5a

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Contents

Appendix A

List of Papers

This thesis is based on the following papers I-IV and supplementary material V, referred to in the text by their Roman numerals:

I. **Synthesis and Inhibitory Activity of a Di- and a Trisaccharide Corresponding to an Erythrocyte Glycolipid Responsible for the NOR Polyagglutination.** Westerlind, Ulrika; Hagback, Per; Duk, Maria; Norberg, Thomas. *Carbohydrate Research* **2002**, *337(17)*, 1517-1522.

II. **Synthesis of Deoxy- and Acylamino-derivatives of Lactose and use of these for Probing the Active Site of *Neisseria meningitidis N*-Acetylglucosaminyltransferase.** Westerlind, Ulrika; Hagback, Per; Tidbäck, Björn; Wiik, Lotta; Blixt, Ola; Razi, Nahid; Norberg, Thomas. *Carbohydrate Research* **2005**, *340(2)*, 221-233.

III. **Ligands of the Asialoglycoprotein Receptor for Targeted Gene Delivery, part 1: Synthesis of and Binding Studies with Biotinylated Cluster Glycosides Containing *N*-Acetylgalactosamine.** Westerlind, Ulrika; Westman, Jacob; Törnquist, Elisabeth; Smith, C. I. Edvard; Oscarson, Stefan; Lahmann, Martina; Norberg, Thomas. *Glycoconjugate Journal* **2004**, *21(5)*, 227-241.

IV. **Chemical Synthesis of Analogues of the Glycopeptide Contulakin-G, an Analgetically Active Conopeptide from *Conus geographus*.** Westerlind, Ulrika; Norberg, Thomas. *Submitted (Carbohydrate Research)*

V. **Supplementary material**. Westerlind, Ulrika

The following paper is not included in the thesis:

VI. **Specificity of Human *anti*-NOR Antibodies, a Distinct Species of "Natural" *anti*-α-Galactosyl Antibodies.** Duk, Maria; Westerlind, Ulrika; Norberg, Thomas; Pazynina, Galina; Bovin, Nicolai N.; Lisowska, Elwira. *Glycobiology* **2003**, *13(4)*, 279-284.

Appendix B

Contribution report

The author wishes to clarify her contributions to the papers I-IV in the thesis:

I. Performed all synthetic work, except the previously known building block **4**, prepared by Per Hagback. Characterized all new compounds; contributed partly to the writing of the manuscript. Dr. Maria Duk (Ludwik Hirsfeld Institute of Immunology and experimental Therapy, Wroclaw, Poland) performed the biological investigations.

II. Performed all synthetic work, with the exception of 6´-deoxy phenyl β-lactoside, which was prepared by Björn Tidbäck and 3´- and 4´-deoxy phenyl β-lactosides which were prepared according to the first route by Lotta Wiik and Per Hagback. Characterized all new compounds; contributed partly to the writing of the manuscript. Dr. Ola Blixt and Dr. Nahid Razi (Scripps institute, La Jolla, CA, USA) performed the enzyme assay experiments.

III. Performed all synthetic work, except for compounds **79** (route B) and **91** which were prepared by Dr. Martina Lahmann (Department of Organic Chemistry, Stockholm University, Sweden). Characterized all new compounds; contributed partly to the writing of the manuscript. Elisabeth Törnqvist (Clinical Research Center, Karolinska institute, Huddinge, Sweden) performed the biological evaluation.

IV. Initiated the project, contributed to its design. Performed all synthetic work, characterized all new compounds; contributed partly to the writing of the manuscript. The first synthetic studies towards intermediate **107** were done as a part of the diploma work by the author.

Appendix C

Abbreviations

5-HT	5-Hydroxy-tryptamine (Serotonin)
Å	Ångström
Ac	Acetyl
All	Allyl
ASGPr	Asialoglycoprotein receptor
Bn	Benzyl
Boc	t*ert*-Butoxycarbonyl
Bz	Benzoyl
CRD	Carbohydrate recognition domain
DBU	Diazabicycloundecan
DCC	Dicyclohexylcarbdiimide
DCM	Dichloromethane
DIPEA	*N*,*N*-Diisopropyl-*N*-ethylamine
DMAP	4-(*N*,*N*-Dimethylamino)pyridine
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DMTST	Dimethyl(thio-methyl)sulfonium trifluoromethanesulfonate
DNA	Deoxyribonucleic acid
E. Coli	*Escherichia coli*
equiv.	Equivalent(s)
Et	Ethyl
FACS	Fluorescence-activated cell sorting
Fmoc	Fluoren-9-ylmethoxycarbonyl
Gal	Galactose
GalNAc	*N*-Acetylgalactosamine
Glc	Glucose
GlcNAc	*N*-Acetylglucosamine
GLUT	Glucose transporter
h	hour(s)
HBTU	[*N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium *N*-oxide]$^{+}$ PF_6^{-}
HOBt	1-*N*-Hydroxybenzotriazole
HPLC	High performance liquid chromatography
i-Pr	*iso*-Propyl
MALDI	Matrix assisted laser desorption ionization
Me	Methyl
MS	Molecular sieves
Mtt	4-Methyltrityl
nAChR	Nicotinic acetylcholine receptor
NBS	*N*-Bromosuccinimide
NMDA	*N*-methyl-D-aspartate

NMR	Nuclear magnetic resonance
NOE	Nuclear overhauser effect
NOR	named from the city of first case, NORton
NTR	Neurotensin receptor
O.N.	overnight
Ph	Phenyl
Phen	Phenacyl
Phth	Phthalimido
PNA	peptide nucleic acid
p-Tol	*para*-Tolyl
PyBOP	[Benzotriazol-1-yl-oxytris(pyrrolidino)phosphonium]$^{+}PF_6^{-}$
RP	Reversed phase
rt	Room temperature (≈ 23° C)
SPE	Solid phase extraction
t-Bu	*tert*-Butyl
TCEP	tris-(2-carboxyethyl)-phosphine hydrochloride
Tf	Trifluoromethanesulfonyl
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TIPS	Triisopropylsilane
TMS	Trimethylsilyl
TOF	Time Of Flight
TSDU	[Succinimidyl-1,3-dimethyl-1,3-trimethyleneuronium]$^{+}BF_4^{-}$
UDP	Uridine diphosphate
Wilkinsons catalyst	$(PPh_3)_3RhCl$

1. Introduction

1.1 Historical background

Carbohydrates represent a unique family of poly-functional compounds possessing a rich chemistry and extraordinary complex biology. As most compound classes, carbohydrates can be chemically manipulated in a multitude of ways. The chemistry of carbohydrates has during its more than hundred years long history developed into a discipline of its own. Emil Fischer, later awarded the Nobel Prize in 1902, laid the basis of understanding of the organic chemistry of the carbohydrates in Germany at the end of 19th century. Fisher established systematic methods for the nomenclature and configurational assignment of carbohydrates.[1] Another great contribution to the whole field of organic chemistry was that Fischer continued the fundamental studies of the phenomenon named *chirality*. Initially discovered by the French scientists Hauy and Biot[2], chirality became a matter of general interest due to Pasteur´s seminal studies during mid 19th century, that postulated that the optical activity of molecules is a consequence of three-dimensional isomerism[3]. Pasteur mainly investigated natural and synthetic derivatives of tartaric acid, while Fischer extended the investigations into the most commonly abundant group of naturally occurring chiral compounds, namely carbohydrates. Fischer showed that enantiomers of a given molecule rotate the plane of polarized light with the same magnitude but in opposite direction. Further, he put forward that (*d*)- and (*l*)-symbols should be independent of the direction of rotation and that they should be used only to denote the spatial orientation of the substituents. Fischer also created the *Fischer projection*, a two-dimensional formula used to relate the configurations of chiral molecules and according to this system an assumption of the absolute configuration of glucose, galactose, mannose, fructose, arabinose and xylose was made.[4, 5] This assumption was 75 years later proven to be correct by X-ray structural analysis.[6] In the early days of carbohydrate chemistry, melting point and optical rotation were essential in the determination of chemical structure and equivalence. Pure crystalline compounds were necessary for identification and the purification of sugar syrups by distillation, the most important purification technique before chromatography, was not a good alternative due to extensive decomposition. This limited the scope of carbohydrate research and it was not until the 1960s, when chromatographic methods and NMR spectroscopy were developed, that isolation of carbohydrate derivatives and their structural analysis became more feasible. During modern times, many scientists have contributed to the field of synthetic carbohydrate chemistry, but no one has had a greater impact than the Canadian scientist Ray Lemieux.[7] His group was responsible for the first synthesis of sucrose and introduction of ^{1}H and ^{13}C NMR spectroscopy to the field for structural elucidation. Lemieux identified endo- and exo-anomeric and reverse anomeric effects and developed key glycosylation reactions such as halide-ion assisted catalysis for the preparation of α-glycopyranosides. Today, carbohydrate research has expanded from only being an obscure area for a small number of dedicated scientist, to become a large scientific subject, located at the interface between chemistry and biology. The main questions addressed by carbohydrate chemists

today differ from those of Emil Fischer´s days, which dealt mainly with fundamental properties of the molecules themselves. Instead, carbohydrate chemistry has entered a new era, were the biological researchers need of complex carbohydrates is the driving force of the synthetic chemistry. Therefore, the method development for synthetic carbohydrate chemistry and the understanding of the chemical biology of carbohydrates is more important than ever.

1.2 Biological Roles of Carbohydrates

In the past, carbohydrates were considered to be solely of use for storage and supply of energy and as skeletal components. Examples of such components are the polysaccharides starch and glycogen as reserve energy substances and cellulose and chitin as structural compounds. This view of carbohydrates was challenged in 1963 when a protein from seeds of *Canavalia ensiformis* (jack bean) was isolated and demonstrated ability to bind to carbohydrates on erythrocytes[8]. In 1982, the first mammalian carbohydrate binding protein was identified[9] and this sparked interest into the wider roles of carbohydrates in biological systems. The importance of glycoconjugates, especially of glycoproteins and glycolipids, were discovered and it was found that the carbohydrate part of glycoconjugates might act e.g. as antigens and receptors for proteins. The study of carbohydrates within biological systems has illustrated that they are involved in a number of biological functions such as cell-cell recognition and cell-external agent interactions. These interactions can initiate biological events such as fertilization, cell growth and cell differentiation, immune responses, inflammation, viral and bacterial infections, cancer metastasis, to mention just a few[10-13](Figure 1).

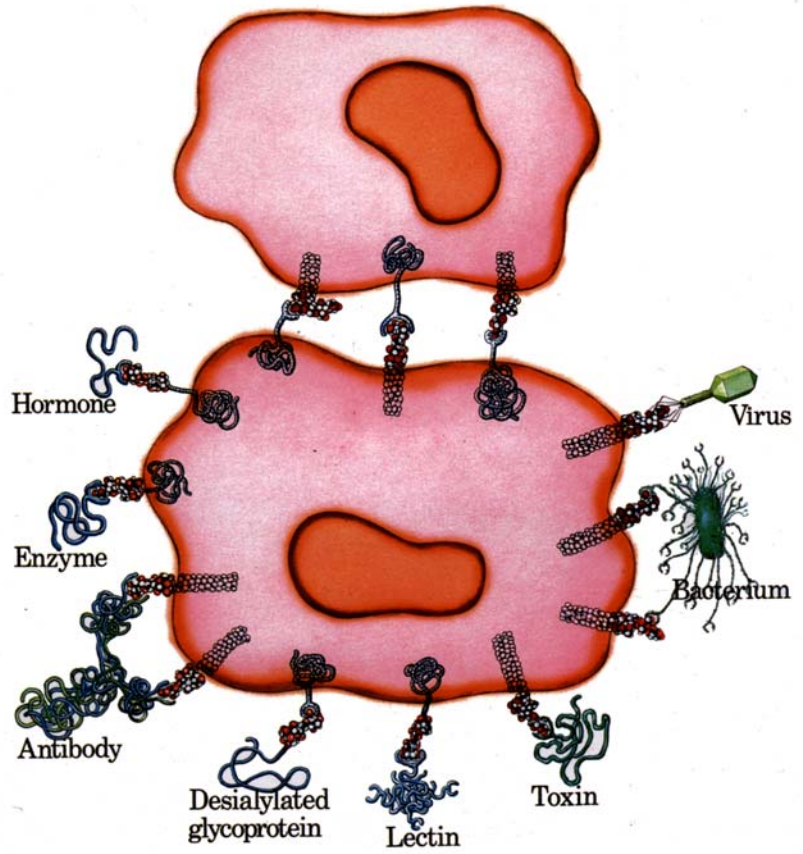


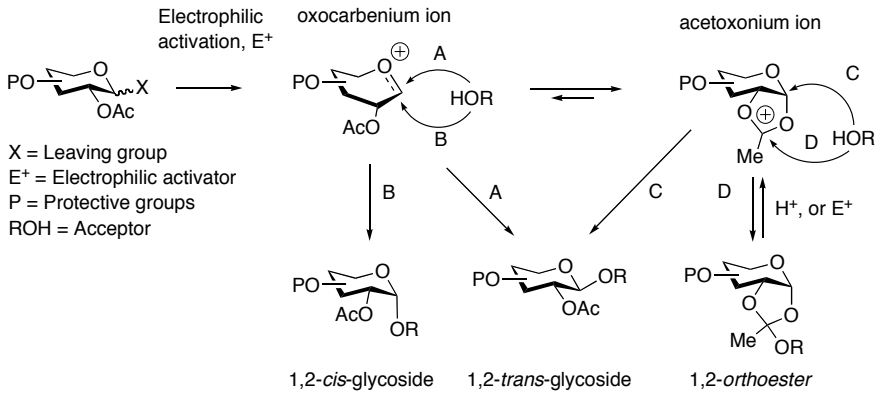
Figure 1. Schematic illustration of cell-cell and cell-external agent carbohydrate interactions. Picture taken from the former BioCarb AB catalogue.

Even short carbohydrate sequences can be used for carrying biological information and as shown in the human blood groups they may only differ in a simple change in oligosaccharide structure. The carbohydrate structure can also modify the physiochemical properties of a protein, influence and stabilize its conformation and protect the protein against proteolytic digestion. Furthermore, the oligosaccharide structure can serve as signals for protein targeting, for example the terminal sialic acid residue is vital for the survival of glycoproteins in blood. Desialylated glycoproteins are rapidly cleared from circulation by carbohydrate specific receptors on hepatocytes and macrophages. The discoveries of the important roles of carbohydrates in biological systems have made carbohydrates pharmacologically interesting as targets for drugs. Therapeutic agents based on carbohydrates or synthetic modifications thereof are used as enzyme inhibitors, immune stimulators, carditonic, anti-ulcer, anticoagulant, antiviral and antibacterials[14-20]. Due to the structural complexity of many natural bioactive carbohydrates, simplified structures and synthetic analogues are desired to create further understanding of their biological role.

1.3 Carbohydrate and Glycopeptide Synthesis

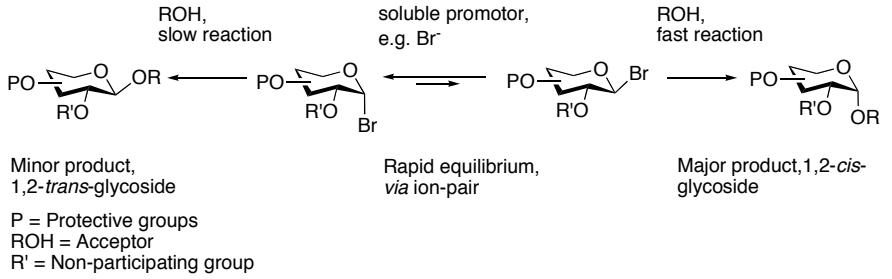
1.3.1 Carbohydrate synthesis

Since unprotected carbohydrates often are capable of existing in both pyranose and furanose forms, and have a number of hydroxyl groups which are often of similar reactivity, its necessary to use protective groups that mask the hydroxyl groups and direct the stereochemistry of glycosylation reactions. Further, the glycosidic bond can exist in two different anomeric forms, usually depicted as the α- and β-forms, it is necessary to control the stereochemistry as well as the reactivity at the anomeric center. The reactivity mainly depends on the configuration of the saccharide unit, and to a large degree also on the substitution pattern[21]. In general, acyl groups, such as acetyl and benzoyl groups, reduce the reactivity at the anomeric center, while ether groups such as benzyl groups increase it. Formation of a glycosidic bond generally involves a donor with a leaving group, an acceptor with a free hydroxyl and an activator. Nearly all methods available for the formation of the glycosidic linkage utilize a glycosyl donor that is a precursor of either an intermediate oxocarbenium ion or at least a species that has significant positive charge at the anomeric carbon atom. This high-energy intermediate is susceptible to nucleophilic attack from the acceptor present in the reaction mixture. If the protective group attached to C-2 position, e.g. an ester group, of the glycosyl donor is capable of giving anchimeric assistance (neighbouring group participation), the oxocarbenium ion, which is transiently formed, will give rise to the more stable 1,2-dioxocarbenium ion (acetoxonium ion) (Scheme 1). The dioxocarbenium ion can be steroselective opened in a *trans*-fashion and generate a 1,2-trans-glycoside (pathway C), or if the nucleophilic attack occurs at the dioxocarbenium ring, the reaction will yield an orthoester[22] (D). Pathway D is reversible, thus orthoesters are easily activated by electrophiles back to the 1,2-dioxocarbenium ions, which makes them versatile intermediates in carbohydrate synthesis.



Scheme 1. Possible reaction pathways in the glycosylation reaction of donors possessing a participating neighbouring group.

This principle of directing the stereoselectivity at the anomeric center towards 1,2-trans-glycosides has generally been very successful. The 1,2-cis-glycosylation, e.g. the formation of an α-glycoside in the galacto- and gluco-series, is much more difficult (Scheme 1). This requires a non-participating neighbouring group, such as an alkyl ether at C-2. The α-glycoside is then favoured due to the anomeric effect. However, solvent and temperature also influences the stereochemical outcome of the reaction, and generally α- and β-mixtures are formed (pathway A and B) [23-25]. α-Glycosides can also be formed by *in situ* anomerisation, a soluble catalyst is employed to cause rapid equilibration between the α- and the less stable β-glycosyl halide and nucleophilic attack of the acceptor form the α-glycoside via an S_N2 mechanism[26] (Scheme 2).



Scheme 2. The 1,2-cis glycosylation reaction, *via* the halide-ion assisted *in situ* anomerisation process.

There are a large number of glycosylating agents and donors that have been developed, the most common donors are the thioglycosides[27-29], trichloracetamidates[30, 31], glycosyl halides[24] and 4-pentenyl glycosides[32, 33]. In this thesis, thioglycosides have mainly been employed as donors, as they are stable compounds that are easy to prepare and react selectively at the anomeric center with soft activating electrophiles such as DMTST. On the other hand, hydroxyl groups of the thioglycosides are hard nucleophiles, which can be selectively functionalised using hard electrophiles, without affecting the alkyl- or arylthio group. Due to this fact, thioglycosides can be considered as highly versatile building blocks for oligosaccharide synthesis. Thioglycosides are also very useful in the preparation of the less stable bromo sugars that are useful for example in α-glycosylations, according to the previously mentioned halide-ion assisted anomerisation mechanism.

Carbohydrates can also be synthesized without using protective group manipulations. Enzymes that catalyse regio- and stereoselective couplings between saccharides are present in all living organisms. Two main classes of glycosylating enzymes exist, the glycosyltransferases using mono- or dinucleotide sugars as donors and the glycosidases, which naturally hydrolyse the glycoside bond[34-36]. Glycosyltransferases are highly specific in the formation of glycosides, however the availability of many of the necessary transferases is a limiting factor. The recent advances in genetic engineering and recombinant techniques have begun to remedy this drawback. Instead, the synthetically complex mono- and dinucleotide sugars needed as donors are often limiting for large-scale preparative applications. The glycosidases have the advantage of wider availability and lower cost, but they

are not as specific in synthetic reactions. Another drawback of glycosidases is that they are dependent of the thermodynamic equilibrium, as the reaction is reversible and therefore generally do not give high yields. In future, enzymatic methods in carbohydrate synthesis can be expected to become standard methodology. In paper II of this thesis, the substrate scope of a synthetically useful glycosyltransfease has been investigated.

1.3.2 Glycopeptide synthesis

The synthesis of glycopeptides can be realized by different strategies. Glycopeptides can be prepared either by direct attachment of the carbohydrate to the completed target peptide or by the use of a glycosylated amino acid building block in a stepwise peptide assembly. The first approach has often failed in solution synthesis of *O*-glycopeptides, due to low solubility of the peptide acceptor in organic solvents. However, peptides can be *N*-glycosylated[37] in solution and polymer bound peptides can be both *N*- and *O*-glycosylated[38-40]. Glycosyltransferases can also be used for direct *O*-glycosylation of peptides[41], but low availability of these enzymes restricts this approach. Synthesis of glycosylated amino acids for use as building blocks in a stepwise fashion is the most common method both in solution and solid phase glycopeptide synthesis.[42-48] Solid phase synthesis has become attractive due to fast assembly, the possibility of automation and reduction of the number of chromatographic purification steps. During the synthesis of building blocks suitable for *O*-glycopeptide synthesis, an activated glycosyl donor is coupled to an *N*- and *C*-terminally protected hydroxy amino acid. The *O*-glycosidic bond is sensitive to both strong acid and base. Due to this, protective groups of the glycosylated amino acid must be removable using mild conditions. The carbohydrate is usually protected with acetyl groups, which can be removed after the peptide synthesis under mild conditions, dilute methoxide in methanol[49-51] or hydrazine hydrate in methanol[52-54]. In solid phase synthesis the Nα-amino group is usually protected with the base labile fluoren-9-ylmethoxycarbonyl (Fmoc) group, which can be removed by treatment with piperidine[55, 56] or morpholine[57]. As *C*-terminal protective groups, *tert*-butyl[47, 58], allyl[59-61], benzyl[62], phenacyl[63, 64] and pentafluorophenyl[65] esters have been frequently used. All Cα-protective groups except the pentafluorophenyl ester are removed before use in the solid phase synthesis. In paper IV, analogues of the analgetically active glycopeptide contulakin-G were synthesized according to the previously mentioned stepwise glycosylated Fmoc-amino acid building block approach.

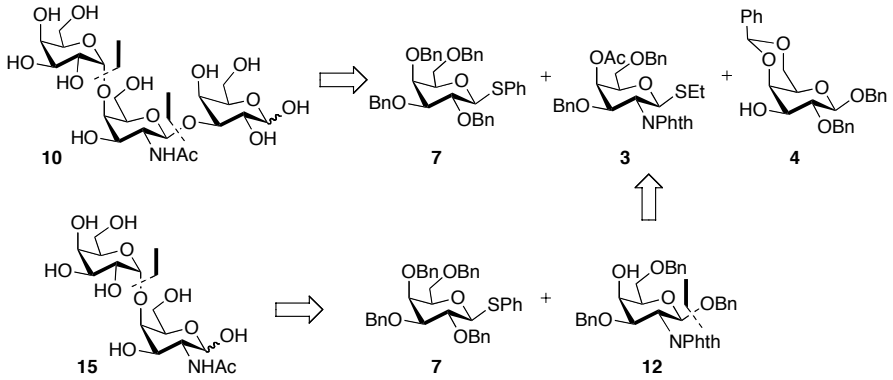
2. Synthesis of the Non-reducing Di- and Trisaccharide End of the NOR-antigen

2.1 Introduction

Human sera contain many "natural" anti-carbohydrate antibodies directed against structures absent on the cells of the host or present in a cryptic form. Polyagglutination of erythrocytes, when antibodies or lectins bind to carbohydrate recognition domains (CRD) and form cell precipitates, occur when an unusual structure is exposed on cell surface of an individual and is recognized by antibodies present in human sera. Well-known examples are the α-galactosyl antibodies in human sera that recognize the α-gal epitope, Gal-α-(1→3)-Gal-β-(1→4)-GlcNAc-R, and which are responsible for the acute rejection of xenotransplants[66, 67]. Another carbohydrate epitope terminated with an α-linked galactose residue is the extremely rare NOR antigen, a neutral glycolipid on the surface of erythrocytes responsible for the NOR polyagglutination[68]. An inherited NOR-polyagglutination has so far been identified in two families[69, 70], when the erythrocytes of these people are treated with human serum (blood group AB) aggregation occurs. This indicates a common presence of novel anti α-galactosyl antibodies in human sera. The structure of the NOR1 antigen has recently been established as Gal-α-(1→4)-GalNAc-β-(1→3)-Gal-α-(1→4)-Gal-β-(1→4)-Glc-Ceramide[68]. The non-reducing terminal end contains the unusual structural element Gal-α-(1→4)-GalNAc-β which has hitherto only been demonstrated in NOR glycolipids and in amphibium oviductal mucins[71] and not been synthesized before. In order to verify the structures and supplying material for further immunological studies, NOR1 di- and trisaccharide structures of the non-reducing terminal end was synthesized.

2.2 Synthetic strategy

Disconnection of the trisaccharide **10** gives three monomeric building blocks; **7**[72], **3**[73] and **4**[74], previously known in the literature (Scheme 3). Benzyl groups were chosen as persistent groups during the synthesis, to be able to form the α-glycosidic bond. The disaccharide building block containing building blocks **3** and **4** had to be formed first, in order to avoid having two different thioglycosides during coupling. The phthalimido group was chosen as nitrogen protecting group of the monosaccharide **3** in order to form a β-glycosidic bond between **3** and **4**. The galactose monomer **3** could be formed by inversion of configuration at the C-4 position of the corresponding easier available gluco-compound by activation and subsequent S_N2 substitution with a suitable O-nucleophile[75]. Retrosynthetic analysis of the disaccharide **15** gives two building blocks **7** and **12**. The thioglycoside donor **7** was chosen in order to form a α-glycosidic bond. The acceptor **12** could be formed from **3** by DMTST coupling using benzyl alcohol as acceptor, followed by deacetylation.

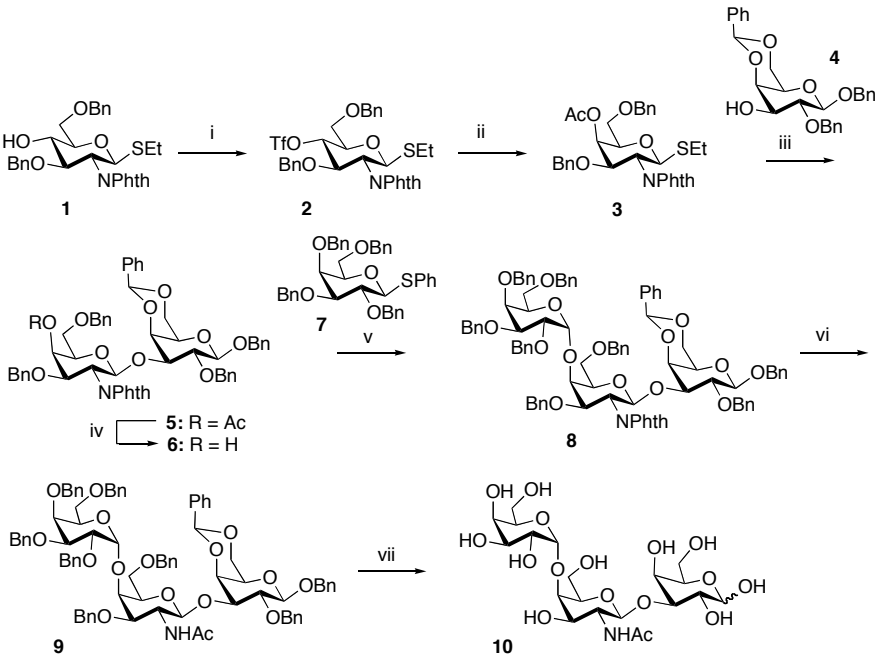


Scheme 3. Retrosynthetic analysis of NOR di- and trisaccharides.

2.3 Synthesis and evaluation

2.3.1 Synthesis

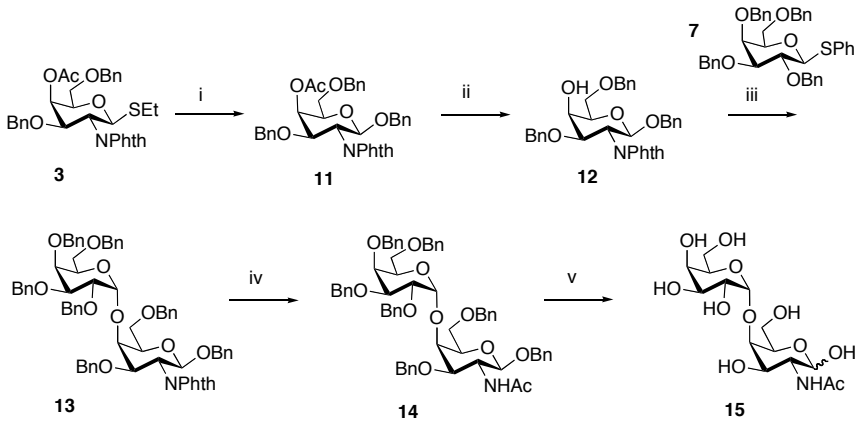
Synthesis of the trisaccharide **10** was carried out using the monosaccharide building blocks **7**[72], **3**[73] and **4**[74] (Scheme 4). The *N*-phthalimido monosaccharide **3** was synthesized using a slightly modified literature procedure. Treatment of **1**[75] with triflic anhydride gave triflate **2**, and subsequent S_N2 substitution of the triflate with cesium acetate in DMF yielded **3**, possessing the correct galacto-configuration in 4-position. Compound **3** was then coupled with acceptor **4**, which formed the β-(1-3)-glycosidic bond of **5** using DMTST as promotor. Deacetylation of the disaccharide **5** using sodium methoxide in methanol gave **6** in 37% yield calculated from **4**.[76] Compound **6** was further α-glycosylated using the tetra-benzylated thioglycoside **7** as donor and DMTST as activator, which gave **8** in 70% yield. Deprotection of the *N*-phtalimido moiety of **8** with hydrazine hydrate and subsequent *N*-acetylation gave **9** in almost quantitative yield. The synthesis of the trisaccharide was completed by catalytic hydrogenation of **9**, which gave **10** in 61% yield. The overall yield of **10** for the synthetic sequence, calculated from **1**, was 11%.



Reagents and Conditions: i) Tf_2O, Pyridine, 0 °C to rt; ii) CsOAc, DMF, rt, 72% over two steps; iii) DMTST, CH_2Cl_2, 0° C, 3 h.; iv) MeOH, NaOMe, rt, 12 h. 37% over two steps.; v) DMTST, MS 4Å, Et_2O, CH_2Cl_2, toluene, rt, 3 h, 70%.; vi) $N_2H_4{\cdot}H_2O$, toluene, EtOH, reflux, 36 h, then Ac_2O, CH_2Cl_2, rt, 2 h, 97%.; vii) Pd(C) (10%), H_2, EtOH, rt, O.N. 61%.

Scheme 4. Synthesis of NOR-trisaccharide **10**.

The disaccharide **15** was synthesized from the monosaccharide building blocks **7**[72] and **12** (Scheme 5). The monosaccharide **12** was prepared by glycosylation of the galactosamine derivative **3** using DMTST and benzyl alcohol as acceptor to give **11** in 97 % yield. Deacetylation of **11** gave **12** in 95% yield. α-Glycosylation using DMTST gave the disaccharide **13** in 66% yield. Further, phthalimido deprotection and N-acetylation gave **14** in 93% yield. Finally, catalytic hydrogenation gave disaccharide **15**. The overall yield of **15** over the synthetic sequence, calculated from **3**, was 40%.



Reagents and Conditions: i) BnOH, DMTST, Et_2O, CH_2Cl_2, toluene, rt, 3 h, 97%.; ii) MeOH, NaOMe, rt, 8 h, 95%.; iii) DMTST, MS 4Å, Et_2O, CH_2Cl_2, toluene, rt, 2 h, 66%.; iv) $N_2H_4 \cdot H_2O$, toluene, EtOH, reflux, 36 h, then Ac_2O, CH_2Cl_2, rt, 2 h, 93%.; v) Pd(C) (10%), H_2, EtOH, rt, O.N., 68%.

Scheme 5. Synthesis of NOR disaccharide **15**.

2.3.2 Biological evaluation

The ability of the synthesized oligosaccharides **10** and **15** to react with anti-NOR antibodies isolated from human sera was investigated by measuring the hemagglutination of papain treated NOR erythrocytes. The NOR-antibodies were weakly inhibited by galactose, and 8- and 2400 times stronger by the di- and trisaccharide, respectively. The reference substance α-Gal-(1→3)-Gal, known as the terminal part of the α-gal epitope, did not show any inhibition at all. To verify the specificity of these reactions, the inhibition of the anti-α-Gal-(1→3)-Gal antibodies was also investigated. These antibodies were weakly inhibited by galactose, slightly more strongly by the α-Gal-(1→3)-Gal disaccharide, while **10** and **15** were not displaying any inhibition. These results show that the disaccharide **15** and the trisaccharide **10** binds specifically to the anti-NOR antibodies and that the galactose reducing end is important for effective binding. Further biolological studies of the NOR di- and trisaccharides have been published.[77-79]

3. Synthesis of Deoxy- and Acylamino Derivatives of Lactose for use in Acceptor Studies of *N. meningitides N*-Acetylglucosaminyltransferase

3.1 Introduction

Glycosylation is a common and complex form of posttranslational modification of proteins and peptides in biological systems. The carbohydrates range from single sugars to elaborate, multibranched structures. Glycosyltransferases[80] are essential components in this post-translational machinery, which transfer monosaccharides from high-energy donors, nucleotide sugars, to saccharide acceptors or proteins. Chemically, glycosyltransferases can be used as preparative tools in laboratory synthesis of biologically active carbohydrates.[81-86] These enzymes give regio- and stereospecific glycosylations without protective group manipulations and reduce the number of synthetic steps. The bacterial β-(1→3)-*N*-acetylglucosaminyltransferase (lgtA gene from *Neisseria meningitidis*) was recently cloned and expressed in *E.coli* and acceptor and donor substrates were investigated.[87] β-(1→3)-*N*-acetylglucosaminyltransferase use galactosyl derivatives as acceptors and UDP-GlcNAc as a donor to catalyse the formation of a β-GlcNAc-(1→3)-Gal glycosidic linkage. By examining different substrates for the enzyme, it was found that only UDP-GlcNAc and UDP-GalNAc of the common nucleotide sugars could be used as donors.[87] On the acceptor side, the enzyme could glycosylate a variety of terminal α-and β-pyranose derivatives of the D-galacto configuration. It was found that *p*-nitrophenyl-β-lactoside was the best acceptor in these experiments and acceptors with a hydrophobic aglycon were more active as substrates. To expand the studies on the acceptor side, deoxy- and acylamino derivatives of phenyl β-lactoside were synthesized and used as acceptors in an enzymatic assay.

3.2 Synthetic Strategy

Retrosynthetic analysis of the 2´-deoxy derivative **24** gives that the deoxy function could be formed by reduction of a thionocarbonate or xanthate starting from a free hydroxyl group[88, 89] (Scheme 6). Selective protection of the 2´-position is readily achieved by starting from two monosaccharide building blocks, **18** and **19**. The donor **19** was selectively protected with an acetyl group in the 2´-position using a participating group during coupling to form a β-glycosidic bond and also to have a protective group that could be easily removed after coupling. The acceptor **18** could be formed by reductive benzylidene acetal opening, under conditions which selectively releases a free hydroxyl in 4´-position.

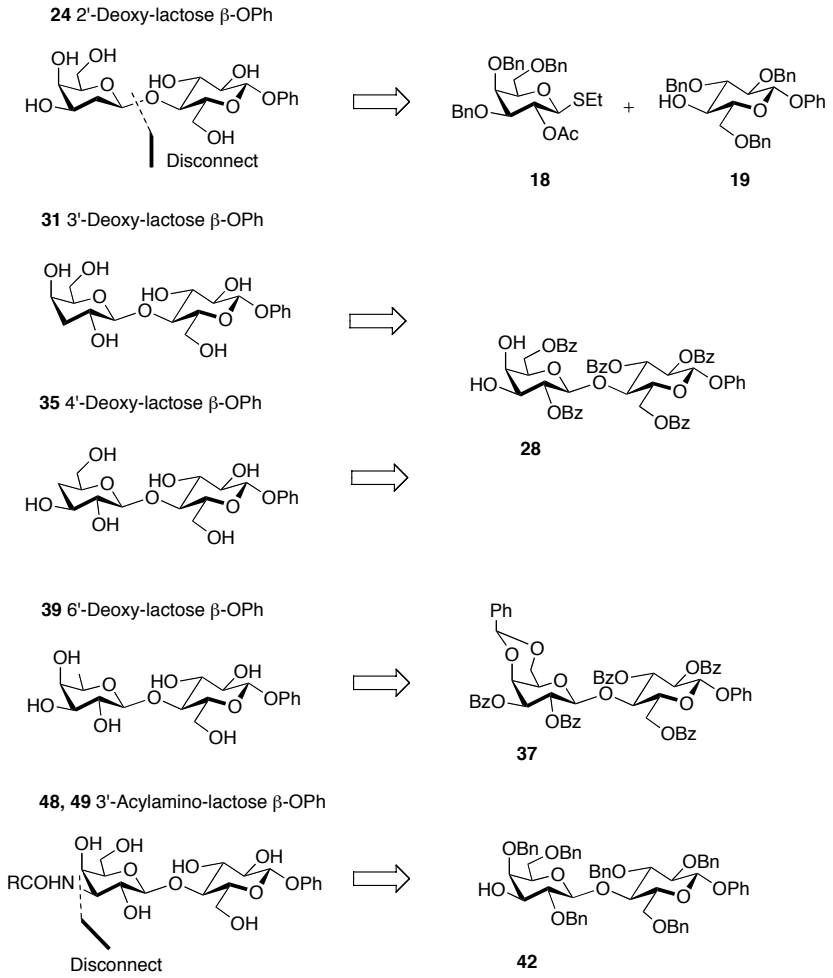
The 3´-deoxy-phenylactoside **31** could be formed using two different approaches; using the greater reactivity of the 3´-hydroxyl than the axial 4´-hydroxyl starting from **28**, or using the benzyl protected disaccharide **42**. The disaccharide **28** could be formed by hydrolysis of an 3´, 4´-isopropylidene acetal.

The benzylated disaccharide **42**, which is also used in the synthesis of the 3´-aminoacyl disaccharides **48** and **49**, could be synthesized by selective allylation in the 3´-position, followed by benzylation of the other hydroxyl groups and finally deallylation.

Compound **28** can also be used for preparation of the 4´-deoxy analogue **35** by the sequential selective 3´-benzylation, 4´-thionocarbonate formation and deoxygenation.

Analysis of the 6-deoxy derivative **39** suggests that an oxidative ring opening of the 4´,6´-benzylidene acetal could be a successful approach. By employing bromide as nucleophile in the oxidative ring opening, and choosing benzoyls as protective groups, the resulting bromine in 6-position could be removed by catalytic hydrogenation.

The 3´-acylamino derivatives **48**, **49** could be obtained by selective reduction of the 3´-oxime followed by acylation. The corresponding ketone is easily prepared from **42** by a suitable oxidation protocol. Compound **42**, as previously mentioned, is prepared by 3´-selective allylation.



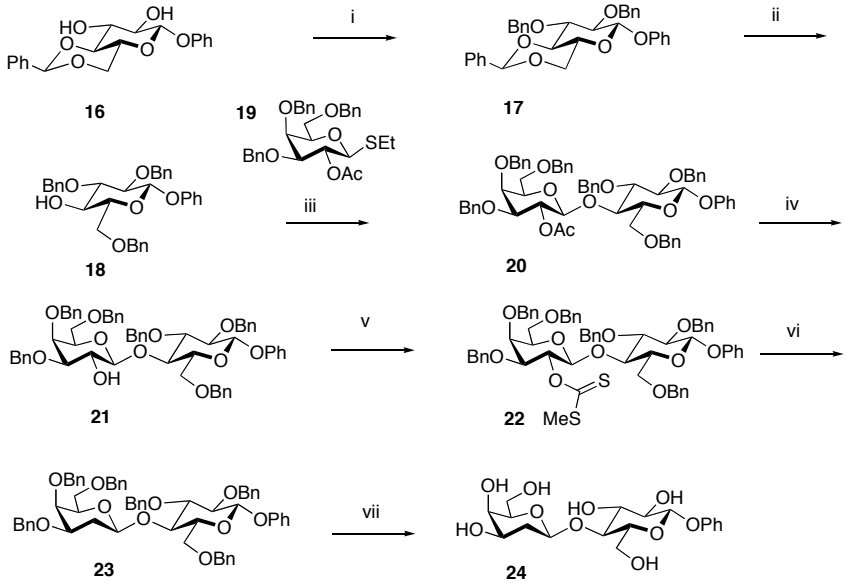
Scheme 6. Retrosynthetic analysis of deoxy- and acylamino derivatives of phenyl-β-lactoside

3.3 Synthesis and Evaluation

3.3.1 Synthesis

Synthesis of the 2´-deoxy-derivative **24** was carried out starting from the monosaccharide building blocks **18**[90] and **19**[91] (Scheme 7). The monosaccharide **18** was prepared from phenyl 4,6-O-benzylidene-β-D-glucopyranoside **16**[92] starting with benzylation using benzyl bromide/NaH in DMF, followed by selective benzylidene acetal opening using $NaCNBH_3$ and HCl, yielding the 2,3,6-tribenzyl derivative **18** in 37% from **16**. DMTST[93] mediated glycosylation of **18** with **19** gave the disaccharide **20** in 70% yield. The disaccharide was then treated with sodium methoxide in methanol to give a free hydroxyl in 2´-position **21** in almost quantitative yield. Formation of the xanthate **22** followed by tributyltin hydride reduction gave **23** in 56% yield. It is worth mentioning that it was found

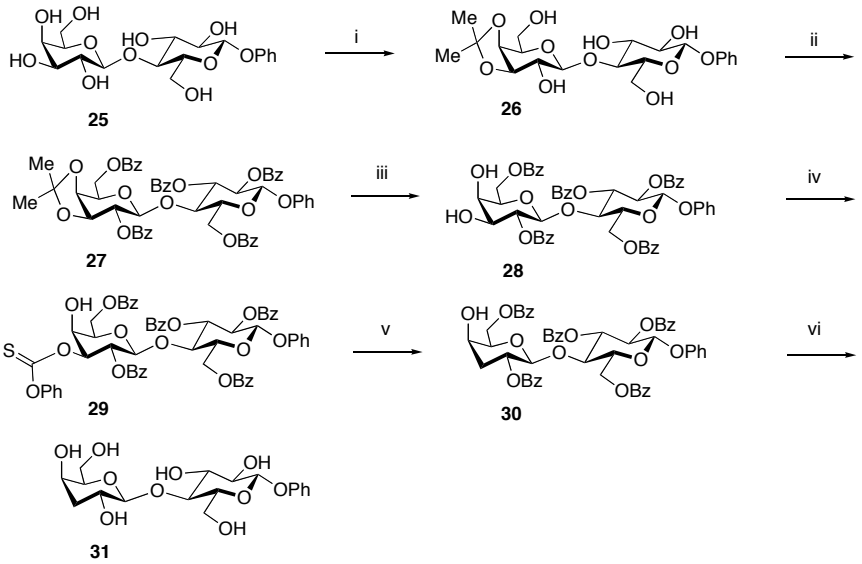
necessary to employ the xanthate as reacting group in all cases when the rest of the hydroxyls were protected as benzyl ethers. In the cases where benzoyls were chosen as protective groups, thionocarbonates worked well in the deoxygenation reactions. Finally, catalytic hydrogenation yielded the 2´-deoxy compound **24** in 94 %. The overall yield of **24** over the synthetic sequence, calculated from **16**, was 13%.



Reagents and Conditions: i) BnBr, NaH, DMF, rt, O.N.; ii) $NaBH_3CN$, HCl, Et_2O, rt, 1 h. 37% over two steps.; iii) DMTST, CH_2Cl_2, Et_2O, toluene, 2 h, 70%.; iv) MeOH, NaOMe, rt, O.N. 98%.; v) NaH, Imidazole, THF, 2 h, then CS_2, O.N. and MeI, 2 h. vi) AIBN, Toluene, 75° C, 8h, 56% over two steps. vii) Pd(C) (10%), H_2, EtOH, O.N., 90%.

Scheme 7. Synthesis of 2´-deoxy Phenyl β-lactoside **24**.

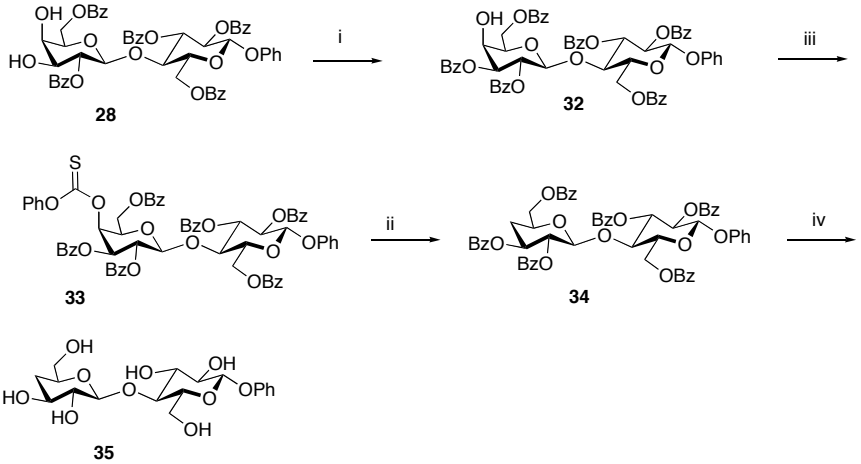
For the synthesis of the 3´-deoxy compound **31**, Phenyl β-lactoside **25**[94] was converted into the 3´,4´-diol **28** (Scheme 8). Synthesis of **28** started with 3´,4´-isopropylidene acetal formation, followed by benzoylation, to give **27** in 63% yield. Acetal hydrolysis under mild acidic conditions gave **28** in 89% yield. Selective 3´-functionalization of the diol **28** with phenylchlorothionoformate and further tributyltin-mediated radical deoxygenation gave **30** in only 8% yield. The poor yield obtained through this strategy was mainly due to the lack of protection on the 4´-position during the tributyltin reduction. Subsequent synthesis of the same compound used a different, unpublished, approach. Debenzoylation of **30** gave the 3´-deoxy target compound **31** in 88% yield. The overall yield of **31** over the synthetic sequence, calculated from **25**, was only 4%.



Reagents and Conditions: i) $Me_2C(OMe)_2$, Me_2CO, H_2SO_4, DMF.; ii) BzCl, Pyridine, 3 h. 63% over two steps.; iii) HOAc, 95° C, 1 h, 89%.; iv) DMAP, PhOC(S)Cl, CH_2Cl_2, 4 h.; v) AIBN, Bu_3SnH, Benzene, 80° C, 1 h. 8% over two steps.; vi) MeOH, NaOMe, rt, O.N., 90%.

Scheme 8. Synthesis of 3´-deoxy phenyl β-lactoside **31**.

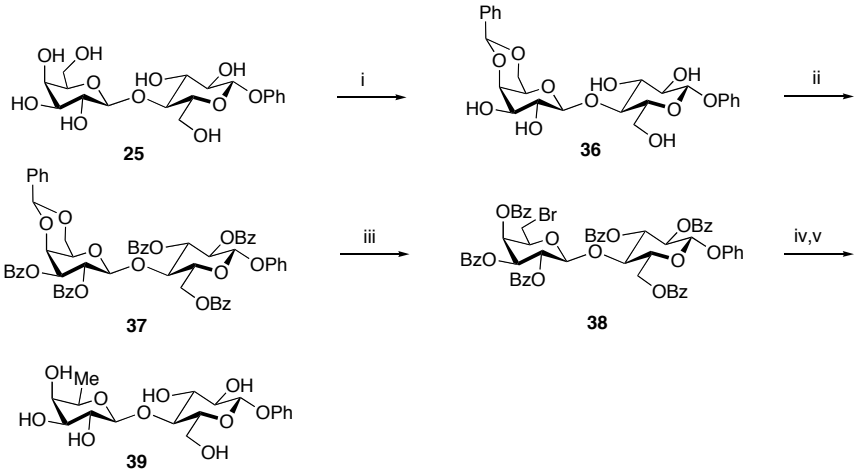
The synthesis of the 4´deoxy derivative **35** starts with monobenzoylation of the 3´-position of the previously used diol **28** (Scheme 9). Employing benzoyl chloride in pyridine, gave the 3´-protected compound **32** in 60% yield. Formation of the 4´-thionoformate, followed by radical mediated deoxygenation gave **34** in 76% yield. Debenzoylation of **34** with sodium methoxide in methanol gave the 4´-deoxy derivative **35** in 90% yield. The overall yield of **35** over the synthetic sequence, calculated from **28**, was 41%.



Reagents and Conditions: i) 1 eq. BzCl, pyridine, 3 h, 60%.; ii) DMAP, PhOC(S)Cl, CH_2Cl_2, O.N.; iii) AIBN, Bu_3SnH, Benzene, 80° C, 1 h. 76% over two steps.; iv) MeOH, NaOMe, rt, O.N., 90%.

Scheme 9. Synthesis of 4´-deoxy phenyl β-lactoside **35**.

The 6´-deoxy derivative **39** was prepared from phenyl β-lactoside **25** (Scheme 10). Starting with 4´,6´-benzylidene acetal formation, followed by benzoylation, gave **37** in high yield. NBS mediated, oxidative ring opening of the 4´,6´-benzylidene acetal[95] gave compound **38** in 29% yield. The 6´-bromo derivative **38** was dehalogenated by hydrogenation over palladium on carbon (Pd/C) and debenzoylated to give the target compound **39** in 40 % yield. The overall yield of **39** over the synthetic sequence, calculated from **25**, was 9%.

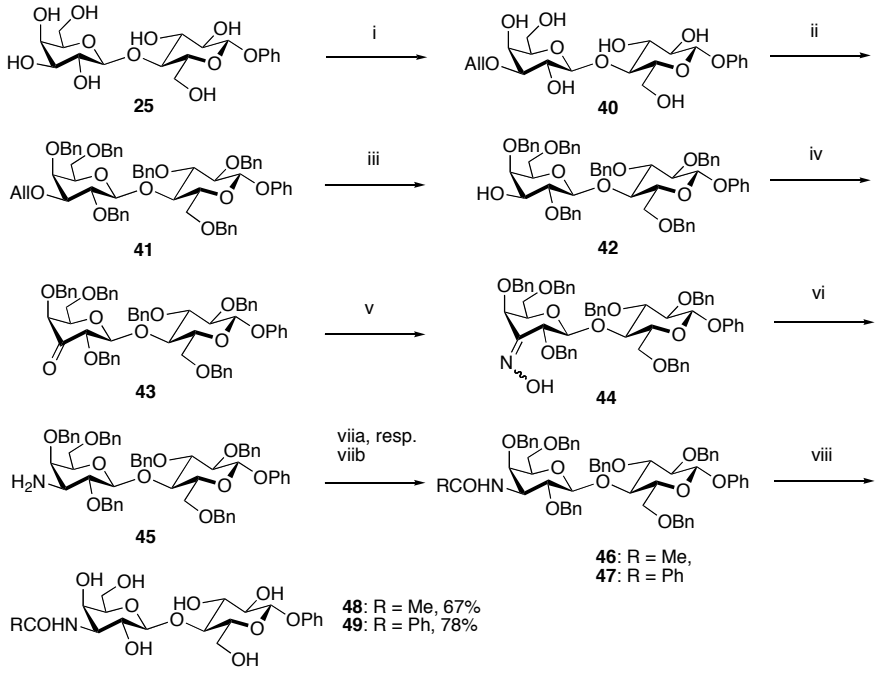


Reagents and Conditions: i) $PhCH(OMe)_2$, TsOH, DMF, 50° C, 200 mbar, 6h.; ii) BzCl, Pyridine, 3 h. 77% over two steps.; iii) CCl_4, $BaCO_3$, 1,2-dichloroethane, 85° C, 3 h, then NBS, 29%.; iv) Pd/C (10%), H_2, EtOAc:MeOH 1:1, 48h.; MeOH, NaOMe, 70° C, 30 min, 40% over two steps.

Scheme 10. Synthesis of 6´-deoxy phenyl β-lactoside **39**.

For the synthesis of the acylamino derivatives **48** and **49**, phenyl β-lactoside **25** was selectively allylated in the 3´-position with allyl bromide and dibutyl tin oxide, which gave **40** in 47% yield (Scheme 11). Benzylation using benzyl bromide/NaH in DMF, followed by a deallylation sequence, employing Wilkinson´s catalyst and DBU for the isomerisation and mild acidic hydrolysis of the resulting enol ether in acetic acid and water, gave **42** in 81 % yield. The 3´ hydroxyl was oxidized into the corresponding ketone **43** with oxalyl chloride and DMSO (Swern oxidation) in 93% yield. No epimerisation of either 2´- or 4´-positions could be detected at this point. Treatment of **43** with hydroxylamine hydrochloride under slightly basic conditions formed oxime **44** in 88% yield. Reduction of the oxime with $LiAlH_4$ in THF gave the equatorial amine **45**. Catalytic heterogeneous reduction methods, like Adams catalyst (PtO_2) and palladium on carbon (Pd/C) were also investigated, but only resulted in incomplete conversions and complex product mixtures. Isolation and purification of the free amine **45** proved to be difficult. Instead, direct treatment with an acylating reagent gave the more easily handled amides; treatment with Ac_2O gave **46** in 38% yield (over two steps), whereas treatment with benzoylchloride gave **47** in 61% (over two steps). At this stage, no axial amide products could be detected. Catalytic hydrogenation gave the 3´NHAc derivative **48** (67%) and 3´NHBz derivative **49**

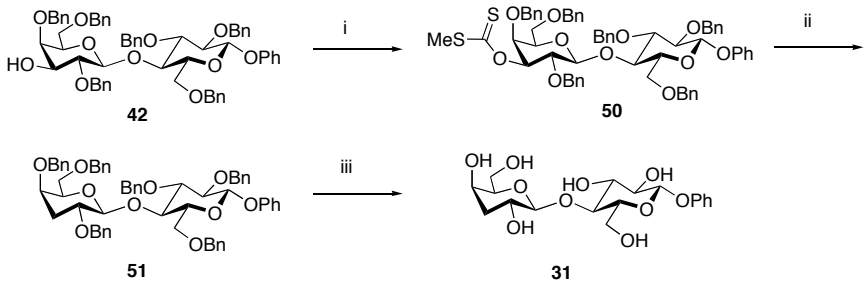
(78%) in good yields. The overall yields of **48** and **49**, starting from **25**, were 5% and 9% respectively.



Reagents and Conditions: i) $(Bu_2SnO)_2$, MS 4Å, MeCN, reflux, O.N., then Et_4NBr, AllBr, reflux, 24 h, 45%.; ii) BnBr, NaH, DMF, rt, O.N., 63%.; iii) $(PPh_3)_3RhCl$, DBU, EtOH, toluene, H_2O, reflux, 24 h, then HOAc, H_2O, 80° C, 1 h, 81%.; iv) Swern oxidation, 93%.; v) $HONH_3Cl$, pyridine, EtOH, rt, O.N., 88%.; vi) $LiAlH_4$, THF, rt, O.N.; viia) Ac_2O, pyridine, rt, O.N., 38% over two steps.; viib) BzCl, pyridine, rt, O.N., 61% over two steps.; viii) Pd(C) (10%), H_2, EtOH, rt, O.N.

Scheme 11. Synthesis of 3´-acylamino derivatives **48** and **49**.

The 3´-deoxy phenyl lactoside **31** was also synthesized by an alternative route starting from compound **42** (Scheme 12). The xanthate **50** was formed using NaH, then treated with carbon disulfide overnight, followed by alkylation with metyl iodide. In presence of the radical intiator AIBN the xanthate **50** reacted readily with tributyltin hydride forming the 3´-deoxy compound **51** in 42% yield. Finally, the benzyl groups were removed by catalytic hydrogenation forming the 3´-deoxy phenyllactoside **31** in almost quantitative yield (V, Supplementary material).



Reagents and Conditions: i) NaH, Imidazole, THF, 2 h, then CS_2, O.N., followed by MeI, 2 h. ii) AIBN, Toluene, 75° C, 8h, 42% over two steps. iii) Pd(C) (10%), H_2, EtOH, O.N., 98%.

Scheme 12. Alternative synthesis of 3´-deoxy phenyl β-lactoside **31**.

3.3.2 Evaluation of Acceptor Activity

The synthesized deoxy- and acylamino-compounds, **24, 31, 35, 39, 48** and **49** were used as acceptors in an enzymatic assay, using *N.menigitidis N*-acetylglucosaminyltransferase and ^{3}H-labeled UDP-GlcNAc as the donor (Figure 2). The radioactivity of the formed and purified trisaccharides was measured. The 3´-deoxy derivative **31**, and the acylamino compounds **48** and **49** did not function as acceptors, this was expected since they have no free hydroxyl group in 3´-position. These derivatives will later be studied as inhibitors of the enzyme. The deoxy derivatives **24, 35** and **39** were then investigated as acceptors. The deoxy derivatives **24** and **35** were less active than the reference compound **25**, while the 6´-deoxy derivative **39** showed an almost threefold increase in activity (Figure 2).

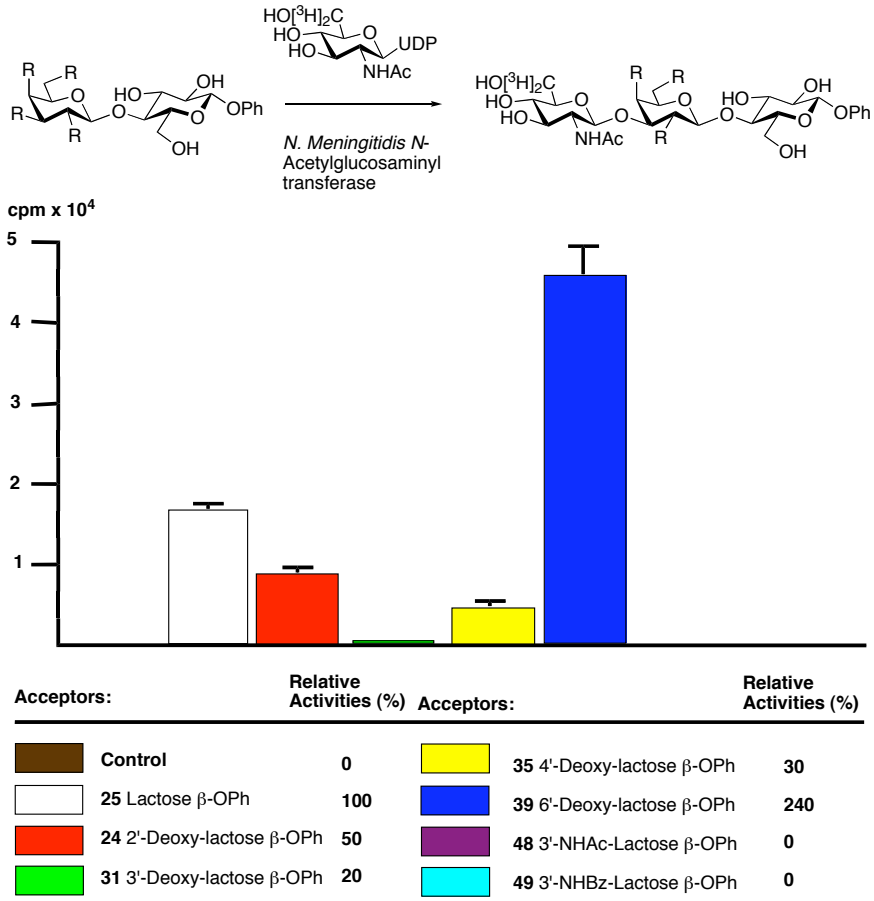
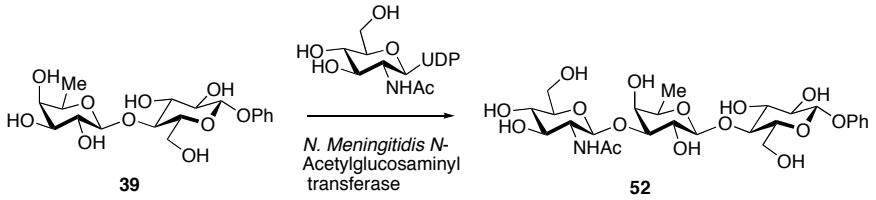


Figure 2. Acceptor activity of synthesized lactose derivatives.

The 6´-position is remote from the glycosidic bond formation and the reason for higher activity could be a product release effect, as it has one less hydrogen bond to break after coupling. To control that the 6´-deoxy-derivative was glycosylated in the normal way, a preparative enzymatic reaction was preformed employing **39** as the acceptor and UDP-GlcNAc as donor (Scheme 13). The resulting 6´-deoxytrisaccharide **52** was isolated and characterized, and proved to be the expected glycosylation product.



Scheme 13. Preparative enzymatic synthesis of 6´-deoxytrisaccharide **52**.

In conclusion, three acceptors **24**, **35** and **39** and three inhibitors **31**, **48** and **49** of *Neissera meningitides N*-acetylglucosaminyltransferase have been synthesized. These analogues of phenyl β-lactoside **25** were tested in a radioactivity based enzymatic assay. The 6´-deoxy derivative **39** was almost three times more active than the phenyl β-lactoside **25** (reference compound), and the 2´-deoxy **24** and 4-deoxy **35** derivatives were less active. As expected, the inhibitors **31**, **48** and **49** were not active. The inhibitory capacity of the synthesized derivatives and the 3-dimentional details of the enzyme–substrate interaction will be further studied, using NMR and X-ray crystallography.

4. Synthesis of Biotinylated Ligands for Optimization of Binding to the Asialoglycoprotein receptor

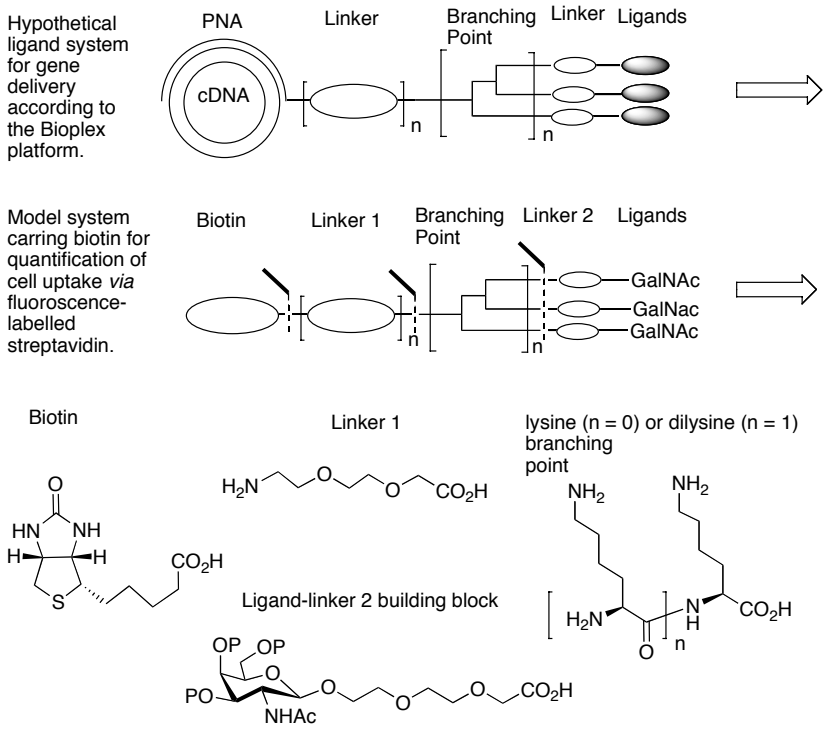
4.1 Introduction

The nature of the terminal sugar residues that cap the carbohydrate chains of glycoproteins is a major factor in carbohydrate mediated cell-cell and receptor-ligand interactions. An example of such a process is the binding and uptake of desialylated serum glycoproteins mediated by the asialoglycoprotein receptor (ASGPr)[9] found on and selectively expressed by hepatocytes. This endosomal uptake leads to clearance of the proteins from circulation.[96-98] The ASGPr interacts with terminal Gal or GalNAc residues of desialylated serum glycoproteins and with synthetic cluster glycoside ligands that mimics these ligands.[99-108] Uptake of glycoproteins by this receptor is both a high-affinity and high-capacity process. This feature, combined with its specific location on the liver cell, makes it an attractive target system for delivery of drugs and genes to this metabolically important cell. In order to direct and deliver foreign DNA in a stable way into cells in vivo and to promote efficient expression, delivery vectors are required. To date, viral vectors have been used with superior efficacy, however, issues concerning the safety of the methods have been raised leading to an interest in developing safer alternatives. The Bioplex system for non-viral gene delivery takes advantage of the specific and stable interaction between peptide nucleic acids (PNA) and DNA for anchoring peptide functions to a plasmid vector.[109-113] This approach provides high flexibility in the composition of the vector and results in well-defined and stable carrier-complexes with the potential of becoming efficient delivery vectors. To adapt the Bioplex vector for hepatocyte targeting, a synthetic high affinity ligand must be synthesized and covalently linked to a PNA.[114] To achieve a specific and high uptake into the target cells and for subsequent gene expression, optimisation of the targeting ligand is necessary. Structure-activity studies of synthetic galactoside ligands have shown that binding to the ASGPr is highly dependent on the number of terminal sugars, the distance and three-dimensional arrangement of the galactosyl residues.[103, 115] The affinity to the ASGPr increases 100-1000 fold for each sugar from mono- to tri-antennary galactosides.[116, 117] Further increasing of galactosyl residues has only shown a modest effect. Gal-containing constructs have shown high affinity for the ASGPr when long spacers of >20Å are used[118]. Targeting of the related macrophage receptor on Kupffer cells was observed if the spacers were too short.[119, 120] To gain further insight in the structure-binding relationship and to measure the binding of ASGPr ligands to target hepatocyte cells, biotin-labelled di-, tri-, and hexa-GalNAc-containing ligands have been synthesized. GalNAc residues was used instead of Gal since they display 10-50 fold higher affinity.[102, 121] The ligands also have spacers with more than 20Å distances between the GalNAc residue to the branching point for further increase of the binding to the ASGPr.

4.2 Synthetic Strategy

Biotinylated clusters containing GalNAc ligands were designed to study and optimize binding and uptake to ASGPr. The reason to synthesize biotinylated clusters was to use their ability to bind to fluorescent labelled streptavidin, and be able to measure the binding and uptake into HepG2 cells using FACS.

The synthetic strategy for the synthesis of biotinylated cluster glycosides was to use biotinylated lysine coupled to one or more dioxaoctanoic acid linkers (linker 1). Further coupling with one ore two lysine branching units, followed by coupling with the GalNAc-linker acid (ligand-linker 2) gave the complete biotin labeled GalNAc construct (Scheme 14).



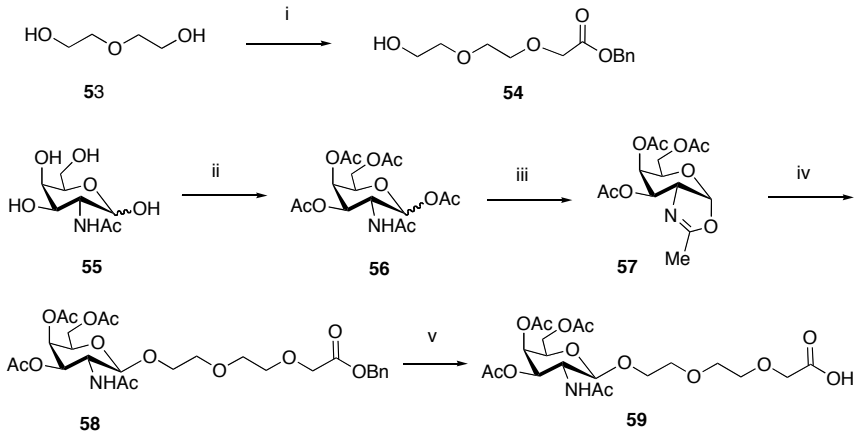
Scheme 14. Conceptual design and retrosynthetic analysis of ligand-linker system for gene delivery.

4.3 Synthesis and Biological Evaluation

4.3.1 Synthesis

The central building block in the solid-phase synthesis was the *O*-acetylated GalNAc residue linked through a diethylene glycol spacer ending with a carboxylic acid function, compound **59** (Scheme 15). This carboxylic acid was synthesised starting from the building block **56** and **54**. The linker **54** was prepared by anion formation of ethylene glycol **53** and treatment with bromoacetic acid obtaining a carboxylate, which was *in situ* benzylated with benzyl bromide to give **54** in modest yield (19%). Due to its simple character and cheap starting materials,

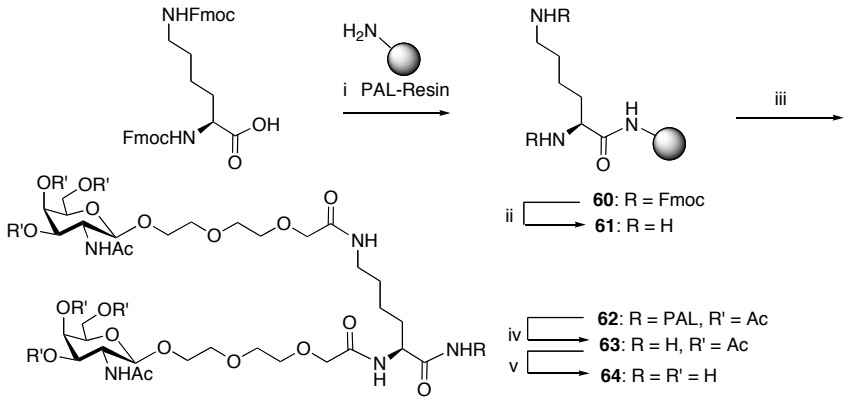
this reaction could be performed on large scale and pure product could easily be isolated by flash chromatography. Per-acetylation of *N*-acetylgalactosamine **55** gave **56** in 95% yield. The oxazoline **57** was prepared by treatment of **56** with TMSOTf in DCM followed by direct coupling with the spacer **54** yielding the GalNAc-linker compound **58** in 79% yield over two steps. Debenzylation of **58** gave the free carboxylic acid **59** in good yield. The overall yield of **59**, calculated from *N*-acetylgalactosamine **55**, was 45%.



Reagents and conditions: i) NaH, $BrCH_2CO_2H$, then BnBr, 19%.; ii) Ac_2O, Pyridine, rt, O.N. 95%.; TMSOTf, rt, CH_2Cl_2.; iii) TMSOTf, Compound **54**, CH_2Cl_2, reflux. 79% over two steps.; v) Pd(C), H_2, EtOAc, 60% after chromatography.

Scheme 15. Synthesis of GalNAc ligand building block **59**.

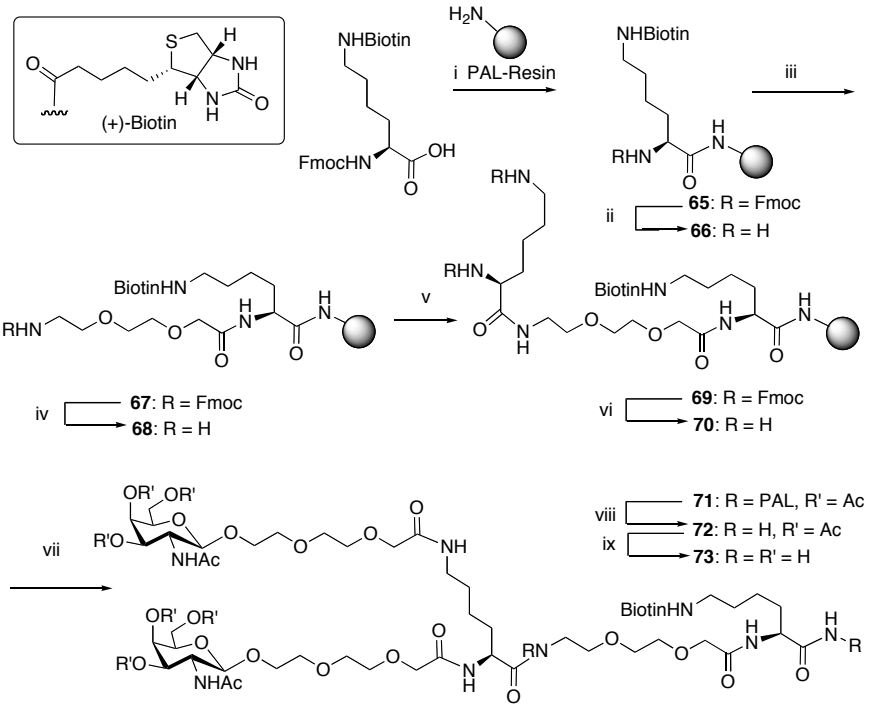
Solid-phase synthesis of the di-GalNAc construct **64**, which should be used as a reference compound in the biological studies, was carried out (Scheme 16). Starting with deprotection of the Fmoc-group from PAL resin (loading level 0.4-0.7 mmol g^{-1}) followed by coupling of di-Fmoc-lysine using PyBOP/DIPEA in DMF gave **60**, again Fmoc deprotection using 20% piperidine to give a di-amine **61**. Coupling of the GalNAc-linker compound **59** and cleavage from resin using 95% TFA, 5% H_2O gave **63**. Deacetylation of the sugar residue gave **64** in 15-29%.



Conditions and Reagents: i) PyBOP, DIPEA, DMF.; ii) Piperidine, DMF.; iii) Carboxylic acid **59**, PyBOP, DIPEA, DMF.; iv) TFA, rt.; v) MeOH, NaOMe, rt.

Scheme 16. Synthesis of di-GalNAc –lysine building block **64**.

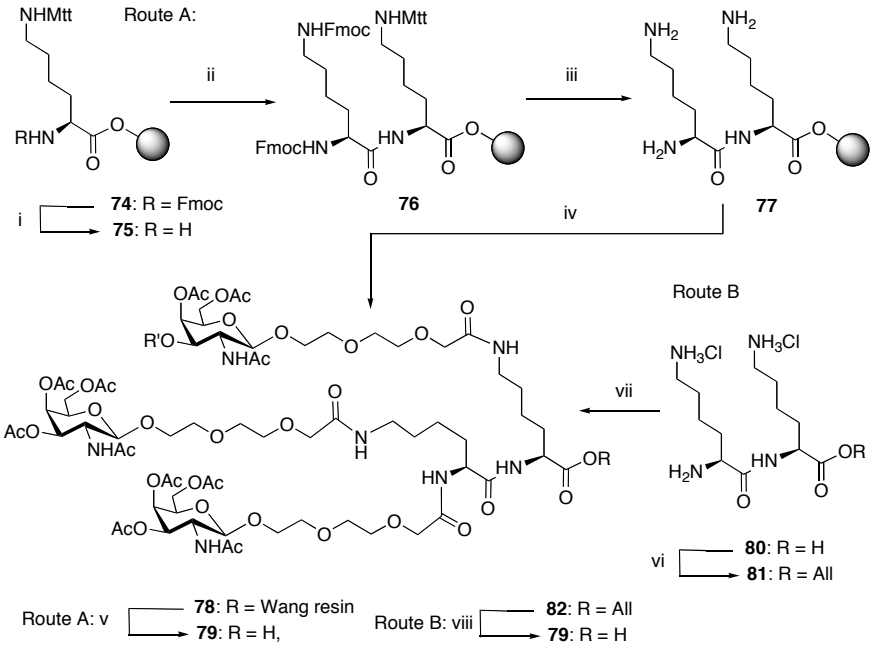
The Biotin-labelled di-GalNAc construct **73** was synthesized starting with coupling of a biotinylated di-Fmoc-lysine to Pal resin followed by Fmoc deprotection to give **66** (Scheme 17). The amine was coupled with the spacer unit, Fmoc-amino-3,6-dioxaoctanoic acid and treated with piperidine to give **68**. Coupling with di-Fmoc-lysine and removal of the Fmoc protecting groups gave **70**. The di-amine was then coupled with the GalNAc-linker compound **59** and then cleavage from resin gave **72**. Finally, deacetylation using sodium methoxide in methanol gave **73** in 25-46% overall yield.



Conditions and Reagents: i) PyBOP, DIPEA, DMF.; ii) Piperidine, DMF.; iii) 8-(fluorenylmethoxycarbonylamino)-3,6-dioxaoctanoic acid, PyBOP, DIPEA, DMF.; iv) Piperidine, DMF.; v) *N*(α),*N*(β)-di-Fmoc-L-lysine, PyBOP, DIPEA, DMF.; vi) Piperidine, DMF.; vii) Carboxylic acid **59**, PyBOP, DIPEA, DMF.; viii) TFA, rt.; ix) MeOH, NaOMe, rt.

Scheme 17. Synthesis of biotin labeled di-GalNAc construct **73**.

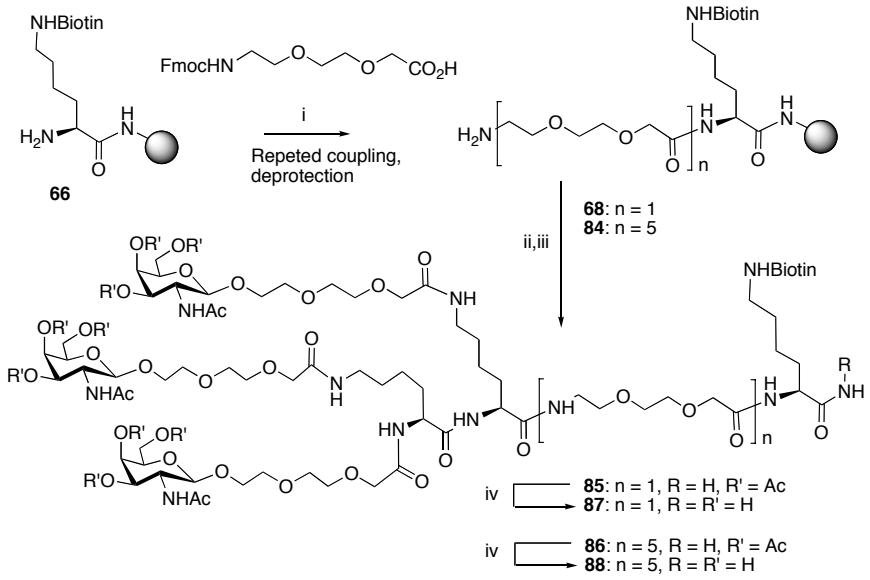
The tri-GalNAc building block **79** was prepared by two different routes, either using solid-phase synthesis or in solution (Scheme 18). For the preparation of **79** by solid-phase synthesis, commercial *N*-α-Fmoc-*N*-ε-Mtt-Lysine-Wang resin **74** was treated with piperidine to give the free α-amine **75** and then coupling with di-Fmoc-Lysine gave **76**. Mtt deprotection using 1% TFA, 5% TIPS in DCM followed by Fmoc removal gave the tri-amine **77**. Coupling with the GalNAc-linker compound **59** gave the resin-bound compound **78**, cleavage from resin gave the tri-GalNAc linker compound **79** in 34% yield, calculated from loading of lysine on the Wang resin. For the preparation of **79** by solution-phase chemistry, commercial di-lysine hydrochloride **80** was treated with TMSCl in the precensce of a large excess allyl alcohol to produce the allyl ester **81** in 98% yield. Coupling with the GalNAc-linker **59** using DCC/HOBt gave compound **82** (80%) and finally deallylation yielded **79** in 69%. As the overall yield of the solution phase route B was 54%, compared with 34% for the solid phase synthesis route A, it is clear that the tri-GalNAc building block **79** should be prepared by solution phase chemistry.



Conditions and Reagents: i) Piperidine, DMF.; ii) *N*(α),*N*(β)-di-Fmoc-L-lysine, PyBOP, DIPEA, DMF.; iii) 1% TFA, 5% TIPS in CH_2Cl_2; then Piperidine, DMF ; iv) Carboxylic acid **59**, PyBOP, DIPEA, DMF.; v) TFA:H_2O 95:5. 34% over 6 steps.; vi) AllOH, TMSCl, 98%.; vii) Carboxylic acid **59**, PyBOP, DIPEA, DMF, 80%.; viii) $(PPh_3)_4Pd$, Morpholine, THF, rt, 69%.

Scheme 18. Two alternative synthetic routes to tri-GalNAc building block **79**, by either solid phase synthesis (route A), or solution phase synthesis (route B).

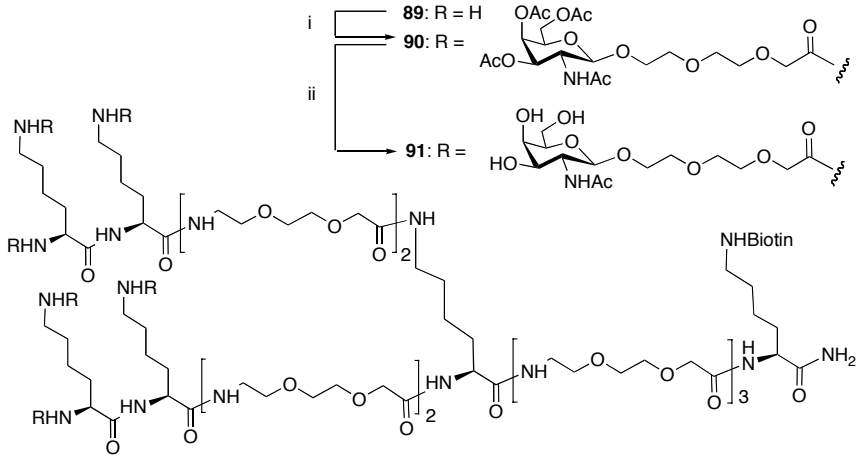
The biotin-labelled tri-GalNAc construct was synthesized using one or five linkers to produce **87** and **88** (Scheme 19). Coupling of compound **66** with one linker followed by Fmoc removal gave compound **68** and coupling with 5 linkers and Fmoc removal gave **84**. Coupling of **68** and **84** with the tri-GalNAc building block **79** followed by cleavage from resin gave **85** and **86** respectively. Deacetylation gave **87** in 13-24% yield (from PAL resin) and providing **88** in 3-5% yield, calculated from PAL resin loading. The low yield of **88** was partly caused by incomplete product separation on gel filtration and only a few relatively pure fractions were selected and pooled. The small scale (5-10 μmol) was also a reason for the lower yield.



Conditions and Reagents: i) 8-(fluorenylmethoxycarbonylamino)-3,6-dioxaoctanoic acid, PyBOP, DIPEA, DMF; then piperidine, DMF.; ii) carboxylic acid building block **79**, PyBOP, DIPEA, DMF.; iii) TFA.; iv) MeOH, NaOMe, rt.

Scheme 19. Synthesis of biotin-labelled tri-GalNAc constructs **87** and **88** with one respective five spacer units.

The biotin-labelled hexa-GalNAc construct **91** was prepared by coupling of the hexamine **89** (obtained by custom synthesis from a commercial source) with the GalNAc linker **59** using TSDU activation to give **90** in 63% yield (Scheme 20). Finally, deacetylation gave the biotinylated hexa-GalNAc construct **91** in 50% yield.



Conditions and Reagents: i) Carboxylic acid **59**, TSDU, DMF.; ii) MeOH, NaOMe, rt.

Scheme 20. Synthesis of biotin-labelled hexa-GalNAc construct **91**.

4.3.2 Biological Evaluation

To evaluate the ability of the biotin-labelled GalNAc constructs **73, 87, 88** and **91** to mediate ASGPr specific binding and uptake into human hepatocytes, fluorescent- labelled streptavidin was functionalized with the ligand constructs and incubated with ASGPr expressing human HepG2 cells. The uptake was monitored by FACS analysis of the cells.[122] The hexa-GalNAc construct **91** resulted in the most efficient uptake, 90%. The di-and tri-GalNAc ligands **73** and **87** showed only a moderate uptake, but the lower uptake was probably not only an effect of the number of GalNAc units. The tri-GalNAc construct **88** containing five linkers showed only slightly lower uptake, 80%, than the hexa-GalNAc **91**.

In conclusion, the biotin-labelled di-, tri-and hexa-GalNAc constructs **73, 87, 88,** and **91** have been synthesized and the selective ASGPr uptake into human liver cells have been studied. The length of the spacer between the GalNAc ligands seems to be important, while increasing the number of GalNAc units above three seems only to have a minor contribution to the overall affinity.

5. Synthesis of *Conus* Glycopeptides

5.1 Introduction

Cone snails (genus *Conus*) are marine predators harpooning their prey, injecting immobilizing venoms. There are over 500 species of cone snails, mainly fish hunting, but also worm and mollusc hunting types, living on or near coral reefs in tropical waters. Cone snails have a highly sophisticated venom production apparatus and delivery system. An example of the latter is the specialized teeth, which in effect serve both as harpoon and disposable needle for venom delivery[123]. Since the cone snails cannot swim and move relatively slowly compared to other ambulatory predators, it became necessary to develop effective defence and hunting strategies. The fast-acting venoms immobilise their prey, which is subsequently engulfed and digested by the snail (Figure 3).

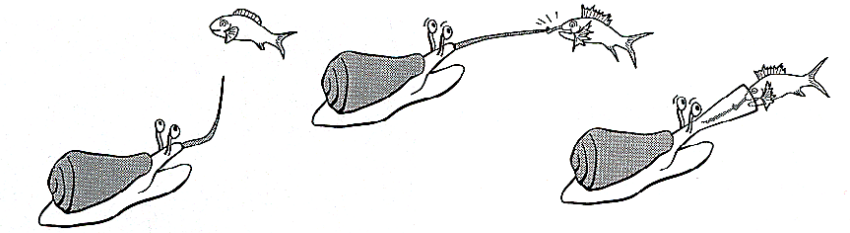


Figure 3. A cartoon representing the harpoon strategy of a fish-hunting cone snail. Picture reproduced from reference 126.

The venoms of these snails are neuropharmacologically interesting, with a composition of 50-200 components per species, mainly small peptides, composed of 10-35 amino acids[124-129]. Each *Conus* species has evolved its own large molecular repertoire of venom component, different from every other *Conus* species. This means that more than 50 000 different pharmacologically active components are present in the venoms of all living cone snail species. Only a small part of the conopeptides have been characterized and physiologically studied, but have shown diverse functionality mainly targeting voltage or ligand gated ion channels or G-protein coupled receptors. *Conus* peptides that target voltage gated ion channels include those that delay the inactivation of sodium channels as well as those that specifically block sodium channels, calcium channels and potassium channels. Peptides that target ligand gated ion channels include antagonists of *N*-methyl-D-aspartate (NMDA) and serotonin receptors (5-HT) as well as competitive and non-competitive nicotinic receptor antagonists (nAChR). Peptides that act on G-protein coupled receptors include neurotensin (NTR) and vasopressin receptor agonists. The peptides ability to discriminate between closely related molecular isoforms of members of a particular receptor family, leading to high specificity and high potency, together with their small size which make them relatively easy to synthesize, have altogether led to that several *Conus* peptides are in various stages of clinical development for treatment of human disorders[130]. Venom peptides need to be sufficiently stable to survive chemical degradation in

solution at ambient temperature and enzymatic degradation by processing proteases present in the venom itself, as well as those in the tissues of prey species. This stability is often achieved naturally through the use of post-translational modifications and/or use of disulfide bonds[131], which fold the peptide into a stabilized structure. Typical post-translational modifications include amidation (C-terminal), sulphation (tyrosine)[132], bromination (tryptophan)[133-135], glycosylation (threonine or serine)[135-137], γ-carboxylation (glutamate)[138-140], hydroxylation (proline)[135, 141, 142], pyroglutamation (cyclization of glutamate)[137, 143], and isomerization to D-amino acids[144, 145]. Glycosylation have hereto only been found in three *Conus* peptides; contulakin G from *C. geographus*[137], κA-conotoxin SIVA from *C. striatus*[136] and tx5a from *C. textile*[135, 146-148].

5.2 Synthesis of Contulakin-G analogues

5.2.1 Introduction

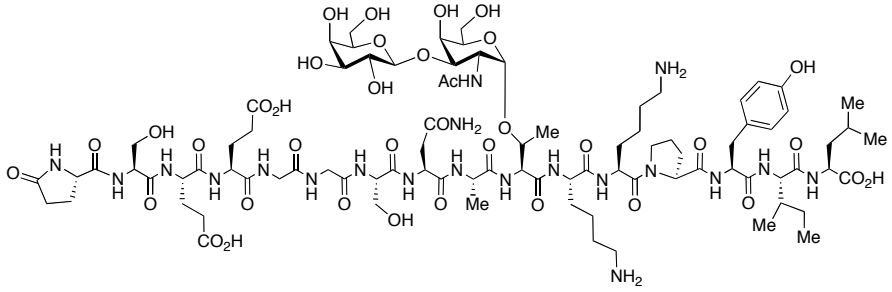


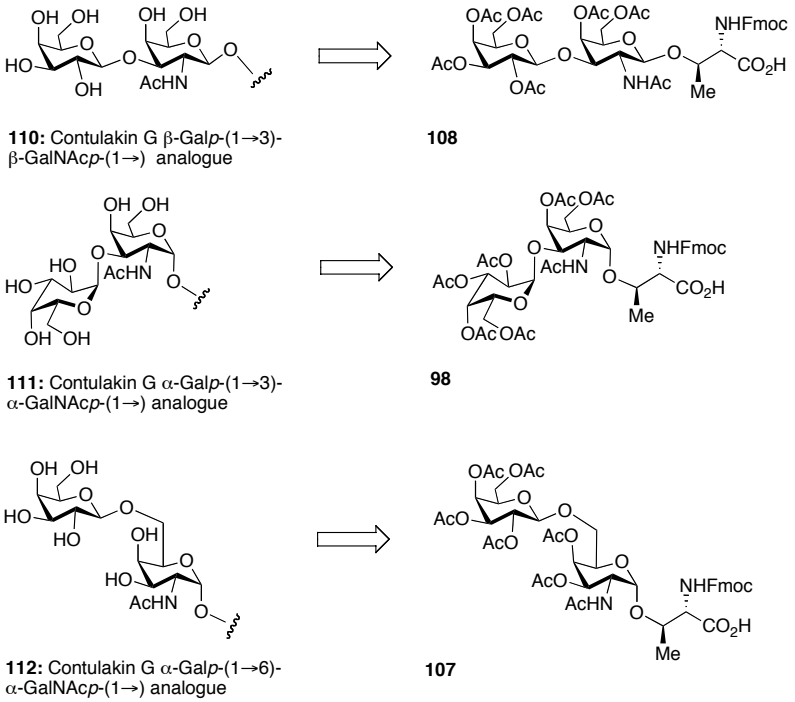
Figure 4. Native contulakin-G.

Contulakin-G is the first *O*-glycosylated *Conus* peptide for which the complete structure of both peptide and glycan has been determined (Figure 4). Contulakin-G was found to be a 16 amino acid glycopeptide with the sequence ZSEEGGSNAT*KKPYIL where the *N*-terminal was blocked with a pyroglutamate residue and Thr 10 was glycosylated with the disaccharide Gal-β-(1→3)-GalNAc-α(1→)[137, 149]. The C-terminal of contulakin-G shows a high degree of sequence similarity to the neurotensin family of peptides. Contulakin-G is the first member of the neurotensin family of peptides isolated from an invertebrate source. It binds to three known neurotensin receptor subtypes, human neurotensin type 1 receptor (hNTR1), rat neurotensin type 1 and type 2 and the mouse neurotensin receptor type 3. Contulakin-G has shown to be a potent broad spectrum analgesic in a number of models of acute pain, and even more potent *in vivo* than the known analgesic neurotensin[150]. The toxicity of contulakin-G also appears to be much lower compared with neurotensin. In recognition of these properties contulakin-G has entered pre-clinical trials for short-term management of post-operative pain[130]. The biological *in vivo* activity of contulakin-G was compared with the peptide lacking the glycan and the activity of glycosylated contulakin-G was found to be significantly higher[137]. The glycan part seems to be important for activity and there are several possible reasons for this fact. For example, increased stability to proteolytic degradation, an enhanced uptake through the blood brain barrier via facilitated diffusion transport (the glucose

transporter, GLUT-1), or a conformational change of the peptide and/or a change in binding to the receptor could be favourable for activity. In order to study the importance of the disaccharide structure of contulakin-G three analogues have been synthesized. Native contulakin-G has a β-glycosidic bond between the Gal-GalNAc unit and a α-glycosidic bond between the GalNAc residue and threonine, the configuration in either of this positions have been altered in order to get information of the sterochemical relevance. By changing from the natural (1→3)-Gal-GalNAc bond to (1→4) or (1→6) the importance of the configurational pattern can be studied. The (1→6) disaccharide was considered as prime synthetic target, since the configurational difference is larger between (1→3) and (1→6) than between (1→3) and (1→4). The 3-dimensional solution structure of the contulakin-G analogues will be investigated by NMR spectroscopy. The biological *in vivo* activity of these analogues will also be studied.

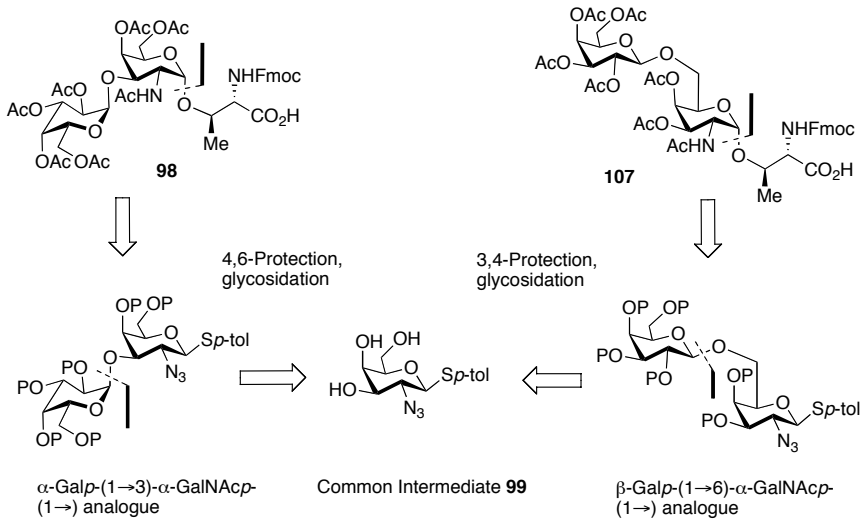
5.2.2 Synthetic Strategy

The contulakin-G analogue glycopeptides **110, 111** and **112** could be synthesized from their respective glycosylated amino acids in a stepwise fashion, starting with the C-terminal amino acid attatched to Wang resin. The β-Thr analogue **110** could be prepared from the amino acid building block **108**, obtained according to the literature[42]. The α-Gal-(1→3)-α-GalNAc-(1→) analogue **111** was prepared from the glycosylated amino acid **98**, which could be formed by coupling of a benzylated disaccharide and a *N*-Boc and *C*-*t*-Bu protected threonine. Use of an Fmoc protected threonine was not suitable, since the benzyl groups of the formed disaccharide amino acid had to be removed after coupling, using catalytic hydrogenation, conditions that Fmoc-protective groups usually do not survive. The disaccharide could be formed by halide ion assisted glycosylation of monosaccharides. Benzyl groups was used on the Gal monomer in order to form a α-glycosidic bond and an azide group was used in C-2 position of the GalN-monosaccharide in order to have a non-participating group during the later coupling with threonine. The GalN monosaccharide used in the disaccharide coupling could be formed by 4,6-benzylidene protection of the intermediate **99**.



Scheme 21. Retrosynthetic analysis of Contulakin-G analogues.

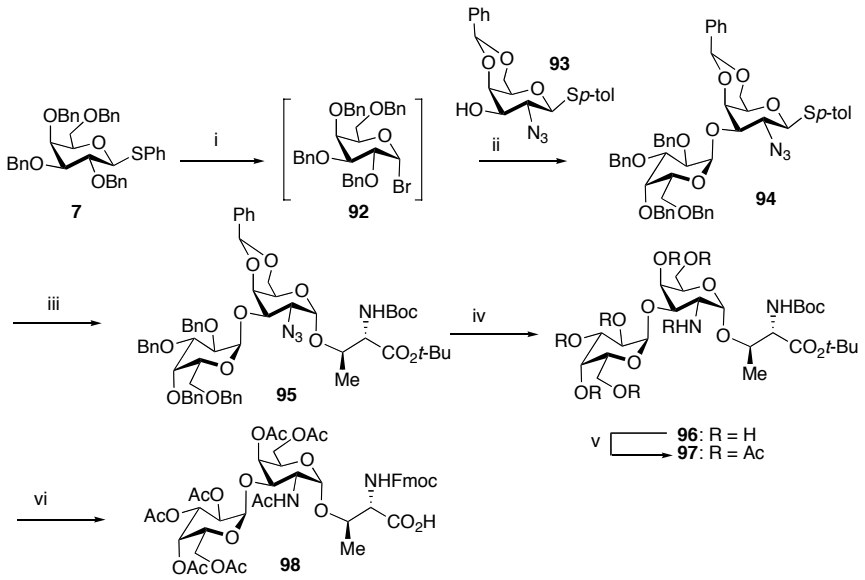
The β-Gal-(1→6)-GalNAc analogue **112** could be prepared from the amino acid building block **107** (Scheme 22). The amino acid **107** could be obtained by coupling of a disaccharide with Fmoc-threonine phenacylester. The disaccharide could be prepared by AgOTf promoted coupling of acetobromogalactose and a 6-hydroxy monosaccharide. The 6-hydroxy monosaccharide building block could be formed by 3,4-isopropylidene protection of **99**. By choosing this strategy, both (1→3)- and (1→6)-analogues could be prepared from the common intermediate **99**.



Scheme 22. Synthesis of both (1→3)- and (1→6)-analogues from the common intermediate **99**, by either 4,6- or 3,4-protection strategy.

5.2.3 Synthesis

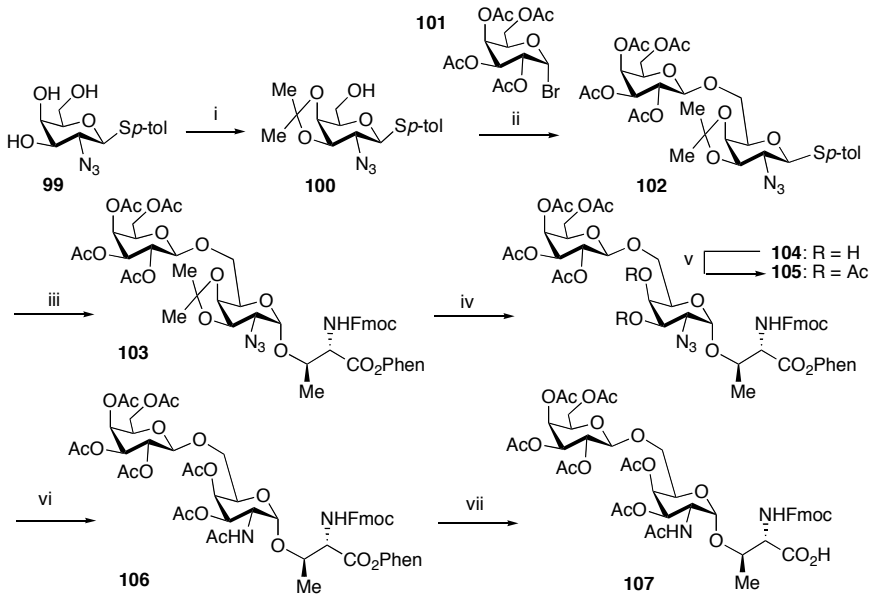
The glycosylated amino acid **108** was prepared according to the literature[42]. The disaccharide-Fmoc amino acid **98** was obtained starting from the monosaccharides **7** and **93** (Scheme 23). The fully benzylated galactosyl bromide **92** was obtained *in situ* from the thioglycoside **7**[72] using bromine, followed by halide ion assisted coupling with **93**[42] forming the disaccharide **94** in 63 % yield. The disaccharide was used in glycosylation of Boc-threonine t-butyl ester[151] employing DMTST as promotor, which gave the glycosylated amino acid **95** in 31% yield. This low yield could not be improved, although many attempts were made to tune the reaction conditions. Catalytic hydrogenation, followed by acetylation yielded compound **97** in 64%. Removal of the *N*-Boc and *t*-Bu protective groups using 50 % TFA in dichloromethane, followed by treatment with Fmoc-*O*-succinimide gave the Fmoc protected amino acid **98** in 77% yield. The overall yield of **98** over the synthetic sequence, calculated from **93** was 10 %.



Reagents and Conditions: i) Br_2, *n*-Bu_4NBr, CH_2Cl_2.; ii) *n*-Bu_4NBr, CH_2Cl_2, O.N., 63%.; iii) DMTST, *N*-Boc-*t*-Bu-threonine, 31%.; iv) Pd(C), H_2, , EtOH.; v) Ac_2O, pyridine, 64% over two steps.; vi) TFA, then FmocOSu, 77%.

Scheme 23. Synthesis of glycosylated amino acid building block **98**.

The glycosylated amino acid **107** was prepared starting from the monosaccharide building blocks **99** and **101** (Scheme 24). The monosaccharide **99** was treated with 2,2-dimethoxypropane under anhydrous acidic conditions to form the 3,4-isopropylidene acetal compound **100** in 39% yield. Silver triflate promoted coupling with acetobromogalactose[152] **101** gave the β-(1→6)-disaccharide **102** in 86 % yield. DMTST promoted coupling with Fmoc-threonine phenacylester gave the disaccharide amino acid **103** in 56% yield. The isopropylidene acetal was hydrolysed using 80% aqueous acetic acid, followed by treatment with acetic anhydride in pyridine to form compound **105**. Reduction of the azide and subsequent *N*-acetylation by treatment with thioacetic acid yielded compound **106**. Finally, removal of the phenacyl group gave the Fmoc-amino acid derivative **107** in 89% yield. The overall yield of **107** over the synthetic sequence, calculated from **99**, was 16%.



Reagents and Conditions: i) $Me_2C(OMe)_2$, DMF, TsOH, 200 mbar, 50° C, 3 h, 39%.; ii) AgOTf, CH_2Cl_2, O.N. 86%.; iii) DMTST, Fmoc-threonine phenacylester, 56%.; iv) HOAc, H_2O, 80° C.; v) Ac_2O, pyridine.; vi) AcSH, rt, O.N. 67% over three steps; vii) Zn, HOAc, 89%.

Scheme 24. Synthesis of glycosylated amino acid building block **107**.

The glycopeptides **110**, **111**, **112** were prepared using the respective glycosylated amino acid building blocks. Manual Fmoc solid-phase chemistry was used, starting with Leu-Wang resin, each coupling was activated with PyBOP/HOBt (5eq) and DIPEA (10 eq) in DMF, the Fmoc group was then removed using 20% piperidine in DMF. The coupling/deprotection cycles were repeated using the appropriate amino acids until the entire peptide sequence had been assembled. During coupling of the glycosylated amino acid only 2 equivalents of activated amino acid was used. The completed glycopeptide was removed from resin using 95% TFA and was treated with sodium methoxide in methanol yielding the glycopeptides **110** (9%), **111** (37%) and **112** (56%). The glycopeptide **110** was obtained in low yield due to leakage of resin from the vessel; during synthesis of the two other peptides agitation of the resin was achieved by nitrogen bubbling instead of rotating of the vessel. The asparagine and pyroglutamate amino acids required longer reaction times and were routinely double coupled. The formed glycopeptides were analyzed with NMR and MALDI and will be further used in biological as well as NMR conformational studies.

5.3 Synthesis of Conotoxin tx5a

5.3.1 Introduction

The 13-amino acid glycopeptide tx5a (Gla-Cys-Cys-Gla-Asp-Gly-Trp*-Cys-Cys-Thr*Ala-Ala-Hyp-OH, where Trp* = 6-bromotryptophan and Thr = Gal-GalNAc-Thr) has recently been isolated from the venom of the mollusk-hunting cone snail, *Conus textile*[135, 147, 148](Figure 5). Tx5a is a conotoxin (a *Conus* peptide containing

disulfide bonds) which has a conserved arrangement of the cysteine residues (--CC--CC--) and a consensus signal sequence of its prepropeptide precursor common for all members of the T-superfamily conotoxins[147]. This conotoxin is one of the most highly modified gene products known today, containing nine of 13 post-translationally modified amino acids. The peptide contain four cysteins forming two disulfide bonds, two γ-carboxyglutamate residues, one 6-bromotryptophan, one hydroxyproline and one glycosylated threonine (Gal-GalNAc). The role of tx5a is not completely known, but seems to be involved in interactions with Ca^{2+} channel subtypes that regulate neurotransmitter release from presynaptic neurons[135]. Native tx5a was reported to cause hyperactivity and spasticity when injected intracranially into mice[147].

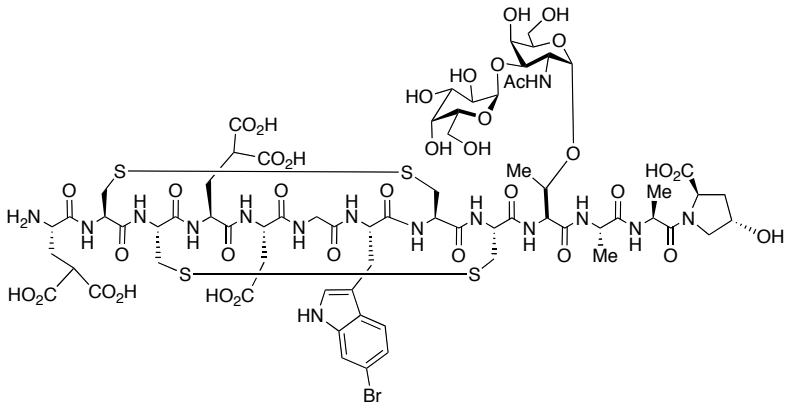
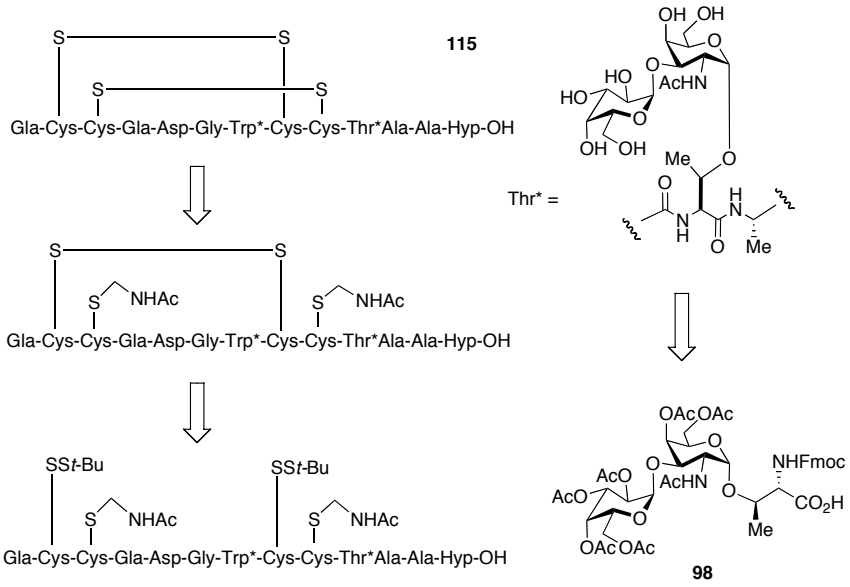


Figure 5. Proposed structure of native *Conus* toxin tx5a (**115**).

In order to verify the structure of tx5a, it was recently chemically synthesized. However, the synthesized product was found to be different from the native peptide[146]. NMR studies of the native tx5a suggested that the glycan part contain a α-Gal-(1→3)-GalNAc glycosidic bond instead of the biologically more common β-glycosidic bond. The stereochemistry of the 6-bromotryptophan amino acid is not known either. To be able to verify the most possible structure of tx5a, a peptide containing the α-Gal-(1→3)-GalNAc glycoside and *L*-6-Br-tryptophan was synthesized.

5.3.2 Synthetic Strategy

The glycopeptide **115** could be prepared from the glycosylated amino acid building block **98** previously synthesized in paper IV of this thesis. The disulfides of the peptide could be formed selectively by orthogonal protection of the cysteine residues using *t*-BuS and Acm groups (Scheme 25).



Scheme 25. Synthetic strategy for *Conus* toxin tx5a.

5.3.3 Synthesis

The tx5a glycopeptide was synthesized manually by Fmoc-solid-phase chemistry using Wang resin (V, Supplementary material). Fmoc-amino acids with appropriate side chain protection was used, *t*-butyl ester protection of γ-carboxglutamic acid and aspartic acid, *t*-butyl ether protection of hydroxy-proline and S-*t*-butyl disulfide protection of $Cys^{2,8}$ and Acm protection of $Cys^{3,9}$. *O*-acetyl groups were used to protect the glycosylated threonine **98**, whose synthesis is described in paper IV of this thesis. The Fmoc-6-bromo-L-tryptophan amino acid was obtained from commercially available 6-bromo-D/L-thryptophan by *N*-acetylation, followed by enzymatic kinetic resolution using an aminoacylase from porcine kidney to yield the L-amino acid, previously known in the literature.[153] Finally, protection using Fmoc-*O*-Su gave the 6-bromo-L-tryptophan[154]. The amino acids were sequently coupled using HBTU/HOBt/ DIPEA in DMF and the Fmoc protecting groups were removed using 20% piperidine in DMF. After the last coupling cycle removal from resin using 95% TFA, 2.5 % TIPS and 2.5% H_2O yielded the acetylated peptide in 6 %. Treatment with methoxide in methanol gave the deacetylated product in 52 % yield. Reduction of the *S*-*S*-*t*Bu protective groups using TCEP gave the free thiols of $Cys^{2,8}$ in 22 % yield. Oxidation using $K_3Fe(CN)_6$ gave the $Cys^{2,8}$ disulfide, which was followed by iodine oxidation to form the final glycopeptide in 38% yield. The structure of the synthesized glycopeptide **115** was confirmed by MALDI mass spectrometry and the material will be compared with tx5a by HPLC co-elution and NMR spectroscopy.

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