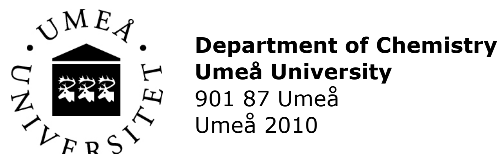
Glycoconjugates
Synthesis and investigation of carbohydrate-protein interactions

Sara Spjut

DOCTORAL THESIS



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Title
Glycoconjugates-Synthesis and Investigation of Carbohydrate-Protein Interactions

Abstract
To study the functions of glycoconjugates in biological systems reliable and efficient protocols for glycoconjugate synthesis are needed. To reach this goal we have developed methods for solid-phase synthesis of glycoconjugates that can be monitored with gel-phase ^{19}F spectroscopy using fluorinated linkers, building blocks, and protecting groups. We have developed a new fluorine containing linker suitable for solid-phase synthesis of glycoconjugates. The linker was more acid-labile than similar linkers in order to enable cleavage under mild conditions of the target compound from the linker resin. A carbamate-based strategy has been applied to attach a spacer carrying an amino group to a fluorinated Wang linker for synthesis of amino-functionalized glycoconjugates using thioglycoside donors with fluorinated protective groups. Cleavage from the solid support was performed with trifluoroacetic acid and subsequent protecting group removal gave the target compound. The terminal amine was conjugated with didecyl squarate and this derivative can be attached to various proteins and solid surfaces carrying primary or secondary amines. To evaluate this methodology we have immobilized glycoconjugates in amino-functionalized microtiter plates and successfully probed them with lectin. In addition, a novel fluorine containing protecting group has been designed, synthesized and evaluated. The protecting group was used for protection of the unreactive 4-OH in a galactose building block that was applied in the synthesis of 6-aminohexyl galabioside and was removed with TBAF in THF.

Adenovirus serotype 8 (Ad8), Ad19, and Ad37 cause the severe ocular infection, epidemic keratoconjunctivities (EKC). During infection, the adenoviruses interact with sialic acid containing glycoconjugates on the epithelial cells via fiber structures extending from the viral particles. The virus particle most likely binds to the host cell in a multivalent way by simultaneously using multiple fiber proteins and binding sites. Multivalent sialic acid containing conjugates could efficiently inhibit Ad37 cell attachment and subsequent infection of human corneal epithelial (HCE) cells. Three compact tri- and tetravalent sialic acid conjugates were prepared and evaluated as inhibitors of adenoviral host cell attachment and subsequent infection and all conjugates were potent as anti-adenoviral agents. The conjugates can readily be synthesized from accessible starting materials. A crystal structure of the Ad37 fiber knob protein and the trivalent sialic acid conjugate showed that the three binding sites were all occupied by one sialic acid residue each.

Keywords
Glycoconjugates, Carbohydrates, Galactose, Glucose, Solid-phase synthesis, SPOS, Glycosylation, Gel-phase ^{19}F NMR spectroscopy, Fluorinated linker, Carbohydrate array, Microtiter plates, Carbohydrate-protein interactions, carbamate linker, Fsec, Protecting group, Fluorinated protecting group, Multivalent glycoconjugates, Adenovirus, Ad37, Epidemic keratoconjunctivitis, EKC, Sialic acid, Adenovirus inhibitor, Trivalent, Tetravalent, X-ray crystal structure.

Contents

LIST OF PAPERS 7

ABBREVIATIONS 9

1. INTRODUCTION 11
 1.1 Carbohydrates and glycoconjugates 11
  1.1.1 Synthesis of glycoconjugates 11
  1.1.2 Carbohydrate-protein interactions and multivalency 13
  1.1.3 Carbohydrate-protein interactions and microarrays 15
 1.2 Solid-phase synthesis and gel-phase ^{19}F NMR spectroscopy 15
  1.2.1 Resins for solid-phase synthesis 16
  1.2.2 Linkers and protecting groups for solid-phase synthesis 16
  1.2.3 Solid-phase synthesis of glycoconjugates 18
 1.3 Gel-phase ^{19}F NMR spectroscopy 19
  1.3.1 Linkers and protecting groups for gel-phase ^{19}F NMR spectroscopy 19
 1.4 Multivalent glycoconjugates targeting viral infections 22

2. AIMS OF THE THESIS 24

3. METHODS FOR OF SOLID-PHASE SYNTHESIS OF GLYCOCONJUGATES (PAPER I AND II) 25
 3.1 Design and synthesis of the acid-labile 2-(2-fluoro-4-hydroxymethyl-5-methoxy-phenoxy)acetic acid linker (Paper I) 25
 3.2 Evaluation of the acid-labile 2-(2-fluoro-4-hydroxymethyl-5-methoxy-phenoxy)acetic acid linker (Paper I) 27
 3.3 The carbamate linker strategy in solid-phase synthesis of amino-functionalized glycoconjugates (Paper II) 31
  3.3.1 Solid-phase synthesis of amino-functionalized biotin derivatives 32
  3.3.2 Solid-phase synthesis of amino-functionalized neoglycoconjugates 33
 3.4 Attachment of amino-functionalized glycoconjugates to solid surfaces and investigation of protein-carbohydrate interactions (Paper II) 35
  3.4.1 Immobilization and detection of amino-functionalized biotin 35
  3.4.2 Immobilization and detection of the neoglycoconjugates 36
  3.4.3 Summary 38

4. SYNTHESIS AND APPLICATION OF A 2-[(4-FLUOROPHENYL)SULFONYL]ETHOXY CARBONYL (FSEC) PROTECTED GLYCOSYL DONOR IN CARBOHYDRATE CHEMISTRY (PAPER III) 39
4.1 Synthesis of 2-[(4-fluorophenyl)sulfonyl]ethoxy carbonyl chloride (Fsec-Cl) 40
4.2 Stability of the *O*-Fsec group 41
4.3 Synthesis of a disaccharide employing an *O*-Fsec protected glycosyl donor 42
4.4 Solid-phase synthesis using an *O*-Fsec protected glycosyl donor 44
4.5 Conclusions 45

5. SYNTHESIS AND EVALUATION OF TRI- AND TETRAVALENT SIALIC ACID INHIBITORS OF EKC-CAUSING ADENOVIRUSES (PAPER IV) 47
5.1 Synthesis of tri- and tetravalent sialic acid conjugates 48
5.2 Inhibition of adenoviral cell attachment and infection 50
5.3 Crystal structure 53
5.4 Conclusions 53

6. POLYPEPTIDE CONJUGATES 55
6.1 Synthesis and evaluation of sialic acid-polypeptide conjugates 56
6.2 Conclusions 59

7. CONCLUDING REMARKS 60

8. SUPPLEMENTARY 62
8.1. Synthesis and application of a 2-[(4-fluorophenyl)sulfonyl]ethoxy carbonyl (Fsec) protected glycosyl donor in solid-phase synthesis 62
Experimental section for solid-phase synthesis 62
8.2. Experimental section for sialic acid-polypeptide conjugates 66
8.2.1 Conjugation of sialic acid the polypeptides 66
8.2.2 Fluorescence screening experiments 66
8.2.3 Conjugation of sialic acid to the polypeptide 4-D25L22 67
8.2.4 Binding assay 67

9. ACKNOWLEDGMENTS 68

10. REFERENCES 70

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Wallner, F.K., **Spjut, S**., Boström, D., and Elofsson, M. (2007) Synthesis and evaluation of 2-(2-fluoro-4-hydroxymethyl-5-methoxy-phenoxy)acetic acid as a linker in solid-phase synthesis monitored by gel-phase ^{19}F NMR spectroscopy. *Organic & Biomolecular Chemistry*, 5, 2464-2471.

II **Spjut, S**., Pudelko, M., Hartmann, M., and Elofsson, M. (2009) Carbamate linker strategy in solid-phase synthesis of amino-functionalized glycoconjugates for attachment to solid surfaces and investigation of protein-carbohydrate interactions. *European Journal of Organic Chemistry,* 2009, 349-357.

III **Spjut, S.**, Qian, W.X., and Elofsson, M.; Synthesis and application of a 2-[(4-fluorophenyl)sulfonyl]ethoxy carbonyl (Fsec) protected glycosyl donor in carbohydrate chemistry. (Manuscript)

IV **Spjut, S.**, Qian, W.X., Bauer, J., Storm, R., Stehle, T., Arnberg, N., and Elofsson, M.; Synthesis and evaluation of tri- and tetravalent sialic acid inhibitors of EKC-causing adenoviruses. (Manuscript)

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Abbreviations

ATP	Adenosine-5´-triphosphate
aq.	Aqueous
BFB	Bromophenol blue
Bn	Benzyl
Boc	*tert*-Butyloxycarbonyl
Bz	Benzoyl
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DIC	*N,N´*-Diisopropylcarbodiimide
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
EDC	*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride
EKC	Epidemic keratoconjunctivities
Fmoc	9-Fluorenylmethoxycarbonyl
Gal	Galactose
Glc	Glucose
h	Hour/hours
HCE	Human corneal epithelial
HOAt	1-Hydroxy-7-azabenzotriazole
HOBt	1-Hydroxybenzotriazole
HSA	Human serum albumin
IR	Infrared
LC-MS	Liquid chromatography-mass spectrometry
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
m-	*meta-*
MAS	Magic angel spinning
MeIm	*N*-Methyl imidazole
MSNT	1-(2-Mesitylenesulfonyl)-3-nitro-1*H*-1,2,4-triazole
NHS	*N*-Hydroxysuccinimide
NIS	*N*-Iodosuccinimide
NMR	Nuclear magnetic resonance
o-	*ortho-*
p-	*para-*
PBS	Phosphate-buffered saline
Ph	Phenyl

PEG	Polyethylene glycol
PS	Polystyrene
RCA_{120}	*Ricinus communis* agglutinin
RINK	*p*-{(R,S)-α-[1-(9H-Fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl}-phenoxyacetic acid
SASRIN	Superacid sensitive resin
SPOS	Solid-phase organic synthesis
TBAF	tetra-*N*-butylammonium fluoride
THF	Tetrahydrofuran
TEA	Triethylamine
TFA	Trifluoroacetic acid
TLC	Thin-layer chromatography
Tsc	2-(4-Trifluoromethylphenylsulfonyl)ethoxycarbonyl

1. Introduction

1.1 Carbohydrates and glycoconjugates

Carbohydrates have for a long time been well known for their ability to form structural components or act as energy source *e.g.* the α(1-4) linked D-glucose polymer in starch or the utilization of D-glucose in glycolysis to form ATP. However, it is now known that they also play important roles through carbohydrate-carbohydrate and carbohydrate-protein interactions in biological systems. They are involved in *e.g.* viral entry, bacteria-host interactions, cell growth, cell-cell adhesion, fertilization, immune defense, and inflammation.[1] The majority of all carbohydrates present in the cells are in the form of glycoconjugates, *i.e.* the carbohydrate is attached to non-carbohydrate moieties, *e.g.* proteins and lipids. In comparison with other important biomolecules such as nucleic acids and proteins that usually are linear, carbohydrates can be highly branched due to their many reactive sites. The many stereocenters and the possibilities to form a variety of linkage types result in complex structures and an extremely high amount of structural variation.

1.1.1 Synthesis of glycoconjugates

Research to elucidate the role of carbohydrate containing molecules in biological systems has led to an increased need for pure and well-defined glycoconjugates. However, obtaining complex structures of carbohydrates is a challenge for glycobiologists since isolation of pure glycoconjugates from biological samples is difficult and often results in very small quantities. In addition, determination of the structure of the carbohydrates is complicated. Organic synthesis of glycoconjugates is also challenging because of their complex structures. The synthesis can be performed with various glycosyl donors, *e.g.* thioglycosides, halides, or trichloroacetimidates, under promotion of *e.g.* NIS-TfOH, AgOTf, or $BF_3{\cdot}Et_2O$, respectively, in various solvents.[2] The main problem for the organic chemist is the regio- and stereoselective formation of new glycosidic bonds. However, by masking selected hydroxyl groups with protecting groups complex carbohydrates can be synthesized. Orthogonality, *i.e.* the ability to selectivity remove one protecting group in presence of another, is extremely important since it offers possibilities to control the regioselectivity. The properties of the

protecting groups are critical, not only from the orthogonality point of view, but also from a steric and electronic point of view since they have a great influence on the reactivity of the building blocks and the outcome of the glycosylation reaction. A useful method to control the stereochemistry in the formation of a new glycosidic linkage is by neighboring group participation. The use of an acyl protecting group, *e.g.* a benzoyl group, on the O-2 position (Scheme 1a) enables the formation of a 1,2-*trans* β-glycosidic linkage. The use of non-participating protecting groups, for example a benzyl ether, facilitate the formation of 1,2-*cis* α-glycosidic linkages (Scheme 1b).

a) Formation of a 1,2-*trans* β-glycosidic bond with a participating group

Oxocarbenium ion   Acyloxonium ion   1,2-*trans* Glycoside

b) Formation of a 1,2-*cis* α-glycosidic bond with a non-participating group

1,2-*trans* Glycoside   1,2-*cis* Glycoside

Scheme 1. Proposed mechanisms of glycosylation. a) When the anomeric leaving group has left and formed the oxocarbenium ion, participation of the carbonyl oxygen in the acyl protecting group at the 2-OH position may stabilize the intermediate glycosyl cation by cyclization. Ring opening of the acyloxonium ion is most likely performed in a S_N2 fashion by the attack of the nucleophile, the acceptor, with inversion of configuration, leading to a β-glycoside. b) The formation of a 1,2-*cis* glycosidic bond is more difficult. Conditions that increase the chances for an α-glycosidic bond are *e.g.* no participating protecting groups on the O-2 position, and unpolar solvents (*e.g.* CH_2Cl_2 or toluene) or ether like solvents (*e.g.* diethyl ether and tetrahydrofuran (THF)).

Acyl groups (*e.g.* acetyl, benzoyl, and pivaloyl), ethers (*e.g.* benzyl), and acetals (*e.g.* benzylidene and isopropylidene) are some of the most common protecting groups for hydroxyls in carbohydrates.[3, 4] Acetylations can be performed with acetic anhydride in pyridine and benzoylations with benzoyl chloride in pyridine. Acyl protecting groups are easily cleaved by the Zemplén procedures, sodium methoxide (NaOMe) in dry methanol.[5] Benzyl ether protecting groups are introduced under Williamson conditions using sodium hydride or sodium hydroxide in dimethylformamide (DMF) with alkyl chloride or alkyl bromide. Typically, the benzyl ethers are cleaved by

catalytic hydrogenation with palladium on charcoal. Acetal protecting groups are used for simultaneous protection of two hydroxyl groups in the saccharide, for example 4,6-*O*-benzylidene protection in a hexapyranoside like glucose and galactose. They are commonly introduced by an acid-catalyzed reaction with aldehydes, ketones or dimethyl acetals and can be completely deprotected by hydrogenation or acidic hydrolysis. Selective opening can be performed under various reducing conditions giving 4-OBn-6-OH[6] or 4-OH-6-OBn derivatives[6, 7]. The 9-fluorenylmethoxycarbonyl (Fmoc) group has frequently been used as a *N*-protecting group in peptide synthesis and recently for *O*-protection in both solution[8] and solid-phase[9, 10] synthesis of carbohydrates. The Fmoc group is acid stable and can be cleaved under mild basic conditions. Typical condition for removal of the Fmoc group in solid-phase synthesis of peptides is 20 % piperidine in DMF. For deprotection of hydroxyl groups the Fmoc group can be cleaved by triethylamine (TEA) in CH_2Cl_2 (1:4).[8] A combinatorial and selective one-pot reaction method for installation of orthogonal protecting groups on D-glucose have been described by Wang *et al.*[11] Additional protecting group strategies have developed in recent years but carbohydrate synthesis is still time consuming and cumbersome due to the high number of protecting group manipulations that frequently are necessary.

1.1.2 Carbohydrate-protein interactions and multivalency

Carbohydrate-protein interactions are important in many biological processes. The individual interaction between a carbohydrate and a protein is generally weak with dissociation constants in the millimolar range. Nevertheless carbohydrate recognition is of outmost importance since they often are the first step in a cascade of further interactions leading to biological responses. Protein binds to carbohydrate structure primarily via multiple hydrogen bonds and hydrophobic interactions. The many hydroxyl groups of carbohydrates interact with polar groups in the protein, most frequently aspartic acid, asparagine, glutamic acid, glutamine, arginine, and serine residues. Water molecules that form hydrogen bond bridges between the protein and carbohydrate can mediate bonding between carbohydrates and proteins. Despite the fact that carbohydrates are very polar molecules they contain hydrophobic regions due to steric disposition of the hydroxyl groups. These regions can to some extent form hydrophobic interactions with aromatic or aliphatic side-chains in the protein, *i.e.* the methyl moiety of the acetamide in sialic acid can interact with aromatic residues in the protein. Carbohydrates and proteins are hydrated in the cells. If a surface on the glycan is complementary to the protein-binding site, water can be displaced and binding occurs. During binding, the protein-water and carbohydrate-water interactions are replaced by protein-carbohydrate interactions and the water molecules are released to the bulk. The surface of

the new carbohydrate-protein complex is also hydrated. The extent of water that has to be displaced from the protein and especially from the carbohydrate in order to maximize the interactions is unfavorable for binding. This, in combination with the amphiphilic nature of carbohydrates and the characteristics of the receptor binding site, *i.e.* the shallow binding site, has been suggested as reasons for the weak binding affinities between carbohydrates and proteins.[12] That carbohydrate protein interactions are important despite their weak affinities has been explained by improvement of the strength and specificity by multivalent interactions.[12, 13] In multivalent interactions numerous copies of the glycoconjugate and the receptor sequentially and/or simultaneously binds to each other resulting in increased binding avidities and thus a biological response. Avidity is the functional affinities for ligand-protein interactions and describes the strength of association in polyvalent interactions. The glycocalyx, *i.e.* the glycoprotein-polysaccharide matrix on the cell surface, consists of a multivalent milieu. The multivalent ability of the carbohydrate-binding site of the protein can vary. There can for example be more than one carbohydrate recognition domain on a polypeptide, the protein may form oligomers, or the lectins can be in close proximity on a cell membrane.[14]

1.1.2.1 Multivalent mechanisms

The mechanisms behind multivalent ligand-receptor interactions are complicated and still not fully understood. One mechanism that can explain enhanced multivalent binding is *the chelate effect*, which can be explained in terms of entropy. After binding of the first ligand of a multivalent conjugate, the binding of following ligands will be faster since they are in close proximity of the binding pocket and hence there is a lower entropic barrier.[15, 16] Enhanced affinity has also been explained by *high local concentration* where the close proximity of additional ligands facilitates binding when the first ligand has been released. Another plausible explanation is the *aggregation* process, where sugar ligands of a multivalent conjugate binds to multiple lectins and thus form cross-linked aggregates.[17] *Steric stabilization* has also been suggested as a mechanism for affinity enhancement. When one or more multivalent ligands have bound to proteins, *e.g.* on a pathogen, it is sterically hindered from attachment to cells. The explanation behind the *bind and slide* mechanism is that the multivalent ligand is involved in multiple ligand-receptor binding interactions in a sequential manner.[18] Each interaction will result in low affinities, typical for carbohydrate-lectin interactions, but that all together they will result in the high affinity that can explain the biological response.

1.1.2.2 Multivalent glycoconjugates

Multivalent glycoconjugates can be formed by conjugation of glycans/glycoconjugates to scaffolds. The scaffolds can *e.g.* be peptides,

proteins, lipids, polymers, nanoparticles, or dendrimers.[12, 16, 19] An early example of the impact of multivalency was published by Lee *et al.*[20] The synthesized simple di- or trivalent glycosides consisted of oligopeptides carrying GalNAc residues and were potent as inhibitors for mammalian hepatic Gal/GalNAc-specific receptors. The IC_{50} values for the di- and trivalent glycosides were approximately 200- and 4000-fold lower, respectively, than their monovalent counterpart.

1.1.3 Carbohydrate-protein interactions and microarrays

In order to study carbohydrate-protein interactions the carbohydrate microarray technique has been developed.[21-26] To a solid surface consisting of *e.g.* multiwall plastic plates or glass slides, small amounts of glycoconjugates are immobilized using different methods. To this surface a labeled analyte, *e.g.* protein or microbe, is added. Binding events between glycan and analyte can then be then examined. This technique has many advantages including high-throughput, miniaturization, and direct characterization of interactions. It is also suitable for investigation of carbohydrate- protein interactions since it overcomes the problem of low affinities typical for these interactions. This is due to the multivalent way the carbohydrates are presented on the solid surface. Typical applications of this technique are detection of carbohydrate binding proteins and pathogens, detection of antibodies for medical diagnosis, and high-throughput screening of inhibitors of carbohydrate binding to proteins and pathogens.[21-26]

1.2 Solid-phase synthesis and gel-phase ^{19}F NMR spectroscopy

In organic chemistry the molecule of interest, the product, is obtained by chemical manipulation involving reactions between different reactants and reagents. The chemical reaction is typically performed as a solution-phase synthesis with all the building blocks, reagents, and catalysts dissolved in a solvent. This type of reaction requires both work-up and purification but the advantages with this method are the ease of reaction monitoring and determination of the outcome of the reaction. In 1963 Merrifield was the first to report a solid-phase synthesis when he synthesized a tetrapeptide on a cross-linked polystyrene resin, an effort for he was later rewarded with the Nobel prize.[27] Solid-phase synthesis have since then been widely applied in peptide (solid-phase peptide synthesis, SPPS)[28, 29] and oligonucleotide synthesis[30]. In solid-phase synthesis the target compound is synthesized on an insoluble polymeric bead by step-wise chemical manipulation with different reactant solutions. The big advantage with this method is that

complicated and time-consuming purification steps are avoided since excess or unreacted reactants and reagents can be removed by filtration after each reaction step. It is not until completion of the synthesis that the target molecule is cleaved from the resin and purified. Also, excess amounts of reagents can be used to drive the reactions to completion. However, the insoluble polymer complicates monitoring of the reaction steps.

There are three main requirements that have to be fulfilled in solid-phase chemistry. The resin (*i.e.* the cross-linked insoluble polymeric material) should be stable during synthesis, the first building block should be readily coupled to the resin in such a way that the target molecule can be cleaved from the resin, and an orthogonal protecting strategy has to be applied so that selective protection and deprotection of functional groups can be performed during synthesis.

1.2.1 Resins for solid-phase synthesis

The structures of the resins allow penetration of solvents and reagents to reaction sites in the beads. Typical resins are based on polystyrene (PS) and polyethylene glycol (PEG) with different degrees of cross-linking by for example divinylbenzene. The amount of cross-linking affects the physical properties of the resin. If there is moderate or high amount of cross-linking, the resin is stable towards mechanical degradation but the swelling ability is decreased which reduces access to the reaction sites and thereby gives a lower loading capacity. Copolymers such as TentaGel are also used in solid supports.[31] TentaGel is composed by PEG attached to low cross-linked PS through an ether link. The reactive sites are situated on the terminal of the long flexible chains of ethylene oxide that are well separated from the rigid polystyrene backbone. The functionality of these reactive groups can for example be $-NH_2$, -OH, -COOH, or –SH. The TentaGel resin has good swelling properties in solvents of medium to high polarity.

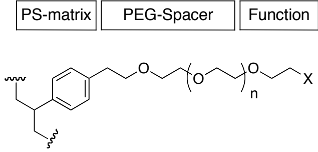
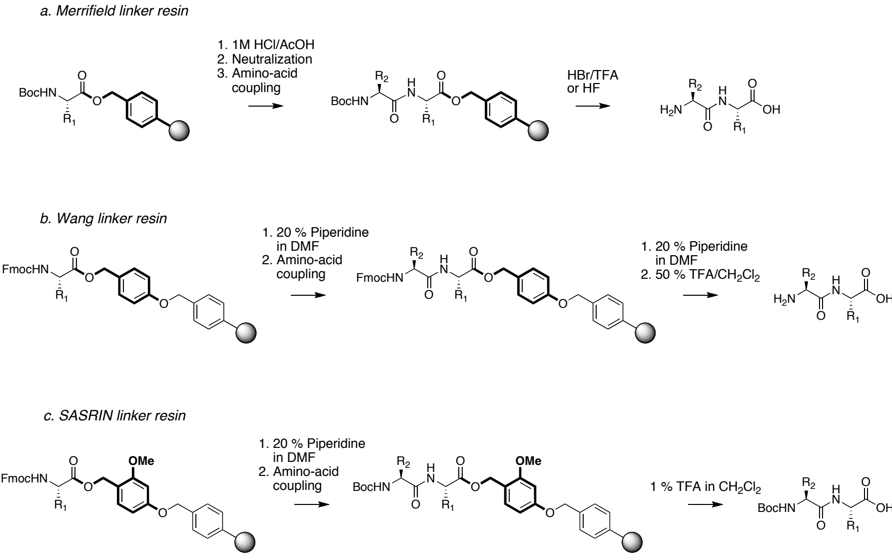


Figure 1. The TentaGel™ resin.

1.2.2 Linkers and protecting groups for solid-phase synthesis

The first reaction step in solid-phase synthesis is the immobilization of the linker to the insoluble support. After attachment to the resin, the linker

should be able to form a covalent bond with the first building block in a quantitative yield. It should be stable under the synthetic conditions during synthesis and after completed synthesis the target compound should be cleavable from the linker in a quantitative yield. In order to allow solid-phase synthesis of target molecules under various conditions (regarding functionality, reagents, and cleavage conditions) a great number of different linkers have been developed (>200) and many are mimics of common protecting groups used in solution-phase synthesis. The linkers are employed to immobilize a range of different functional groups to the solid support, *e.g.* acids, amides, amines, etc. However, each functionality can be cleaved only under specific conditions; under harsh or mild conditions by acid, base, reduction, oxidation, or light.[32] The cleavage of the target compound from the linker resin either retains a previously protected functionality or introduces a new functionality. In the synthesis of Bradykinin, Merrifield developed a strategy employing N^{α}-*tert*-butyloxycarbonyl (Boc) amino-protected amino acids that were coupled to a chloromethylpolystyrene resin (Scheme 2a).[33]



Scheme 2. Examples of linkers cleaved under acidic conditions; a) Merrifield resin, b) Wang linker, and c) SASRIN linker.

This linker resin is now known as the Merrifield resin. However, the strong acid hydrofluoric acid (HF) was required for cleavage of the product from the Merrifield resin. HF is extremely hazardous and not applicable in some cases where the product may be sensitive towards strong acids. By introducing methoxy or alkoxy groups milder acidic conditions could be

used, as in the example of the Wang linker[34] (Scheme 2b). This linker is popular in solid-phase peptide synthesis (SPPS) and solid-phase organic synthesis (SPOS). The SASRIN (superacid sensitive resin) linker,[35, 36] which essentially is a Wang linker with a methoxy group, is more acid sensitive due to increased stabilization of the benzylic cation during cleavage (Scheme 2c). This linker can be cleaved under very mild conditions, only 1% TFA in CH_2Cl_2 is needed.

There are many protecting groups suitable for solid-phase synthesis.[37] The 9H-fluoren-9-ylmethoxycarbonyl (Fmoc) group is routinely used for protection of amines in solid-phase synthesis and there are numerous Fmoc-protected building blocks commercially available. The Fmoc group can easily be removed, typically with 20 % piperidine in DMF, and are compatible with many linkers (Scheme 2a and 2b).

1.2.3 Solid-phase synthesis of glycoconjugates

Solid-phase synthesis of glycoconjugates has become an area of increasing interest during the last 20 years. Solid-phase synthesis of glycoconjugates utilizing glycosylated building blocks has been successful but the obstacles faced in solution-phase synthesis of carbohydrates remain. Glycosylation of an acceptor on a solid support is a major challenge and since reaction conditions suitable for solution-phase synthesis of glycoconjugates are not commonly applicable for solid-phase synthesis of glycoconjugates and methodological development is thus needed. The methods can either be acceptor- or donor-based. The acceptor-based strategy is more popular and more applicable since it allows an excess of glycosylating reagents to drive the reaction to completion. The glycosyl acceptor is attached to the linker and the glycosyl donor and the promotors are in solution. The use of excessive amounts of glycosyl donor can however be very expensive. When the solid-phase synthesis technique was new, reaction conditions for *O*-glycosylations were not compatible with solid-phase reaction conditions. Since then, several new glycosyl donors suitable for solid-phase synthesis have been developed, *e.g.* trichloroacetimidates[38, 39], thioglycosides[40], sulfoxides[41], and phosphates[42].[43] The linker properties are also of great importance since the linker must be possible to cleave under mild conditions that do not influence the saccharide backbone, but still stable enough to withstand the glycosylation conditions. Linkers that fulfill these criteria are for example based on ether[44], amide[45], or silyl ether[46] bonds.[43, 47]

The synthesis of peptides and oligonucleotides has for a long time been practically automated and attempts to automate oligosaccharide synthesis have been performed.[48-50]

1.3 Gel-phase ^{19}F NMR spectroscopy

Despite many improvements there are still some disadvantages with solid-phase synthesis of glycoconjugates, especially the monitoring of the reaction progress, which is difficult while the product is still attached to the solid support. Two different strategies to analyze products attached to the solid support can be employed; either destructive or non-destructive techniques, and they can be performed on-bead or demand cleavage from the resin. Destructive techniques lower the yield since a small fraction of the product-resin is destroyed by the analytical method after each reaction step. Some examples of destructive techniques are the colorimetric Kaiser test[51] and mass spectrometric tests. Despite the disadvantages some of these methods are routinely used in SPPS since they are fast and robust. In solid-phase synthesis gel-phase NMR (primarily ^{13}C), magic angel spinning (MAS) NMR, Raman and IR spectroscopy, and MALDI-TOF techniques have been applied.[52] These methods are either time demanding and/or require specialized and expensive instrumentation. To address this problem gel-phase ^{19}F NMR spectroscopy has been employed as a fast, sensitive, and reliable monitoring technique for solid-phase synthesis. The ^{19}F nucleus has a high sensitivity and the signal-to-noise ratio is good since the ^{19}F nucleus has 100 % natural abundancy. The risk of peak overlapping is low due to the large chemical shift dispersion. Spectra can be recorded on standard NMR spectroscopy equipment and the spectra allow calculation of conversion and in many cases also diastereomeric ratios. This monitoring technique has been successfully employed in both oligosaccharide and small-molecule SPOS.[53-67] However, in order to be able to use gel-phase ^{19}F NMR spectroscopy the molecule attached to the solid support has to contain fluorine atoms, *e.g.* as a permanent substitution on building blocks or by use of fluorinated protecting groups. In addition a ^{19}F reference signal is needed preferably in the form of a fluorinated linker.

1.3.1 Linkers and protecting groups for gel-phase ^{19}F NMR spectroscopy

Several linkers have been developed for SPOS and gel-phase ^{19}F NMR spectroscopy. The fluorinated linkers in linker resin **1**[66] and **4**[55, 64] (Scheme 3) have high structural similarities with the Wang linker (Scheme 2b). The selenium linker in linker resin **7** was designed to synthesize *n*-pentyl glycosides.[63]

*a. 2-Fluoro-4-(hydroxymethyl)-phenoxyacetic acid linker resin **1***

95 % TFA in water, 60 °C

1 **2** **3**

*b. 3-Fluoro-4-(hydroxymethyl)phenoxyacetic acid linker resin **4***

90 % TFA in water, 60 °C

4 **5** **6**

*c. 2-Fluoro-4-[5-(tert-butyldiphenylsilanyloxy)-pentylselenyl]-phenoxyacetic acid linker resin **7***

1. *t*BuOOH 2. Washing 3. 90 °C

7 **8** **9**

Scheme 3. Fluorine containing linker resin **1**, **4**, and **7**.

In resemblance with the Wang linker rather harsh conditions were demanded for cleavage, *e.g*. removal of the carboxylic acid from linker resin **2** required 95 % trifluoroacetic acid (TFA) in water at 60 °C for 3.5 hours[66] and linker resin **5** required TFA:water:thioanisole:ethanedithiol 87.5:5:5:2.5[55], LiOH (1M, aq.) in THF:MeOH:water (3:1:1)[55], or 90 % TFA in water at 60 °C for three hours[64]. The selenide resin **8** was first treated with anhydrous *t*-butyl hydroperoxide (*t*BuOOH) and the resulting selenoxide derivative demanded 90 °C for four hours to achieve complete elimination.[63]

During solid-phase synthesis reaction monitoring can be performed with gel-phase ^{19}F NMR spectroscopy since the ^{19}F resonances derived from fluorinated protecting groups (Table 1a) and linkers (Table 1b) are spread over a wide spectral range.

Table 1. ^{19}F NMR chemical shift for fluorinated protecting groups attached to monosaccharides and gel-phase ^{19}F NMR chemical shift for fluorinated linker resins.

	^{19}F NMR shift δ [ppm]		
a. Protecting group	*ortho*	*meta*	*para*
FBz[a]	-109.0 to -110.0	-111.6 to -112.6	-103.9 to -105.5
FBn[b]	-119.0 to -119.4	-113.5 to -113.8	-114.9 to -115.8
FPhCH[c]	-121.1	-113.4	-112.7
b. Linker resin			
1		-117.2[d]	
4		-134.5[e] or -134.3[f]	
7		-133.4[g]	

[a] *o-, m-, p*-F-perbenzoates of D-glucopyranoside;[62] [b] *o-, m-, p*-F-benzyl ethers attached to HO-2, -3, -4, and -6 of methyl α-D-glucopyranoside;[62] [c] *o-, m-, p*-F-4,6-benzylidene acetals on 4-methylphenyl 1-thio-β-D-galactopyranoside;[62] [d] Linker attached to ArgoGel-NH_2;[64] [e] Linker attached to ArgoGel-NH_2;[66] [g] Linker attached to TentaGel-NH_2;[67] Linker attached to ArgoGel-NH_2.[63]

Recently a new fluorine containing protecting group, the *N*-[1-(4-(4-fluorophenyl)-2,6-dioxocyclohexylidene)ethyl] (Fde) group, **12** (Figure 2) for protection of α-amino acids (**13**) for solid-phase synthesis of peptides was developed.[68] The Fde group is based on the (4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde) group **10**, since Dde has proved to be relatively stable towards TFA and piperidine but can easily be cleaved by 2 % hydrazine.[69-71] The Fde group can be removed with 2 % hydrazine or 5 % hydroxylamine in DMF.

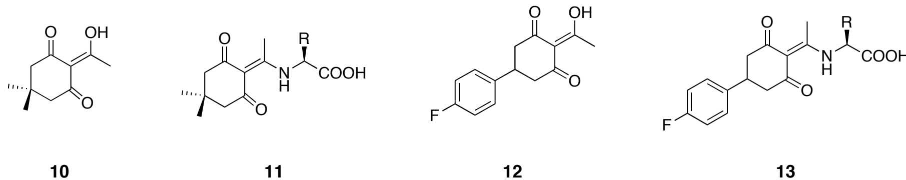


Figure 2. The Dde protection group reagent **10**, a Dde protected amino acid **11**, the Fde protection group reagent **12**, and a Fde protected amino acid **13**.

1.4 Multivalent glycoconjugates targeting viral infections

Viruses of the family *Adenoviridae* cause numerous infections, for example respiratory, gastrointestinal, and eye infections in humans.[72-75] Adenoviruses of serotypes Ad8, Ad19, and Ad37 are responsible for epidemic keratoconjunctivitis (EKC), a severe ocular infection.[76, 77] EKC is highly contagious and viruses are readily spread by contact. Red, swollen, and tearing eyes as well as sensitivity towards light are typical symptoms of infection. There are no anti-viral agents available for treatment of EKC but eventually, most patients will recover. However, permanent reduction of sight has been reported in some severe cases.[76] The viral particle has twelve fiber proteins extended from their surface. At the end of each fiber protein there is a fiber knob consisting of a homotrimer that presents three binding sites.[78] It has been shown that Ad37 uses sialic acid containing oligosaccharides as cellular receptors.[79, 80] The virus particle is most likely binding to sialic acid residues on the host cell in a multivalent fashion, using the multiple fiber proteins and binding sites to be able to infect the cell.

To find new anti-adenoviral substances multivalent glycoconjugates have been synthesized and evaluated as inhibitors of Ad37 (Figure 3).[81, 82] The multivalent 3’-sialyllactose and sialic acid human serum albumin (HSA) conjugates could efficiently inhibit Ad37 binding and subsequent infection of human corneal epithelial (HCE) cells. 3´-Sialyllactose and sialic acid conjugates with the same level of valency (3-19 saccharides per HSA molecule) were equally efficient as Ad37 inhibitors.[82] It was also shown that the glycoconjugates trapped the viral particles by cross-linking and aggregation.[82]

Figure 3. Multivalent 3´-sialyllactose-HSA conjugates[81] (left), multivalent sialic acid HSA conjugates[82] (right, R = CH_3), and the multivalent *N*-acyl modified sialic acid-conjugates[83] (right, R = CH_2CH_3, $CH_2CH_2CH_3$ or $CH(CH_3)_2$). The valency *n* is typically 3-19.

The acetamide functionality in sialic acid was varied (Figure 3, right) in order to improve the potency of the multivalent sialic acid conjugates.[83] From the crystal structure of the fiber knob domain in complex with sialyl lactose it was evident that the sialic acid acetamide is positioned in a relatively large and hydrophobic pocket.[83] From structure-based design and docking studies, a library of ten *N*-acyl modified sialic acid derivatives were designed and synthesized. The overall goal was to improve hydrophobic interactions and thus affinity and efficacy.[83] Initially the monosaccharide derivatives was evaluated and based on these results a set of multivalent HSA conjugates was prepared. None of the multivalent derivatives were as potent as the original multivalent sialic acid. Apparently it is challenging to optimize sialic acid to improve the binding affinity between sialic acid and the Ad37 fiber knob.

Multivalent sialic acid conjugates have the potential to be developed into a drug against adenovirus even though the substances are carbohydrate-based. Generally carbohydrates are not suitable as drugs due to several reasons, *e.g.* weak affinities to target proteins, high polarity that will lead to low absorption, and metabolic instability. These compounds are multivalent and hence the affinities are much higher than for monovalent carbohydrates/glycoconjugates. Also, since they target a virus present in the eye the administration and degradation problems are reduced.

2. Aims of the thesis

The general aim with this thesis was to establish efficient methods for synthesis of glycoconjugates and to study carbohydrate protein interactions. To be able to study these interactions glycoconjugates must be easy to get hold of. To achieve this, strategies for solution- and solid-phase synthesis of glycoconjugates in particular novel linker strategies and protecting groups were developed. To study carbohydrate proteins interactions efficient biological assays are necessary. We aimed to develop a strategy for an efficient synthesis of glycoconjugates that easily could be covalently attached to microtiter plates for biological evaluation with *e.g.* lectins. The latter part of the project came to include synthesis of sialic acid conjugates that inhibit adenovirus binding and infection to human corneal epithelial (HCE) cells.

3. Methods for of solid-phase synthesis of glycoconjugates (Paper I and II)

To study the functions of glycoconjugates in biological assays efficient glycoconjugate synthesis protocols are needed. The synthesis of carbohydrate containing molecules is challenging but during the last years reliable methods for solid-phase synthesis of glycoconjugates have been developed.[9, 47, 84, 85, 43] The absence of fast and efficient methods for monitoring the reaction outcome to some extent limits further developments of e.g. glycosylations. However, by using fluorine containing building blocks and linker in combination with gel-phase ^{19}F NMR spectroscopy glycosylation and protecting group manipulations can be monitored during solid-phase synthesis.

3.1 Design and synthesis of the acid-labile 2-(2-fluoro-4-hydroxymethyl-5-methoxy-phenoxy)acetic acid linker (Paper I)

The linker and building blocks must contain fluorine atoms in order to allow yields and sometimes also diastereomeric ratios to be determined from the ^{19}F spectra. Several fluorine containing linkers for solid-phase syntheses have been published (Scheme 3, section 1.3.1). However, quite harsh acidic conditions were necessary for cleavage of the products from the linkers. For example, cleavage of a carboxylic acid from **5** (Scheme 3b) demanded 90 % TFA in water at 60 °C for three hours.[64] This can be compared with cleavage of carboxylic acids from the fluorine deficient version of **5**, *i.e.* the classical Wang linker (Scheme 2b), which is performed with TFA at room temperature. However, introduction of an electron-withdrawing fluorine atom decreases the acid lability of the linker.[55] Basic cleavage conditions are not preferable since β-elimination is a risk.[86] Introduction of an electron-donating group could possibly increase the acid lability due to stabilization of the benzylic cation.[32] The desired properties could possibly be achieved by introduction of an additional methoxy group giving the 2-(2-fluoro-4-hydroxymethyl-5-methoxy-phenoxy)acetic acid linker **14** (Figure 4).

Figure 4. The novel fluorinated linker **14** (R = OMe). The fluorinated analog **15** (R = H) of the Wang linker.[55, 66]

Initially the plan to synthesis the fluorinated dialkoxy linker **14** was based on two nucleophilic aromatic substitutions on *tert*-butyl trifluorobenzoate **16** (Scheme 4). However, both nucleophilic substitutions gave low yield due to decomposition of the starting material and transesterification.

Scheme 4. Initial attempts to synthesize the fluorinated linker **14**. *Reagents and conditions:* a) Methyl glycolate, *t*-BuOK, THF, 0 °C; b) MeOH, *t*-BuOK, THF, 0 °C→ room temperature.

Linker **14** was instead prepared from trifluorobenzonitrile **19** (Scheme 5). Two nucleophilic aromatic substitutions, first with potassium benzoxide and then with methoxide gave **21** in 66 % yield over two steps. Hydrolysis of the nitrile (92 % yield) and hydrogenolysis of the benzyl ether (86 % yield) gave the benzoic acid **23**. Reduction of the carboxylic in **23** gave alcohol **24** in 92 % yield. However, **24** was very sensitive, possibly due to oxidation, and was therefore only briefly characterized before alkylation using ethyl bromoacetate and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) that gave **25** in 72 % yield. Finally basic ester hydrolysis gave the target linker **14** in 81 % yield. Linker **14** was prepared in a total yield of 29 % over seven steps

Scheme 5. Synthesis of linker **14**. *Reagents and conditions:* a) *t*-BuOK, BnOH, THF, -78 °C → room temperature, 84 %; b) *t*-BuOK, MeOH, THF, -50 °C → 0 °C, 79 %; c) NaOH, EtOH, reflux, 92 %; d) H_2 (g), Pd/C, AcOH, 86 %; e) BH_3·DMS, $B(OMe)_3$, THF, 92 %; f) Ethyl bromoacetate, DBU, MeCN, reflux, 72 %; g) LiOH, THF/MeOH/water 3:1:1, 81 %.

3.2 Evaluation of the acid-labile 2-(2-fluoro-4-hydroxymethyl-5-methoxy-phenoxy)acetic acid linker (Paper I)

First, suitable cleavage conditions for the linker were investigated. The fluorinated linker **14** and its monoalkoxy analog **15** (Figure 4) were attached to Tentagel HL-NH_2 through amide bonds formed by reaction with activation with *N′N*-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) in DMF to form linker resin **26** and **27** (Scheme 6). The reactions were followed by bromophenol blue (BFB). Fmoc-glycine was esterified to the two linker resins with 1-(2-mesitylenesulfonyl)-3-nitro-1*H*-1,2,4-triazole (MSNT) and *N*-methyl imidazole (MeIm) to give **28** and **29** in quantitative yield according to gel-phase ^{19}F NMR spectroscopy. Amide formation with DIC and HOBt, first with Fmoc-4-fluorophenylalanine, and secondly, after Fmoc deprotection with 20 % piperidine in DMF, 2,4-difluorobenzoic acid gave the resin bound peptides **30** and **31** in quantitative yields according to gel-phase ^{19}F NMR spectroscopy. Based on the cleavage conditions suitable for the very acid-labile non-fluorinated version of **14** (*i.e.* the SASRIN linker, Scheme 2c, section 1.2.2; 1 % TFA in CH_2Cl_2) and the impact of the electron withdrawing fluorine, 5 % TFA in CH_2Cl_2 were used for cleavage of the peptide from **30**.

14 R = OMe
15 R = H
a
26 R = OMe
27 R = H
b
28 R = OMe
29 R = H
c
30 R = OMe
31 R = H
d
32

Scheme 6. Synthesis and cleavage of test peptide. *Reagents and conditions:* a) TentaGel HL-NH_2, DIC, HOBt, DMF; b) Fmoc-Gly-OH, MSNT, MeIm, CH_2Cl_2; c) *i*. Piperidine, 20 % in DMF; *ii*. Fmoc-*p*-F-Phe-OH, DIC, HOBt, DMF; *iii*. Piperidine, 20 % in DMF; *iv*. 2,4-difluorobenzoic acid, DIC, HOBt, DMF; d) 5 % TFA in CH_2Cl_2 (from **30**).

The resins were submitted to the cleaving solution for 30 minutes at room temperature. It was found that approximately 10 % of the peptide in **30** was cleaved after 30 min of treatment with 5 % TFA according to gel-phase ^{19}F NMR spectroscopy (Figure 5). An additional portion of the cleavage solution was added to the resin and after 90 minutes ~40 % was cleaved. The procedure was repeated and after a total of four hours 90 % was cleaved from the resin. The same treatment with linker resin **31** gave no cleavage. When **30** was treated with 20 % TFA in CH_2Cl_2 for 30 minutes ~90 % of the peptide was cleaved. Despite prolonged reaction times 5 % of the peptide could not be cleaved from the linker neither with 5 % nor 20 % TFA, which indicates that some of the peptide was bound to the resin matrix in a non-cleavable way. Cleavage of resin **30** with 5 % TFA and 20 % TFA in CH_2Cl_2 resulted in ~95 % yield based on the Fmoc determination of the linker resin **28**. Resin **31** was then cleaved with 90 % TFA in water at 60 °C.[67] The introduction of the electron donating methoxy group has thus resulted in a more acid labile linker.

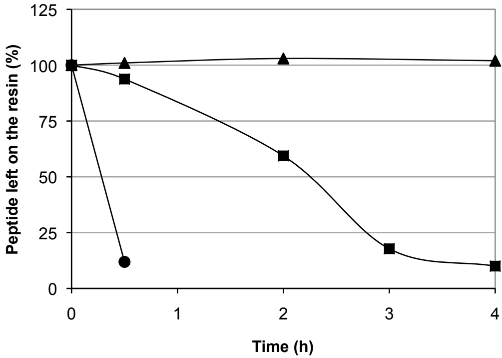
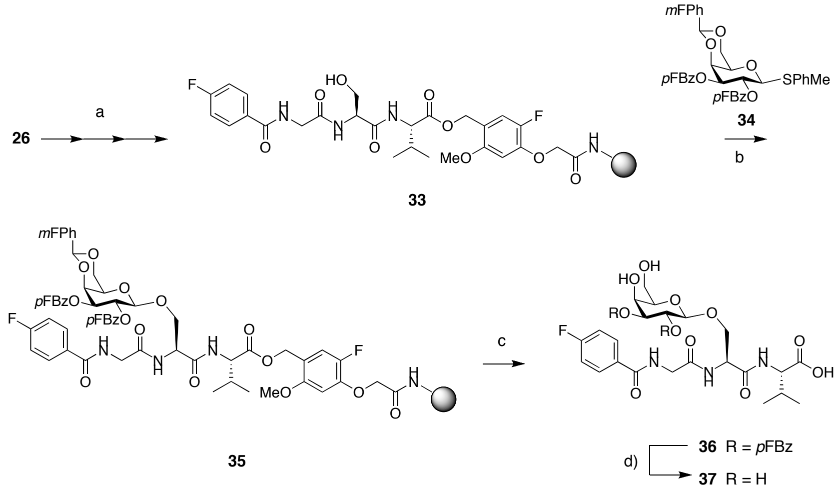


Figure 5. Cleavage of peptide **32** from resins **31** (black triangles) and **30** (black squares) using 5 % TFA in CH_2Cl_2 and **30** using 20 % TFA in CH_2Cl_2 (black circles). Figure adopted from Paper I.

The novel linker **14** was further examined in solid-phase glycosylation. First a model tripeptide **33** was synthesized on the linker resin **26**, followed by glycosylation of the serine hydroxyl in **33** with the galactose donor **34** (2 eq.) under promotion of *N*-iodosuccinimide (NIS) and trifluoromethanesulfonic acid (TfOH) in 89 % yield (Scheme 7).



Scheme 7**.** Synthesis of glycopeptide **37**. *Reagents and conditions:* a) *i*. Fmoc-Val-OH, MSNT, MeIm, CH_2Cl_2, room temperature, 14 h; *ii*. Peptide synthesis according to previously reported procedure;[87] b) NIS, TfOH, CH_2Cl_2; c) TFA-H_2O (9:1), room temperature, 2 h, 29 % yield based on the resin loading; d) LiOH (20 mM in MeOH/water 4:1), room temperature, 47 %.

The yield was determined by comparison of the gel-phase ^{19}F NMR signals from the *N*-terminal *p*-fluorobenzoyl amide groups with signals from the fluorines in the protecting groups in the galactose moiety (Figure 6). The yield is remarkably high in regard to the low amount of donor used. For comparison, in solid-phase glycosylations 2 × 5 eq. of the glycosyl donor is commonly used.[88] Under these conditions no cleavage of the peptide from the resin could be detected. The glycosylation was β-selective since no additional signals could be detected in the gel-phase ^{19}F NMR spectrum. The glycopeptide **36** was cleaved from the resin with simultaneous deprotection of the benzylidene protecting group using TFA-H_2O (9:1) at room temperature in 29 % yield based on the loading capacity of the resin. Finally, debenzoylation gave the deprotected glycopeptide **37** in 47 % yield.

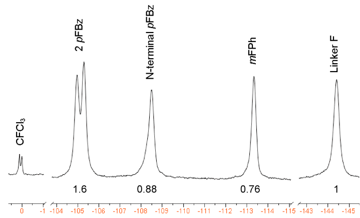


Figure 6. Gel-phase ^{19}F NMR spectra with integral values for **35**. $CFCl_3$ was used as internal standard. Figure from Paper I.

The potential of the linker was further explored by direct glycosylation of the linker resins **26** and **27** with galactose donor **34** (4 eq.) under promotion of NIS and TfOH (Scheme 8). It was concluded from the gel-phase ^{19}F NMR spectrum that 100 % of the more acid-stable resin **27** was glycosylated and 40 % of the acid-labile linker **26**. After a second glycosylation of **26** resin **38** was obtained in 63 % yield. The spectra also revealed that the direct glycosylation of the novel linker gave rise to an anomeric mixture (α:β 1:10). Treatment of resin **39** with 5 % TFA in CH_2Cl_2 gave no cleavage. Under the same conditions the hemiacetal **40** was cleaved in approximately 72 % from resin **38**. Despite an additional portion of the cleavage solution no further cleavage was obtained. The mild cleavage conditions make it possible to maintain sensitive protecting groups such as benzylidene acetals during the cleavage step from the linker resin. The cleaved hemiacetals can then be converted into new donors for further glycosylations in a convergent synthesis.

Scheme 8. Glycosylation of the linker resins **26** and **27**. *Reagents and conditions:* a) 4 or 6 equivalents of **34** and NIS, TfOH (catalytic amount), CH_2Cl_2, room temperature, 4h or 6 h; b) 5% TFA in CH_2Cl_2, room temperature, 7 h.

The yield and diastereomeric ratio could be determined from the gel-phase ^{19}F NMR spectrum implying the applicability of the novel linker for solid-phase glycoconjugate synthesis. The linker was found to be stable during synthesis and could be cleaved under mild conditions that allowed cleavage of substances with *e.g.* sensitive protecting groups. However, glycosylation of the novel acid-labile linker resulted in lower yield compared to the more acid-stable linker.

3.3 The carbamate linker strategy in solid-phase synthesis of amino-functionalized glycoconjugates (Paper II)

Glycosphingolipids like the α-galactosylceramide **41** (Figure 7) are involved in many biological events. Glycosphingolipids consists of a ceramide part (hydrophobic) and a carbohydrate (hydrophilic) part and are integrated in cell membranes. The ceramide part is embedded in the lipid layer and the carbohydrate part is exposed to the surroundings for recognition, extracellular or intracellular. When they are located on the outside of the cell they can for example bind to lectins and mediate recognition events, *e.g.* as blood-group substances or as binding sites for bacteria, viruses or toxins.[89]

Figure 7. β-Galactosylceramide **41** and the serine-based analog **42**.

Since there are two stereocenters and one double bond in the lipid part of **41** simplified analogs have been synthesized, *e.g.* the serine-based neoglycolipid **42**[66, 90] (Figure 7). In order to study protein-carbohydrate interactions **42** was conjugated to amino-functionalized plates and then probed with a biotin-labeled lectin. Conjugation of the carboxylic acid in **42** to a secondary amine in CovaLink™ microtiter plates was performed with *N*-hydroxysuccinimide (NHS) and *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC) for six hours. This method demanded high concentrations (mM) of the valuable neoglycolipid in the coupling buffer. A strategy that would be more efficient is based on two amines efficiently cross-linked by a dialkyl squarate under mildly basic conditions.[91-93] The amino-functionalized glycoconjugate is first attached to a dialkyl squarate under mild basic conditions (TEA) and after purification the squaric amide ester glycoconjugate can be immobilized to amino-functionalized surfaces under basic conditions (pH 9) (Scheme 9).

Scheme 9. Example of conjugation of an amino-functionalized glycoconjugate with an alkyl squarate and then to an amino-functionalized surface. *Reagents and conditions:* a) TEA, DMF; b) $NaHCO_3$ (aq., pH 9) R = alkyl group.

Based on this strategy, we designed the amino-functionalized neoglycoconjugate **43** (Figure 8). Solid-phase synthesis of this compound

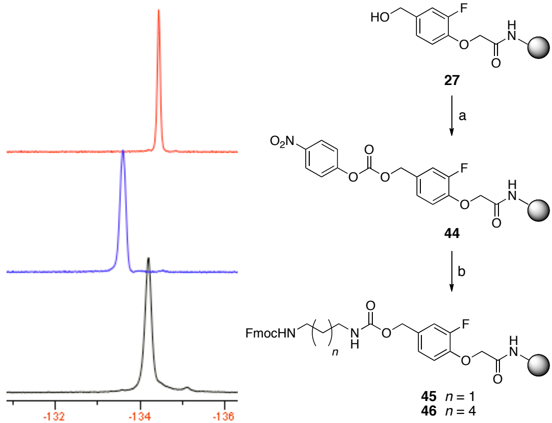
demanded a new strategy for coupling of the amine functionality to the linker resin. We envisioned that the lipid terminal amine could be coupled to the fluorinated linker resin **27**[66] (Scheme 6, section 3.2) via a carbamate linkage[94, 95].

43

Figure 8. The amino-functionalized serine-based neoglycolipid **43**.

3.3.1 Solid-phase synthesis of amino-functionalized biotin derivatives

Two biotin model compounds **51** and **52** (Scheme 11) were synthesized to evaluate the carbamate linker strategy. The model compounds were equipped with biotin, providing the opportunity to optimize array conditions that could later be used for the more valuable compound **43**. First the linker resin **27** was converted into the carbonate resin **44** through base-catalyzed reaction with *p*-nitrophenyl chloroformate (Scheme 10, right). According to the gel-phase ^{19}F NMR spectrum the conversion was complete (Scheme 10, left) and the signal from the fluorine in the linker was shifted from -133.5 ppm to -134.2 ppm. Next, *N*-Fmoc-1,3-diaminopropane and *N*-Fmoc-1,6-diaminohexane were reacted with the carbonate **44** giving the carbamate resins **45** and **46** in quantitative yield.



Scheme 10. Synthesis of the carbamate resin **45** and **46** (right), and gel-phase ^{19}F NMR spectra recorded for resin **27**, **44**, and **46** (left, figure adopted from Paper II). *Reagents and conditions*: a) *N*-methyl morpholine, *p*-nitrophenyl chloroformate, CH_2Cl_2; b) *N*-Fmoc-1,3-diaminopropane or *N*-Fmoc-1,6-diaminohexane hydrochloride, DIPEA, DMF.

Next, resins **45** and **46** were treated with 20 % piperidine in DMF in order to remove the Fmoc group (Scheme 11). Biotin was coupled to the amine with DIC and 1-hydroxy-7-azabenzotriazole (HOAt) in DMF. Cleavage from the linker resin was performed with 90 % TFA in water, which gave **49** and **50** in quantitative and 70 % yield, respectively. Functionalization to dimethyl squarate in DMF and TEA finally gave **51** and **52** in quantitative yields.

Scheme 11. Synthesis of biotin model compounds **51** and **52**. *Reagents and conditons*: a) *i*. Piperidine (20 % in DMF); *ii*. HOAt, DIC, D-biotin, DMF; b) 3 × TFA (90 % in H_2O), 70 % yield; c) Dimethyl squarate, TEA, DMF, quantitative yield.

3.3.2 Solid-phase synthesis of amino-functionalized neoglycoconjugates

In the synthesis of the neoglycolipids **43** and **58**, the lipid part was prepared under standard peptide synthesis conditions starting from resin **46** (Scheme 12).

Scheme 12. Synthesis of the neoglycolipids. *Reagents and conditions* a) *i*. Piperidine (20 % in DMF); *ii*. *N*-Fmoc-6-aminohexanoic acid, HOBt, DIC, DMF, BFB; *iii*. Piperidine (20 % in DMF); *iv*. Fmoc-L-serine HOBt, DIC, DMF, BFB; *v*. Piperidine (20 % in DMF); *vi*. Hexanoic acid, HOBt, DIC, DMF, BFB; b) **61** or **62**, QOTf, NBS, MS 3Å, CH_2Cl_2, 65 % and 57 %, respectively; c) TFA:H_2O 9:1, 24 % and 12 %, respectively; d) NaOMe (0.20 M in MeOH), MeOH; e) Didecyl squarate, TEA, DMF, 20 % and 27 %, respectively.

The hydroxyl group in **53** was glycosylated with **61** or **62** (Figure 9) under promotion of *N*-bromosuccinimide (NBS) and tetrabutylammonium trifluoromethanesulfonate (QOTf). According to the gel-phase ^{19}F NMR spectra, the yield was 50 % for glycosylation of **53** with the galactose donor **61** (3 eq.) and after a repeated glycosylation the yield increased to 65 %. Glycosylation of **53** with the glucose donor **62** (2 × 3.4 eq.) under similar conditions afforded **55** in 57 % yield. Cleavage of the resins **54** and **55** by addition of TFA-H_2O (9:1) gave the partially deprotected **56** and **57** in 24 %

and 12 % yield, respectively based on the loading capacity of the resin. Removal of the remaining *p*-fluorobenzoate was performed with NaOMe in methanol. The deprotected neoglycolipids **43** and **58** were conjugated to didecyl squarate[93] (3.3 eq.) in 20 % and 27 % yield respectively. Didecyl squarate was used since it is more lipophilic than dimethyl squarate and hence, purification could be facilitated. The total yield was ~5 % for **59** and ~2 % for **60** over 13 steps (excluding steps required to synthesis **61** and **62** based on the initial loading of TentaGel™.

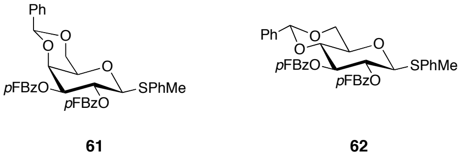


Figure 9. Galactose and glucose thioglycoside donors.

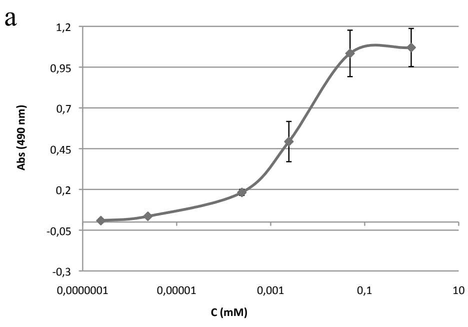
3.4 Attachment of amino-functionalized glycoconjugates to solid surfaces and investigation of protein-carbohydrate interactions (Paper II)

The model compounds were attached to amino-functionalized plates to optimize conditions for immobilization and detection. The glycoconjugates were then attached as described for the model compounds and detected with lectin in order to investigate carbohydrate protein interactions.

3.4.1 Immobilization and detection of amino-functionalized biotin

The biotin model compounds **51** and **52** were dissolved in sodium hydrocarbonate buffer ($NaHCO_3$) (pH 9) and serially diluted (1.0 mM to 0.24 nM and 1.1 mM to 2.2 nM, respectively) in amino-functionalized microtiter plates and incubated for 18 hours in room temperature. The immobilized model compounds were detected with avidin-horseradish peroxidase (avidin-HRP) and substrate (Figure 10). A clear dose-response is observed and the result indicates that the carbamate linker and squarate array strategy is a promising approach.

a



b

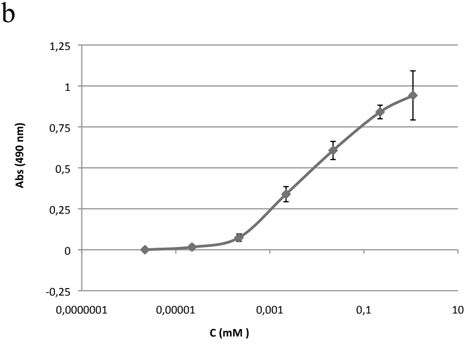


Figure 10. Binding of avidin-HRP to the immobilized biotin model compounds **51** (a) and **52** (b). The compounds were serially diluted and covalently immobilized in wells of clear amino-functionalized microtiter plates and probed with the avidin-HRP and substrate. Figure adopted from Paper II.

Compounds **51** and **52** were attached to white amino-functionalized microtiter plates and subsequently probed with fluorescein isothiocyanate labeled avidin (avidin-FITC) in order to investigate the potential for detection by fluorescence (data not shown). Both absorbance and fluorescence technique show potential but detection with avidin-HRP was superior to avidin-FITC. The background level and signal to noise ratio were clearly better for avidin-HRP. Fluorescence detection is generally performed in black microtiter plates since that reduces the detected background signal. However, to our knowledge no black amino-functionalized microtiter plates were commercially available at that point of time.

3.4.2 Immobilization and detection of the neoglycoconjugates

The neoglycolipids **59** and **60** were dissolved in $NaHCO_3$ buffer (pH 9) and serially diluted (0.1 mM to 48 nM) on the microtiter plates as described for the biotin model compounds **51** and **52** (Scheme 13).

59 (Gal) 60(Glc) → a → 63 (Gal) 64 (Glc)

Scheme 13. Immobilization of squaric amide ester neoglycolipid **59** and **60** in amino-functionalized microtiter plates. *Reagents and conditions:* a) $NaHCO_3$ buffer (pH 9), CovaLink^{TM} (Nunc A/S, Denmark) microtiter plate.

Detection of the immobilized galactose derivative **63** was performed with biotin-conjugated lectin from *Ricinus communis* (RCA_{120}) and avidin-HRP. Maximum absorbance was observed at 0.01 mM concentration (Figure 11 a). This concentration is low, especially in comparison to a previously published method[66]. In that study **42**, an analog to **41**, (Figure 7, section 3.3) was immobilized as an amide in the same type of microtiter plates and ~1 mM concentration was necessary for a maximal response with the same lectin. The squarate immobilization method is clearly more efficient, as observed from the clear dose-response curve (Figure 11a). The lectin RCA_{120} is specific for α- and β-galactose and can therefore not bind to glucose. The galactose and glucose derivatives **59** and **60** were diluted to 0.025 mM and immobilized and detected as described for **63** (Figure 11b). No signal was detected from the wells with immobilized glucose conjugate **64** verifying that there was no unspecific binding of the lectin. This method is suitable for immobilization of glycoconjugates and detection with lectins, which is in agreement with a previous publication[93].

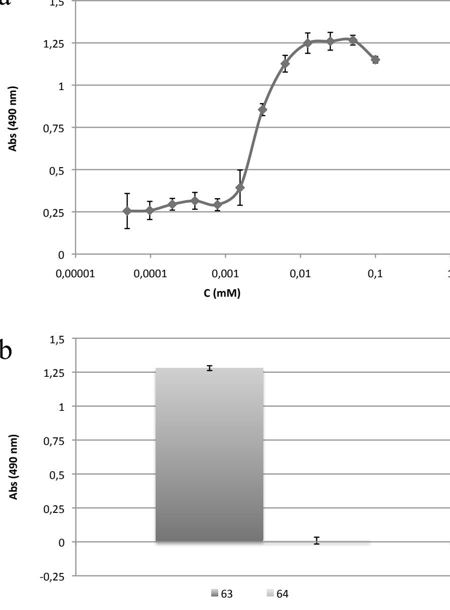


Figure 11. a) Binding of galactose selective lectin from *Ricinus communis* (RCA_{120}) to the immobilized neoglycolipid **63**. The compound was serially diluted in the wells and covalently immobilized to the amino-functionalized wells. The immobilized neoglycolipid was detected with biotin-conjugated RCA_{120} and avidin-HRP. b) Binding of the galactose selective lectin RCA_{120} to the immobilized neoglycolipids **63** and **64** (0.025 mM). No binding of the lectin was observed in the wells reacted with the glucose neoglycolipid **64**. Figures adopted from Paper II.

3.4.3 Summary

Synthesis of carbohydrates and glycoconjugates, in particular on solid phase, is complicated. In order to develop straightforward synthetic protocols for solid-phase synthesis of glycoconjugates we have developed a novel fluorinated linker **14** (Figure 4). The fluorine enables reaction monitoring with gel-phase ^{19}F NMR spectroscopy. The linker was equipped with an electron donating methoxy group in order to facilitate cleavage of a carboxylic acid using an acid of moderate strength. In comparison with the previously developed linker **15**, cleavage was easily performed with 20 % TFA in water. In addition, a carbamate linker strategy was employed in order to enable mild cleavage of amino-functionalized products based on resin **45** and **46** (Scheme 10). By attachment to dialkyl squarate the conjugate can be to crosslinked to amino-functionalized surfaces such as microtiter plates or proteins. This method is suitable for immobilization of glycoconjugates and detection with labeled proteins and possibly pathogens such as bacteria and viruses.

4. Synthesis and application of a 2-[(4-fluorophenyl)sulfonyl]ethoxy carbonyl (Fsec) protected glycosyl donor in carbohydrate chemistry (Paper III)

The fluorine containing protecting group N-[1-(4-(4-fluorophenyl)-2,6-dioxocyclohexylidene)ethyl] (Fde) (Figure 12) was recently developed for protection of α-amino acids for solid-phase synthesis of peptides.[68] The Fde group was based on a similar protecting group [(4,4.dimethyl-2,6-dioxocyclohexylidene)ethyl] (Dde) group (Figure 12), since Dde has proved to be relatively stable towards TFA and piperidine and can easily be cleaved by 2 % hydrazine in DMF[71].[69, 70] The two methyl groups in the dimedone ring in Dde was replaced by a *p*-fluorophenyl ring. Gel-phase ^{19}F NMR spectroscopy can be applied during the solid-phase synthesis due to the fluorine atom in the Fde group. Cleavage of the Fde group was performed with 2 % hydrazine or 5 % hydroxylamine in DMF. The 2-(4-trifluoromethylphenylsulfonyl)ethoxycarbonyl (Tsc) (Figure 12) group have been used for *N*-protection of amino acids in solid-phase synthesis of peptides.[96] The Tsc group can be removed under mild basic conditions, *e.g.* 0.1 N LiOH in THF:H_2O (1:1) at 0 ° C. Even though the protecting group contains fluorine atoms gel-phase ^{19}F NMR spectroscopy was not performed in order to monitor reaction progress. The methylsulfonylethoxycarbonyl (Msc)[97] group has been synthesized and evaluated for *O*-protection in carbohydrate chemistry[98] but was originally developed and used for *N*-protection of amino acids[97]. The Msc group is an alternative to the Fmoc group for *O*-protection and it is less lipophilic and sterically demanding than the bulky Fmoc group. The Msc group was cleaved with *e.g.* TBAF (0.1 eq.) in THF (30 min.) and DBU (0.1 eq.) in DMF (25 min.) in quantitative yields.[98] The fluorous propylsulfonylethoxycarbonyl (FPsc) group, has been synthesized and evaluated for *O*-protection in carbohydrate chemistry.[98] The FPsc group is a fluorinated analog to the Msc group and was used for synthesis of a trisaccharide.[98] The fluorous component in the FPsc group ($F_{17}C_8$-) was introduced and exploited for purification purposes. The trisaccharide was purified by fluorous solid-phase extraction (FSPE)[99].

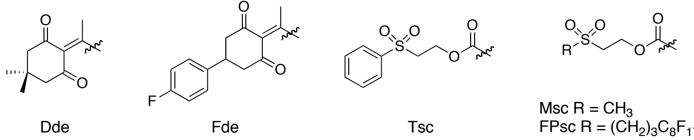


Figure 12. The Dde, Fde, Tsc, Msc, and FPsc protecting groups.

The Fmoc group is used to some extent for *O*-protection in solution and solid-phase synthesis of carbohydrates.[8-10, 100] The Fmoc group is however sterically demanding and we have not been able to introduce it at unreactive and sterically hindered hydroxyl groups in carbohydrates (not published). We therefore designed, synthesized, and evaluated a new fluorinated *O*-protecting group suitable for solution and solid-phase synthesis of glycoconjugates. The new protecting group, 2-[(4-fluorophenyl)sulfonyl]ethoxy carbonyl (Fsec) group (Scheme 14), was designed for mild cleavage under basic conditions. In addition, the Fsec protecting group is less sterically demanding compared to Fmoc, which might be advantageous for protection of unreactive and sterically hindered hydroxyl groups *e.g.* the 4-OH in galactose. The fluorine atom allows reaction monitoring with gel-phase ^{19}F NMR spectroscopy, which is very convenient.

4.1 Synthesis of 2-[(4-fluorophenyl)sulfonyl]ethoxy carbonyl chloride (Fsec-Cl)

2-[(4-Fluorophenyl)sulfonyl]ethoxy carbonyl chloride (Fsec-Cl) **68** was synthesized in 91 % yield over three steps (Scheme 14). First, 4-fluorothiophenol **65** was reacted with 2-bromoethanol under basic conditions giving 2-[(4-fluorophenyl)thio]ethanol **66**. Subsequent oxidation with *m*-CPBA produced compound **67** in 92 % yield. Compound **67** was reacted with triphosgene to afford Fsec-Cl **68** in quantitative yield

65 —a→ **66** —b→ **67** —c→ Fsec-Cl **68**

Scheme 14. Synthesis of Fsec-Cl **68**. *Reagents and conditions:* a) 2-Bromoethanol (1.1 eq.), Et_3N (4 eq.), CH_3CN, room temperature, over night; b) *m*-CPBA (3 eq.), CH_2Cl_2, room temperature, 22 h, 92 % over two steps; c) Triphosgene (19 eq.), DMF (1 droplet), CH_3CN, -50 $^{\circ}$C-room temperature, 24 h, 99 %.

4.2 Stability of the *O*-Fsec group

The stability of the *O*-Fsec protecting group was tested under both basic and acidic conditions. Since glycosyl donors are very time-consuming to synthesize a model substance **69**, 4-fluorobenzyl 2-[(4-fluorobenzyl)sulfonyl]ethyl carbonate, was used for the stability studies (Scheme 15). Compound **69** was synthesized from 4-fluorobenzyl alcohol and Fsec-Cl **68** under basic conditions in 85 % yield.

Scheme 15. Synthesis of the model compound **69**. *Reagents and conditions:* a) 4-Fluorobenzyl alcohol (0.83 eq.), pyridine (0.83 eq.), CH_2Cl_2, room temperature, over night, 85 %.

The Fsec group could be completely removed by *e.g.* 1.1 eq. TBAF in THF or 20 % piperidine in DMF in three minutes at room temperature according to analysis of the reaction mixtures with LC-MS (Entry 1 and 2, Table 2). The chromatogram showed no formation of by-products. It was found that the Fsec group is stable under acidic conditions (entry 8-10) and this is important since glycosylation reactions are generally performed under acidic conditions.

Table 2. Stability test of the *O*-Fsec group in **69**.

Entry	Reagents	Solvents	Time (min.)	Removal[a]
1	1.1 eq. TBAF	THF	3	Quant.
2	20 % piperidine	DMF	3	Quant.
3	20 % piperidine	CH_2Cl_2	6	Quant.
4	20 % morpholine	DMF	> 60	Quant.
5	20 % morpholine	CH_2Cl_2	360	~ 90 %
6	2 % DBU	DMF	3	Quant.
7	2 % DBU	CH_2Cl_2	3	Quant.
8	5 % AcOH	THF	300	Stable
9	5 % TFA	THF	300	Stable
10	Neat AcOH	-	300	Stable

[a] Estimated from the LC chromatogram.

4.3 Synthesis of a disaccharide employing an *O*-Fsec protected glycosyl donor

Since the Fsec protecting group met our requirements concerning stability and cleavage conditions the *O*-Fsec protected glycosyl donor **71** was synthesized (Scheme 16). The protected galactose donor **61** (Figure 9)[101] was treated with trimethylamino borane and aluminium chloride[7] to selectively open the acetal ring and give **70** in 85 % yield. We had earlier failed in our efforts to introduce Fmoc on the free 4-OH in **70** *e.g.* with Fmoc-Cl and pyridine in room temperature[100] or Fmoc-Cl and 4-dimethylaminopyridine (DMAP) under microwave irradiation. However, when Fsec-Cl was reacted with **70** in neat pyridine the expected product **71** was gratifyingly formed in 68 % yield.

61 → (a) → 70 (HO, OBn, *p*FBzO, *p*FBzO, SPhMe) → (b) → 71 (FsecO, OBn, *p*FBzO, *p*FBzO, SPhMe)

Scheme 16. Synthesis of the Fsec protected galactose donor **71**. *Reagents and conditions:* a) Trimethylaminoborane (4 eq.), aluminium chloride (6 eq.), water (2 eq.), THF, room temperature, over night, 84 %; b) Fsec-Cl **68** (3 eq.), pyridine, 45 °C for 1 h, 56-58 °C for 6 h, 68 %.

In order to further investigate the properties of the Fsec group, 5-aminohexyl galabiose **78** was synthesized. The amino functionality in 6-amino-1-hexanol was protected with CBz-Cl in 82 % yield. Next, the hydroxyl moiety in **73** (4 eq.) was glycosylated with the Fsec *O*-protected galactose donor **71** (1 eq.) under promotion with *N*-iodosuccinimide (NIS) (4 eq.) and trifluoromethane sulfonic acid (TfOH) giving **74** in 83 % yield (Scheme 17).

Scheme 17. Synthesis of the disaccharide **78**. *Reagents and conditions:* a) Benzyl chloroformate (CbzCl) (1.1 eq.), NaOH (aq.), (1.1 eq), CH_2Cl_2, 0 °C→ room temperature, 3.5 h, 82 %; b) **71** (donor) (0.25 eq.), *N*-iodosuccinimide (NIS) (1 eq.), TfOH (cat), dry CH_2Cl_2, room temperature, 2 h 83 %; c) TBAF in THF (1.1 eq.), room temperature, 11 min., 72 %; d) **80** (donor) (2 eq.), NIS (2 eq.), TfOH (cat.), dry THF:CH_2Cl_2, (1:1), room temperature, 39 %; e) NaOMe (10 eq.), dry MeOH, 1.5 h, 89 %; f) H_2 (g), Pd(C), AcOH, 31 h, room temperature, 50 %.

Removal of the Fsec was performed with TBAF in THF (1.1 eq.) for ~10 min yielding **75** in 72 % yield. The free hydroxyl group was glycosylated with the galactose donor **80** (2 eq.) under promotion with NIS (2 eq.) and TfOH giving **76** in 39 % yield. The galactose donor **80** was synthesized by benzylation of **79**[62] with *o*-fluorobenzyl bromide by addition of NaH in 83 % yield (Scheme 18).

Scheme 18. Synthesis of the galactose donor **81**. *Reagents and conditions*: a) *o*-Fluorobenzyl bromide (2.4 eq.) NaH (2.4 eq.), DMF, 18 h, 83 %.

The benzoyl protecting groups in the disaccharide **76** was removed with NaOMe in MeOH and the remaining protecting groups were removed with Pd on charcoal in AcOH giving **78** in 45 % yield over two steps (Scheme 17).

4.4 Solid-phase synthesis using an *O*-Fsec protected glycosyl donor

In order to further evaluate the properties of the Fsec group in solid-phase synthesis a model disaccharide was prepared. First the spacer 5-aminopentyl-4-fluorobenzoate **83** was synthesized in 60 % yield over two steps.

Scheme 19. Synthesis of 5-aminopentyl-4-fluorobenzoate hydrochloride **83**. *Reagent and conditions:* a) *p*-Fluorobenzoyl chloride (1.1 eq.), TEA (1.3 eq.), CH_2Cl_2, 0 °C → room temperature, 1 h; b) TFA (5.8 eq.), CH_2Cl_2, room temperature, 2 h, 60 % yield over two steps.

Next the spacer amine **83** was attached to the carbonate resin **44** (Scheme 10, section 3.3.1) forming the protected carbamate resin **84** (Scheme 20) in 90 % according to gel-phase ^{19}F NMR spectroscopy. The *p*-fluorobenzoyl was removed by addition of NaOMe in MeOH. The reaction was repeated twice, but there was still a small fraction of the *p*-fluorobenzoyl protecting group attached to hydroxyl groups. The reaction resulted in **85** in 95 % yield according to gel-phase ^{19}F NMR spectroscopy.

Scheme 20. Synthesis of the hydroxyl resin. *Reagent and conditions:* a) **83** (1.6 eq.), DIPEA (1.6 eq.), DMF, 20 h, 90 %; b) 3 × NaOMe (0.9-1.8 eq.), MeOH, 1 h, ~95 % yield, MeOH, 3 h, 95 %.

The resin was then glycosylated with the Fsec protected galactose donor **71** (2 eq.) under promotion with NIS (2 eq.) and TfOH (cat.) in 35 % yield according the ^{19}F NMR spectra (Scheme 21). The reaction was repeated two more times to get **86** in 80 % yield. In order to cap any unreacted hydroxyl groups *m*-fluorobenzoyl chloride and pyridine was added. Apparently the capping resulted in two benzoylated functional groups since the gel-phase ^{19}F NMR spectrum contained two new signals (-111.9 and -112.8 ppm). After repeated reactions approximately 9 + 11 % had been blocked. These signals were detected throughout the rest of the synthesis. Next, the Fsec protecting group was completely cleaved by repeated addition of 20 % piperidine in DMF. The 4-OH deprotected glycoconjugate was glycosylated

with the galactose donor **80** (4 eq.) using NIS (2 eq.) and TfOH (cat.). According to gel-phase ^{19}F NMR spectroscopy the yield was higher than 100 %, indicating that something more then 4-OH was glycosylated. This was very puzzling since all possible free hydroxyl groups were blocked with *m*-fluorobenzoyl chloride in a previous step. However, the synthesis proceeded by debenzoylation with NaOMe in MeOH. The reaction resulted in **89** in quantitative yield according to gel-phase ^{19}F NMR spectroscopy. Cleavage of the glycoconjugate from the linker resin by simultaneous deprotection of the benzylidene was performed by TFA:H_2O (9:1) and purification by HPLC resulted in **90** in 2.6 % total yield (based on the initial loading capacity of the resin). Due to the minimal amount of **90** (3 mg), the final deprotection step was not performed.

Scheme 21. *Reagents and conditions*: a) 3 × **71** (2 eq.), NIS (2 eq.), TfOH (cat.), CH_2Cl_2, 3 h, 35 % → 55 % → 80 % yield; b) *i*. *m*-fluorobenzoyl chloride (0.84 eq.), pyridine (0.84), CH_2Cl_2, 2 h; *ii*. 2 × 20 % piperidine in DMF, 10 min. quantitative yield; c) **80** (4 eq.), NIS (4 eq.), TfOH (cat.), CH_2Cl_2, 1 h; d) 2 × NaOMe (5 eq.), MeOH, 1 h, quantitative yield; e) 2 × TFA:H_2O (9:1), 1 h, 2.6 % total yield (based on the loading capacity of the resin); f) nor attempted.

4.5 Conclusions

The Fsec group, a fluorine containing *O*-protecting group, has been designed and synthesized. Removal of the Fsec group was performed under mild basic conditions. The Fsec group was stable under acidic conditions, which is necessary since glycosylation reactions are generally performed in acidic milieu. The new protecting group was easily introduced on the 4-OH in galactose, where attempts to introduce an Fmoc group failed.

The glycosyl acceptor (Cbz-6-amino-1-hexanol) was glycosylated with the Fsec-protected galactose donor in 83 % yield. After mild Fsec deprotection, a second glycosylation, and subsequent deprotection an amino-functionalized galabiose derivative was obtained in 9 % total yield.

The Fsec group was also evaluated for solid-phase synthesis of a disaccharide derivative. Glycosylation of the spacer-linker resin with the Fsec-protected galactose donor (2 eq.) under promotion by NIS and TfOH gave unfortunately only gave 35 % yield according to gel-phase ^{19}F NMR spectroscopy. The reaction was repeated two times, which increased the yield to 80 %. After mild Fsec deprotection, a second glycosylation was performed. According to gel-phase ^{19}F NMR spectroscopy the yield was >100 %. The reason for this is unclear but nucleophiles of some kind in the solid-phase that did not react with the linker or the unreactive β-glycosyl donor, possibly reacted with the α-glycosyl donor. The capping procedure was also intricate since it gave rise to two signals in the ^{19}F spectrum. The problems encountered might stem from batch variations in the solid support. It has been shown that resin quality differ considerably even between different batches of resin from the same source.[102] Such variations in resin quality is a challenge for future development of solid-phase synthesis. Nevertheless, subsequent deprotection and cleavage resulted in a benzyl protected galabiose derivative in 2.6 % total yield.

5. Synthesis and evaluation of tri- and tetravalent sialic acid inhibitors of EKC-causing adenoviruses (Paper IV)

Previous studies have shown that multivalent 3´-sialyllactose- and sialic acid-HSA conjugates (Figure 13) can inhibit adenovirus binding and infection of human corneal epithelial (HCE) cells.[81-83] The acetamide functionality in sialic acid was varied in order to further improve the potency of the sialic acid conjugates by increased hydrophobic interactions.[83] The *N*-acyl modified sialic acids were tested in an Ad37 cell binding assay. The compounds with highest activity were synthesized as multivalent conjugates and evaluated for inhibition of infection. Unfortunately none of them were as potent as the original multivalent sialic acid-HSA conjugate. A comparison of the crystal structures of the two *N*-acyl modified sialic acids in complex with the sialic acid interacting domain of the Ad37 fiber protein (the knob domain) in complex with sialyllactose in complex with Ad37 fiber knob revealed that *N*-acyl modified sialic acids binds in a similar manner as sialic acid.

Figure 13. Multivalent 3´-sialyllactose-HSA conjugates (above)[81], multivalent sialic acid-HSA conjugates (right, R = CH_3)[82], and the multivalent *N*-acyl modified sialic acid conjugates (below, R = CH_2CH_3, $CH_2CH_2CH_3$ or $CH(CH_3)_2$)[83]. The valency *n* is typically 3-19.

The Ad37 fiber knob consists of a homotrimer that presents three separate binding sites.[78] Since the multivalent sialic acid-HSA conjugate are relatively big (74,381 g/mol) it was hypothesized that only one sialic acid from a multivalent sialic acid-HSA conjugate binds to one binding site in a

fiber knob domain, and another sialic acid on the same conjugate binds in the same manner to another virion.[82] This will then lead to crosslinking of the virions and formation of aggregates that cannot adhere to HCE cells.

Since the binding pocket has three binding sites we aspired to synthesize smaller conjugates that possibly could fit and fill one, two, or even three of the binding sites. Also, the HSA conjugates are quite big (mw > 70,000 g/mol) and not economical to synthesize since large amounts of sialic acid building blocks are required.

5.1 Synthesis of tri- and tetravalent sialic acid conjugates

Since the distance between the binding sites in the Ad37 fiber knob domain is relatively short we anticipated difficulties in designing and synthesizing derivatives that would fit perfectly. Instead we aimed to synthesize flexible molecules that hopefully would fill at least two binding sites. Three scaffolds were selected for conjugation to the sialic acid building block **97**[82] (Scheme 23) that was used in the synthesis of the multivalent sialic acid-HSA conjugate. The scaffolds were chosen due to their length, valency, functionality, flexibility, and ease of access (Figure 14).

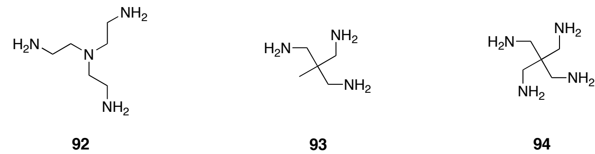


Figure 14. Tri- and tetravalent scaffolds selected for conjugation with sialic acid.

Scaffold **92** has previously been used for conjugation to mannose squaric ethyl ester and evaluated for its anti-adhesive properties against type 1 fimbriated *E. coli*.[103] Scaffold **92** and **93** are commercially available and **94** can be synthesized in two steps from pentaerythrityl tetrabromide according to a previously reported procedure[104] (Scheme 22). The pentaerythrityl tetrazide was obtained by treatment of pentaerythrityl tetrabromide with sodium azide. Since alkylazides are explosive compounds they should be handled with great care.[105] The conversion of the tetrazide into pentaerythrityl tetramine was performed by Staudinger ligation and the tetraiminophosphorane was hydrolyzed under acidic conditions yielding **94** in 76 % over two steps.

Scheme 22. Synthesis of the tetravalent scaffold **95**[104]. a) NaN_3 (8 eq.), DMSO, 96 °C, 20 h; b) PPh_3 (5 eq.), NH_3 (aq.), dioxane, HCl (aq.) room temperature, 20 h 76 % yield.

Synthesis of the tri- and tetravalent conjugates were performed by conjugation of the sialic acid squaric decyl ester **97** to the scaffolds in presence of DIPEA in methanol according to a previously reported procedure (Scheme 23).[81]

Scheme 23. Coupling of the sialic acid squaric decyl ester **97**[82] to the scaffolds to form the conjugates **98**-**100**. *Reagents and conditions:* a) **92, 93**, or **94**, DIPEA, MeOH, room temperature, 2-8 days, 29-69 % yield.

Attempts to synthesize divalent conjugates were also performed. The amino-functionalized sialic acid **101** was reacted with the sialic acid squaric decyl ester **97** in presence of DIPEA but no conversion was observed (Scheme 24). Previously, **97** was conjugated to amines on HSA in $NaHCO_3$-buffert in the synthesis of the sialic acid-HSA conjugates (Figure 13)[82]. When **97** was reacted with ethylene diamine **103** in $NaHCO_3$-buffer the divalent conjugate **104** was formed in small amounts (< 10 %) after four hours according to LC-MS (Scheme 24). After four days there was still a large amount of the starting material left and when the reaction mixture was heated (30 °C) for five hours conversion did not increase. Since the syntheses of the tri- and tetravalent conjugate were successful no further attempts to synthesize a divalent conjugate were done.

Scheme 24. Failed syntheses of two divalent sialic acid conjugates. *Reagents and conditions:* a) Didecyl squarate, DIPEA, MeOH, room temperature; b) **97** (2.1 eq.), $NaHCO_3$ (pH 9).

5.2 Inhibition of adenoviral cell attachment and infection

The inhibitory effects of **98-100** were evaluated in a cell-based virus binding assay[81-83]. Dilution series of sialic acid, the tri- and tetravalent sialic acid conjugates **98-100**, and a 17-valent sialic acid-HSA conjugate were pre-incubated with ^{35}S-labeled Ad37 virions for one hour. The virions were added to HCE cells and after one hour unbound virions were washed away and cell-associated radioactivity was measured. All conjugates were efficient as inhibitors of binding of Ad37 to HCE cells and were at least two orders of magnitude more efficient then sialic acid (Figure 15). The most potent conjugate **98** was as efficient as the 17-valent sialic acid-HSA conjugate.

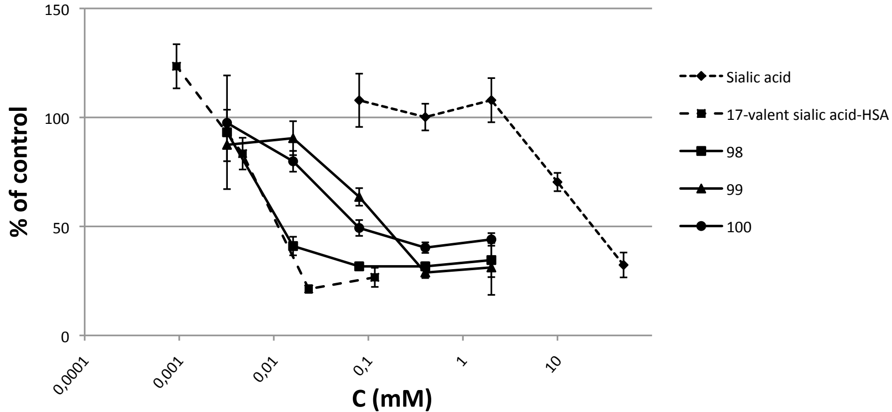


Figure 15. Conjugates **98-100** inhibit binding of Ad37 to HCE cells to the same extent as 17-valent sialic acid-HSA conjugate. ^{35}S-labeled Ad37 virions were pre-incubated (1 h) with sialic acid, tri- or tetravalent glycoconjugates **98-100**, and 17-valent sialic acid-HSA conjugate at different concentrations and further incubated with HCE cells (1 h), and analyzed in a scintillation counter for measurement of cell-associated radioactivity. On the Y axis 100 % of control corresponds to viral attachment in absence of an inhibitor. Mean values and standard deviations (calculated with the Gauss approximation formula) are from duplicates, and experiments were reproduced three times; except from the 17-valent sialic acid-HSA conjugate, which was only performed once. Figure adopted from Paper IV.

The potential of the most potent conjugate **98** was further evaluated in a cell-based infection assay[81-83]. Dilution series of **98** or sialic acid were pre-incubated with Ad37 virions for one hour and then let to infect monolayers of HCE cells. After cell-fixation and staining with anti-adenoviral antibody, infected cells were identified in a fluorescence microscope (Figure 16a and 16b). Conjugate **98** proved to be very potent and efficiently inhibited Ad37 from infecting HCE cells (Figure 16b). The IC_{50} value for **98** was approximately 0.5 μM, about four orders of magnitude better than sialic acid.

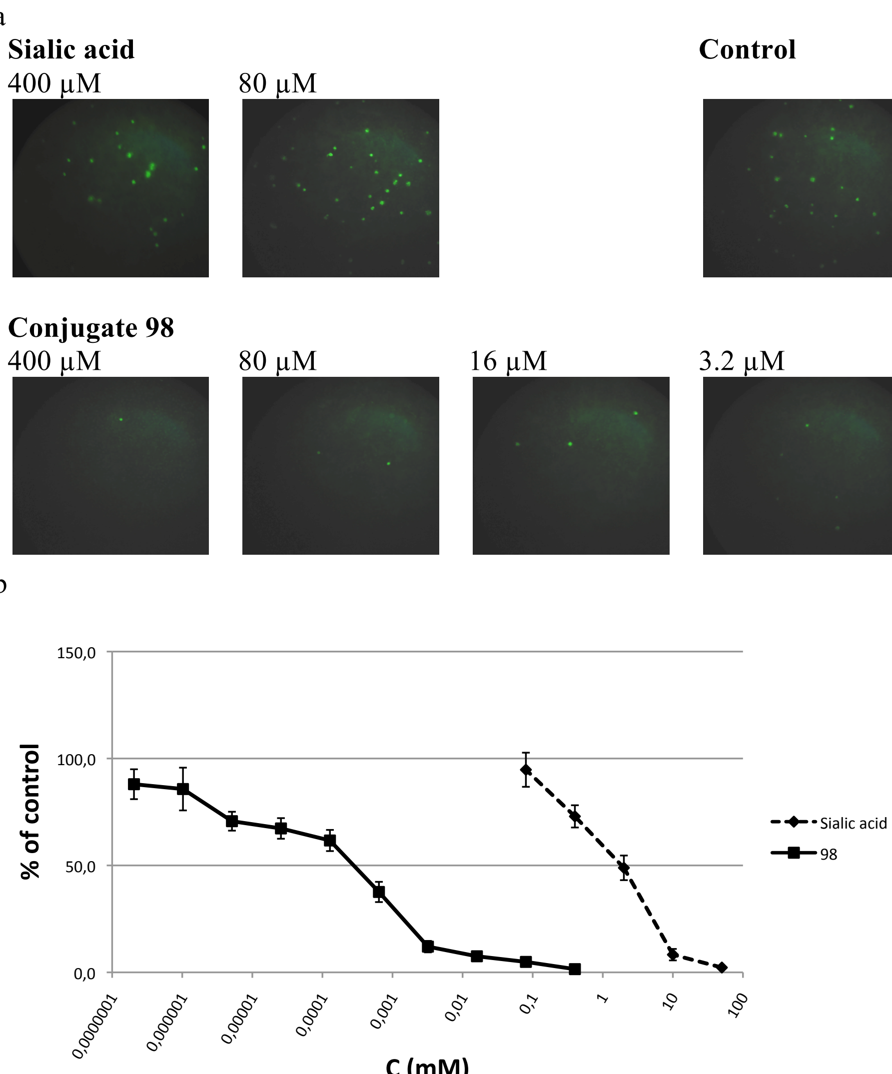


Figure 16. Conjugate **98** protects HCE cells from infection by Ad37. Sialic acid (diamonds) or **98** (squares) were pre-incubated with non-labeled Ad37 (on ice, 1 h) and further incubated with a monolayer of HCE cells, first on ice (1 h) and after removal of unbounded virions in 37 °C (44 h). Control experiments were performed without any inhibitor. The cells were then fixed, labeled, and analyzed in a fluorescence microscope. a) Pictures showing infected cells (green) in absence (control) and presence of different concentrations of sialic acid and **98**. b) Graph showing quantitative data representing an average of two independent experiments. On the Y axis 100 % control corresponds to viral infection in absence of inhibitor. Mean values and standard deviations (calculated with the Gauss approximation formula) are from duplicates of two independent experiments. Figure adopted from Paper IV.

5.3 Crystal structure

To investigate the binding of the trivalent sialic acid conjugate **98** to Ad37 fiber knob domain, X-ray crystallography was used. The structure of an Ad37 fiber knob domain in complex with the inhibitor trivalent sialic acid conjugate **98** was solved at 2.4 Å resolution (Figure 17). No ordered structure were found for the spacers and scaffolds. The result shows that all three sialic acids bind simultaneously to the fiber knob.

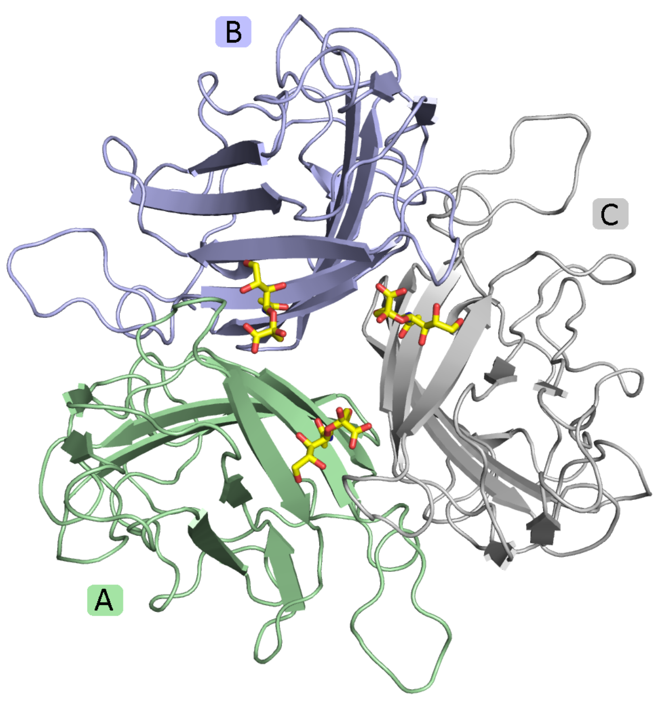


Figure 17. Overall structure of the Ad37 fiber knob in complex with **98**. The three Ad37 chains A, B and C are shown as ribbon tracings and colored green, blue and grey. The sialic acids of **98** are bound on top of the fiber head and are shown in stick representations with carbons drawn in yellow, oxygens in red, and nitrogens in blue. The spacers and scaffold are not shown. Figure from Paper IV.

5.4 Conclusions

We successfully synthesized three new small multivalent sialic acid conjugates **98-100** (mw: 1533.63-2020.81 g/mol). These conjugates were easily synthesized in preperatively useful yields. We have also showed that

they efficiently inhibited binding of Ad37 to HCE cells in the same range as 17-valent sialic acid-HSA conjugate. Importantly, they are more drug-like and less sialic acid is consumed which is very advantageous. The trivalent conjugate **98** proved to be a very potent anti-adenoviral agent with an approximate IC_{50} value of 0.5 μM in the cell-based infection assay. The conjugate is four orders of magnitude more potent than sialic acid and substantially more efficient than previously described multivalent 3'-sialyllactose and sialic acid-HSA conjugates[81-83]. A crystal structure revealed that all three of the sialic acids in conjugate **98** simultaneously bind to the fiber knob domain. The results support the hypothesis that small compact sialic acid conjugates have the potential to be developed into anti-adenoviral drugs for eye administration to prevent or possibly cure EKC.

6. Polypeptide conjugates

Most biological processes rely on molecular interactions, *e.g.* between a protein and a small molecule ligand, cell membranes or another protein. These interactions are very complex and more knowledge would increase the possibilities to find new therapeutics. Drugs, often low-molecular weight substances, must have multiple functional groups to enable strong and specific binding to the target protein. The process to develop potent compounds is very tedious, difficult, and expensive. In interactions between two proteins the number of participating atoms can be 200 and depend on several types of "weak" non-covalently interactions (hydrogen bonds, and hydrophobic and electrostatic interactions), which together form a relatively strong binding.

The research group of Prof. Lars Baltzer (Uppsala University) have described a concept for protein recognition, where a small molecule ligand is attached to synthetic polypeptide consisting of 42 amino acids (Figure 18).[106] The ligand provides specificity and the peptide gives affinity and the overall result is a conjugate with both specificity and potency**.** This conjugate is called a binder and the method has been applied in order to find for example new diagnostic tools. To the polypeptide a fluorescent probe is attached in order to be able to monitor binding to a target. Ideally the binder, the ligand-polypeptide conjugate, should have a structure that corresponds to the target protein considering shape and charge but that is difficult to achieve. Instead, they have designed a library of 16 polypeptides with different properties, for example charge, to which the ligand and the probe can be coupled in different positions. They all contain a helix-loop-helix motif as a base structure and the aim was to use this small set of polypeptide scaffolds and combine them with small molecules especially selected for the protein in question, to form specific high-affinity binders in simple procedures. This set of polypeptides is supposed to be applicable to a wide range of proteins. By chemical modification of the side-chains in the polypeptides a drug-like molecule, a ligand, can be introduced at different positions. The affinity can be increased up to 100-10,000 times with affinities in the pM to nM range, compared to the ligand itself.[107] In their work they have studied C-reactive protein, Carbonic anhydrase, and acetylcholine esterase with promising results.[106]

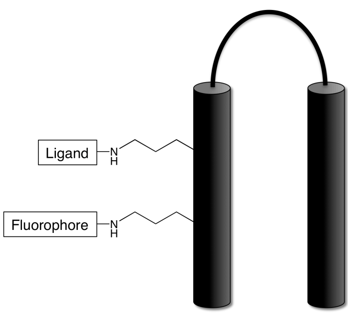


Figure 18. A schematic representation of a binder where a ligand and a fluorophore are attached to the polypeptide. If the binder interacts with to the target protein the fluorescence will be different compared to unbound binder since the environment for the fluorescent probe will be changed.

6.1 Synthesis and evaluation of sialic acid-polypeptide conjugates

Since EKC is an infection in the eye, an active binder can potentially be used as an antiviral agent, since it will be administered locally through an eye cream or eye drops, and therefore not be degraded by *e.g.* peptidases. To find high-affinity binders for Ad37 fiber knob, binders with the naturally ligand sialic acid was synthesized. Under basic conditions ($NaHCO_3$, pH 9) the activated sialic acid **97** (Scheme 23, section 5.1) (2 eq.) was conjugated to lysine side chains in 14 different polypeptides (1 eq.) (Figure 19).[81-83] Some of the reactions were monitored by HPLC and MALDI-TOF. The reactions were stopped when the conversion to conjugate reached approximately 95-100 %. The reaction mixtures were not purified before biological evaluation.

1-D15L8	*Ac*-NEADLEA**K**IRHLAEKLEARGPEDAEQLAEQLARAFEAFARAG-*COOH*	-7
2-D15L8	*Ac*-NEADLEA**K**IRHLAEKLAARGPVDAAQLAEQLARAFEAFARAG-*COOH*	-4
3-D15L8	*Ac*-NAADJEA**K**IRHLAEKJAARGPVDAAQJAEQLARRFEAFARAG-$CONH_2$	-1
4-D15L8	*Ac*-NAADJEA**K**IRHLREKJAARGPRDAAQJAEQLARRERFARAG-*CONH2*	2
1-D10L17	*Ac*-NAADLEAAIKHLAEAL**K**ERGPEDCEQLAEQLARAFEAFARAG-*COOH*	-7
2-D10L17	*Ac*-NAADLEAAIKHLAEAL**K**ARGPVDAAQLAEQLARAFEAFARAG-*COOH*	-4
3-D10L17	*Ac*-NAADJEARIKHLAERJ**K**ARGPVDAAQJAEQLARAFEAFARAG-$CONH_2$	-1
1-D25L22	*Ac*-NEADLEAAIRHLAEALEARGP**K**DAKQLAEQLARAFEAFERAG-*COOH*	-6
2-D25L22	*Ac*-NEADLEAAIRHLAEALAARGP**K**DAKQLAEQLARAFEAFARAG-*COOH*	-4
3-D25L22	*Ac*-NAADJEAAIRHLAERJAARGP**K**DAKQJAEQLARAFEAFARAG-$CONH_2$	-1
4-D25L22	*Ac*-NAADJEARIRHLRERJAARGP**K**DAKQJAEQLARAFERFARAG-$CONH_2$	2
1-D37L34	*Ac*-NEADLEAAIRHLAERLEARGPADAAQLAEQLAA**K**FEKFARAG-*COOH*	-5
2-D37L34	*Ac*-NAADLEAAIRHLAERLAARGPVDAAQLAEQLAA**K**FEKFARAG-*COOH*	-3
4-D37L34	*Ac*-NAADJEARIRHLRERJAARGPRDAAQJAEQLAR**K**FEKFARAG-$CONH_2$	2

Figure 19. The amino acid sequence of the polypeptides in the library with the total charge of the binder conjugates are displayed to the right. The fluorophore Dansyl is incorporated into one lysine side chain before the peptide is cleaved from the solid support and thereby only one lysine is available for ligand conjugation in solution. The site of ligand incorporation is shown in bold face.

Evaluation of interactions between the binders (the sialic acid-polypeptide conjugates) and adenovirus fiber knob protein[108] was done in a fluorescence-based assay. If any of the sialic acid-polypeptide conjugates interacts with a protein the environment for the Dansyl probe will change leading to a change in fluorescence, compared to the control (polypeptide only). For the sialic acid-polypeptide conjugate (4-D25L22) the fluorescence from the control (sialic acid-polypeptide conjugate only) differs from sample with sialic acid-polypeptide conjugates and protein (Figure 20). Hence, a binding might have occurred since there is a shift in fluorescence. However, in all of these experiments, the fluorescence signal was considerably low and noisy. It appears as 1 or 2 μM of the binder carrying a Dansyl is too low concentration for reliable results. However, since there was a limited access of fiber knob protein, no higher concentration could be used at this point. In the studies performed by Tollstoy Tegler *et al.* 7-Methoxycoumarine-3-carboxylic acid (coumarine) was used as a fluorophore and the concentration of the binder was 0.5 μM.[106]

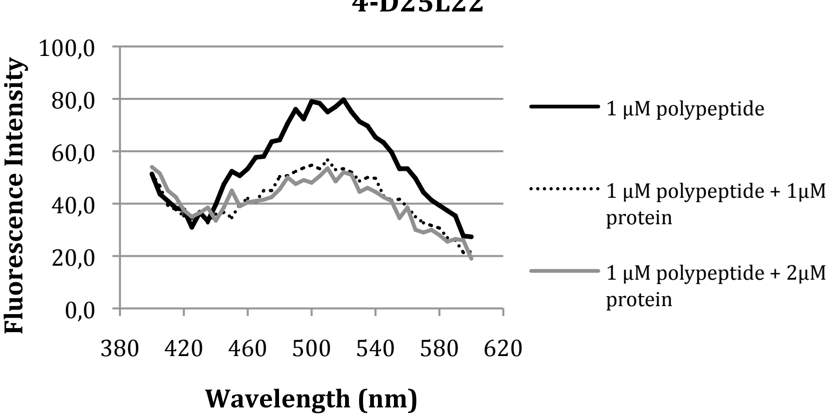


Figure 20. The binder (sialic acid conjugated to the polypeptide 4-D25L22) (1µM in phosphate-buffered saline (PBS)-buffer) was mixed with the protein (0, 1 µM, or 2 µM in PBS-buffer). The binder show activity since the fluorescence has changed when the binder has bound to the protein compared to the control (0 µM protein). X-axis show different wavelengths (400-600 nm) and the y-axis show fluorescence intensity.

The most promising sialic acid-polypeptide conjugate (4-D25L22) was evaluated in a cell-based virus binding assay[81-83]. Dilution series of sialic acid and the sialic acid-polypeptide conjugate were pre-incubated with ^{35}S-labeled Ad37 virions for one hour. The virions were added to human corneal epithelial (HCE) cells and after one hour unbound virions were washed away and cell-associated radioactivity was measured (Figure 21). Some dose-response relationship can be seen but the inhibitory effect of the sialic acid-polypeptide conjugate at 1 mM is relatively low compared to the trivalent conjugate **98** or the 17-valent sialic acid-HSA conjugate (section 5.2). Unfortunately, too low amount of the binder was obtained for further evaluation.

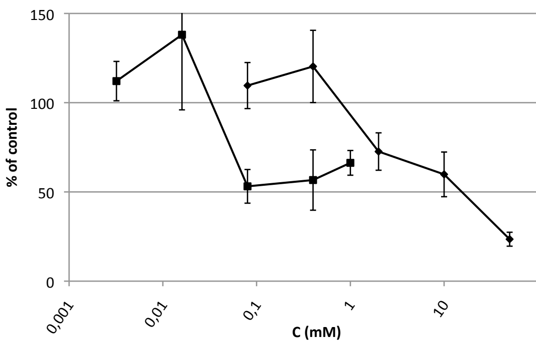


Figure 21. ^{35}S-labeled Ad37 virions were pre-incubated with 4-D25L22 (1, 0.4, 0.08, 0.016, and 0.0032 mM) (squares) and sialic acid (50 mM-0.08 mM) (diamonds) (1 h) and further incubated with HCE cells (1 h), and analyzed in a scintillation counter for measurement of cell-associated radioactivity. On the Y axis 100 % of control corresponds to viral attachment in absence of an inhibitor. Mean values and standard deviations (calculated with the Gauss approximation formula) are from duplicates, and experiments were reproduced two times.

6.2 Conclusions

Even though these results are not convincing and the binder is considerably less active than the trivalent sialic acid conjugate, cf. chapter 5, we still believe that there are some potential for this project. If a binder is active, the binder is preferably re-synthesized without the fluorescent probe, and then evaluated again. Since the probe most likely influence the conformation of the polypeptide the activity might be affected. Instead of the evaluation of the binders in the fluorescent-based assay, binders without a fluorescent probe should be conjugated to the ligand and then directly evaluated in the cell-based virus binding assay. That procedure would increase the probability to find potent anti-adenoviral sialic acid polypeptide conjugates.

7. Concluding remarks

Glycoconjugates have important roles in biological systems. To further elucidate important carbohydrate protein interactions synthetic glycoconjugates are needed.

The general aim with this thesis was to establish efficient methods for synthesis of glycoconjugates and to study carbohydrate protein interactions.

- A new acid-labile fluorinated 2-(2-fluoro-4-hydroxymethyl-5-methoxy-phenoxy)acetic acid linker was designed, synthesized, and evaluated. The linker allows reaction monitoring of *e.g.* glycosylation reactions with the product still attached to the resin. Thanks to the linker, the product can be cleaved from the resin under mild acidic conditions, which is useful for sensitive compounds.

- The novel fluorinated protecting 2-[(4-fluorophenyl)sulfonyl]ethoxy carbonyl (Fsec) group was designed and applied for *O*-protection in solution and solid-phase synthesis. The group can be used to protect unreactive and sterically demanding positions in a carbohydrate. The protecting group was removed under mild basic conditions, which is advantageous for orthogonal protecting strategies.

- A protocol for solid-phase synthesis of amino-functionalized glycoconjugates was developed. The resulting conjugates can easily be attached to solid surfaces for biological evaluation, for example as carbohydrates microarrays.

- Three tri- or tetravalent sialic acid compounds have been designed, synthesized and evaluated for their ability to inhibit adenovirus binding to HCE cells. One trivalent conjugate proved to be very potent as inhibitor of adenoviral infection of HCE cells. A X-ray crystal structure for the trivalent conjugates in complex with the fiber knob protein was solved and more knowledge of the carbohydrate protein interactions has been gained.

Solid-phase synthesis of glycoconjugates is still complicated and far from routine synthesis. More research and ideas are demanded before solid-phase synthesis can be routinely used for assembly of glycoconjugates.

The trivalent sialic acid conjugate proved to be a very potent anti-adenoviral agent with great affinity for the Ad37 fiber knob. This conjugate has potential to be further optimized and developed into an anti-adenoviral drug for eye administration to prevent or possibly cure EKC.

8. Supplementary

8.1. Synthesis and application of a 2-[(4-fluorophenyl)sulfonyl]ethoxy carbonyl (Fsec) protected glycosyl donor in solid-phase synthesis

Experimental section for solid-phase synthesis

General

Solid-phase synthesis was performed on TentaGel HL-NH_2 resin (0.42 mmol/g) from Rapp Polymere. CH_2Cl_2 was distilled from calcium hydride, THF from potassium, and DMF was distilled under vacuum. TLC was run on Silica Gel 60 F_{254} (Merck) and the spots were detected in UV-light and stained with H_2SO_4 in ethanol and heat. Silica gel (Matrex, 60 Å, 35-70 mm, Grace Amicon) and solvents of analytical grade were used for flash column chromatography. ^1H and ^{13}C NMR spectra were recorded at 298 K on a Bruker DRX-400 at 400 and 100 MHz, respectively, with D_2O or pyridine-d_5 as solvent and residual D_2O (δ_H 4.79 ppm) or pyridine-d_5 (δ_H 8.74, 7.58, and 7.22 ppm) as internal standard for ^1H and pyridine-d_5 (δ_C 50.3, 135.9, and 123.9 ppm) as internal standard for ^{13}C. Gel-phase proton decoupled ^{19}F NMR spectra were recorded at 298 K on a Bruker DRX-400 at 376 MHz on resin suspensions in $CDCl_3$ with $CFCl_3$ (δ_F 0.00 ppm) as internal standard. Two peaks appear in the spectra around 0 ppm. One is originating from $CFCl_3$ inside the polymer and one from $CFCl_3$ outside the polymer. The peak with higher shift was used as internal standard. Positive and negative electrospray mass analyses were carried out on a Waters Micromass ZG 2000. Preparative HPLC separations were performed on a Beckman System Gold HPLC, using a Supelco Discovery Biowide Pore C18 column (250 * 212 mm, 5 μm) eluted with a linear gradient of MeCN in water, both of which contained trifluoroacetic acid (0.1 %). The flow rate was 11 ml min-1 and detection at 214 nm. Analytical HPLC were performed on a Beckman System Gold HPLC, using a Supelco Discovery Biowide Pore C18 column (250*46 mm, 5 μm) with a flow rate of 1.5 ml min-1 and detection at 214 nm. The yield was determined by comparison of the integral of the linker fluorine with fluorines originating from newly attached building blocks.

5-aminopentyl-4-fluorobenzoate hydrochloride (83)

To a solution of 5-(Boc-amino)-1-pentanol **81** (3.94 g, 19.4 mmol) and triethylamine (2.62 g, 26.02 mmol) in CH_2Cl_2 (30 ml) cooled to 0 °C was added 4-fluorobenzoyl chloride (3.38 g, 21.4 mmol). The reaction mixture was stirred at 0 °C for 1 hr. and then at room temperature for 1 hr. The mixture was partitioned between CH_2Cl_2 and water. The aqueous layer was extracted with CH_2Cl_2. The combined organic phase was washed with brine and dried over Na_2SO_4. After filtration and concentrated in vacuo, the residue **82** was pure enough for next step. Compound **82** (6.31 g, 19.39 mmol) was dissolved in CH_2Cl_2 (100 ml) and treated with trifluoroacetic acid (13.0 g, 114 mmol) at room temperature for 2 hr. The mixture was partitioned between CH_2Cl_2 and water, and basified with NaOH (2.0 M) to pH=12-13. The aqueous layer was extracted with CH_2Cl_2. The combined organic phase was washed with brine and dried over Na_2SO_4. After filtration and concentrated in vacuo, the residue was dissolved in ether (80.0 ml). Methanolic hydrogen chloride was added to form salt, and then the solution was removed in vacuo. The crude solid was recrystallized from benzene to give **83** (3.05 g) in 60 % yield. ^1H NMR (D_2O): δ 7.95-8.00 (m, 2H, Ar-H), 7.17-7.23 (m, 2H, Ar-H), 4.30 (t, 2H, J = 7.6Hz, -$OC\mathit{H_2}CH_2CH_2CH_2CH_2NH_2$), 3.04 (t, 2H, J = 7.6Hz, -$CH_2CH_2CH_2CH_2C\mathit{H_2}NH_2$), 1.71-1.83 (m, 4H, -$CH_2C\mathit{H_2}CH_2C\mathit{H_2}CH_2$-), 1.50-1.57 (m, 2H, -$CH_2CH_2C\mathit{H_2}CH_2CH_2$-); ^{13}C NMR ($CDCl_3$): δ 167.9, 165.8 (d, J = 250.6 Hz), 132.1 (d, J = 9.1 Hz), 125.8 (d, J = 2.9 Hz), 115.6 (d, J = 22.6 Hz), 65.5, 39.4, 27.4, 26.4, 22.3; ^{19}F NMR (D_2O): δ −105.8 ppm; MS(ES^+) calculated for $C_{12}H_{17}FNO_2$ ($M+H^+$) 226.12, found 226.19.

Resin **84**

The carbonate linker resin **44**[101] (0.314 mmol) was washed with dry DMF (2 × 5 ml) and swelled in dry DMF (4 ml) for 10 min. 5-Aminopentyl-4-fluorobenzoate hydrochloride (0.132 g, 0.504 mmol) and DIPEA (0.088 ml, 0.505 mmol) were added and the suspension was agitated over night at room temperature. The resin was washed with DMF and CH_2Cl_2 (3 × 5 ml) to give **84**. According to gel-phase ^{19}F NMR spectrum the conversion was ~91 %. ^{19}F NMR ($CDCl_3$): δ -106.4 (*p*FBz-), -134.0 (linker-F).

Resin **85**

The resin **84** (0.314 mmol) was washed with dry MeOH followed by addition of NaOMe in dry MeOH (7.2 ml, 0.576 mmol, 0.08 M). The suspension was agitated at room temperature for 3 h. The resin was washed with MeOH and CH_2Cl_2 (3 × 5 ml). According to gel-phase ^{19}F NMR ~29 % of the *p*-fluorobenzoyl was not cleaved. Reaction was repeated. According to

gel-phase ^{19}F NMR spectrum ~12 % of the *p*-fluorobenzoyl was not cleaved. Reaction was repeated with NaOMe in dry MeOH (3.6 ml, 0.288 mmol, 0.08 M). According to gel-phase ^{19}F NMR ~5% of the *p*-fluorobenzoyl was not cleaved from resin **85**. ^{19}F NMR ($CDCl_3$): δ -106.4 (*p*FBz-), -134.0 (linker-F).

Resin **86**

The resin **85** (0.157 mmol), the Fsec protected galactose donor **71** (0.266 g, 0.313 mmol), and NIS (0.070 g, 0.311 mmol) were separately dried under vacuum over night and in absence of light. The donor and NIS were dissolved in dry CH_2Cl_2 (~0.5 ml) and then transferred to the resin. TfOH (1M solution in CH_2Cl_2) (0.15 ml, 0.002 mmol) was added to the resin. The suspension was agitated in absence of light for 3 h. Washed with CH_2Cl_2, DMF, and CH_2Cl_2 (3 × 5 ml). Gel-phase ^{19}F NMR showed ~35 % yield. The reaction was repeated. Gel-phase ^{19}F NMR showed ~55 % yield. Reaction was repeated with **71** (0.112 g, 0.132 mmol) and NIS (0.031 g, 0.138 mmol) and TfOH (1M solution in CH_2Cl_2) (0.15 ml, 0.002 mmol). Gel-phase ^{19}F NMR spectrum showed ~84 % yield. ^{19}F NMR ($CDCl_3$): δ -103.3 (Fsec), -104.8 (*p*FBz-), 105.2 (*p*FBz-), -134.0 (linker-F).

Resin **87**

The resin **86** (0.157 mmol) was dried under vacuum over night. Distilled CH_2Cl_2 (3.5 ml) was added to the resin followed by *m*-fluorobenzoyl chloride (0.016 ml, 0.132 mmol) and pyridine (0.011 ml, 0.136 mmol) were added. The suspension was agitated for 2 h at room temperature. Washed with CH_2Cl_2, DMF, and CH_2Cl_2 (3 × 5 ml). Gel-phase ^{19}F NMR spectrum showed that 3-fluorobenzoyl was bound to the resin at two positions (in 9 +11 % yield). ^{19}F NMR ($CDCl_3$): δ -103.3 (Fsec), -104.7 (*p*FBz-), -105.2 (*p*FBz), -111.9 (capping reagent), -112.8 (capping reagent), -134.0 (linker-F) ppm. The resin (0.157 mmol) was treated with piperidine (20 % in DMF, 10 min, 2 × 5 ml). The resin was washed with piperidine (20 % in DMF, 5 ml), DMF, and CH_2Cl_2 (5 × 5 ml). ^{19}F NMR ($CDCl_3$): δ -105.4 (*p*FBz-), -105.7 (*p*FBz), -111.9 (capping reagent), -112.8 (capping reagent), -134.0 (linker-F).

Resin **88**

The resin **87** (0.157 mmol), the galactose donor **80** (0.371 g, 0.629 mmol), and NIS (0.141 g, 0.627 mmol) were dried under vacuum over night and in absences of light. Distilled CH_2Cl_2:THF (1:1, 5 ml) was added to the resin. TfOH (1M solution in CH_2Cl_2) (0.15 ml, 0.002 mmol) was added to the resin. Agitated at room temperature for 3 h in absences of light. Washed with

CH_2Cl_2, DMF, and CH_2Cl_2. According to the gel-phase ^{19}F NMR spectrum the yield > 100 % indicating that something more the acceptor was glycosylated. ^{19}F NMR ($CDCl_3$): δ -104.4 (*p*FBz-), -105.2 (*p*FBz), -112.0 (capping reagent), -112.9 (capping reagent), -118.6 (*o*FBn-), -119.3 (*o*FBn-), -134.0 (linker-F).

Resin **89**

The resin **88** (0.157 mmol) was treated with NaOMe in MeOH (4.0 ml, 0.8 mmol). Agitated at room temperature for 1 h. Washed with MeOH, CH_2Cl_2, DMF, and CH_2Cl_2 (5 × 5 ml). The reaction was repeated. ^{19}F NMR ($CDCl_3$): δ -112.0 (capping reagent), -112.9 (capping reagent), -118.6 (*o*FBn-), -119.3 (*o*FBn-), -134.0 (linker-F).

Glycoconjugate 90

The resin **89** was treated with TFA:H_2O (9:1, 20 ml). The suspension was agitated at room temperature for 3 h. The resin was washed with TFA:H_2O (9:1, 4 ml), CH_2Cl_2, THF, and CH_2Cl_2. The filtrate was collected and concentrated. The reaction was repeated. The combined crude fractions (63 mg) were purified with preparative HPLC yielding **90** in 3 mg (2.6 % yield over 7 steps. ^1H NMR (Pyridine): δ 8.07-7.98 (m, 1H, ArH), 7.75-7.63 (m, 2H, ArH), 7.57-7.52 (m, 1H, ArH), 7.46-7.40 (m, 2H, ArH), 7.38-7.34 (m, 2H, ArH), 7.31-7.26 (m, 1H, ArH), 7.22-7.01 (m, 4H, ArH), 5.64 (dd, 1H, J = 3.4 Hz), 5.61 (dd, 1H, J = 2.1 Hz), 5.30 (s, 1H), 5.12-4.99 (m, 3H), 4.95-4.83 (m, 2H), 4.72-4.42 (m), 4.20-4.13 (m, 2H), 4.11-3.93 (m, 4H), 3.66 (s, 2H), 3.61-3.54 (m, 1H, -$OCH_2CH_2CH_2$-), 3.18-3.14 (m, 2H, -CH_2NH_2), 1.89-1.80 (m, 2H, -$CH_2CH_2NH_2$), 1.60-1.44 (m, 4H, -$CH_2CH_2CH_2CH_2$-); ^{13}C NMR (Pyridine): δ 162.0 (-*C*F-), 161.7 (-*C*F-), 131.2, 130.6, 13.2, 130.0, 129.4, 129.2, 128.5, 128.3, 128.1, 128.0, 127.7, 126.8, 126.1, 115.0 (J = 18.7 and 39.2 Hz), 104.9, 100.2 (J = 38.5 Hz), 79.5 (J = 15.3 Hz), 77.9, 77.5, 76.9, 73.3 (J = 74.4 and 267.4 Hz), 72.9, 70.5, 69.5, 69.1, 68.7, 67.7, 67.2, 65.6, 65.3, 64.7, 64.4, 62.3, 39.3, 29.1, 27.8, 23.3; MS(ES^+) calculated for $C_{38}H_{49}F_2NO_{11}$ ($M+H^+$) 734.33, found 734.60.

8.2. Experimental section for sialic acid-polypeptide conjugates

Sara Spjut[a], Johan Rydberg[b], Lars Baltzer[c], and Mikael Elofsson[a]

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General

The polypeptides were synthesized on ABI 433A (Applied Biosystems) according to standard Fmoc-protocol. The Dansyl group was attached to the peptides by addition of Dansyl chloride (6 eq.) and DIPEA (12 eq.) to the resin in DMF and agitated for 3 h. Analytical HPLC was performed on Varian ProStar HPLC, using a Vydac 214 MS C4 column (150 * 4.6 mm, 5 μm) or on a Beckman System Gold HPLC, using a Supelco Discovery Biowide Pore C18 column (250 * 46 mm, 5 μm). The gradient was 10-90 % acetonitrile in water with 0.1 % TFA at a flow rate of 1 ml/min. Collected fractions were identified by MALDI-TOF mass spectrometry on a Voyager DE-Pro (Applied Biosystems) with the matrix α-cyano-4-hydroxycinammic acid. Fluorescence spectra were recorded using a GeminiXPS plate-reader or a Tecan Infinite® 200 plate reader. NUNC™ polystyrene 384 plates were coated with Pluronic ® F108NF Prill Poloxamer338 (BASF) (1 % aqueous solution over night) before use. The plates were thoroughly washed in water and dried before use. $NaHCO_3$ buffer (pH 9) consisted of 20 g $NaHCO_3$ in 1000 ml H_2O.

8.2.1 Conjugation of sialic acid the polypeptides

The binders were formed by addition of sialic acid squaric decyl ester **97** (2 eq., from 10 mM stock solution in $NaHCO_3$) to 14 different polypeptides (0.5 mM in $NaHCO_3$) (Table x). The reactions were left over night and the degree of functionalization in some of the reactions was confirmed with analytical HPLC and MALDI-TOF-MS. The reaction mixtures were used in the fluorescence screening experiments without further purification.

8.2.2 Fluorescence screening experiments

The Ad37 fiber knob protein[108] (1 or 2 eq., 1 or 2 μM final concentration in PBS) was added to the binder (1 μM final concentration in PBS) and incubated for 20 minutes. Control wells contained binder alone. The plate

was incubated for 20 minutes. The Dansyl fluorophore was excited at 337 nm and emission was recorded at 400-600 nm.

8.2.3 Conjugation of sialic acid to the polypeptide 4-D25L22

Performed as in 8.2.1 except from that the concentration of the peptide 4-D25L22 was 5 mM. The reaction mixture was used in the fluorescence screening experiments without further purification.

8.2.4 Binding assay

The assay was carried out essentially as described previously.[81-83] ^{35}S-labeled Ad37 virions (1x10^9 per well) were incubated in 50 µl binding buffer (BB: DMEM containing 1 % bovine serum albumin [Roche AB, Stockholm, Sweden] and HEPES [EuroClone, Milan, Italy], pH 7.5) at 4 °C, together with different concentrations of commercial sialic acid (50, 10, 2, 0.4, and 0.08 mM in BB) or binder (1, 0.4, 0.08, 0.016, and 0.0032 mM in BB) in 96-well plates for 1 h. The mixtures were transferred to V-shaped 96-well plates with 50 µl HCE cells in BB (2 x 10^5 cells per well and incubated for 1 h at 4 °C. After two washes in BB, the cell-associated radioactivity was measured in a Wallac 1409 liquid scintillation counter (Perkin Elmer). Data are presented as % of control, control is the value obtained in absence of inhibitor. The experiment was repeated one time.

9. Acknowledgments

Högst upp på listan av alla jag vill tacka kommer min handledare **Mikael Elofsson**. Jag har lärt mig otroligt mycket av dig! Du har inspirerat och uppmuntrat mig med ditt ständiga engagemang, kreativitet, optimism och envishet. Tack för att du gav mig chansen att doktorera och för att du alltid har haft tid att svara på mina frågor! Och tack till hela **familjen Elofsson** för alla trevliga fester hemma hos er!

Ytterligare tack till…

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