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The Netherlands

Synthesis & biological applications of glycosylated iminosugars

Duivenvoorden, B.A.

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1

General Introduction and Outline

1.1 Iminosugars: Structures, Activities and Applications

Alkaloids are nitrogen containing molecules which are widely distributed in nature. They are produced by a wide variety of organisms such as plants, fungi, bacteria, marine animals, amphibians, some birds and a few mammals.¹⁻⁷ Over the years the group of polyhydroxylated alkaloids has gained considerable interest as potential therapeutic agents and as tools to gain a better insight in biological processes. This specific group of alkaloids can be considered as carbohydrate mimics in which the endocyclic oxygen is replaced by a nitrogen. This alteration in combination with their structural resemblance to normal sugars makes that they are often evaluated as inhibitors of glycosidases⁸ and glycosyltransferases.⁹ These enzymes in turn, both play an essential role in various biological processes including carbohydrate catabolism, maturation, transport and secretion of glycoproteins and cell recognition processes.^{10,11} Polyhydroxylated alkaloids, often referred to as iminosugars, can be divided in several different classes depending on their ring structures (Figure 1.1).¹²

Nojirimycin **1** (NJ) is the first iminosugar isolated from natural sources (*S. roseo* R-468 and *S. laven*. SF-425), and shows remarkable biological activity. In subsequent studies NJ was shown to be a good inhibitor of various α - and β -glycosidases.^{13,14} Nojirimycin contains a hemiaminal function, which renders it rather unstable under neutral and acidic conditions at room temperature, therefore it is usually stored as bisulphite adduct or reduced to the more stable 1-deoxynojirimycin **2** (DNJ).^{13,15} Over the years a wide range of iminosugar and related alkaloids were isolated from the leaves, root bark and fruits of the mulberry tree

(*Morus* spp.). Prominent examples are DNJ **2**,¹⁶ fagomine **4**, *N*-methyl-DNJ **3** and 1,4-dideoxy-1,4-imino-D-arabinitol **7** (DAB) (Figure 1.1).^{17–19}

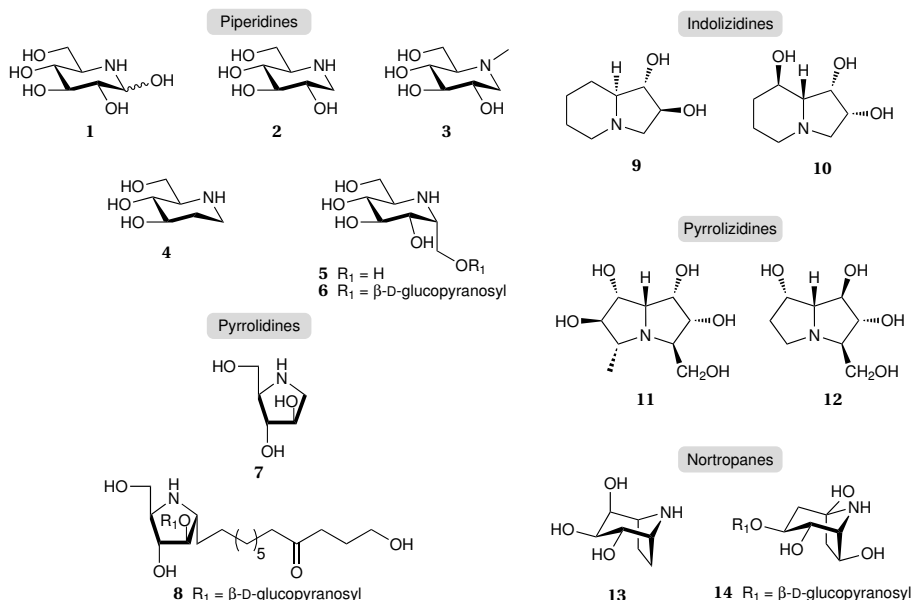


Figure 1.1: Five classes of iminosugars with some examples.

Nojirimycin (NJ, **1**); 1-Deoxynojirimycin (DNJ, **2**); *N*-methyl-DNJ (**3**); Fagomine (**4**); α -Homonojirimycin (α -HNJ, **5**); 7-*O*- β -D-glucopyranosyl- α -HNJ (**6**); 1,4-Dideoxy-1,4-imino-D-arabinitol (DAB, **7**); Broussonetin B (**8**); Lentiginosine (**9**); Swainsonine (**10**); Hyacinthacine C₁ (**11**); Australine (**12**); Calystegine B₄ (**13**); Calystegine B₁-3-*O*- β -D-glucopyranoside (**14**).

In the field of iminosugar research many *N*-alkylated derivatives of DNJ have been synthesized. Miglitol (**15**, Figure 1.2) is the first α -glucosidase inhibitor based on DNJ **2** and is used as drug for diabetes mellitus type 2.^{20,21} By inhibition of α -glucosidase, **15** slows down the rate by which large carbohydrates (poly- and oligomers) are processed in the gut.^{21,22} Fleet *et al.*²³ synthesized *N*-butyl-1-deoxynojirimycin **16** (NB-DNJ or Miglustat) which was found to be an inhibitor of glucosylceramide synthase (GCS).^{24,25} GCS plays an essential role in the biosynthesis of glucosylceramide, the precursor for more complex glycosphingolipids (Figure 1.2C). Inhibitory properties of NB-DNJ **16** are used to the full extent in the so called substrate reduction therapy (SRT)^{26–28} to prevent the accumulation of glucosylceramide (GC) in cells (Figure 1.2B). NB-DNJ is the first orally administered drug to be active in the treatment of type 1 Gaucher disease.²⁹ Gaucher disease is a rare lysosomal storage disorder in which GC is inefficiently hydrolyzed by mutant glucocerebrosidase (GBA1, Figure 1.2B). This causes accumulation of GC-laden macrophages which results in enlargement of organs (spleen and liver) and inflammation. The first therapy developed for the treatment of Gaucher dis-

ease was enzyme replacement therapy (ERT), in which recombinant GBA1 (called cerezyme) is intravenously administrated to patients.³⁰ This functional GBA1 enzyme ends up in the Gaucher cells where it temporarily restores the efflux of GC (Figure 1.2B). The disadvantages of ERT are the intravenous administration and the high costs of enzyme production. Substrate reduction therapy offers a useful alternative. Inhibition of GCS alters the influx of GC thereby restoring the influx/-efflux balance of GC in Gaucher cells (Figure 1.2B).^{31–33}

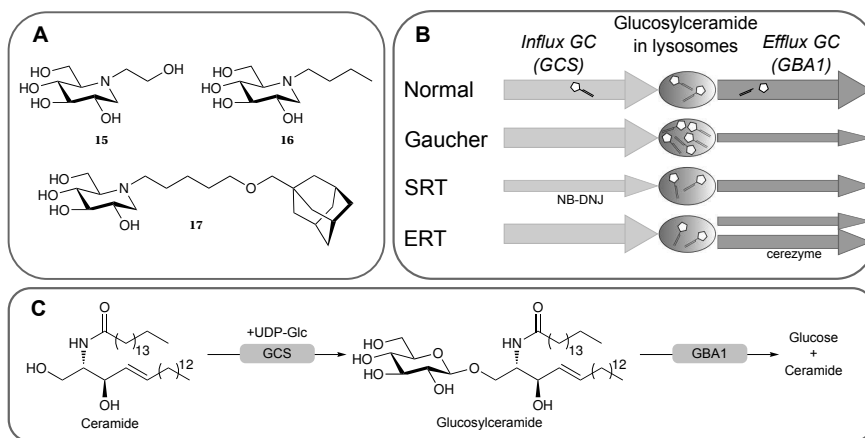


Figure 1.2: **A:** Structures of Miglitol (**15**), NB-DNJ (**16**), AMP-DNJ (**17**); **B:** Schematic overview of Gaucher disease and currently used therapies; **C:** Anabolism and catabolism of glucosylceramide.

Compound **17**, also known as AMP-DNJ, bears a *N*-5-(adamantan-1-yl-methoxy)-pentyl (AMP) chain on the ring nitrogen and has been found to be a better inhibitor of GCS as compared to NB-DNJ.³⁴ AMP-DNJ has great potential as a novel drug for Gaucher disease and other sphingolipidoses^{25,35} and shows promising results regarding treatment of diabetes mellitus type 2,³⁶ hepatosteatosis and inflammatory bowel disease.³⁷ Oral administration of AMP-DNJ has also been found to result in prevention of atherosclerosis³⁸ and neurodegeneration in Sandhoff disease.³⁹

Next to decorating iminosugars by alkylation of its endocyclic nitrogen to gain better or more selective inhibitors, iminosugars can also be glycosylated to yield a new class of potential inhibitors. Glycosylated iminosugars may be closer mimics of the natural substrates for the enzyme of interest, thereby making them potentially more selective inhibitors than the non-glycosylated iminosugars. Glycosylated iminosugars can also give a better insight in the mechanism of action of glycosidases, as well as potentially being prodrugs or slow-releasing agents that have to undergo an enzymatic transformation to liberate the active inhibitor. There are several examples of naturally occurring glycosylated iminosugars, which are mostly found in iminosugar producing plants. Isolation is often done by extrac-

tion of leaves, bark or roots with aqueous MeOH or EtOH, after which the extracts are purified by a variety of ion-exchange chromatography steps. After isolation and purification careful characterization, is done by Nuclear Magnetic Resonance Spectroscopy (NMR), High Resolution Mass Spectroscopy (HRMS) and enzymatic assays to confirm their structure. Glycosylated iminosugars have been found to contain, amongst others, α - and β -glucosides, α -galactosides, apiosides, β -xylosides, β -mannosides and β -fructofuranosyl glycosides. Some examples are given in Figure 1.1 and Figure 1.3.^{8,12,17,40–44} Biological evaluation show that most glycosylated iminosugars are selective inhibitors, probably due to their close resemblance of the enzymes natural substrates.^{8,12,17,40–44}

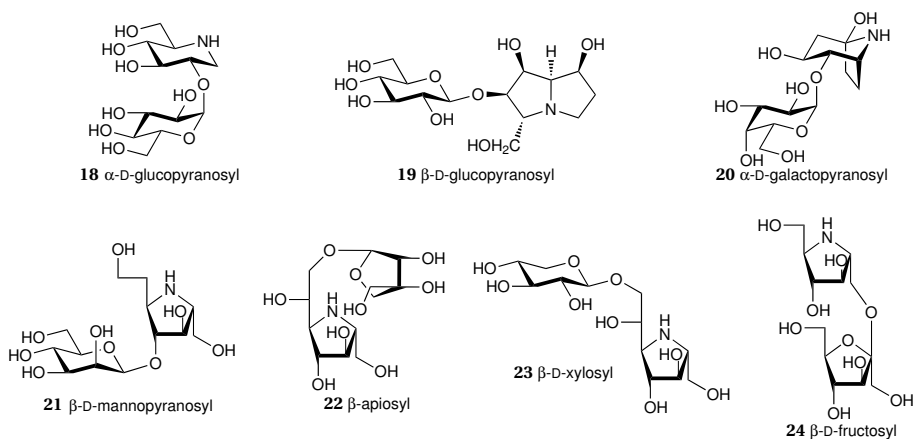


Figure 1.3: Structures of natural occurring glycosylated iminosugars.

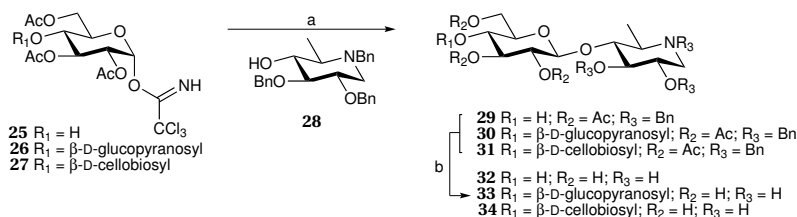
2-*O*- α -D-glucopyranosyl-1-deoxynorjirimycin (**18**), 1-*epi*-australine-2-*O*- β -D-glucopyranoside (**19**), 4-*O*- α -D-galactopyranosyl-calystegine B₂ (**20**), 4-*O*- β -D-mannopyranosyl-6-deoxy-homoDMDP (**21**), homoDMDP-7-*O*-apioside (**22**), homoDMDP-7-*O*- β -D-xylopyranoside (**23**); DMDP-7-*O*- β -D-fructofuranoside (**24**).

1.2 Synthesis of *O*-Glycosylated Iminosugars

1.2.1 Chemical Synthesis

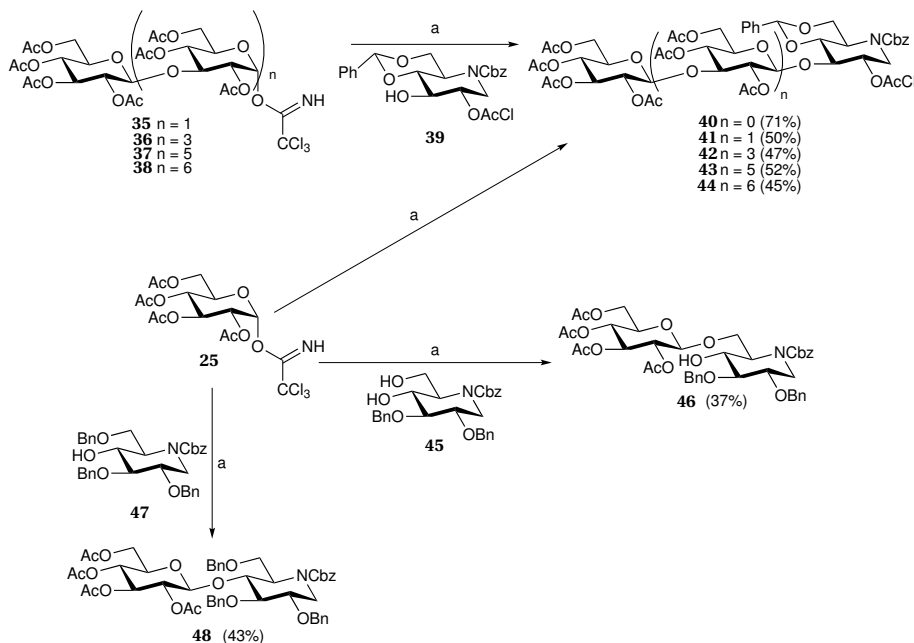
The natural abundance of *O*-glycosylated iminosugars is extremely low and most of them are potent inhibitors of several glycosidases.¹⁹ To fully explore the potential of *O*-glycosylated iminosugars larger quantities are needed. This goal can be achieved through chemical or enzymatic synthesis.

One of the first syntheses of glycosylated iminosugars was reported by Ganem *et al.*⁴⁵ who prepared a cellulase inhibitor. Using the trichloroacetimidate method⁴⁶ a glucose mono-, di- or trimer was coupled in a β -1,4 fashion to an iminosugar (Scheme 1.1). Biological evaluation of the resulting glycosylated iminosugars **32**, **33** and **34** showed potent inhibitory effects towards different endo-cellulases from *T. fusca*.^{47,48}

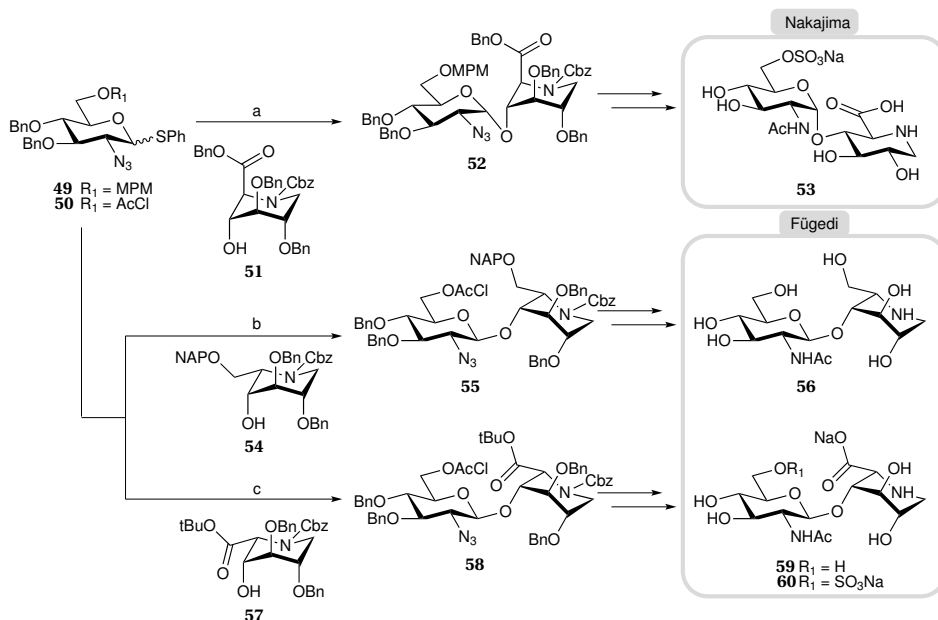
Scheme 1.1: Synthesis of cellulase inhibitors **32**, **33**, **34** as reported by Ganem.⁴⁵

Reagents and conditions: a) BF₃·OEt₂, DCM, 0°C; b) (1) KOH, MeOH; (2) Pd/C, H₂, EtOH:HCl, **32** (50% overall), **33** (38% overall), **34** (40% overall).

To get a better insight in the processing of cross-linked polysaccharides Blatter and co-workers⁴⁹ *O*-glycosylated DNJ at various positions (Scheme 1.2) and evaluated several β-1,3, β-1,4 and β-1,6 linked DNJ oligo-glucosides as potential fungicides. For the synthesis several *O*-acetylated glycosyl trichloroacetimidate donors were condensed with a protected DNJ derivative. A regioselective coupling was achieved in the synthesis of β-1,6 linked disaccharide **46**.⁵⁰ All compounds were tested, after deprotection, on a wide variety of fungi and small organisms of which only the brine shrimp (*Artemia salina*) showed to be vulnerable to most of compounds.

Scheme 1.2: Synthesis of fungicides, based on DNJ glucosylated at various positions.⁴⁹

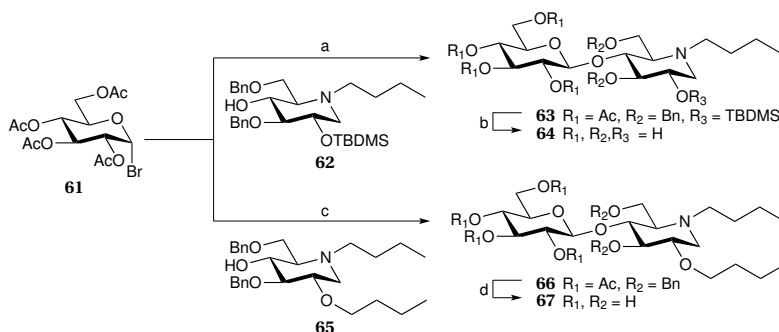
Reagents and conditions: a) TMSOTf, DCM, 0°C.

Scheme 1.3: Synthesis of heparanase inhibitors with D-Glu or L-Ido configuration.^{51–53}

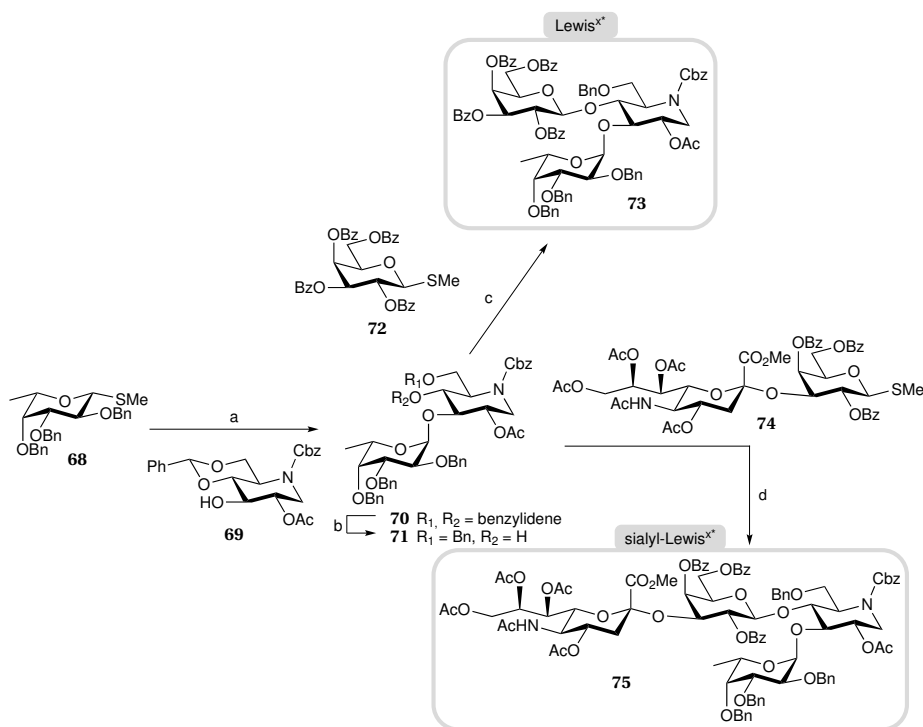
Reagents and conditions: a) NIS, TMSOTf, DCM:Et₂O, -50°C, 72%; b) DMTST, DCM:Et₂O, 59%; c) Me₂S₂-Tf₂O, DCM:Et₂O, 80%.

Glycosylated iminosugars have also been used as inhibitors for heparanase, as a potential antimetastatic cancer drug.^{54,55} The groups of Nakajima⁵¹ and Fügedi^{52,53} independently synthesized a set of iminosugar containing heparanase inhibitors using 2-azido-2-deoxy-D-glucopyranosyl donors **49** and **50**. Condensing **49** with iminosugar **51**, having the D-glucuronic acid configuration,⁵¹ afforded, after deprotection, inhibitor **53**. Compound **53** showed to inhibit heparanase, thereby preventing the degradation of heparan sulfate.^{56,57} Fügedi and co-workers^{52,53} based their design on the use of iminosugars having the L-ido configuration. Condensation of the iminosugars having a L-idose (**54**) or L-iduronic acid configuration (**57**) with donor **50** using DMTST or Me₂S₂-Tf₂O led to the pseudo disaccharides **55** and **58** which were transformed into **56** and **60**. No biological data were reported on these compounds.

To assess if iminosugars can act as a ceramide mimic in β -glucocerebrosidase (GBA1), Martin and Compain⁵⁸ developed two GBA1 inhibitors, featuring a N-alkylated DNJ derivative bearing two alkyl chains (**65**) and a glucose, to fully mimic the natural substrate of GBA1. Condensation of 2,3,4,6-tetra-O-acetyl- α -D-glucopyranosyl bromide donor **61** with DNJ acceptor **62** or **65** under Koenings-Knorr conditions afforded, after deprotection, inhibitors **64** and **67**. Biological results show improved affinity of **67** towards GBA1 (IC₅₀ 56 μM) as compared to the mono-alkylated **64** (no inhibition) and even as compared to DNJ **2** (IC₂₇₀ 56 μM).

Scheme 1.4: Synthesis of β -glucocerebrosidase inhibitors.⁵⁸

Reagents and conditions: a) AgOTf, DCM, -78°C , 34%; b) (1) $n\text{Bu}_4\text{NF}$, THF, 0°C , 70%, (2) NaOMe, MeOH, 86%, (3) Pd/C, H_2 , $i\text{PrOH}/\text{AcOH}$, (4) DowexTM OH^- , 44%; c) AgOTf, DCM, -78°C , 55%; d) (1) NaOMe, MeOH, quant., (2) Pd/C, H_2 , $i\text{PrOH}/\text{AcOH}$, (3) DowexTM OH^- , quant.

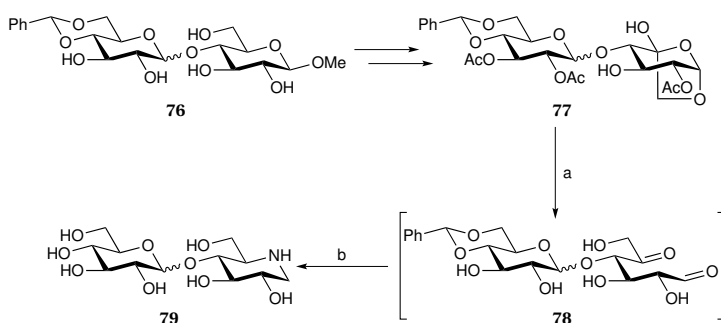
Scheme 1.5: Synthesis of Lewis^x **73** and sialyl-Lewis^x **75**.⁵⁹

Reagents and conditions: a) DMTST, benzene, 7°C , 92%; b) NaCNBH_4 , Et_2O , 81%; c) NIS, TfOH, DCM, 70%; d) NIS, TfOH, DCM, 61%; * protected forms of Lewis^x and sialyl-Lewis^x iminosugar analogs.

Next to iminosugars that are glycosylated on one position, various iminosugars have been synthesized that bear more than one carbohydrate. Furui and

co-workers⁵⁹ reported the synthesis of Lewis^x **73** and sialyl-Lewis^x **75** iminosugar analogs in which DNJ is di-glycosylated (Scheme 1.5). Coupling of L-fucose **68** to the 3-position of DNJ **69** followed by selective opening of the benzylidene in **70** gave acceptor **71**. Mono glycosylated DNJ acceptor **71** was then condensed with D-galactose **72** under influence of NIS and TfOH to yield trimer **73**, which after deprotection gave the DNJ derivative of Lewis^x. By coupling of thio donor **74** to DNJ acceptor **71**, using similar conditions as in the assembly of **73**, tetramer **75** was gained, which after deprotection afforded DNJ analog of sialyl-Lewis^x.

Scheme 1.6: Synthesis of glucosidase inhibitors β -**79** and α -**79** starting with cellobiose and maltose.⁶⁰

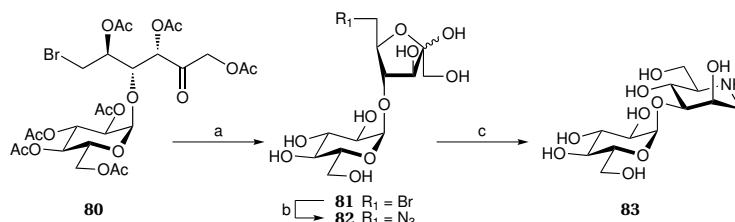


Reagents and conditions: a) NaOMe, MeOH; b) Pd(OH)₂, H₂, NH₄OH, H₂O, 24% over two steps.

A different approach for the synthesis of glycosylated iminosugars is to first synthesize a carbohydrate oligomer, after which the reducing end sugar is converted in the corresponding iminosugar. By using naturally occurring oligomers as starting material, this approach circumvents the use of a glycosylation steps and lengthy protective group manipulations. The group of Stütz reported three syntheses in which they use cellobiose, maltose or maltulose as starting materials for the synthesis of glucosylated iminosugars (Scheme 1.6 and Scheme 1.7).^{60,61} Conversion of cellobiose and maltose into their 1,6-anhydrosugar derivatives (β -**77** and α -**77**), followed by deprotection of the acetyl functions and concomitant ring opening afforded di-carbonyl β -**78** and α -**78** (Scheme 1.6). Double reductive amination using Pearlman's catalyst in aqueous ammonia under a hydrogen atmosphere yielded target compounds β -**79** and α -**79**.

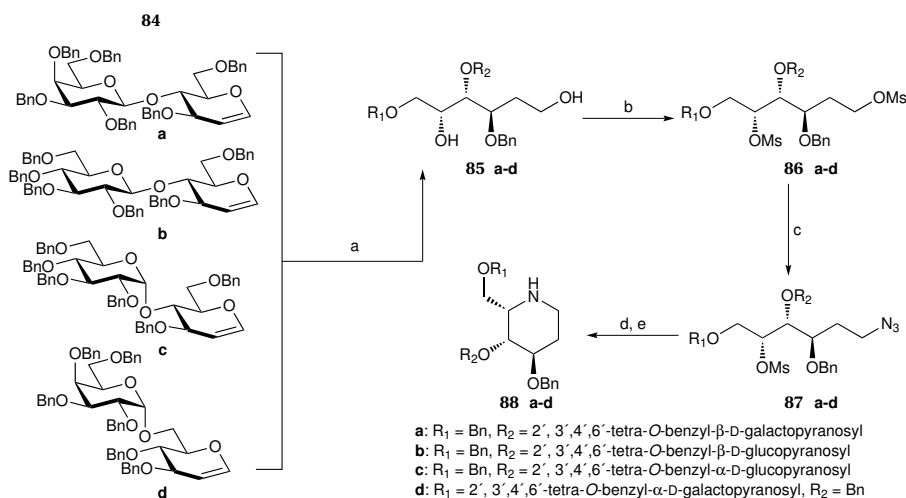
The maltulose derivative was synthesized *via* open-chain bromide **80**⁶² (Scheme 1.7), which cyclized under Zemplén conditions to give **81**, which was subsequently reacted with NaN₃ in DMF to gain compound **82**. Conventional catalytic hydrogenation of azidodeoxysugar **82** in dry methanol using Pd(OH)₂ furnished title compound **83**.

The group of Piancatelli⁶³ used glycosyl glycals (D-lactal **84a**, D-cellobial **84b**, D-maltal **84c** and D-melibial **84d**) for the synthesis of glycosylated L-fagomine

Scheme 1.7: Synthesis of glucosidase inhibitor **83**.^{61,62}

Reagents and conditions: a) NaOMe, MeOH; b) NaN₃, DMF; c) Pd(OH)₂, H₂, MeOH, 26% over 3 steps.

derivatives. Opening of the glycals **84a-d** by mercury(II) acetate/sodium borohydride,^{64,65} gave compounds **85a-d** which were converted in *N*-heterocyclized compounds **88a-d** in a three-step sequence: 1) formation of the 2,6-di-*O*-mesylates (**86a-d**), 2) regioselective azidation by treatment with NaN₃ in DMF (**87a-d**), 3) cyclization by reduction of the azide (**88a-d**).

Scheme 1.8: Synthesis of glycosylated imonosugars *via* D-lactal (**84a**), D-cellobial (**84b**), D-maltal (**84c**) and D-melibial (**84d**).⁶³

Reagents and conditions: a) Hg(OAc)₂, NaBH₄, DCM, **85 a-d** ~90%; b) Et₃N, MsCl, DCM, **86 a-d** ~80%; c) NaN₃, DME, 70°C, **87 a-d** ~80%; d) P(Ph)₃, THF:H₂O; e) Et₃N, 40°C **88 a-d** ~70%.

1.2.2 Enzymatic Synthesis

Enzymatic synthesis using glycosidases and glycosyltransferases can be a useful alternative for the synthesis of iminosugar containing oligomers. There are a few examples in which DNJ or *N*-protected DNJ is used as acceptor for enzymatic syntheses of glycosylated DNJs.

For the syntheses of the DNJ derivatives of sialyl Lewis^x and Lewis^a on a large scale, Kojima *et al.*⁶⁶ used a β -galactosidase to gain a large amount of the galactosylated DNJ building block. Mixing of lactose (50 kg), DNJ (5 kg) and β -galactosidase (250 mL) in H₂O (250 L) for 18 hours gave 8.2 kg of product as a mixture of galactosyl-DNJ derivatives. Purification by strong base anion-exchange resin yielded 300 grams of different galactosyl-DNJ in the following ratio; unreacted DNJ **2** (32%), 1,2-linked **89** (6%), 1,3-linked **90** (20%), 1,4-linked **91** (25%), 1,6-linked **92** (7%) and other unidentified transgalactosylated DNJs (Figure 1.4).

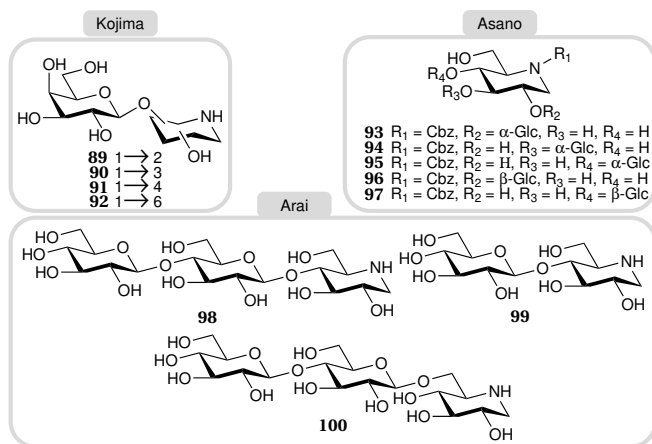


Figure 1.4: Structures of glycosylated iminosugars described in section 1.2.2.

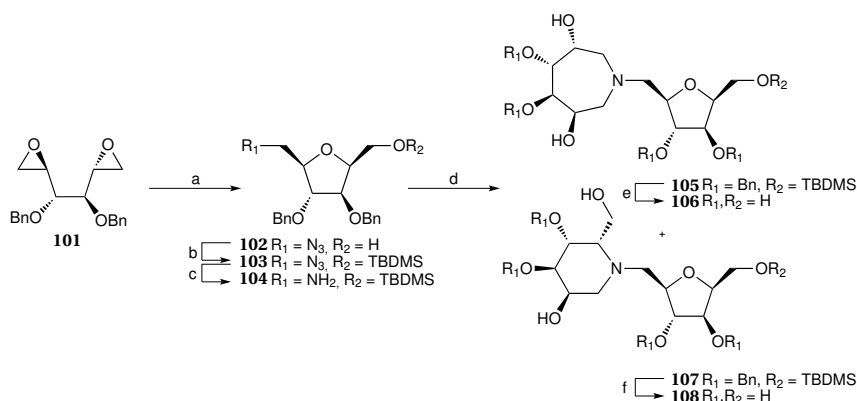
By using α - and β -glucosidases and *N*-benzyloxycarbonyl protected DNJ, Asano and co-workers⁶⁷ made a series of α - and β -linked glucosylated DNJ derivatives. Maltose and DNJ were stirred with rice α -glucosidase yielding 1,4-linked (**95**), 1,3-linked (**94**) and 1,2-linked (**93**) α -glucosyl DNJ in yields of 40, 13 and 2% respectively (Figure 1.4). No 1,6-linked coupling was observed, probably due to steric hindrance of the *N*-benzyloxycarbonyl group. Cellobiose was used as glucose donor in the coupling effected by yeast β -glucosidase to give 1,2-linked (**96**) and 1,4-linked (**97**) β -glucosylated DNJ in yields of 69% and 3% respectively (Figure 1.4). After deprotection the glucosylated iminosugars were tested for their biological activity showing that α -1,2-linked (**93**) and α -1,3-linked (**94**) were more effective than DNJ against trehalases and rice α -glucosidase, respectively.

To elucidate the mechanism of hydrolysis of cellulase, the group of Arai⁶⁸ synthesized cellulase inhibitors by condensing cellobiose and DNJ using a transglycosylase. They synthesized three inhibitors, two bearing a disaccharide either on the 4- (**98**) or the 6-position (**100**) of DNJ and one bearing a glucose on the 4-position of DNJ (**99**) (Figure 1.4). Trimer **98** (1,4) was found to be the best inhibitor for several fungal and bacterial cellulases as it best resembles natural cellulose.⁶⁹

1.3 Synthesis of Different Linked Glycosylated Iminosugars

Aside from the *O*-glycosylated iminosugars there are a few examples in which the endocyclic nitrogen of an iminosugar is linked to a carbohydrate by a non-hydrolyzable bond. The group of Merrer reported^{70,71} the synthesis of DNJ which bears D-glucitol on the ring nitrogen. First bis-epoxide **101** was reacted with NaN_3 and SiO_2 , directly followed by an *O*-cyclization according to a 5-*exo-tet* process giving D-glucitol **102**.^{72,71}

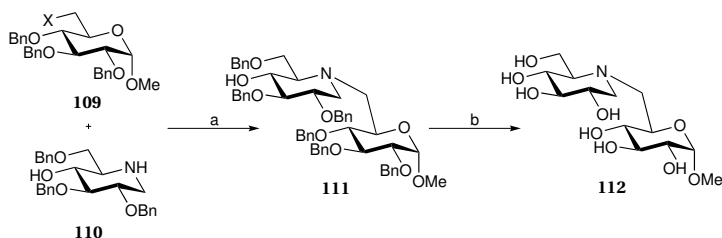
Scheme 1.9: Synthesis of *N*-glycosylated iminosugars.⁷¹



Reagents and conditions: a) NaN_3 , SiO_2 , ACN, Δ , 95%; b) TBDMSCl, imidazole, DME, 95%; c) Pd black, H_2 , EtOAc, 95%; d) **101**, EtOH, **105** 40%, **107** 30%; e) (1) nBuN_4F , THE, 85%, (2) Pd black, H_2 , AcOH, 70%; f) (1) nBuN_4F , THE, 80%, (2) Pd black, H_2 , AcOH, 75%.

Next the primary hydroxyl was protected to give **103**, followed by reduction of the azide moiety in **103** to give **104**. The free amine in **104** was subsequently reacted with another equivalent of bis-epoxide **101** to form azepane derivative **105** and DNJ derivative **107**, via an *N*-cyclyzation in 40% and 30% yield respectively.

Scheme 1.10: Synthesis of MDL 7395.⁷³

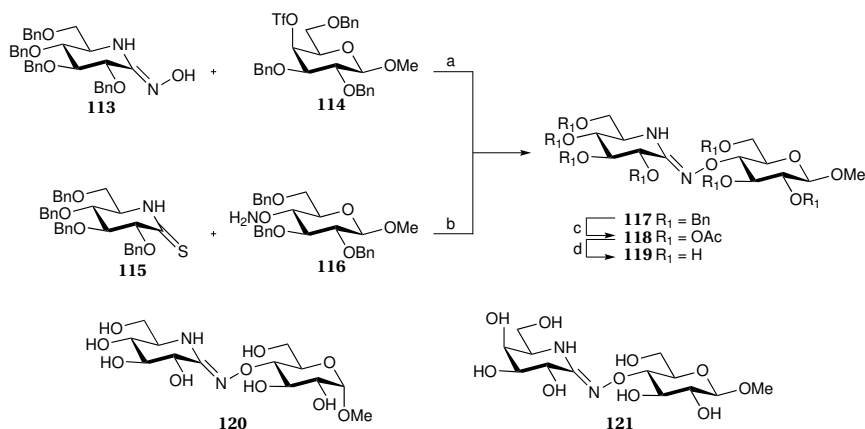


Reagents and conditions: a) DME, Δ , 80%; b) Pd/C, H_2 , EtOH, 79%.

Another example of a *N*-glycosylated iminosugar is *N*-[6-deoxy-1-*O*-methyl-6- α -glucopyranosyl]-1-deoxynojirimycin or MDL 7395 (**112** Scheme 1.10), which was synthesized by the pharmaceutical company Merrel Dow (Strasbourg, France).⁷³ Coupling using an excess glucosyl halide (**109**) with DNJ acceptor (**110**) yielded, after deprotection, **112**.⁷³ Biological evaluation of MDL 7395 (**112**) showed that it reduced the glycemic response, by inhibition of the intestinal α -glucohydrolase, which makes it a potential diabetes mellitus drug.⁷⁴

Vasella and co-workers⁷⁵ used anomeric oximes such as **113** to link monosaccharides to iminosugars, gaining selective α - and β -glycosidase inhibitors (Scheme 1.11). They used two approaches to synthesize methyl β -cellobioside analog **119**: one by alkylation of the hydroximolactam **113**⁷⁶ with triflate **114**⁷⁷ and the other by condensation of the thiogluconolactam **115**⁷⁸ with hydroxylamine **116**. By use of the latter method compounds **120** and **121** were also synthesized. It was found that compounds **119**, **120** and **121** were strong inhibitors of several different β -glucosidases.⁷⁵

Scheme 1.11: Synthesis of compounds **120**, **119**, **121**.⁷⁵



Reagents and conditions: a) NaOH, Et_4NBr , Tol, 59%; b) $\text{Hg}(\text{OAc})_2$, $\text{Et}(\text{iPr})_2\text{N}$, THF, 72%; c) (1) Li, EtNH_2 , THF, (2) Ac_2O , pyr., 80%; d) NH_3 , MeOH, 77%.

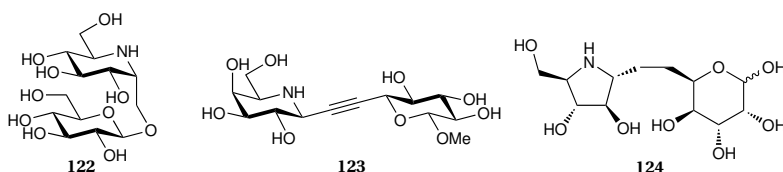


Figure 1.5: Examples of C-glycosylated iminosugars.**122**⁷⁹, **123**⁸⁰, **124**.⁸¹

A different class of iminosugars with promising biological and therapeutic properties are iminosugars bearing C-glycosides. An overview of the synthesis and

strategical design of this class of stable iminosugar analogs is given in several reviews.^{82–84}

1.4 Thesis Outline

The ongoing research in the field of lysosomal storage diseases (LSD), and more specific Gaucher disease is the basis for the research described in this thesis. The progress of Gaucher disease and the effect of therapeutic intervention is correlated to the level of chitotriosidase (CHIT1), the first identified human chitinase. Measurement of plasma CHIT1 activity in man is done by an assays using fluorogenic substrate **125**. The ability of CHIT1 to transglycosylate can complicate the enzyme assay, however compound **125** is not prone to be transglycosylated. And gives a proportional fluorophore to active enzyme ratio read-out. Because of this umbelliferone 4'-deoxychitobioside **125** has become a popular fluorogenic substrate for the measurement of human chitinases, an improved scalable route towards **125** is described in **Chapter 2**.

Chapter 3 describes the synthesis and biological evaluation of three novel fluorogenic substrates, containing substituents of different sizes on the 4'-OH of the non-reducing sugar.

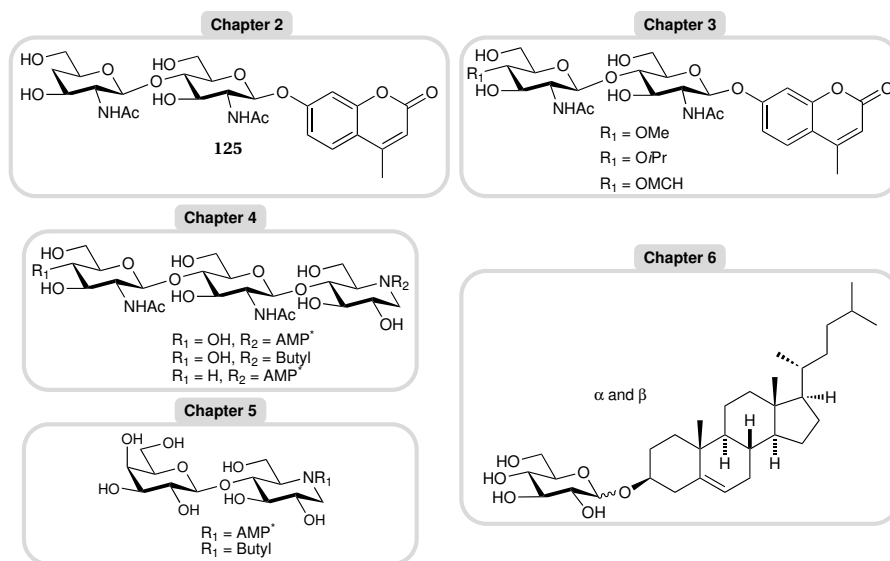


Figure 1.6: Overview of the compounds described in this thesis.

* AMP = *N*-5-(adamantan-1-yl-methoxy)-pentyl

The locally elevated activity of CHIT1 allows site-specific drug delivery *via* the prodrug approach. **Chapter 4** describes the design and synthesis of novel prodrugs in which a chitobiose core, the substrate for CHIT1, is coupled to known in-

hibitors of GCS which are able to restore the influx/efflux balance of GC in Gaucher cells.

It is known that some iminosugars and *N*-alkylated derivatives thereof have a taste bitter. In **Chapter 5** attempts are made to palliate this bitter taste by appending a galactosyl moiety to DNJ. Aside from potentially masking the bitter taste this modification will also help to direct the inhibitors to the colon where they will be processed by lactase.

Cholesteryl- α -glucoside and cholesteryl- β -glucoside, the synthesis of which is described in **Chapter 6**, will be used as internal standards to get a better insight in the biosynthesis of the potentially neurotoxic steryl-glucosides, which are potentially linked to a high level of glycosylceramide. **Chapter 7** summarizes the research described in chapters 2 to 6 and future prospects based on these results are presented.

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