Synthesis of the Repeating Unit of the O-Specific Polysaccharie of *Shigella sonnei*

theses of doctoral (PhD) dissertation

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1 Introduction and objectives of the dissertation

In this dissertation the synthesis of the repeating unit of the O-specific polisaccharide of *Shigella sonnei* is presented. Our primary, long-range objective is the development of a carbohydrate-protein conjugate vaccine against enteric infections caused by the Gram-negative bacterium *Shigella sonnei*.

Shigellosis, an inflammatory disease of the lower intestine, is a continuing public health problem in both the developing and industrialized countries. *Shigellae* are the leading cause of dysentery throughout the world. It is dysentery (fever, mucous and blood in the stool) which is the leading cause of growth retardation in the world. In the United States $\sim$60.000 cases are reported annually, and the rate of disease is increasing e.g. among patients infected with the human immunodeficiency virus. The incidence in Israel is $\sim$20 times higher than in the United States, and affects $\sim$0.15% of the population. The attack rate is especially high in the military field units of the Israeli Defense Force and among children between 1 and 4 years of age. Four groups of *Shigellae* (*Sh. dysenteriae* type 1, *Sh. flexneri* type 2a, *Sh. boydii*, and *Sh. sonnei*) account for almost all cases of shigellosis throughout the world. *Sh. sonnei* is responsible for $\sim 3/4$ of the cases of shigellosis among the recruits of the IDF, while the other quarter is caused by *Sh. flexneri* type 2a.

An approach to combat this disease can be vaccine development. In spite of the fact, that the causative organisms have been identified a century ago, there is no licensed vaccine against shigellosis. The need for such vaccines is documented by a World Health Organisation document which accords priority to the development of vaccines against Shigellosis.

The essential role of the O-specific polysaccharides of *Sh. sonnei*, and also other enteropathogenic bacteria led to the hypothesis of Robbins and co-workers, that serum IgG antibodies to the O-SPs confer protective immunity to the host. The validity of this hypothesis was substantiated by experiments with conjugate vaccines containing purified, O-specific polysaccharides covalently linked to an immunogenic protein. In mice, subcutaneous, injection of a conjugate containing the O-SP of *Sh. sonnei* elicited O-specific polysaccharide IgG and IgM antibodies.

2 Methods applied

The macro- and micro methods of the modern preparative organic chemistry where applied in the synthetic work.

Thin layer-, high pressure liquid- and gas chromatography where applied tofollow the reactions, to control the purity of substances and to determine the ratios of the products. Besides classical crystallization, column chromatography was usedfor the purification of the crude products and for the separation of the isomers.

Besides classical methods (elemental analysis, melting point ans optical rotation determination) modern spectroscopic methods (one- and two dimensional NMR and mass spectrometry) where applied for the verification of the structures of the synthesized compounds.

3 New scientific results of the dissertation

The O-specific polysaccharide of *Shigella Sonnei* is composed of the disaccharide repeating unit consisting of the rare sugars 2,4,6-trideoxy-2-acetamido-4-amino-D-galactose and 2-acetamino-2-deoxy-L-altruonic acid that are connected through 1,2-trans interglicosidic linkages (Figure 1). This disaccharide is quite unique since it posesses the zwitterionic

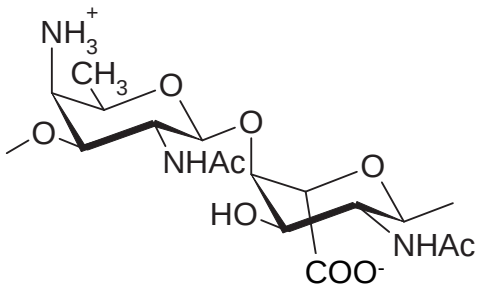


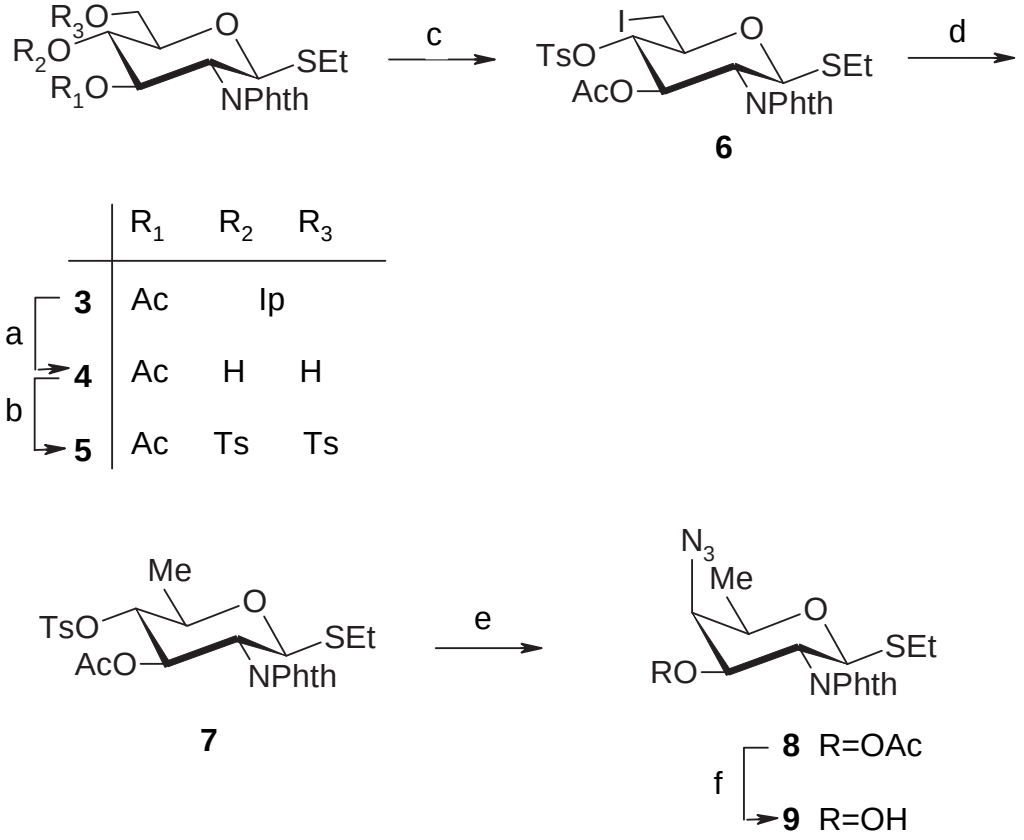
Figure 1:

structure of the peptides/proteins, as well as the interglycosidic (α-L and β-D) linkage characteristic of the oligosaccharides, two different types of linkage [(1→3) and (1→4)], as well as the NHAc-2 moiety present in both saccharide units.

3.1 Synthesis of 4-amino-2,4,6-trideoxy-2-acetamido-D-galactose

To design the synthetic route to this compound the compatibility of the protecting groups and selection of a strict oder of the steps in the reaction sequence were essentially important. When selecting the *N*-protecting group, essential requirements were to ensure the development of an 1,2-*trans*-glycosidic bond, and also an easy exchange to an *N*-acetyl function in a later phase of the synthesis. For such purposes a great majority of the literature examples suggest the application of the *N*-phthaloyl group. Thus, the

starting sugar D-glucoseamine **2** was conventionally converted into ethyl 3-*O*-acetyl-2-deoxy-4,6-*O*-isopropylidene-2-phthalimido-1-thio-β-D-glucopyranoside (**3**), described earlier by the van Boeckel group. Following removal of the isopropylidene function under mild acidic conditions, the resulting diol **4** was treated with *p*-toluenesulfonyl chloride to obtain ethyl 3-*O*-acetyl-2-deoxy-2-phthalimido-1-thio-4,6-di-*O*-(p-toluenesulfonyl)-β-D-glucopyranoside (**5**) (Figure 2). Since direct conversion of the primary *p*-toluenesulfonyl



Reagensek és reakciókörülmények: (a) CF_3COOH, H_2O, CH_2Cl_2, 2 óra, 96%; (b) TsCl, piridin, 4 nap, 77%; (c) NaI, MeCOEt, reflux, 16 óra, 69%; (d) H_2, Pd/C, EtOH, Et_3N, 2 óra, 86%; (e) NaN_3, DMF, 120 ^oC, 16 óra, 61%; (f) NaOMe, MeOH, 30 perc, 88%.

Figure 2:

group of **5** into a deoxy moiety failed, it was exchanged to an iodo function, to allow to isolate ethyl 3-*O*-acetyl-2,6-dideoxy-6-iodo-2-phthalimido-l-thio-4-*O*-(p-toluenesulfonyl)-β-D-glucopyranoside (**6**). Reduction of the iodosugar **6** could be best achieved by using the procedure of Wessel. Thus, hydrogenation over Pd/C in the presence of Et_3N resulted in 86% of ethyl 3-*O*-acetyl-2,6-dideoxy-2-phthalimido-1-thio-4-*O*-(*p*-toluenesulfonyl)-D-galactopyranosyde (**7**). Reduction with Zn powder in acetic acid, or with tri-*n*-butyltin

hydride proceeded with 76% and 74% yield, respectively, but workup of the reaction mixtures were much more tedious. Nucleophilic substitution of the 4-*O*-(p-toluenesulfonyl) derivative **7** with sodium azide furnished crystalline ethyl 3-*O*-acetyl-4-azido-2,4,6-trideoxy-2-phthalimido-1-thio-β-D-galactopyranoside (**8**) in acceptable (61%) yield. Either this compound itself, or its 1-*O*-trichloroacetimidate derivative, which are available from **8**, were considered to be promising glycosyl donors for the subsequent glycosylation reactions. At the same time, the azidosugar **8** was an excellent starting material for the synthesis of the aglycones: methyl 4-azido-2,4,6-trideoxy-2-phthalimido-β-D-galactopyranoside (**12**) and methyl 2-acetamido-4-azido-2,4,6-trideoxy-β-D-galactopyranoside (**14**), and also of methyl 4-amino-2,4,6-trideoxy-2-acetamido-β-D-galactopyranoside (**15**) (Figure 3) which was required for the biological studies. Although the lowest yields in the applied reaction sequence were higher than 60%, the overall yield for the synthesis of compound **15** was still near 1-2% because of the numerous reaction step.

3.2 Synthesis of the disaccharides

Since the biosynthesis of the natural polysaccharides (i.e. also the O-PS of *Shigella sonnei*) is not under genetic-control, their structure is primarily determined by the specificity of the enzymes (e.g. glycosyltransferases) participating in the biosynthetic process. In the present case it is impossible to claim that which is the monosaccharide building unit the sequence is started with. Therefore-taking the inhomogenity by the molecular mass also into consideration-we can suppose that either of the following two structures may describe the correct structure of the O-PS:

$\rightarrow$ 3)-4-NH_2-2,4,6td-β-D-GalpNAc-(1$\rightarrow$4)-2d-α-L-AltpNAcA-(1$\rightarrow$ (I)

$\rightarrow$ 4)-2d-α-L-AltpNAcA-(1$\rightarrow$3)-4-NH_2-2,4,6td-β-D-GalpNAc-(1$\rightarrow$ (II)

It is of historical interest that in the Arrhenius Laboratory (Stockholm, Sweden), one of the finest carbohydrate research team of Europe, attempted to realize the synthesis of structure I in 1990. However, the trial was unsuccessful, and although this team elucidated the structure of many natural polysaccharides, they gave up related research. The severe difficulty in the synthesis of the disaccharides with structure I and II is encountered with the presence of the 2-acetamido-2-deoxy unit in both monosaccharides. This is why generation of the interglycosidic likage can not be carried out with the participating 2-acetamido groups, and also, the zwitterionic structure of both units is much more characteristic of pep-

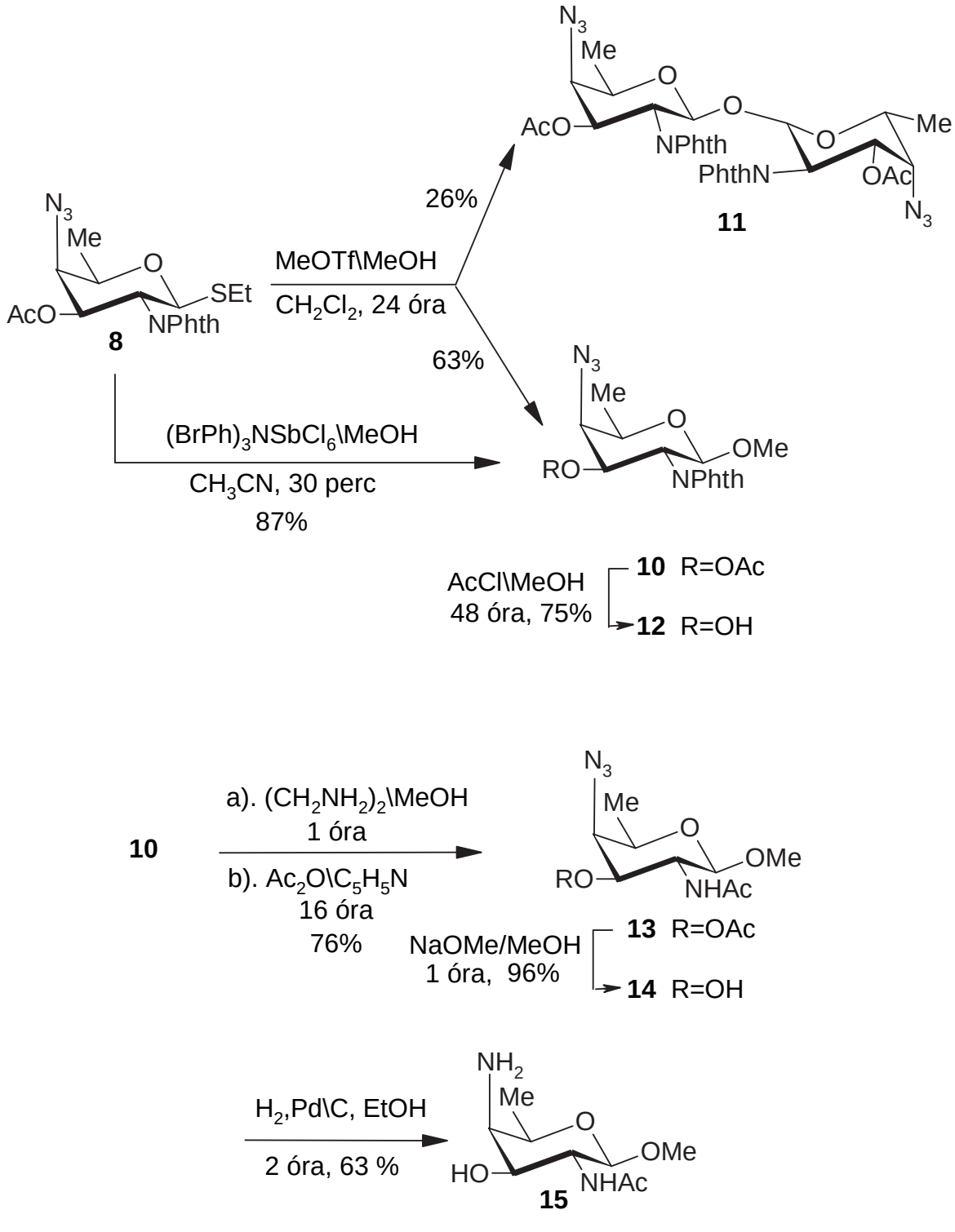

N3
Me
O
AcO
SEt
NPhth
8
MeOTf\MeOH
CH2Cl2, 24 óra
26%
63%
11
PhthN
OAc
Me
(BrPh)3NSbCl6\MeOH
CH3CN, 30 perc
87%
RO
OMe
AcCl\MeOH
48 óra, 75%
10 R=OAc
12 R=OH
a). (CH2NH2)2\MeOH
1 óra
b). Ac2O\C5H5N
16 óra
76%
NHAc
NaOMe/MeOH
1 óra, 96%
13 R=OAc
14 R=OH
H2,Pd\C, EtOH
2 óra, 63 %
NH2
HO
15

Figure 3:

tides than of the carbohydrate oligomers.

For the production of 1,2-*trans* interglycosidic linkages built up from 2-acetamido sugars *N*-phthaloyl (NPhth) and *N*-tetrachlorophtaloyl (NTCP) protecting groups are generally applied, which work well also for *N*-glycoproteins, although removal of the imide-type protecting groups calls for rather drastic conditions. Activation of the anomeric centers of the monosaccharide units is usually facilitated with various leaving groups, represented by the trichloroacetimidate, thioglycoside, halogeno derivatives. With these literature experiences in mind, we designed our oligosaccharide syntheses mainly with the NPhth and the NTCP protecting groups, and based on the thio, imidate, and halogeno leaving groups.

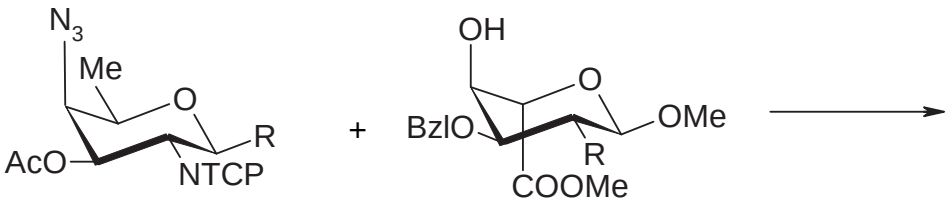
3.2.1 Synthesis of disaccharides with structure I.

For the preparation of the disaccharide represented by structure **I**, methyl (methyl 3-*O*-benzyl-2-deoxy-2-phthalimido-α-L-altropyranoside)uronate (**33**), the corresponding 2-tetrachlorophthalimido derivative (**34**), and methyl (methyl 2-acetamido-3-*O*-benzyl-2-deoxy-α-L-altropyranoside)uronate (**32**) were applied as the aglycone.

The glycosyl donors to the syntheses were ethyl 3-*O*-acetyl-4-azido-2,4,6-trideoxy-2-phthalimido-1-thio-β-D-galactopyranoside (**8**), its 2-tetrachlorophthalimido derivative (**28**), and the 1-trichloroacetimidate **38**. The prepared building blocks were employed in the following combinations: the NHAc uronate (**32**), *N*-phthaloyl uronate and (**33**) *N*-tetrachlorophthaloyl (**34**) uronate were glycosylated with the *N*-tetrachlorophthaloyl trichloroacetimidate (**38**), and afforded the disaccharides **39** in 32% yield, **40** in 89% yield and **41** in 93% yield (Figure 4).

Although we strictly kept the literature suggestions, i.e. 1) hydrolysis of the uronic ester with LiOH or LiI in pyridine; 2) treatment with the base; 3) acetylation, removal of the *N*-protecting group of both disaccharides (**39** and **40**) either with hidrazine hydrate or ethylenediamine proceeded with rather low yields.

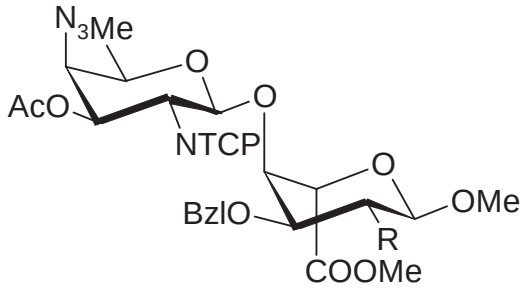
Literature data, and our investigations with the monosaccharide models showed that the *N*-tetrachlorophthaloyl group can be much quickly removed with a considerably lower diamine concentration than the *N*-phthaloyl function. The above difficulties with the removal of the protecting groups led us to prepare structure **I** also with the *N*-tetrachlorophthaloyl protecting group. By removal of the methyl ester moiety with LiOH (Figure 5), followed by refluxing with 6 equivalents of ethylenediamine in methanol for 14h, we could get rid of the tetrachlorophthaloyl group. However, acetylation of the prod-



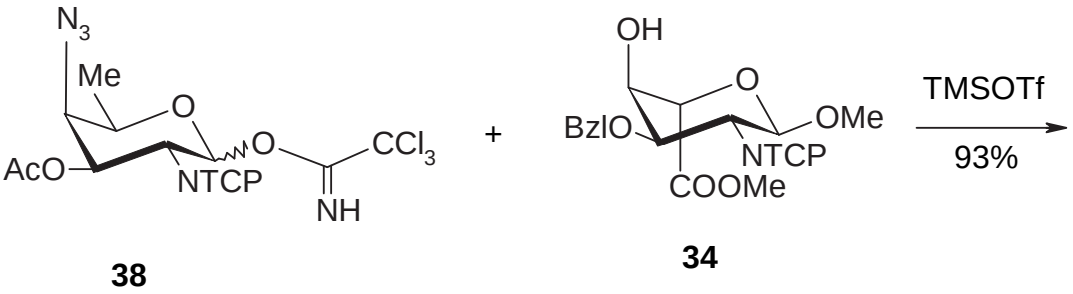
34

38 R= OC(=NH)CCl_3 (α/β)
28 R= SEt

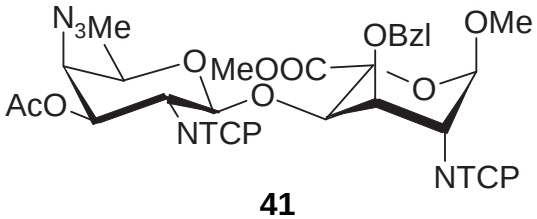
32 R= NHAc
33 R= NPhth
34 R= NTCP



38 + **32** ⟶ **39,** R= NHAc, 32%
38 + **33** ⟶ **40,** R= NPhth, 89%
28 + **34** ⟶ ø



38 **34**



41

Figure 4:

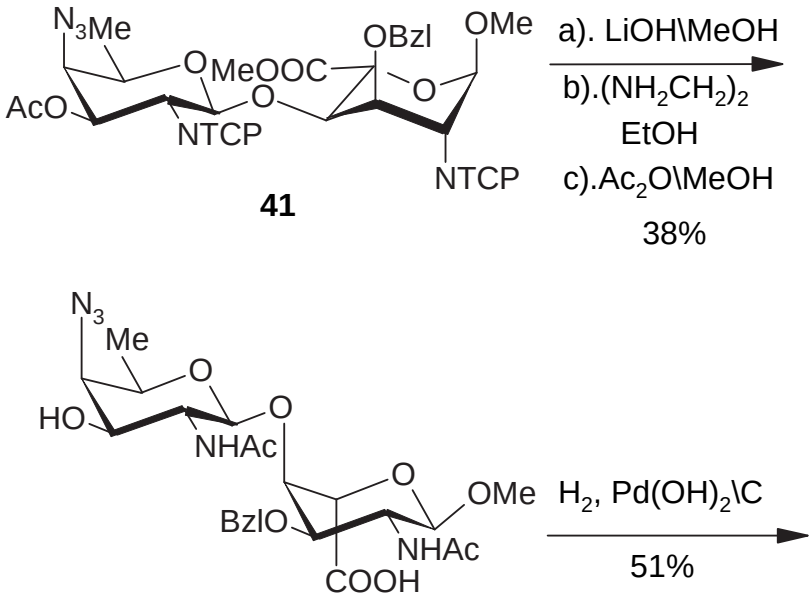

N3Me
OBzl
OMe
O
MeOOC
O
O
AcO
NTCP
NTCP
a). LiOH\MeOH
b).(NH2CH2)2
EtOH
c).Ac2O\MeOH
38%
41
N3
Me
O
HO
O
NHAc
O
OMe
BzlO
NHAc
COOH
H2, Pd(OH)2\C
51%
42
NH2
Me
O
HO
O
NHAc
O
OMe
HO
NHAc
COOH
43

Figure 5:

uct could be achieved only with 38% yield, to obtain the di-N-acetyl derivative **42**. Splitting off of the benzyl group, and reduction of the azide function into amino was effected with catalytic hydrogenation, to obtain the target disaccharide **43** with 51% yield. Full characterization of each of the disaccharide derivatives was carried out by means of ^{1}H and ^{13}C-NMR measurements.

3.2.2 Synthesis of disaccharides with structure II.

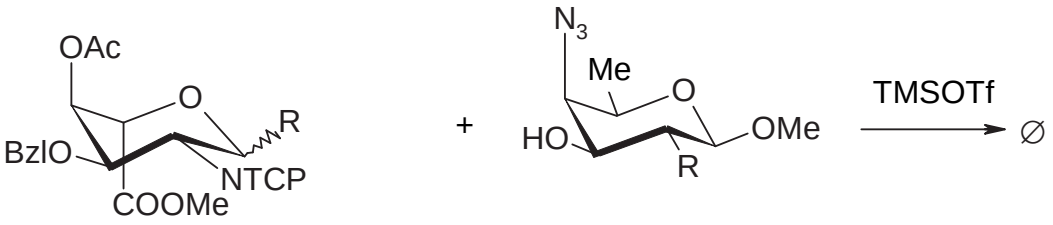
In this case we applied methyl 4-azido-2,4,6-trideoxy-2-phthalimido-β-D-galactopyranoside (**12**), and its 2-tetrachlorophthalimido analogue (**31**) as the aglycones, and methyl (4-*O*-acetyl- 3-*O*-benzyl- 2-deoxy-2- phthalimido- 1-*O*- trichloroacetimidoyl- α-L- altropyranosid)uronate (**35**) as the glycosyl donor.

Coupling of **12** with **35** in the presence of trimethysilyl triflate catalyst furnished 69% of methyl[methyl(4-*O*-acetyl-3-*O*-benzyl-2-deoxy-2-tetrachlorophthalimido- α-L- altropyranosyl)uronate]-(1→3)-4-azido-2,4,6-trideoxy-2-phthalimido-β-D-galactopyranoside (**44**). Under equal conditions, the two 2-tetrachlorophthalimido components **31** and **35** afforded methyl[methyl(4-*O*-acetyl-3-*O*-benzyl-2-deoxy-2-tetrachlorophthalimido-α-L-altropyranosyl)uronate]-(1→3)-4-azido-2,4,6-trideoxy-2-tetrachlorophthalimido-β-D-galactopyranoside (**45**) in an essentially lower yield (44%). The **36** bromid glycosyl donor and the **31** 2-*N*-tetrachlorophthaloyl alglycone afforded the **45** disaccharide also in rather low yield. (35%) (Figure 6).

The removal of the *N*-phthaloyl and *N*-tetrachlorophtaloyl groups from the disaccharide **44** and **45**, could not be performed (Figure 7).

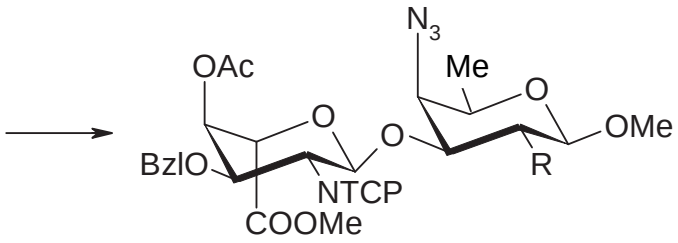
Due to the observed low yields, this route was somewhat modified.

Thus, glycosylation of the *N*-tetrachlorophthaloyl derivative **31** with the donor: 4,6-di-*O*-acetil-3-*O*-benzyl-2-deoxy-2- tetrachlorophthalimido-1-*O*-trichloroacetimidoyl-α-L-altropyranose (**48**) furnished methyl (4,6-di-*O*-acetyl-3-*O*-benzyl-2-deoxy-2- tetrachlorophthalimido-α-L-altropyranosyl)-(1→3)-4-azido-2,4,6-trideoxy-2-tetrachlorophthalimido-β-D-galactopyranoside (**49**) with good (60%) yield (Figure 8). This latter compound, after acidic de-*O*-acetylation, was oxidized with NaOCl and 2,2,6,6-tetramethylpiperidin-1-oxide (TEMPO), in the presence of KBr and tetrabutylammonium bromide in dichloromethane. The reaction mixture was slightly acidified, and the disaccharide **51** was isolated from the organic phase over 60% yield. Following removal of the tetrachlorophthaloyl groups, acetylation afforded the disaccharide **52** with 56% yield carrying C-4 azido and *O*-3'-benzyl groups. Catalytic



35 R= OC(=NH)CCl_3
36 R= Br

14 R= NHAc
12 R= NPhth
31 R= NTCP



35 + **14** ⟶ ø
35 + **12** ⟶ **44,** R= NPhth, 69%
35 + **31** ⟶ **45,** R= NTCP, 44%
36 + **31** ⟶ **45**, R= NTCP, 35%

Figure 6:

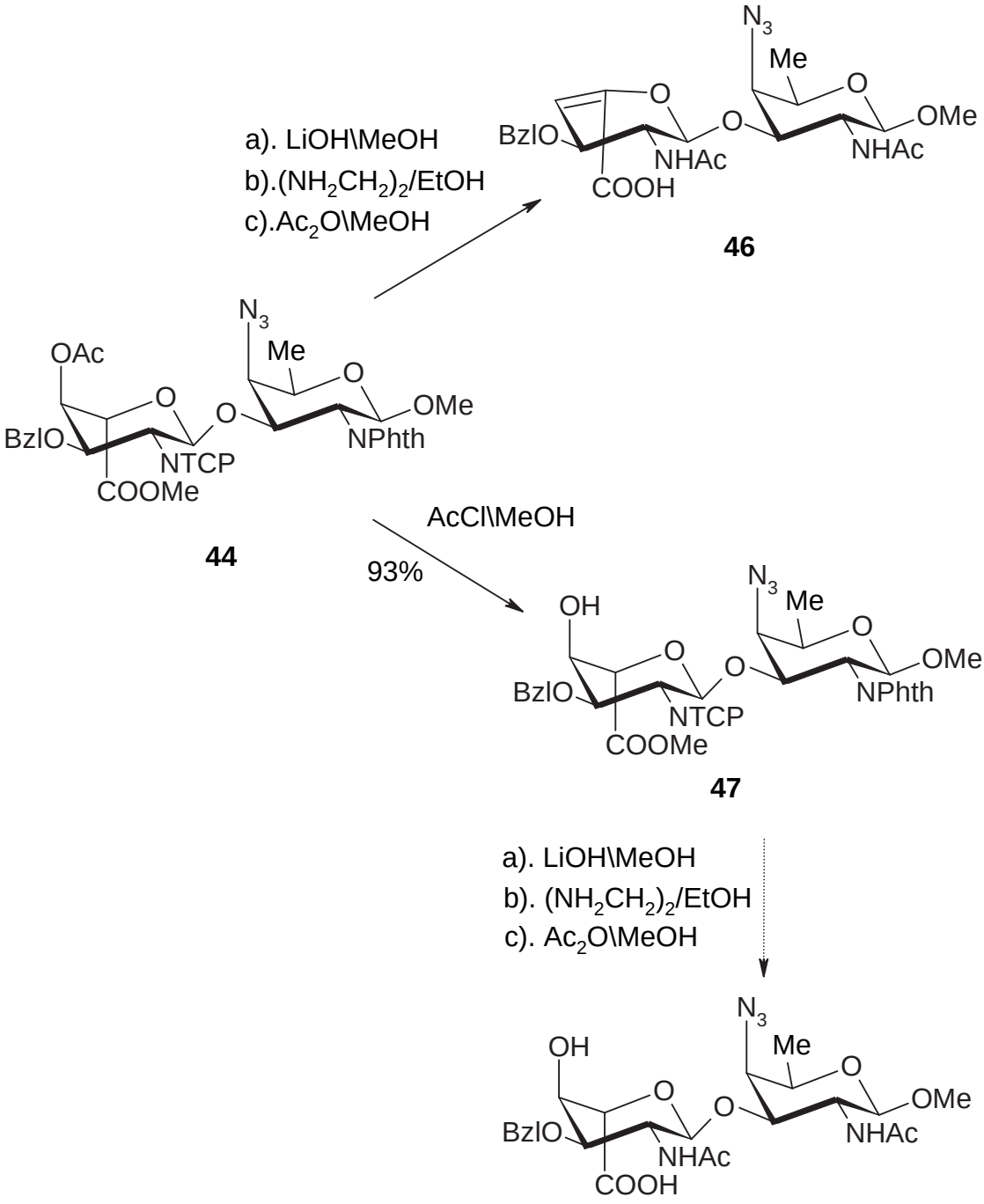
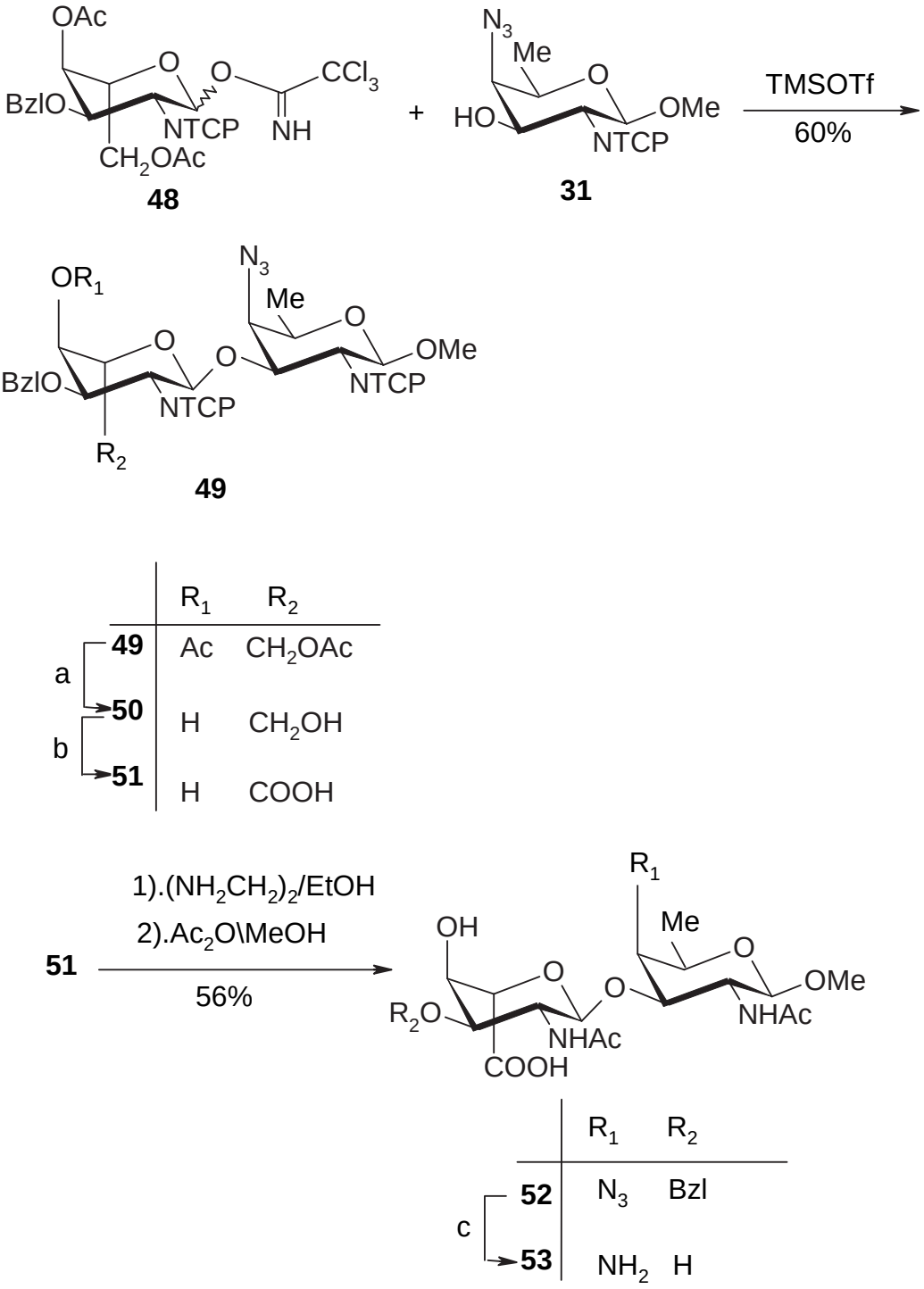

a). LiOH\MeOH
b).(NH2CH2)2/EtOH
c).Ac2O\MeOH
46
44
AcCl\MeOH
93%
47
a). LiOH\MeOH
b). (NH2CH2)2/EtOH
c). Ac2O\MeOH

Figure 7:



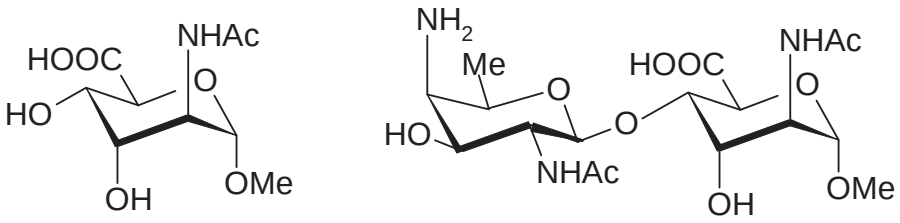
Reagensek és reakciókörülmények: (a) HCl, MeOH, 1 nap, 98%; (b) TEMPO, NaOCl, 2 óra, 61%; (c) H_2, $Pd(OH)_2/C$, EtOH, CH_3COOH, 4 nap, 51%.

Figure 8:

hydrogenation then led us to the target disaccharide **53** with 53% yield. The structure of the products were demonstrated by means of ^{1}H and ^{13}C-NMR measurements.

3.3 Immunological study

The antigenicity of the disaccharide was assayed by the passive hemolysis inhibition test as described by Kontrohr and Péterffy. The method is based on complement-mediated hemolysis of lipopolysaccharide-sensitized erythrocytes after anti-LPS antibody binding. Using sheep erythrocytes, guinea pig complement, phase I lipopolysaccharide of *S. sonnei*, and hyper-immune polyclonal rabbit serum raised against *Shigella Sonnei*, the concentration of the disaccharide **43** and those of the component monosaccharides **57** (Figure 9) and **15** were



57 **58**

Figure 9:

determined that were necessary for 50% inhibition of hemolysis. The results show that the disaccharide **43** is a better inhibitor than either one of its monosaccharide components of which the altruronic acid derivative **57** is superior. Less than 20% inhibition could be observed with the unnatural disaccharide **58** up to 15 mM concentration and no inhibition was seen with unrelated saccharides.

4 List of publications

Publications in the field of the dissertation

- Synthesis of the Monosaccharide Units of the O-Specific Polysaccharide of *Shigella sonnei*, A. Medgyes, E. Farkas, A. Lipták, V. Pozsgay, *Tetrahedron*, **53**, (1997), 4159.
- Synthetic Studies Towards the O-specific Polysaccharide of *Shigella sonnei*, A. Medgyes, I. Bajza, E. Farkas, V. Pozsgai, A. Lipták, *J. Carbohydr. Chem.*, **19(3)**, (2000), 285.
- Synthesis of the Repeating Unit of the O-specific Polysaccharide of *Shigella sonnei* and Quantitation of its Serologic Activity, A. Tóth, A. Medgyes, I. Bajza, A. Lipták, Gy. Batta, T. Kontrohr, K. Péterffy, V. Pozsgay, *Bioorg. Med. Chem. Lett.*, **10**, (2000), 19.

Publications in other fields

- The Use of a New Magnesium-Derived Hydride Reagent for Carbohydrate Derivatives, G. Szabovik, A. Medgyes, Zs. Antal, Zs. Varga, W. Knott, A. Lipták, *Polish J. Chem.*, **73**, 1999, 1003.
- Synthesis of 4-substituted phenyl 2,5-anhydro-1,6-dithio-α-D-gluco- and α-L-guloseptanosides possessing antithrombotic activity, É. Bozó, A. Medgyes, S. Boros, J. Kuszmann, *Carbohydr. Res.*, **329**, (2000), 25.

Lectures

- Új megközelítés az *N*-glikoproteinek törzs régiójának előállításában, A. Medgyes, A. Lipták, *Magyar Szénhidrátkémiai Munkabizottsági Ülés*, május 22-24, 1995.
- Unusual Opening of Sugar Oxiranes with a New Magnesium-Derived Hydride Reagent, A. Medgyes, Zs. Antal, G. Szabovik, A. Lipták, W. Knott, *XVIII International Carbohydrate Symposium*, July 21-26, 1996, Milano, Italy.
- Synthesis of the Monosaccharide Units of the O-Specific Polysaccharide of *Shigella sonnei*, A. Medgyes, E. Farkas, A. Lipták, and V. Pozsgay, 1. *German-East-European- Carbohydrate Workshop for Field Researchers*, March 22-24, 1997, Güstrow.
- Synthesis of the Monosaccharide Units of the O-Specific Polysaccharide of *Shigella sonnei*, A. Medgyes, E. Farkas, A. Lipták, and V. Pozsgay, *Magyar Szénhidrátkémiai Munkabizottsági Ülés*, május 24-27, 1997, Mátrafüred.

Posters

- A *Shigella sonnei* O-specifikus oldallánca monoszaccharid egységeinek szintézise, E. Farkas, A. Medgyes, A. Lipták, *Vegyészkonferencia* 1995, augusztus 29-31, 1995, Debrecen.
- Synthesis of the Monosaccharide Constituents of the Mimetic Antigen of *Shigella sonnei*, A. Lipták, A. Medgyes, E. Farkas, V. Pozsgay, *XVIII International Carbohydrate Symposium*, July 21-26, 1996, Milano, Italy.
- The Use of a new Derived Magnesium-Derived Hydride Reagent for Carbohydrate Derivatives, A. Medgyes, G. Szabovik, Zs. Antal, W. Knott and A. Lipták, *XVIII International Carbohydrate Symposium*, July 21-26, 1996, Milano, Italy.