

The application of HPLC to carbohydrate chemistry and biochemistry

1. Introduction

Carbohydrates and glycoconjugates are very important biological species involved in many life processes. Because of the structural diversities and the multilateral importance of carbohydrates, the analytical methodologies used to analyse them continue to evolve. Over the last two decades, high performance liquid chromatography (HPLC) has been extensively used in the separation and isolation of carbohydrates. The objective of this work is to demonstrate the use of HPLC in synthetic and enzymatic research of carbohydrates. The results are classified to the next chapters:

Separations in connection with the synthetic work

- Preparative separations
- Analytical separations
 - Synthesis of methyl glycosides
 - Separation of maltooligosaccharides

Study of α -amylase enzymes

- Chemoenzymatic synthesis of substrates
- Examination of the active sites of Human Salivary α -Amylase
- Subsite mapping of *Bacillus licheniformis* α -Amylase

2. Methods

The main experimental methods was HPLC. Other chromatographic methods (column chromatography and thin layer chromatography) were used during the experimental work. NMR and MALDI-TOF methods were used for the identification and purity control of products. The classical enzymological methods were make up with HPLC product analysis for enzyme investigation.

3. New results of dissertation

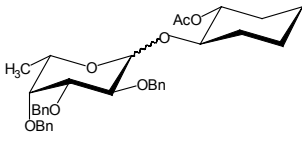
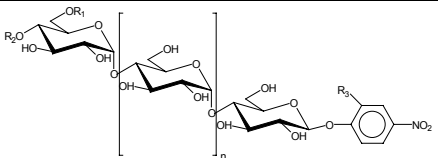
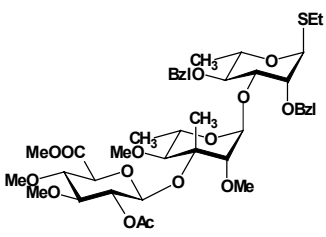
3.1. Preparative separations

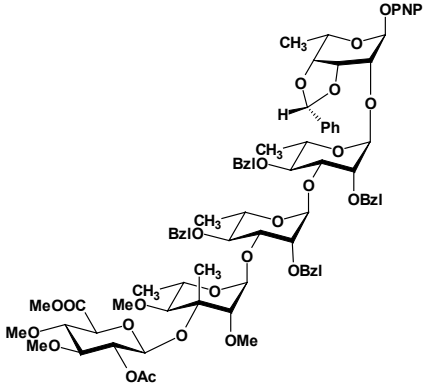
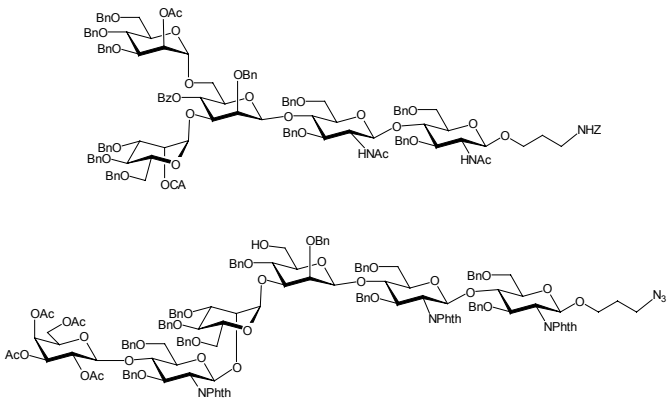
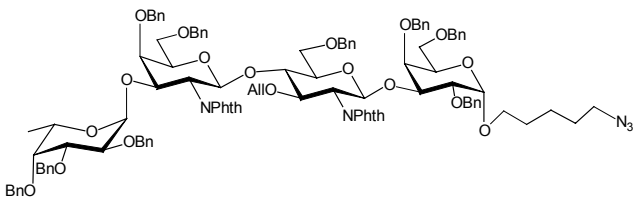
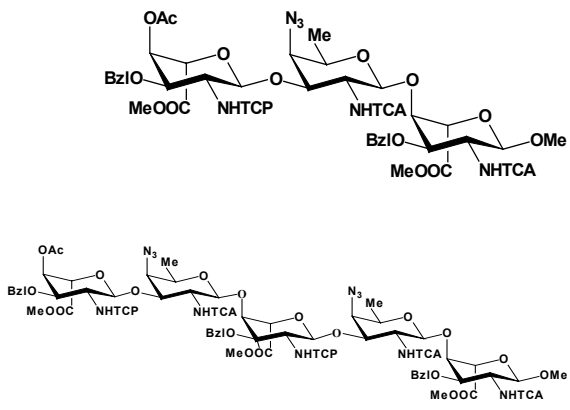
The aim of preparative HPLC was to promote the goal of synthetic project with the means of chromatography. A few mg oligosaccharide for NMR structural investigation or higher amount for further synthesis were separated. The semi-preparative isolations of synthetic oligosaccharides were carried out on silica, amino and C18 stationary phase. The separations were presented in accordance with biological role of synthesised oligosaccharides:

- Synthesis of Sialil LewisX analogue
- *Mycobacterium avium* antigen
- Synthesis of core oligosaccharide of N-glycoproteins
- Diagnosis of *Shistosoma mansoni* infection
- Preparation of vaccine against *Shigella sonnei* infection
- Synthesis of maltooligosaccharide substrates

These separations demonstrate the wide variety of the chromatographic problems in connection with carbohydrate syntheses.

1. Table

Subject	Separated compound	System
Synthesis of Sialil LewisX analogs		C18 MeCN:water=9:1 3 ml/min DAD 254 nm
Synthesis of maltooligosaccharide substrates	 <p style="text-align: center;">$n = 5-9, R_{1,2} = H \quad R_3 = Cl$</p>	Amino MeCN:water=7:3 3 ml/min DAD 302 nm
Synthesis of oligosaccharide repeating unit of <i>Mycobacterium avium</i> antigen		C18 MeCN:water=9:1 3 ml/min DAD 200 nm

		<p>Silica Hexan:EtOAc=6:4 3 ml/min DAD 294 nm</p>
<p>Synthesis of core oligosaccharide of N-glykoprotein antennas</p>		<p>Silica DKM:MeOH=98:2 3 ml/min DAD 254 nm Silica Hexan:EtOAc=1:1 3 ml/min DAD 254 nm</p>
<p>Synthesis of oligosaccharid part of <i>Shistosoma mansoni</i> glyocalix</p>		<p>Silica Hexan:EtOAc=7:3 3 ml/min DAD 254 nm</p>
<p>Vaccina against <i>Shigella sonnei</i> infection</p>		<p>Silica Hexan:EtOAc=1:1 3 ml/min DAD 254 nm Silica Hexan:aceton=6:4 3 ml/min DAD 214 nm</p>

3.2. Analytical separations

3.2.1. Synthesis of methyl glycosides

The product distribution of the iodine-catalysed methyl glycosidation of four pentoses (D-ribose, D-arabinose, D-xylose, and D-lyxose) and two 6-deoxyhexoses (L-rhamnose, and D-fucose) was studied by HPLC in an APS column (sulphate form) with different acetonitrile-water mobile phases. In general, pentoses require 4–5 h to reach a nearly complete conversion into glycosides, the major (and in some cases the exclusive) products are furanosides, and the anomer-selectivity is rather low. The results are summarised in Figure 1. In agreement with earlier results, a temperature dependent on-column isomerization was observed for all the investigated aldoses, except for ribose.

3.2.2. Separation of maltooligosaccharide substrates

The separation of different oligosaccharide series by HPLC using amino, diol and C18 reversed phase column was evaluated. Amino and C18 columns performed well in separating the member of the maltooligosaccharide glycoside series. The separation of oligomer peracetates were successful on the amino and diol column. It was found that the retention sequence was reversed on the C18 column compared with the amino column. Linear relationship was found between the logarithm of retention time and number of monosaccharide unit of the oligosaccharides or oligosaccharide glycosides on the amino and diol column. The relationship was not linear on C18 stationary phase in all case investigated.

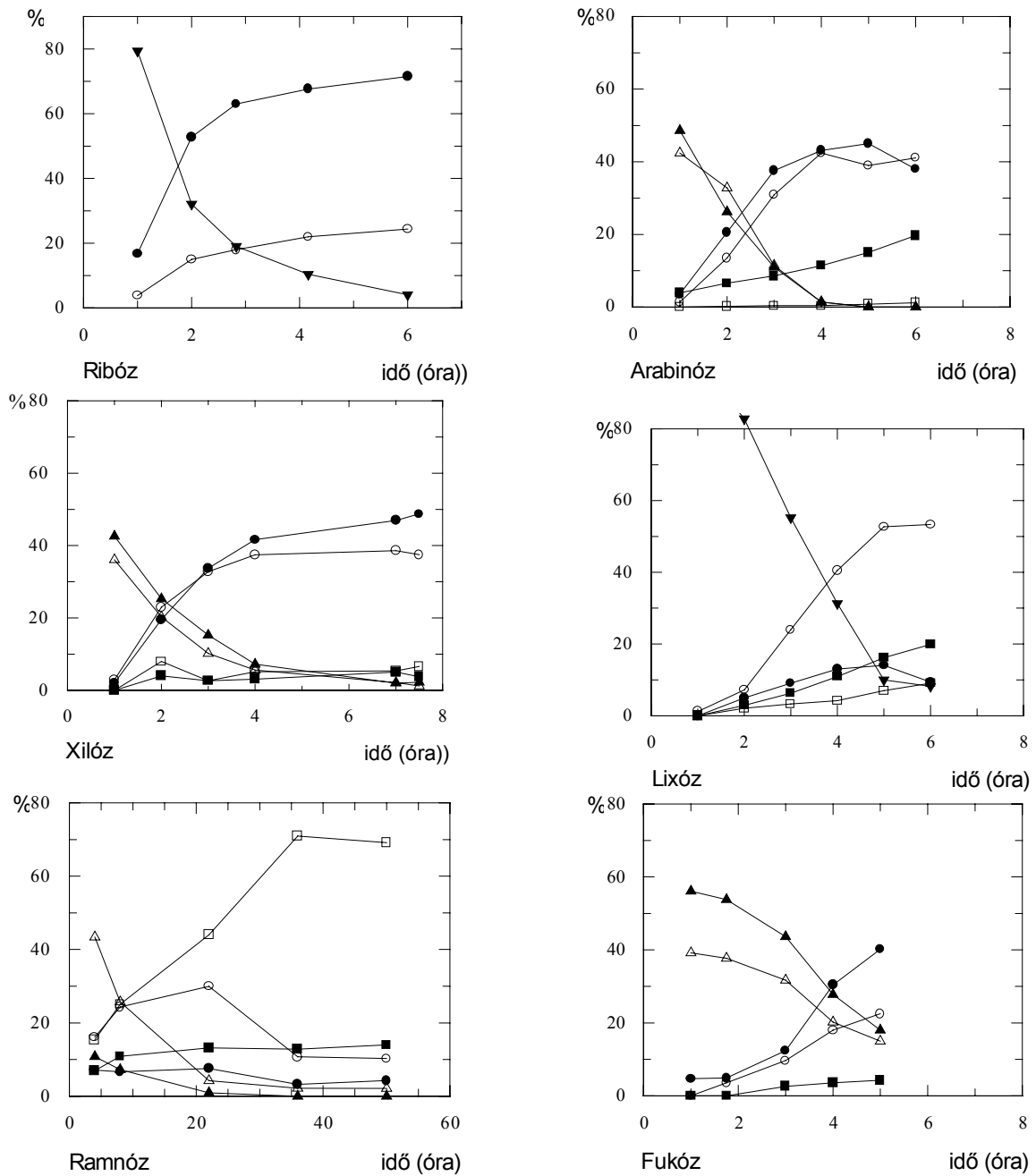


Figure 1. Composition of reaction mixtures plotted against the reaction time

○ α -furanoside, ● β -furanoside, □ α -pyranoside, ■ β -pyranoside, △ α -pyranose, ▲ β -pyranose, ▼ α - és β -pyranose together

3.3. Study of α -amylase enzymes

α -Amylase (α -1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) is a classical calcium-containing enzyme, which constitutes a family of endo-amylases catalyzing the cleavage of α -(1,4) glycosidic bonds in starch and related carbohydrates with retention of the α -anomeric configuration in the products. α -Amylase is one of the major secretory products of the pancreas and salivary glands in humans, playing a role in digestion of starch and glycogen. Human α -amylases, both salivary and pancreatic (HSA and HPA, respectively) have been extensively studied enzymes from the view point of clinical chemistry because they are important as indicators of dysfunction tissue from which they originate. *Bacillus licheniformis* produces a highly thermostable α -amylase. Therefore, it is among the most important enzymes and is of great significance in the present-day biotechnology. It is widely used in alcohol, sugar and brewing industries for the initial hydrolysis of starch to dextrin, which are then converted to glucose by glucoamylases. Enzymic hydrolysis of starch has now replaced acid hydrolysis in over 75% of starch hydrolysing processes, due to the many advantages, not least its higher yields. The homologous maltooligosaccharide substrates are indispensable tools in the investigation of the binding site and the action of different depolymerising enzymes. In these studies well defined, high purity, low-molecular weight substrates are preferred because the purity of these substances and their reaction patterns can be exactly determined.

3.3.1. Chemoenzymatic synthesis of substrates

In the course of our studies of convenient substrates for alpha-amylases, 2-chloro-4-nitrophenyl (CNP) and 4,6-O-benzylidene modified 4-nitrophenyl (Bnl-NP) β -maltooligosaccharides, dp 4 to 10 and dp 4 to 8, respectively were synthesised and used for the study of the active centre amylases. Unfortunately, there is no efficient chemical method for carbohydrate chemists to form glycosidic linkages stereospecifically, or to generate higher-molecular-weight oligosaccharide glycosides with chromogenic aglycons. Therefore,

we developed a chemoenzymatic procedure for the synthesis of CNP- β -maltooligosaccharide glycosides.

Preparation of substrates DP 4-6 by phosphorolytic cleavage

Shorter chain length CNP-maltooligosaccharides in the range of dp 4 to 6 were prepared using rabbit skeletal muscle glycogen phosphorylase b (EC 2.4.1.1). Detailed enzymological investigations revealed that the conversion of G₇-CNP was highly dependent on the conditions of phosphorolysis. A 100 % conversion of G₇-CNP was achieved during 10 minutes in 1 M phosphate buffer (pH 6.8) at 30 °C with the tetramer glycoside (77 %) as the main product. Phosphorolysis at 10 °C for 10 minutes resulted in 89 % conversion and the formation of G₄-, G₅-, G₆-CNP oligomers were detected with the ratio of 29, 26, 34 %, respectively. The reaction pattern was investigated using an HPLC system. The preparative scale isolation of G_{3→6}-CNP glycosides was achieved by size exclusion column chromatography on Toyopearl HW-40 matrix. The productivity of the synthesis was improved in yields up to 70-75 %.

Preparation of substrates DP 8-11 by transglycosilation

CNP-maltooligosaccharides of longer chain length, in the range of dp 8-11, were obtained by a transglycosylation reaction using α -D-glucopyranosyl-phosphate (G-1-P) as donor. Detailed enzymological studies revealed that the conversion of G₇-CNP catalysed by rabbit skeletal muscle glycogen phosphorylase b could be controlled by acarbose and was highly dependent on the conditions of transglycosylation. The reaction pattern was investigated using an HPLC system. The preparative scale isolation of G_{8→12}-CNP glycosides was achieved on a semi-preparative HPLC column. The productivity of the synthesis was improved by yields up to 70-75%. The structures of the oligomers were confirmed by their chromatographic behaviours and MALDI-TOF MS data.

3.3.2. Examination of the active sites of Human Salivary α -Amylase

The action pattern of human salivary amylase (HSA) was examined by utilising as model substrates 2-chloro-4-nitrophenyl (CNP) β -glycosides of maltooligosaccharides of dp 4-8 and some 4-nitrophenyl (NP) derivatives modified at the non-reducing end with a 4,6-*O*-benzylidene (Bnl) group. The product pattern and cleavage frequency were investigated by the method of product analysis, using HPLC.

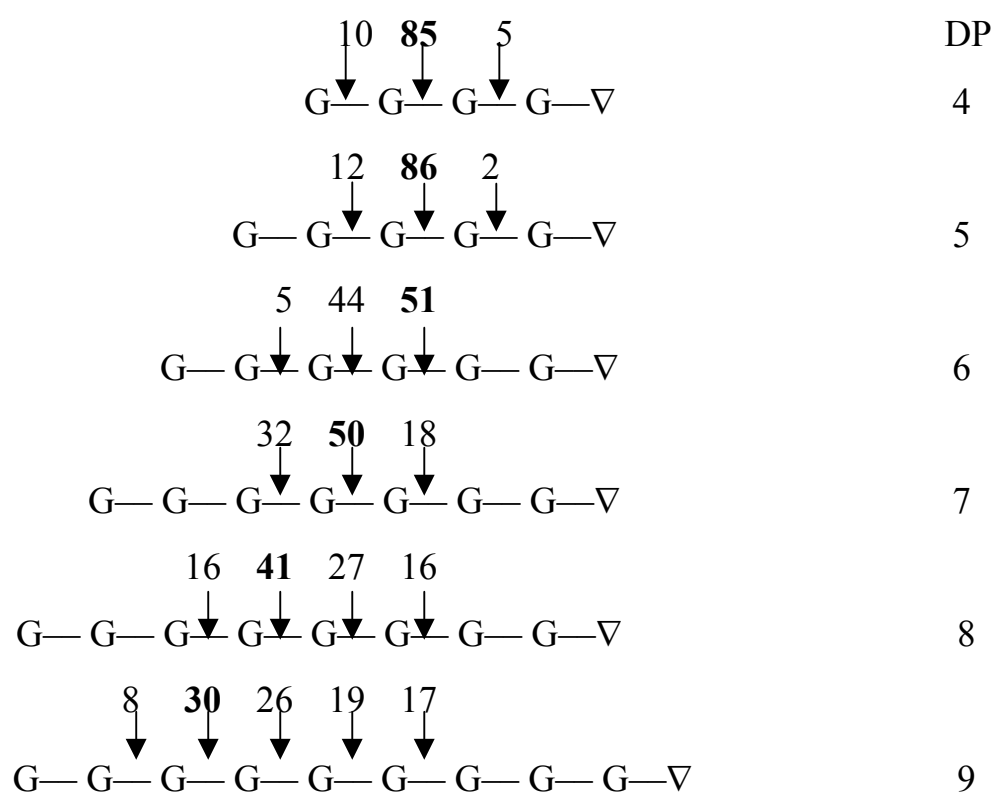


Figure 2. Bond cleavage frequencies of CNP-glycosides cleaved by HSA

G: glucose unit, ∇ : 2-chloro-4-nitrophenyl group, —: glycosydic linkage

The results revealed that the binding region in HSA is longer than five subsites usually considered in the literature and suggested the presence of at least six subsites; four glycone-binding sites (-4, -3, -2, -1) and two aglycone-binding sites (+1, +2). The existence of -4 subsite was confirmed by the comparison of cleavage frequencies of PNP- and benzylidene modified PNP-glycosides, in that binding mode, which all subsite were occupied.

3.3.3. Subsite mapping of *Bacillus licheniformis* α -Amylase

The action pattern and product specificity of the amylase from *Bacillus licheniformis* (BLA) was examined by utilising as model substrates the 2-chloro-4-nitrophenyl (CNP) β -glycosides of maltooligosaccharides of dp 5-10 and two 4-nitrophenyl (NP) derivatives modified at the nonreducing end with a 4,6-O-benzylidene (Bnl) group. The product pattern and cleavage frequency were investigated by product analysis using HPLC.

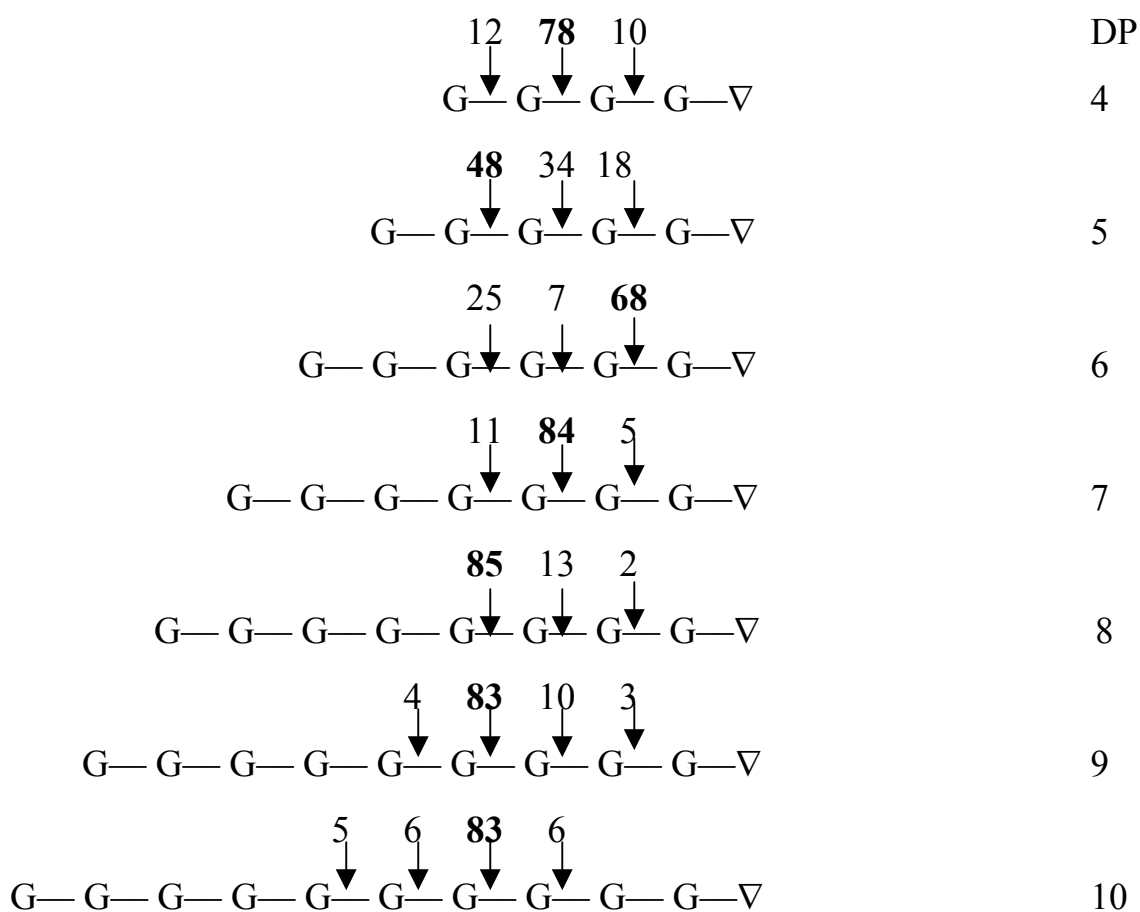


Figure 3. Bond cleavage frequencies of of CNP-glycosides cleaved by HSA

G: glucose unit, ∇ : 2-chloro-4-nitrophenyl group, —: glycosydic linkage

The results revealed that the binding region of BLA is longer than that of human α -amylases and suggested the presence of at least eight subsites; five glycone (-5, -4, -3, -2, -1) and three aglycone binding sites (+1, +2, +3). In the ideal arrangement, the eight subsites are filled by a glucopyranosyl unit. The release of maltopentaose (G_5) from the nonreducing end is dominant in the shorter substrates ($G_8 \rightarrow G_6$), and in the case of the longer substrates ($G_8 \rightarrow G_{10}$), the cleavage of CNP/NP- G_3 from the reducing end becomes preferred. The binding modes of the benzylidene derivatives indicated an unfavourable interaction between the Bnl group and subsite (-6). The calculated subsite map energies confirm the eight subsite model of BLA. There are a barrier subsite at the end of aglycon binding site. This barrier subsite causes the interesting dual product specificity of BLA.

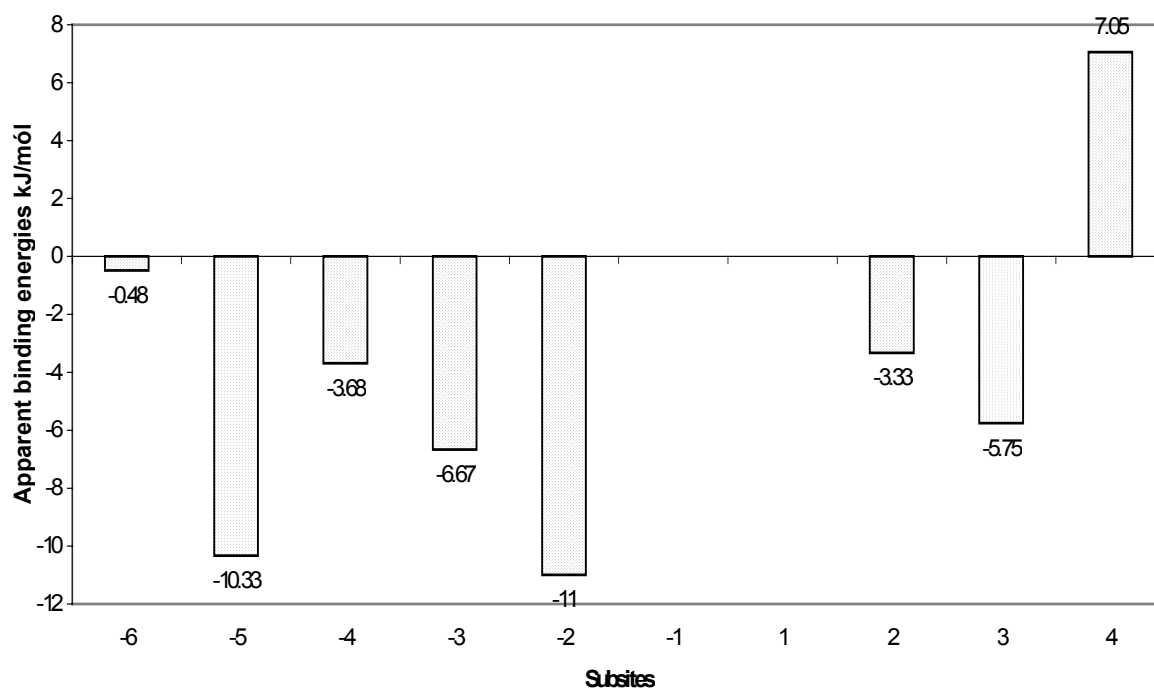


Figure 4. Subsite map of BLA

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