**Helicobacter pylori** binding to new glycans based on N-acetyllactosamine

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Previously we reported binding of **Helicobacter pylori** to various nonacid and sialylated neolacto carbohydrate structures using a wide range of natural chemical modified sequences. A novel nonsialylated neolacto-based binding epitope, GlcNAcβ3Galβ4GlcNAc, and analogous structures carrying terminal GalNAcβ3, GalNAcα3, or Galα3 showed the binding activity (Miller-Podraza H, Lanne B, Ångström J, Teneberg S, Abul-Milh M, Jovall P-Å, Karlsson H, Karlsson K-A. 2005. Novel binding epitope for **Helicobacter pylori** found in neolacto carbohydrate chains. J Biol Chem. 280:19695–19703). The present work reports two other **H. pylori**-binding nonsialylated neolacto-based structures, GlcAβ3Galβ4GlcNAcβ3-R and Glcβ3Galβ4GlcNAcβ3-R, and two amide derivatives (N-methyl and N-ethyl) of GlcAβ3Galβ4GlcNAcβ3-R which were bound by **H. pylori**. The latter structures turned out to be more effective as **H. pylori** binders than the parent saccharide. New reducing-end variants of the neolacto epitope including species containing N-acetyllactosamine linked β6 to GlcNAc or Gal with similarity to branched polyacetylated mannans were prepared and tested. The results extend our previous findings on binding specificities of **H. pylori** and show that this pathogen is able to interact with an array of N-acetyllactosamine/neolacto structures, which may be of importance for the in vivo interaction of the bacterium with human cells. The information gained in this work may also be of value for rational design of anti-**H. pylori** drugs.

**Keywords:** binding epitope/**Helicobacter pylori*/neolacto/structure

**Introduction**

**Helicobacter pylori** is a common human gastric pathogen associated with chronic gastritis and duodenal and gastric ulcers (Dunn et al. 1997; McGee and Mobley 1999; Figueiredo et al. 2005). The infection increases the risk of neoplastic diseases of the gastrointestinal tract (Peek and Blaser 2002; Marshall and Windsor 2005; Fuccio et al. 2007), and in 1994, the bacterium was classified by the World Health Organization as a class 1 carcinogen (IARC 1994). Like many other microbes, **H. pylori** has the ability to adhere to cell surfaces of target tissues, and the binding is mediated by host carbohydrates (Karlsson 2000). Table I shows saccharide structures which are known to bind the bacterium. The significance of the multiple binding specificities is at present not clear. Lewis b epitope (Van de Bovenkamp et al. 2003), sulfated structures (Natomi et al. 1993), and acto epitope (Teneberg et al. 2002) were found in the stomach epithelium and are believed to be of importance for colonization. Neu5Ac may also have an important role since **H. pylori**-induced inflammation is associated with higher expression of this monosaccharide in the mucosa layer (Syder et al. 1999; Chaturvedi et al. 2001; Mahdavi et al. 2002). Neu5Ac and neolacto saccharides are also present in human neutrophils (Fukuda et al. 1985; Muthing et al. 1996; Stroud et al. 1996; Miller-Podraza et al. 1999) and human saliva (Prakobphol et al. 1998; Thompson et al. 2002), which are of relevance for the maintenance of chronic **H. pylori** inflammations in the stomach (Blaser 1992; Karlsson 2000) and for binding of **H. pylori** in the oral cavity. Nonsialylated neolacto structures have not yet been found as binding epitopes in the stomach mucosa. However, they are expected to be present in this tissue because the mucosa layer contains a wide range of complex glycoproteins including highly glycosylated mucins and proteoglycans.

**H. pylori** can be eradicated from the stomach using antibiotics. However, this treatment may lead to development of antibiotic resistance and is not recommended for prevention. Knowledge about binding specificities of **H. pylori** and detailed information on binding epitopes may be of importance for development of antiadhesion therapy (Karlsson 2000).

**Results**

In a previous paper, we described binding of **H. pylori** to various neutral neolacto-based glycolipid sequences, and the minimum epitope was proposed to be GlcNAcβ3Galβ4 GlcNAcβ (Miller-Podraza et al. 2005). In the present paper, we report two new neolacto nonsialylated compounds with binding activity with the terminal trisaccharide GlcAβ3Galβ4 GlcNAcβ3 and Glcβ3Galβ4GlcNAcβ3 (Nos. 1 and 2 in Table II), respectively. Binding of **H. pylori** to GlcAβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer and its reduced counterpart Glcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer is shown in Figures 1 and 2B. Both glycolipids were active; however, the detection level was higher for the GlcA-containing variant indicating lower affinity for the bacterium. Interestingly, amidation of GlcA could improve the binding. N-Methyl- and N-ethyl-amides of the GlcAβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer (Nos. 3 and 4 in Table II) turned out to be more potent than the parent glycolipid. The
binding by \textit{H. pylori} of these amides was on TLC plates about 20 times stronger than that of the unmodified glycolipid (Figure 2A). The detection level for \textit{N}-methyl- and \textit{N}-ethyl-amides was between 10 and 50 pmol per spot which is similar to detection levels of the most potent previously described glycolipids (Miller-Podraza et al. 2004, 2005; Johansson et al. 2005). Structures and MS data of the new neolacto-based epitopes are shown in Figures 3 and 4 and Table III.

We also investigated the importance of GlcNACs and N-acetamido groups by binding of \textit{H. pylori} to de-N-acetylated variants of the Gal\beta4GlcNACβ3Galβ4GlcNACβ3Galβ4Glcβ1Cer. As shown in Table II (Nos. 8 and 9), the modified structures were inactive indicating that \textit{N}-acetylation is crucial for the interaction with \textit{H. pylori}. Negative binding results were also obtained when the ring of the terminal Gal of Galβ4GlcNACβ3Galβ4GlcNACβ3Galβ4Glcβ1Cer was opened by periodate oxidation (Table II, No. 7, and Figure 5). In the latter experiment, the aldehyde groups generated in the molecule by NaIO\textsubscript{4} were reduced to alcohols. The experiment confirmed that a modified neighboring group may restrict access to the GlcNACβ3Galβ4GlcNACβ.

Binding experiments in this work were performed on both glycosphingolipids and their derivatives (Table II) and neoglycolipids (Table IV). The latter were prepared by chemical coupling of free neolacto oligosaccharides to selected aminated lipids. Using neoglycolipids, we could confirm that

### Table I. Carbohydrate structures recognized by \textit{H. pylori}

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactosylceramide</td>
<td>Galβ1Cer</td>
<td>(Abul Mill et al. 2001)</td>
</tr>
<tr>
<td>Lactosylceramide</td>
<td>Galβ4Glcβ1-Cer</td>
<td>(Ångström et al. 1998)</td>
</tr>
<tr>
<td>Lactotetraosylceramide</td>
<td>Galβ3GlcNACβ3Galβ4Glcβ1Cer</td>
<td>(Teneberg et al. 2002)</td>
</tr>
<tr>
<td>Neolacto structures</td>
<td>Rβ3→Galβ4GlcNACβ3Galβ4Glc-R</td>
<td>(Miller-Podraza et al. 2005)</td>
</tr>
<tr>
<td>Some blood group antigens, e.g. Lewis b antigen</td>
<td>Fucα2Galβ3(Fucα4)GlcNACβ-R (Lewis b)</td>
<td>(Børø et al. 1993)</td>
</tr>
<tr>
<td>Ganglio structures</td>
<td>Galβ3Galβ4Glcβ4Glcβ1Cer</td>
<td>(Lingwood et al. 1992)</td>
</tr>
<tr>
<td>Some sulfated structures</td>
<td>HS\textsubscript{2}O\textsubscript{3}R</td>
<td>(Slomiany et al. 1989; Ascencio et al. 1993; Natomi et al. 1993)</td>
</tr>
</tbody>
</table>

### Table II. Binding activity of carbohydrate structures investigated in this work

<table>
<thead>
<tr>
<th>Structure</th>
<th>Obtained from</th>
<th>Chemical derivatization</th>
<th>Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Glcβ3Galβ4GlcNACβ3Galβ4Glcβ1Cer</td>
<td>None (commercial product)</td>
<td>R-COOH → R-CH\textsubscript{2}OH</td>
<td>+</td>
</tr>
<tr>
<td>2. Glcβ3Galβ4GlcNACβ3Galβ4Glcβ1Cer</td>
<td>1</td>
<td>R-COOH → R-CH\textsubscript{2}OH</td>
<td>++</td>
</tr>
<tr>
<td>3. Glcβ3Galβ4GlcNACβ3Galβ4Glcβ1Cer</td>
<td>1</td>
<td>R-COOH → R-CH\textsubscript{2}OH</td>
<td>+++</td>
</tr>
<tr>
<td>4. Glcβ3Galβ4GlcNACβ3Galβ4Glcβ1Cer</td>
<td>1</td>
<td>R-COOH → R-CH\textsubscript{2}OH</td>
<td>++</td>
</tr>
<tr>
<td>5. Glcβ3Galβ4GlcNACβ3Galβ4Glcβ1Cer</td>
<td>1</td>
<td>R-COOH → R-CH\textsubscript{2}OH</td>
<td>+</td>
</tr>
<tr>
<td>6. Galβ4GlcNACβ3Galβ4GlcNACβ3Galβ4Glcβ1Cer</td>
<td>6</td>
<td>None (prepared from rabbit thymus)</td>
<td>++</td>
</tr>
<tr>
<td>7. Galβ4GlcNACβ3Galβ4GlcNACβ3Galβ4Glcβ1Cer</td>
<td>6</td>
<td>R-Gal → R-C\textsubscript{2}O\textsubscript{2}H\textsubscript{2}</td>
<td>−</td>
</tr>
<tr>
<td>8. Mixtures of: Galβ4GlcNACβ3Galβ4GlcNACβ3Galβ4Glcβ1Cer</td>
<td>6</td>
<td>Mono de-N-acetylation (GlcNAC → GlcN)</td>
<td>−</td>
</tr>
<tr>
<td>9. Galβ4GlcNACβ3Galβ4GlcNACβ3Galβ4Glcβ1Cer</td>
<td>6</td>
<td>Di de-N-acetylation (2GlcNAC → 2GlcN)</td>
<td>−</td>
</tr>
</tbody>
</table>

“+++” means strong binding (detection level on TLC plates between 10 and 60 pmol/spot or 1–6 pmol/mm\textsuperscript{2}).

“++” and “+” mean less strong bindings (60–200 pmol/spot and more on TLC plates).

“−” means no binding on TLC plates.

The modified monosaccharides are underlined.

### Table III. Summary of mass spectrometry data (FAB+ MS)

<table>
<thead>
<tr>
<th>Numbers as in Table II</th>
<th>[\textit{M}_{\text{mol}}–\textit{H}]\textsuperscript{−} calculated</th>
<th>[\textit{M}_{\text{mol}}–\textit{H}]\textsuperscript{−} observed</th>
<th>Δ[\textit{M}_{\text{mol}}–\textit{H}]</th>
<th>\textit{M}_{\text{terminal sugar}} calculated</th>
<th>\textit{M}_{\text{terminal sugar}} observed</th>
<th>Δ\textit{M}_{\text{terminal sugar}}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1429.9 (d18:1–22:0)</td>
<td>1429.7</td>
<td>−0.2</td>
<td>162.1</td>
<td>162.0</td>
<td>−0.1</td>
</tr>
<tr>
<td>2</td>
<td>1415.9</td>
<td>1415.8</td>
<td>−0.1</td>
<td>162.1</td>
<td>162.0</td>
<td>−0.1</td>
</tr>
<tr>
<td>3</td>
<td>1442.9</td>
<td>1442.7</td>
<td>−0.2</td>
<td>189.1</td>
<td>189.1</td>
<td>0.0</td>
</tr>
<tr>
<td>4</td>
<td>1456.9</td>
<td>1456.7</td>
<td>−0.2</td>
<td>203.1</td>
<td>203.1</td>
<td>0.0</td>
</tr>
<tr>
<td>5</td>
<td>1518.9</td>
<td>1518.8</td>
<td>−0.1</td>
<td>265.2</td>
<td>265.1</td>
<td>−0.1</td>
</tr>
<tr>
<td>6</td>
<td>1591.6 (d18:1–16:0)</td>
<td>1591.5</td>
<td>−0.1</td>
<td>162.1</td>
<td>162.1</td>
<td>0.0</td>
</tr>
<tr>
<td>7</td>
<td>1563.6</td>
<td>1563.0</td>
<td>−0.6</td>
<td>134.1</td>
<td>134.1</td>
<td>0.0</td>
</tr>
<tr>
<td>8</td>
<td>1549.6</td>
<td>1549.5</td>
<td>−0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1507.6</td>
<td>1507.5</td>
<td>−0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textit{M}, mass; \textit{M}_{\text{mol}}, molecular mass; Δ\textit{M}, mass difference between calculated and observed masses.
neolacto structures carrying terminal GlcNAc or GlcAMe (methyl amide of GlcA) are among the best binders, which is in agreement with the results obtained from experiments performed on natural glycolipids and their derivatives (compare with Table II). Neoglycolipids carrying terminal GlcA displayed lower binding strength as compared to other neolacto-based neoglycolipid variants, which also is in line with studies performed on ceramide-based glycolipids.

Studies using neoglycolipids also confirmed the importance of more distal sugars for the binding. As shown, active epitopes were influenced by subtle changes in the core chain. The glycolipids were most active when the trisaccharide epitope was coupled to GlcNAc or Gal by the β6-linkage (Table IV, Nos. 1, 4, and 7). Further, the replacement of the β6-linkage by α6- or β3-linkage negatively influenced the binding (Table IV, Nos. 2 and 8). Even lipid parts were important, as shown by the use of neoglycolipids containing different hydrophobic moieties (see differences in binding between HDA and C42 derivatives of

Fig. 1. Binding of Helicobacter pylori to GlcAβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer (GlcA-R, lanes 1) on TLC plates. The left plate (Anis) shows staining for carbohydrates with anisaldehyde. The middle and right plates show detection of the glycolipids by 35S-labeled H. pylori (strain CCUG 17875 and CCUG 17874, respectively). Globoside (lanes 2) was used as a negative control. The silica gel TLC plates were run in C/M/0.25% KCl in water, 50:40:10.

Fig. 2. Relative strength of binding of H. pylori to GlcAβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer (GlcA-R), N-methylamide of GlcA-R, GlcA(N-Me)β3Galβ4GlcNAcβ3Galβ4Glcβ1Cer (GlcA(N-Me)-R, panel A) and its reduced derivative, Glcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer (Glc-R, panel B), as estimated by the detection level. The left plate in each panel shows staining for carbohydrates with anisaldehyde, and the right plate shows detection of the glycolipids by 35S-labeled H. pylori (strain CCUG 26695). The investigated glycolipids were applied on the plates in decreasing amounts in the form of 2-fold dilution series. Lane 1 contains globoside: 2 μg (negative control). Lanes 2–8 contain the decreasing amounts of (GlcA(Me)-R) and Glc-R in panels A and B, respectively. The amount of the material in lane 2 is about 2 nmol for each glycolipid. The silica gel TLC plates were run in C/M/0.25% KCl in water, 50:40:10.

Fig. 3. FAB−MS of GlcAβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer (panel A) and its reduced counterpart Glcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer (panel B) (Table II, Nos. 1 and 2). The sequence of monosaccharides is confirmed by the Y series of ions (Domon and Costello 1988). The ceramide structure is indicated by the ion at m/z 564.4 corresponding to d18:1–22:0.

Fig. 4. FAB−MS spectra of the methyl (A) and ethyl (B) amides of GlcAβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer, respectively (Table II, Nos. 3 and 4). As in Figure 2, the main fragment ions are of the Y series (Domon and Costello 1988). The fragment ion at m/z 564.3 indicates the ceramide d18:1–22:0.
Glcramides with binding activity for *H. pylori*
present paper, we report new neolacto-based pentaglycosyl-
Xenoglycan (without the loss of the activity. In the
β-Glc
reduced (by NaBH4) and (Miller-Podraza et al. 2005). GlcNAc
based on nonsialylated neolacto carbohydrate chains
H. pylori
Discussion
issue how in Figure 6.
Table IV). An example of mass spectrometry of neoglycolipids
H. pylori
plates using as a solvent C/M/H2O 60:35:8. Right panel: the FAB
MS of GlcA(+CO-Me), and GlcA(-CO-N, Et) as a terminal
monosaccharide (Table II and Figures 1–4), respectively. These
structures broaden the spectrum of neolacto epitopes recognized
by *H. pylori*. The specificity includes two different epimers at
C4 (Gal- resp. Glc) and possibly N-acetyl substituent at C2, as
well as tolerance of both α- and β-linkages (Miller-Podraza et al.
2005). Similar specificity was described for *Clostridium difficile*
toxin A and Galα3-Xenoantibodies (Teneberg et al. 1996).

The Glcβ3 terminal has not yet been found on natural neolacto
glycans; however, the terminal GlcAβ3 is part of HK-1 epitope
precursors present on natural killer (NK) cells. Terminal
GlcAβ3Galβ4GlcNAcβ6Galβ4 is part of the HK-1 epitope which
carries a sulfate ester at 3-position of the glucuronic acid.
The precursor of the HK-1 epitope may be a rare natural glyco-
form. It has been shown that a nonsulfated form of the HK-1
precursor was present in mouse kidney (Tagawa et al. 2005), and it has
been suggested that the sulfated epitope is partially replaceable
in carbohydrate–protein interactions by a nonsulfated precursor
(Chou et al. 2002). It should be noted that *H. pylori* has been
reported to interact with NK cells (Tarkkanen et al. 1993; Kuo et
al. 2005; Yun et al. 2005). The potential role of HK-1 precursors
in microbial interactions requires however further studies.

Chemical or enzymatic synthesis using cloning techniques as
well as characterization of the biosynthetic enzymes could
contribute to the in vitro production of rare HK-1 variants for
further investigation of host–pathogen interactions (Terayama
et al. 1997; Kornilov et al. 2000; Ong et al. 2002; Kizuka et al.
2006).

In a previous paper, we reported a new binding specificity of
*H. pylori* based on nonsialylated neolacto carbohydrate chains
(Miller-Podraza et al. 2005). GlcNAcβ3Galβ4GlcNAcβ3Galβ4
Glc-βCer is an effective binder of the bacterium on TLC
plates and we have proven that the terminal GlcAβ3 of this
glycolipid can be replaced by GalNAcβ3 (part of X-2 epitope),
GalNAcβ3 (part of A-antigen), or Galα3 (part of B
antigen/xenoantigen) without the loss of the activity. In the
present paper, we report new neolacto-based pentaglycosyl-
ramides with binding activity for *H. pylori*, carrying GlcAβ3,
Glcβ3, GlcA(-CO-N, Me), and GlcA(-CO-N, Et) as a terminal
The results show that H. pylori has the ability to interact with a wide range of terminal carbohydrate structures associated with neolacto core chains. Galβ4GlcNAcβ3Galβ4GlcNAc is a common component of human cells. However, other neolacto-based structures discussed in this paper have not yet been described as regular parts of carbohydrates in human stomach or other potential H. pylori target tissues. It is not likely that they serve as natural binding epitopes for the bacterium. On the other hand, some inner sequences of carbohydrate chains present in the gastric mucosa and other glycocomponents of human cells and body fluids can be identical or resemble these structures and can be of importance for the interaction with H. pylori. As previously discussed (Miller-Podraza et al. 2005), GlcNAcβ3Galβ4GlcNAc is present as an internal sequence in neolacto carbohydrate chains with repeated N-acetyllactosamine units and occurs in various carbohydrate chains of complex glycoproteins, glycolipids, and glycosaminoglycans.

Several research groups have indicated gastric mucins as binding sites for H. pylori (Hirmo et al. 1998; Van de Bovenkamp et al. 2003). MUC5Ac has been proposed as the main receptor for the bacterium in the stomach (Van de Bovenkamp et al. 2003), and the Lewis b antigen has been pointed out as a binding part of this protein (Linden et al. 2002). However, H. pylori can bind to epithelial cells in the absence of the Lewis b epitope (Rubinstei-Dunlop et al. 2005) indicating that other sequences are also involved in the binding (Linden et al. 2004). Experiments with neoglycolipids (Table IV) reveal that the terminal neolacto-type epitope structures linked by β6-linkages to Gal/GlcNAc have a high binding activity. This implies that GlcNAcβ3Galβ4GlcNAcβ6GalNAc of human gastric mucin (Hanisch et al. 1993) may be a potential mucosal receptor for H. pylori. Neolacto-based structures may also be of importance, together with other epitopes, for the interaction with H. pylori in the oral cavity. It has been proposed that mucins MG1 and MG2, which are present in human saliva, bind H. pylori through sialyl-Lewis x, Lewis b, and sialyl-Lewis a epitopes (Prakobphol et al. 2005). MG1 and MG2 contain a variety of structures based on lacto, neolacto, and GalNAc-containing carbohydrate chains, creating an enormous repertoire of potential binding sites for microorganisms (Prakobphol et al. 1998; Thompson et al. 2002).

It should be noted that gastric mucosa layer contains heparan sulfate, dermatan sulfate, and hyaluronic acid (Theocharis et al. 2003) and glycosaminoglycans made up of hexoses, hexosamines, and uronic acids. Hexoses, hexosamines, and an uronic acid (glucuronic acid, GlcA) are also present in carbohydrate epitopes discussed in this paper. It is possible that H. pylori binds to proteoglycans present in human stomach which is supported by studies on binding of H. pylori to chondroitin sulfate (Linden et al. 2004) and heparan sulfate (Ascencio et al. 1993).

On the other hand, we know that not all neolacto-based saccharides are recognized by H. pylori and that particular side groups and vicinal sugars may hamper the activity. Previously we have shown, using a large number of carbohydrate structures, that the only terminal extensions of the GlcNAcβ3Galβ4GlcNAc epitope which are tolerated by the bacterium are Galβ4 and NeuAca3Galβ4 (Miller-Podraza et al. 2005). In the present study, we have shown that oxidized/reduced Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer is also inactive as a binding molecule (Table II, No. 7 and Figure 5). Using periodate oxidation followed by reduction with NaBH₄, we could open the terminal Gal ring without modification of other sugars, leaving the internal GlcNAcβ3Galβ4GlcNAcβ intact. Various de-N-acetylated variants of Galβ4GlcNAcβ3Galβ4GlcNAcβ3 Galβ4Glcβ1Cer (Table II, Nos. 8 and 9) were also inactive indicating that the intact core chain of GlcNAcs is required.

Using neoglycolipids with different carbohydrate core structures, we have further confirmed the importance of distal monosaccharides in more extended chains. We have shown that both glycosidic linkages and types of monosaccharides are of importance for the interaction (Table IV). It seems that structural conditions required for optimal interactions of H. pylori with carbohydrate sequences on TLC plates are defined by both binding areas (actual epitopes) and carrier parts of the molecules. We have previously examined the role of GlcNAc in NeuAca3Galβ4GlcNAcβ-R for H. pylori binding (Johansson et al. 2005) and came to the conclusion that this monosaccharide is of importance for spatial orientation of the epitope. The hydrophobic layers of TLC plates mimic natural conditions of cell surfaces, and one may speculate that saccharide core chains as well as glycosylons could be of importance for the in vivo adhesion of H. pylori.

Because of the shortage and hydrophobic nature of chemical derivatives used in this work, the binding experiments were limited to TLC overlay tests. TLC binding assays are proper for work with small amounts of lipid substrates, both pure and in mixtures, and in a previous paper we have confirmed the reliability of TLC overlay assays in H. pylori studies comparing this approach with hemagglutination inhibition assay and another binding assay based on the use of carbohydrate epitopes in water milieu (Johansson et al. 2005). The TLC method is an excellent approach for the survey of activities; however, the results should be considered as semi-quantitative, and further investigation of selected molecules is necessary.

As discussed, the use of antibiotics is not recommended for the prevention of H. pylori diseases in large human populations because resistant strains are emerging (Van Der Wouden et al. 2000; Wu et al. 2005). However, anti-H. pylori therapy is rational and effective for patients with developed disease (Pilottato and Malfertheiner 2002; Ford et al. 2004; Bytzer 2005). Regarding potential anti-adhesion therapy, the best effects may be obtained using mixtures of molecules with different active epitopes including natural molecules and their analogs. Novel glycan libraries from chemoenzymatic synthesis, as presented here, as well as novel array technologies open a new era for studies of pathogen–host interactions and screening of pharmaceuticals against infections (Disney and Seeberger 2004; Stevens et al. 2006).

**Material and methods**

**Preparation of glycolipid derivatives**

Glcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer (Table II, No. 2). This structure was prepared from GlcAβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer (glucuronylneolactotetraosylceramide, 18, 18, Wako Pure Chemical Industries) (Table II, No. 1), by reduction of R-COOH to RCH₂OH. The glycolipid (100 µg) was dissolved in DMSO (250 µL, Fluka/Sigma-Aldrich, Sweden), mixed with CH₃(l 50 µL, Fluka, Steinheim, Germany), and sonicated at room temperature for 1 h, followed by freeze drying.
The produced methyl ester was dissolved in 300 μL of methanol and incubated at room temperature in the presence of 4 mg of NaBH₄ overnight. The sample was next evaporated, dissolved in 2 mL of C/M/H₂O (60:30:4.5), and desalted using a small column filled with Sephadex G-25 (Miller-Podraza et al. 2005). The yield of the derivatization was about 50%, as estimated by TLC chromatography using as a developing solvent C/M/0.25% KCl in H₂O, 50:40:10 (vol by vol). Finally, the material was separated by phase partition using the Folch’s system (Folch et al. 1957), C/M/H₂O (8:4:3), and Glcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer was recovered from the lower phase.

Amide forms prepared from GlcAβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer

Methyl Amide (Table II, No. 3). Hundred micrograms of the parent glycolipid was converted to methyl ester using CH₃I as described above and divided into two portions (samples 1 and 2). Each sample was dissolved in 500 μL of methanol, and mixed with 500 μL of 40% of methylamine (Fluka/Sigma-Aldrich, Sweden) in water. The sample was incubated at room temperature overnight and evaporated under nitrogen.

Ethyl and Benzyl Amides (Table II, Nos. 4 and 5). One hundred and forty micrograms of GlcAβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer was converted to the methyl ester form as described above and divided into two portions (samples 1 and 2). Each sample was dissolved in 500 μL of methanol, and the amidation was induced by adding 1 mL of ethylamine (Aldrich Chemical Company, Steinheim, Germany) or 100 μL of benzylamine (Fluka/Sigma-Aldrich, Sweden) in water. The samples were then incubated at room temperature overnight, evaporated under nitrogen, and purified on small (Pasteur pipette) silica gel 60 column. The material was recovered from the silica gel TLC chromatography as above. The material was collected and used for further analyses.

Synthesis of neoglycolipids

Neoglycolipids were synthesized by reductive amination using two kinds of aminated lipids: (a) Hexadecylamine (Aldrich Chemical Company, Milwaukee, USA), CH₃(CH₂)₁₂CH₂NH₂, HDA and (b) a branched palmiate-lysine-based conjugate, Pal-Lys(Pal)CONH(CH₂)₂-NH₂ (called later C42) obtained through custom synthesis (RAPP Polymere GmbH, Germany). The core structure of this lipid was made up of palmitic acid (Pal) and lysine (Lys). The conditions for coupling of HDA to free oligosaccharides were as described before (Miller-Podraza et al. 2004). The conditions for coupling of C42 to reducing sugars were in a typical experiment as follows. A free oligosaccharide (200–600 nmol in 300 μL of M/H₂O, 2:1) was mixed with C42 (20 mg/mL in C/M, 5:1, 280 μL) and NaBH₃CN (62 mg/mL in MeOH, 100 μL) (Fluka/ Sigma-Aldrich, Sweden), and the sample was kept at 50°C for 1–2 days. The progress of the reaction was monitored by TLC chromatography using a developing solvent C/M/0.25% KCl in H₂O, 50:40:10. The final yield of the products varied between 30% and 70%. The samples were then evaporated, redissolved in a small volume of C/M/H₂O, 60:30:5, and mixed with 10 volumes of C/M 3:1. Each sample was applied on a small (Pasteur pipette) silica gel 60 column. The material was eluted using solvents with increasing polarity: C/M, 2:1; CM, 1:1; C/M/H₂O, 60:35:8; and C/M/H₂O, 50:40:10. The eluates were evaporated, redissolved in small volumes of the respective solvents, and tested for the presence of neoglycolipids using the silica gel TLC chromatography as above. The material was collected and used for further analyses.

Synthesis of oligosaccharides

Galβ4GlcNAcβ6GlcNAc. GalNAcβ6GlcNAc was prepared by HF-reversion of N-acetylglucosamine (Defaye et al. 1989) and isolated by gel filtration chromatography on a column of Superdex 30 (5 × 95 cm). The disaccharide was then β-galactosylated by bovine milk β-galactosyltransferase (Calbiochem) (Breu et al. 1968). The title trisaccharide was isolated in pure form by chromatography on a column of graphitized carbon (Hypercarb, Thermo-Hypersil Ltd, UK) using for elution gradient 1 (see chromatographic methods below) and characterized by MALDI-TOF MS and 1H-NMR spectroscopy.

GlcNAcβ3Galβ4GlcNAcβ6GlcNAc. Galβ4GlcNAcβ6GlcNAc obtained as above was incubated with UDP-GlcNAc and human serum containing a β3-GlcNAc transferase essentially as described previously (Piller and Cartron 1983). The reaction mixture was desalted and deproteinized by passing through a mixed bed column of AG 1-X8 (Ac⁻) and AG 50W-X8 (H⁺) resins (200–400 mesh, Bio-Rad). The tetrasaccharide was finally isolated by chromatography on a column of graphitized carbon (Hypercarb) using gradient 2 (see chromatographic methods below) and characterized by MALDI-TOF MS and 1H-NMR spectroscopy.

GlcNAcβ3Galβ4GlcNAcβ6GlcNAc. GlcNAcβ6GlcNAc was prepared by HCl-reversion of N-acetylglucosamine (Blumberg et al. 1982) followed by β4-galactosylatation and β3-GlcNAc transferase reactions as above. The title tetrasaccharide was isolated in pure form by Hypercarb chromatography using gradient 1 and characterized by MALDI-TOF MS and 1H-NMR spectroscopy.
GlcNAcb3Galβ4GlcNAcb3Man. GlcNAcb3Man (Sigma) was β4-galactosylated by bovine milk β4-galactosyltransferase as described above. A crude oligosaccharide fraction was isolated by gel filtration chromatography on a column of Superdex Peptide and subjected to β3-N-acetylglucosaminylolation using human serum. The serum reaction mixture was desalted and deproteinized by passing through a mixed bed column of AG 1-X8 (Ac⁻) and AG 50W-X8 (H⁺) resins. The title tetrascaracide was isolated by Hypercarb chromatography with gradient 1 and characterized by MALDI-TOF MS and 1H-NMR spectroscopy.

GlcNAcb3Galβ4GlcNAcb6Gal. GlcNAcb6Gal (Sigma) was β4-galactosylated by bovine milk β4-galactosyltransferase as described above. A crude oligosaccharide fraction was isolated by gel filtration chromatography on a column of Superdex Peptide and subjected to human serum β3-GlcNAc transferase as above. The serum reaction mixture was desalted and deproteinized by passing through a mixed bed column of AG 1-X8 (Ac⁻) and AG 50W-X8 (H⁺) resins. The title tetrascaracide was isolated by Hypercarb chromatography with gradient 1 and characterized by MALDI-TOF MS and 1H-NMR spectroscopy.

GlcABβ3Galβ4GlcNAb3Galβ4Glc. Galβ4GlcNAbβ3Galβ4Glc (IsoSep, Lund, Sweden) was subjected to glucuronolysis using β-glucuronidase transglycosylation reaction essentially as described previously (Yasukochi et al. 1997). Excessive nitrophenol was removed by solid-phase extraction with BondE押C-18 columns (Varian), and the oligosaccharide products were isolated by gel filtration chromatography on a column of Superdex 30 (5 × 95 cm). The title tetrasaccharide was isolated in pure form by ion-exchange chromatography with ResourceQ and characterized by MALDI-TOF MS and 1H-NMR spectroscopy.

GlcAβ3Galβ4GlcNAcβ3Galβ4Glc. GlcAβ3Galβ4GlcNAcβ6GlcNAc obtained as above was subjected to β-glucuronolysis using β-glucuronidase transglycosylation reaction essentially as described previously. The excess nitrophenol was removed by solid-phase extraction with BondElut C-18 columns (Varian), and the oligosaccharide products were isolated by gel filtration chromatography on a column of Superdex 30 (5 × 95 cm). The title pentascaracide was isolated in pure form by ion-exchange chromatography with ResourceQ and characterized by MALDI-TOF MS and 1H-NMR spectroscopy.

GlcA(N-Me)β3Galβ4GlcNAcβ3Galβ4Glc. GlcAβ3Galβ4GlcNAcβ6GlcNAc obtained as described above was dissolved in 90% aqueous pyridine containing 3 x molar excess of methylamine, HBTU (Novabiochem) and diisopropylethylene (Fluka). The reaction was conducted at room temperature for 3 days, and the crude oligosaccharide fraction was isolated by gel filtration chromatography on Superdex Peptide. The methylated species were isolated by anion-exchange chromatography (neutral fraction), further purified by gel filtration chromatography, and characterized by MALDI-TOF MS and 1H-NMR spectroscopy.

GlcA(N-Me)β3Galβ4GlcNAcβ3Galβ4Glc. GlcAβ3Galβ4GlcNAcβ3Galβ4Glc was amidated with methylamine in a reaction containing HBTU and diisopropylethylene as above. The methylated species were isolated by anion-exchange chromatography (neutral fraction), further purified by gel filtration chromatography, and characterized by MALDI-TOF MS and 1H-NMR spectroscopy.

Chromatographic methods

Gel filtration chromatography was performed with Superdex™ Peptide HR 10/30 (10–300 mm) (Amersham Pharmacia Biotech, Sweden) with 50 mM NH₄HCO₃ as eluent, at a flow rate of 1 mL/min or with Superdex 30 (5 × 95 cm) (Amersham Pharmacia Biotech, Sweden), with 50 mM NH₄HCO₃ as eluent, at a flow rate of 5 mL/min. All experiments were monitored at 214 nm. Fractions of 10 mL were collected in Superdex 30 runs, while product peaks from the Superdex peptide column were collected manually.

Chromatography on graphitized carbon was performed using a Hypercarb column (5 μm, 250 × 4.6 mm; Thermo-Hypersil Ltd, UK). Ammonia was used in the solvents to catalyze mutarotation since separations of the anomeric forms of oligosaccharides were often observed. Two different gradients were employed. Gradient 1: the column was equilibrated with 10 mM NH₃, and elution was performed with a linear gradient of acetonitrile (0–40% over 60 min). Gradient 2: the column was equilibrated with 10 mM NH₃, and elution was performed with a linear gradient of acetonitrile (8–40% over 80 min). Absorbance at 214 nm was recorded.

Anion-exchange chromatography was performed on a column of ResourceQ Q (Amersham Pharmacia Biotech AB, Sweden). The column was eluted with water for 4 min, and then with a linear gradient of 0–50 mM NaCl over 8 min, followed by a steeper linear gradient of 50 –500 mM NaCl over 8 min. Absorbance at 214 nm was recorded.

Mass spectrometry

MALDI-TOF mass spectra of oligosaccharides were recorded on a Voyager-DE™ STR BioSpectrometry™ (PerSeptive Biosystems) time-of-flight instrument. Samples were analyzed in either positive-ion delayed extraction mode using 2,5-dihydroxybenzoic acid (DHB) (Alrich) matrix (10 μg/mL in H₂O) or in negative-ion linear delayed extraction mode using 2,4,6-trihydroxyacetophenone (Fluka) (3 μg/mL in acetonitrile/20 mM aqueous diammonium citrate, 1:1, by volume). Negative-ion FAB MS was performed as described before (Miller-Podraza et al. 2004). Helicobacter pylori strains

The following strains were used: CCUG 17874, CCUG 17875, and CCUG 26695 (from Culture Collection Göteborg University, Sweden); the BabA1A2-knockout mutant derivative of CCUG 17875 (from Dr. Thomas Borén, Umeå University, Sweden); and the strain S-032 (obtained from a patient with duodenal ulcer at the Örebro Medical Center, Örebro, Sweden). Growth of H. pylori cells was as previously described (Miller-Podraza et al. 1996).

TLC methods

Chromatogram binding assays using 35S-labeled H. pylori cells were performed as described before (Miller-Podraza et al. 2004, 2005; Johansson et al. 2005). silica gel HPTLC plates were from Merck, Germany (silica gel 60 on aluminum sheets).
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Conflict of interest statement
H.M.-P., J.H., J.N., and K.-A.K. are inventors of patent applications related to the article.

Abbreviations
C, chloroform; DMSO, dimethyl sulfoxide; Et, ethyl; FAB-MS, fast atom bombardment mass spectrometry; Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcA, glucuronic acid, GlcN, glucosamine; GlcNAc, N-acetylgulcosamine; M, methanol; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; Me, methyl; Neu5Ac, N-acetylmuramic acid; PBS, phosphate buffer saline; TLC, thin-layer chromatography. Solvent mixtures were by volume, unless otherwise stated. The carbohydrate and glycolipid nomenclature is according to recommendations of IUPAC-IUB Joint Commission on Biochemical Nomenclature, 1998, Eur J Biochem., 257, 293.

References


