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(71) Hakija - Sökande - Applicant

1 • **Carbion Oy,** Viikinkaari 9, 00710 Helsinki, SUOMI - FINLAND, (FI)

(72) Keksijä - Uppfinnare - Inventor

1 • **Miller-Podraza, Halina,** Västra Frölunda, SVERIGE, (SE)
2 • **Teneberg, Susann,** Hindås, SVERIGE, (SE)
3 • **Ångström, Jonas,** Göteborg, SVERIGE, (SE)
4 • **Karlsson, Karl-Anders,** Göteborg, SVERIGE, (SE)

(74) Asiamies - Ombud - Agent

Oy Jalo Ant-Wuorinen Ab, Iso Roobertinkatu 4 - 6 A, 00120 Helsinki

(54) Keksinnön nimitys - Uppfinningens benämning - Title of the invention

Uudet helicobacter pylori reseptorit ja niiden käyttö

Nya receptorer för helicobacter pylori och deras användning

Novel receptors for *Helicobacter pylori* and use thereof.

FIELD OF THE INVENTION

The present invention relates to a substance or a receptor binding to *Helicobacter pylori*, and the use thereof in, e.g., pharmaceutical and nutritional compositions and methods for treatment. The invention is also directed to the use of the receptor for diagnostics of *Helicobacter pylori*.

BACKGROUND OF THE INVENTION

Helicobacter pylori has been implicated in several diseases of the gastrointestinal tract including chronic gastritis, non-steroidal anti-inflammatory drug (NSAID) associated gastı disease, duodenal and gastric ulcers, gastric MALT lymphoma, and gastric adenocarcinom (Axon, 1993; Blaser, 1992; DeCross and Marshall, 1993; Dooley, 1993; Dunn *et al.*, 1997; Lin *et al.*, 1993; Nomura and Stemmermann, 1993; Parsonnet *et al.* 1994; Sung *et al.*, 200(Wotherspoon *et al.*, 1993). Totally or partially non-gastrointestinal diseases include sudder infant death syndrome (Kerr *et al.*, 2000 and US 6,083,756), autommune diseases like autoimmune gastritis and pernicious anaemia (Appelmelk *et al.*, 1998; Chmiela *et al*, 1998 Clayes *et al.*, 1998; Jassel *et al.*, 1999; Steininger *et al.*, 1998) and some skin diseases (Rebora *et al.*, 1995), pancreatic disease (Correa *et al.*, 1990), liver diseases including adenocarcinoma (Nilsson *et al.*, 2000; Avenaud *et al.*, 2000) and heart diseases such as atherosclerosis (Farsak *et al.*, 2000). Multiple diseases caused or associated with *Helicobacter pylori* has been reviewed (Pakodi *et al.*, 2000). Of prime interest with respect to bacterial colonization and infection is the mechanism(s) by which this bacterium adheres to the epithelial cell surfaces of the gastric mucosa.

Glycoconjugates, both lipid- and protein-based, have been reported to serve as receptors fo the binding of this microorganism as, e.g., sialylated glycoconjugates (Evans *et al.*, 1988), sulfatide and GM3 (Saitoh *et al.*, 1991), Le^b determinants (Borén *et al.*, 1993), polyglycosylceramides (Miller-Podraza *et al.*, 1996; 1997a), lactosylceramide (Ångström *e al.*, 1998) and gangliotetraosylceramide (Lingwood *et al.*, 1992; Ångström *et al.*, 1998). Other potential receptors for *Helicobacter pylori* include the polysaccharide heparan sulphate (Ascensio *et al..*, 1993) as well as the phospholipid phosphatidylethanolamine (Lingwood *et al.*, 1992).

US patents of Zopf *et al.*: 5,883,079 (March 1999), 5,753,630 (May 1998) and 5,514,660 (May, 1996) describe Neu5Acα3Gal- containing compounds as inhibitors of the *H. pylori*

adhesion. The sialyl-lactose molecule inhibits *Helicobacter pylori* binding to human gastrointestinal cell lines (Simon *et al.*, 1999) and is also effective in a rhesus monkey animal model of the infection (Mysore *et al.*, 2000). The compound is in clinical trials.

US patent Krivan *et al.* 5,446,681 (November 1995) describes bacterium receptor antibiot conjugates comprising an asialo ganglioside coupled to a penicillin antibiotic. Especially i claimed the treatment of *Helicobacter pylori* with the amoxicillin-asialo-GM1 conjugate. The oligosaccharide sequences/glycolipids described by the invention does not belong to ganglioseries of glycolipids.

US patents of Krivan *et al.*: 5,386,027 (January 1995) and 5,217,715 (June 1993) describe use of oligosaccharide sequences or glycolipids to inhibit several pathogenic bacteria, however the current binding specificity is not included and *Helicobacter pylori* is not amo the bacteria studied or claimed.

The saccharide sequence GlcNAcβ3Gal has been described as a receptor for *Streptococcu* (Andersson *et al.*, 1986). Some bacteria may have overlapping binding specificities, but it not possible to predict the bindings of even closely related bacterial adhesins. In case of *Helicobacter pylori* the saccharide binding molecules, except the Lewis b binding protein are not known.

SUMMARY OF THE INVENTION

The present invention relates to a substance or a receptor binding to *Helicobacter pylori* comprising the oligosaccharide sequence

$$[Gal(NAc)_r/Glc(NAc)_r\,\alpha 3/\beta 3]_s\,[Gal\beta 4GlcNAc\beta 3]_t\,Gal\beta 4Glc(NAc)_u$$

wherein r, s, t, and u are each independently 0 or 1, so that when both t = 0 and u = 0, then the oligosaccharide sequence is linked to a polyvalent carrier or present as a free oligosaccharide in high concentration, and analogs or derivatives of said oligosaccharide sequences having binding activity to *Helicobacter pylori*.

Among the objects of the invention are the use of the *Helicobacter pylori* binding oligosaccharide sequences of the invention as a medicament, and the use of the same for the manufacture of a pharmaceutical composition, particularly for the treatment of any condition due to the presence of *Helicobacter pylori*.

The present invention also relates to the methods for the treatment of conditions due to the presence of *Helicobacter pylori*. The invention is also directed to the use of the receptor(s) according to the invention as *Helicobacter pylori* binding or inhibiting substance, e.g., for diagnostics of *Helicobacter pylori*.

Another object of the invention is to provide pharmaceutical compositions and nutritional additives or compositions containing *Helicobacter pylori* binding substance(s) according to the invention.

Other objects of the invention are the use of the above-mentioned *Helicobacter pylori* binding substances for the identification of bacterial adhesin, the typing of *Helicobacter pylori*, and the *Helicobacter pylori* binding assays.

Yet another object of the invention is the use of the above-mentioned *Helicobacter pylori* binding substances for the production of a vaccine against *Helicobacter pylori*.

DETAILED DESCRIPTION OF THE INVENTION

The present invention describes a family of specific oligosaccharide sequences binding to *Helicobacter pylori*. Numerous naturally occuring glycosphingolipids were screened by th layer overlay assay (Table 2). The structures of the glycosphingolipids used were characterized by proton NMR and mass spectrometric experiments. Molecular modeling w used to compare three dimensional structures of the substances binding to *Helicobacter pylori*.

The novel binding specificity was demonstrated by comparing four pentasaccharide glycolipids. It was found that the exchange of the non-reducing end terminal saccharide in GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer by either GalNAcβ3 (short name x2 GSL), GalNAcα3 or Galα3 (B5) all resulted in binding of *Helicobacter pylori*, despite difference in anomerity, absence or presence of an acetamido moiety and axial/equatorial position of the 4-OH. The specificity also includes structures with weaker binding to *Helicobacter pylori*: a shorter form Galβ4GlcNAcβ3Galβ4GlcβCer and β4-elongated forms of the glycolipid with terminal N-acetylglucosamine: Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer and NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer. In contrast to previously know sialic acid depending specificities (Evans *et al.*, 1988; Miller-Podraza *et al.*, 1996; 1997a),

the N-glycolyl neuraminic acid of the last mentioned glycosphingolipid could be released without effect to the binding of *Helicobacter pylori.*

The binding to GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer was very reproducible, though general saccharide bindings of *Helicobacter pylori* suffer from phase variations of the bacterium, and high affinity of the binding was visible in the overlay assay at low picomol amounts of the glycolipid.

The length of the binding epitope was indicated by experiments showing that GlcNAcβ3Galβ4GlcβCer, Galβ4GlcNβ3Galβ4GlcβCer, and Galα3Galβ4GlcNβ3Galβ4GlcβCer (a shortened form and N-deacetylated forms of the active species) were not binding to *Helicobacter pylori.* The data reveal that the inner GlcNAc residue participites in binding but does not create strong enough binding alone. Tl binding epitope was considered to be the terminal trisaccharide in the pentasaccharide epitopes discussed above. When only two of the residues are present as in Galβ4GlcNAcβ3Galβ4GlcβCer, binding is weaker, and in the hexasaccharide glycolipid Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer the terminal Galβ4 inhibits the binding, explaining the weaker activity. A heptasaccharide glycolipid having Galα3 on the less acti hexasaccharide glycolipid strucure, Galα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβC had higher activity also indicating that terminal trisaccharide epitopes are required for goo binding activity.

Specificity of the binding was characterized by assaying isomers and modified forms of th active species. Elongated forms of Galβ4GlcNAcβ3Galβ4GlcβCer having the following modifications on the terminal Gal : Fucα2 (short name H5-2), Fucα2 and Gal/GalNAcα3 (B6-2, A6-2), Neu5Acα3 or Neu5Acα6 (sialylparaglobosides), or Galα4 (P_1) were inactiv in the binding assays with *Helicobacter pylori.* The binding was also destroyed by having a β6-linked branch inner galactose, shown by the structure Galβ4GlcNAcβ3(Galβ4GlcNAcβ6)Galβ4GlcβCer. The branch has been shown to change the presentation of the Galβ4GlcNAcβ3-epitope and the disaccharide binding site is probably sterically hindered (Teneberg *et al.*, 1994). (However the result shows that the inner galactose residue to which the disaccharide- or trisaccharide binding epitopes are bound by β3-linkage may also contribute to binding.) Furthermore Neu5Acα3GalNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer (an elongated form of the binding active x_2-glycosphingolipid) or GalNAcβ3Galα3Galβ4GlcNAcβ3Galβ4GlcβCer (elongate B5 GSL) did not appear to bind to *Helicobacter pylori.*

Molecular modeling was used to compare the active binding structures and inactive specie Calculated minimum energy conformers of the four pentasaccharide glycosphingolipids (Galβ4GlcNAcβ3Galβ4GlcβCer with elongation by either GlcNAcβ3, GalNAcβ3, GalNAcα3 or Galα3) show that conformations of the compounds may closely mimic each other. The conformations of the inactive glycolipids were different. Despite the fact that th terminal saccharides differ also in their anomeric linkage (two alfa- and two beta-linked), molecular modeling revealed that the minimum energy structures are topographically very similar. The differences of the terminal structures are that Galα3 lacks an acetamido group present in the other three, Gal and GalNAc have the 4-OH in the axial position and GlcNA in the equivatorial position, and the ring planes of the alfa anomeric terminal are raised slightly above the corresponding plane in the beta anomeric ones. The elongation of the terminal is allowed on position 4 of GlcNAc, also indicating that the 4-OH is not very important for the binding, though the Galβ4 elongation causes steric interference. In conclusion, neither the position of 4-OH nor the absence/presence of an acetamido group nor the anomeric structure of terminal monosaccharide residue appear to be crucial for binding to occur, since all the four pentasaccharide glycolipids have similar affinities for th *Helicobacter pylori* adhesin.

In the light of these rules of binding four other terminal monosaccharides in the binding substance may also provide trisaccharide binding epitopes: Galβ3Galβ4GlcNAc, GlcNAcα3Galβ4GlcNAc, Glcβ3Galβ4GlcNAc and Glcα3Galβ4GlcNAc. These are analogous to the sequences studied only having differences in the anomeric, 4-epimeric or on C2 NAc/OH structures. The first one is present on a glycolipid from human erythrocyte while the last three are not known from human tissues so far, but could rather represent analogues of the natural receptor.

The binding epitope was shown to include the terminal trisaccharide element of active pentasaccharide glycolipids, and at least in larger repetitive *N*-acetyllactosamines the epito may be also in the middle of the saccharide chain. The inventors realize that the binding epitopes can be presented in numerous ways on natural or biosynthetically produced glycoconjugates and oligosaccharides such as O-linked or N-linked glycans of glycoprotein and on poly-N-acetyllactosamine oligosaccharides. Chemical and enzymatic synthesis methods, especially in the carbohydrate field, allow production of almost an infinite numbe of derivatives and analogs. The size of the binding epitope allows some modifications, as exemplified on the C1, C2 and C4 of the terminal monosaccharide, by loss of the non-reducing terminal monosaccharide or elongation from C4 of terminal GlcNAc of GlcNAcβ3Galβ4GlcNAc, e.g., the position C4 of GlcNAcβ3 can be linked to an oligosaccharide chain by a glycosidic bond. When the oligosaccharide sequence is

GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc, position C4 of terminal GlcNAcβ3 can be linked tc Galβ1- or an oligosaccharide chain by a glycosidic bond. Especially the C2 and C4 positic of the non-reducing terminal monosaccharide residue in the trisaccharide epitope and the reducing ends of the epitopes can be used for making derivatives and oligomeric or polymeric conjugates having binding activity to *Helicobacter pylori*. The C6 positions of t monosaccharide residues can also be used to produce derivatives and analogs, especially t C6 position of the non-reducing terminal residue in trisaccharide sequence and the reducir end residue of di- and trisaccharide binding substances are preferred.

Also non-carbohydrate mimetics or analogs of the compounds can be made. The structure the active carbohydrate epitope can be used to design compounds which have the configuration of chemical groups participating binding to the counter receptor (adhesin) o the saccharide on *Helicobacter pylori*. The invention describes the acetamido group at the reducing end GlcNAc of the di- and trisaccharide epitopes as a part of the binding epitope. The binding epitope did not tolerate modification by neutral monosaccharide residues on C or C4, or acidic Neu5Ac on C3 or C6 of the Gal in Galβ4GlcNAc, or by acidic or neutral monosaccharide on C3 of the trisaccharide epitopes.

Steric hindrance by the lipid part or the proximity of the silica surface probably limits the measurement of the epitope GlcNAcβ3Galβ4Glc in current TLC-assay. Using the assay activity of this sequence could not be obtained in recent study of toxin A from *Clostridium difficile*, which specifically recognizes the same four trisaccharide epitopes described here for *Helicobacter pylori* (Teneberg *et al.*, 1996). However, the binding of Galα3Galβ4Glc t the toxin A was demonstrated by others using a large polymeric spacer modified conjugate of the saccharide (Castagliuolo *et al.*, 1996). Also considering the contribution of the terminal monosaccharide to the binding indicates that Glc could be allowed at the reducing end of the epitope; in the non-active N-deacetylated form the positive charge of the free amine group is probably more destructive to the binding than the presence of the hydroxyl group. The trisaccharide epitopes with Glc at reducing end are considered as effective analogs of the *Helicobacter pylori* binding substance when present in oligovalent or more preferably in polyvalent form. One embodiment of the present invention is the saccharides with Glc at reducing end, which are used as free reducing saccharides with high concentration, preferably in the range 1 – 100 g/l, more preferably 1 – 20 g/l. It is realized that these saccharides may have minor activity in the concentration range 0,1 – 1 g/l.

In the following the *Helicobacter pylori* binding sequence is described as an oligosacchari sequence. The oligosaccharide sequence defined here can be a part of a natural or synthetic glycoconjugate or a free oligosaccharide or a part of a free oligosaccharide. Such

oligosaccharide sequences can be bonded to various monosaccharides or oligosaccharides or polysaccharides on polysaccharide chains, for example, if the saccharide sequence is expressed as part of a bacterial polysaccharide. Moreover, numerous natural modifications of monosaccharides are known as exemplified by O-acetyl or sulphated derivative of oligosaccharide sequences. The *Helicobacter pylori* binding substance defined here can comprise the oligosaccharide sequence of the invention as a part of a natural or synthetic glycoconjugate or a corresponding free oligosaccharide or a part of a free oligosaccharide. The *Helicobacter pylori* binding substance can also comprise a mix of the oligosaccharide sequences of the invention.

Several derivations of the receptor oligosaccharide sequence reduced the binding below the limit of detection in current assay, showing the specificity of the recognition. The binding data shows that if the said oligosaccharide sequences have GalNAcβ3 linked to Galα3Galβ4GlcNAc (substituted sequence: GalNAcβ3Galα3Galβ4GlcNAc), or Neu5Acα3 linked to GalNAcβ3Galβ4GlcNAc (substituted sequence: Neu5Acα3GalNAcβ3Galβ4GlcNAc) the compounds are not active. When the said oligosaccharide sequence is Galβ4GlcNAc, it is not α4-galactosylated (sequence is not Galα4Galβ4GlcNAc), α3-, or α6-sialylated (sequence is not Neu5Acα3/6Galβ4GlcNAc), α2- or α3-fucosylated [said oligosaccharide sequence is not Fucα2Galβ4GlcNAc or Galβ4(Fucα3)GlcNAc or Fucα2Galβ4(Fucα3)GlcNAc, α3-fucosylation referring to fucosylation of GlcNAc residues of lactosamine forming Lewis x, Galβ4(Fucα3)GlcNAc]. Saccharides having structures where Galβ3 is linked to GlcNAcβ3 (such as Galβ3GlcNAcβ3Galβ4GlcNAc/Glc) have different conformations in comparision to the substances according to the invention and their binding specificies have been studied separately. The substances according to the invention may be part of a saccharide chain or a glycoconjugate or a mixture of glycocompounds containing other known *Helicobacter* binding epitopes, with different saccharide sequences and conformations, such as Lewis b (Fucα2Galβ3(Fucα4)GlcNAc) or Neu5Acα3Galβ4Glc/GlcNAc. Using several binding substances together may be beneficial for therapy.

The oligosaccharide sequences of the invention can be synthesized enzymatically by glycosyltransferases, or by transglycosylation catalyzed by glycosidase or transglycosidase enzymes (Ernst *et al.*, 2000). Specifities of these enzymes and the use of co-factors can be engineered. Specific modified enzymes can be used to obtain more effective synthesis, for example, glycosynthase is modified to do transglycosylation only. Organic synthesis of the saccharides and the conjugates of the invention or compounds similar to these are known (Ernst *et al.*, 2000). Saccharide materials can be isolated from natural sources and modified chemically or enzymatically into the compounds according to the invention. Natural

oligosaccharides can be isolated from milks produced by various ruminants. Transgenic organisms, such as cows or microbes, expressing glycosylating enzymes can be used for tl production of saccharides.

One embodiment of the present invention is a substance or a receptor binding to *Helicobacter pylori* comprising the oligosaccharide sequence

$[Gal(NAc)_r/Glc(NAc)_r\ \alpha 3/\beta 3]_s\ [Gal\beta 4GlcNAc\beta 3]_t\ Gal\beta 4Glc(NAc)_u$

wherein r, s, t, and u are each independently 0 or 1, so that when both t = 0 and u = 0, then the oligosaccharide sequence is linked to a polyvalent carrier or present as a free oligosaccharide in high concentration, and analogs or derivatives of said oligosaccharide sequences having binding activity to *Helicobacter pylori.*

The following oligosaccharide sequences are among the preferable *Helicobacter pylori* binding substances of the invention

Galβ4GlcNAc,

GalNAcα3Galβ4GlcNAc, GalNAcβ3Galβ4GlcNAc, GlcNAcα3Galβ4GlcNAc, GlcNAcβ3Galβ4GlcNAc, Galα3Galβ4GlcNAc, Galβ3Galβ4GlcNAc, Glcα3Galβ4GlcNA Glcβ3Galβ4GlcNAc,

Galβ4GlcNAcβ3Galβ4GlcNAc, Galβ4GlcNAcβ3Galβ4Glc,

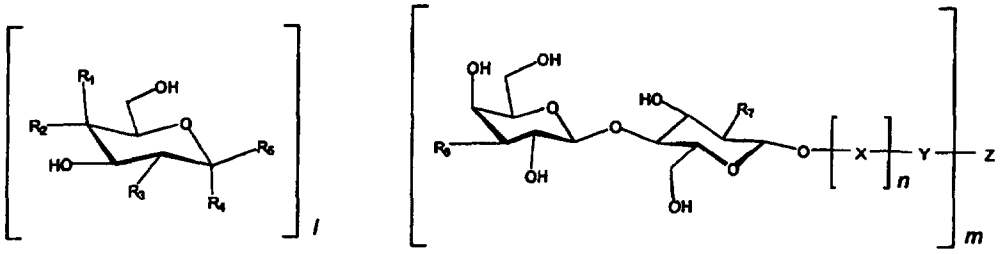
GalNAcα3Galβ4GlcNAcβ3Galβ4Glc, GalNAcβ3Galβ4GlcNAcβ3Galβ4Glc, GlcNAcα3Galβ4GlcNAcβ3Galβ4Glc, GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc, Galα3Galβ4GlcNAcβ3Galβ4Glc, Galβ3Galβ4GlcNAcβ3Galβ4Glc, Glcα3Galβ4GlcNAcβ3Galβ4Glc, Glcβ3Galβ4GlcNAcβ3Galβ4Glc,

Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc, and reducing-end polyvalent conjugates thereof,

as well as GalNAcα3Galβ4Glc, GalNAcβ3Galβ4Glc, GlcNAcα3Galβ4Glc, GlcNAcβ3Galβ4Glc, Galα3Galβ4Glc, Galβ3Galβ4Glc, Glcα3Galβ4Glc, and Glcβ3Galβ4Glc.

Another embodiment of the invention is described in Formula 1.

Formula 1:



Among the preferable *Helicobacter pylori* binding substances or mixtures of the substances of the invention are the oligosaccharide structures according to Formula 1, wherein integers l, m, and n have values $m \geq 1$, l and n are independently 0 or 1, and wherein R_1 is H and R_2 is OH or R_1 is OH and R_2 is H or R_1 is H and R_2 is a monosaccharidyl- or oligosaccharidyl- group preferably a beta glycosidically linked galactosyl group, R_3 is independently -OH or acetamido ($-NHCOCH_3$) or an acetamido analogous group. R_7 is acetamido ($-NHCOCH_3$) or an acetamido analogous group. When l = 1, R_4 is -H and R_5 is oxygen linked to bond R_6 and forms a beta anomeric glycosidic linkage to saccharide B or R_5 is -H and R_4 is oxygen linked to bond R_6 and forms an alpha anomeric glycosidic linkage to saccharide B, when l = 0 R_6 is -OH linked to B. X is monosaccharide or oligosaccharide residue, preferably X is lactosyl- , galactosyl- , poly-N-acetyl-lactosaminyl, or part of an O-glycan or an N-glycan oligosaccharide sequence; Y is a spacer group or a terminal conjugate such as a ceramide lipid moiety or a linkage to Z. Z is an oligovalent or a polyvalent carrier. The binding substance may also be an analog or derivative of said substance according to Formula 1 having binding activity with regard to *Helicobacter pylori*, e.g., the oxygen linkage (-O-) between position C1 of the B saccharide and saccharide residue X or spacer group Y can be replaced by carbon (-C-), nitrogen (-N-) or sulphur (-S-) linkage.

The bacterium binding substances are preferably represented in clustered form such as by glycolipids on cell membranes, micelles, liposomes, or on solid phases such as TCL-plates used in the assays. The clustered representation with correct spacing creates high affinity binding.

According to the invention it is also possible to use the *Helicobacter pylori* binding epitopes or naturally occurring, or a synthetically produced analogue or derivative thereof having a similar or better binding activity with regard to *Helicobacter pylori*. It is also possible to use a substance containing the bacterium binding substance such as a receptor active ganglioside described in the invention or an analogue or derivative thereof having a similar or better binding activity with regard to *Helicobacter pylori*. The bacterium binding substance may be a glycosidically linked terminal epitope of an oligosaccharide chain. Alternatively the bacterium binding epitope may be a branch of an oligosaccharide chain, preferably a polylactosamine chain.

The substance according to the invention may be conjugated to an antibiotic substance, preferably a penicillin type antibiotic. The substance according to the invention targets the antibiotic to *Helicobacter pylori*. Such conjugate is beneficial in treatment because a lower amount of antibiotic is needed for treatment or therapy against *Helicobacter pylori*, which leads to lower side effect of the antibiotic. The antibiotic part of the conjugate is aimed at killing or weaken the bacteria, but the conjugate may also have an antiadhesive effect as described below.

The bacterium binding substances, preferably in oligovalent or clustered form, can be used to treat a disease or condition caused by the presence of the *Helicobacter pylori*. This is done by using the substances of the invention for anti-adhesion, i.e. to inhibit the binding of *Helicobacter pylori* to the receptor epitopes of the target cells or tissues. When the substance or pharmaceutical composition according to the invention is administered it will compete with receptor glycoconjugates on the target cells for the binding of the bacteria. Some or all of the bacteria will then be bound to the substance according to the invention instead of the receptor on the target cells or tissues. The bacteria bound to the substances according to the invention are then removed from the patient (for example by the fluid flow in the gastrointestinal tract), resulting in reduced effects of the bacteria on the health of the patient. Preferably the substance used is a soluble composition comprising the substances of the invention. The substance according to the invention can be attached to a carrier substance which is preferably not a protein. When using a carrier molecule several molecules of the substance according to the invention can be attached to one carrier and inhibitory efficiency is improved.

The target cells are primarily epithelial cells of the target tissue, especially the gastrointestinal tract, other potential target tissues are for example liver and

pancreas. Glycosylation of the target tissue may change because of infection by a pathogen (Karlsson *et al.*, 2000). Target cells may also be malignant, transformed or cancer/tumour cells in the target tissue. Transformed cells and tissues express altered types of glycosylation and may provide receptors to bacteria. Binding of lectins or saccharides (carbohydrate-carbohydrate interaction) to saccharides on glycoprotein or glycolipid receptors can activate cells, in case of cancer/malignant cells this may be lead to growth or metastasis of the cancer. Several of the oligosaccharide epitopes of the invention, such as GlcNAcβ3Galβ4GlcNAc (Hu, J. *et al.*, 1994), Galα3Galβ4GlcNAc (Castronovo *et al.*, 1989), and neutral and sialylated polylactosamines from malignant cells (Stroud *et al.*, 1996), have been reported to be cancer-associated or cancer antigens. Oligosaccharide chains containing substances according to invention have also been described from lymphocytes (Vivier *et al.*, 1993). *Helicobacter pylori* is associated with gastric lymphoma. The substances according to the invention can be used to prevent binding of *Helicobacter pylori* to premalignant or malignant cells and activation of cancer development or metastasis. Inhibition of the binding may cure gastric cancer, especially lymphoma.

Target cells also includes blood cells, especially leukocytes. It is known that *Helicobacter pylori* strains associated with peptic ulcer, as the strain mainly used here, stimulates an inflammatory response from granulocytes, even when the bacteria are nonopsonized (Rautelin *et al.*, 1994a,b). The initial event in the phagocytosis of the bacterium most likely involves specific lectin-like interactions resulting in the agglutination of the granulocytes (Ofek and Sharon, 1988). Subsequent to the phagocytotic event oxidative burst reactions occur which may be of consequence for the pathogenesis of *Helicobacter pylori*-associated diseases (Babior, 1978). Several sialylated and non-acid glycosphingolipids having repeating N-acetyllactosamine units have been isolated and characterized from granulocytes (Fukuda *et al.*, 1985; Stroud *et al.*, 1996) and may thus act as potential receptors for *Helicobacter pylori* on the white blood cell surface. Furthermore, also the X_2 glycosphingolipid has been isolated from the same source (Teneberg, S., unpublished). The present invention confirms the presence of receptor saccharides on human erythrocytes and granulocytes which can be recognized by an N-acetyllactosamine specific lectin and by a monoclonal antibody (X_2, GalNAcβ3Galβ4GlcNAc-). The substances according to the invention can be useful to inhibit the binding of leukocytes to *Helicobacter pylori* and in prevention of the oxidative burst and/or inflammation following the activation of leukocytes.

It is known that *Helicobacter pylori* can bind several kinds of oligosaccharide sequences. Some of the binding by specific strains may represent more symbiotic interactions which do not lead to cancer or severe conditions. The present data about binding to cancer-type saccharide epitopes indicates that the substance according to the invention can prevent more pathologic interactions, in doing this it may leave some of the less pathogenic *Helicobacter pylori* bacteria/strains binding to other receptor structures. Therefore total removal of the bacteria may not be necessary for the prevention of the diseases related to *Helicobacter pylori*. The less pathogenic bacteria may even have a probiotic effect in the prevention of more pathogenic strains of *Helicobacter pylori*.

It is also realized that *Helicobacter pylori* contains large polylactosamine oligosaccharides on its surface which at least in some strains contains non-fucosylated epitopes which can be bound by the bacterium as described by the invention. The substance according to the invention can also prevent the binding between *Helicobacter pylori* bacteria and that way inhibit bacteria for example in process of colonization.

According to the invention it is possible to incorporate the substance according to the invention, optionally with a carrier, in a pharmaceutical composition, which is suitable for the treatment of a condition due to the presence of *Helicobacter pylori* in a patient or to use the substance according to the invention in a method for treatment of such conditions. Examples of conditions treatable according to the invention are chronic superficial gastritis, gastric ulcer, duodenal ulcer, non-Hodgkin lymphoma in human stomach, gastric adenocarcinoma, and certain pancreatic, skin, liver, or heart diseases, sudden infant death syndrome, autoimmune diseases including autoimmune gastritis and pernicious anaemia and non-steroid anti-inflammatory drug (NSAID) related gastric disease, all, at least partially, caused by the *Helicobacter pylori* infection.

The pharmaceutical composition according to the invention may also comprise other substances, such as an inert vehicle, or pharmaceutically acceptable carriers, preservatives etc, which are well known to persons skilled in the art. The substance according to invention can be administered together with other drugs such as antibiotics used against *Helicobacter pylori*.

The substance or pharmaceutical composition according to the invention may be administered in any suitable way, although an oral administration is preferred.

The term "treatment" used herein relates both to treatment in order to cure or alleviate a disease or a condition, and to treatment in order to prevent the development of a disease or a condition. The treatment may be either performed in a acute or in a chronic way.

The term "patient", as used herein, relates to any human or non-human mammal in need of treatment according to the invention.

It is also possible to use the substance according to the invention to identify one or more adhesins by screening for proteins or carbohydrates (by carbohydrate-carbohydrate interactions) that bind to the substance according to the invention. The carbohydrate binding protein may be a lectin or a carbohydrate binding enzyme. The screening can be done for example by affinity chromatography or affinity cross linking methods (Ilver *et al.*, 1998).

Furthermore, it is possible to use substances specifically binding or inactivating the substances of the invention present on human tissues and thus prevent the binding of *Helicobacter pylori*. Examples of such substances include plant lectins such as *Erythrina cristagalli* and *Erythrina corallodendron* (Teneberg *et al.*, 1994). When used in humans, the binding substance should be suitable for such use such as a humanized antibody or a recombinant glycosidase of human origin which is non-immunogenic and capable of cleaving the terminal monosaccharide residue/residues from the substances of the invention. However, in the gastrointestinal tract, many naturally occuring lectins and glycosidases originating for example from food are tolerated.

Furthermore, it is possible to use the substance according to the invention as part of a nutritional composition including food- and feedstuff. It is preferred to use the substance according to invention as a part of so called functional or functionalized food. The said functional food has a positive effect on the person's or animal's health by inhibiting or preventing the binding of *Helicobacter pylori* to target cells or tissues. The substance according to the invention can be a part of a defined food or functional food composition. The functional food can contain other acceptable food ingredients accepted by authorities such as Food and Drug Administration in the USA. The substance according to invention can also be used as a nutritional additive, preferably as a food or a beverage additive to produce a functional food or a functional beverage. The food or food additive can also be produced by having

,e.g., a domestic animal such as a cow or other animal produce the substance according to invention in larger amounts naturally in its milk. This can be accomplished by having the animal overexpress suitable glycosyltransferases in its milk. A specific strain or species of a domestic animal can be chosen and bred for larger production of the substance according to the invention. The substance according to invention, and especially the substance according to invention for a nutritional composition or nutritional additive can also be produced by a micro-organisms such as a bacteria or a yeast.

It is especially useful to have the substance according to the invention as part of a food for an infant, preferably as a part of an infant formula. Many infants are fed by special formulas in replacement of natural human milk. The formulas may lack the special lactose based oligosaccharides of human milk, especially the elongated ones such as lacto-N-neotetraose, Galβ4GlcNAcβ3Galβ4Glc, and its derivatives. The lacto-N-neotetraose and para-lacto-N-neohexaose (Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc) as well as Galβ3Galβ4Glc are known from human milk and can therefore be considered as safe additives or ingredients in an infant food. *Helicobacter pylori* is especially infective with regard to infants or young children, and considering the diseases it may later cause it is reasonable to prevent the infection. *Helicobacter pylori* is also known to cause sudden infant death syndrome, but the strong antiobiotic treatments used to eradicate the bacterium may be especially unsuitable for young children or infants.

Preferred concentrations for human milk oligosaccharides in functional food to be consumed (for example, in reconstituted infant formula) are similar to those present in natural human milk. It is noted that natural human milk contains numerous free oligosaccharides and glycoconjugates (which may be polyvalent) comprising the oligosaccharide sequence(s) described by the invention, wherefore it is possible to use even higher than natural concentrations of single molecules to get stronger inhibitory effect against *Helicobacter pylori* without harmful side effects. Natural human milk contains lacto-N-neotetraose at least in range about 10 – 210 mg/l with individual variations (Nakhla *et al.*, 1999). Consequently, lacto-N-neotetraose is preferably used in functional food in concentration range 0,01 – 10 g/l, more preferably 0,01 – 5 g/l, most preferably 0,1 – 1 g/l. When the free oligosaccharides of the invention are trisaccharides or the disaccharide with sequence Galβ4Glc at the reducing end, they are preferably consumed in concentrations 1 – 100 g/l, more preferably in the concentration range 1 – 20 g/l. Alternatively, the total concentration of the saccharides used in functional food is the same or similar to the total

concentration of natural human milk saccharides, which bind *Helicobacter pylori* like the substances according to the invention, or which contain the binding epitope/oligosaccharide sequence according to the invention. At least in one case human milk has been reported to contain Galβ3Galβ4Glc as a major neutral oligosaccharide with high concentration (Charlwood *et al.*, 1999).

Furthermore, it is possible to use the substance according to the invention in the diagnosis of a condition caused by an *Helicobacter pylori* infection. Diagnostic uses also include the use of the substance according to the invention for typing of *Helicobacter pylori*. When the substance according to the invention is used for diagnosis or typing, it may be included in, e.g., a probe or a test stick, optionally constituting a part of a test kit. When this probe or test stick is brought into contact with a sample containing *Helicobacter pylori*, the bacteria will bind the probe or test stick and can be thus removed from the sample and further analyzed.

Glycolipid and carbohydrate nomenclature is according to recommendations by the IUPAC-IUB Commission on Biochemical Nomenclature (Carbohydrate Res. 1998, 312, 167; Carbohydrate Res. 1997, 297, 1; Eur. J. Biochem. 1998, 257, 29).

It is assumed that Gal, Glc, GlcNAc, and Neu5Ac are of the D-configuration, Fuc of the L-configuration, and all the monosaccharide units in the pyranose form. Glucosamine is referred as GlcN or $GlcNH_2$ and galactosamine as GalN or $GalNH_2$. Glycosidic linkages are shown partly in shorter and partly in longer nomenclature, the linkages of the Neu5Ac-residues α3 and α6 mean the same as α2-3 and α2-6, respectively, and with other monosaccharide residues α1-3, β1-3, β1-4, and β1-6 can be shortened as α3, β3, β4, and β6, respectively. Lactosamine refers to N-acetyllactosamine, Galβ4GlcNAc, and sialic acid is N-acetylneuraminic acid (Neu5Ac) or N-glycolylneuraminic acid (Neu5Gc) or any other natural sialic acid. Term glycan means here broadly oligosaccharide or polysaccharide chains present in human or animal glycoconjugates, especially on glycolipids or glycoproteins. In the shorthand nomenclature for fatty acids and bases, the number before the colon refers to the carbon chain lenght and the number after the colon gives the total number of double bonds in the hydrocarbon chain. Abbreviation GSL refers to glycosphingolipid. Abbreviations or short names or symbols of glycosphingolipids are given in the text and in Tables 1 and 2. *Helicobacter pylori* refers also to the bacteria similar to *Helicobacter pylori*.

The present invention is further illustrated by the following examples, which in no way are intended to limit the scope of the invention:

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. EI/MS of permethylated oligosaccharides obtained from hexaglycosylceramide by endoglycoceramidase digestion. Gas chromatogram of the oligosaccharides (top) and EI/MS spectra of peaks A and B, respectively (bottom).

Fig. 2. Negative-ion FAB mass spectra of hexa- (A) and pentaglycosylceramide (B).

Fig. 3. Proton NMR spectra showing the anomeric region of the six-sugar glycolipid (A) and the five-sugar glycolipid (B). Spectra were acquired overnight to get good signal-to-noise for the minor type 1 component.

Fig. 4. Enzymatic degradation of rabbit thymus glycosphingolipids. Silica gel thin layer plates were developed in C/M/H_2O, (60:35:8, by vol.). **A** and **B**, 4-methoxybenzaldehyde visualized plates. **C**, autoradiogram after overlay with ^{35}S-labeled *Helicobacter pylori*. 1, heptaglycosylceramide (structure 1, Table I); 2, desialylated heptaglycosylceramide (obtained after acid treatmet); 3, desialylated heptaglycosylceramide treated with β4-galactosidase; 4, heptaglycosylceramide treated with sialidase and β4galactosidase; 5, reference glycosphingolipids from human erythrocytes (lactosylceramide, trihexosylceramide and globoside); 6, desialylated heptaglycosylceramide treated with β4-galactosidase and β-hexosaminidase; 7, heptaglycosylceramide treated with sialidase, β4-galactosidase and β-hexosaminidase.

Fig. 5. TLC of products obtained after partial acid hydrolysis of rabbit thymus heptaglycosylceramide (structure 1, Table I). Developing solvent was as for Fig. 4. **A**, 4-methoxybenzaldehyde-visualized plate; **B**, autoradiogram after overlay with ^{35}S-labeled *Helicobacter pylori*. 1, heptaglycosylceramide; 2, desialylated heptaglycosylceramide (acid treatment); 3, pentaglycosylceramide; 4, hydrolysate; 5, reference glycosphingolipids (as for Fig. 4).

Fig. 6. Dilution series of glycosphingolipids. The binding activity on TLC plates was determined using bacterial overlay technique. TLC developing solvent was as for Fig. 4. Different glycolipids were applied to the plates in equimolar amounts.

Quantification of the glycolipids was based on hexose content. A, hexa- and pentaglycosylceramides (structures 2 and 3, Table I); B, penta- and tetraglycosylceramides (structures 4 and 5, Table I). The amounts of glycolipids (expressed as pmols) were as follows: 1, 1280 (of each); 2, 640; 3, 320; 4, 160; 5, 80; 6, 40; 7, 20 pmols (of each).

Fig. 7. Thin-layer chromatogram with separated glycosphingolipids detected with 4-methoxybenzaldehyde (**A**) and autoradiogram after binding of radiolabeled *Helicobacter pylori* strain 032 (**B**). The glycosphingolipids were separated on aluminum-backed silica gel 60 HPTLC plates (Merck) using chloroform/methanol/water 60:35:8 (by volume) as solvent system. The binding assay was done as described in the "Materials and methods" section. Autoradiography was for 72 h. The lanes contained:
lane 1) Galβ4GlcNAcβ3Galβ4Glcβ1Cer (neolactotetraosylceramide), 4 μg;
lane 2) Galα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer (B5 glycosphingolipid), 4 μg;
lane 3) Galα3Galβ4GlcNH$_2$β3Galβ4Glcβ1Cer, 4 μg;
lane 4) Galα3(Fucα2)Galβ4GlcNAcβ3Galβ4Glcβ1Cer (B6 type 2 glycosphingolipid), 4 μg;
lane 5) GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer, 4 μg;
lane 6) Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer, 4 μg;
lane 7) GalNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer (x_2 glycosphingolipid), 4 μg;
lane 8) NeuAcα3GalNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer (NeuAc-x_2), 4 μg;
lane 9) Fucα2Galβ4GlcNAcβ3Galβ4Glcβ1Cer (H5 type 2 glycosphingolipid), 4 μg;
lane 10) NeuAcα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer (sialylneolactotetraosylceramide), 4 μg. The sources of the glycosphingolipids are the same as given in Table 2.

Fig. 8. Calculated minimum energy conformations of three glycosphingolipids which bind *Helicobacter pylori*: GalNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer (**A**), GalNAcα3Galβ4GlcNAcβ3Galβ4GlcβCer (**B**) and Galα3Galβ4GlcNAcβ3Galβ4GlcβCer (**C**). Also shown is the non-binding Galα3Galβ4GlcNH$_2$β3Galβ4GlcβCer structure (**D**). Top views of the oligosaccharide part of each of the calculated minimum energy structures are also shown. Despite differences in anomerity, absence or presence of an acetamido group, axial or equatorial position of the 4-OH of the terminal sugar and the fact that the ring plane of the terminal α3-linked compounds is raised somwhat above the corresponding plane of the one being β3-linked, a

substantial topographical similarity exists between these structures and also the GlcNAcβ3-terminated structure derived from rabbit thymus (see Fig. 9A), thus explaining their similar affinities for the bacterial adhesin. In contrast, the acetamido group of the internal GlcNAcβ3 is essential for binding (*cf.* **C** and **D**).

Fig. 9. Calculated minimum energy conformations of the binding-active glycosphingolipids GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer (**A**) and Galβ4GlcNAcβ3Galβ4-GlcNAcβ3Galβ4GlcβCer (**B**) and the non-binding glycosphingolipids NeuAcα3GalNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer (**C**) and Galα3(Fucα2)Galβ4GlcNAcβ3Galβ4GlcβCer (**D**). The latter two extensions (**C** and **D**) abolish binding of *Helicobacter pylori* while the former (**B**) is tolerated but results in a reduced affinity. Together with the finding that de-*N*-acylation of the acetamido moiety of the internal GlcNAc of B5 (Fig. 8) completely abolishes binding, the part constituting the binding epitope must consist of the terminal trisaccharide of B5 shown in Fig. 8C since the acetamido group of a terminally situated *N*-acetylgalactosamine is non-essential.

Fig. 10. Minimum energy conformer of the seven-sugar compound NeuGcα3Galβ4-GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer shown in two projections rotated 90 degrees relative each other. The terminal carbon atom of the glycolyl moiety of the sialic acid as well as the methyl carbon atoms of the acetamido groups of the two internal GlcNAc residues are indicated in black only in order to facilitate the viewer's orientation. For the Glcβcer linkage the extended conformation was arbitrarily chosen for presentation but the minimum binding sequence GlcNAcβ3Galβ4GlcNAcβ3 is most likely better exposed toward an approaching adhesin in GlcβCer conformations other than the one shown here.

Fig. 11. Binding of the monoclonal antibody TH2 (**B**) and the lectin from *E. cristagalli* (**C**) to total non-acid glycosphingolipid fractions from epithelial cells from human gastric mucosa, human granulocytes and human erythrocytes separated on thin-layer chromatograms. In (**A**) the same fractions are shown with 4-methoxybenzaldehyde staining Autoradiography was in cases (**B**) and (**C**) performed for twelve hours. In lanes 1-6 80 μg c the total non-acid fractions from epithelial cells from human gastric mucosa of five differer blood group A individuals were applied, whereas in lane 6 40 μg from the total non-acid fraction from human granulocytes and in lane 7 40 μg from the total non-acid fraction from human erythrocytes were applied. The overlay assays were performed as described in "Materials and methods".

EXAMPLES

Materials and methods

Materials - TLC silica gel 60 (aluminum) plates were from Merck (Darmstadt, Germany). All investigated glycosphingolipids were obtained in our laboratory. β-Galactosidase (*Escherichia coli*) was purchased from Boehringer Mannheim (Germany), Ham's F12 medium from Gibco (U.K.), ^{35}S-methionine from Amersham (U.K.) and FCS (fetal calf serum) was from Sera-Lab (England). β4-Galactosidase (*Streptococcus pneumoniae*), β-N-acetylhexosaminidase (*Streptococcus pneumoniae*) and sialidase (*Arthrobacter ureafaciens*) were from Oxford GlycoSystems (Abington, U.K.). The clinical isolates of *Helicobacter pylori* (strains 002 and 032) obtained from patients with gastritis and duodenal ulcer, respectively, were a generous gift from Dr. D. Danielsson, Örebro Medical Center, Sweden. Type strain 17875 was from Culture Collection, University of Göteborg (CCUG).

Glycosphingolipids. The pure glycosphingolipids of the experiment shown in Fig. 7 wer prepared from total acid or non-acid fractions from the sources listed in Table 2 as describe in (Karlsson, 1987). In general, individual glycosphingolipids were obtained by acetylatior (Handa, 1963) of the total glycosphingolipid fractions and separated by repeated silicic aci column chromatography, and subsequently characterized structurally by mass spectrometry (Samuelsson *et al.*, 1990), NMR (Falk *et al.*, 1979a,b,c; Koerner Jr *et al.*, 1983) and degradative procedures (Yang and Hakomori, 1971; Stellner *et al.*, 1973). Glycolipids derived from rabbit thymys are described below.

Purification of glycolipids. Acid glycosphingolipids were isolated from 1000 g acetone powder of rabbit thymus (Pel-Freeze Biological Inc., North Arkansas, Ark. US). The acetone powder was extracted in a Soxhlet apparatus with chroloroform/methanol 2/1 (vol/vol unless otherwise stated) for 24 h followed by chloroform/methanol/water 8/1/1 for 36 h. The extracted lipids, 240 g, were subjected to Folch separation (Folch *et al.*, 1957) and the collected hydrophilic phase to ion-exchange gel chromatography on DE23 cellulose (DEAE, Whatman, Maidstone, UK). These isolation steps gave 2.5 g of acid glycosphingolipids. The gangliosides were separated according to number of sialic acids by ion-exchange gel with open-tubular chromatography on a glass column (50 mm i.d). The column was connected to an HPLC pump producing a concave gradient (pre-programmed gradient no 4, System Gold Chromatographic Software, Beckman Instruments Inc., CA, USA) starting with methanol and ending with 0.5 M CH_3COONH_4 in methanol. The flow rate was 4 ml/min and 200 fractions with 8 ml in each were

collected. 300-400 mg of ganglioside mixture was applied at a time to 500 g of DEAE Sepharose, (CL6, Pharmacia, Uppsala, Sweden, bed height approx. 130 mm). The monosialylated gangliosides were further separated by HPLC on a silica column, 300 mm x 22 mm i.d., 120 Å pore size, 10 μm particle size (SH-044-10, Yamamura Ltd., Kyoto, Japan). Approximately 150 mg of monosialylated gangliosides were applied at time and a streight eluting gradient was used (chloroform/methanol/water from 60/35/8 to 10/103, 4 ml/min, 240 fractions).

Partial acid hydrolysis - Desialylation of gangliosides was performed in 1.5% CH_3COOH in water at 100°C after which the material was neutralized with NaOH and dried under nitrogen. For partial degradation of the carbohydrate backbone the glycolipid was hydrolyzed in 0.5M HCl for 7 min in a boiling water bath. The material was then neutralized and partitioned in C/M/H_2O, (8:4:3, v/v)[2]. The lower phase was collected, evaporated under nitrogen and the recovered glycolipids were used for analysis.

Preparation of pentaglycosylceramide from hexaglycosylceramide by enzyme hydrolysis - Hexaglycosylceramide (structure 2, Table 1) obtained from heptaglycosylceramide (4 mg, from rabbit thymys) (structure 1, Table 1) by acidic desialylation (see above) was redissolved in C/M (2:1) and applied to a small silica gel column (0.4 x 5 cm). The column was eluted with C/M/H_2O (60:35:8, v/v). Fractions of about 0.2 ml were collected and tested for the presence of carbohydrates. The recovered hexaglycosyleramide (2.0 mg) was dissolved in 1.5 ml of 0.1 M potassium phosphate buffer, pH 7.2, containing sodium taurodeoxycholate (1.5 mg/ml), $MgCl_2$ (0.001M) and β-galactosidase (*E. coli*, 500 U when assayed with 2-nitrophenyl-β-D-galactoside as a substrate), and the sample was incubated overnight at 37°C. The material was next partitioned in C/M/H_2O (10:5:3) and the glycolipid contained in the lower phase was purified using silica gel chromatography (0.4 x 5 cm columns) as described above for hexaglycosylceramide. To remove all contaminating detergent the chromatography was repeated twice. The final recovery of pentaglycosylceramide was 0.7 mg.

Endoglycoceramidase digestion of glycolipids (Ito and Yamagata, 1989) - The reaction mixture contained 200 μg of glycolipid, 80 μg of sodium taurodeoxycholate and 0.8 mU of enzyme in 160 μl of 50 mM acetate buffer, pH 6.0. The sample was incubated overnight at 37°C, after which water (140 μl) and C/M, (2:1, by vol., 1500 μl) were added, and the sample was shaken and centrifuged. The upper phase was dried under nitrogen, redissolved in a small volume of water and desalted on a Sephadex G-25 column (0.4x10 cm), which had been equilibrated in H_2O, and eluted with water. Fractions of about 0.1 ml were collected and tested for the presence of sugars.

Permethylation of saccharides - Permethylation was performed according to Larson *et al.*, 1987. Sodium hydroxide was added to samples before methyl iodide as suggested by Needs and Selvendran 1993. In some experiments the saccharides were reduced with $NaBH_4$ before methylation. In this case the amount of methyl iodide was increased to a final proportion of DMSO (dimethylsulfoxide)/methyl iodide of 1:1 (Hansson and Karlsson, 1990).

Gas chromatography/mass spectrometry - Gas chromatography was carried out on a Hewlett-Packard 5890A Series II gas chromatograph equipped with an on-column injector and a flame ionization detector. Permethylated oligosaccharides were analyzed on a fused silica capillary column (Fluka, 11m x 0.25 mm i.d.) coated with cross-linked PS264 (film thickness 0.03 μm). The sample was dissolved in ethyl acetate and injected on-column at 80°C. The temperature was programmed from 80°C to 390°C at a rate of 10°C/min with a 2 min hold at the upper temperature. Gas chromatography-mass spectrometry of the permethylated oligosaccharides was performed on a Hewlett-Packard 5890A Series II gas chromatograph interfaced to a JEOL SX-102 mass spectrometer (Hansson and Karlsson, 1990). FAB-MS analyses were performed on a JEOL SX-102 mass spectrometer. Negative FAB spectra were produced using Xe atom bombardment (10 kV) and triethanolamine as matrix.

NMR spectroscopy - Proton NMR spectra were recorded at 11.75 T on a Jeol Alpha 50C (Jeol, Tokyo, Japan) spectrometer. Samples were deuterium exchanged before analysis and spectra were then recorded at 30 °C with a digital resolution of 0.35 Hz/pt. Chemical shifts are given relative to TMS (tetramethylsilane) using the internal solvent signal.

Analytical enzymatic tests - Oxford GlycoSystems enzymatic tests were performed according to the manufacturer's recommendations except that Triton X-100 was added to each incubation mixture to final concentration of 0.3%. When a mixture of sialidase and β4-galactosidase were taken for digestion the incubation buffer from β4–galactosidase kit was used. If β-hexosaminidase was present in the digestion mixture the buffer from this enzyme kit was employed. The enzyme concentrations in the incubation mixtures were: 80 mU/ml for Hexβ4HexNAc-galactosidase (*S. pneumoniae*), 120 mU/ml for β-N-Acetylhexosaminidase (*S. pneumoniae*) and 1 U/ml for sialidase (*Arthrobacter ureafaciens*) The concentration of substrate was about 20 μM. Enzymatic digestion was performed overnight at 37°C. After digestion the samples were dried and desalted using small columns of Sephadex G-25 (Wells and Dittmer, 1963), 0.3 g, equilibrated in C/M/H_2O, (60:30:4.5, by vol.). Each sample was applied on the column in 2 ml of the same solvent and eluted with 2.5 ml of C/M/H_2O, (60:30:4.5) and 2.5 ml of C/M, (2:1). Application and washing solutions were collected and evaporated under nitrogen.

Other analytical methods - Hexose was determined according to Dubois *et al.* 1956.

De-N-acylation. Conversion of the acetamido moiety of GlcNAc/GalNAc residues into amine was accomplished by treating various glycosphingolipids with anhydrous hydrazine as described previously (Ångström *et al.*, 1998).

Bacterial growth. The *Helicobacter pylori* strains were stored at -80 °C in tryptic soy broth containing 15% glycerol (by volume). The bacteria were initially cultured on GAB-CAMP agar (Soltesz *et al.*, 1988) under humid (98%) microaerophilic conditions (O_2: 5-7% CO_2: 8-10% and N_2: 83-87%) at 37 °C for 48-72 h. For labeling colonies were inoculated GAB-CAMP agar, except for the results presented in Fig.1 where Brucella agar (Difco, Detroit, MI) was used instead, and 50 μCi ^{35}S-methionine (Amersham, U.K.), diluted in 0. ml phosphate-buffered saline (PBS), pH 7.3, was sprinkled over the plates. After incubatio for 12-24 h at 37 °C under microaerophilic conditions, the cells were scraped off, washed three times with PBS, and resuspended to 1x10^8 CFU/ml in PBS. Alternatively, colonies were inoculated (1x10^5 CFU/ml) in Ham's F12 (Gibco BRL, U.K.), supplemented with 10 heat-inactivated fetal calf serum (Sera-Lab). For labeling, 50 μCi ^{35}S-methionine per 10 m medium was added, and incubated with shaking under microaerophilic conditions for 24 h. Bacterial cells were harvested by centrifugation, and purity of the cultures and a low conte of coccoid forms was ensured by phase-contrast microscopy. After two washes with PBS, the cells were resuspended to 1x10^8 CFU/ml in PBS. Both labeling procedures resulted in suspensions with specific activities of approximately 1 cpm per 100 *Helicobacter pylori* organisms.

TLC bacterial overlay assay. Thin-layer chromatography was performed on glass- or aluminum-backed silica gel 60 HPTLC plates (Merck, Darmstadt, Germany) using chloroform/methanol/water 60:35:8 (by volume) as solvent system. Chemical detection wa accomplished by anisaldehyde staining (Waldi, 1962). The bacterial overlay assay was performed as described previously (Hansson *et al.*, 1985). Glycosphingolipids (1-4 μg/lane or as indicated in the figure legend) were chromatographed on aluminum-backed silica gel plates and thereafter treated with 0.3-0.5% polyisobutylmethacrylate in diethylether/*n*-hexane 1:3 (by volume) for 1 min, dried and subsequently soaked in PBS containing 2% bovine serum albumin and 0.1% Tween 20 for 2 h. A suspension of radio-labeled bacteria (diluted in PBS to 1x10^8 CFU/ml and 1-5x10^6 cpm/ml) was sprinkled over the chromatograms and incubated for 2 h followed by repeated rinsings with PBS. After drying the chromatograms were exposed to XAR-5 X-ray films (Eastman Kodak Co., Rochester, NY, USA) for 12-72 h.

TLC protein overlay assays. ^{125}I-labeling of the monoclonal antibody TH2 and the lect from *Erythrina cristagalli* (Vector Laboratories, Inc., Burlingame, CA) was performed b the Iodogen method (Aggarwal *et al.*, 1985), yielding an average of 2 x 10^3 cpm/μg. Th

overlay procedure was the same as described above for bacteria except Tween was not u and that ^{125}I-labeled protein, diluted to approximately 2 x 10^3 cpm/μl with PBS contain 2% bovine serum albumin, was used instead of a bacterial suspension.

Molecular modeling. Minimum energy conformers of the glycosphingolipids listed in Table 1 were calculated within the Biograf molecular modeling program (Molecular Simulations Inc.) using the Dreiding-II force field (Mayo *et al.*, 1990) on a Silicon Graphi 4D/35TG workstation. Partial atomic charges were generated using the charge equilibratic method (Rappé and Goddard III, 1991), and a distance dependent dielectric constant (ε=3. was used for the Coulomb interactions. In addition a special hydrogen bonding term was used in which the maximal interaction (D_{hb}) was set to -4 kcal mol^{-1}. The dihedral angles the Glcβ1Cer linkage are defined as follows: Φ = H-1 - C-1 - O-1 - C-1, Ψ = C-1 - O-1 - 1 - C-2 and θ = O-1 - C-1 - C-2 - C-3 starting from the glucose end (see Nyholm and Pascher, 1993).

RESULTS

The heptaglycosylceramide NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer was purified from rabbit thymus by HPLC as described above. The structure was characterized by NMR and mass spectrometry (data not shown). The heptasaccharide ganglioside was bound by most *Helicobacter pylori* isolates (about 60) tested in the laboratory of the inventors.

In order to detect possible minor isomeric components in the heptaglycosylceramide material, the ganglioside was desialylated, treated with endoglycoceramidase after which the released oligosaccharides were permethylated and analyzed by gas chromatography and EI/MS, (Fig. 1). Two saccharides were identified in the six-sugar region which showed the expected carbohydrate sequence of Hex-HexNAc-Hex-HexNAc-Hex-Hex, as confirmed by fragment ions at *m/z* 219, 464, 668, 913 and 1118. When the carbohydrates were converted to alditols (by reduction with $NaBH_4$) before methylation distinct fragment ions at *m/z* 235, 684 and 1133 were found in addition to the previously listed ions (data not shown). The predominant saccharide, which accounted for more than 90% of the total material (peak B, Fig. 1), was characterized by a strong fragment ion at *m/z* 182 confirming the presence of β4GlcNAc (neolacto series, type 2 carbohydrate chain). The minor saccharide (peak A, Fig.1) gave a spectrum typical for type-1 chain (lacto series) with a very weak fragment ion at m/z 182 and a strong fragment ion at *m/z* 228. The preparation also contained traces of other sugar-positive substances which might be 4- and 5-sugar-containing saccharides of the same series. Fucose-containing saccharides were not found in the mixture. The purity of the asialoganglioside was

tested also by FAB/MS and NMR spectroscopy. The negative FAB/MS of the hexaglycosylceramide (Fig.2, A) confirmed the predicted carbohydrate sequence and showed that the ceramides were composed mainly of sphingosine and C16:0 fatty acid (*m/z* 536.5). The NMR spectrum obtained of hexaglycosylceramide (Fig. 3, A) showed four major doublets in the anomeric region with β-couplings (J~8 Hz). They had an intensity ratio of 2:2:1:1. The signals at 4.655 ppm (GlcNAcβ3), 4.256 ppm (internal Galβ4), 4.203 ppm (terminal Galβ4) and 4.166 ppm (Glcβ) were in agreement with results previously published for nLcOse6-Cer (Clausen *et al.*, 1986). There was also a small doublet at 4.804 ppm, which together with a small methyl signal at 1.81 ppm (seen as a shoulder on the large type 2 methyl resonance) indicated the presence of a small fraction of type 1 chain. Due to the overlap in the 4.15 to 4.25 ppm region the position and distribution of this type 1 linkage could not be determined. The total amount of type 1 linkage was roughly 10%. As the amount of type 1 chain in the pentaglycosylceramide obtained from hexaglycosylceramide by β-galacosidase digestion also was approximately 5% (Fig 3, B) it seems likely that the type 1 linkage was evenly distributed between the internal and external parts of the saccharide chain, i.e. 5% of the glycolipids could be type1-type1.

To find out if the binding activity of the glycolipid was associated with the predominant neolacto (type 2) structure the asialo-glycolipid was treated with β4-galactosidase and β-hexosaminidase, and the products were investigated by TLC and by overlay tests (Fig. 4). As expected, the first enzyme converted the hexaglycosylceramide to a pentaglycosylceramide (A, lane 3) and the mixture of the two enzymes degraded the material to lactosylceramide (B, lane 6). According to visual evaluation of the TLC plates both reactions were complete or almost complete. The same results were obtained for sialidase- and acid-treated material. The β4-galactosidase degradation of hexaglycosylceramide was accompanied by disappearance of the *Helicobacter pylori* binding activity in the region of this glycolipid on TLC plates with simultaneous appearance of a strong activity in the region of pentaglycosylceramides (C, lane 3). Further enzymatic degradation of the pentaglycosylceramide resulted in the disappearance of binding activity in this region. Appearance of binding activity in the four-sugar region was not observed. The sensitivity of the chemical staining of TLC plates is too low to allow trace substances to be observed.

In a separate experiment the parent ganglioside was subjected to partial acid degradation and the released glycolipids were investigated for *Helicobacter pylori* binding activity. Fig. 5 shows TLC of the hydrolyzate (A) and the corresponding autoradiogram (B) after overlay of the hydrolyzate with ^{35}S-labeled *Helicobacter pylori*. Glycolipids located in the regions of hexa-, penta-, tetra- and

diglycosylceramides displayed binding activity, whereas triglycosylceramide was inactive.

The binding of the hexa-, penta-, tetraglycosylceramides were similar when tested with at least three *Helicobacter pylori* strains (17875, 002 and 032).

The strongly binding pentaglycosylceramide produced after detachment of the terminal galactose from hexaglycosylceramide and purification by silica gel chromatography was investigated in greater detail. The negative ion FAB/MS spectrum of this glycolipid confirmed a carbohydrate sequence of HexNAc-Hex-HexNAc-Hex-Hex- and showed the same ceramide composition as the hexaglycosylceramide (Fig 2, B). The proton NMR spectrum obtained for the pentaglycosylceramide (Fig. 3, B) had five major β-doublets in the anomeric region: at 4.653 ppm (internal GlcNAcβ3), 4.615 ppm (terminal GlcNAcβ3), 4.261 ppm (double intensity, internal Galβ4), 4.166 (Glcβ), consistent with GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer and also in perfect agreement with the six sugar compound having been stripped of its terminal Galβ. There is also a small β-doublet at 4.787 ppm corresponding to 3-substituted GlcNAcβ (type 1 chain). The expected methyl signal was also seen as a shoulder on a much larger methyl signal at 1.82 ppm, but overlap prohibits quantitation of these signals. From the integral of the anomeric proton it can be calculated that 6% of the glycolipid contained type 1 chain. Thus the relative proportion of type 2 and type 1 carbohydrate chains was similar to that of the six sugar glycolipid. The two spots visible on TLC plates both in the hexa- and pentaglycosyl fractions reflected a ceramide heterogeneity rather than differences in sugar chain composition as judged by their susceptibility to β4-galactosidase. The upper penta-region spot appeared both after unselective hydrolysis of the asialoganglioside and selective splitting of 4-linked galactose from the asialoproduct. Furthermore, when hexaglycosylceramide with a high content of the upper chromatographic subfraction was degraded by β4-galactosidase and β-hexosaminidase the resulting lactosylceramide gave two distinct chromatographic bands. Chromatographically homogenous hexaglycosylceramide resulted in only one lactosylceramide band. Both upper and lower subfractions in the penta-region were highly active as shown by overlay tests.

Glycosphingolipids of the neolacto series with 6, 5 and 4 sugars (structures 2, 4 and 5, Table I) were examined by semi-quantitative tests using the TLC overlay procedure. The glycolipids were applied on silica gel plates in series of dilutions and their binding to *Helicobacter pylori* was evaluated visually after overlay with labeled bacteria and autoradiography (Fig. 6). The most active species was pentaglycosylceramide, which gave a positive response on TLC plates in amounts down to 0.039 nmol/spot (mean value calculated from 7 experiments, standard

deviation δ_{n-1} = 0.016 nmol). Hexa- and tetraglycosylceramides bound *Helicobacter pylori* in amounts of c:a 0.2 and 0.3 nmoles of glycolipid/spot, respectively.

The binding of *Helicobacter pylori* to higher glycolipids of the investigated series was highly reproducible. The binding frequency for *Helicobacter pylori*, strain 032, recorded for pentaglycosyl- and hexaglycosylceramides was ~ 90% (total number of plates was about 100).

Binding assays revealing the isoreceptors and specificity of the binding (Fig 7.) In addition to the seven-sugar glycosphingolipid from rabbit thymus having a neolacto cor NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer, and tetra- to hexaglycosylceramides derived thereof, the binding specificity could involve other glycolipids from the neolacto series.

The binding of *Helicobacter pylori* (strain 032) to purified glycosphingolipids separatec on thin-layer plates using the overlay assay is shown in Fig. 7. These results together with those from an additional number of purified glycosphingolipids are summarized in Table 2 The binding of *Helicobacter pylori* to neolactotetraosylceramide (lane 1) and the five- and six-sugar glycosphingolipids (lanes 5 and 6) derived from NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer is identical to results above. Unexpectedly, however, binding was also found for GalNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer (x_2 glycosphingolipid, lane 7) and the de-fucosylated A6-2 glycosphingolipid GalNAcα3Galβ4GlcNAcβ3Galβ4GlcβCer (no. 12, Table 2). Together with the finding that Galα3Galβ4GlcNAcβ3Galβ4GlcβCer (B5 glycosphingolipid, lane 2) also is binding-active, these results suggest the possibility of cross-binding rather than the presence of multiple adhesins specific for each of these glycosphingolipids (see below). Furthermore, the only extension of the different five-sugar containing glycosphingolipids just mentioned that was tolerated by the bacterial adhesin wa Galβ4 to the thymus-derived GlcNAcβ3-terminated compound (lane 6). Other elongated structures, as the NeuAc-x_2 (lane 8) and GalNAcβ3-B5 (no. 25, Table 2), were thus all found to be non-binding. It may be further noticed that the acetamido group of the internal GlcNAcβ3 in B5 is essential for binding since de-*N*-acylation of this moiety by treatment with anhydrous hydrazine leads to complete loss of binding (lane 3) as is the case also whei neolactotetraosylceramide is similarly treated (no. 6, Table 2).

Cross-binding of five-sugar glycosphingolipids. In order to understand the binding characteristics of the different neolacto-based glycosphingolipid molecules used in this stuc the conformational preferences of active as well as inactive structures were investigated by molecular modeling. Fig. 8 shows the x_2 glycosphingolipid together with three other sequences: defucosylated A6-2, B5 and de-*N*-acylated B5, which, except for the chemically modified B5 structure, show similar binding strengths. Also the five-sugar glycosphingolipi from rabbit thymus (see Fig. 9A) should be included in this comparison since this structure

differs only at position four of the terminal residue compared with the x_2 structure and is equally active. The four active structures all have neolacto cores which thus are terminate by GalNAcβ3, GalNAcα3, Galα3 and GlcNAcβ3, respectively. The minimum energy conformers of these structures were generated as descibed previously (Teneberg *et al.*, 1996). Other minimum energy structures given in Table 2 are based on earlier results foun in the literature (Bock *et al.*, 1985; Meyer, 1990; Nyholm *et al.*, 1989). Regarding sialic acid-terminated glycosphingolipids the *synclinal* conformation was adopted for the glycosidic dihedral angles of α3-linked residues as seen in, e.g., Fig. 9C, but the effect of other conformations (Siebert *et al.*, 1992), in particular the *anticlinal* one, was also tested. Also for the α6-linked variant several low energy conformers (Breg *et al.*, 1989) were generated for the same purpose.

As mentioned above, the fact that there are four binding-active five-sugar glycosphingolipids (nos. 10-13, Table 2), all having a neolacto core, suggests that cross-binding to the same adhesin site may be the reason behind these observations. At first glance, however, it might seem surprising that the B5 glycosphingolipid, which differs at t terminal position in comparison with the five-sugar compound obtained from rabbit thymu the former having a Galα3 and the latter a GlcNAcβ3, is equally active and should be included within the binding specificity of the neolacto series. Despite the fact that these tw terminal saccharides differ also in their anomeric linkage it is seen (Figs. 8C and 9A) that t minimum energy structures topographically are very similar, the differences being that Galα3 lacks an acetamido group, has the 4-OH in the axial position and its ring plane raise slightly above the corresponding plane in the five-sugar compound. However, neither the 4 OH position nor the absence/presence of an acetamido group appear to be crucial for bindi to occur, since also the x_2 and defucosylated A6-2 glycosphingolipids (Fig. 8A, B), which are terminated by GalNAcβ3 and GalNAcα3, respectively, have similar affinities for the *Helicobacter pylori* adhesin. In the light of these findings also Galβ3Galβ4GlcNAcβ3Galβ4GlcβCer, which has been isolated from human erythrocytes (Stellner and Hakomori, 1974), would be expected to bind the bacterial adhesin. In the ligh of the rules of binding also three other terminal monosaccharides in *Helicobacter pylori* binding epitopes are possible trisaccharide binding epitopes, namely GlcNAcα3Galβ4GlcNAc, Glcβ3Galβ4GlcNAc and Glcα3Galβ4GlcNAc. Such compound are not known from human tissues so far, but could rather represent analogues of the natura receptor. Neither the Galβ3Galβ4GlcNAc-glycolipid nor the three analogs were unfortunately available for testing.

The neolacto seven-sugar compound,NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer, was also subjected to molecular modeling. Fig 10 shows two different projections of the minimum energy structure with the GlcβCer linkage in an extended conformation.

The sialic acid was given the *syn clinal* conformation but the *anti* conformer is also likely in unbranched structures (Siebert *et al.*, 1992). The sialic acid appears to have little influence on the binding activity towards *Helicobacter pylori* as compared with the six-sugar compound, 9B. Comparision of the first projection with Fig. 9A and 9 B suggests that the same binding epitope is also available in the seven-sugar structure.

Delineation of the neolacto binding epitope. The relative binding strength of the structures obtained by chemical and enzymatic degradation of the rabbit thymus seven-sug compound (nos. 1, 5, 10, and 21, Table 2) suggest that the three-sugar sequence GlcNAcβ3Galβ4GlcNAcβ3 may constitute the minimal binding sequence. Thus, in the six sugar compound an inhibitory effect from the terminal Galβ4 is expected, whereas for neolactotetraosylceramide lack of a terminal GlcNAcβ3 reduces the binding strength since only two out of three sugars in the epitope are present. The essentiality of the internal GlcNAcβ3 is clearly shown by the loss of bacterial binding both to neolactotetraosylceramide and B5 following de-*N*-acylation of the acetamido group to an amine (nos. 6 and 14, Table 2). This non-binding may occur either by loss of a favorable interaction between the adhesin and the acetamido moiety and/or altered conformational preferences of these glycosphingolipids. However, it is difficult to envision a situation wh an altered orientation of the internal Galβ4 would sterically hinder access to the binding epitope. Thus, having established that the minimal binding sequence must encompass the GlcNAcβ3Galβ4GlcNAcβ3 sequence it is now easy to rationalize the absence of binding f P_1, H5-2 and the two sialylparagloboside structures (nos. 15, 18-20, Table 2) since these extensions interfere directly with the proposed binding epitope. Also the glycosphingolipic from bovine buttermilk (Teneberg *et al.*, 1994), which has a β6-linked branch of Galβ4GlcNAcβ attached to the internal Galβ4 of neolactotetraosylceramide (no. 26, Table 2), is non-binding due to blocked access to the binding epitope.

Elongation of the different binding-active five-sugar sequences in Table 2 shows that or addition of Galβ4 to the thymus-derived structure is tolerated, in accordance with the observation that the 4-OH position may be either equatorial or axial, but with an ensuing lo of binding affinity due to steric interference. Addition of either NeuAcα3 to x_2 or GalNAc to B5 thus results in complete loss of binding (nos. 24 and 25, Table 2). It is further seen th the negative influence of a Fucα2 unit as in H5-2 is confirmed by the non-binding of *Helicobacter pylori* both to A6-2 and B6-2 (nos. 22 and 23, Table 2). Concerning the elongated structure (no. 28, Table 2), terminated by the same trisaccharide found in B5, it must, as in B5, be this terminal trisaccharide that is responsible for the observed binding although a second internal binding epitope also is present. However, binding to the internal epitope can most likely be excluded since the penultimate Galβ4 would be expected to reduce the binding strength similarly to what is observed for the six-sugar compound from

rabbit thymus relative to the five-sugar structure. It should also be pointed out that the sial acid residue of the seven-sugar compound from rabbit thymus does not have an influence (the binding for the bacterial strains used in this study and must consequently be outside th epitope area. Whether sialic acid-dependent or -independent binding of *Helicobacter pylo* is obtained or not depends, however, both on the type of strain and growth conditions (Miller-Podraza *et al.*, 1996,1997a,b).

To summarize, the binding epitope of the neolacto series of glycosphingolipids has to involve the three-sugar sequence GlcNAcβ3Galβ4GlcNAcβ3 in order to obtain maximal activity. From a comparison of the binding pattern of the potential isoreceptors used in this study it can be deduced from the structures shown in Figs. 8 and 9 that nearly all of this trisaccharide is important for binding to occur, excepting the acetamido group of the terminal GlcNAcβ3 and the 4-OH on the same residue, which are non-crucial.

Biological presence of the receptors. Of the four five-sugar glycosphingolipids that *in vitro* may function interchangeably as receptors for *Helicobacter pylori* only x_2 occurs naturally in human tissue but has as yet not been found to be present in the gastric mucosa, excepting a case of gastric cancer where it was identified in the tumor tissue (Kannagi *et a* 1982b). A study by Thorn *et al.*, 1992, showed, however, that the x_2 glycosphingolipid and elongated structures having a terminal GalNAcβ3Galβ4GlcNAcβ sequence are present in several human tissues, but gastric epithelial tissue was unfortunately not among the ones investigated. Thin-layer chromatogram overlay with the GalNAcβ3Galβ4GlcNAcβ-specifi monoclonal antibody TH2 of preparations of total non-acid glycosphingolipids from epithelial cells of human gastric mucosa of several blood group A individuals (lanes 1-6) was therefore performed (Fig. 11B). No detectable binding, however, was observed to the glycosphingolipids derived from stomach epithelium using this assay. The corresponding overlay using the Galβ4GlcNAc-binding lectin from *E. cristagalli* is shown in Fig. 11. Of the different glycosphingolipid preparations of gastric epithelial origin the first three lanes show weak binding to bands in the four-sugar region, which probably correspond neolactotetraosylceramide, but no detectable binding of *Helicobacter pylori* to these bands was discerned due to the low amounts of this glycosphingolipid (Teneberg *et al.*, 2001).

Furthermore, the sequence Galα3Galβ4GlcNAcβ, whether present in B5 glycosphingolipid or in the elongated structure discussed above (no. 28, Table 2), is possib not found in normal human tissue due to non-expression of the transferase responsible for the addition of Galα3 (Larsen *et al.*, 1990). One is therefore left with the conclusion that if target receptor(s), carrying the binding epitope identified above, are present on the surface the gastric epithelial cells they may be based on repetitive *N*-acetyllactosamine elements in glycoproteins and not on lipid-based structures.

However, it is known that *Helicobacter pylori* strains associated with peptic ulcer, as the strain mainly used here, stimulates an inflammatory response from granulocytes, even whe

the bacteria are nonopsonized (Rautelin *et al.*, 1994a,b). The initial event in the phagocytc of the bacterium most likely involves specific lectin-like interactions resulting the agglutination of the granulocytes (Ofek and Sharon, 1988). Subsequent to the phagocytoti event oxidative burst reactions occur which may be of consequence for the pathogenesis o *Helicobacter pylori*-associated diseases (Babior, 1978). Several acid and non-acid glycosphingolipids from granulocytes, having both a neolacto core and repeating lactosamine units, including no. 21 in Table 2 and the sialylated seven-sugar compound (n 27, Table 2), where the acetamido group of the sialic acid is in the acetyl form, have been isolated and characterized (Fukuda *et al.*, 1985; Stroud *et al.*, 1996) and may thus act potential receptors for *Helicobacter pylori* on the white blood cell surface. Furthermore, al the x_2 glycosphingolipid has been isolated from the same source (Teneberg, S., unpublished).

Returning to Fig. 11B it is seen that the monoclonal antibody TH2 indeed binds to band in the five-sugar region, both for granulocytes and erythrocytes (lanes 7 and 8, respectively which may correspond to the x_2 glycosphingolipid (Teneberg, S., unpublished; Thorn *et a* 1992; Teneberg *et al.*, 1996). Similarly, neolactotetraosylceramide is found to be present both in granulocytes and erythrocytes when using the *E. cristagalli* lectin instead in the overlay assay (Fig. 11C, lanes 7 and 8). In these two cases *Helicobacter pylori* binds to neolactotetraosylceramide (Bergstöm, J., unpublished). For granulocytes a further rather weak band in the six-sugar region, probably corresponding to neolactotetraosylceramide extended by one *N*-acetyllactosamine unit (*cf.* no. 21, Table 2), is found in accordance with the results of Fukuda *et al.*, 1985. Whether these glycosphingolipids are prime targets in th agglutination process referred to above remains, however, to be elucidated.

Table 1

Structures of glycosphingolipids discussed in the application. The designation is according to recommendations of IUPAC-IUB Joint Commissions on Biochemical Nomenclature (Lipids 1977 12, 455; Eur. J. Biochem. 1998 257, 293).

Structure	Symbol
1. NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer	VI^3NeuGc-nLcOse$_6$Cer
2. Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer	nLcOse$_6$Cer
3. Galβ3GlcNAcβ3Galβ3GlcNAcβ3Galβ4GlcβCer	LcOse$_6$Cer
4. GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer	nLcOse$_5$Cer
5. Galβ4GlcNAcβ3Galβ4GlcβCer	nLcOse$_4$Cer
6. Galβ3GlcNAcβ3Galβ4GlcβCer	LcOse$_4$Cer

Table 2

Binding of *Helicobacter pylori* to glycosphingolipids separated on thin-layer chromatograms.

No.	Trivial name	Glycosphingolipid structure[a]	*H. pylori* binding[b]	Source[c]	References
1	Lactotri	GlcNAcβ3Galβ4Glcβ1Cer	-	RT	(Miller-Prodraza *et al.*, 2001)
2	GgO3	GalNAcβ4Galβ4Glcβ1Cer	(+)	GPE	(Yamakawa, 1966)
3	GgO3 (de-*N*-acylated)	$GalNH_2$β4Galβ4Glcβ1Cer	-	GPE[e]	(Ångström *et al.*, 1998)
4	Le^y-6	Fucα2Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	-	DSI	(McKibbin *et al.*, 1982)
5	Neolactotetra	Galβ4GlcNAcβ3Galβ4Glcβ1Cer	(+)	HE[f]	
6	Neolactotetra (de-*N*-acylated)	Galβ4$GlcNH_2$β3Galβ4Glcβ1Cer	-	HE[e]	
7	GgO4	Galβ3GalNAcβ4Galβ4Glcβ1Cer	+	HB[g]	
8	GgO4 (de-*N*-acylated)	Galβ3$GalNH_2$β4Galβ4Glcβ1Cer	-	HB[e]	(Ångström *et al.*, 1998)
9	Le^x-5	Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	-	DSI	(Teneberg *et al.*, 1996)
10		GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+	RT[d]	(Miller-Podraza *et al.*, 2001)
11	x_2	GalNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+	HE	(Teneberg *et al.*, 1996; Thorn *et al.*, 1992)
12		GalNAcα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+	HE[h]	
13	B5	Galα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+	RE	(Eto *et al.*, 1968)
14	B5 (de-*N*-acylated)	Galα3Galβ4$GlcNH_2$β3Galβ4Glcβ1Cer	-	RE[e]	
15	P_1	Galα4Galβ4GlcNAcβ3Galβ4Glcβ1Cer	-	HE	(Naiki *et al.*, 1975)
16	H5-1	Fucα2Galβ3GlcNAcβ3Galβ4Glcβ1Cer	-	HM	(Karlsson and Larson, 1981a)
17	Le^b-6	Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	-	HM	(Karlsson and Larson, 1981b)
18	H5-2	Fucα2Galβ4GlcNAcβ3Galβ4Glcβ1Cer	-	HE	(Koscielak *et al.*, 1973)
19	NeuAcα3-SPG	NeuAcα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	-	HE	(Ledeen and Yu, 1978)

Table 2 (continued)

20	NeuAcα6-SPG	NeuAcα6Galβ4GlcNAcβ3Galβ4Glcβ1Cer	-	HM	(Nilsson *et al.*, 1981)
21		Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	(+)	RT[d]	(Miller-Podraza *et al.*, 2001)
22	A6-2	GalNAcα3(Fucα2)Galβ4GlcNAcβ3Galβ4Glcβ1Cer	-	HE	(Laine *et al.*, 1974)
23	B6-2	Galα3(Fucα2)Galβ4GlcNAcβ3Galβ4Glcβ1Cer	-	HE	(Koscielak *et al.*, 1973)
24	NeuAc-x_2	NeuAcα3GalNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	-	HE	(Watanabe and Hakomori, 1979)
25		GalNAcβ3Galα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	-	RCC	(Thurin *et al.*, 1989)
26		Galβ4GlcNAcβ6(Galβ4GlcNAcβ3)Galβ4Glcβ1Cer	-	BB	(Teneberg *et al.*, 1994)
27		NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	(+)	RT	(Lanne *et al.*, 2001)
28		Galα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+	RT	(Lanne *et al.*, 2001)
29	A7-2	GalNAcα3(Fucα2)Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	-	DSI	(Falk *et al.*, 1979c)
30	B7-2	Galα3(Fucα2)Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	-	HE	

Footnotes to Table 2

a The glycosphingolipid shorthand nomenclature follows recent recommendations (Nomenclature of glycoproteins, 1988).

b The following abbreviations are used for the glycosphingolipid sources: RT, rabbit thymus; HE, human erythrocytes; RE, rabbit erythrocytes; HM, human meconium; RCC, rat colon carcinoma; BB, bovine buttermilk; DSI, dog small intestine.

c Definition of binding strength is as follows: + denotes a significant darkening of the autoradiogram with 4 μg applied on the TLC plate, (+) indicates a weak to intermediate darkening while a minus sign signifies no binding.

Footnotes to Table 2 (continued)

d Prepared from No. 27 by mild acid hydrolysis and No. 10 by subsequent treatment with β-galactosidase.

e Glycosphingolipid Nos. 3, 6, 8 and 14 were prepared from Nos. 2, 5, 7 and 13, respectively, by treatment with anhydrous hydrazine.

f Prepared from no. 19 by neuraminidase treatment.

g Prepared by mild acid hydrolysis of GM1 ganglioside from human brain.

h Prepared from No. 22 by incubation in 0.05 M HCl at 80°C for 2 h.

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What is claimed:

1. A *Helicobacter pylori* binding substance comprising the oligosaccharide sequence

$[Gal(NAc)_r/Glc(NAc)_r\,\alpha 3/\beta 3]_s\,[Gal\beta 4GlcNAc\beta 3]_t\,Gal\beta 4Glc(NAc)_u$

wherein r, s, t, and u are each independently 0 or 1,

so that when both t = 0 and u = 0, then the oligosaccharide sequence is linked to a polyvalent carrier or present as a free oligosaccharide in high concentration, and analogs or derivatives of said oligosaccharide sequences having binding activity to *Helicobacter pylori*.

2. The *Helicobacter pylori* binding substance according to claim 1 comprising the oligosaccharide sequence

GlcNAcβ3Galβ4GlcNAc or GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc

where position C4 of terminal GlcNAcβ3 is optionally linked to Galβ1- or an oligosaccharide chain by a glycosidic bond.

3. The *Helicobacter pylori* binding substance according to claim 1 comprising one or several of the following oligosaccharide sequences

Galβ4GlcNAc,

GalNAcα3Galβ4GlcNAc, GalNAcβ3Galβ4GlcNAc, GlcNAcα3Galβ4GlcNAc, GlcNAcβ3Galβ4GlcNAc, Galα3Galβ4GlcNAc, Galβ3Galβ4GlcNAc, Glcα3Galβ4GlcNAc Glcβ3Galβ4GlcNAc,

Galβ4GlcNAcβ3Galβ4GlcNAc, Galβ4GlcNAcβ3Galβ4Glc,

GalNAcα3Galβ4GlcNAcβ3Galβ4Glc, GalNAcβ3Galβ4GlcNAcβ3Galβ4Glc, GlcNAcα3Galβ4GlcNAcβ3Galβ4Glc, GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc, Galα3Galβ4GlcNAcβ3Galβ4Glc, Galβ3Galβ4GlcNAcβ3Galβ4Glc, Glcα3Galβ4GlcNAcβ3Galβ4Glc, Glcβ3Galβ4GlcNAcβ3Galβ4Glc,

Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc, and reducing-end polyvalent conjugates thereof.

4. The *Helicobacter pylori* binding substance according to claim 1 comprising one or several of the following oligosaccharide sequences

GalNAcα3Galβ4Glc, GalNAcβ3Galβ4Glc, GlcNAcα3Galβ4Glc, GlcNAcβ3Galβ4Glc, Galα3Galβ4Glc, Galβ3Galβ4Glc, Glcα3Galβ4Glc, and Glcβ3Galβ4Glc.

5. The *Helicobacter pylori* binding substance according to claim 3 comprising one or several of the following oligosaccharide sequences

Galβ4GlcNAcβ3Galβ4Glc (lacto-N-neotetraose), Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc (para-lacto-N-neohexaose), and reducing-end polyvalent conjugates thereof.

6. The *Helicobacter pylori* binding substance according to any one of claims 1 – 5, wherein the substance is conjugated to a polysaccharide, preferably to a polylactosamine chain or a conjugate thereof.

7. The *Helicobacter pylori* binding substance according to any one of claims 1 – 5, wherein the substance is a glycolipid.

8. The *Helicobacter pylori* binding substance according to any one of claims 1 – 5, wherein the substance is an oligomeric molecule containing at least two or three oligosaccharide chains.

9. The *Helicobacter pylori* binding substance consisting of a micelle comprising one or more of the substances of any one of claims 1 – 8.

10. The *Helicobacter pylori* binding substance according to any one of claims 1 – 9, wherein the substance(s) is/are conjugated to a carrier.

11. The *Helicobacter pylori* binding substance according to any one of claims 1 - 10, wherein the substance is covalently conjugated with an antibiotic effective against *Helicobacter pylori*, preferably a penicillin type antibiotic.

12. The *Helicobacter pylori* binding substance or a mixture of substances according to any one of claims 1 – 10, wherein position C1 of reducing end terminal Glc or GlcNAc of the oligosaccharide sequence (OS) is oxygen linked (–O–) to an oligovalent or a polyvalent carrier (Z), via a spacer group (Y) and optionally via a monosaccharide or oligosaccharide residue (X), forming the following structure

$$[OS\text{–}O\text{–}(X)_n - Y]_m - Z$$

where integers m, and n have values $m \geq 1$, and n is independently 0 or 1; X is preferably lactosyl-, galactosyl-, poly-N-acetyl-lactosaminyl, or part of an O-glycan or an N-glycan oligosaccharide sequence, Y is a spacer group or a terminal conjugate such as a ceramide lipid moiety or a linkage to Z;

or an analog or a derivative of the substance of said structure having binding activity to *Helicobacter pylori.*

13. The substance according to any one of claims 1 - 12 for use as a *Helicobacter pylori* binding or inhibiting substance.

14. The substance according to any one of claims 1 - 12 for use as a medicament.

15. Use of the substance according to any one of claims 1 - 12 for the production of a composition having *Helicobacter pylori* binding or inhibiting activity.

16. Use of the substance according to any one of claims 1 - 12 for the production of a pharmaceutical composition for the treatment of any condition due to the presence of *Helicobacter pylori.*

17. A pharmaceutical composition comprising the substance according to any one of claims 1 - 12 for the treatment of any condition due to the presence of *Helicobacter pylori.*

18. The pharmaceutical composition according to claim 17, for the treatment of chronic superficial gastritis, gastric ulcer, duodenal ulcer, gastric adenocarcinoma, non-Hodgkin lymphoma in human stomach, liver disease, pancreatic disease, skin disease, heart disease, or autoimmune diseases including autoimmune gastritis and pernicious anaemia and non-steroid anti-inflammatory drug (NSAID) related gastric disease, or for prevention of sudden infant death syndrome.

19. A method for the treatment of a condition due to presence of *Helicobacter pylori*, wherein a pharmaceutically effective amount of the substance according to any one of claims 1 – 12 or the composition according to claims 17 or 18 is administered to a subject in need of such treatment.

20. The method according to claim 19, when said condition is caused by the presence of *Helicobacter pylori* in the gastrointestinal tract of a patient.

21. The method according to claim 19, for the treatment of chronic superficial gastritis, gastric ulcer, duodenal ulcer, gastric adenocarcinoma, non-Hodgkin lymphoma in human stomach, liver disease, pancreatic disease, skin disease, heart disease, or autoimmune diseases including autoimmune gastritis and pernicious anaemia and non-steroid anti-inflammatory drug (NSAID) related gastric disease, or for prevention of sudden infant death syndrome.

22. Use of the substance according to any one of claims 1 - 12, for the diagnosis of a condition due to infection by *Helicobacter pylori*.

23. A nutritional additive or composition containing the substance according to any one of claims 1 - 12.

24. The nutritional additive or composition according to claim 23 for use in infant food.

25. The method of treatment according to any one of claims 19 – 21 or the use according to claims 15 or 16, wherein the substance according to the invention is a nutritional additive or as part of a nutritional composition.

26. The method of treatment according to claim 25 wherein the substance is used in infant food.

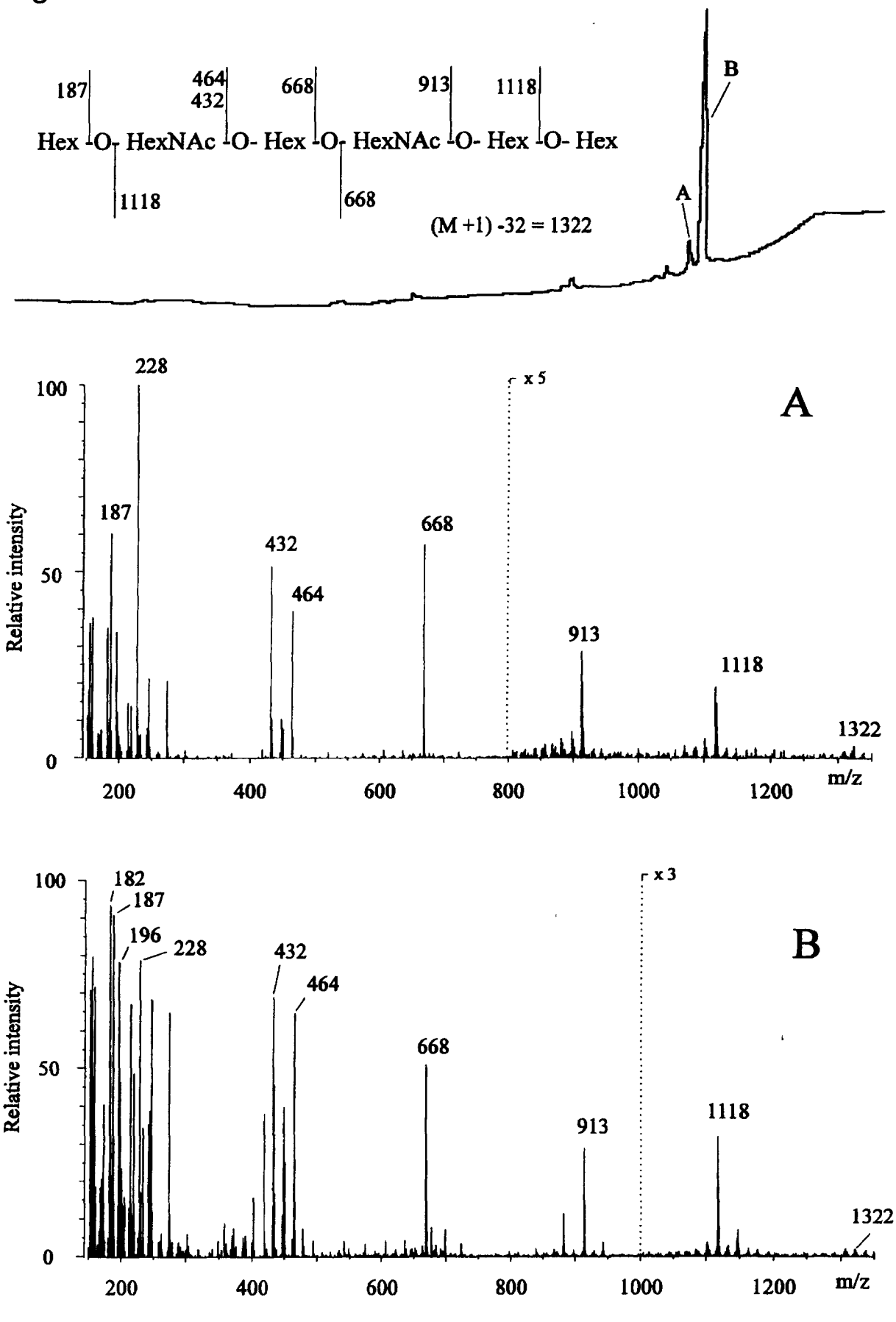
27. Use of the substance according to any one of claims 1 – 12, for the identification of bacterial adhesin.

28. Use of the substance according to any one of claims 1 – 12 or a substance identified according to claim 27, for the production of a vaccine against *Helicobacter pylori*.

29. Use of the substance according to any one of claims 1 – 12, for typing *Helicobacter pylori.*

30. The substance according to any one of claims 1 – 12, for use in *Helicobacter pylori* binding assays.

Fig. 1


187
464
432
668
913
1118
Hex -O- HexNAc -O- Hex -O- HexNAc -O- Hex -O- Hex
1118
668
(M +1) -32 = 1322
A
B
Relative intensity
100
50
0
228
187
432
464
668
913
1118
1322
x 5
A
200
400
600
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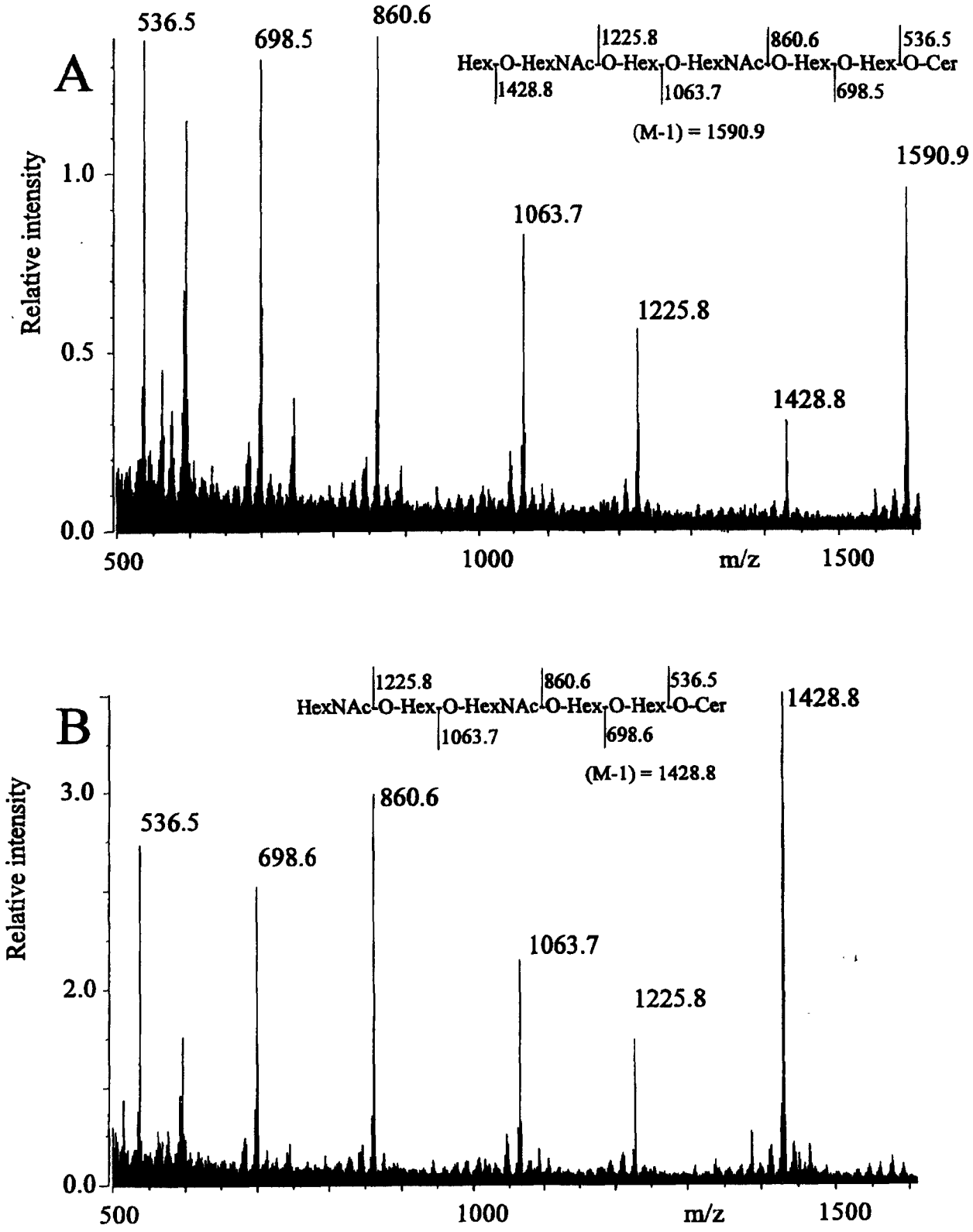
1322
x 3
B

Fig. 2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698.5
860.6
1225.8
860.6
536.5
Hex-O-HexNAc-O-Hex-O-HexNAc-O-Hex-O-Hex-O-Cer
1428.8
1063.7
698.5
(M-1) = 1590.9
1590.9
1063.7
1225.8
1428.8
Relative intensity
1.0
0.5
0.0
500
1000
m/z
1500
B
1225.8
860.6
536.5
HexNAc-O-Hex-O-HexNAc-O-Hex-O-Hex-O-Cer
1063.7
698.6
(M-1) = 1428.8
1428.8
860.6
536.5
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1225.8
Relative intensity
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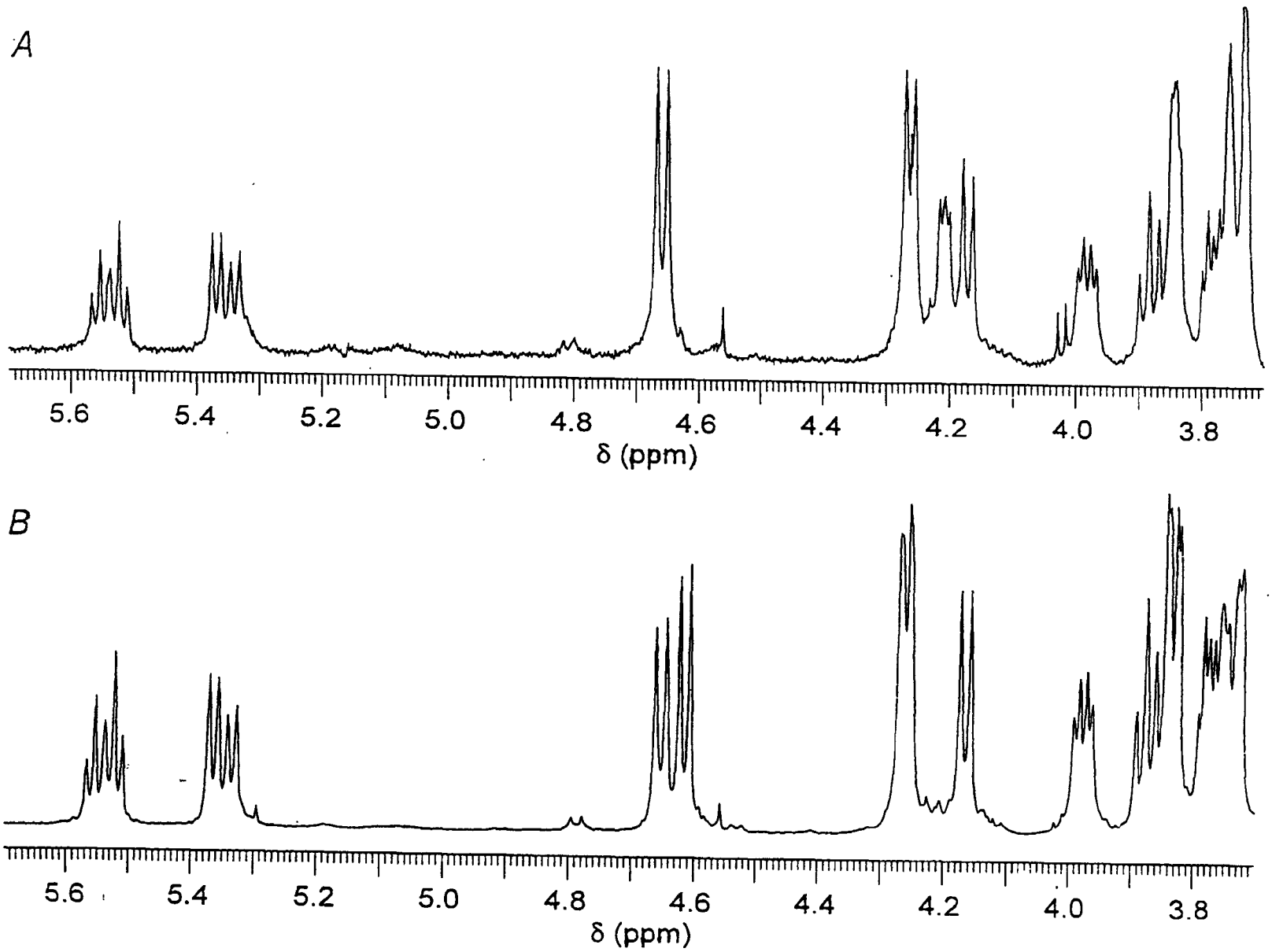
500
1000
m/z
1500

Fig. 3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δ (ppm)

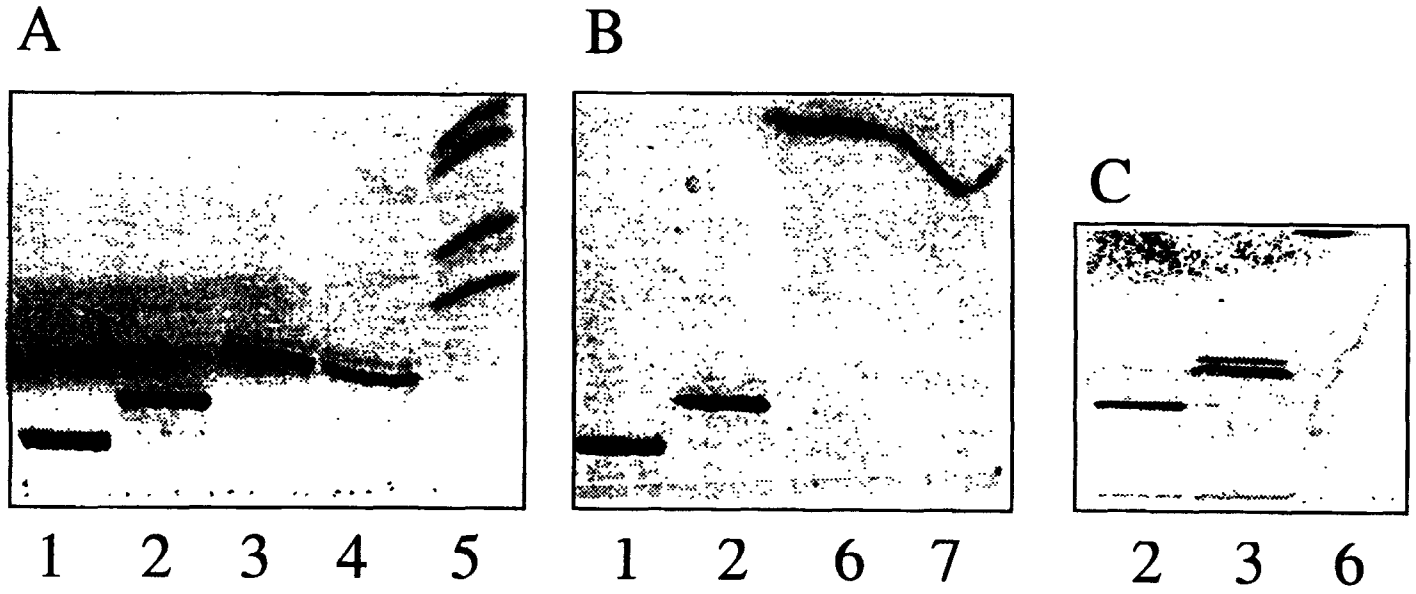


Fig. 4

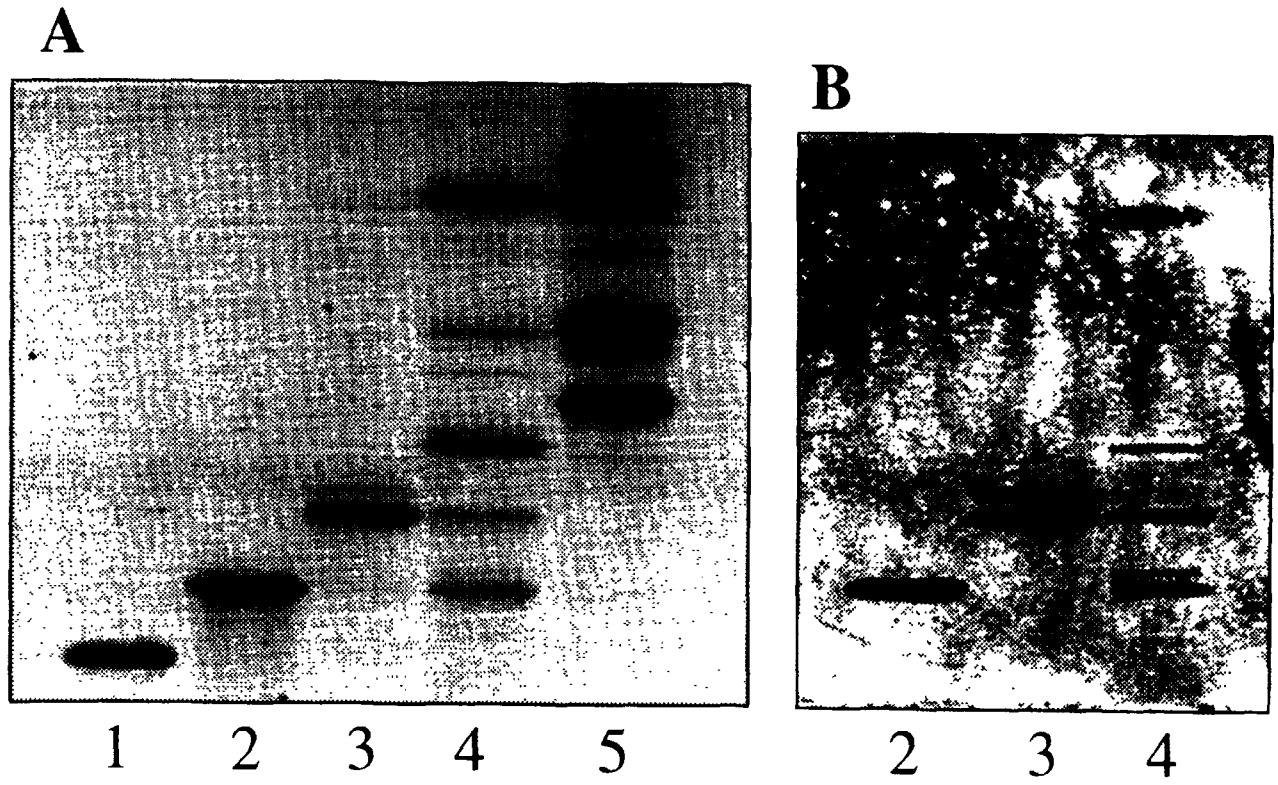
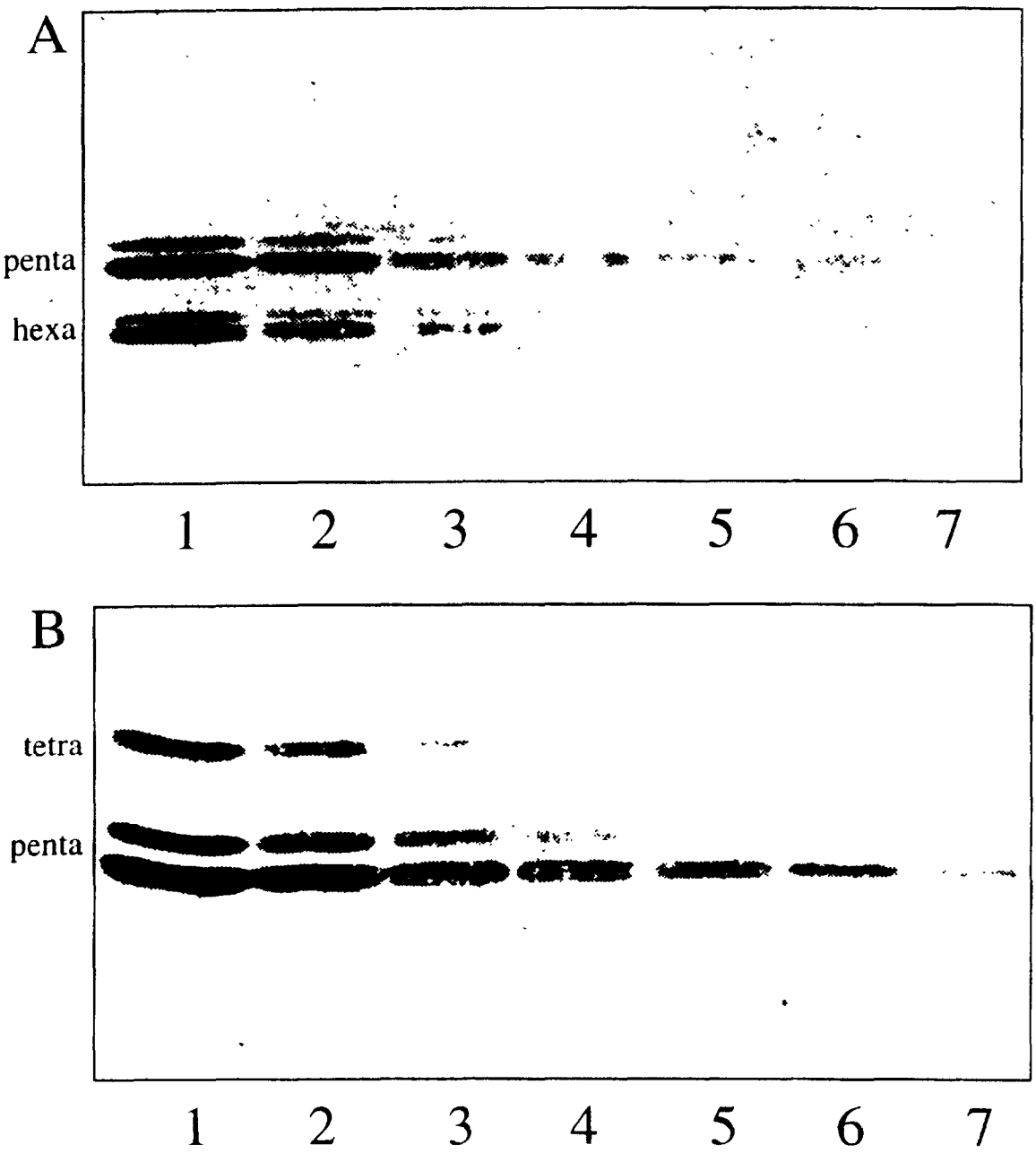


Fig. 5

Fig. 6



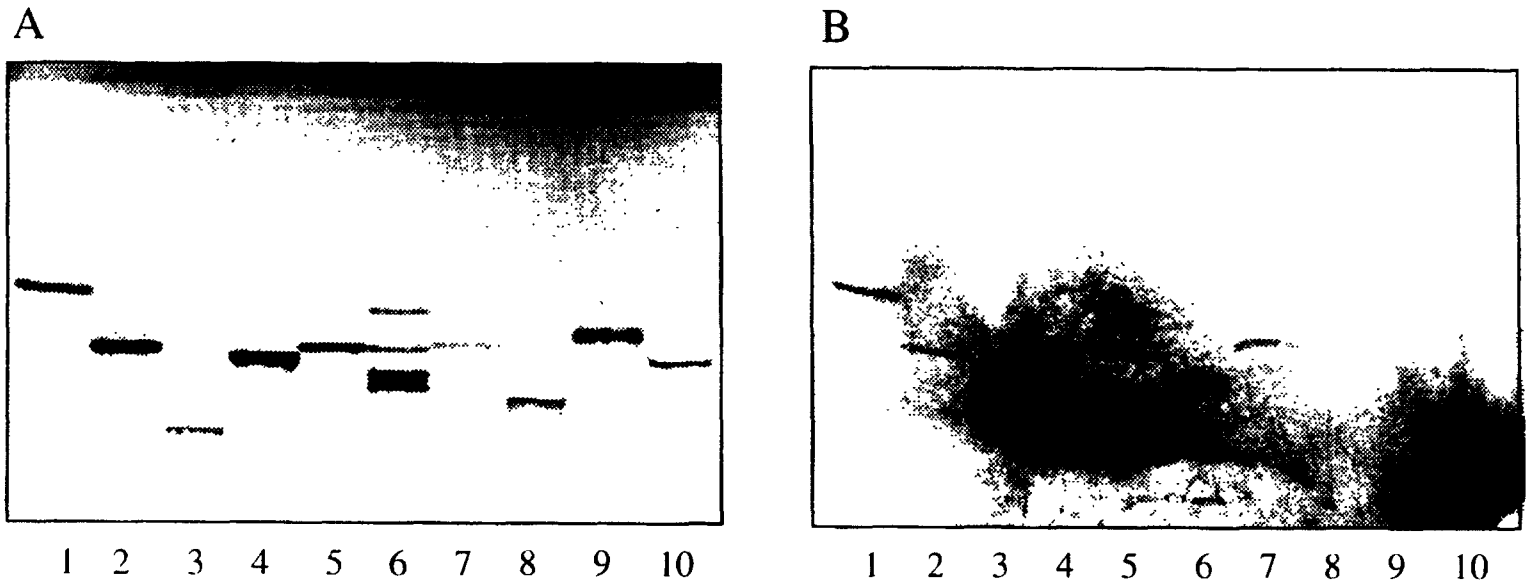


Fig. 7

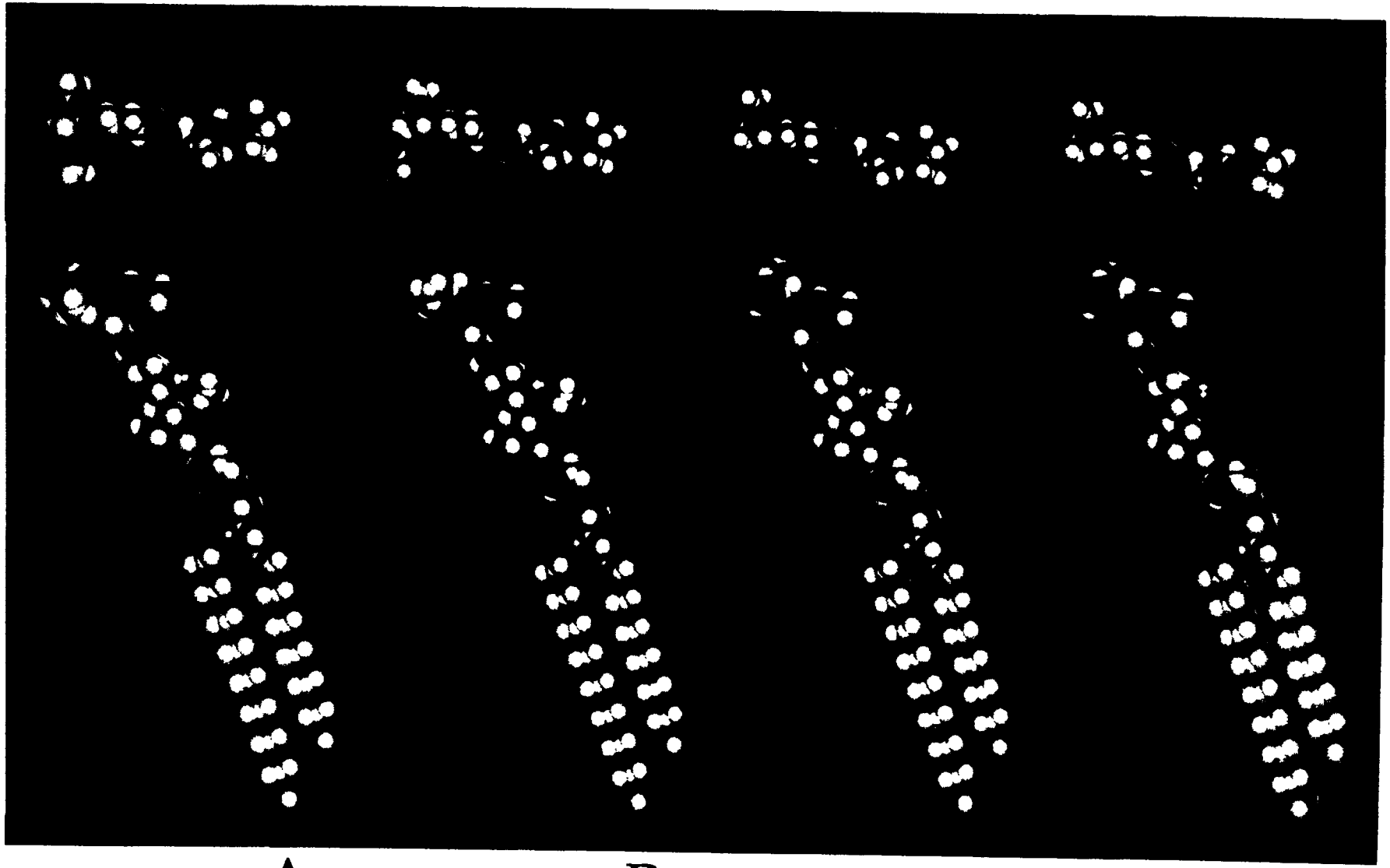


Fig. 8

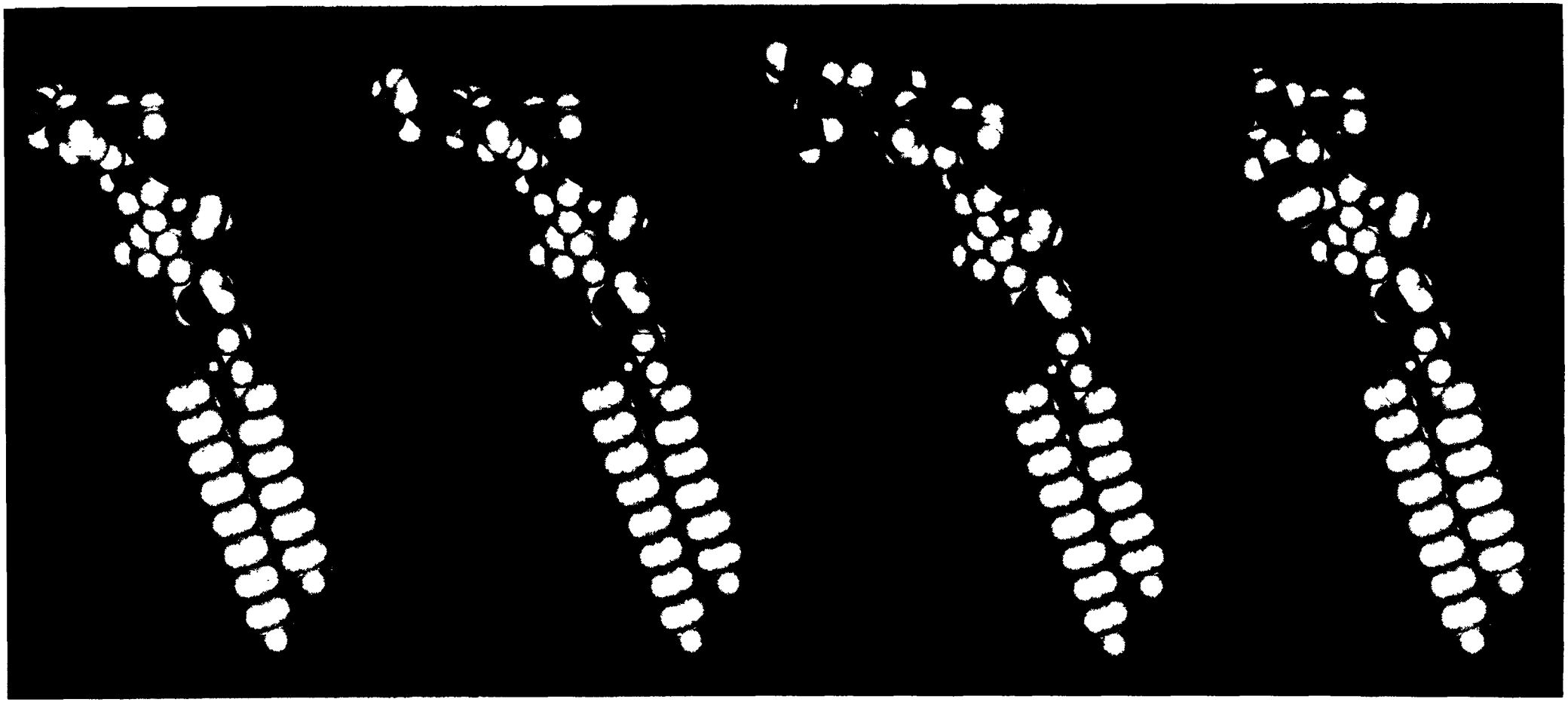
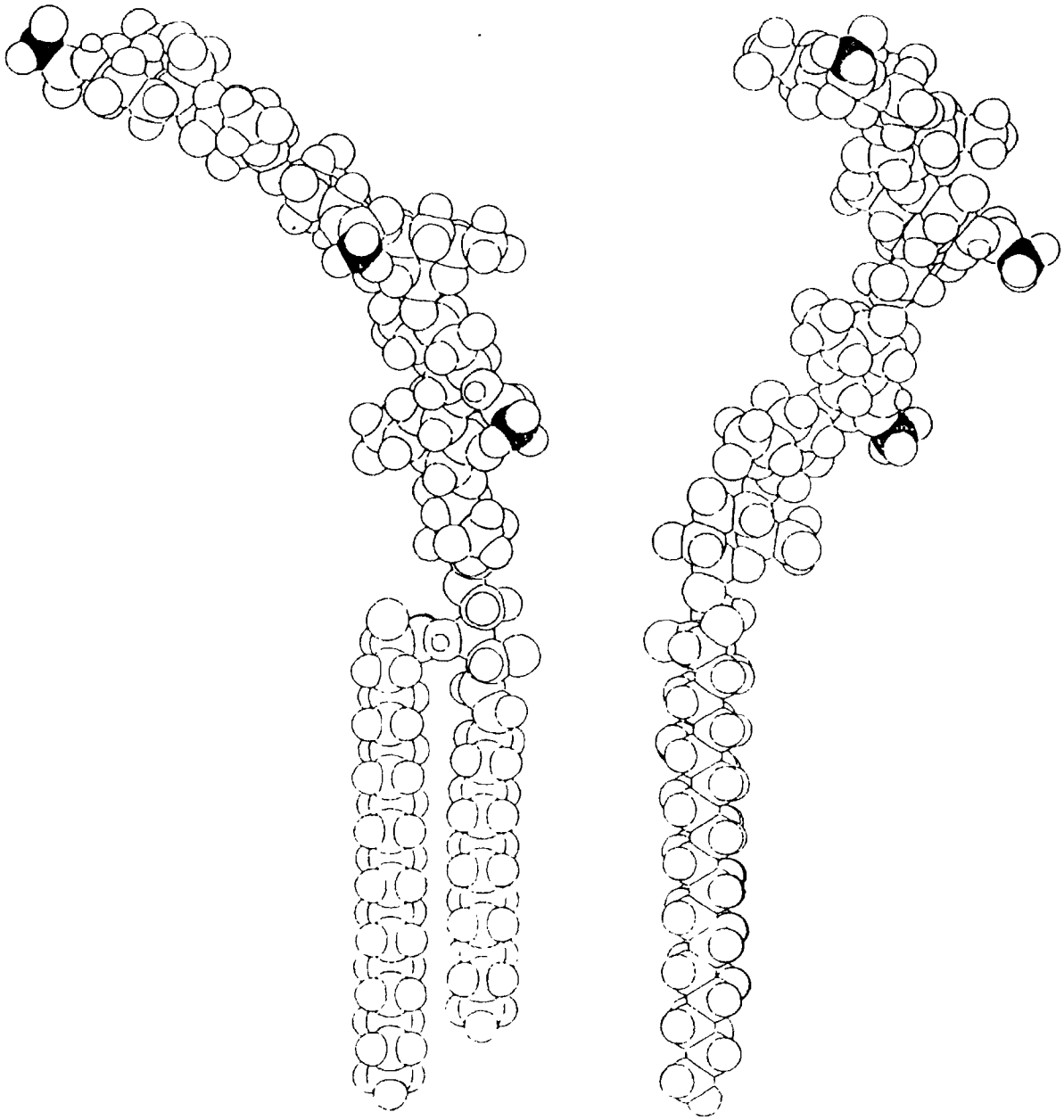


Fig. 9

Fig. 10



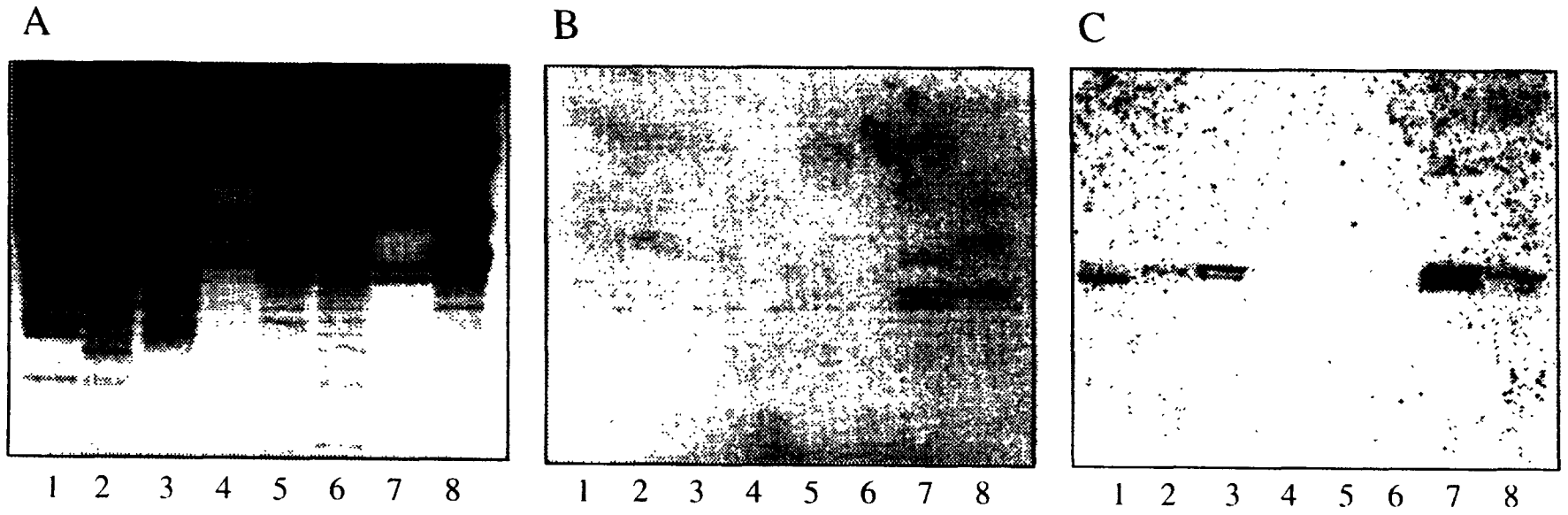


Fig. 11

PATENTTI- JA REKISTERIHALLITUS **TUTKIMUSRAPORTTI**

Patentti- ja innovaatiolinja

PATENTTIHAKEMUS NRO	LUOKITUS
20010118	C 07 H 15/04, 5/06

TUTKITTU AINEISTO

Tutkitut luokat : patenttijulkaisukokoelma (FI, SE, NO, DK), EPOQUE/Epodoc (DE, CH, FR, EP, WO, GB, US), Patent Abstracts of Japan

C07H 3/06, 5/04 - 5/06, 15/04 - 15/06

C08B 37/00

A61K 31/73, 31/7016 - 31/702, 31/7032, 31/726

epodoc lisäksi : ecla A61K 31/70J, C07H 13/04(C), C08B 37/00P(2)

VIITEJULKAISUT

Kategoria*)	Julkaisun tunnistetiedot	Koskee vaatimuksia
X	WO - A1 - 86/04065 (C07H 15/04), erityisesti sivun 16 rivit 28-35, sivun 25 riviltä 13 sivun 27 riville 12 sekä sivun 29 rivit 3-9.	1 - 10, 12, 14, 27
X	EP - B1 - 98 252 (C07H 15/04), erityisesti sivun 3 riviltä 37 sivun 4 riville 51.	1 - 10
X	EP - A1 - 89 938 (A61K 31/70)	1 - 10, 12, 14, 27
X	WO - A1 - 01/43751 (A61K 31/702)	1 - 30

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Y Patentoitavuuden kannalta merkittävä julkaisu, kun otetaan huomioon tämä ja yksi tai useampi samaan kategoriaan kuuluva julkaisu
A Yleistä tekniikan tasoa edustava julkaisu, ei kuitenkaan patentoitavuuden este

Päiväys	Tutkija
17.7.2001	