Pergamon

Bioorganic & Medicinal Chemistry, Vol. 4, No. 11, pp 1809–1817, 1996

PII: S0968-0896(96)00163-0
0968-0896/96 $15.00 + 0.00

PapG Adhesin from *E. coli* J96 Recognizes the Same Saccharide Epitope when Present on Whole Bacteria and as Isolated Protein

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Abstract—Purified PapG adhesin from the genetically well-defined uropathogenic *Escherichia coli* strain J96, as well as whole bacteria, were bound to microtiter plates that carried covalently bound globotetraose and galabiose. The binding was inhibited by soluble saccharide derivatives corresponding to the globoseries of glycolipids, including all di-, tri-, tetra-, and pentasaccharide fragments of the Forssman antigen and all monodeoxy analogues of galabiose. Analysis of the inhibition pattern showed no significant difference between purified adhesin and whole bacteria. The glucose unit at the reducing end of the natural saccharides was detrimental to PapG binding since deletion of the glucose unit increased the inhibitory power 10–20 fold. The five hydroxyl groups HO-6, -2′, -3′, -4′, -6′ of the galabiose unit were shown to be important for PapG binding, presumably via intermolecular hydrogen bonds. Copyright © 1996 Elsevier Science Ltd

Introduction

Adherence of bacteria to host cells constitutes the first step in many infections.[1] A well-known example is *Escherichia coli*, which is considered to be the most common cause of nonobstructive pyelonephritis in humans.[2] Adhesion of these uropathogenic *E. coli* bacteria to epithelial cells in the urinary tract is mediated by long thin hair-like fibers called P pili.[3] The detailed mechanism for P pili biogenesis has been determined, based on selective deletion of the genes responsible for the expression of pilus subunit proteins.[4–8] In short, P pili are made up of a large number of subunits arranged into a helical rod, which at the distal end exposes the adhesin subunit PapG, well situated for binding to glycolipids present at the surface of the host's epithelial cells.

Three classes (I–III) of PapG adhesins have been defined according to differences in the *E. coli*-erythrocyte agglutination patterns.[9,10] The prototype class I adhesin $PapG_{J96}$ from the pyelonephritic *E. coli* strain J96,[11] is the most extensively studied.[12–15] The class II adhesin $PapG_{AD110}$ was recently shown to be critical for bacterial binding to human kidney tissue[16] and, therefore, very likely a prerequisite for human disease.[17]

Following an extensive synthetic effort,[18–20] a panel of galabioside analogues (including all the monodeoxy derivatives) was tested for inhibition of hemagglutination of an *E. coli* strain (HB101/pPAP5) expressing only the $PapG_{J96}$ adhesin.[13] It was thus demonstrated that five hydroxyl groups (HO-6, -2′, -3′, -4′, and -6′) were important for the binding between saccharide and bacterium. It is presumed that the binding was mediated via the adhesin present at the pilus tips.

The $PapG_{J96}$ adhesin has later been purified as a stable, water soluble complex with the chaperone $PapD_{J96}$, using affinity chromatography on a column with galabiose covalently anchored to Sepharose beads.[21] The ability to isolate the GD complex where $PapG_{J96}$ is in a native-like conformation has facilitated chemical studies of the carbohydrate specificity of the adhesin (see below). We now report an investigation of the potential differences between the adhesin present as a soluble GD complex, and the adhesin present at the tip of bacterial pili. The results showed that the galabiose moiety of the various globoseries saccharides (Chart 1) is hydrogen bonded by the adhesin via the same five hydroxyl groups that were identified by hemagglutination for bacterial binding.[13] Furthermore, the glucose moiety present in the natural glycolipids interacts unfavorably with the adhesin. No significant differences in structural requirements for the saccharides were observed between the purified adhesin and the pilus-associated adhesin present on intact bacteria.

Results and Discussion

P-piliated HB101/pPAP5 bacteria and the corresponding purified adhesin[21] (GD complex) were compared in their binding to globotetraoside **1** and galabioside **2**, covalently attached to microtiter plates

Key words: *E. coli* J96, PapG adhesin, galabiose binding epitope, microtiter plate ELISA, Forssman-related saccharides.

(Glycoplate I and Glycoplate II; Fig. 1 and Experimental). Investigation of the bacterial binding was performed with Glycoplate I, whereas binding of the GD complex was evaluated with Glycoplate II; the GD complex bound more avidly to the latter than to the former.

The soluble saccharides **3–24** (see Experimental) were used as inhibitors of the binding, which led to a detailed picture of the saccharide binding epitope as discussed below. In the inhibition experiments, the optical density at 405 nm was plotted against the concentration of the inhibitor. The data were fitted to the standard binding function as described in the Experimental section. The IC_{50} values (Tables 1–4) were obtained as the inflexion points of the binding curves.

This work represents a refined methodology for studying the molecular basis of adhesin–receptor interactions using a chemically defined ELISA assay. An important advantage of this assay is that purified adhesins in native-like conformations (in this case the adhesin–chaperone GD complex) can be studied, since no significant differences in structural requirements for the receptor was observed between the purified adhesin and the pilus-associated adhesin. The ELISA assay has the added advantage over hemagglutination inhibition in that the reading is performed by spectroscopy and not by human judgement. Therefore, more accurate IC_{50} values can be obtained via curve fitting to the data points. In hemagglutination inhibition, an IC_{50} value is estimated from the interval between the lowest inhibitor concentration that inhibits and the highest concentration that does not inhibit.

ELISA assays typically use glycolipids or neo-glycoproteins that have been adsorbed onto plastic surfaces to test the binding of a lectin and the effect of soluble inhibitors. Adsorbing glycoconjugates on microtiter plates have two disadvantages. First, isolation or synthesis of glycolipids or synthesis of neo-glycoproteins needed in the experiments is laborious. Second, the binding of G adhesins to glycolipids is very sensitive to how the glycolipid is presented.[9,10,22] To circumvent these disadvantages, we coupled **1** and **2**, via a flexible spacer-arm covalently to microtiter plates provided with secondary amino groups. The chemically well-defined and stable amide linkage was hypothesized to allow a homogeneous presentation of all significant epitopes to the adhesin.

The synthetic inhibitors **3–24** (Chart 1) were submitted to four sets of experiments: (i) definition of essential monosaccharide units, using compounds **3–11** (Table 1); (ii) definition of essential hydroxyl groups in galabiosides, using compounds **12–19** (Table 2); (iii) investigation of the importance of hydrophobic aglycons, using compounds **9**, **12**, and **20–22** (Table 3); (iv) inhibitor efficiency of thio-glycoside analogs, using compounds **9**, **23**, and **24** (Table 4). The galabiosides **9** and **12** were used as reference compounds ($K_{rel} = 100\%$).

The use of synthetic analogues of natural saccharides for biological investigations requires that both analogues and natural compounds have similar conformations; only then can it be assumed that differences in inhibitory power reflect the involvement of individual monosaccharides in the interaction with the adhesin. We recently reported the conformations, by NMR in water solution and by calculations, of the Forssman fragments **3–11**,[23] the deoxy galabiosides **12–19**,[24] and the thio-galabiosides **23** and **24**.[25] No significant deviations from the natural saccharides were found.

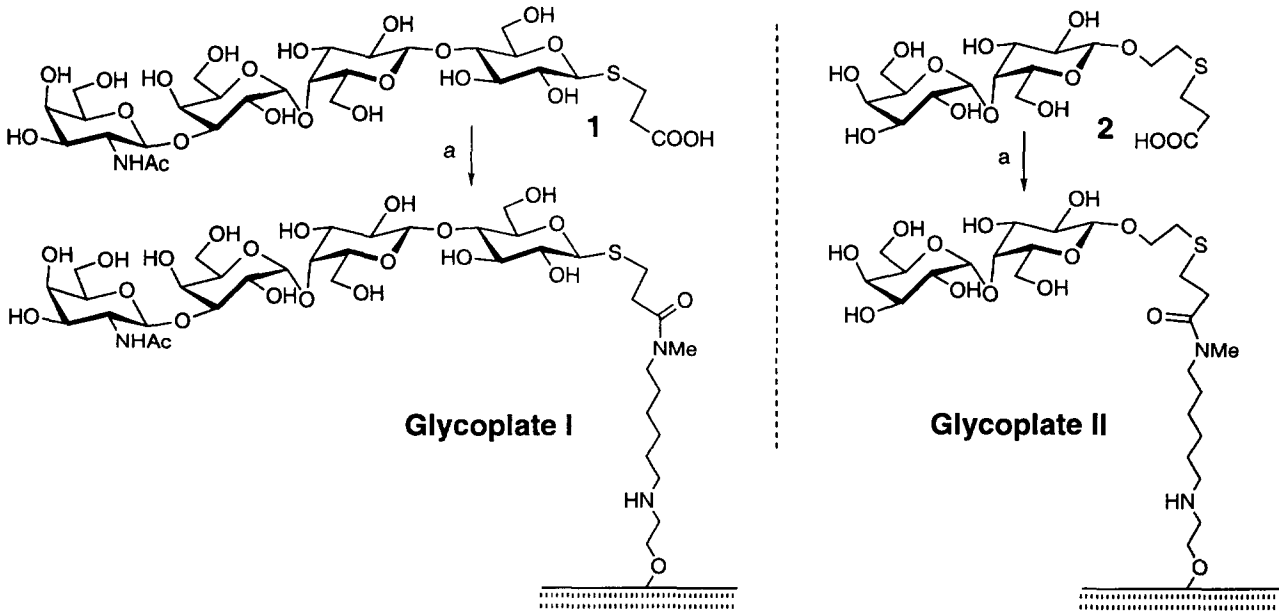

Figure 1. Covalent coupling of the spacer glycosides **1** and **2** to aminoalkyl-modified microtiter plates. (a) CovaLink® plate; *N*-hydroxysuccinimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide.

Definition of essential monosaccharide units

The glucose unit of the globoseries of saccharides interacts unfavorably with the $PapG_{J96}$ adhesin. Forssman fragments devoid of the galabiose disaccharide moiety (**6**, **10**, **11**) were all inactive confirming the well-established hypothesis[26] that galabiose is the minimum epitope required for binding (Table 1). Elongation of the galabioside **9** in the 3′ position with GalNAcβ or GalNAcα3GalNAcβ (**9**

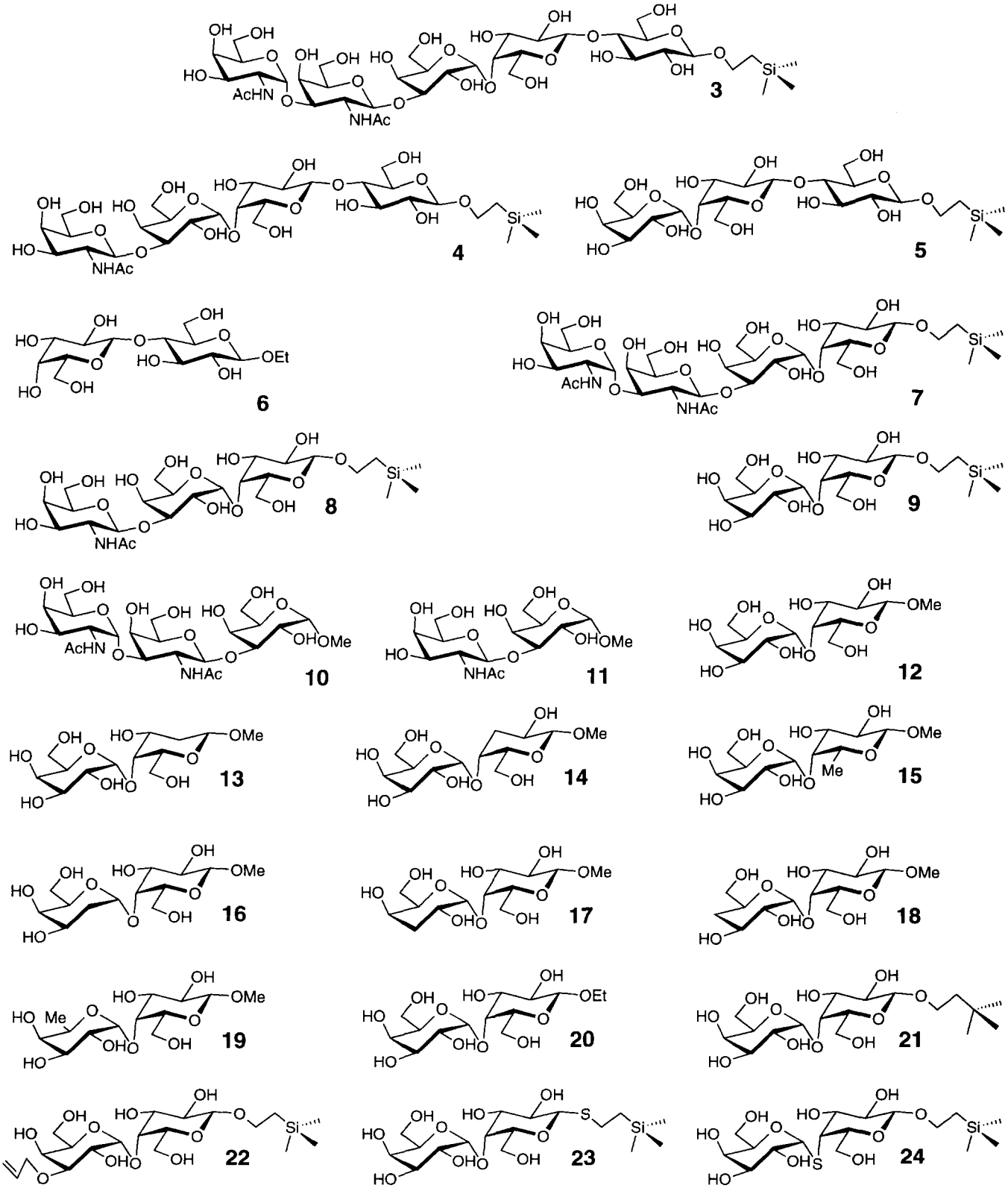

Chart 1. Synthetic, water-soluble analogues of the globoseries of glycolipids, used as inhibitors of $PapG_{J96}$ binding to Glycoplates I and II.

Table 1. Inhibition of *E. coli* HB101/pPAP5 bound to Glycoplate I and GD complex bound to Glycoplate II, using as inhibitors the Forssman pentasaccharide **3** and the fragments **4–11**[a]

Inhibitor	HB101/pPAP5			GD complex		
	IC_{50} (mM)	K_{rel} (%)	$\Delta\Delta G$ (kJ mol^{-1})	IC_{50} (mM)	K_{rel} (%)	$\Delta\Delta G$ (kJ mol^{-1})
3	4.0±0.9	18	4.2	>10	<2	>9.7
4	2.8±0.5	26	3.4	>10	<2	>9.7
5	6.4±2.1	11	5.4	>10	<2	>9.7
6	>50	<1.4	>10.5	n.d.	n.d.	n.d.
7	0.73±0.14	99	0.0	0.13±0.03	146	−0.94
8	1.30±0.35	55	1.5	0.22±0.05	86	0.36
9	0.72±0.14	100	0.0	0.19±0.05	100	0.0
10	>50	<1.4	>10.5	>10	<2	>9.7
11	>50	<1.4	>10.5	>10	<2	>9.7

[a]The K_{rel} and $\Delta\Delta G$ values were related to the TMSEt galabioside **9**.

Table 2. Inhibition of *E. coli* HB101/pPAP5 bound to Glycoplate I and GD complex bound to Glycoplate II, using as inhibitors the methyl galabioside **12** and the deoxy analogues **13–19**[a]

Inhibitor	HB101/pPAP5			GD complex		
	IC_{50} (mM)	K_{rel} (%)	$\Delta\Delta G$ (kJ mol^{-1})	IC_{50} (mM)	K_{rel} (%)	$\Delta\Delta G$ (kJ mol^{-1})
12	4.2±1.8	100	0.0	0.54±0.04	100	0.0
13	9.0±3.3	47	1.9	1.09±0.06	60	1.3
14	15.0±5.1	28	3.2	1.95±0.15	33	2.7
15	>40	<9.5	>5.8	9.00±0.70	7	6.6
16	15.8±3.0	27	3.2	3.40±0.15	19	4.1
17	>40	<9.5	>5.8	>20	<3	>8.5
18	>60	<7.0	>6.6	>20	<3	>8.5
19	>60	<7.0	>6.6	>20	<3	>8.5

[a]The K_{rel} and $\Delta\Delta G$ values were related to the methyl galabioside **12**.

Table 3. Inhibition of *E. coli* HB101/pPAP5 bound to Glycoplate I and GD complex bound to Glycoplate II, using as inhibitors the galabiosides **9**, **12**, and **20–22**, having aglycons of varying hydrophobicity[a]

Inhibitor	HB101/pPAP5			GD complex		
	IC_{50} (mM)	K_{rel} (%)	$\Delta\Delta G$ (kJ mol^{-1})	IC_{50} (mM)	K_{rel} (%)	$\Delta\Delta G$ (kJ mol^{-1})
9	0.54±0.17	161	−1.2	0.25±0.04	236	−2.1
12	1.05±0.35	100	0.0	0.59±0.09	100	0.0
20	0.90±0.28	115	−0.3	0.44±0.07	134	−0.70
21	0.66±0.15	159	−1.1	±0.30±0.05	196	−1.7
22	0.66±0.16	159	−1.1	±0.24±0.04	246	−2.2

[a]The K_{rel} and $\Delta\Delta G$ values were related to the methyl galabioside **12**.

Table 4. Inhibition of *E. coli* HB101/pPAP5 bound to Glycoplate I and GD complex bound to Glycoplate II, using as inhibitors the TMSEt galabiosides **9**, **23**, and **24**[a]

Inhibitor	HB101/pPAP5			GD complex		
	IC_{50} (mM)	K_{rel} (%)	$\Delta\Delta G$ (kJ mol^{-1})	IC_{50} (mM)	K_{rel} (%)	$\Delta\Delta G$ (kJ mol^{-1})
9	0.65±0.17	100	0.0	0.16±0.04	100	0.0
23	0.92±0.27	70	0.9	0.31±0.06	52	1.6
24	n.d.	n.d.	n.d.	4.40±1.10	4	8.0

[a]The K_{rel} and $\Delta\Delta G$ values were related to the TMSEt galabioside **9**.

versus **7** and **8**) did not affect the inhibitory power significantly, indicating that the binding site of $PapG_{J96}$ probably does not expand beyond the Galα unit. The class II adhesin $PapG_{AD110}$, considered to be important in human infections,[17] has recently been shown to be somewhat more efficiently inhibited by compounds carrying a GalNAcβ unit.[16]

Elongation at the reducing end of **9** with a Glcβ unit (compounds **3**, **4**, and **5**) significantly reduced the inhibitory power for both the bacteria and the GD complex. The effect was more pronounced with the latter. These results were consistent with previous hemagglutination inhibition data[13] and also with the ability of the GD complex to bind galabioside (Glycoplate II, Fig. 1) more strongly than globotetraoside (Glycoplate I), covalently coupled to the microtiter plates.

Reduction of the inhibitory effect by the Glcβ unit was surprising since the proposed natural receptor[9,10] for $PapG_{J96}$, globotriosyl ceramide (GbO_3), contains the Glcβ unit. In contrast, the adhesin considered to be important in human infections, class II $PapG_{AD110}$, requires the Glcβ unit for efficient binding.[16]

Earlier reports on the specificity of $PapG_{J96}$ are contradictory, since *E. coli* J96 binds erythrocytes which predominantly express GbO_3 (rabbit) and GbO_4 (human), but not human kidney tissue[16] or human uroepithelial cells,[27] which carry substantial amounts of both GbO_3 and GbO_4. The $PapG_{J96}$ adhesin may be rare and it is the only representative of class I adhesins known. Since it binds soluble GbO_3 poorly and does not bind human kidney tissue, its status as a virulence factor in human pyelonephritis is questionable. Possibly, the $PapG_{J96}$ adhesin was evolved for colonization of an unknown ecological niche with an as of yet unknown natural galabiose-containing receptor, as earlier suggested.[17] The natural receptor might be galabiosyl ceramide. However, the unique agglutination of rabbit erythrocytes by $PapG_{J96}$ has not been demonstrated to be mediated via binding to galabiosyl ceramide.[10]

Definition of essential hydroxyl groups

The hydrogen bonding pattern is the same for whole bacteria and the GD complex. The inhibition data for the deoxygenated galabiosides **13–19** are shown in Table 2. The 2-deoxy analogue **13** retained ~55% ($\Delta\Delta G \approx 1.5$ kJ mol^{-1}) inhibitory power compared with the reference compound **12**, suggesting that HO-2 of Galα4Gal is probably not involved in hydrogen bonding to the adhesin. [Fersht et al.[28] found that deletion (by site-specific mutagenesis) of a hydrogen-bonding residue in tyrosyl-tRNA synthetase resulted in decreased binding strength of the ligand by 2.1–6.3 kJ mol^{-1}.] The 3-deoxy and 2′-deoxy galabiosides **14** and **16** retained moderate inhibitory powers of ~30% and ~25%, respectively, corresponding to $\Delta\Delta G \approx 3$ kJ mol^{-1}. The 6-deoxy analogue **15** only retained a small fraction of the binding strength (7% binding to the GD complex; $\Delta\Delta G = 6.6$ kJ mol^{-1}) and the 3′-, 4′-, and 6′-deoxy analogues **17**, **18**, and **19** had virtually lost the inhibitory power. These results are almost identical to the previously reported hemagglutination inhibition data[13] and no significant binding differences between whole bacteria and the GD complex were detected.

The results from the inhibition experiments show that the 6-, 3′-, 4′-, and 6′-hydroxyl groups (cf. **15**, **17**, **18**, **19**) form critical hydrogen bonds to the adhesin, the 2′-hydroxyl group (cf. **16**) forms a somewhat weaker hydrogen bond, and the 2-hydroxyl group (cf. **13**) is probably not in direct contact with the adhesin. The moderate inhibitory power of the 3-deoxy analogue **14** indicates that the 3-hydroxyl group is not involved in a strong hydrogen bond but rather situated in a nonpolar pocket in the adhesin. This interpretation is augmented by the finding that the 3-C-methyl analogue (3-OH replaced by 3-CH_3) was a stronger inhibitor than the 3-deoxy analogue **14** in the hemagglutination assay.[13] The hydroxyl groups of intermediate importance for binding (HO-3, -6, and -2′) are probably involved in intramolecular hydrogen bonding; a 3-OH→O-5′ bond was found in the galabiose crystal[29] and a 2′-OH—6-OH bond was shown by NMR and indicated by calculation of the conformation of **12**.[25]

The overall hydrogen bonding pattern between the galabiose moiety and the $PapG_{J96}$ adhesin is identical to that of the class II $PapG_{AD110}$ adhesin.[16] This suggests that the two different adhesins recognize the same epitope of galabiose and that the difference between them is due to other interactions, such as additional binding of the $PapG_{AD110}$ adhesin to the glucose unit as discussed above. It is also interesting to note that the Gram-positive organism *Streptococcus suis* recognizes a different galabioside epitope, where the 2-, 3-, 4′- and 6′-hydroxyl groups are used for hydrogen bonding to the bacterial adhesin.[30]

Importance of hydrophobic substituents

Hydrophobic aglycons contribute favorably to binding. Inhibition data for galabiosides having aglycons of varying hydrophobicity (**12**, **20**, **21**, **9**) are shown in Table 3. With both bacteria and the GD complex, a small increase in inhibitory power was seen with increasing hydrophobicity (**12**→**20**→**21**→**9**). The quite similar inhibitory power of **9** and **21** shows that the silicon atom of **9** has no effect *per se*. The 3′-*O*-allyl galabioside **22** had the same inhibitory power as **9**, showing that substituents in the 3′ position are unimportant (see also **9** versus **7** and **8**, Table 1). In the hemagglutination assay, a 3′-*O*-methyl galabioside was a somewhat stronger inhibitor than the reference galabioside.[13]

Inhibitor efficiency of thio-glycoside analogs

Replacing O with S in the galabiose intersaccharidic bond decreases the inhibitory power significantly. Inhibition data for the thioglycosides **23** and **24** are shown in Table 4. Thioglycosides are often used as

hydrolytically stabilized analogues of receptor-active saccharides. Substitution of O-1 by sulfur (→**23**) reduced the inhibitory power only slightly, whereas the hemagglutination experiment[13] indicated a small increase in inhibitory power by such substitution. On the other hand, replacing the oxygen atom of the intersaccharidic linkage (O-1′) with a sulfur atom (→**24**) almost abolished the binding to the GD complex. This is probably due to a change in the spatial arrangement of HO-6 relative to HO-2′, thus destroying the 2′-OH—6-OH intramolecular hydrogen bond required for optimal hydrogen bonding to the adhesin (cf. Fig. 2). An intramolecular hydrogen bond between HO-2′ and HO-6 was found by NMR to be present in **9** in DMSO-d6-solution but absent in **24**. The conformational difference between **9** and **24** was also found by molecular mechanics calculations.[25]

Conclusions

The binding of galabiosides by the $PapG_{J96}$ adhesin was virtually the same, whether the adhesin was present on whole bacteria or in the form of the adhesin–chaperone GD complex. Therefore, the saccharide-binding domain of the $PapG_{J96}$ adhesin has a similar fold in both modes of presentation. A combination of the results from this and previous investigations[13,16,25] reveals a plausible model of the binding epitope of galabiose-containing saccharides, exemplified in Figure 2 by a globotetraoside.

The unfavorable interaction of the class I adhesin $PapG_{J96}$ with the Glc unit of GbO_3 and GbO_4 is in sharp contrast to the class II adhesins common in human pyelonephritic *E. coli*, such as $PapG_{AD110}$, which require the presence of a Glc unit for strong binding. Due to the differences between class I and class II adhesins, human erythrocytes should be used with caution in screening for human uropathogenic *E. coli* adhesins, since they are agglutinated by both class I and class II adhesin-carrying bacteria, while only class II adhesins bind to human kidney tissue. In addition, epidemiological screening for G adhesins should be performed with these differences in mind.

Experimental

Abbreviations used: TS: tryptic soy agar; PBS: phosphate buffered saline; BSA: bovine serum albumin; Covabuffer: PBS with 2 M NaCl, 0.04 M $MgCl_2$, 0.05% Tween 20.

Saccharides **3**–**20** and **22**–**24** were synthesized as reported: **3**,[31] **4**,[32] **5**,[13] **6** (commercial), **7**,[31] **8**,[32] **9**,[33] **10**,[31] **11**,[32] **12**,[34] **13**,[19] **14**,[18] **15**,[19] **16**,[20] **17**,[20] **18**,[20] **19**,[20] **20**,[35] **22**,[32] **23**,[25] **24**,[25].

2-Carboxyethyl 4-*O*-{4-*O*-[3-*O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-α-D-galactopyranosyl]-β-D-galactopyranosyl}-1-thio-β-D-glucopyranoside (1). To a solution of 2-(trimethylsilyl)ethyl 2,3,6-tri-*O*-acetyl-4-*O*-{2,3,6-tri-*O*-acetyl-4-*O*-[2,4,6-tri-*O*-acetyl-3-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-galactopyranosyl)-α-D-galactopyranosyl]-β-D-galactopyranosyl}-β-D-glucopyranoside[32] (1.12 g, 0.85 mmol) in dichloromethane (4.1 mL) was added acetic anhydride (372 μL) and boron trifluoride etherate (221 μL).[33] After 3 h the mixture was diluted with dichloromethane, washed with satd sodium hydrogencarbonate, dried (Na_2SO_4), and concentrated. The crude β-acetate was dissolved in dichloromethane (12.6 mL) and 2-mercaptopropionic acid (287 μL) and boron trifluoride etherate (221 μL) were added. After 6 h, the reaction was quenched with triethyl amine (0.5 mL), diluted with dichloromethane, washed with 1 M hydrochloric acid in satd sodium chloride, dried (Na_2SO_4), and concentrated. Flash chromatography (CH_2Cl_2:MeOH 20:1, 1% AcOH) of the residue yielded acetylated **1** (806 mg). A major portion of this material (791 mg, 0.61 mmol) was de-*O*-acetylated with methanolic sodium methoxide (0.06 mM, 26 mL) overnight, neutralized with Amberlite IR-120 (H^+) resin, filtered and concentrated to give **1** (498 mg, 73%), $[\alpha]_D^{25}$ +78° (*c* 0.44, H_2O). ^1H NMR data (300 MHz, D_2O): δ 4.86 (d, 1 H, *J* = 3.7 Hz, H-1″), 4.59 (d, 1 H, *J* = 8.3 Hz, H-1‴), 4.54 (d, 1 H, *J* = 10.0 Hz, H-1), 4.47 (d, 1 H, *J* = 7.6 Hz, H-1′), 4.33 (br t, 1 H, *J* = 6.0 Hz, H-5″), 4.20 (br d, 1 H, *J* = 2.2 Hz, H-4″), 2.00 (s, 3 H, Ac), 2.92 (m, 2 H, SCH_2), 2.63 (m, 2 H, CH_2CO). ^{13}C NMR data (75 MHz, D_2O, 1,4-dioxane as internal standard): δ 178.6, 176.0, 104.1, 101.2, 86.2, 79.6, 79.5, 79.3, 78.0, 76.7, 76.3, 75.8, 73.0, 72,9, 71.7, 71.6, 71.1, 69.8, 68.6, 68.5, 61.8, 61.2, 61.1, 61.0, 53.4, 26.7, 23.1, 22.0. HRMS: calcd for $C_{29}H_{49}NNaO_{22}S$ (M + Na): *m/z* 818.2365; found: *m/z* 818.2349.

2-(2-Carboxyethylthio)ethyl 4-*O*-(α-D-galactopyranosyl)-β-D-galactopyranoside (2). 2-(2-Methoxycarbonylethylthio)ethyl 4-*O*-(α-D-galactopyranosyl)-β-D-galactopyranoside[35] (257 mg, 0.53 mmol) was hydrolysed in aq. sodium hydroxide (30 mL, 0.04 mM) for 1 h, neutralized with Duolite C26 (H^+) resin, filtered, and concentrated. Flash chromatography (CH_2Cl_2:MeOH:H_2O 65:35:5, 1% AcOH) of the residue gave quantitatively **2**; $[\alpha]_D^{25}$ +76° (*c* 0.53, H_2O). ^1H NMR data (300 MHz, D_2O): δ *4.96 (d, 1 H, J* = 3.9 Hz, H-1′), 4.49 (d, 1 H, *J* = 7.8 Hz, H-1), 4.37 (br t, 1 H, *J* = 6.0 Hz, H-5′), 2.84 (m, 4 H, CH_2SCH_2), 2.58 (t, 2 H, *J* = 7.2 Hz, CH_2CO). HRMS: calcd for $C_{17}H_{31}O_{13}S$ (M + 1): *m/z* 475.1485; found: *m/z* 475.1493.

3,3-Dimethylbutyl 4-*O*-(α-D-galactopyranosyl)-β-D-galactopyranoside (21). 2,3,6-Tri-*O*-acetyl- 4-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-β-D-galactopyranose[33] (426 mg, 0.63 mmol) was treated with boron trifluoride etherate (229 μL) and 3,3-dimethylbutanol (101 μL) in dichloromethane (30 mL) for 2 h, then diluted with dichloromethane, washed with satd sodium hydrogencarbonate, dried (Na_2SO_4), and concentrated. Flash chromatography (heptane:EtOAc 2:1) of the

residue yielded acetylated **21** (282 mg). A portion of this material (268 mg, 0.37 mmol) was de-*O*-acetylated with methanolic sodium methoxide (0.06 mM, 40 mL) for 6 h, neutralized with Amberlite IR-120 (H^+) resin, filtered and concentrated to give **1** (159 mg, 62%), $[\alpha]_D^{25}+83°$ (*c* 0.43, H_2O). ^1H NMR data (300 MHz, D_2O): δ 4.93 (d, 1 H, $J=3.9$ Hz, H-1′), 4.42 (d, 1 H, $J=7.7$ Hz, H-1), 4.33 (br t, 1 H, $J=6.0$ Hz, H-5′), 1.53 (m, 2 H, CH_2CMe_3), 0.88 (s, 9 H, 3 Me). HRMS: calcd for $C_{18}H_{35}O_{11}$ (M+1): *m/z* 427.2179; found: *m/z* 427.2172.

Covalent coupling of compound 1 or 2 to microtiter wells

Microtiter plates functionalized with secondary amino groups (CovaLink® microtiter plates, A/S Nunc, P.O. Box 280, Kamstrup, DK4000 Roskilde, Denmark) were

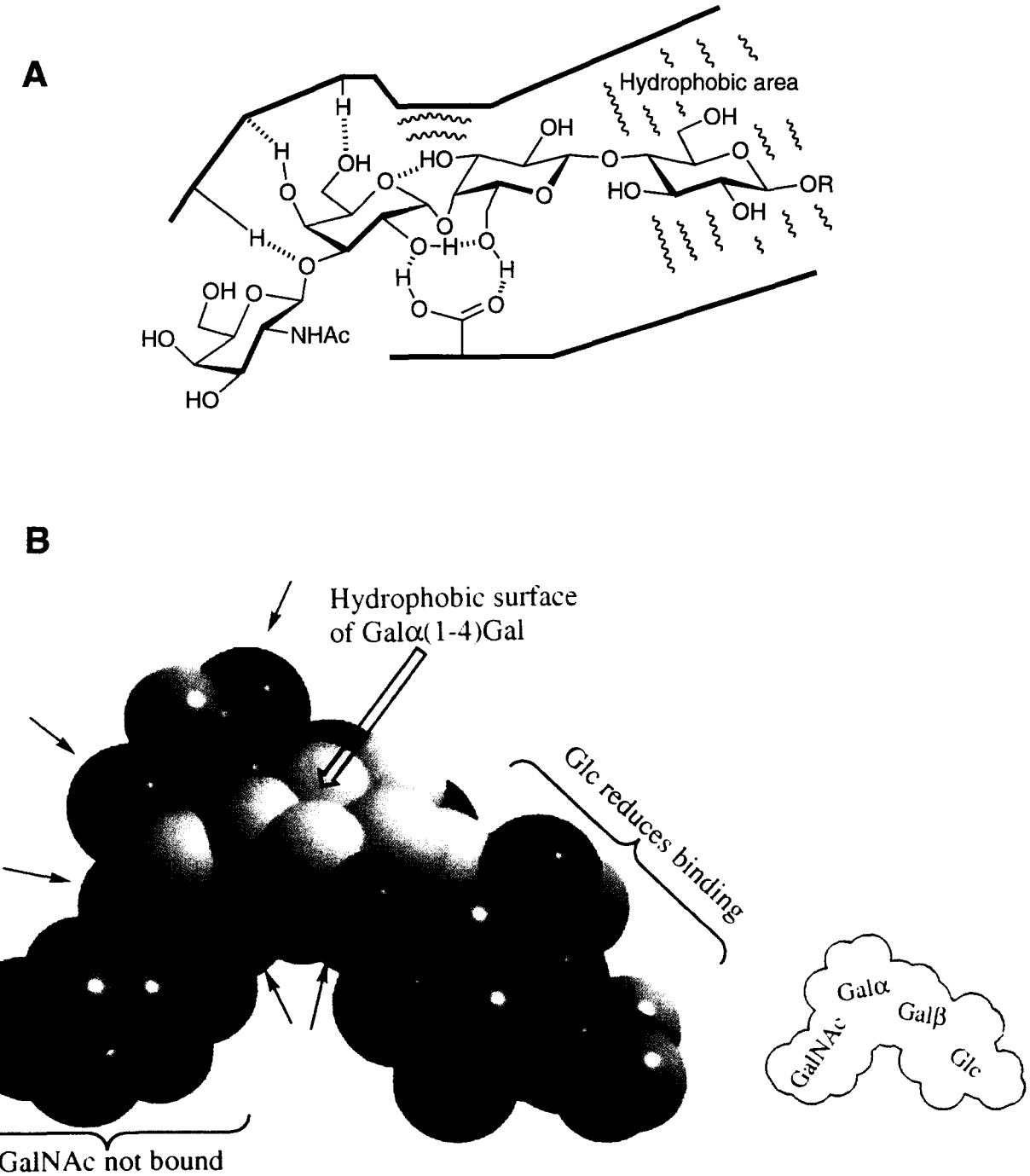

Figure 2. (a) Schematic model of the globotetraose binding epitope. Suggested hydrogen bonds are shown as dashed lines. (b) Space-filling model of methyl globotetraoside (atomic coordinates were taken from ref. 23). Hydroxyl group hydrogens are not shown. The oxygen atoms involved in hydrogen bonding with the $PapG_{J96}$ adhesin (O-6, 2′, 3′, 4′, 6′) are indicated by arrows. The hydrogens shown in light grey form a large hydrophobic surface suitable for contact with a nonpolar surface of the adhesin.

used for covalent coupling of the carboxylic acid-functionalized saccharides **1** and **2**, essentially as described by Nunc (Fig. 1). To each well was added 50 μL of an aq. soln containing **1** or **2** (4 mM) and *N*-hydroxysuccinimide (8 mM) was added, followed by 50 μL of a solution containing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (8 mM). The plate was shaken for 2–6 h, then washed three times with Covabuffer. The resulting Glycoplate I and Glycoplate II were fully functional after storage at 4 °C for at least 8 weeks (no long-term storage test was conducted). The optimal coupling concentrations of **1** and **2** were obtained by performing the coupling with a dilution series of **1** and **2**. Incubation and detection of bacteria or GD complex were then performed as in the inhibition experiments described below.

Bacterial strains and plasmids

Escherichia coli HB101[36] was used as a host in all experiments. Plasmid pPAP5 contains the entire *pap* gene cluster cloned from the human urinary tract *E. coli* isolate J96 and encodes for Galα4Gal-binding P pili.[37] Plasmid pPAP24 is a derivative of pPAP5 containing a deletion of the *papG* gene.[38]

Competitive inhibition ELISA assay for bacteria

Microtiter plates with covalently linked **1** or **2** (Glycoplate I and II) were blocked for nonspecific bacterial binding by incubation overnight with 0.4 mL of 2% bovine serum albumin in phosphate buffered saline (BSA/PBS) having *E. coli* HB101 cells (devoid of P pili) resuspended to a klett of 50 at 4 °C. The bacteria were grown on TS agar (Baxter) with 100 μg mL^{-1} Carbenicillin and passaged for 3 days. On the third day they were suspended in PBS to a klett of 120. A known volume of this suspension was pelleted by centrifugation, and then resuspended in 1/20th this volume of 2% BSA/PBS. This homogeneous suspension (50 μL per well) was added to plates having 50 μL of inhibitor serially diluted three times between each well. Every inhibitor was used two or three times. The light absorption at 405 nm due to residual nonspecific binding was obtained by incubating a plate with HB101/pPAP24 bacteria (devoid of the PapG adhesin). These background values were subtracted from all other values, to give the IC_{50} values in Tables 1–4. The plates were shaken once in the Thermomax microplate reader (Molecular Devices) and then incubated for 45 min. The plates were washed three times with Covabuffer. Bacteria were detected with a primary anti-pili rabbit antiserum (WU93, adsorbed against HB101/pBR322 to remove antibodies against PapA, diluted 1/200 in 2% BSA/PBS, 100 μL/well) for 1 h, whereafter the plates were washed three times with Covabuffer. Alkaline phosphatase-conjugated anti-rabbit IgG (Sigma A7539, diluted 1/500) and phosphatase substrate 104 (Sigma 104) was, according to the manufacturer's instructions, added to the plates and the optical density was read at 405 nm. Incubations were at 24 °C unless otherwise stated.

Competitive inhibition ELISA assay for GD complex

The GD ($PapG_{J96}/PapD_{J96}$) complex was purified as previously described.[21] The GD complex (0.1 mg mL^{-1} in 2% BSA/PBS, 50 μL per well) was added to Glycoplate I or II, which had 50 μL of inhibitor serially diluted three times between each well. Every inhibitor was used two or three times. After 45 min incubation, the plates were washed three times with PBS and then blocked for nonspecific binding (as opposed to the bacterial assay where blocking was done prior to the addition of bacteria) by a 1 h incubation with 0.4 mL of 2% PBS/BSA. The GD complex was detected by incubation with primary GD rabbit antiserum (diluted 1/500 in 2% PBS/BSA, 100 μL per well) for 1 h followed by washing (three times) with Covabuffer and addition of alkaline phosphatase-conjugated antirabbit IgG (Sigma A7539, diluted 1/5000) and phosphatase substrate 104 (Sigma 104). The optical density was read at 405 nm. Incubations were at 24 °C unless otherwise stated.

Data analysis

Optical densities were read at 405 nm, plotted against the inhibitor concentrations, and the data points were least-squares fitted (using the Levenberg–Marquardt algorithm) to the standard binding function $OD = OD_0\{1 - IC/(IC_{50} + IC)\}$, were OD_0 is the OD in the absence of inhibitor, IC is the inhibitor concentration, and IC_{50} is the inhibitor concentration at 50% inhibition. Curve fitting was performed using the program KaleidaGraph (Abelbeck Software Inc.) on a Macintosh computer. In Tables 1 and 4 compound **9** is used as reference and in Tables 2 and 3 compound **12** is used as reference. The relative equilibrium constants (K_{rel}) were obtained from the ratio of the IC_{50} value of the reference compound and each of the other compounds. The K_{rel} values were used for calculations of difference free energies ($\Delta\Delta G$), using the expression $\Delta\Delta G = -RT \ln K_{rel}$.[39]

Acknowledgments

This work was supported by grants from the Swedish Natural Science Research Council (to G.M.) and from the National Institutes of Health (to S.J.H.; Support Grant 1RO1A129549).

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(*Received in U.S.A.* 11 *January* 1996; *accepted* 15 *May* 1996)