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Aromatic Alpha-Glycosides of Mannose Are Powerful Inhibitors of the Adherence of Type 1 Fimbriated *Escherichia coli* to Yeast and Intestinal Epithelial Cells

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Adherence of bacteria via their surface lectins to host epithelial cells is considered an important initial event in bacterial pathogenesis. Mannose-specific (type 1) fimbriae are among the most commonly found lectins in enterobacteria. We studied the effect of aromatic α-glycosides of mannose on the agglutination of mannan-containing yeasts by different strains of *Escherichia coli* and on the adherence of the bacteria to guinea pig ileal epithelial cells. In both systems these compounds were considerably more effective inhibitors than methyl α-mannoside, with 4-methylumbelliferyl α-mannoside and *p*-nitro-*o*-chlorophenyl α-mannoside being the strongest inhibitors. Both compounds were approximately 400-times stronger inhibitors of yeast agglutination by *E. coli* O128 than was methyl α-mannoside and 1,000- and 470-fold stronger, respectively, than was methyl α-mannoside in inhibiting the adherence of the bacteria to ileal epithelial cells. 4-Methylumbelliferyl α-mannoside was 540 to 1,000 times more effective in inhibiting yeast agglutination by four additional strains of mannose-specific *E. coli*. It was also more efficient than methyl α-mannoside in removing adherent *E. coli* O128 from ileal epithelial cells. Our results provide further evidence that type 1 fimbriae of *E. coli* possess a hydrophobic region next to the mannose-binding site. The results suggest that 4-methylumbelliferyl α-mannoside and *p*-nitro-*o*-chlorophenyl α-mannoside are good candidates for the design of therapeutic agents that may prevent adherence in vivo and infection by *E. coli* strains that express type 1 fimbriae.

Considerable evidence has accumulated during the last decade to show that lectins on bacterial surfaces may be involved in the initiation of infection by mediating bacterial adherence to epithelial cells, especially in the urinary and gastrointestinal tracts (2, 5, 19, 21, 22). This has been best documented for *Escherichia coli* carrying type 1 (mannose-specific) or type P [galactosyl(α1→4)galactose-specific] fimbriae and for type 1 fimbriated *Klebsiella pneumoniae*. Although both type 1 and type P fimbriae seem to play a role in the initiation of ascending pyelonephritis, in other forms of urinary tract infection type 1 fimbriae appear to play a predominant role. This notion is supported by both human studies and experimental urinary tract infection in animals (6–8, 15). In particular, the incidence of urinary tract infection in experimental animals is markedly decreased by sugars that specifically inhibit the bacterial surface lectins (3, 10, 20) and by antibodies to the bacterial lectins (1, 23, 24) or to the lectin receptors (1), all of which inhibit the adherence of the bacteria to epithelial cells. It is thus of importance to investigate in detail the sugar specificity of the bacterial surface lectins in order to find highly effective inhibitors of adherence that may be useful in preventing infection.

In our studies on the sugar specificity of the mannose-specific *E. coli* and *K. pneumoniae* lectins, we have found that pNPαMan and PαMan (for definitions of abbreviations for sugars, see Table 1) are much stronger inhibitors (30 to 75 times) of lectin activity than is MeαMan (11–13). We have therefore concluded that the lectins of these organisms possess a hydrophobic binding region close to the mannose-binding site. The investigation of additional α-linked aromatic derivatives of mannose reported in this study revealed that some of these compounds are about 500 to 1,000 times more effective than MeαMan in inhibiting the yeast agglutinating activity of the bacteria as well as their adherence to ileal epithelial cells.

MATERIALS AND METHODS

Materials. MeαMan was a product of Pfanstiehl (Waukegan, Ill.); pNPαMan and MeUmbαMan were products of Koch Light (Haverhill, Suffolk, United Kingdom). The other aromatic mannosides were a gift from F. G. Loontiens of the University of Ghent, Belgium (17). All other chemicals were of analytical grade obtained from commercial sources.

Bacteria. *E. coli* O128:H4 was obtained from N. Garber of Bar-Ilan University, Ramat Gan, Israel. *E. coli* serotypes O25 (previously designated as *E. coli* 346 [11–13]), O22, O62, O75A, and O75B and *K. pneumoniae* were isolated from patients with urinary tract infections. *Salmonella typhimurium* was from the National Center of *Enterobacteriaceae*, Government Central Laboratories, Jerusalem, Israel. The bacteria were grown for 20 h at 37°C in a stationary culture in a medium containing 1% Bacto-Peptone (Difco Laboratories, Detroit, Mich.), 0.5% yeast extract, and 0.5% NaCl to obtain cultures enriched with type 1 fimbriated organisms (12). Mannose-specific activity was tested before each experiment by agglutination of yeast cells (see below). *E. coli* H10407 and E1393-75 were obtained from J. Holmgren, Göteborg, Sweden. They were isolated from humans with watery diarrhea; they possess colonization factor antigens (CFA) I and II, respectively, and were checked and grown as described previously (9, 14).

Radiolabeled bacteria were prepared by adding [D-^{14}C]glucose (346 mCi/mmol; Radiochemical Centre (Amersham, United Kingdom) to the culture medium as previously described (16). The radioactivity of the labeled bacteria was counted in a liquid scintillation spectrometer

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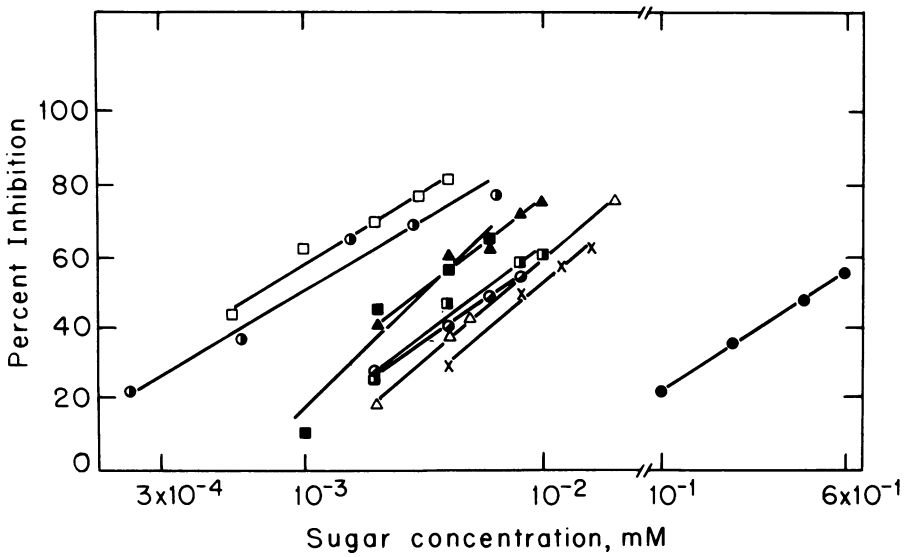

FIG. 1. Inhibition by aromatic mannosides of yeast agglutination by *E. coli* O25. All data are derived from experiments with the same bacterial culture. Symbols: ●, MeαMan; ×, PαMan; △, pNPαMan; ◒, pBrPαMan; ◨, pEtPαMan; ■, pMetOPαMan; ▲, pEtOPαMan; □, pNoClPαMan; ◑, MeUmbαMan.

(model 3255; Packard Instrument Co., Inc., Rockville, Md.) with 10 ml of scintillation fluid (Lumax-xylene [Lumec BV, Schaesberg, the Netherlands] at 1:3 [vol/vol]). The average specific radioactivity obtained was approximately 1 cpm/10^3 bacteria.

Yeast agglutination assay. Agglutination of mannan-containing yeasts (*Saccharomyces cerevisiae*, commercial bakers' yeast [Fleischmann, Standard Brands, Canada Ltd.]; 0.5 mg [dry weight]/1.0 ml of phosphate-buffered saline [PBS; 0.02 M sodium phosphate in 0.15 M NaCl [pH 7.4]) was monitored in a Payton aggregometer (12, 18). The inhibitory sugars were added in 10 to 50 μl of PBS to the yeast cell suspension followed by the addition of the bacteria in 10 μl of PBS. Percent inhibition by the sugar was calculated from the decrease in the yeast agglutination rate as previously described (12).

Bacterial adherence to epithelial cells. Dunkin-Hartley noninbred guinea pigs (1 month old) were sacrificed by cervical dislocation. The intestinal epithelial cells were released by the procedure of Weiser (25) with slight modification (4); a suspension of epithelial cells (200 μl containing 10^6 cells) was incubated with radiolabeled bacteria (100 μl containing 10^8 to 2×10^9 bacteria per ml; 10 to 200 bacteria per epithelial cell) at 37°C in a rotating rack (16 rpm). When inhibitory sugars were used, the bacteria were preincubated in morpholinoethane sulfonate buffer (50 mM [pH 6.2]) containing 1 mM $CaCl_2$ (MES-Ca^{2+}) with the desired sugar concentration for 15 min at 37°C in the same rotating rack. The subsequent steps were all done at 37°C. After 45 min of incubation, 2.5 ml of PBS was added, the epithelial cells were collected by centrifugation (500 × *g*, 5 min), and the supernatant containing the free bacteria was discarded. The epithelial cells were separated from the remaining unattached bacteria by discontinuous density gradient centrifugation with Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) (16). The gradient was built on top of the sediment, which consisted of epithelial cells and bacteria, by layering 0.5 ml of 100% Percoll followed by 1 ml of 60%, 2 ml of 40%, 1 ml of 30%, and 0.5 ml of 10% Percoll, all diluted with saline. After centrifugation (500 × *g*, 20 min), the free bacteria formed a layer between 100 and 60% Percoll, whereas the epithelial cells banded at 30%. The epithelial cell layer with the adherent bacteria was collected, and its radioactivity was counted.

Removal of adherent bacteria. Radiolabeled *E. coli* O128 (10^8 cells per 100 μl of MES-Ca^{2+}) was incubated with 200 μl of 10^6 ileal epithelial cells at 37°C for 10 min in a rotating rack. At the end of the incubation period, inhibitory sugar or saline was added to the reaction mixture and incubated with the cells for 30 min at 37°C. The number of bacteria that remained attached to the epithelial cells was determined as described above.

RESULTS

Yeast agglutination. The percent inhibition of yeast agglutination by *E. coli* O25 obtained with the various α-linked aromatic mannosides as a function of their concentration is given in Fig. 1. In all cases, there was a linear correlation within the range of sugar concentrations tested between the percentage of inhibition and the logarithm of the sugar concentration. For each sugar, the concentration causing 50% inhibition was derived from its inhibition line (12) and the relative inhibitory activity compared with that of MeαMan was calculated (Table 1). All the aromatic α-mannosides were potent inhibitors of yeast agglutination by the bacteria, up to 600 to 720 times more effective than MeαMan. Interestingly, the disaccharide MeUmbα-Manα2Man was a poor inhibitor, being 300-fold weaker than the corresponding glycoside of mannose.

When the inhibitory potencies of the strong inhibitor, MeUmbαMan, and of the corresponding disaccharide, MeUmbαMan2Man, were similarly tested with five additional strains of *E. coli* belonging to different serotypes, a similar specificity pattern was observed (Table 2). pNoClPαMan was also tested with *E. coli* O128 and found to be 400 times more inhibitory than MeαMan. Also, with *K. pneumoniae*, MeUmbαMan was 600 times more inhibitory than MeαMan. In control experiments, methylumbelliferone and methylumbelliferyl α-glucoside at concentrations up to 2.5 mM were not inhibitory.

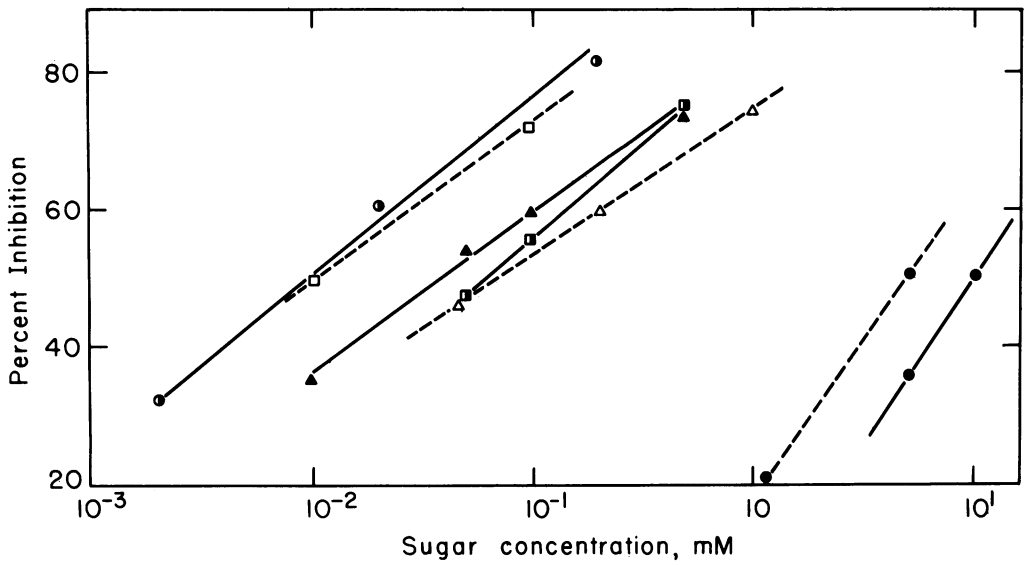

FIG. 2. Inhibition by aromatic mannosides of *E. coli* O128 adherence to guinea pig ileal epithelial cells. For symbols of the sugars tested, see legend to Fig. 1. Two bacterial cultures of *E. coli* O128 were used. pNPαMan and pNoClPαMan were tested with one culture for which the concentration of MeαMan causing 50% inhibition was 4.7 mM (– – –), and MeUmbαMan, pEtOPαMan, and pEtPαMan were tested with another culture for which the concentration of MeαMan causing 50% inhibition was 9.5 mM (——).

Adherence to guinea pig intestinal cells. The adherence of radioactive *E. coli* O128 at various concentrations to guinea pig ileal epithelial cells was dose dependent when the cells were incubated with the bacteria for 15 min. Thus, with 5×10^7 bacteria per ml in the incubation mixture, 4 to 5 bacteria bound per cell; with 10^8 bacteria per ml, 21 to 25 bacteria bound per cell; and with 2×10^8 bacteria per ml, 40 to 45 bacteria bound per ileal epithelial cell. Binding was inhibited by 80 to 90% when incubation was in the presence of 0.1 M MeαMan (data not shown), demonstrating that adherence to the ileal epithelial cells was mannose specific. Based on the above results, we chose a concentration of 10^8 bacteria for the examination of the effect of other inhibitory sugars on the binding of the bacteria to the ileal epithelial cells. It can be seen that in two series of experiments using different bacterial cultures, the concentrations of MeαMan required for 50% inhibition were 4.7 and 9.5 mM (Fig. 2). We have previously reported (12) variations in the concentration of MeαMan required for 50% inhibition of yeast agglutination. The data presented in Fig. 2 served for the calculation of the inhibitory activity of each sugar relative to MeαMan in the corresponding series of experiments (Table 3). In this system, too, the sugar specificity pattern was very similar to that obtained in the yeast agglutination assay, with pNoClPαMan and MeUmbαMan being the best inhibitors, 470 and 1,015 times stronger, respectively, than MeαMan.

MeUmbαMan was much more effective than MeαMan when tested for its ability to remove adherent bacteria that were preincubated with epithelial cells for 10 min (Fig. 3).

With mannose-specific *S. typhimurium* (13), MeUmbα Man, pMetOPαMan, and pNoClPαMan were less effective (by 10 to 60%) than MeαMan in inhibiting both yeast agglutination by the bacteria and the adherence of the bacteria to ileal epithelial cells (data now shown). The adherence of *E. coli* CFA/I and CFA/II, known to be

TABLE 1. Inhibition by aromatic mannosides of yeast agglutination by *E. coli* O25

Sugar[a]	Abbreviation	Relative inhibitory activity ± SD[b]
Methyl α-mannoside	MeαMan	1
p-Nitrophenyl β-mannoside	pNPβMan	1
Phenyl α-mannoside	PαMan	40 ± 7[c]
p-Nitrophenyl α-mannoside	pNPαMan	69 ± 4[c]
p-Bromophenyl α-mannoside	pBrPαMan	72
p-Ethylphenyl α-mannoside	pEtPαMan	77
p-Methoxyphenyl α-mannoside	pMetOPαMan	140
p-Ethoxyphenyl α-mannoside	pEtOPαMan	154
p-Nitro-*o*-chlorophenyl α-mannoside	pNoClPαMan	717
4-Methylumbelliferyl α-mannoside	MeUmbαMan	600[d]
4-Methylumbelliferyl α-mannobioside	MeUmbαManα2Man	2

[a] Because of the scarcity of material, most of the compounds were tested only in one series of experiments (at least four inhibitory concentrations were used [see Fig. 1]).

[b] The inhibitory activities of the various sugars are relative to that of the concentration (0.43 mM) of MeαMan causing 50% inhibition, which was arbitrarily set as 1.

[c] Average of three sets of experiments, only one of which is presented in Fig. 1.

[d] A 2.5 mM concentration of 4-methylumbelliferone or of 4-methylumbelliferyl α-glucoside caused no inhibition of yeast agglutination.

TABLE 2. Relative inhibition by MeUmbαMan and MeUmbαManα2Man of yeast agglutination by different strains of *E. coli*

Sugar	Relative inhibition (fold) by *E. coli* serotype:				
	O128	O22	O62	O75A	O75B
MeαMan[a]	1	1	1	1	1
MeUmbαMan	395	830	544	720	1,000
MeUmbαManα2Man	11	2	3.5	NT[b]	5

[a] The concentrations of MeαMan causing 50% inhibition of yeast agglutination were 0.8 mM for *E. coli* O128, 1.2 mM for *E. coli* O22 and *E. coli* O75A, 0.9 mM for *E. coli* O62, and 1.8 mM for *E. coli* O75B.

[b] NT, Not tested.

TABLE 3. Inhibition by aromatic α-mannosides of *E. coli* O128 adherence to guinea pig ileal epithelial cells

Sugar	Relative inhibitory activity[a]
MeαMan	1
pNPαMan	70
pEtPαMan	150
pEtoPαMan	240
pNoClPαMan	470
MeUmbαMan	1,015

[a] Calculated from data from the two series of experiments presented in Fig. 2. The inhibitory activities are relative to that of MeαMan (see Table 1, footnote *b*).

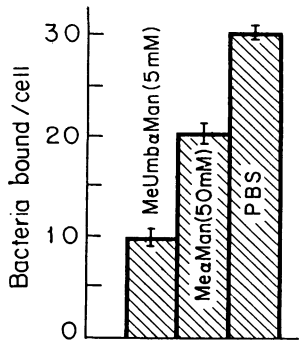

FIG. 3. Removal of *E. coli* O128 from ileal epithelial cells by MeαMan and MeUmbαMan. Bacteria (10^8/100 μl of MES-Ca^{2+}) were incubated with guinea pig ileal cells (10^6) for 10 min. At the end of the incubation period, the reaction was either stopped (PBS) or inhibitory sugars were added to the reaction mixture and incubated with the cells for 30 min at 37°C. Incubation of the bacteria with the cells in PBS alone for an additional 30 min at 37°C did not cause their removal, since the number of bacteria bound per cell at the end of the incubation was 41.2 ± 0.5 (standard deviation) compared with 37.9 ± 1.3 at the start of the incubation.

mannose resistant (9, 14), was not inhibited by a concentration as high as 1 mM MeUmbαMan.

DISCUSSION

In previous studies (11–13), we characterized the sugar specificities of various mannose-specific enterobacteria that belong to different species and genera by comparing the inhibitory potencies on the lectin activity of bacteria of a variety of mannose derivatives and mannose-containing oligosaccharides. We found that with all the *E. coli* strains tested, as well as with *K. pneumoniae*, pNPαMan is a strong inhibitor, indicating that the mannose-specific fimbrial lectins of these bacteria possess a hydrophobic region in or close to their sugar-combining sites (12, 13).

In this study we tested the inhibitory potencies of additional α-linked aromatic derivatives of mannose on the yeast agglutinating activity and on adherence to epithelial cells of several *E. coli* strains. The method used for adherence to ileal cells is suitable for such studies, since we obtained a dose-dependent curve of adherence and straight inhibition lines for all the sugars tested. In all cases, MeUmbαMan was found to be a most potent inhibitor. Another aromatic derivative of mannose, pNoClPαMan, was nearly as effective as MeUmbαMan (Table 1). A comparison between the inhibitory activities of pNPαMan and pNoClPαMan led to the conclusion that the presence of an *ortho*-halogen substituent in the *p*-nitrophenyl ring attached via an α-linkage to the mannose residue markedly increased the affinity of the glycoside for the *E. coli* lectin. Similar patterns of inhibition were observed with the other *E. coli* strains tested (Table 2), as well as with one strain of *K. pneumoniae*.

The strict specificity of the bacterial lectin for mannose was manifested by the following findings. (i) The aromatic moiety by itself was not sufficient for binding to the sugar-combining site, since 4-methylumbelliferone was inactive even at a concentration which was well over 100 times higher than the concentration of MeUmbαMan required for 50% inhibition of the *E. coli* lectin. (ii) Methylumbelliferyl α-glucoside was also inactive at the concentration tested. (iii) Mannose-resistant CFA/I and CFA/II strains of *E. coli* were not inhibited by MeUmbαMan. The fact that MeUmbαManα2Man was considerably less inhibitory than MeUmbαMan (Tables 1 and 2), together with the earlier observation that *p*-nitrophenyl β-mannoside is a poor inhibitor (13), indicates that the presence in a compound of both a mannose residue and an aromatic residue was not sufficient for endowing it with strong inhibitory activity.

MeUmbαMan and pNoClPαMan were the most potent inhibitors of the adherence of *E. coli* O128 to guinea pig ileal epithelial cells, in line with the results obtained with yeast agglutination (Table 3). MeUmbαMan was also more effective in removing adherent bacteria from ileal cells than was MeαMan (Fig. 3). After incubations longer than 10 min, removal of the bacteria was less efficient (data not shown), most likely since secondary interactions leading to irreversible binding occurred.

The concentration of MeαMan required for 50% inhibition of the adherence of *E. coli* O128 to ileal cells varied from one culture to another, probably because of variations in the expression of the mannose-specific lectin on the bacterial cell surface. We noted the same phenomenon before with yeast agglutination by *E. coli* (12). Despite this variation, the relative inhibitory activity of the same compound was quite constant, even when different strains of the same organism were examined and in different assay systems (11–13).

With *S. typhimurium*, which we showed to be devoid of a hydrophobic region in its sugar combining site (12, 13), the aromatic glycosides tested were all less effective than MeαMan in inhibiting yeast agglutination or adherence of the bacteria to epithelial cells. The results emphasize again the difference in the sugar specificity patterns between the *E. coli* strains and *K. pneumoniae* on one hand and the *Salmonella* species on the other and demonstrate that the inhibition of adherence was based upon specific recognition between the bacterial lectin and the sugar residue(s) on the target cell. As pointed out in the introduction, it is now well established that it is possible to prevent experimental infection by inhibitors of bacterial adherence. Our results indicate that the α-linked aromatic glycosides of mannose, in particular MeUmbαMan and pNoClPαMan, are suitable candidates for therapeutic agents that may prevent bacterial infections by *E. coli* strains and by *K. pneumoniae* that express type 1 fimbriae.

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