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Squaric Acid Monoamide Mannosides as Ligands for the Bacterial Lectin FimH: Covalent Inhibition or Not?

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Bacteria use long proteinaceous appendages, called fimbriae or pili, to adhere to the surfaces of their host cells. Widely distributed among the Enterobacteriacae are type 1 fimbriae that mediate mannose-specific bacterial adhesion through the lectin FimH, located at the fimbrial tips. It is possible to design synthetic mannosides such that they show high affinity for FimH and can thus inhibit mannose-specific bacterial adhesion in a competitive manner. It has been found that mannosidic squaric acid monoamides serve especially well as inhibitors of type 1 fimbriae-mediated bacterial adhesion, but it has remained unclear whether this effect is due to specific inhibition of the bacterial lectin FimH or to unspecific bioconjugation between the lectin's carbohydrate binding site and a squaric acid monoamide. A bioconjugation reaction would result in a covalently crosslinked squaric acid diamide. Here it is shown that covalent inhibition of FimH by mannosidic squaric acid derivatives is very unlikely and that compounds of this type serve rather as excellent specific candidates for low-molecular-weight inhibitors of bacterial adhesion. This has been verified by testing the properties of glycosidic squaric acid monoamides in diamide formation, by two different adhesion assays with a series of selected control compounds, and by molecular docking studies that further support the results obtained in the bioassays.

Introduction

Bacterial infections constitute a major global health problem, especially threatening the health of young children.[1] The most common serious neonatal infections involve bacteremia, meningitis, and respiratory-tract infections.[2] Key pathogens in these infections are *Escherichia coli*, *Klebsiella* spp., *Staphylococcus aureus*, and *Streptococcus pyogenes*. It is therefore of great interest to understand the mechanisms that facilitate pathogenicity of bacteria: that is, carbohydrate-specific adhesion to glycosylated surfaces (Figure 1).

To cause infection, bacteria usually need to adhere to their target cells. For their attachment to cell surfaces and colonization most bacteria depend on the expression of specialized adhesive organelles, called fimbriae or pili.[3] Fimbriae are hair-like (1–2 μm long and ~7 nm wide) protein structures produced on the bacterial surface. Much studied examples include P fimbriae and type 1 fimbriae that provide uropathogenic *E. coli* (UPEC) with the ability to attach to specific niches in the urinary tract.[4] There, a wide range of high-mannose type glycoproteins serve as receptor molecules for type 1 fimbriae. Specificity for terminal α-D-mannosyl residues is mediated by the fimbrial lectin FimH, which is located at the type 1 fimbrial tip, as revealed by studies on fimbriae assembly.[5] FimH-mediated bacterial adhesion can be prevented to a greater or lesser extent by competitive mannosidic inhibitors, such as natural oligosaccharides[6] or synthetic mannosides and cluster mannosides.[7] Studies on inhibition of bacterial adhesion have been of importance for functional investigations[8] as well as for applications in medicine, such as in the development of anti-adhesives and antiadhesive surfaces.[9] For any application, however, it is essential to distinguish specific inhibition from unspecific inhibition, as well as competitive inhibition from ir-

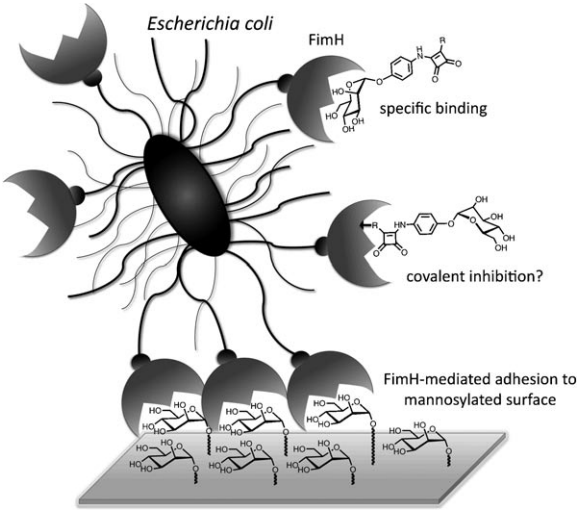

Figure 1. Adhesion of *E. coli* to glycosylated surfaces: the drawing (not to scale) shows type 1 fimbriae-mediated bacterial adhesion that is enabled by the α-D-mannoside-specific lectin FimH located at the fimbrial tips. Adhesion can be inhibited by addition of appropriate mannosides (specific binding). With reactive mannosides it is possible that unspecific covalent binding to the FimH CRD could also occur, leading to covalent inhibition.

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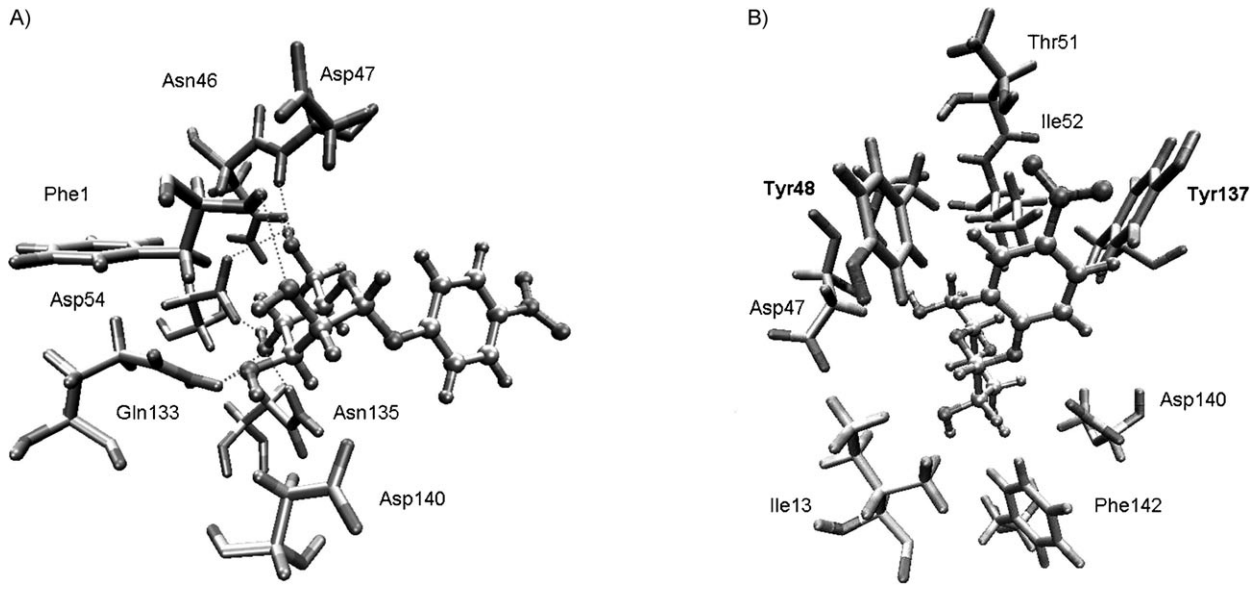


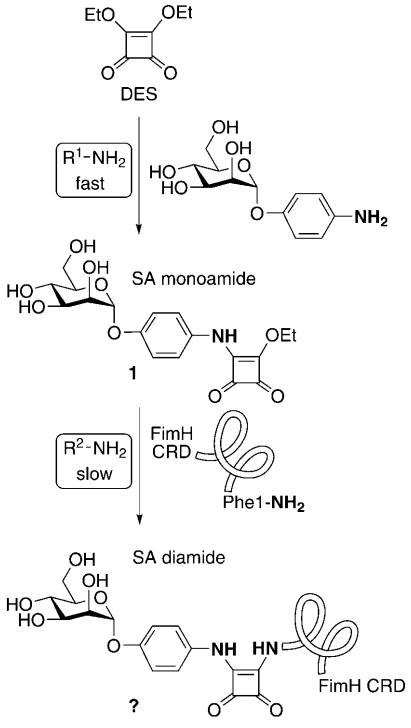
Figure 2. A) The bottom site of the FimH CRD with docked *p*-nitrophenyl α-D-mannoside (*p*NPMan), depicted in ball and stick. The CRD contains the N-terminal Phe1 amino acid of the protein. Prominent hydrogen bonds between the carbohydrate binding site and the ligand are depicted as dashed lines. B) The amino acid residues at the entrance of the FimH CRD comprise a "hydrophobic ridge". The aromatic side chains of Tyr48 and Tyr137 enter into π–π interactions with docked *p*NPMan. Both graphics show the amino acid residues oriented as in the crystallized protein (PDB ID: 1KLF).[11] Pictures were generated with VMD[14] and rendered in Pov-Ray.[15]

reversible covalent blocking of the lectin's carbohydrate binding site (Figure 1). The latter mechanism has parallels with affinity labeling of proteins.[10] In this account, the details of lectin inhibition have been investigated with the aid of a particularly interesting example of ligand binding to the bacterial lectin FimH.

FimH is a mannose-specific, two-domain adhesin with a "lectin domain", $FimH_L$, comprising the mannose-binding pocket, and a "pilin domain", $FimH_P$, that performs an organelle integration function, needed for fimbriae assembly. The mannose-binding pocket (carbohydrate recognition domain, CRD) of FimH perfectly accommodates one α-mannosidic glycon moiety. Its bottom site consists mainly of hydrophilic amino acid side chains: those of Asn46, Asp47, Asp54, Gln133, Asn135, Asp140 and the N-terminal Phe1 (Figure 2A).[11] These amino acid residues form a stabilizing hydrogen-bond network with the glycon moiety of a complexed α-D-mannoside ligand.

The periphery of the FimH CRD, on the other hand, is characterized by amino acids with rather lipophilic side chains, defining a "hydrophobic ridge" at the entrance of the CRD (Figure 2B).[11,12] The aromatic side chains of Tyr48 and Tyr137 form what has been called the "tyrosine gate" at the entrance of the mannose binding pocket.[9a,11–13] Mannosides with an aromatic aglycon, such as *p*-nitrophenyl α-D-mannoside (*p*NPMan), can establish favorable π–π interactions with these two residues, thus leading to significantly improved affinities relative to methyl α-D-mannoside (MeMan).

We have recently published a systematic study on carbohydrate binding of type 1-fimbriated *E. coli* in which we utilized a series of α-D-mannosidic squaric acid monoamides.[16] There, it was shown that the squaric acid monoamide **1** (Scheme 1) clearly exceeds *p*NPMan in potency as an inhibitor of type 1 fimbriae-mediated bacterial adhesion according to an ELISA (enzyme-linked immunosorbent assay). This finding was inter-



Scheme 1. Squarates such as diethyl squarate (DES) form squaric acid (SA) monoamides in a fast reaction and the corresponding SA diamides in a much slower subsequent reaction step. This feature can be exploited for conjugation of two amines R^1–NH_2 and R^2–NH_2. Accordingly, the mannosidic SA monoamide **1** can be formed and might, in the second reaction step, covalently attach to the N terminus of the bacterial lectin FimH (Phe1) located within the CRD to yield a crosslinked ligand–lectin conjugate.

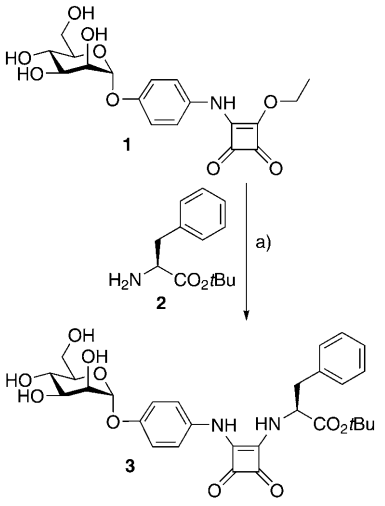
preted in terms of additional interactions of the extended aglycon moiety in **1**, relative to *p*NPMan, at the entrance of the FimH CRD. It has been asked, however, whether the reason for the high inhibitory potency of the squaric acid derivative **1** might be found in the formation of a covalent bond within the FimH CRD (Scheme 1). This hypothesis is justified by the special reactivity of squaric acid monoesters, which are frequently exploited for bioconjugation.[17]

Sequential treatment of a squarate such as diethyl squarate (DES) with two amines results in their conjugation through two vinylogous amides. In these reactions, the formation of the first amide bond is much faster than the reaction between the squaric acid (SA) monoamide formed in the first process and a second amine. Nevertheless, once a mannosidic squaric acid monoamide such as **1** is complexed within the FimH CRD, its reaction with the N-terminal amino group of Phe1 might lead to a crosslinked ligand–lectin SA diamide conjugate (Scheme 1). It could be this crosslinking reaction that accounts for the high inhibitory potency of the squaric acid monoamide **1**.[16]

Results and Discussion

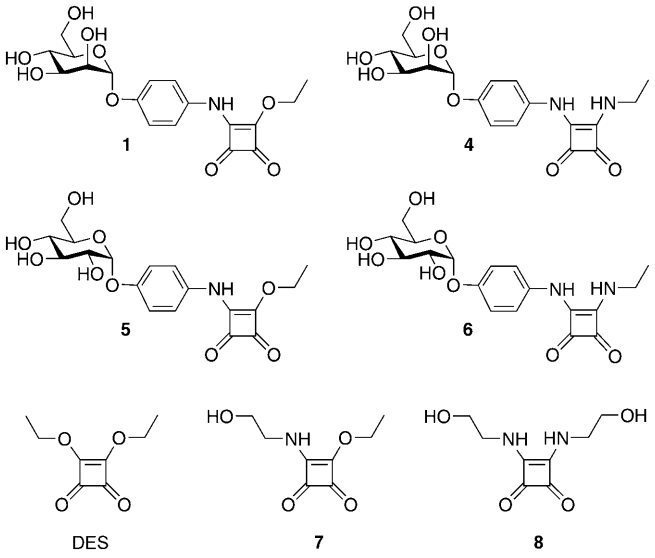
Synthesis of squaric acid derivatives

In order to test the ability of the SA monoamide **1** to form a diamide under the conditions used for the bioassay, it was treated with the L-phenylalanine ester **2** (Scheme 2), as a model for the N-terminal Phe1 of FimH. When this reaction was carried out in PBS buffer under physiological conditions (pH 7.2), the squaric acid diamide **3** was formed in quantitative yield. Conversion of the reaction partners was complete after 45 min and the reaction outcome did not change further for the next 24 h according to MADLI-TOF-MS monitoring. When the same reaction was carried out in citric acid buffer at pH 4.5 no reaction occurred over 24 h.



Scheme 2. Treatment of the squaric acid monoamide **1** with the phenylalanine derivative **2** under bioassay conditions led to quantitative formation of the SA diamide **3**. a) PBS buffer (pH 7.2), 37 °C, 45 min–24 h, quant. (no reaction at pH 4.5).

This result indicates that the SA monoamide **1** can undergo the predicted reaction to form the corresponding diamide **3** (Scheme 2), and so it had to be investigated whether the analogous reaction can also occur within the lectin's CRD. A number of control compounds were therefore synthesized and tested in parallel with **1** as inhibitors of type 1 fimbriae-mediated bacterial adhesion (Scheme 3). As the most obvious control mannoside, the SA diamide **4**, lacking the potential for covalent crosslinking within the FimH CRD, was required. The mannoside **4** was synthesized by treatment of **1** with ethylamine under triethylamine catalysis. In addition, the glucosides **5** and **6** were prepared as analogues of the SA monoamide **1** and its diamide **4**, respectively, in order to test the carbohydrate specificity of the inhibition. The synthesis of **5** and **6** started from *p*-aminophenyl α-D-glucoside and proceeded analogously to the preparation of **1**[16] and **4**. Finally, diethyl squarate (DES) and its known monoamide and diamide derivatives, **7** and **8**, respectively,[18] were synthesized and included in the biological study as unspecific control compounds.



Scheme 3. Control compounds for the bioassay: Squaric acid diamide **4** lacks the crosslinking ability of SA monoamide **1**; glucosides **5** and **6** were required to test carbohydrate specificity of ligand binding and squaric acid derivatives DES, **7** and **8** are unspecific control compounds.

Bioassay: Inhibition of bacterial adhesion

All of the squaric acid derivatives under investigation (Scheme 3) were tested as inhibitors of type 1 fimbriae-mediated bacterial adhesion to mannan-coated 96-well microtiter plates by using uropathogenic *Escherichia coli* cells. Serial dilutions of each inhibitor were incubated with fluorescent *E. coli* cells.[19] If possible, inhibition curves were determined for each tested inhibitor, from which IC_{50} values were deduced. The IC_{50} values in this case reflect the inhibitor concentrations that

cause 50% inhibition of bacterial binding to mannan. On each individual test plate *p*NPMan was tested in parallel to allow referencing of the IC_{50} value obtained for each tested ligand to the IC_{50} of *p*NPMan. This procedure leads to relative inhibitory potencies (RIPs) for every tested compound, which are consistently referenced and can therefore be compared even if they were not examined in the same experiment.

This bioassay revealed no inhibitory potency for the glucosides **5** and **6**, or for the squaric acid derivatives DES, **7**, and **8**. From this finding it can be concluded that the presence of a squaric acid moiety is not sufficient for preventing adhesion of bacteria, even though compounds such as **5**, DES, and **7** possess the capacity to crosslink to amines. The mannosides **1** and **4**, on the other hand, showed good inhibitory potency, as expected (Table 1). Interestingly, though, the squaric acid diamide **4**, without any crosslinking capability, exceeds the inhibitory potency of the corresponding SA monoamide **1**. Whereas the mannoside **1** showed an inhibitory potency approximately 16 times higher than that of *p*NPMan, **4** works 50 times better. This result makes the initial hypothesis—that **1** performs especially well as an inhibitor of type 1 fimbriae-mediated bacterial adhesion because it undergoes crosslinking within the CRD of the bacterial lectin, thus irreversibly blocking the carbohydrate binding site—rather unlikely.

Table 1. Inhibition of bacterial adhesion to a mannoside-coated surface: IC_{50} values for the squaric acid-functionalized mannosides **1** and **4** were compared to the reference mannoside *p*NPMan in three independent bacterial adhesion assays.[a][19]

	1	**4**	pNPMan
IC_{50} [μM]	17.3 ± 6.5	6.38 ± 3.7	274 ± 110
RIP[b]	16 ± 1.5	50 ± 19	1

[a] All substances were tested as duplicates in all assays. [b] Relative inhibitory potencies are based on the IC_{50} value for *p*NPMan measured on the same microtiter plate.

Computer docking with FimH

In order to deepen our understanding of binding of the different squaric acid derivatives to FimH, docking studies with the FimH lectin domain were performed. Carbohydrate ligands were docked into the CRD of FimH by using FlexX[20] flexible docking and consensus scoring[21,22] as implemented in Sybyl6.9.[23] During the docking process the FimH CRD was held fixed, whereas the ligand was allowed to change its conformation. To allow for false-positive solutions a two-stage strategy was employed. The conformations delivered by FlexX were regarded as "unrelaxed". These conformations were minimized to obtain "relaxed" conformations and ranked.[24] All solutions were then docked and screened for the most reasonable structures. For each docked conformation a FlexX scoring value was obtained, which is correlated to its free binding energy, a more negative score correlating with higher binding affinity. For validation of the obtained results, consensus scoring was employed. In order to address the conformational flexibility of the tyrosine gate at the entrance of the FimH CRD, docking was based on two different FimH X-ray structures, reflecting two extreme relative conformations of Tyr48 and Tyr137: the tyrosine gate of FimH in complexation with mannose was found to be in an "open" conformation,[11] whereas a "closed-gate" structure was seen in complexation with *n*-butyl α-D-mannoside.[13]

Docking of mannosides **1** and **4** into the FimH CRD showed that the sugar glycon portion is always complexed within the binding pocket, with the aglycon moiety sticking out of the binding site (Figure 3). To enable crosslinking of the SA monoamide ligand **1** to the N-terminal end of FimH (Phe1), "upside-down" complexation of the mannoside would be required. This is not seen in the docking studies. Instead, computer modeling reveals favorable hydrophobic interactions established by the aglycon moiety of **1** at the entrance of the FimH CRD (Figure 3 A and B) as reported earlier.[16] Complexation of **1** within the CRD leads to typical scoring values of −29.6 to −33.1 depending on the X-ray structure used for the docking.

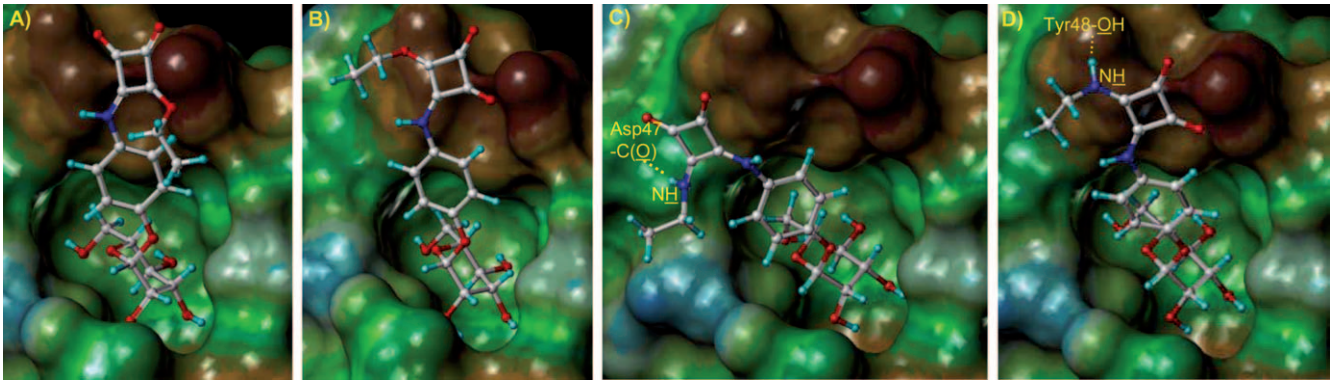


Figure 3. Representative conformations of the docked SA monoamide **1** and SA diamide **4**. A) Fit of the top scoring conformation of the SA monoamide **1** with a FlexX score of −33.1; B) docking solution No. 8 for the SA monoamide **1** with a FlexX score of −32.8; C) fit of the top scoring conformation of the SA diamide **4** with a FlexX score of −35.3; D) docking solution No. 5 for the SA diamide **4** with a FlexX score of −34.3. The SA diamide **4** can establish hydrogen bonds (indicated in yellow) to the amino acid side chains of Asp47 (in C) or Tyr48 (in D), which are not seen in the case of **1**. The depicted docking results are based on the closed-gate crystal structure of FimH.[13]

Analogous docking of the squaric acid diamide **4** led to scoring values of −30.9 for the open-gate structure of FimH[11] (see the Supporting Information) and up to −35.3 for the closed-gate structure[13] (Table 2). The docking results thus predict a higher affinity to FimH for **4** than for **1** and this is in accordance with the experimental findings (Table 1). The observed higher affinity of the SA diamide **4** relative to the SA monoamide **1** can be explained by the formation of additional hydrogen bonds at the entrance of the CRD, especially when the closed-gate structure of FimH is used for docking. Hydrogen-bridging of **4** is due to the NH-ethyl hydrogen bond donor function, which is absent in case of the SA monoamide **1**. As hydrogen bond acceptors the side chains of Asp47 (Figure 3C) and also of Tyr48 (Figure 3D) can be identified.

Table 2. FlexX scoring values for seven different ligands obtained from docking based on two different crystal structures.

Ligand	Scores (hit no.)[a] based on open-gate structure[11]	Scores (hit no.)[a] based on closed-gate structure[13]
1	−29.6 (1)	−33.1 (1)
4	−30.9 (1)	−35.3 (1)
5	−15.1 (3)	[b]
6	−12.8 (4)	[c]
DES	−10.1 (1)	[c]
7	−17.5 (1)	−17.1 (3)
8	−18.5 (1)	−21.2 (1)

[a] In each case scoring values are listed for the most reasonable ligand conformation. [b] Only 7 of 30 hits are complexed within the CRD, so no representative conformation can be deduced. [c] None of 30 hits is complexed within the CRD.

Docking studies with glucosides **5** and **6**, on the other hand, suggested some "upside-down" complexation, in which the squaric acid aglycon moiety is complexed within the mannose binding pocket of the open-gate structure (17 hits out of 30 in the case of **5** and 14 hits out of 30 in case of **6**). Docking based on the closed-gate structure of FimH hardly showed any complexation of these two glucosides (Table 2). For complexation of the SA monoamide **5**, docking predicts an extended conformation for the complexed ligand, whereas for complexation of **6** a more bent binding mode is indicated (Figure 4), possibly due to a hydrogen bond between the NH-ethyl donor function in **6** and the side chain of Asp140 (not shown), which is not possible in the case of the glucoside **5**. The scoring values for all conformations complexed in an upside-down mode, however, are extremely poor, with values between −15.3 (**5**, hit No. 3 and 16 other solutions) and −12.8 (**6**, hit No. 4 and 13 other solutions). FlexX docking with the low-affinity ligand MeMan, for example, gives scoring values of around −23.

It can thus be concluded that glucosides **5** and **6** do not serve as ideally specific ligands for FimH. Likewise, docking studies with the SA derivatives DES, **7**, and **8** suggested extremely weak affinities for FimH in all cases (Table 2). However, for the SA monoamide **7**, possessing a hydroxyethyl moiety, hydrogen-bond-supported complexation within the CRD was predicted with scoring values of −17.5 (open-gate) and −17.1 (closed-gate structure of FimH). For the SA diamide **8**, scores of −18.5 for the open-gate and −21.2 for the closed-gate structure were obtained, most likely resulting from further hydrogen bond interactions with the second hydroxyethyl moiety (see the Supporting Information). Even docking of DES to the open-gate structure of FimH revealed hits, albeit with a very poor FlexX score of −10.1 (Table 2).

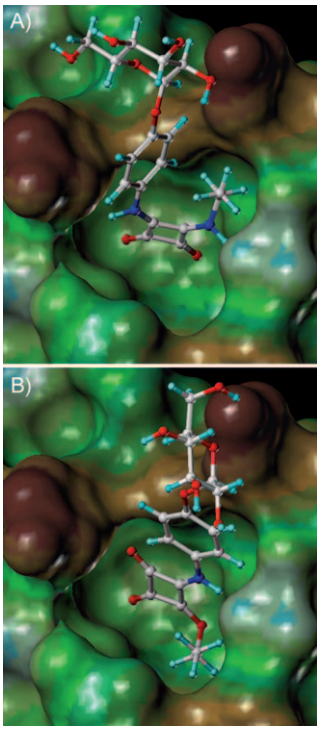


Figure 4. Comparison of "upside-down" complexation of the glucosides **5** and **6** within the FimH CRD. A) Representative conformations (here hit No. 3 with a FlexX score of −15.1) of the SA monoamide **5** are rather extended; B) representative conformations (here hit No. 4 with a FlexX score of −12.8) of the SA diamide **6** are more likely to be bent. The depicted docking results are based on the open-gate crystal structure of FimH.[11]

Preincubation of bacteria with ligands

The obtained computer-aided docking results are in good accordance with the experimental testing results. However, some—though small—affinity for FimH was even predicted for the glucosides **5** and **6**, as well as for **7**, **8**, and DES, a finding not reflected in the inhibition adhesion assay employed so far. This might be due to the fact that in this assay inhibitors have to compete against a highly mannosylated surface presenting a high concentration of specific ligands for the fimbrial lectin FimH. To test crosslinking ability in a noncompetitive setup, serial dilutions of all three classes of ligands—the mannosides **1** and *p*NPMan, the glucosides **5** and *p*NPGlc, and the non-glycosidic DES—were preincubated with type 1-fimbriated *E. coli* bacteria. After 1 h of incubation under physiological conditions, the mixtures were transferred into mannan-coated test plates and the preincubated bacteria were allowed to bind to the mannosylated surface. In this case, bacterial binding to the

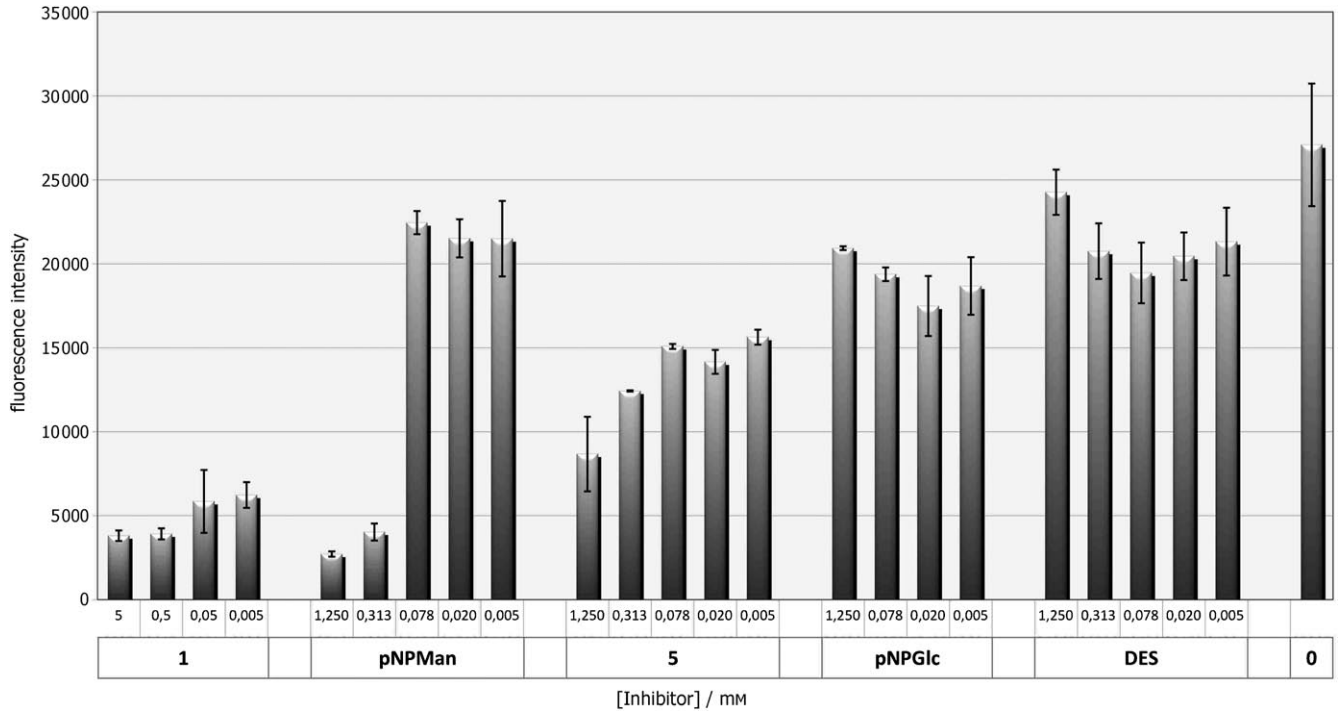


Figure 5. Preincubation assay with GFP-expressing *E. coli*: after preincubation with the indicated inhibitors at the given concentrations, the ligand/*E. coli* mixtures were transferred to mannan-coated plates, to allow adhesion to the mannan surface. Non-adhered bacteria were washed away and adhered bacteria were detected by fluorescence. Lower fluorescence intensity thus reflects more effective inhibition of bacterial adhesion to the microtiter surface due to preincubation with the tested ligand. Right bar (0 control): bacterial adhesion without added inhibitor.

mannan surface is only possible if the fimbrial carbohydrate binding sites are not ligand-saturated.

This assay showed that when ligand concentrations between 1.25 mM and 5 μM were applied for preincubation of bacteria, concentration-dependent inhibition of bacterial adhesion was seen in the cases of the mannosides **1**, *p*NPMan, and to some extent with the glucoside **5** (Figure 5). The mannosidic SA monoamide **1** prohibits the attachment of bacteria very effectively at all concentrations applied, whereas inhibition with *p*NPMan follows a typical sigmoidal concentration dependence curve (see the Supporting Information). With the glucoside **5**, low, but also concentration-dependent inhibition was detected. In contrast, a concentration gradient of the reference glucoside *p*NPGlc or of DES had no effect on the extent of bacterial adhesion to mannan (Figure 5).

Because the applied concentrations of the SA monoamides **1** and **5** are high enough to saturate all lectin binding sites of the employed type 1-fimbriated bacteria, the observed concentration dependency of inhibition once again proves that no covalent blocking of the FimH binding site occurred. Even DES showed no inhibitory effect, although it should fit into the FimH carbohydrate binding site. It can thus be concluded that neither DES nor the SA monoamides **1** and **5** lead to covalent binding within the FimH CRD. Probably protonation of the N-terminal Phe1 amino group prevents crosslinking in these cases. Our initial finding that the mannosidic SA monoamide **1** serves as an especially potent inhibitor of type 1 fimbriae-mediated bacterial adhesion[16] can in fact be attributed to the extended aglycon moiety that enhances affinity of this mannoside to the lectin. This is seen by comparison of the testing results obtained with **1** and *p*NPMan, on one hand, and the SA monoamide **5** and *p*NPGlc on the other.

Conclusions

The mannosidic SA monoamide **1** can covalently crosslink to amines such as the phenylalanine ester **2**, leading to the corresponding SA diamide. Such a crosslinking reaction might also occur with the N terminus (Phe1) of the bacterial lectin FimH, located within the lectin's mannose binding site (CRD). According to the results presented here, however, it can be concluded that the relatively high affinity of the SA monoamide **1** for the bacterial lectin FimH[16] must be due to its extended aglycon moiety and not to covalent crosslinking within the bacterial binding site.

This conclusion was confirmed by an adhesion inhibition assay in which the affinity of the SA diamide **4** towards FimH was found to exceed that of its monoamide analogue **1**. The SA diamide **4** reproducibly showed an inhibitory potency three times greater than that of the SA monoamide **1** even though it lacks the potential to form a covalent bond to a free amino function. A covalently crosslinked ligand–inhibitor complex after incubation with **1** is hence very unlikely. Furthermore, covalent crosslinking of **1** within the FimH CRD could only occur if the mannoside were complexed in an "upside-down" mode, an option that is not supported by the performed docking

studies. Modeling instead provides an explanation for the higher affinity of the SA diamide **4** in relation to the monoamide **1**. For the glucosides **5** and **6**, on the other hand, molecular docking studies suggested some probability for "upside-down" complexation of these ligands into the FimH CRD. Therefore, an assay was performed in which type 1-fimbriated *E. coli* were preincubated with different ligands prior to adhesion. If covalent crosslinking were to occur within the FimH CRD, no bacterial adhesion should be possible after preincubation with ligands at appropriate concentrations. This was not found; rather, concentration-dependent inhibition of bacterial adhesion was seen in the case of the mannosides **1** and *p*NPMan and to some extent with the glucoside **5**. These results once again support the affinity-promoting properties of the aglycon moiety in **1** and **5** and at the same time confirm that no covalent crosslinking occurs within the FimH CRD with SA monoamides. Interestingly, the strict mannoside-specificity of FimH-mediated bacterial adhesion might be put into perspective under the conditions of the preincubation assay. Here, the α-D-glucoside **5** had an effect on bacterial adhesion, lower than that of the analogous mannoside **1**, but in a concentration-dependent manner.

Finally, crosslinking of SA monoamides to the N-terminal Phe1 residue of FimH is completely unlikely if the N terminus is protonated. In this case, no diamide formation can occur (cf. Scheme 2).

In summary, it has been shown that the high inhibitory potency of **1** is not the result of covalent linkage of the SA monoamide to the N terminus of the FimH CRD but is due to the specific structure of this synthetic mannoside. The affinity for FimH found with **1** is further enhanced in its SA diamide analogue **4**. Mannosides such as **1** and **4** and similar derivatives that can be obtained from them[16] thus constitute promising candidates for a new class of low-molecular-weight antiadhesives for type 1-fimbriated bacteria, exceeding the inhibitory potencies of many other mannosides, in particular those of longer-chain alkyl mannosides.[25] The squaric acid inhibitor **4** was shown to perform ~50 times better than *p*NPMan, whereas *n*-heptyl α-D-mannoside has been reported to be a ~1.6-times better inhibitor.[25c] Competitive binding of mannosiolic SA diamides to type 1-fimbriated *E. coli* will therefore be further investigated in our laboratory.

Experimental Section

Reagents and methods: Commercially available starting materials (phenylalanine *tert*-butyl ester from Fluka, DES and *p*-nitrophenyl α-D-glucopyranoside from Aldrich) were used without further purification. *p*-Aminophenyl α-D-mannopyranoside and *p*-aminophenyl α-D-glucopyranoside were prepared by catalytic hydrogenation of the corresponding *p*-nitrophenyl glycosides.[19,26] All solvents used were purified by distillation. Methanol was dried over magnesium turnings with subsequent distillation. Monitoring of reactions was performed by TLC on silica gel F_{254} (Merck) with detection by UV light and/or by charring with ethanolic sulfuric acid (10%) or ninhydrin solution [ninhydrin (300 mg) in butanol (100 mL) and glacial acetic acid (3.00 mL)] and subsequent heating. Flash chromatography was performed on Merck silica gel 60 (0.040–0.063 mm). NMR spectra were recorded with 500 or 600 MHz Bruker DRX 500 or AV 600 instruments. Chemical shifts (δ) are calibrated relative to internal solvent. Full assignment was achieved with 2D NMR techniques (^{1}H,^{1}H COSY and ^{1}H,^{13}C HSQC). ESI-MS measurements were performed with a Mariner instrument, MALDI-TOF mass spectra were recorded with a Bruker Biflex III instrument with 19 kV acceleration voltage and an ionization laser at 337 nm. As matrices, 2,5-dihydroxybenzoic acid and α-cyano-4-hydroxycinnamic acid were used. For measurement of optical rotations a Perkin–Elmer 241 polarimeter was used (10 cm cells, Na D-line: 589 nm). Purities of employed products were checked by HPLC. Analytical HPLC was performed with a Merck–Hitachi LaChrom instrument with D-7000 interface and L-7455 diode array detector and a LiChrosorb RP-8 silica column. Preparative HPLC was performed with a Shimadzu system, an SPD-M10A diode array detector, and a Merck Hibar RT250–25 mm column with LiChrosorb RP-8 silica. (For HPLC chromatograms see the Supporting Information.) For bacterial adhesion studies, a TECAN infinite 200 multifunction microplate reader was employed.

See the Supporting Information for additional procedures and for supplementary analytical and graphical material.

***p*-[*N*-(4-Ethylamino-2,3-dioxocyclobut-1-enyl)amino]phenyl α-D-mannopyranoside (4)**: A methanolic solution of ethylamine (2.0 M, 240 μL, 480 μmol) and NEt_3 (134 μL, 960 μmol) were added to a solution of the monoamide **1** (95.0 mg, 240 μmol) in dry MeOH (10 mL). The reaction mixture was stirred at room temperature for 12 h, followed by neutralization with Amberlite IR120 ion-exchange resin, filtration, and concentration in vacuo. The crude product was purified by silica gel chromatography (MeOH/AcOEt 1:1) to provide the diamide **4** (60 mg, 63%) as a colorless lyophilizate. $[\alpha]_D = +100$ ($c = 0.10$ in DMSO); ^{1}H NMR (500 MHz, [D_6]DMSO, 298 K): $\delta = 9.70$ (br s, 1H; NH), 7.75 (br s, 1H; NH), 7.35 (d, $J = 9.0$ Hz, 2H; 2Ha_{Ar}), 7.06 (d, $J = 9.0$ Hz, 2H; 2Hb_{Ar}), 5.28 (d, $J_{1,2} = 1.8$ Hz, 1H; H-1), 4.91–4.42 (m, 4H; 4OH), 3.82 (dd, $J_{1,2} = 1.8$ Hz, $J_{2,3} = 3.3$ Hz, 1H; H-2), 3.67 (dd, $J_{2,3} = 3.3$ Hz, $J_{3,4} = 9.4$ Hz, 1H; H-3), 3.63–3.59 (m, 3H; H-6a, SA-NHCH_2CH$_3$), 3.51–3.46 (m, 2H; H-4, H-6b), 3.43 (ddd, $J_{4,5} = 9.6$ Hz, $J_{5,6a} = 1.8$ Hz, $J_{5,6b} = 5.8$ Hz, 1H; H-5), 1.21 ppm (t, $J = 7.1$ Hz, 2H; SA-NHCH$_2$CH_3); ^{13}C NMR (150 MHz, [D_6]DMSO, 298 K): $\delta = 183.6$, 180.2, 168.8, 163.6 (C_{SA}), 152.2 (man-O-C_{Ar}), 133.6 (C_{Ar}-NHSA), 119.4 (Ca_{Ar}), 117.9 (Cb_{Ar}), 99.5 (C-1), 74.8 (C-5), 70.6 (C-3), 70.1 (C-2), 66.8 (C-4), 61.1 (C-6), 38.7 (CH_2), 16.4 ppm (CH_3); ESI MS: *m/z* calcd for $C_{18}H_{22}N_2O_8Na$: 417.1268 $[M+Na]^+$; found: 417.1246.

***p*-[*N*-(4-Ethoxy-2,3-dioxocyclobut-1-enyl)amino]phenyl α-D-glucopyranoside (5)**: *p*-Aminophenyl α-D-glucopyranoside[26] (400 mg, 1.48 mmol) was dissolved in dry MeOH (20 mL), DES (432 μL, 2.95 mmol) was added, and the reaction mixture was stirred at room temperature for 12 h. The solvent was then removed under reduced pressure and the resulting syrup was subjected to purification by column chromatography (MeOH/AcOEt 1:3) to provide the title compound (308 mg, 65%) as a colorless lyophilizate. $[\alpha]_D = +126$ ($c = 0.19$ in DMSO); ^{1}H NMR (600 MHz, [D_6]DMSO, 300 K): $\delta = 10.67$ (br s, 1H; NH), 7.29 (br s, 2H; 2Ha_{Ar}), 7.08 (d, $J = 8.9$ Hz, 2H; 2Hb_{Ar}), 5.33 (d, $J_{1,2} = 3.6$ Hz, 1H; H-1), 4.77 (q, $J = 7.1$ Hz, 2H; SA-OCH_2CH$_3$), 4.41–3.78 (m, 4H; 4OH), 3.63–3.56 (m, 2H; H-3, H-6a), 3.49–3.45 (m, 2H; H-5, H-6b), 3.36 (dd, $J_{1,2} = 3.6$ Hz, $J_{2,3} = 9.6$ Hz, 1H; H-2), 3.18 (t, $J_{3,4} = J_{4,5} = 9.0$ Hz, 1H; H-4), 1.40 ppm (t, $J = 7.1$ Hz, 3H; SA-OCH$_2$CH_3); ^{13}C NMR (125 MHz, [D_6]DMSO, 323 K): $\delta = 187.9$, 183.2, 177.7, 169.3 (C_{SA}), 154.0 (man-O-C_{Ar}), 132.1 (C_{Ar}-NHSA), 121.0 (Ca_{Ar}), 117.5 (Cb_{Ar}), 98.3 (C-1), 73.6 (C-5), 73.0 (C-3), 71.5 (C-2), 70.0 (C-4), 69.2 (CH_2), 60.7 (C-6), 15.4 ppm (CH_3); ESI MS: *m/z* calcd for $C_{18}H_{21}NO_9Na$: 418.1109 $[M+Na]^+$; found: 418.1135.

***p*-[*N*-(4-Ethylamino-2,3-dioxocyclobut-1-enyl)amino]phenyl α-D-glucopyranoside (6)**: A methanolic solution of ethylamine (2.0 M, 709 μL, 354 μmol) and NEt_3 (199 μL, 142 mmol) were added to a solution of the SA monoamide **5** (140 mg, 345 μmol) in a mixture of MeOH (10 mL) and DMSO (2 mL). After having been stirred at room temperature for 12 h the reaction mixture was neutralized with Amberlite IR120 ion-exchange resin and filtered, the solvent was removed under reduced pressure, and the crude product was purified by silica gel chromatography (MeOH/AcOEt 1:2) to provide the title squaric acid diamide (132 mg, 95%) as a colorless lyophilizate. $[\alpha]_D = +155$ ($c = 0.10$ in DMSO); ^{1}H NMR (600 MHz, [D$_6$]DMSO, 298 K): $\delta = 7.34$ (d, $J = 8.4$ Hz, 2H; 2Ha_{Ar}), 7.06 (d, $J = 8.4$ Hz, 2H; 2Hb_{Ar}), 5.29 (d, $J_{1,2} = 3.6$ Hz, 1H; H-1), 3.63–3.55 (m, 4H; H-3, H-6a, SA-NHCH_2), 3.51–3.46 (m, 2H; H-5, H-6b), 3.36 (dd, $J_{1,2} = 3.6$ Hz, $J_{2,3} = 9.7$ Hz, 1H; H-2), 3.18 (t, $J_{3,4} = J_{4,5} = 9.2$ Hz, 1H; H-4), 1.21 ppm (t, $J = 6.9$ Hz, 3H; SA-OCH$_2$CH_3); ^{13}C NMR (150 MHz, [D$_6$]DMSO, 298 K): $\delta = 183.4$, 180.6, 168.9, 163.8 (C_{SA}), 152.9 (man-O-C_{Ar}), 134.0 (C_{Ar}-NHSA), 119.5 (Ca_{Ar}), 117.9 (Cb_{Ar}), 98.6 (C-1), 73.6 (C-5), 73.0 (C-3), 71.6 (C-2), 70.0 (C-4), 60.6 (C-6), 38.7 (CH_2), 16.5 ppm (CH_3); MALDI-TOF MS: *m/z* calcd $C_{18}H_{22}N_2O_8Na$: 417.13 $[M+Na]^+$; found: 417.79.

Bioassay: Media and buffer solutions: LB medium (+AMP, +CAM): tryptone (10.0 g), sodium chloride (10.0 g), and yeast extract (5.00 g) were dissolved in doubly distilled water (1.00 L); after sterilization, ampicillin (100 mg) and chloramphenicol (50.0 mg) were added. Carbonate buffer solution (pH 9.5): sodium carbonate (1.59 g) and sodium hydrogen carbonate (2.52 g) were dissolved in doubly distilled water (1.00 L) with subsequent pH adjustment. PBS buffer solution (pH 7.2): sodium chloride (8.00 g), potassium chloride (200 mg), sodium hydrogen phosphate dihydrate (1.44 g), and potassium dihydrogen phosphate (200 mg) were dissolved in doubly distilled water (1.00 L). PBST buffer solution (pH 7.2): PBS buffer + Tween(R) 20 (0.05%, *v/v*); pH values were adjusted with HCl (0.1 M) or NaOH (0.1 M).

Liquid bacterial culture: *E. coli* of the strain PKL1162 were grown in LB medium (+AMP, +CAM) overnight, washed with PBS buffer and suspended to a concentration of 2 mg mL^{-1} in PBS buffer.

Mannan coating: Black 96-well plates (Nunc Maxisorp) were filled with a solution of mannan from *Saccharomyces cerevisiae* (1.2 mg mL^{-1} in carbonate buffer, pH 9.5; 120 μL solution per well) and allowed to dry in at 37 °C overnight. The plates were washed with PBST (3×150 μL per well) and stored at 4 °C. Before use the wells were blocked with BSA (5% in PBS, 150 μL per well) for 2 h at 37 °C and then washed with PBST (3×150 μL per well).

Ligand solutions: All tested ligands were dissolved in PBS buffer. The glucosides **5** and **6** and the SA derivatives DES, **7**, and **8** were tested at the highest possible concentrations with respect to their solubility.

GFP-based bacterial adhesion assay: A serial dilution of the examined inhibitor was prepared and 50 μL was transferred into each well of a mannoside-coated, BSA-blocked test plate. The bacterial suspension in PBS buffer (2 mg mL^{-1}) was added (50 μL per well) and the plates were agitated (120 rpm) and incubated for 1 h at 37 °C. After washing with PBS (3×150 μL), the wells were filled with PBS (100 μL per well) and the fluorescence intensity (485 nm/535 nm) was determined.

GFP-based bacterial adhesion assay with preincubation: A serial dilution of the examined inhibitor was prepared and mixed with an equal volume of the bacterial suspension in PBS (2 mg mL^{-1}). These mixtures were incubated at 37 °C with agitation (120 rpm) for 1 h. Then, 100 μL of the preincubated mixture was transferred into each well of a mannoside-coated, BSA-blocked 96-well microtiter plate and the plates were agitated (120 rpm) and incubated for 1 h at 37 °C. After washing with PBS (3×150 μL), the wells were filled with PBS (100 μL per well) and the fluorescence intensity (485 nm/535 nm) was determined.

Docking: Two different crystal structures were used for docking studies: FimH in complexation with α-D-mannose (PDB ID: 1KLF; open-gate structure)[11] and FimH in complexation with *n*-butyl α-D-mannoside (PDB ID: 1UWF; closed-gate structure).[13] For docking, flexible FlexX 1.11.1L[20] as implemented in Sybyl6.9[23] was employed. The mannose binding pocket (CRD) was specified as a sphere around the carboxyl C atom of Asp54 with a radius of 10 Å. This procedure results in a CRD consisting of 26 amino acids available for interactions with a docked ligand.

For every docking run 30 conformations (hits) were scored by FlexX. Consensus scoring was used for validation of docked conformations. Default parameters for formal charges and CScore calculations[21,22] were employed for consensus scoring, including the following scoring functions: FlexX original score, ChemScore,[27] DockScore,[28] GoldScore,[29] PMFScore.[30] The conformational hits were relaxed with the aid of the Tripos Force Field with default parameters and scored.

For graphical representation of docked ligand conformations (Figures 3 and 4), the protein FimH is depicted as a Connolly surface, with a color scale reflecting the lipophilic potential of the surface (brown correlates with high lipophilicity, blue with high hydrophilicity).

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