Design and Evaluation of Pilicides: Potential Novel Antibacterial Agents Directed Against Uropathogenic *Escherichia coli*

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The heavy use of antibiotics during the second half of the last century has resulted in widespread bacterial resistance.[1, 2] Overcoming resistance requires the development of antibiotics aimed at new targets in microorganisms.[3] Preferably, such targets should be highly conserved in bacteria and required for pathogenesis, but not found in humans. Herein, we describe the design of a class of potential novel antibacterial agents called "pilicides". Pilicides target periplasmic chaperones, that is proteins required for the assembly of organelles (pili) that allow pathogenic Gram negative bacteria to adhere to host tissue. Synthetic routes that enable the combinatorial synthesis of two families of pilicides have been developed and used to prepare an initial set of eight pilicides. Surface plasmon resonance showed that the pilicides bind to two bacterial chaperones from uropathogenic *Escherichia coli* and that the binding was in agreement with affinities predicted by calculations. Importantly, the most potent compounds were able to dissociate chaperone – pilus protein complexes which are required for the assembly of pili.

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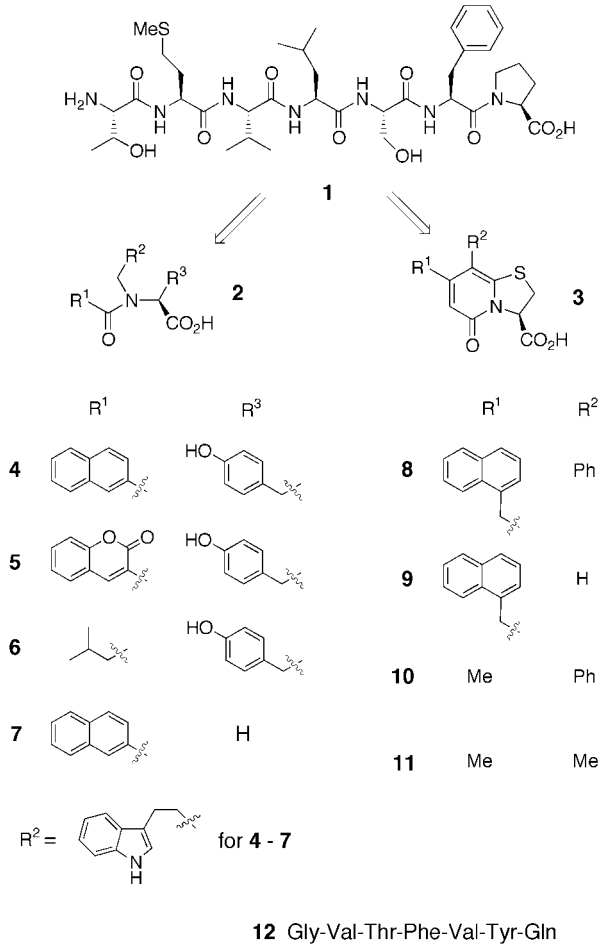
A large number of infectious Gram negative bacteria produce pili, which are a family of extracellular, supramolecular protein organelles that mediate attachment to host tissue.[4, 5] *E. coli* is the main cause of urinary tract infections and the bacterium assembles two types of disease-associated pili. Type 1 pili are important for the development of common urinary tract infections affecting the bladder (cystitis), while P pili are expressed in more severe infections that lead to kidney damage (pyelonephritis).[5, 6] Type 1 and P pili have the saccharide-binding adhesins FimH and PapG, respectively, located at the tip of the rodlike pilus, which is composed of approximately 1000 pilus proteins of a few different types.[5] These two complex organelles are assembled by the molecular chaperones FimC and PapD, respectively. The chaperones bind to the pilus proteins as they cross the cytoplasmic membrane and bring them across the periplasmic space to assembly sites at the outer membrane of the bacterium. Formation of chaperone – pilus protein complexes is required for assembly of pili, without which colonization of host tissue can not occur. Bacterial periplasmic chaperones therefore appear to be ideal targets for the development of novel antibacterial agents.

Synthetic peptides from the conserved C-termini of pilus proteins are bound by the PapD chaperone,[7] and the structures of complexes between PapD and two of these peptides have been solved by X-ray crystallography.[8, 9] The structure of the complex formed between PapD and a C-terminal peptide from the pilus adhesin PapG (**1**, Scheme 1) reveals that the C-terminus of the peptide is anchored to the Arg 8 and Lys 112 residues in PapD (Figure 1 a).[8] Interestingly, these two charged residues are invariant in all known periplasmic chaperones and are critical for the ability of chaperones to assemble pili.[10] The peptide – PapD complex is further stabilized by backbone hydrogen bonding and by interaction of hydrophobic peptide side chains, such as those of Leu and Phe at positions 2 and 4, with complementary pockets in the chaperone (Figure 1 a). Identical interactions are found in the complex formed between PapD and a peptide from the pilus adaptor protein PapK.[9] Moreover, the key interactions found in the peptide – chaperone complexes are also present in the native PapD – PapK and FimC – FimH protein – protein complexes.[11, 12]

The direct use of peptides as drugs has several severe drawbacks, namely, peptides are poorly absorbed after oral administration, they undergo rapid enzymatic degradation, and are often quickly excreted.[13] Based on the structure of the complex between PapD and the PapG peptide,[8] we have therefore designed two families of pilicides: amino acid derivatives **2** and pyridinones **3** (Scheme 1). These families target the active site of periplasmic chaperones, and the two generic structures were chosen to allow synthesis and optimization by combinatorial chemistry. Pilicides **4** and **8**, which predominantly have aromatic substituents in the R^1 – R^3-positions, were docked manually into the active site of PapD so as to get insight into the binding properties of PapD by computational studies. The docking was done in such a way that the carboxyl group and the amide backbone overlapped with the corresponding moieties in the Phe – Pro part of the complex between peptide **1** and PapD. Energy minimization of the pilicide – PapD complexes in



Scheme 1. *The structure of peptide* ***1*** *(which originates from the PapG adhesin), in a complex with the* E. coli *chaperone PapD[8] was used to design two classes of pilicides: amino acid derivatives* ***2*** *and pyridinones* ***3****. From each class four pilicides (****4*** *–* ***7*** *and* ***8*** *–* ***11****) were selected for synthesis, based on predicted differences in their binding affinity for PapD. Peptide* ***12*** *corresponds to* ***1*** *but originates from the FimH adhesin.*

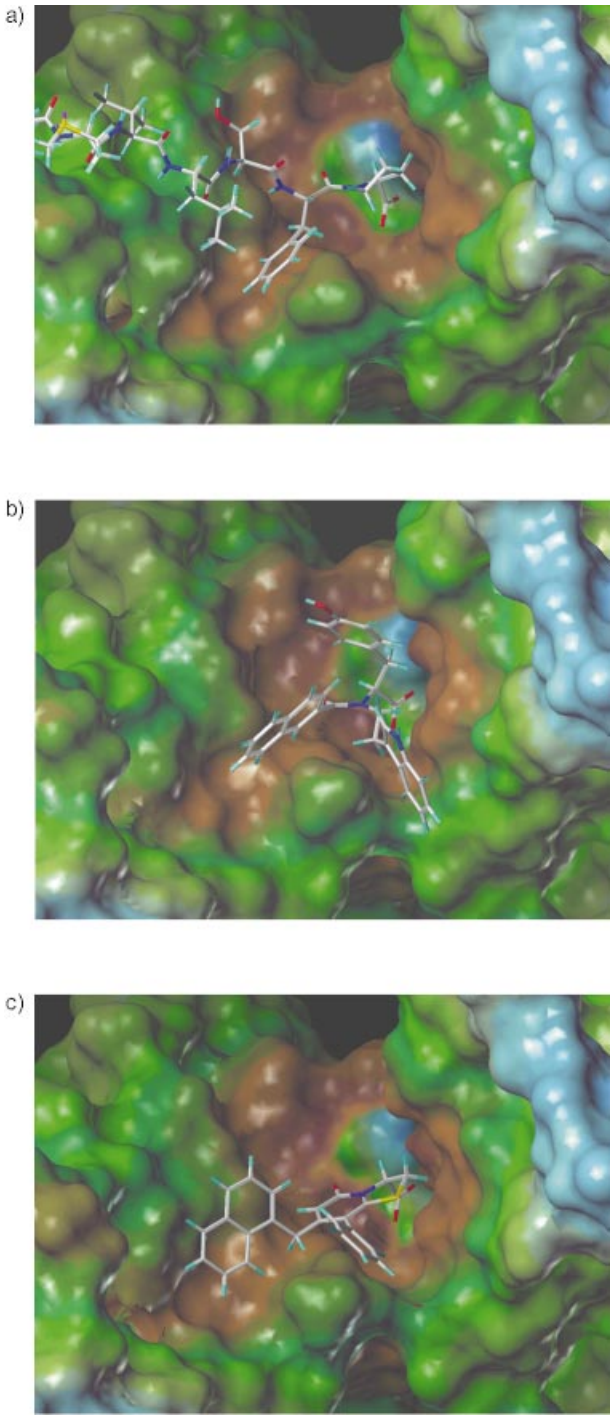


Figure 1. *a) Structure of the crystalline complex formed between the chaperone PapD and a C-terminal peptide from the pilus adhesin PapG.[8] b), c) Energy-minimized models of the complexes formed between PapD and pilicides* ***4*** *and* ***8****, respectively. Polar parts of the PapD surface are blue, nonpolar parts are brown, while green represents intermediate polarity. (The figures were generated with the Sybyl program.)*

MacroModel[14] revealed that the anchoring of the pilicide carboxyl group to the key residues Arg 8 and Lys 112 in PapD was maintained. The naphthyl group in the R^1-position of **4** and **8** then filled the hydrophobic pocket in PapD that was occupied by the Leu and Phe side chains in the cocrystal with peptide **1** (Figure 1). Simultaneously, the pilicide R^2 groups appeared to fit well into a hydrophobic pocket in PapD, which was not occupied by peptide **1**. In addition, the R^3 side chain of **4** was stacked against a nonpolar face in PapD. To probe the validity of this model, a further six pilicides (**5** – **7** and **9** – **11**, Scheme 1), with groups of different size at positions R^1, R^2, and R^3, were modeled into the active site of PapD in the same manner as for **4** and **8**. A calculation of the affinities of **4** – **11** for PapD by the program VALIDATE,[15] suggested differences of more than two orders of magnitude within each family in binding to PapD (Table 1).[16]

The synthesis of the eight pilicides was performed as outlined for **4** and **8** in Scheme 2. Thus, protected tyrosine **13** was first alkylated with 3-(2-bromoethyl)-1*H*-indole (→**14**) and then acylated with 2-naphthoyl chloride to give **15**. Removal of the *tert*-butyl protecting groups by treatment with trifluoroacetic acid gave **4** in 30% overall yield from **13**. Pyridinone **8** was obtained in five steps through a novel procedure based on the

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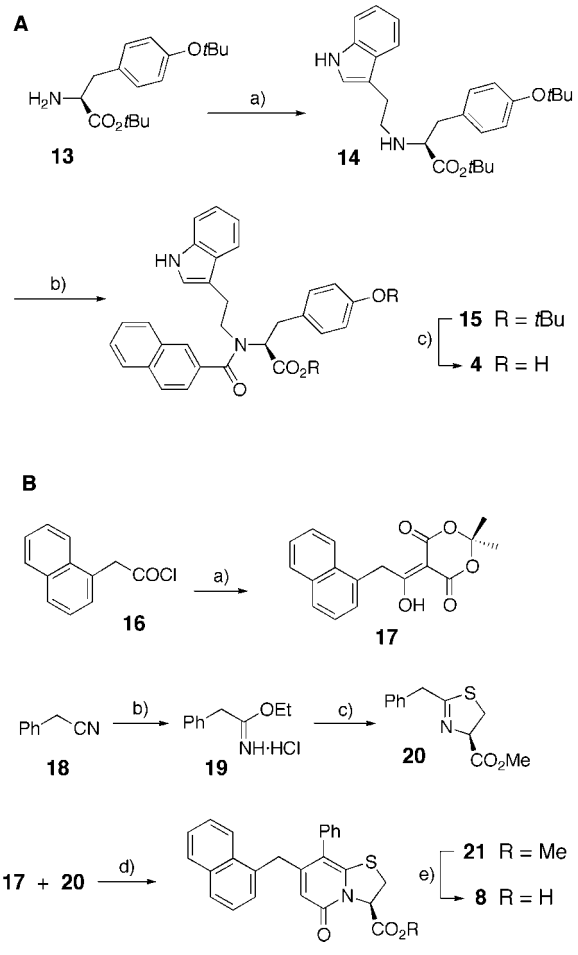
Table 1. *Affinities of pilicides **4** – **11** for the PapD and FimC chaperones, as well as the ability of pilicides to dissociate the FimC – FimH complex. Peptides **1** and **12** were included for comparison.*

Peptide/Pilicide	Calculated affinity [$-\log K_{dis}$][a]	Norm. Response for PapD [%][b]	Norm. Response for FimC [%][b]	CH complex dissociation [%][c]
4	10.8	57	47	15
5	10.1	36	21	8[d]
6	9.5	14	20	0
7	8.9	19	23	5[d]
8	10.0	100	100	86
9	8.7	18	23	7
10	8.3	12	16	5
11	7.1	7	20	1
1	–[e]	25	10	–[e]
12	–[e]	19	11	–[e]

[a] Calculated for binding to PapD with the program VALIDATE.[15] [b] Determined by surface plasmon resonance by using a Biacore 3000 instrument. The two chaperones were immobilized on the dextrane surface of the sensor chip by using a standard thiol coupling procedure. The binding of the ligands, which had been dissolved in phosphate running buffer (pH 7.4) containing dimethylsulfoxide (5%), was then monitored in real time at a fixed concentration (30 μM). The normalized responses were calculated using pilicide **8** as a reference and have been corrected for differences in molecular weight. [c] Each pilicide was incubated with the purified FimC – FimH complex in 20 mM MES buffer (pH 6.8) for 1 h at 37 °C. The amounts of the FimC – FimH complex and free FimC were then determined by FPLC. A pilicide:FimC – FimH ratio of 150:1 was used unless otherwise stated. [d] A pilicide:FimC – FimH ratio of 176:1 was used. [e] Not determined.



Scheme 2. *A) a) 3-(2-Bromoethyl)-1H-indole, Et_3N, DMF, 65 °C, 18 h (46%); b) 2-naphthoyl chloride, DIPEA, CH_2Cl_2, 0 °C→RT, 75 min (74%); c) 2:1 TFA:H_2O, RT, 90 min (87%). B) a) Meldrum's acid, DMAP, CH_2Cl_2, −12 °C→RT, 4 h (91%); b) HCl(g), EtOH, 0 °C, 4 h; c) (R)-cysteine methyl ester hydrochloride, TEA, CH_2Cl_2, 0 °C→RT, 17 h, (83% from **18**); d) HCl(g), 1,2-dichloroethane, 0→64 °C, 11 h (85%); e) 0.1 M aq NaOH, MeOH, RT, 17 h (92%). DMF = N,N-dimethylformamide; DIPEA = N,N-diisopropylethylamine; TFA = trifluoroacetic acid; DMAP = 4-dimethylaminopyridine; TEA = triethylamine.*

use of acid chlorides and nitriles as starting materials.[17] First, acid chloride **16** was treated with Meldrum's acid to give **17**, while nitrile **18** was converted into imidate **19** and then, by reaction with L-cysteine methyl ester, to thiazoline **20**. Condensation of key building blocks **17** and **20** to give pyridinone **21** was achieved in 1,2-dichloroethane which had been saturated with gaseous HCl, and is assumed to proceed by reaction of a ketene derived from **17** with thiazoline **20**.[17] Basic hydrolysis of the methyl ester in **21** then gave **8** in 64% yield based on nitrile **18**.

The binding of pilicides **4** – **11** to PapD and FimC was investigated by surface plasmon resonance[18] after immobilization of the chaperones on a dextrane-coated sensor chip. In this assay tyrosine derivative **4** and pyridinone **8** showed strong binding to both PapD and FimC (Table 1). Pilicide **5** was also bound well by PapD, while the five remaining pilicides displayed lower affinities for the two chaperones. The C-terminal heptapeptides from the adhesins PapG and FimH (**1** and **12**, respectively) bound to PapD and FimC with affinities comparable to the more weakly bound pilicides. Several drugs and druglike compounds, for example, amoxicillin, terbutaline, and L-tryptophan, were used as controls and did not display any affinity for the two chaperones. Interestingly, the binding of the pilicides to PapD was in good qualitative agreement with the calculated affinities, which supported the structural model for the binding to PapD (Figure 1b,c). The pilicides showed no or only weak binding to Protein A and an anti-myoglobin monoclonal antibody, which suggested that they were specific for their chaperone targets. The binding of pilicide **4** to PapD was also investigated by ^{1}H NMR spectroscopy (Figure 2).[19] Addition of one equivalent of PapD to a mixture of **4** and 1-naphthylacetic acid led to a substantial reduction in the intensity of the signals for **4**. No reduction was found for the 1-naphthylacetic acid signals, thus revealing that only **4** bound to PapD.

The studies based on surface plasmon resonance and NMR spectroscopy do not reveal if the pilicides bind in the active site of PapD and FimC, or if they interfere with the formation of

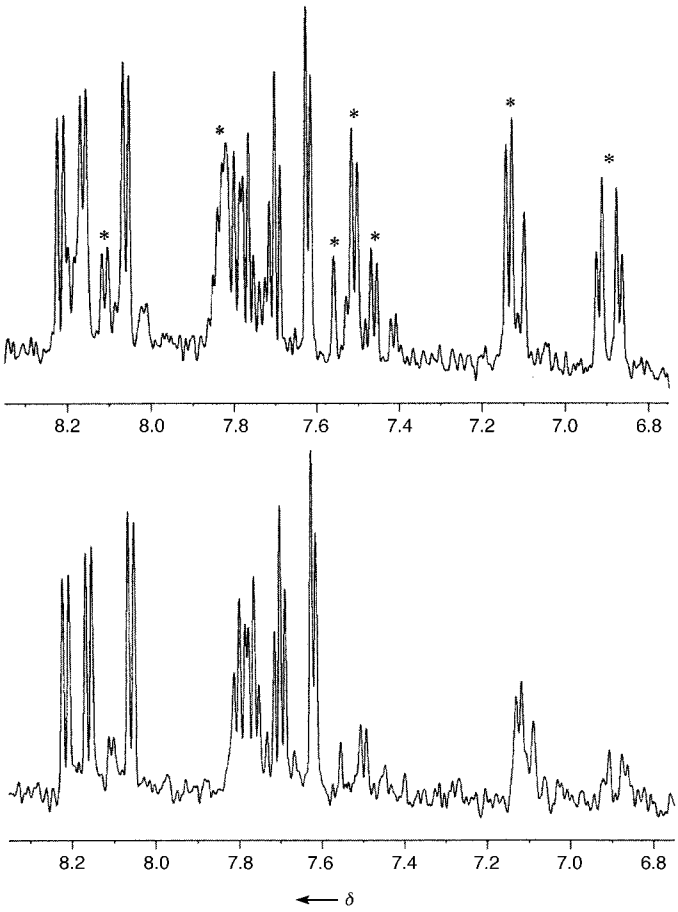


Figure 2. *^{1}H NMR spectra of a 1:1 mixture of pilicide **4** and 1-naphthylacetic acid in the absence (top) and presence (bottom) of one equivalent of PapD. Both spectra were recorded with a delay of 200 ms. Resonances originating from **4** are marked with an asterisk in the top spectrum.*

complexes with pilus proteins. These questions were addressed by incubation of each of the pilicides **4** – **11** with the FimC – FimH complex. The amount of dissociation of the FimC – FimH complex was determined after 1 h at 37 °C by fast-protein liquid chromatography (FPLC, Table 1).[20] Pilicides **4** and **8**, which bound best to PapD and FimC, were both able to dissociate the FimC – FimH complex. Pyridinone **8** was superior and effected almost complete dissociation of the complex when employed at a 150-fold excess. Some of the other pilicides also displayed lower levels of complex dissociation.[21] It can therefore be concluded that **4** and **8**, at least, bind in the active site of the chaperones.

Protein – protein interactions have been considered as attractive but difficult targets for drug development.[22] As described herein, members of the two families of pilicides bind to periplasmic chaperones from uropathogenic *E. coli*, and dissociate chaperone – pilus subunit complexes. Generic structures **2** and **3** therefore constitute leads for the development of a completely new type of antibacterial agents that targets bacterial colonization of host tissue by interfering with chaperone-mediated pilus assembly. Such drug development efforts should be facilitated by the structural model for the binding of pilicides to the PapD chaperone and by the preparation of combinatorial libraries focused on **2** and **3**. Chaperones with highly conserved structures are involved in pilus assembly in a large number of bacteria, which, in addition to urinary tract infection, cause disease such as otitis media and meningitis (H. influenzae), whooping cough (B. pertussis), and gastrointestinal disorders (S. typhimurium).[5, 10] Pilicidal antibacterial agents may therefore find general use for the treatment of infections caused by Gram negative bacteria. Since a complex virulence mechanism is targeted, mutants will most likely be avirulent and the development of resistance is therefore less likely.

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