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Synthesis of Cationic Antimicrobial $\beta^{2,2}$-Amino Acid Derivatives with Potential for Oral Administration

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We have prepared a series of highly potent achiral cationic $\beta^{2,2}$-amino acid derivatives that fulfill the Lipinski's rule of five and that contain the basic structural requirements of short cationic antimicrobial peptides. Highest antimicrobial potency was observed for one of the smallest $\beta^{2,2}$-amino acid derivatives (M_w 423.6) exhibiting a MIC of 3.8 μM against methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *Staphylococcus epidermidis* (MRSE), and *Staphylococcus aureus*, and 7.7 μM against *Escherichia coli*. The $\beta^{2,2}$-amino acid derivatives were shown to have similar absorption properties as several commercially available drugs, and the results implied a resembling membrane disrupting mechanism of action as reported for much larger cationic antimicrobial peptides. By their high potency, nontoxicity, absorption properties, and ease of synthesis, the $\beta^{2,2}$-amino acid derivatives demonstrate a way to modify a vastly investigated class of cationic antimicrobial peptides into small drug-like molecules with high commercial potential.

Introduction

Rapid increase in bacterial resistance has become a major public concern by escalating alongside a lack of development of new anti-infective drugs.[1] During the last few decades, only four new antimicrobial drugs, linezolid, daptomycin, tigecycline, and most recently telavancin have been approved for treatment of serious pathogenic infections.[2] At present, only a handful of the major pharmaceutical companies have R&D programs on anti-infective agents, and the low interest has been defended based on simple economics.[3] A modern drug-development program for an antimicrobial agent is estimated to cost more than 1 billion US$, which should be balanced within a 10−15 year time frame. In addition, development of new anti-infective agents is associated with high risks of inducing bacterial resistance within a few years, as shown by history, or to be restricted for use as a drug of last resort.[4,5] Meanwhile, the Gram-positive bacterium methicillin-resistant *Staphylococcus aureus* (MRSA)[a] has become a leading hospital- and community-acquired pathogen causing life-threatening cutaneous infections, and MRSA is currently outnumbering deaths in US hospitals caused by HIV/AIDS and tuberculosis together.[6−9] Multiresistant Gram-negative enterobacteriaceae *Escherichia coli* and *Klebsiella pneumoniae* containing the New Dehli metallo-β-lactamase 1 (NDM-1) gene were recently isolated from patients who had been hospitalized in India and Pakistan.[10] The plasmid associated NDM-1 gene may easily be transferred to other bacteria through horizontal gene transfer and confers resistance to a number of antimicrobial drugs, such as fluoroquinolones, aminoglycosides, and all β-lactams, including the carbapenems which belong to the drugs of last resort. There is therefore an urgent need for new anti-infective drugs with new targets, distinctive modes of action, and low probability of inducing resistance in order to face the present and forthcoming challenges of multiresistant bacteria.[1,4,11,12]

An extensively investigated class of anti-infective candidates is cationic antimicrobial peptides (AMPs), also known as host defense peptides (see Giuliani for an excellent review).[13] So far, the Antimicrobial Peptide Database includes more than 1200 AMPs originating from the innate immunity of mammals, amphibians, and insects.[14,15] AMPs show a unique mode of action by targeting and rapidly destroying the bacterial cell membrane and are thereby less prone to induce bacterial resistance compared to conventional antimicrobial drugs.[13,16,17] Encouragingly, a number of small to medium-large pharmaceutical companies have been established as a result of the extensive research on these promising molecules, and several drug candidates are in clinical trials.[18]

However, an imperative obstacle to success when developing clinical useful AMP-based drugs is to solve the inappropriate pharmacokinetic properties of AMPs involving low metabolic stability, troublesome absorption kinetics, and risks of unwanted immunological side effects unrelated to their antimicrobial activity.[19] As a result, the majority of AMP-based drug candidates that are currently in clinical trials are directed toward topical treatment of bacterial infections, to be used as antiseptics for implants, or for sterilization of catheter sites.[13,18,20] Noteworthy, Brouwer et al. have demonstrated target-identification by AMPs in vivo by showing that certain AMPs are able to localize at the site of infection in mice.[21]

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[a] Abbreviations: AMP, cationic antimicrobial peptide; DIPEA, diisopropyethylamine; MBC, minimal bactericidal concentration; MRSA, methicillin-resistant *Staphylococcus aureus;* MRSE, methicillin-resistant *Staphylococcus epidermidis*; NDM-1, New Dehli metallo-β-lactamase 1; Ra/Ni, Raney nickel catalyst; RBC, red blood cells; TEA, triethylamine; TFFH, fluoro-*N,N,N′,N′*-tetramethylformamidinium hexafluorophosphate; TI, therapeutic index; TIS, triisopropylsilane.

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There is therefore a great potential for systemic treatment of bacterial infections by AMPs once the pharmacokinetic issues have been solved.

We have recently reported the antimicrobial activity of a series of highly potent β-peptidomimetics consisting of a lipophilic $\beta^{2,2}$-amino acid coupled to a C-terminal L-arginine amide residue that fulfill the minimal pharmacophore requirements for anti-*Staphylococcal* activity of short cationic AMPs, that is, having an overall net charge of +2 and containing two sufficiently bulky and lipophilic residues.[22] The β-peptidomimetics display exceptional proteolytic stability and low hemolytic activity, and by the ease of synthesis allowing amendment of their structural properties, the β-peptidomimetics demonstrate a way to solve many of the obstacles associated with developing small AMP-based drugs.

In the present study, we have further addressed the "drug-likeness" of small antimicrobial β-peptidomimetics by preparing a series of small $\beta^{2,2}$-amino acid derivatives that, in addition to fulfilling the pharmacophore model of short AMPs, also fulfill the Lipinski's rule of five which is used as guidelines for design of peroral active drugs.[23] A series of 16 achiral cationic $\beta^{2,2}$-amino acid derivatives were synthesized and screened for antimicrobial activity against the Gram-positive bacteria MRSA, methicillin-resistant *Staphylococcus epidermidis* (MRSE), *Staphylococcus aureus*, and the Gram-negative bacterium *Escherichia coli*. The seven most potent $\beta^{2,2}$-amino acid derivatives against *E. coli* were furthermore screened for possible lead-compound identification against the Gram-negative bacterium *Pseudomonas aeruginosa*, which is associated with cystic fibrosis. The peroral drug potential of the $\beta^{2,2}$-amino acid derivatives was furthermore addressed both through theoretical calculations and by using a recently developed phospholipid vesicle based barrier model for studying passive diffusion across biological membranes, such as the intestinal epithelia.[24]

The present study demonstrates how a vastly investigated class of peptides can be turned into small drug-like molecules by confining the pharmacophore of small AMPs into a single achiral $\beta^{2,2}$-amino acid derivative. The derivatives displayed very low hemolytic activity and comparable or even improved antimicrobial potency compared to much larger AMPs. The $\beta^{2,2}$-amino acid derivatives demonstrate furthermore the potential for oral administration of future AMP-based drugs by having improved pharmacokinetic properties, as revealed by the results from the permeability studies and by fulfilling the Lipinski's rule of five.[23]

Results

Synthesis. The $\beta^{2,2}$-amino acid derivatives (Figure 1) were synthesized according to the strategies shown in Scheme 1.[22,25−27] For an excellent review covering a wide range of methods for preparing β-amino acids, we highly recommend the review by Abele and Seebach.[28]

The strategies chosen in the current study enabled synthesis of the $\beta^{2,2}$-amino acid derivatives by similar reaction conditions and allowing the use of parallel synthesis equipment ensuring high-throughput synthesis. The Boc-protected $\beta^{2,2}$-amino acids **3a**−**d** were synthesized by the same overall method that we have recently reported, and in the following step coupled to four different C-terminal cationic groups (Scheme 1).[22] In order to improve the yields, 1.5 equiv of the coupling reagent fluoro-*N,N,N′,N′*-tetramethylformamidinium hexafluorophosphate (TFFH) was used in the final coupling step, and the $\beta^{2,2}$-amino acids were also preactivated for 2 h with TFFH instead of the standard 10 min.[27] The coupling reactions were followed by mass spectrometric analysis and terminated by addition of aqueous Na_2CO_3. However, due to sterical hindrance the coupling time had to be extended up to 7 days for attachment of the C-terminal cationic groups. Despite these measures and addition of excessive TFFH, small amounts of uncoupled $\beta^{2,2}$-amino acids were nevertheless detected during purification by RP-HPLC.

Antimicrobial Activity. With a few exceptions, the antimicrobial potency against all the test bacteria increased with decreasing size of the C-terminal cationic groups. As shown in Table 1, there was an increasing number of $\beta^{2,2}$-amino acid derivatives displaying MIC values below 10 μM against the Gram-positive bacteria and *E. coli* when comparing series **4** and **5** with the smaller compounds of series **6** and **7**. Important exceptions were observed against MRSA, which in general was surprisingly susceptible to nearly all of the $\beta^{2,2}$-amino acid derivatives. Thus, all four series **4**−**7** of $\beta^{2,2}$-amino acid derivatives included compounds with MIC values below 10 μM against MRSA. Noteworthy, the two most potent $\beta^{2,2}$-amino acid derivatives **4c** and **7d** against MRSA belonged to two structurally quite different classes of derivatives.

As a general trend, the antimicrobial potency against the Gram-positive bacteria and *E. coli* increased also with respect to side-chain structure in the order **a**, **b**, **c**, and **d**. The $\beta^{2,2}$-amino acid derivatives **4a**, **5a**, **6a**, and **7a** displayed in general poor antimicrobial activity and were also among the least lipophilic derivatives, as observed by measuring the overall lipophilicity of the derivatives on an analytical RP-HPLC C_{18}-column (Figure 2). Highest antimicrobial potency against all test bacteria was displayed by the $\beta^{2,2}$-amino acid derivatives **4d**, **5d**, **6d**, and **7d**, which belonged to the most lipophilic derivatives. Both our previous report on β-peptidomimetics and reports by Haug et al. have shown a strong correlation between high overall lipophilicity and high antimicrobial potency.[22,29] The results of the present study was however unable to detect a similar relationship, as observed when comparing overall lipophilicity as displayed in Figure 2 with antimicrobial potency shown in Table 1. Clearly, overall lipophilicity was not the sole determinant for antimicrobial potency.

When considering antimicrobial potency against the Gram-positive test bacteria MRSE and *S. aureus*, **6d**, **7c**, and **7d** were the most potent $\beta^{2,2}$-amino acid derivatives against MRSE, and the two latter compounds **7c** and **7d** were also the most potent derivatives against *S. aureus*. We observed a somewhat lower potency against *E. coli* compared to the Gram-positive bacteria, although the trends in antimicrobial potency were similar. The two most potent $\beta^{2,2}$-amino acid derivatives against *E. coli* were **6d** and **7d**, which displayed similar potency as against the Gram-positive bacteria.

On the basis of the results from screening of the $\beta^{2,2}$-amino acid derivatives against the Gram-negative bacterium *E. coli*, the seven most potent $\beta^{2,2}$-amino acid derivatives with MIC values below 25 μM against *E. coli* were also screened for antimicrobial activity against the Gram-negative bacterium *P. aeruginosa* for possible lead-compound identification (Table 1). The antimicrobial potency against *P. aeruginosa* was in general lower than against *E. coli*, but three $\beta^{2,2}$-amino acid derivatives **6d**, **7c**, and **7d** were identified as promising lead molecules against *P. aeruginosa*. The remaining $\beta^{2,2}$-amino

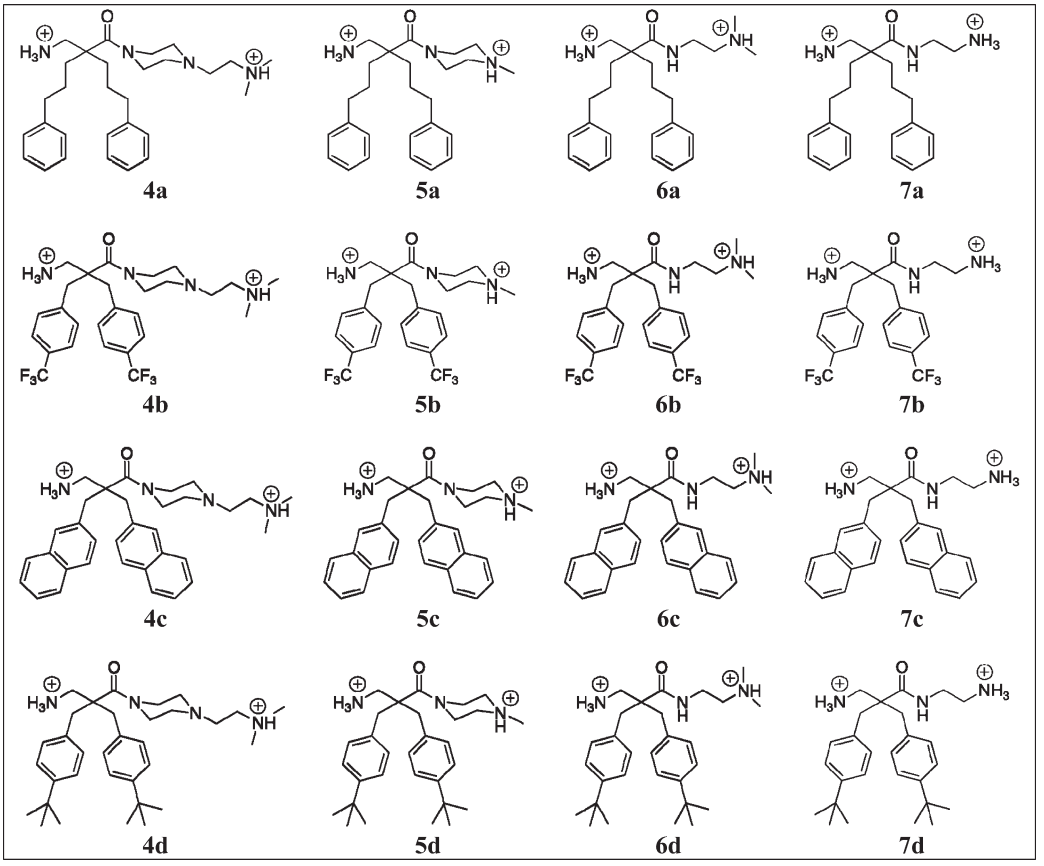
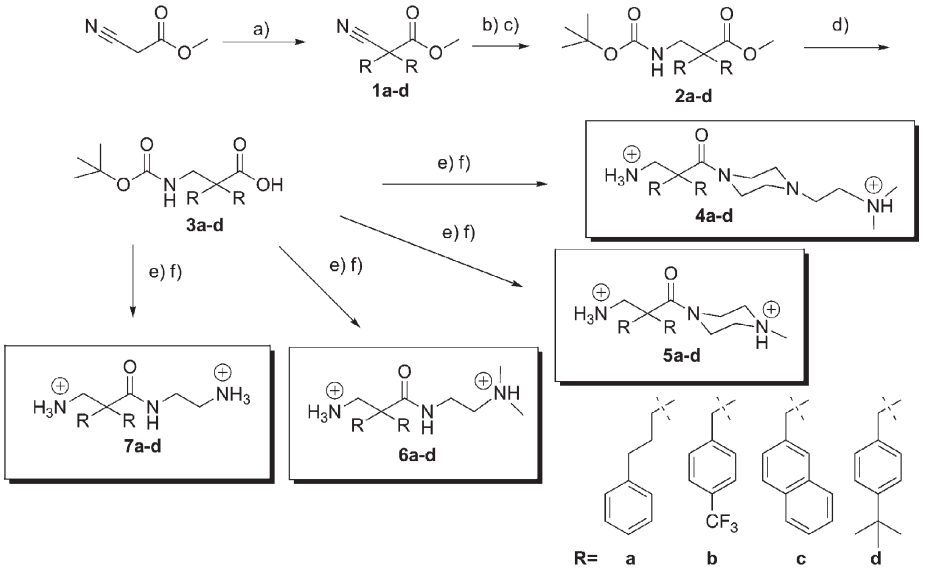


Figure 1. Structure of the antimicrobial $\beta^{2,2}$-amino acid derivatives that were prepared for development of more drug-like AMP-based molecules. All the $\beta^{2,2}$-amino acid derivatives were isolated as their ditrifluoroacetate salts.

Scheme 1. Strategies for Synthesis of All the Antimicrobial $\beta^{2,2}$-Amino Acid Derivatives[a]



[a] (a) NaOMe (1 equiv), R-Br (1 equiv), performed twice, 78 °C s: MeOH. (b) Ra/Ni, H_2(g), 45 °C, 5 days, s: MeOH containing 2% acetic acid. (c) TEA, pH 8, Boc_2O (1.2 equiv), r.t, 18 h, s: H_2O/dioxane (1:5). (d) LiOH (6 equiv), 18 h, 100 °C, s: H_2O/dioxane (1:3). (e) DIPEA (3 equiv), TFFH (1.5 equiv), r. t., 2 h, then addition of the desired amine (2 equiv), up to 7 days, s: DMF. (f) TFA/TIS/H_2O (95:2.5:2.5), r.t, 2 h, s: DCM. All $\beta^{2,2}$-amino acid derivatives were isolated as their di-trifluoroacetate salts.

acid derivatives tested against *P. aeruginosa* showed no or poor antimicrobial potency.

Selectivity. The hemolytic activity of the $\beta^{2,2}$-amino acid derivatives was in general very low, and except for **7d** all derivatives displayed hemolytic activity above 100 μM, which was well above their MIC values. A therapeutic index (TI) was calculated by dividing the hemolytic activity (EC_{50}) of the $\beta^{2,2}$-amino acid derivatives by the MIC value against

Table 1. Minimal Inhibitory Concentration (MIC in μM) against MRSA, MRSE, *S. aureus*, *E. coli*, and *P. aeruginosa*, and Hemolytic Activity (EC_{50} in μM) against Human RBC for a Series of Small $\beta^{2,2}$-Amino Acid Derivatives Prepared for Development of More Drug-like AMP-Based Molecules[a]

	MIC[b]					EC_{50}[c]	therapeutic index				
entry	MRSA[d]	MRSE[e]	*S. aureus*[f]	*E. coli*[g]	*P. aeruginosa*[h]	*RBC*[i]	MRSA	MRSE	*S. aureus*	*E. coli*	*P. aeruginosa*
4a	72	72	289	289		-[j]					
4b	6.5	65	45	129		1116	172	17	25	8.6	
4c	3.4	14	20	48		409	120	29	20	8.5	
4d	13	13	13	13	255	337	26	26	26	26	1.3
5a	315	315	315	315		-					
5b	14	70	49	70		525	38	7.5	11	7.5	
5c	7.4	15	15	22	-	303	41	20	20	14	
5d	15	7.2	15	15	289	348	23	48	23	23	1.2
6a	56	56	56	160		507	9.1	9.1	9.1	3.2	
6b	7.1	21	14	50		468	66	22	33	9.4	
6c	7.5	7.5	7.5	23	300	117	16	16	16	5.1	0.4
6d	7.4	3.7	7.4	7.4	74	274	37	74	37	37	3.7
7a	59	25	71	336		1377	23	55	19	4.1	
7b	7.4	15	15	52		425	57	28	28	8	
7c	7.8	3.9	3.9	24	55	457	59	117	117	19	8.3
7d	3.8	3.8	3.8	7.7	23	18	4.7	4.7	4.7	2.3	0.8

[a] A therapeutic index was calculated by dividing the EC_{50} RBC values by the MIC values against each bacterial strain. Highest concentrations tested were [b]200 μg/mL and [c]1000 μg/mL. All $\beta^{2,2}$-amino acid derivatives were isolated as their ditrifluoroacetate salts, and the molar concentrations were calculated as such. [d] Methicillin-resistant *Staphylococcus aureus* (ATCC 33591). [e] Methicillin-resistant *Staphylococcus epidermidis* (ATCC 27626). [f] *Staphylococcus aureus* (ATCC 25923). [g] *Escherichia coli* (ATCC 25922). [h] *Pseudomonas aeruginosa* (ATCC 27853). [i] Human red blood cells. [j] The notation "-" denotes no detectable activity (MIC or EC_{50}) within the concentration range tested. Please note that the aminoglycoside antibiotic gentamicin was used as positive control and had a MIC value of 8.4 μM against *S. aureus* (ATCC 25923).

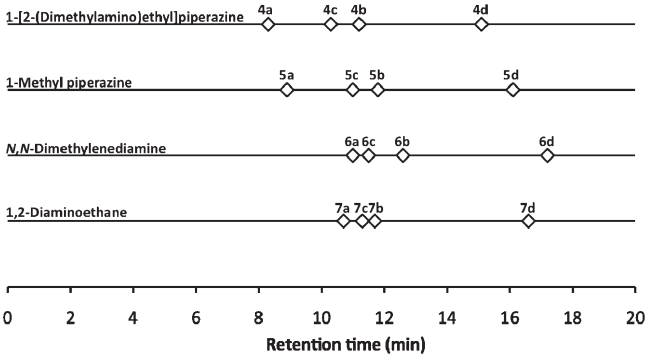


Figure 2. Retention time (Rt) on an analytical RP-HPLC C_{18}-column for all $\beta^{2,2}$-amino acid derivatives prepared as an estimation of overall lipophilicity. The Rt was determined using a linearly increasing gradient of acetonitrile in water containing 0.1% trifluoroacetic acid.

each bacterial strain (Table 1). The results showed that the $\beta^{2,2}$-amino acid derivatives **4b** and **4c** displayed the uppermost selectivity for MRSA compared to human red blood cells (RBC). Although we observed a general trend of increased antimicrobial potency of the $\beta^{2,2}$-amino acid derivatives by decreasing size of the C-terminal cationic group, in the case of MRSA, a large C-terminal cationic group was advantageous and resulted in both high antimicrobial potency and very low RBC toxicity. Further inspections of the TI with respect to MRSA revealed that **6b**, **7b**, and **7c** also displayed profound potency and selectivity for MRSA compared to human RBC and are thereby promising $\beta^{2,2}$-amino acid derivatives for future studies.

A fairly different TI-profile was observed for the $\beta^{2,2}$-amino acid derivatives with respect to the other two Gram-positive strains MRSE and *S. aureus*, in which the highly potent compounds **6d** and **7c** displayed the highest selectivity for these strains. In case of the Gram-negative bacterium *E. coli*, the highly potent $\beta^{2,2}$-amino acid derivative **6d** displayed the highest TI, but **4d**, **5d**, and **7c** were also promising compounds for future studies based on their high TIs. Among the seven $\beta^{2,2}$-amino acid derivatives tested against *P. aeruginosa*, **7c** displayed the highest TI followed by **6d**, whereas the TI for the remaining four active compounds was poor.

Drug-likeness and Oral Absorption. By using the Schrödinger's QikProp application that is included in the Schrödinger's Maestro software v9.1, an evaluation of the drug-likeness of the $\beta^{2,2}$-amino acid derivatives with respect to the Lipinski's rule of five was assessed together with an estimation of percentage oral absorption in humans (Table 2).

The calculations concerning hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA) groups were based on the number of hydrogen bonds that could be formed with water molecules in an aqueous solution, as determined by the software. This explains the noninteger values in Table 2 for the HBA groups of $\beta^{2,2}$-amino acid derivatives confined in the **6a**−**d** and **7a**−**d** series.

The results from the calculations revealed that all the $\beta^{2,2}$-amino acid derivatives fulfilled the Lipinski's rule of five since none of the compounds violated more than one of the four rules.[23] A single rule was violated by 5 of the 16 $\beta^{2,2}$-amino acid derivatives prepared, in which three of these violations were due to a molecular mass above 500 (**4b**, **4c**, and **4d**), while the other two were due to a calculated log *P* value (of the un-ionized compound) above the limit of 5 (**5d** and **6d**).

Calculation of percentage oral absorption in humans by the software concluded that all the $\beta^{2,2}$-amino acid derivatives were likely to be absorbed rather well by having a calculated oral absorption in the range of 62−89% (Table 2). Highest percentage oral absorption was calculated for the $\beta^{2,2}$-amino acid derivatives **5a**, **5b**, **5c**, **6b**, and **6c**, which all were in the range of 86−89% oral absorption.

Table 2. Evaluation of Drug-Likeness of the Prepared $\beta^{2,2}$-Amino Acid Derivatives with Respect to the *Lipinski's Rule of Five* and Calculation of Potential Oral Absorption in Humans

	Lipinski's rule of five (highest permitted value)				calc oral
comp	M_w[a] (500)	HBD[b] (5)	HBA[c] (10)	CLogP[d] (5)	abs[e] (%)
4a	464.7	1	7	3.8	72
4b	544.6	1	7	4.2	62
4c	508.7	1	7	4.3	63
4d	520.8	1	7	4.8	66
5a	407.6	1	5	3.9	86
5b	487.5	1	5	4.4	88
5c	451.6	1	5	4.3	87
5d	463.7	1	5	5.1	79
6a	395.6	2	4.5	3.8	84
6b	475.5	2	4.5	4.6	89
6c	439.6	2	4.5	4.7	89
6d	451.7	2	4.5	5.3	80
7a	367.5	4	3.5	2.9	73
7b	447.4	4	3.5	3.4	71
7c	411.5	4	3.5	3.4	72
7d	423.6	4	3.5	3.7	73

[a] Molecular weights were calculated for nonionized $\beta^{2,2}$-amino acid derivatives. [b] Calculated number of hydrogen bond donor (HBD) groups. [c] Calculated number of hydrogen bond acceptor (HBA) groups. The values were averages over a number of configurations, hence the noninteger values. [d] Calculated octanol−water partition coefficient CLogP of the neutral species of the compounds [e] Calculated percent oral absorption in humans. All predictions were calculated using the Schrödinger QikProp application included in the Schrödinger's Maestro software v9.1.

Permeability. Encouraged by the theoretical calculations, the permeability of the $\beta^{2,2}$-amino acid derivatives was further investigated using a recently established phospholipid vesicle based barrier model.[24] The model has been developed for estimation of drug permeability by passive diffusion across biological barriers such as the intestinal epithelia. Four $\beta^{2,2}$-amino acid derivatives (**4c**, **5c**, **6c**, and **7c**), which showed similar potency against MRSA and against *E. coli* were investigated (Figure 3). All test compounds contained the same lipophilic 2-naphthalene methylene side-chains but different C-terminal cationic groups. On the basis of the models classification of absorption, all four compounds showed permeability equivalent to being *moderately absorbed* in humans.[24]

However, the rank order of the compounds based on permeability through the barrier was a bit different from the rank order based on the calculations of human absorption, as observed when comparing Figure 3 and Table 2. For the experimental permeability values, compound **5c** showed highest permeability followed by compounds **6c**, **4c**, and **7c**. Especially compound **7c** showed a much lower experimental permeability than expected from the calculated absorption values, but we also experienced difficulties with dissolving **7c** in the buffer solution used in the experiment. Because of the low solubility of **7c** the experiment had therefore to be performed with pH 5.5 in the donor compartment, and not pH 7.4 as for the other compounds. The lower pH in the donor compartment increased the solubility of compound **7c**, but might also have resulted in a somewhat higher ionization of **7c** disfavoring permeation across the phospholipid barrier. Together, the low solubility of **7c** and lowered pH in the donor compartment may explain the discrepancy between the experimental permeability studies and the calculations.[30]

Transmission Electron Microscopy. As a preliminary study of the mechanism of action of the $\beta^{2,2}$-amino acid derivatives, *S. aureus* was incubated with two different concentrations of **7c** and investigated by transmission electron microscopy (TEM) (Figure 4). In order to get representative pictures, a much higher number of bacteria had to be used compared to what was used in the MIC determinations, and this also explains the high concentration of compound **7c** that was necessary in the TEM experiments.

The TEM experiments revealed formation of mesosome structures arising from the cell wall or the septum of the treated bacteria, both at the lowest concentration of **7c** treated for 3 h and at the highest concentration of **7c** when treated for 2 h. The mesosome structures indicated membrane damage and were absent in pictures of the untreated *S. aureus* control cells. In addition to mesosome structures, lysed bacteria and debris were observed in the pictures of *S. aureus* treated for 3 h with the highest concentrations of **7c**.

Discussion

Synthesis. We have recently reported the synthesis of a series of highly potent small β-peptidomimetics designed to fulfill the minimal pharmacophore model of short cationic AMPs with anti-*Staphylococcal* activity.[22] During this work, we discovered that lipophilic 2,2-disubstituted propionic acid derivatives, or $\beta^{2,2}$-amino acids, that are linked to an L-arginine amide residue are excellent building blocks for preparing highly potent AMP-based molecules. The β-peptidomimetics had the size of a dipeptide but the side-chain functionalities of a tripeptide and showed superior proteolytic stability compared to many naturally occurring AMPs.

In the present study, we have investigated whether even simpler scaffolds based on the same lipophilic $\beta^{2,2}$-amino acids, and which fulfilled the same pharmacophore model, could provide even more drug-like AMP-based compounds while retaining the high antimicrobial potency of the previously reported β-peptidomimetics (Figure 1). A prerequisite when designing the scaffolds was therefore that the $\beta^{2,2}$-amino acid derivatives should fulfill the Lipinski's rule of five, which is used as a guideline to identify drug candidates with potential for high bioavailability following oral administration.[23]

The scaffolds prepared in the present study were based on four different lipophilic $\beta^{2,2}$-amino acid residues selected among the most potent β-peptidomimetics from our previous study, and coupled to four different C-terminal cationic groups: 1-[2-(dimethylamino)ethyl]piperazine (**4a**−**d**), *N*-methylpiperazine (**5a**−**d**), *N,N*-dimethylendiamine (**6a**−**d**), and 1,2-diaminoethane (**7a**−**d**).

It should be noted that the $\beta^{2,2}$-amino acid building blocks were themselves achiral, and when selecting cationic groups this benefit was kept by choosing four achiral cationic groups from a commercial supplier. Compared to synthesis of larger AMPs in which control of epimerization in each coupling step is crucial, achirality is clearly an advantage and excludes the necessity of separating steroisomers that may be the source of unfavorable pharmacological effects. Furthermore, preparation of the compounds was accomplished from cheap starting materials and avoiding expensive resins and equipment used for solid phase peptide synthesis of AMPs. The reported antimicrobial $\beta^{2,2}$-amino acid derivatives should

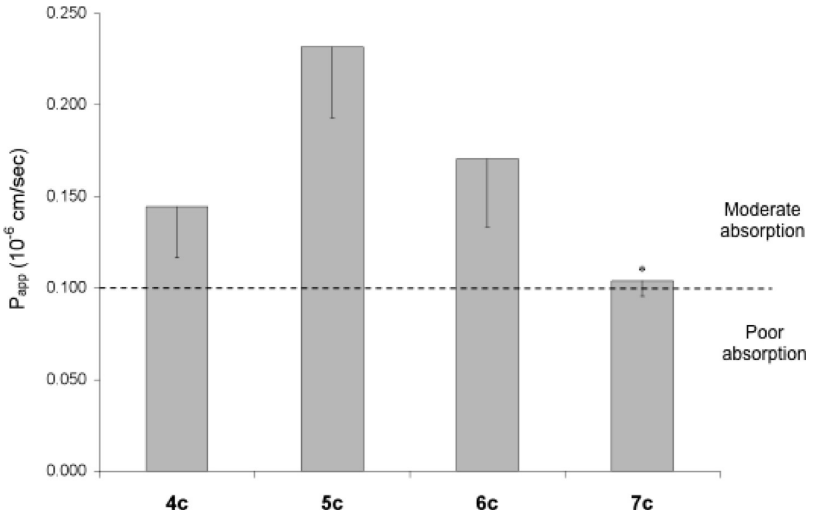


Figure 3. Permeability with standard deviation of the four $\beta^{2,2}$-amino acid derivatives **4c**, **5c**, **6c**, and **7c** across a phospholipid vesicle based barrier as a model for estimation of passive diffusion across, for example, intestinal epithelia.[24] *Because of low solubility of **7c** at pH 7.4, the experiment had to be run with pH 5.5 in the donor compartment. The pH in the acceptor compartments was always 7.4.

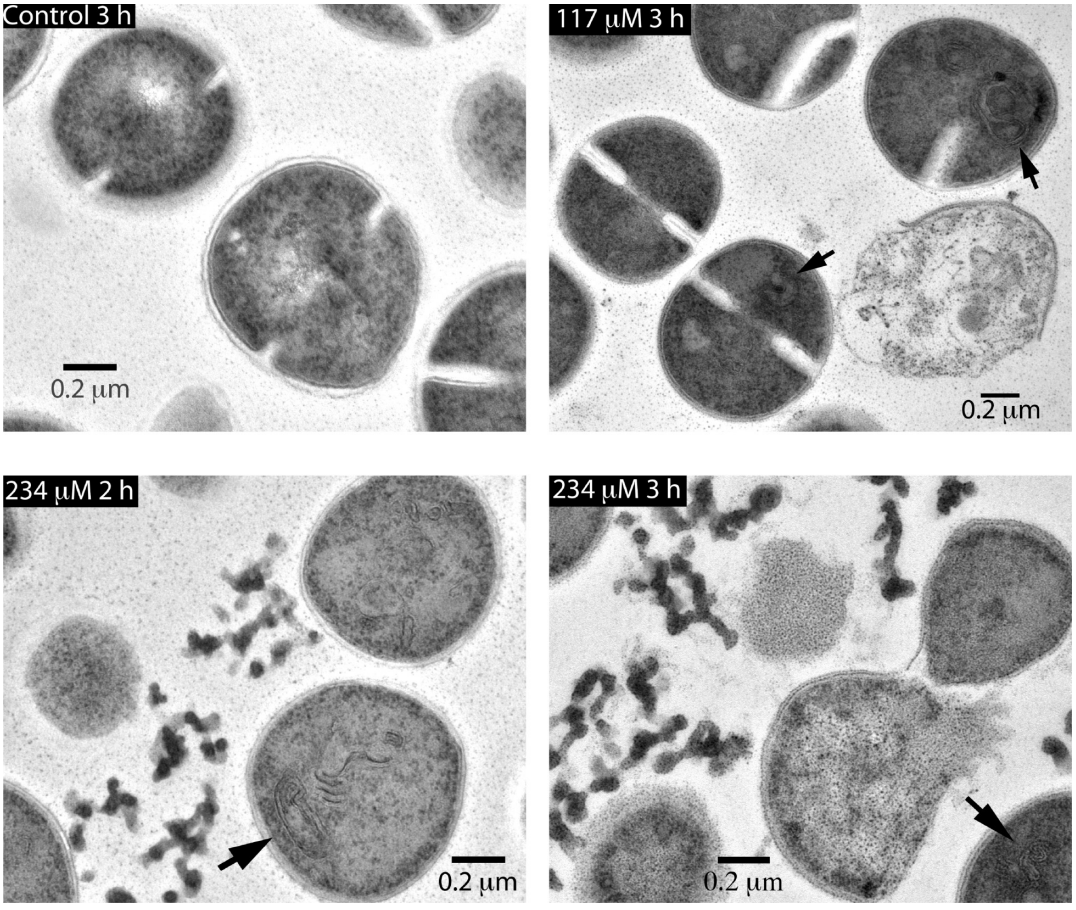


Figure 4. Transmission electron microscopy (TEM) study of the effects of the $\beta^{2,2}$-amino acid derivative **7c** on *S. aureus*. Top left: untreated *S. aureus* control cells. Top right: *S. aureus* incubated with 117 μM of **7c** (30 × MIC) for 3 h. Bottom left: *S. aureus* incubated with 234 μM of **7c** (60 × MIC) for 2 h. Bottom right: *S. aureus* incubated with 234 μM of **7c** (60 × MIC) for 3 h. Mesosome structures (arrows) as an indication of membrane damage were observed at all stages of incubation with **7c**, whereas debris from lysed bacteria was only observed in pictures with the highest concentration of **7c**.

therefore be highly suitable for large-scale industrial manufacture.

Antimicrobial Activity. The results of the study were a series of highly potent $\beta^{2,2}$-amino acid derivatives against both Gram-positive and Gram-negative bacteria. The antimicrobial potency of the most potent compounds **4c**, **6d**, **7c**, and **7d** revealed MIC values below 4 μM against MRSA, MRSE, and *S. aureus*, and MIC values of less than 8 μM against *E. coli* for **6d** and **7d** (Table 1). With respect to *E. coli*, the high potency of the smallest $\beta^{2,2}$-amino acid derivatives may have

been a result of easier penetration through the porins of the outer LPS barrier of *E. coli*, and thereby easier accessibility to the cytoplasmic membrane. However, the Gram-negative bacterium *P. aeruginosa* was somewhat less susceptible with a MIC value of 23 μM for the most potent derivative **7d**. Whether the lower potency against *P. aeruginosa* was an effect of differences in membrane composition or defensive mechanisms was not investigated. However, *P. aeruginosa* is reported to have much lower outer membrane permeability than *E. coli*, and to possess a rich arsenal of efflux pumps that can transport anti-infective agents such as tetracyclines and fluoroquinolones out of the periplasmic space, and together these effects could explain the reduced susceptibility to our small compounds.[31,32]

Selectivity. Hemolytic activity was used as a measurement of toxicity of the prepared $\beta^{2,2}$-amino acid derivatives. The erythrocyte surface consists of glycoproteins bearing sialic acids that provide a negative surface charge, which contributes to efficient blood flow and repulsive forces between blood cells and endothelial cells.[33−35] The negative charge on the erythrocytes are also anchoring sites for positively charged compounds such as AMPs to initiate potential hemolytic action. However, the only $\beta^{2,2}$-amino acid derivatives that could be classified as hemolytic was **7d**. All the other $\beta^{2,2}$-amino acid derivatives displayed EC_{50} values above 100 μM, which was well above their MIC values (Table 1).

We were a bit surprised by the high hemolytic activity displayed by **7d** compared to **4d**, **5d**, and **6d**, which all contained the same *tert*-butyl benzylic side-chains, but different C-terminal cationic groups (Figure 1). The high hemolytic activity of **7d** emphasizes an observation we made from our series of β-peptidomimetics showing that small changes in structure can have a surprisingly high impact on hemolytic activity, whereas antimicrobial potency is much less affected.[22]

The little hemolytic activity that was detected was however used to calculate a preliminary therapeutic index (TI) by dividing the EC_{50} values by the MIC values against each bacterial strain. On the basis of antimicrobial potency and TI, the most promising candidate against MRSA infections was thereby **4b**, with **4c** as a good second choice (Table 1). When regarding the other two Gram-positive bacterial strains MRSE and *S. aureus*, compound **7c** emerged as the most promising candidate based on its high potency and TI. In case of the Gram-negative bacteria, compound **6d** displayed the highest TI with respect to *E. coli*, while for *P. aeruginosa* compound **7c** displayed the highest TI.

C-Terminal Cationic Groups. When considering target interactions, the cationic groups in AMPs certainly have an important role by attracting AMPs to the negatively charged cell surfaces of bacteria, and thereby giving rise to the specificity for bacterial cells over mammalian cells.[36] In most synthetic derivatives of naturally occurring AMPs, lysine, ornithine, and arginine residues are used to provide the cationic charge since these residues are relatively cheap and are easily incorporated into AMPs.[13] Noteworthy, Svenson et al. have recently investigated unnatural cationic amino acid side-chains in short AMPs and have shown that the choice of cationic group plays an important role for antimicrobial potency and proteolytic stability.[37,38]

In the current study, we investigated four different C-terminal cationic groups, and as a general rule, the antimicrobial potency increased with reduction in the size of the C-terminal cationic groups. However, not only the size differed between the four C-terminal cationic groups, but also the structure, primary vs tertiary amine, pK_a, rotational freedom and flexibility of the cationic groups, and the ability to form additional noncharged interactions, such as reinforced hydrogen-bond interactions with the bacterial surface.

The $\beta^{2,2}$-amino acid derivatives **4a**−**d** contained a C-terminal 1-[2-(dimethylamino)ethyl]piperazine group, which was probably capable of forming the most complex charge−hydrogen bond interactions with the bacterial surface, and thereby perhaps being the one closest to resembling the arginine residue of the recently reported β-peptidomimetics.[22] As described above, this motif was highly successful against MRSA, and **4b** and **4c** were both highly potent and displayed the overall highest TI with respect to MRSA. The $\beta^{2,2}$-amino acid derivatives **5a**−**d** contained a C-terminal *N*-methylpiperazine group which resulted in a more constrained C-terminus. The reduced rotational freedom and lack of other HBD groups could explain the somewhat overall low antimicrobial potency of the **5a**−**d** series. The C-terminal *N,N*-dimethylendiamino group of $\beta^{2,2}$-amino acid derivatives **6a**−**d** ensured enhanced flexibility and an extra HBD group that could explain some of the improved potency of **6a**−**d** compared to the $\beta^{2,2}$-amino acid derivatives in series **4** and **5**. The smallest series of $\beta^{2,2}$-amino acid derivatives **7a**−**d** contained a 1,2-diaminoethane group and were the only compounds containing a C-terminal primary amino group. The profound high antimicrobial potency displayed especially by compounds **7b**, **7c**, and **7d** may be a combination of small size, flexible C-terminal primary amino group, and two extra HBD groups.

Although a complex system, investigating different cationic groups can be a powerful tool for optimizing AMP-based compounds, and may improve potency, stability, safety, strain specificity, and last but not least, the pharmacokinetic properties of AMPs as discussed more in detail below.

Lipophilic Side-Chains. The overall lipophilicity of the $\beta^{2,2}$-amino acid derivatives was estimated by investigating the retention time on a RP-HPLC C_{18} column (Figure 2). The results showed that compounds **4d**, **5d**, **6d**, and **7d** with two *para*-*tert*-butyl benzyl side-chains were most lipophilic, whereas $\beta^{2,2}$-amino acid derivatives **4a**, **5a**, **6a**, and **7a** with two benzyl propyl side-chains were the least lipophilic derivatives, which also correlated well with antimicrobial potency. Thus, **4d**, **5d**, **6d**, and **7d** displayed high antimicrobial potency, whereas the potency of **4a**, **5a**, **6a**, and **7a** was poor. However, except for these derivatives, there was little correlation between overall lipophilicity and antimicrobial potency, as observed by the generally higher potency of $\beta^{2,2}$-amino acid derivatives **4c**, **5c**, **6c**, and **7c** containing two 2-naphthyl methylene side-chains compared to the more lipophilic derivatives **4b**, **5b**, **6b**, and **7b** containing two *para*-trifluoromethyl benzyl side-chains (Table 1 and Figure 2). The results were thereby somewhat contradictory with respect to earlier reported studies on small AMP-based compounds and our own β-peptidomimetics, in which much stronger correlations between antimicrobial potency and overall lipophilicity have been observed.[22,29]

However, there are other effects to consider, such as the structure and conformations of the lipophilic side-chains of the $\beta^{2,2}$-amino acids. The close proximity of the lipophilic side-chains causes steric repulsions between the ortho-protons of the aromatic side-chains **b**, **c**, and **d**.[39] This may induce quite rigid conformations with close to perpendicular orientation of these side-chains that affect antimicrobial

potency. The low potency of **4a**, **5a**, **6a**, and **7a** can in part be explained by the reduced ability of their side-chains to form such rigid conformations together with an overall too low lipophilicity. Noteworthy, Tew et al. observed a major improvement in antimicrobial potency by including groups that enabled formation of intramolecular hydrogen bonds, and which thereby rigidified their foldamers.[40]

Furthermore, the *para*-trifluoromethyl benzyl side-chains of **4b**, **5b**, **6b**, and **7b** had a somewhat profound effect on MRSA, resulting in a 2−10 times higher potency against MRSA compared to the two other Gram-positive bacteria MRSE and *S. aureus* (Table 1). We made the same observation against MRSA for our β-peptidomimetics containing this specific side-chain.[22] In drug development, substitution of aromatic or benzylic hydrogen atoms with fluorine is frequently used to optimize the pharmacokinetic properties of drug candidates.[41−43] The contributions of fluorine may involve improved membrane permeability due to increased lipophilicity and altered electronic distribution due to the high electronegativity of the fluorine atom. The high antimicrobial potency displayed by **4b**, **5b**, **6b**, and **7b** against MRSA may have been a result of a combination of these effects. Additionally, by having an effective van der Waals radius similar to an isopropyl group, the trifluoromethyl group increased the bulk of the $\beta^{2,2}$-amino acid side-chains which may have affected the membrane interaction of the compounds.[44,45] Noteworthy, Tew et al. have reported a series of small amphipathic foldamers, in which one of their most potent compounds currently undergoing clinical trials contains two trifluoromethyl phenyl groups.[40] Thus, trifluoromethyl substitution may be an efficient way of improving potency, safety, and pharmacokinetic properties of AMP-based drug candidates.

Lipinski's Rule of Five and Permeability Across Biological Barriers. The drug-likeness and potential for oral administration of the $\beta^{2,2}$-amino acid derivatives was estimated by the Lipinski's rule of five as well as calculation of oral absorption using the Schrödinger QikProp application (Table 2). The rules state that an oral active drug should not violate more than one of the following four criteria: (1) the octanol−water partition coefficient log *P* should be less than 5, (2) the molecular mass (M_w) should not exceed 500 Da, (3) a maximum of 5 hydrogen bond donor (HBD) groups is tolerated, and (4) there should be no more than 10 hydrogen bond acceptor (HBA) groups. However exceptions to the Lipinski's rule of five are known and involve drugs that are transported across membranes by carrier proteins, such as the highly polar antibiotic erythromycin.[46] The issue of active transport of the $\beta^{2,2}$-amino acid derivatives was not investigated in the current study.

The calculations showed that all the $\beta^{2,2}$-amino acid derivatives fulfilled the Lipinski's rule of five even if five compounds violated one single rule (Table 2).[23] Three of these violations were due to molecular weights above 500 (**4b**, **4c**, and **4d**), whereas two were owing to a calculated log *P* above 5 (**5d** and **6d**). Furthermore, the calculated oral absorption was good and ranged from 62 to 89%, with only three $\beta^{2,2}$-amino acid derivatives having a calculated oral absorption below 70% (Table 2).

On the basis of these encouraging theoretical results, we measured the permeability of the $\beta^{2,2}$-amino acid derivatives by passive diffusion through a phospholipid vesicle based barrier model that has proven to give strong coherence between experimental and measured oral bioavailability of commercial drugs.[24] The permeability barrier consisted of phospholipid vesicles made from Lipoid E 80, which is an egg lipid extract with 82% (w/w) phosphatidylcholine in addition to other zwitterionic phospholipids, triglycerides, and cholesterol.[24] Four $\beta^{2,2}$-amino acid derivatives (**4c**, **5c**, **6c**, and **7c**) with comparable potency against MRSA and against *E. coli* were selected since they represented each of the C-terminal cationic groups, but all contained the same lipophilic 2-naphthyl methylene side-chains.

The results showed that all the tested $\beta^{2,2}$-amino acid derivatives were within the models range of *moderate absorption*, in which highest drug-permeability was displayed by the $\beta^{2,2}$-amino acid derivative **5c** followed by **6c** and **4c** (Figure 3). The smallest derivative **7c** was just above the borderline between poor and moderate absorption. However, due to low solubility the permeability of **7c** was measured with pH 5.5 in the donor compartment compared to pH 7.4 for the others, which may have decreased the permeability through the barrier due to increased ionization of **7c** (as described in detail in the Results section).

The measured absorption of the $\beta^{2,2}$-amino acid derivatives was nevertheless in the same range as for several commercial available drugs that have been investigated by the same model, such as the antihypertensive drug enalapril, the β-blocker atenolol, and the antiulcer drug ranitidine, which all are administered by the oral route.[24]

Noteworthy, we observed no lysis of the phospholipid vesicles during the experiments, as we have occasionally observed for more hemolytic peptides, which supported the hemolytic results showing that the $\beta^{2,2}$-amino acid derivatives were safe to eukaryotic cell membranes (results not shown).[37]

Transmission Electron Microscopy. We have so far not identified any intracellular targets for the $\beta^{2,2}$-amino acid derivatives, but at the present stage we cannot exclude possible inhibition of metabolic enzymes in the bacteria, or a mechanism of action involving a combination of membrane disruption and inhibition of crucial bacterial enzymes. Certainly, the cationic groups of the $\beta^{2,2}$-amino acid derivatives would be susceptible to interact with negatively charged intracellular molecules, such as DNA and RNA once diffused across the membrane.

As a brief study of the mechanism of action, the effects of treating *S. aureus* bacteria with the $\beta^{2,2}$-amino acid derivative **7c** was studied by transmission electron microscopy (TEM) (Figure 4). Compound **7c** was selected due to its high potency and selectivity for *S. aureus*. As described in the Results section, we had to increase the number of bacteria and hence also the concentration of **7c** in order to get representative TEM pictures.

By using TEM to assess the impact of peptide treatment, we observed a membrane effect on *S. aureus* treated with **7c** by the appearance of mesosome structures (Figure 4). Mesosome structures have earlier been regarded as structural artifacts thought to be caused by chemical fixation of the bacteria prior to resin embedding and investigation by TEM.[47] However, several groups have pointed out that mesosome structures must be regarded as cytoplasmic membrane alterations, and have demonstrated similar mesosome structures after treatment of *S. aureus* with different types of antimicrobial peptides such as the 26 amino acid α-helical peptide CP29, the cecropine derivative CP11CN, and the linear bactenecin derivative Bac2A-NH_2.[48,49] None of the control cells displayed mesosome structures although they were prepared in the same way as the treated bacteria. We

therefore suspect that the mesosome structures were precursors of membrane disintegration. TEM pictures of *S. aureus* treated with the highest concentrations of **7c** revealed lysed cells and formation of debris. We therefore anticipate that the TEM pictures of mesosome structures, lysed bacteria, and formation of debris reflected subsequent stages in the mechanism of action of **7c**, and that membrane disruption was a plausible mode of action. As determined by reincubating treated bacteria on agar plates, the $\beta^{2,2}$-amino acid derivatives were bactericidal and not bacteriostatic (results not shown).

Conclusion

Naturally occurring cationic AMPs have a general size of 12−50 amino acids and are composed of approximately 50% lipophilic amino acids.[50] The current $\beta^{2,2}$-amino acid derivatives forced this motif to its limits, in which the structural requirements of 50% lipophilic amino acids and a net positive charge was certified by two lipophilic side-chains within a single $\beta^{2,2}$-amino acid unit, and with two cationic groups of diverse structures on each sides (Figure 1).

Both $\beta^{2,2}$-amino acid derivatives with a broad spectrum and a narrow spectrum of activity were developed, and all compounds but one were nontoxic toward human RBC (Table 1). Most notable was the high potency and therapeutic index displayed by the $\beta^{2,2}$-amino acid derivatives against MRSA, especially for **4b** and **4c**. The $\beta^{2,2}$-amino acid derivatives displayed high specificity, in which the most potent and selective compound against MRSE and *S. aureus* was **7c**, whereas **6d** displayed the highest potency and selectivity against *E. coli*. The TEM studies on *S. aureus* treated with **7c** indicated a similar membrane disrupting mode of action as reported for much larger AMPs, although interaction with intracellular targets, or a combination of both, cannot be ruled out at the present stage (Figure 4).

All $\beta^{2,2}$-amino acid derivatives fulfilled the Lipinski's rule of five, which is used as guidelines to ensure high peroral bioavailability of drug candidates.[23] Studies using a phospholipid vesicle based barrier model supported these results and revealed that the absorption properties of the $\beta^{2,2}$-amino acid derivatives were comparable to several commercially available drugs.

The $\beta^{2,2}$-amino acid derivatives were achiral and prepared from cheap starting materials by methods that can easily be scaled up for industrial manufacture. The high potency, nontoxicity, absorption properties, and ease of synthesis emphasize the commercial potential of this new class of antimicrobial compounds.

Experimental Section

All chemicals used in the synthesis and biological evaluation of the $\beta^{2,2}$-amino acid derivatives were purchased from Sigma-Aldrich and used without further purification. Lipoid E-80 was obtained from Lipoid GmbH, Ludwigshafen, Germany. Filter inserts (Transwell, d = 6.5 mm) and plates were purchased from Corning Inc. (Lowell, MA, USA), and the mixed cellulose ester filters (0.65 μm pore size) were purchased from Millipore (Billerica, MA, USA). ^{1}H and ^{13}C NMR spectra were recorded on a 600 MHz Varian spectrometer, and chemical shifts are expressed in ppm relative to methanol (^{1}H 3.31 ppm, ^{13}C 39.0 ppm). The values are given in δ scale. Mass spectra were obtained on a Micromass Quattro LC (Micromass, Manchester, UK). High-resolution mass spectra were obtained on a Waters Micromass LCT Premier (Micromass, Manchester, UK). Preparative RP-HPLC was carried out on a Waters system equipped with a Sunfire C_{18} 100 Å, 5 μm, 19 × 250 mm column, and eluted with acetonitrile and water, both containing 0.1% TFA. Analytical HPLC was carried out on a Waters 2695 HPLC equipped with a Kromasil C_{18}, 300 Å, 5 μm, 4.6 × 250 mm column with PDA detector spanning from wavelength 210 to 310 nm. Compounds undergoing biological evaluation were at least 95% pure as determined by analytical HPLC at 214 and 254 nm. All compounds were prepared by using parallel reaction carousels from Radleys.

Antimicrobial Activity. The antimicrobial screening was conducted at TosLab A/S (Tromsø, Norway). Each compound was tested in duplicate at 200, 100, 50, 35, 15, 10, 5, 2.5, 1, 0.5 μg/mL. All tested compounds were ditrifluoroacetic acid salts.

Hemolytic Activity. The hemolytic activity was assessed as described earlier.[22] In brief, the plasma fraction of heparinized human blood was first removed by centrifugation and three additional washing steps with 37 °C prewarmed phosphate buffered saline (PBS). Subsequently, the human RBCs were diluted to 10% hematocrit and the $\beta^{2,2}$-amino acid derivatives were dissolved in PBS providing concentrations ranging from 1 to 1000 μg/mL. The diluted RBC were added to the compound solutions to a final erythrocyte concentration of 1% v/v. PBS and Triton X-100, in a final concentration of 0.1% v/v, were included as negative and positive control. After 1 h agitated incubation at 37 °C the samples were centrifuged at 4000 rpm for 5 min. Release of hemoglobin was determined by measuring the absorbance of the supernatant at 405 nm. Hemolytic activity was calculated as the ratio of treated sample with $\beta^{2,2}$-amino acid derivative and Triton X-100 treated sample according to the formula:

$$[\%]\text{hemolysis} = \frac{\text{Abs}[\beta^{2,2}\text{-amino acid}] - \text{Abs}[\text{negative control}]}{\text{Abs}[\text{positive control}] - \text{Abs}[\text{negative control}]} \times 100$$

Permeability Experiments. The phospholipid vesicle-based barriers were prepared according to previous reported procedures.[24,51] The lipid used was Lipoid E 80, which is an egg lipid extract with approximately 82% (w/w) phosphatidylcholine, 9% phosphatidylethanolamine, 3% lysophosphatidylcholine, 2% sphingomyelin, 3% triglycerides, and 1% cholesterol (according to the manufacturer Lipoid GmbH, Ludwigshafen, Germany). In brief, liposome dispersions extruded through filters with a pore size of 800 nm and 800 + 400 nm were deposited on a filter support by use of centrifugation. The liposomes were added in consecutive steps, first the smaller liposomes and then the larger ones. Freeze−thaw cycling was then used to promote fusion of the liposomes and hence produce a tight permeation barrier.

Permeability experiments were performed at room temperature by moving inserts, containing the different $\beta^{2,2}$-amino acid derivatives dissolved in phosphate buffer, at certain time intervals to wells containing an equal quantity of fresh phosphate buffer pH 7.4.[24] For **4c**, **5c**, and **6c** phosphate buffer pH 7.4 was also used on the donor side while for **7c** phosphate buffer pH 5.5 was used to be able to dissolve **7c**. UV absorbance (Spectramax190; Molecular devices, Molecular Device Corporation, Sunnyvale, CA, USA) at a wavelength of 230 nm was used to quantify the amount of peptides in the different acceptor compartments giving values for the time points 1, 2, 3, 3.5, 4, 4.5, and 5 h. The electrical resistance across the lipid barriers was measured (Millicell-ERS, Millipore, Billerica, MA, USA) immediately after completion of the permeation studies to control the integrity of the barrier.

Transmission Electron Microscopy (TEM). Approximately 4×10^8 bacteria were incubated with the $\beta^{2,2}$-amino acid derivative **7c** for a maximum time range of 3 h. Suspensions were prefixated with Karnovsky's fixative and stored in pure fixative

at 4 °C overnight. After postfixating with ferrocyanide-reduced osmium tetroxide and dehydration in a graded series of ethanol, samples were infiltrated with a 1:1 mixture of acetonitril/Epon resin (AGAR 100, DDSA, MNA, and DMP-30) overnight. Pure resin was applied the following day and after polymerization ultrathin sections were prepared. Microscopy was carried out on a JEOL JEM-1010 TEM and representative pictures were taken.

Synthesis of $\beta^{2,2}$-Amino Acid Derivatives. Synthesis of the Boc-protected $\beta^{2,2}$-amino acids was conducted in accordance with our previous publication; please see Hansen et al. for details and yields, and the Supporting Information for general procedures.[22]

General Procedure for Synthesis of 4a−d, 5a−d, 6a−d, and 7a−d (GP1). The synthesis was based on the textbook of Chan and White.[27] In brief, the Boc-protected $\beta^{2,2}$-amino acids (**3a−d**) (typically 0.2 mmol) were dissolved in DMF (0.02 M) and DIPEA (3 equiv) was added along with TFFH (1 equiv). The $\beta^{2,2}$-amino acids were activated for 2 h before the desired amine was added (2 equiv). Each reaction was followed by MS, and allowed to react for up to 7 days before it was diluted with ethyl acetate and washed with brine. The organic phase was dried over $MgSO_4$, filtered, and evaporated to dryness. The Boc-protected $\beta^{2,2}$-amino acid derivatives were deprotected by dissolving them in DCM, adding an equivalent volume of TFA/TIS/water (95:2.5:2.5) and stirred at r.t. for 2 h before evaporation to dryness. The crude products were purified by preparative RP-HPLC and lyophilized. The purity of the compounds was checked by analytical HPLC with a PDA detector spanning from 210 to 310 nm. All compounds possessed purity above 95%, as determined by analytical HPLC-PDA at 214 and 254 nm.

Synthesis of 3-Amino-1-(4-(2-(dimethylamino)ethyl)piperazin-1-yl)-2,2-bis(4-(trifluoromethyl)benzyl)propan-1-one (4b). The synthesis was conducted according to GP1. ^{1}H NMR (CD_3OD): δ 2.94−3.04 (9H, m); 3.12 (2H, d, J = 14.9 Hz); 3.19 (2H, br t); 3.50 (2H, br t); 3.55 (2H, d, J = 14.9 Hz); 7.44 (4H, d, J = 8.1 Hz); 7.67 (4H, d, J = 8.1 Hz). ^{13}C NMR (CD_3OD): δ 39.6; 43.9; 45.1; 51.9; 52.3; 53.5; 118.2; 124.7; 126.7; 130.7; 131.0; 132.0; 140.9; 172.5. HRMS-ESI+: $[M + H]^+$ calcd: 545.2713 found: 545.2718, $C_{27}H_{35}F_6N_4O$.

Synthesis of 3-Amino-1-(4-(2-(dimethylamino)ethyl)piperazin-1-yl)-2,2-bis(naphthalen-2-ylmethyl)propan-1-one (4c). The synthesis was conducted according to GP1. ^{1}H NMR (CD_3OD): δ 2.98 (6H, s); 3.07 (2H, s); 3.25 (2H, br d); 3.44 (2H, t, J = 6.9 Hz); 3.61 (2H, t, J = 6.9 Hz); 3.67 (2H, d, J = 14.8 Hz); 7.36 (2H, dd J = 8.4 Hz, J = 1.1 Hz); 7.46−7.52 (4H, m); 7.72 (2H, s); 7.83−7.88 (6H, m). ^{13}C NMR (CD_3OD): δ 40.3; 43.9; 45.8; 52.0; 52.4; 52.9; 53.4; 127.3; 127.6; 128.6; 128.8; 129.1; 129.5; 130.1; 133.9; 134.0; 134.9; 173.6. HRMS-ESI+: $[M + H]^+$ calcd: 509.3278 found: 509.3283, $C_{33}H_{41}N_4O$.

Synthesis of 3-Amino-2,2-bis(4-*tert*-butylbenzyl)-*N*-(2-(dimethylamino)ethyl)propanamide (6d). ^{1}H NMR (CD_3OD): δ 1.28 (18H, s); 2.89−2.92 (8H); 2.97 (2H, s); 3.17 (2H, d, J = 14.2 Hz); 3.30 (2H, t, J = 6.1 Hz); 3.60 (2H, t, J = 6.0 Hz); 7.14 (4H, d J = 8.1 Hz); 7.36 (4H, d, J = 8.1 Hz). ^{13}C NMR (CD_3OD): δ 31.7 (q, J = 21 Hz); 35.2; 36.2 (t, J = 20 Hz); 40.5; 43.2; 43.8; 50.6; 58.4 (t, J = 20 Hz); 126.6; 131.2; 133.3; 151.4; 177.8. Multiplets probably occurring due to insufficient carbon decoupling. HRMS-ESI+: $[M + H]^+$ calcd: 452.3639 found: 452.3650, $C_{29}H_{46}N_3O$.

Synthesis of 3-Amino-*N*-(2-aminoethyl)-2,2-bis(4-*tert*-butylbenzyl)propanamide (7d). The synthesis was conducted accord ing to GP1. ^{1}H NMR (CD_3OD): δ 1.31 (18H, s); 2.91 (2H, d, J = 14.1 Hz); 2.96 (2H, s); 3.10 (2H, t, J = 6.2 HZ); 3.16 (2H, d, J = 14.1); 3.51 (2H, t, J = 6.2 Hz); 7.15 (4H, d, J = 8.2 Hz); 7.38 (4H, d, J = 8.3 Hz). ^{13}C NMR (CD_3OD): δ 31.7; 35.3; 38.6; 40.6; 43.4; 50.5; 126.6; 131.2; 133.5; 151.5; 177.3. HRMS-ESI+: $[M + H]^+$ calcd: 424.3326 found: 424.3324, $C_{27}H_{42}N_3O$.

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Supporting Information Available: Spectroscopic data for all the compounds **4a−d**, **5a−d**, **6a−d**, and **7a−d**, as well as general procedures for the synthesis of **1a−d**, **2a−d**, and **3a−d**. This material is available free of charge via the Internet at http://pubs.acs.org.

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