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Synthesis and Antimicrobial Activity of the Symmetric Dimeric Form of Temporin A Based on 3-*N*,*N*-di(3-aminopropyl)amino Propanoic Acid as the Branching Unit

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Abstract—Dimeric derivative of antimicrobial peptide amide Temporin A (TA) was synthesized by using a new branching unit 3-*N*,*N*-di(3-aminopropyl)amino propanoic acid (DAPPA), which allows building of the parallelly symmetric α-helical structures. Antimicrobial effect of the original peptide amide, its monomeric carboxy (TAc) and novel dimeric (TAd) analogues were tested against *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative). Both TA and TAd completely inhibited the growth of *S. aureus* at the concentrations of 5 and 10 μM, respectively, whereas TAc did not show any inhibitory activity. The activities of TAc, TA and TAd correlate directly with the net charges of the molecules, +1, +2 and +4, respectively. Interestingly, TAd displayed antibacterial effect against *E. coli* at a concentration of 10 μM, where as monomeric TA did not show any activity at concentration as high as 20 μM. The results indicate that the novel structural modification improves the antibacterial properties of Temporin A especially towards Gram-negative bacteria.

Introduction

Antimicrobial peptides play a very important role in innate immunity. They have been isolated from plants, insects, amphibians and vertebrates, including humans.[1,2] Amphibian skin has proven to be an especially rich source of antibacterial peptides. For example bradykinin, brevinins, esculentins, magainin, caerulein, xenopsin and laevitide have been isolated from the skin extracts of several frog species.[3–5] Temporins, a group of antimicrobial peptides, were isolated from the skin of European red frog *Rana temporaria*. These peptides (temporins A–H, K and L) form a group of short (10–13 amino acid residues), alpha helical, amphipathic, hydrophobic peptides with amidated C-terminus and free N-terminus. Temporins are reported to have antibacterial activity without hemolysis to human red cells.[4] They have antimicrobial activity against a broad spectrum of bacteria including clinically important methicillin resistant *Staphylococcus aureus* and vancomysin resistant *Enterococcus faecium*.[6] However, their lethal concentrations toward Gram-negative bacteria are poor.[4,7]

Antibacterial peptides are thought to kill cells by causing leaking of water, ions, and even small macromolecules through the cell membrane. There are currently two alternative hypotheses concerning the mechanisms of action by which amphipathic, α-helical, lytic peptides, such as Temporin A, permeate the cell membrane. These models, 'barrel stave' and 'carpet-like', share a conceptual difference. In the former, the peptides form transmembrane pores by inserting into the hydrophobic core of the membrane. In the 'carpet-like' model the peptides do not insert into the membrane core but the charged hydrophilic regions of the peptide interact with anionic heads of bacterial phospholipids and the hydrophobic regions with the hydrocarbon

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tails.[8–10] It has been proposed, too, that there is not necessarily direct association between the ability to permeabilize the cytoplasmic membrane and peptide antimicrobial activity, but some peptides may act also intracellular by binding to DNA or altering enzyme activities.[11,12] Recently Temporin A all-D enantiomer has been shown to have the same antibacterial activity as Temporin A,[13] indicating that the antimicrobial mechanism of Temporin A is not chiral, but is based on simple hydrophobic interactions.

A substitution of a single amino acid can enhance antibacterial activity, if it increases amphiphilic or amphipathic nature of the peptide and enhances the formation of secondary structure, as shown, for example with Temporin A[13] and ESF1.[14] Modifications, which disturb the secondary structure or amphipathic nature of antimicrobial peptides, usually reduce their ability to permeabilize the zwitterionic or slightly anionic membranes, e.g., blood cells. Nevertheless, if a peptide is sufficiently cationic, it can still permeate highly anionic bacterial cell membranes. Other crucial parameters that affect biological activity of antimicrobial peptides are the hydrophobicity and the hydrophobic moment.[15] Both carboxy and amidated C-terminals of helical antimirobial peptides have been reported. In some cases replacing the amide group with free acid abolished their activity,[16] in the other cases it did not affect activity.[17]

In this paper, we describe the synthesis and the antimicrobial activity of symmetrical dimeric Temporin A analogue. We also report a comparison of the activities of Temporin A, its carboxy analogue (TAc) and the dimeric analogue (TAd) (Fig. 4 A–C) against Gram-positive and Gram-negative bacteria and show that the TAd owns an improved activity toward Gram-negative bacteria.

Results

The bacterial assays were performed in Bioscreen C (Labsystems, Finland). The initial titres of both *S. aureus* and *E. coli* were 1×10^4 cfu/mL and the total growth time was 21 h. The growth of the bacteria was followed turbidometrically every 10 min after a vigorous shaking for 10 s. After 21 h incubation, the numbers of bacteria in the control wells were about 1×10^8 for *S. aureus* and 1×10^9 *E. coli*.

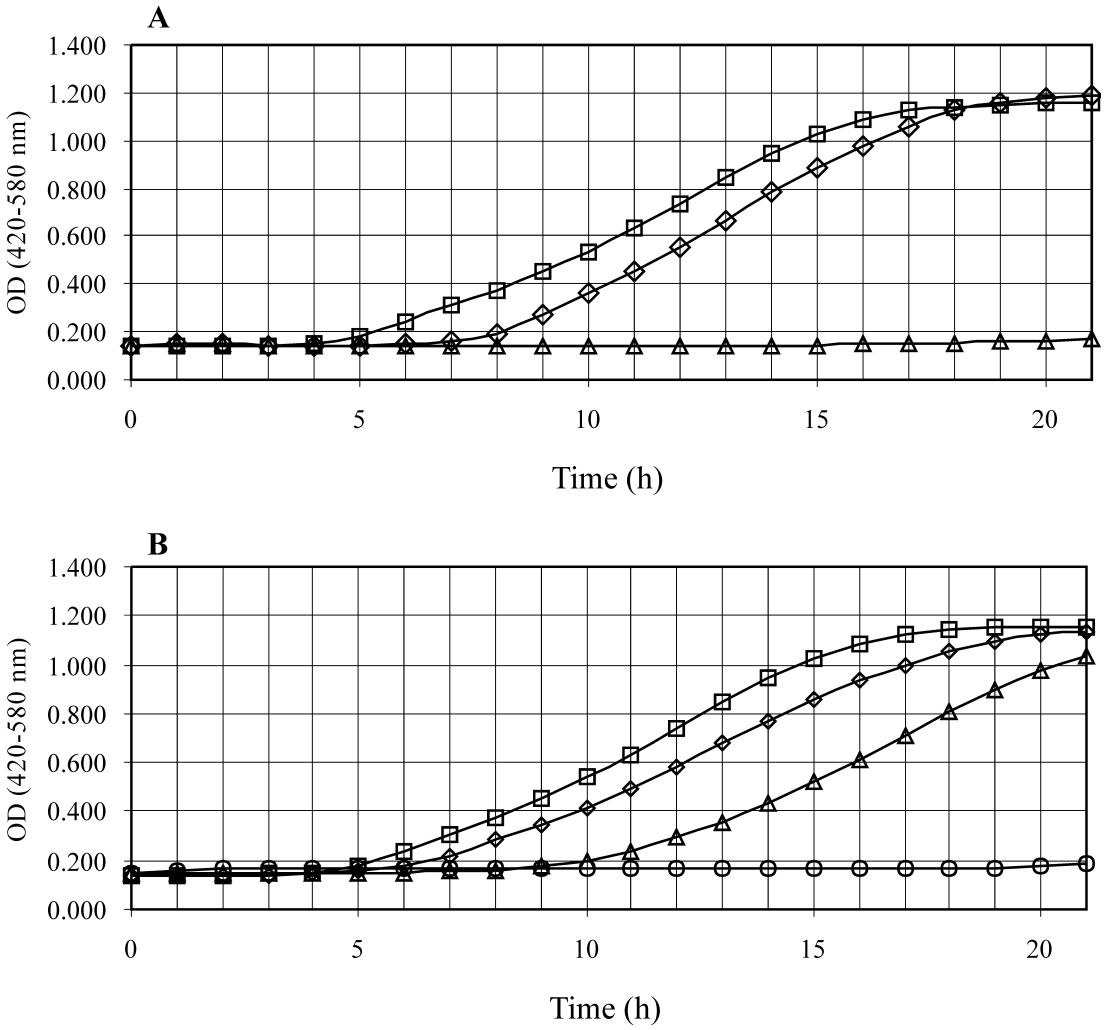

Figure 1. The effect of Temporin A, TA, (A) and its dimeric derivative, TAd, (B) on growth of *S. aureus*. Peptide concentrations were 0 (-□-), 2.5 (-◇-), 5 (-△-) and 10 (-○-) μM. The initial bacterial concentration was 1×10^4 cfu/mL.

The monomeric carboxy analogue of Temporin A (TAc) was totally inactive against both test bacteria at concentrations up to 20 μM. The growth of Gram-positive bacterium *S. aureus* was inhibited totally with TA and TAd at 5 and 10 μM concentration, respectively (Fig. 1). For TAd, some inhibitory effect was seen already at 5 μM. The situation was different with Gram-negative *E. coli*. TA was totally inactive at concentrations up to 20 μM, whereas TAd inhibited the growth totally at 10 μM concentration and the inhibitory effect was already seen at 2.5 μM (Fig. 2).

Discussion

We synthesized a novel, symmetrical backbone, 3-*N*,*N*-di(3-aminopropyl)amino propanoic acid (DAPPA), which enables the building up of symmetrical dimeric peptides. Commonly, branching of multiple peptide chains is based on lysine, which instead allows nonsymmetric[18] and pseudosymmetric[19] structures. In this work we show that DAPPA increases the positive charge of the Temporin A peptide in structurally optimal site for its antibiotic activity.

DAPPA was modified for conventional Fmoc peptide synthesis strategy. The possibility to add the molecule directly to automated peptide synthesizer, without any manual preparations, makes its use convenient. There are some advantages in employment of branched peptide dimers for pharmaceutical purposes. The preorganizing of the chains reduces the binding entropy of the effector peptide and the increase of molecular weight, although generally considered as a disadvantage

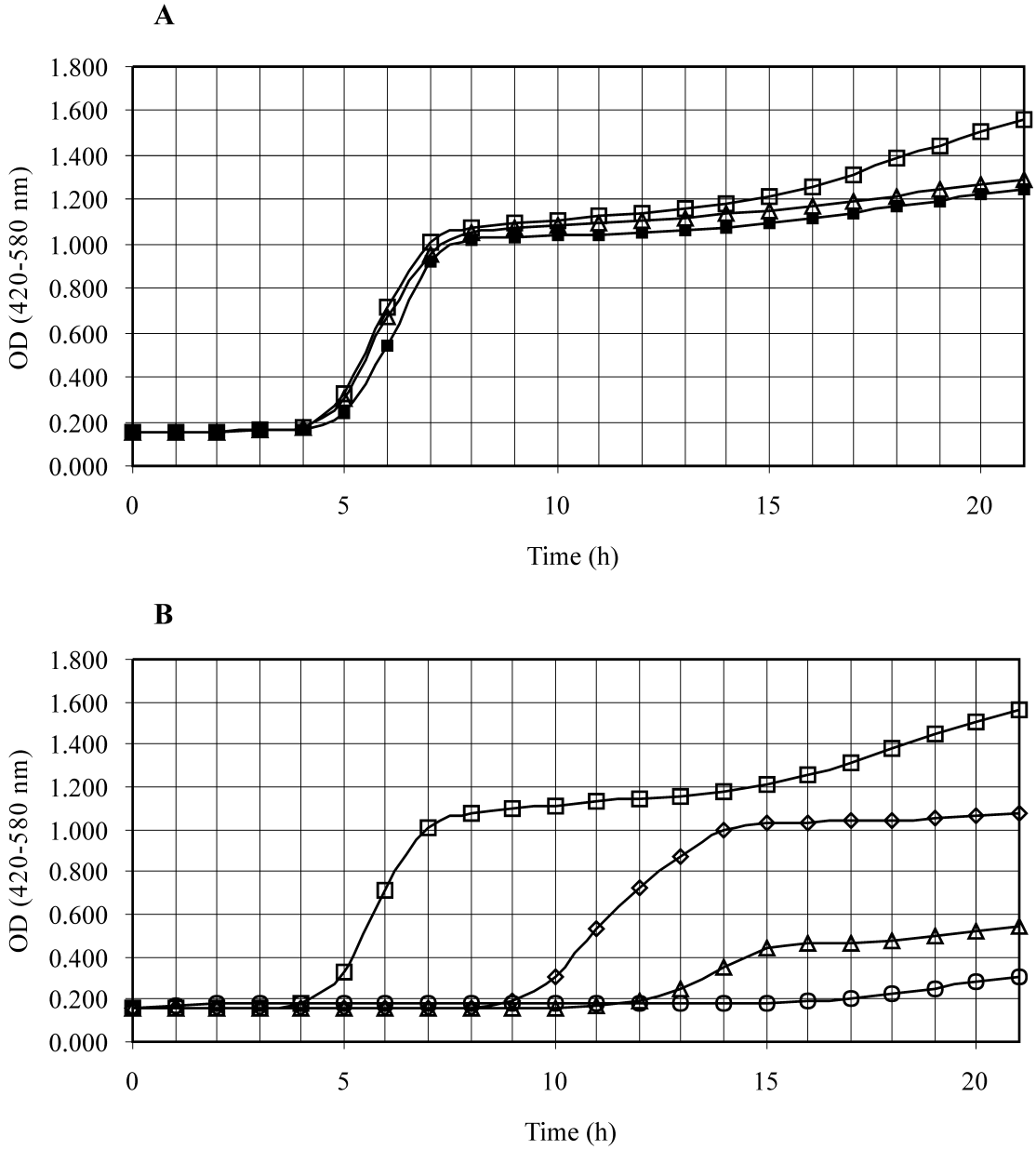

Figure 2. The effect of Temporin A, TA, (A) and its dimeric derivative, TAd, (B) on growth of *E. coli*. Peptide concentrations were 0 (-□-), 2.5 (-◇-), 5 (-△-), 10 (-○-) and 20 (-■-) μM. The initial bacterial concentration was 1×10^4 cfu/mL.

for a drug, would decrease the diffusion of a compound in local use.

The antibiotic peptides, Temporins, contain 10 to 13 amino acids, and according to the CD studies, they adapt an α-helical structure in hydrophobic environment.[7] It has been suggested that Temporins form barrel-stave structures[20] and due to the amphipathic nature of the peptide may form pores where the hydrophobic side of the helix is adjacent to the hydrophobic tails of fatty acids.[21] From the mode of action point of view it is required that the antibiotic peptides penetrate deep enough into the cell membrane in order to lyse the cell. The length of the helical Temporin A is about 24 Å, which is only about half of the thickness of the lipid bilayer. While the mechanism by which Temporins lyse bacteria is not known, we argued that by addition of a spacer tripeptide GGA between DAPPA and the Temporin part would provide the Temporin more freedom for its proposed alignment to the bacterial membrane. The spacer allows also the structure to penetrate deeper into the membrane and its flexibility allows 10–20 Å distance between the C-terminals of the Temporin parts.

The positive charge at the amino terminus is not necessary for the lytic activity of short helical antimicrobial peptides and can be replaced with hydrophobic groups like Fmoc. Unfortunately, such a modification also increases the undesirable lytic activity of Temporin A against eucaryotic cells (erythrocytes).[22] On the contrary, the total positive charge generally improves the antibiotic peptide activity, obviously due to the fact that the surface of the bacteria is negatively charged. This appears to be true also for TA as exemplified by our modifications. The formal total net charges of TA, TAc and TAd are +2, +1 and +4, respectively. It is notable that DAPPA contains one positively charged amino group in its branching point, and this leads to the total net charge +4, instead of +3, of TAd. Also the locations of the charges are of essential importance. It is notable that TA contains a neutral amide group, TAc a negatively charged carboxy group, and TAd a positively charged amino group in that part of the molecule which can be assumed to locate close to the surface of the cell membrane after the peptide is embedded in it for lysis.

The interaction between the negatively charged bacterial inner membrane and the C-terminus of TAc is unfavorable and could render TAc totally inactive against *S. aureus*. The negative charge of TAd at the end of the arm of DAPPA seems to be of less importance for its activity against the Gram-positive bacterium. TA is not able to penetrate the outer membrane (OM) of Gram-negative *E. coli* at the concentrations used. However, the positive charge of TAd makes it active also against *E.coli*, suggesting that it has structural properties that facilitate its OM-penetration. It is known that anions are able to brake OMs of Gram-negative bacteria and that their net total charge is a very essential property for the permeabilization.[23] Our results suggest that the net charge of TA (+2) is not enough, but the net charge of +4 enables total penetration through the outer membrane.

Conclusion

The two Temporin A derivatives (TA and TAd) have similar antimicrobial effects against *S. aureus*, the Gram-positive test strain. However, only the dimeric derivative of Temporin A (TAd) has antimicrobial effect against *E. coli*, the Gram-negative test strain. The permeability of TAd through the outer membrane of *E. coli* can be explained by higher positive net charge of TAd. It is also possible that the positive amino group of the DAPPA unit enhances the activity by interactions between the cell membrane. However, the activity of the TAd is only half that of TA against Gram-positive strains if calculated per one TA unit. This suggests that the present structures are not completely optimized, yet, but provide a good starting point for a further work.

Experimental

Synthesis of the 3-*N*,*N*-di(3-aminopropyl)amino propanoic acid (DAPPA)

According to the synthesis protocol, *N*-(3-aminopropyl)-1,3-propanediamine (**1**) was converted to the corresponding symmetrical backbone **4**. A selective protection of the primary amino-groups of **1** with phthalic anhydride in acetic acid,[24] followed by utilization of methyl acrylate (Michael's addition), afforded pure **3** in a fair yield. Deprotection of the amino and acid functions with concentrated HCl to compound **4** and its Fmoc-protection resulted in the desired Fmoc protected symmetrical molecule **5**, which has a free acid function readily available for the automated solid phase peptide synthesis (Fig. 3).

Procedure for the synthesis of Fmoc-DAPPA (5)

Norspermidine (8.96 g, 68.3 mmol) was dissolved in glacial acetic acid (180 mL). Phthalic anhydride (20.23 g, 136.6 mmol) was added neat and the reaction mixture was refluxed for 1 h. The solvent was distilled in vacuo to give quantitative yield of **2** with the following spectral properties: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (m, 4H), 7.73 (m, 4H), 3.79 (t, J = 6.7 Hz, 4H), 2.97 (t, J = 7.1 Hz, 4H), 2.06 (m, J = 6.9 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.42, 134.13, 131.97, 123.43, 45.60, 34.92, 25.86. Phthalic anhydride protected **2** was dissolved into methyl acrylate (80 mL) and the mixture was stirred at room temperature for 6 h and then refluxed for 4 h. The solvent was distilled in vacuo and the residue was mixed with methanol (50 mL). The precipitate was filtered and washed with cold methanol (20 mL). The yield 26.0 g (80 %) of **3**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 (m, 4H), 7.70 (m, 4H), 3.71 (t, J = 7.3 Hz, 4H) 3.66 (s, 3H), 2.79 (t, J = 7.1 Hz, 2H), 2.51 (t, J = 7.0 Hz, 4H), 2.45 (t, J = 7.1 Hz, 2H), 1.81 (m, J = 7.2 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.10, 168.38, 133.82, 132.21, 123.17, 51.61, 51.25, 49.12, 36.30, 32.18, 26.23. Compound **3** (4.0 g, 8.4 mmol) was mixed with concentrated HCL (30 mL) and refluxed for 20 h. The mixture was allowed to cool to room temperature and the precipitate was filtered. The aqueous phase was

extracted with diethyl ether (5×20 mL) and evaporated to give 2.62 g (99%) of DAPPA (**4**) as a colourless thick oil: ^{1}H NMR (400 MHz, D_2O) δ 3.57 (t, J = 6.8 Hz, 2H), 3.36 (m, 4H), 3.11 (t, J = 7.6 Hz, 4H), 2.94 (t, J = 6.8 Hz, 2H), 2.19 (m, 4H); ^{13}C NMR (100 MHz, D_2O) δ 176.65, 53.41, 51.60, 39.41, 31.38, 24.53. DAPPA (1.54 g, 4.9 mmol) was dissolved in water (15 mL) and neutralized with 10 % NaOH solution (2.4 mL). Ten percent Na_2CO_3 (60 mL) and dioxan (10 mL) was added to the mixture and cooled in an ice bath during the slowly addition of Fmoc-Cl (3.2 g, 10.8 mmol) in dioxan (37 mL). The resulting mixture was stirred for 2 h in an ice bath and 46 h at room temperature. Water (225 mL) was added and the aqueous phase was extracted with diethyl ether (4×50 mL). The pH was set to 6 with concentrated HCl and the aqueous phase was extracted with ethyl acetate (6×60 mL). The organic phase was dried over $MgSO_4$ and evaporated. Yield 2.52 g (79%) of Fmoc-DAPPA (**5**) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (bd, J = 6.6 Hz, 4H), 7.55 (bd, J = 6.8 Hz, 4H), 7.34 (bt, J = 6.7 Hz, 4H), 7.24 (bt, J = 6.7 Hz, 4H), 4.31 (bd, J = 6.1 Hz, 4H), 4.12 (bt, J = 6.1 Hz, 2H), 3.18 (bs, 4H), 2.94 (bs, 2H), 2.78 (bs, 4H), 2.53 (bs, 2H), 1.80 (bs, 4H); MALDI-TOF MS m/z 648.2 (M^+)

Peptide design

The branched peptide was designed based on the proposed amphipatic α-helical structure of Temporin A. The size of the helix structure is proposed to be 24 Å in length and about 12 Å diameter. To facilitate the correct orientation of parallel amphipatic helices, an additional AGG was added between Leu(13) and DAPPA. The distance between carboxy carbon of Leu(13) and the central nitrogen of the DAPPA unit was estimated to be ca. 15 Å in extented conformation and ca. 17 Å to the carboxy group of DAPPA. The Leu(13) carboxy carbons in the two branches are separated by ca. 27 Å.

Peptide synthesis

The monomeric Temporin A peptide amide (NH_2-FLPLIGRVLSGIL-$CONH_2$), the monomeric carboxy (NH_2-FLPLIGRVLSGIL-COOH) and the dimeric derivative [2×(NH_2-FLPLIGRVLSGILAGG)-dappa-G-COOH] (Fig. 4) were synthesized using PerSeptive 9050 Plus automated peptide synthesizer, with Fmoc strategy and TBTU/DIPEA as the coupling reagent. For the synthesis of the peptide amide NovaSyn TGR resin with a modified Rink linker and for the peptide acids NovaSyn TGA with 4-hydroxymethyl-phenoxyacetic acid linker were used (Novabiochem, Läufelfingen, Switzerland). The side-chain protecting groups used in synthesis were trityl (Trt) for Asn and His, *O*-*tert*-Butyl (OtBu) for Glu, *t*-Butyloxycarbonyl (t-Boc) for Lys and *tert*-Butyl (*t*Bu) for Ser. The peptides were purified by HPLC (Shimadzu, Japan) with C_{18} reverse phase column and acetonitrile (ACN) as eluent (0.1%TFA in H_2O/0–60% ACN gradient for 60 min) and verified with MALDI-TOF mass spectrometer (Bruker, Germany). The concentrations of the peptides were confirmed by using amino acid analysis (Protein analysis center, Karolinska Institutet, Sweden).

Microorganisms and growth conditions

The organisms used in these experiments were *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli*

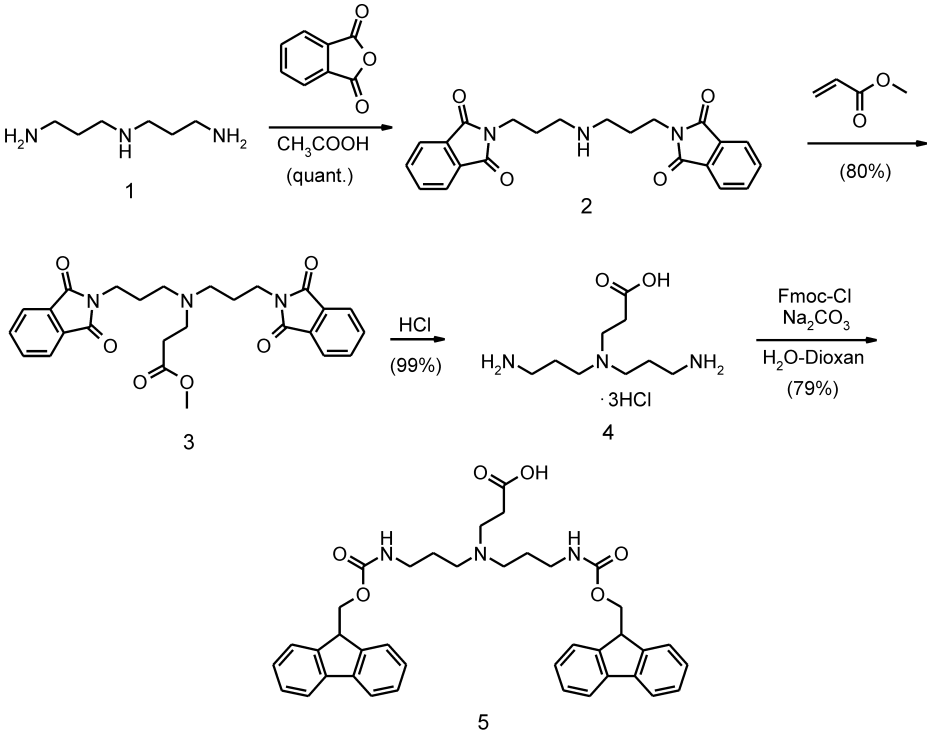

Figure 3. The synthesis strategy of Fmoc-DAPPA (**5**).

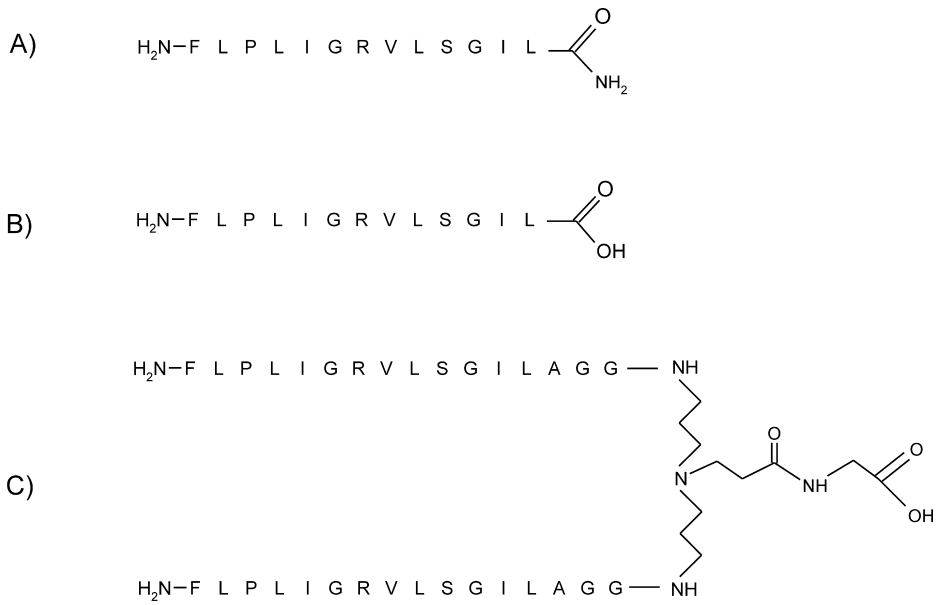

Figure 4. . Schematic representation of original Temporin A, TA, (A), its monomeric carboxy, TAc, (B), and the novel dimeric, TAd, (C) analogues.

(ATCC 25922). The bacteria were stored on blood agar plates at +4 °C and subcultured every second week.

To obtain a suspension culture, a bacterial inoculum was suspended into 5 mL of tryptic soy broth (TSB) medium (Difco, USA), and the suspensions were incubated at +37 °C for 20 h. After the incubation, bacterial cells were centrifuged at $1680\times g$ for 10 min (Br-i, Jouan, France), resuspended into 50 mL of fresh TSB-medium and stored at +4 °C. Part of the suspension was diluted from 10^{-1} to 10^{-9} in buffered peptone water (OXOID, UK) and 100 µl of each dilution was spread on two parallel plate count agar (PC agar) plates (LAB M, UK). The plates were incubated at +37 °C for 24 h, and the number of colony forming units (cfu) were counted. For the antimicrobial assays the stored suspension was diluted with TSB to obtain the desired bacterial concentrations.

Antimicrobial assays

Antimicrobial assays were performed using an automated incubator and a turbidity reader Bioscreen C. Bioscreen C enables the simultaneous testing of 200 samples and monitoring of bacterial growth in real time in the test. 50 µL of bacterial cell suspension (1×10^4 cfu/mL) was incubated in microplate wells containing 18 µL of peptide solution, diluted in water, in a total volume of 350 µL of TSB. The peptide concentrations tested were 0, 0.625, 1.25, 2.5, 5, 10 and 20 µM, which were tested in three parallel wells. The plates were incubated at +37 °C for 21 h and the turbidity of each well was measured every 10 min using wide-band filter (420–580 nm). The plates were shaken for 10 seconds just prior to the measurement to achieve a homogenous suspension. The data obtained were transferred to a spreadsheet program and the growth curves were drawn as averages of three parallel wells.

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