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Synthesis of short cationic antimicrobial peptidomimetics containing arginine analogues

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Worldwide efforts are underway to develop new antimicrobial agents against bacterial resistance. To identify new compounds with a good antimicrobial profile, we designed and synthesized two series of small cationic antimicrobial peptidomimetics (1–8) containing unusual arginine mimetics (to introduce cationic charges) and several aromatic amino acids (bulky moieties to improve lipophilicity). Both series were screened for *in vitro* antibacterial activity against a representative panel of Gram-positive (*Staphylococcus aureus* and *Staphylococcus epidermidis*) and Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) bacterial strains, and *Candida albicans*. The biological screening showed that peptidomimetics containing tryptophan residues are endowed with the best antimicrobial activity against *S. aureus* and *S. epidermidis* in respect to the other synthesized derivatives (MIC values range 7.5–50 μg/ml). Moreover, small antimicrobial peptidomimetics derivatives 2 and 5 showed an appreciable activity against the tested Gram-negative bacteria and *C. albicans*. The most active compounds (1–2 and 5–6) have been tested against Gram-positive established biofilm, too. Results showed that the biofilm inhibitory concentration values of these compounds were never up to 200 μg/ml. The replacement of tryptophan with phenylalanine or tyrosine resulted in considerable loss of the antibacterial action (compounds 3–4 and 7–8) against both Gram-positive and Gram-negative bacterial strains. Furthermore, by evaluating hemolytic activity, the synthesized compounds did not reveal cytotoxic activities, except for compound 5. Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antimicrobial peptidomimetics; arginine analogues; antibacterial activity; antifungal activity; microbial biofilm

Introduction

The discovery of penicillin in 1928 by Alexander Fleming opened the so-called *antibiotic era*, prior to which infections were life-threatening diseases. Currently, conventional antibiotics possess growth-inhibitory or toxic activity on a broad range of pathogenic bacteria. However, recently an increased frequency of bacterial mutations has resulted in a significantly augmented incidence of antibiotic resistance, and infections caused by antibiotic-resistant bacteria have become a global health care problem of the 21st century [1]. Indeed, if resistance was initially a problem only for hospitalized patients, gradually it extended into the community, causing severe infections difficult to diagnose and treat [2].

Moreover, bacteria often choose a sessile biofilm lifestyle characterized by single cells or micro colonies embedded in a hydrated matrix – made of extracellular polymeric substances [3] – which confers to the bacterial population a significant antimicrobial tolerance when compared with the free-living counterparts [4,5]. The biofilm state represents a natural part of the life cycle of microorganisms, and the recognized presence in human infections poses serious concerns in term of eradication. In fact, biofilm-related infections can tolerate antimicrobial agents at concentrations of 10–1000 times higher than that needed to kill the equivalent planktonic bacteria [6]. Thus, the development of novel antimicrobial pharmaceuticals capable of overcoming these difficulties is a major challenge of modern medicinal chemistry.

In the search for new and alternative sources of antibiotics, a place of primary importance is occupied by AMPs [7–9]. AMPs are ribosomally synthesized peptides that constitute a key component of innate immune system of a wide number of organisms, from bacteria to mammals. AMPs show antimicrobial activity against several microorganisms such as bacteria, yeasts, fungi, and protozoa [10]. Currently, more than 850 AMPs have been characterized and most of them can be classified in alpha-helical peptides, peptides with three disulfide bonds, and proline or tryptophan-rich peptides [11]. Despite this wide range of structures, most of the AMPs share common structural features. They are composed of 12–50 amino acids, 50% of which possess a lipophilic chain and have a positive net charge [12]. Such structural arrangement confers them high affinity and selectivity for bacterial membranes. The frequent mode of action of AMPs is partly related to cell membrane permeabilization and lysis. The exact mechanism of membrane disruption by AMPs is not completely known, even though several models have been proposed to explain the effect of these compounds. In this context – using membrane potential sensitive dyes and fluorescently labeled

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Abbreviations used: *AMP, antimicrobial peptides; ATCC, American tissue culture collection; BEC, biofilm eradication concentration; BIC, biofilm inhibitory concentration; CLSI, clinical laboratory standards institute; DIPEA, N,N-diisopropylethylamine; DMF, dimethylformamide; HBTU, O-benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluoro-phosphate; HOBt, hydroxybenzotriazole; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; SAMPs, small antimicrobial peptidomimetics; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran.*

peptides – AMPs have been found to interact with membranes and tend to divide peptides into two classes: (1) membrane disruptive (barrel stave, toroidal, carpet, and micellar aggregate models) and (2) non-membrane disruptive (intracellular targets) [13,14]. In membrane disruptive models, AMPs have been proposed to disrupt the membrane bilayer with consequent loss of bacterial membrane functionality. Independently of the chosen membrane perturbation model, it is important to consider the interaction that peptides are able to establish in a complex environment of a cell membrane. In fact, because AMPs commonly fold up into different amphipathic structures – separating the hydrophobic and the cationic parts – they are able to adhere to the surface of the bacteria through electrostatic interactions, resulting in a rapid lysis of a broad range of bacteria, which decreases the likelihood of developing resistance [15].

As a result, much attention has been given to AMPs, which are rightfully thought to be a novel class for antibacterial treatment. Nevertheless, therapeutic application of AMPs is hampered by many unresolved problems, such as low bioavailability, limited metabolic and proteolytic stability, high production cost, and allergenic properties [16]. In order to increase peptides' half-life, many strategies involving several formulations, ways of administration, and different levels of chemical modification are possible. Introduction of D-amino acids, amidation at the *N*-terminus, non-natural amino acids, and peptide cyclization are the most common strategies to increase peptide stability [15]. Because there are few studies about the pharmacokinetics of AMPs and the effects of all the aforementioned modifications, considerable additional researches on these issues are urgently needed. Strøm *et al.* [17] – before synthesizing small peptidomimetics with antimicrobial activity – focused their attention on the research of the pharmacophore for antibacterial activity in cationic peptides. They reported that three lipophilic bulky moieties and two positively charged residues were necessary for the activity against Gram-negative bacteria; whereas two positively charged residues and two lipophilic bulky residues were required for the activity toward Gram-positive bacteria. On the basis of this pharmacophore motif, several molecules have been designed and synthesized, thus originating a novel class of SAMPs [18–20]. They retained the AMPs antimicrobial activity and were active toward staphylococcal biofilms [21], also showing improved pharmacokinetic properties [22]. In particular, high antibacterial activity was obtained with the introduction of bulky groups as aromatics but with a larger size than a phenyl group [23], and of charged moieties as the side chains of arginine or lysine [17,18,24]. Other research groups have lately reported successful implementations of this pharmacophore. Knappe *et al.* [25,26] found that replacing arginine residues with mimetic analogues protected the AMPs from serum degradation without affecting their antimicrobial activity, whereas Karstad *et al.* [27] reported that the introduction of unnatural amino acids side chain in the sequence of small cationic antimicrobial peptide increased their stability toward chymotryptic degradation.

On the basis of these data, we designed and synthesized new SAMPs derivatives (**1–8**). Starting from the most active compound reported by Strom *et al.* [17] – the dipeptide L-arginine-L-tryptophan benzyl ester – and in order to identify compounds with a good antimicrobial profile, we designed and synthesized two series of SAMPs containing arginine analogues and several aromatic amino acids.

In the first series (peptidomimetics **1–4**), we chose the 1-(4-(aminomethyl)benzyl)guanidine and aromatic α-guanidino amino acids (such as L-tryptophan, L-tyrosine, and L-phenylalanine), respectively, in order to confer both lipophilic properties and positive-charged portions. The second series of SAMPs derivatives (**5–8**) contains the same set of three aromatic amino acids, but the positive charges are conferred to the molecule by another guanidine derivative such as 3,5-diguanidino benzoic acid. Both series were screened for *in vitro* antibacterial activity against a representative panel of Gram-positive (*Staphylococcus aureus* and *Staphylococcus epidermidis*) and Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) bacterial strains, and *Candida albicans*.

Materials and Methods

Substances

L-Arginine-L-tryptophan benzyl ester, *N,N'*-bis(*tert*-butoxycarbonyl)-*S*-methyl-isothiourea, Boc-L-tryptophyl-L-tryptophan, L-tryptophyl-L-tryptophan methyl ester trifluoacetate salt, and 3,5-bis ((*tert*-butoxycarbonyl)amino)benzoic acid were prepared as described in literature [17,28–30]. All other chemicals and all solvents were purchased from Sigma-Aldrich (Sigma-Aldrich Corp., Saint Louis, MO, USA). Mueller–Hinton broth and microtiter plates were obtained from Becton Dickinson (Becton Dickinson, Franklin Lakes, NJ, USA). Mueller–Hinton agar and tryptic soy broth were from Oxoid (Oxoid, Dardilly, France), and glucose was purchased from Sigma-Aldrich.

Synthesis and Purification

Microanalyses were performed on a 1106 Carlo Erba CHN analyzer (Carlo Erba, Milan, Italy). ^{1}H and ^{13}C NMR spectra were recorded on a Varian VXR 300-MHz spectrometer (Varian Medical Systems, Inc., Palo Alto, CA, USA). Chemical shifts are reported in parts per million (δ) downfield from the internal standard Me_4Si. The LC-MS/MS system consisted of an LCQ (Thermo Finnigan, San Jose, CA, USA) ion trap mass spectrometer equipped with an electrospray ionization (ESI) source. The capillary temperature was set at 300 °C and the spray voltage at 4.25 kV. The fluid was nebulized using nitrogen (N_2) as both the sheath gas and the auxiliary gas. The identity of new compounds was confirmed by ^{1}H and ^{13}C NMR data and LC-MS/MS measurements; homogeneity was confirmed by TLC on silica gel Merck 60 F_{254} (Merck & Co., Inc., Whitehouse Station, NJ, USA). Solutions were routinely dried over anhydrous sodium sulfate prior to evaporation. Chromatographic purifications were performed using Merck 60 70–230 mesh ASTM silica gel column. Analytical HPLC was carried out on a Waters 600 HPLC (Waters Co., Milford, MA, USA) equipped with an X-Bridge BEH130 C18, 5 μm, 4.6 × 250 mm column with Waters 2996 PDA detector, using a solvent system of H_2O/CH_3CN (0.1% TFA) in the form of a linear gradient from 10 to 80% of CH_3CN in 30 min and a flow rate of 1 ml/min. All the final products (**1–8**) were obtained as trifluoacetate salts with purity greater than 95%, determined by analytical HPLC at 254 and 280 nm.

*Boc-L-tryptophyl-L-tryptophan-4-((Boc)aminomethyl)benzylamide (**1a**)*

To a stirred solution of Boc-L-tryptophyl-L-tryptophan (1.69 g, 3.44 mmol) in dry DMF (15 ml), HOBt (567 mg, 4.20 mmol), HBTU (1.56 g, 4.16 mmol), DIPEA (1.32 ml, 7.57 mmol), and 4-(*tert*-butoxycarbonyl-aminomethyl)benzylamine (813 mg, 3.44 mmol) were added at 0 °C. After 10 min at 0 °C and 2 h at room temperature, the reaction mixture was evaporated under vacuum. The residue was taken up in EtOAc and washed twice with 5% citric acid, saturated aqueous $NaHCO_3$, and brine. The organic layer

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was dried over Na_2SO_4 and the solvent evaporated in vacuum. The product was purified by column chromatography eluting with $CHCl_3$/MeOH (99 : 1 to 95 : 5) yielding **1a** (2.09 g, 86%). $R_f = 0.43$, $CHCl_3$/MeOH (95 : 5); ^{1}H NMR (DMSO-d_6) δ: 1.23 and 1.84 (18H, 2 × s, OBu^t), 2.90–3.30 (4H, m, 2 × Trp β-CH_2), 4.06–5.57 (5H, m, 1H Trp α-CH, 4H Ar CH_2), 4.57 (1H, m, Trp α-CH), 6.82 (1H, d, $J = 8.1$ Hz, Trp NHCOBu^t), 6.93–7.54 (15H, m, 5H Trp IndH, 5H Trp IndH, 4H ArH, and 1H NHCOBu^t), 7.91 (1H, d, $J = 7.8$ Hz, Trp NHCO), 8.36 (1H, t, NHCO), 10.78 and 10.82 (1H, 2 × br s, Trp IndNH). ^{13}C NMR (DMSO-d_6) δ: 28.25 and 28.50 (2 × Trp β-CH_2), 28.75 and 29.93 (2 × OBu^t), 42.57 and 43.80 (2 × Ar CH_2), 54.13 and 56.16 (2 × Trp α-CH), 78.42 and 79.86 (2 × OBu^t), 110.39–139.26 (aromatics), 155.93 and 156.46 (2 × OCONH), 171.84 and 172.31 (2 × *C*O). Anal. Calcd for $C_{40}H_{48}N_6O_6$: C, 67.78; H, 6.83; N, 11.86. Found: C, 67.80; H, 6.80; N, 11.87.

*N-Amidino-L-tryptophyl-L-tryptophan-4-((N-amidino)aminomethyl) benzylamide (**1**)*

To a solution of **1a** (1.83 g, 2.58 mmol) in dry CH_2Cl_2 (28 ml), TFA was added (2.8 ml). The mixture was stirred at room temperature under N_2. After 1.5 h, the solution was evaporated to dryness and the residue was repeatedly evaporated with ethyl ether to give **1b** in quantitative yield. The product was used without further purification (1.86 g, 98%).

To a stirred solution of **1b** (770 mg, 1.04 mmol) in dry THF (10 ml), 3,4-dimethyl-1*H*-pyrazole-1-carboximidamidenitrate salt (2.1 g, 10.45 mmol) and TEA (1.75 ml, 12 mmol) were added. After 16 h at reflux, the solvent was evaporated in vacuum and the crude product purified by a Waters 600 HPLC system equipped with an X-Bridge® Prep BEH130 C18, 5 μm (10 × 250 mm) column. The purification was carried out using a solvent system of H_2O/CH_3CN (0.1% TFA) in the form of a 30 min gradient: 10–90% CH_3CN in 20 min, 90% CH_3CN for 5 min, and 90–10% CH_3CN in 5 min, yielding 248 mg (40%) of **1**. ^{1}H NMR (DMSO-d_6) δ: 2.95–3.19 (4H, m, 2 × Trp β-CH_2), 4.22 (2H, dd, $J = 5.7$, 13.2 Hz Ar CH_2), 4.30 (2H, d, $J = 6.0$ Hz, Ar CH_2), 4.45 (1H, m, Trp α-CH), 4.63 (1H, m, Trp α-CH), 6.93–7.73 (22H, m, 5H Trp IndH, 5H Trp IndH, 4H ArH, and 8H NH), 7.72 (1H, d, $J = 8.1$ Hz, NHCN) 8.09 (1H, t, $J = 5.7$ Hz, NHCO), 8.42 (1H, d, $J = 8.1$ Hz,Trp NHCO), 8.51 (1H, t, $J = 5.4$ Hz, NHCN), 10.82 and 10.92 (1H, 2 × br s, Trp IndNH). ^{13}C NMR (DMSO-d_6) δ: 28.61 and 28.90 (2 × Trp β-CH_2), 42.53 and 44.39 (2 × Ar CH_2), 54.49 and 55.40 (2 × Trp α-CH), 109.30–139.03 (aromatics), 157.02, and 157.61 (2 × *C*N), 170.10 and 171.61 (2 × CO). Anal. Calcd for $C_{36}H_{38}F_6N_{10}O_6$: C, 52.68; H, 4.67; N, 17.07. Found: C, 52.65; H, 4.68; N, 17.10. MS (ESI): *m/z* 297.4 $[M + 2H]^{++}$.

*General procedure for guanylation reactions of compounds **2b–8b***

Guanylation reactions of compounds **2b–8b** were carried out according to the following general procedure. To a stirred solution of peptidomimetic trifluoroacetate (1 mmol) in dry DMF (6 ml), TEA (0.93 ml, 6.6 mmol), *N,N′*-bis(*tert*-butoxycarbonyl)-*S*-methyl-isothiourea (310 mg, 2.1 mmol), and $HgCl_2$ (598 mg, 2.2 mmol) were added under N_2 at 0 °C. After 3.5 h, the mixture was diluted with EtOAc, filtered through a pad of Celite, and washed with water. The aqueous layer was extracted twice with EtOAc. The combined organic layers were washed with brine, dried over Na_2SO_4, and evaporated.

*Boc-L-tryptophane-4-((Boc)aminomethyl)benzylamide (**2a**)*

Coupling of 4-(*tert*-butoxycarbonyl-aminomethyl)benzylamine (777 mg, 3.29 mmol) with Boc-L-tryptophan (1.00 g, 3.29 mmol) was performed as described for compound **1a**. The crude product obtained was purified by column chromatography using $CHCl_3$/MeOH (95 : 5) as eluant to give **2a** (1.40 g, 81%). $R_f = 0.56$, $CHCl_3$/MeOH (95 : 5). ^{1}H NMR ($CDCl_3$) δ: 1.42 and 1.46 (18H, 2 × s, OBu^t), 3.06 (1H, dd, $J = 5.1$, 15.0 Hz, Trp β-CH_A), 3.29 (1H, dd, $J = 4.8$, 14.4 Hz, Trp β-CH_B), 4.06 (1H, dd, $J = 6.9$, 14.7 Hz, $ArCH_A$), 4.26 (2H, d, $J = 5.7$ Hz, $ArCH_2$), 4.30 (1H, $J = 7.2$, 15.5 Hz, $ArCH_B$), 4.44 (1H, m, Trp α-CH), 5.20–5.41 (2H, 2 × m, N*H*COBu^t), 5.96 (1H, m, N*H*CO), 6.57–7.64 (9H, m, 5H Trp IndH, and 4H ArH), 8.67 (1H, br s Trp IndN*H*). ^{13}C NMR ($CDCl_3$) δ: 28.55 and 28.67(2 × OBu^t), 29.00 (Trp β-CH_2), 43.29 and 44.61 (2 × Ar CH_2), 55.33 (Trp α-CH), 80.08 and 80.27 (2 × OBu^t), 109.73–138.37 (aromatics), 155.65 and 156.53 (2 × OCONH), 171.76 (*C*O). Anal. Calcd for $C_{29}H_{38}N_4O_5$: C, 66.64; H, 7.33; N, 10.72. Found: C, 66.68; H, 7.34; N, 10.73.

*N,N′-bis(Boc)amidino-L-tryptophan-4-((N,N′-bis(Boc)amidino) aminomethyl)benzylamide (**2c**)*

Deprotection of **2a** (615 mg, 1.18 mmol) was performed as described for **1a** to give **2b** (640 mg, 98%). The product was used without further purification.

Guanylation of **2b** (680 mg, 1.24 mmol) was carried out according to the general procedure. The crude product was purified by column chromatography eluting with CH_2Cl_2/EtOAc (9 : 1) yielding **2c** (479 mg, 48%). $R_f = 0.52$, CH_2Cl_2/EtOAc (9 : 1). ^{1}H NMR ($CDCl_3$) δ: 1.43, 1.44, 1.49 and 1.50 (36H, 4 × s, OBu^t), 3.09 (1H, dd, $J = 9.3$, 14.1 Hz, Trp β-CH_A), 3.38 (1H, dd, $J = 5.1$, 14.1 Hz, Trp β-CH_B), 3.97 (1H, dd, $J = 4.5$, 14.4 Hz, Ar CH_A), 4.49 (1H, $J = 6.9$, 14.7 Hz, Ar CH_B), 4.52 (2H, d, $J = 5.1$ Hz, Ar CH_2), 4.83 (1H, m, Trp α-CH), 6.03 (1H, t, $J = 5.4$ Hz, NHCO), 6.70–7.97 (9H, m, 5H Trp IndH, and 4H ArH), 8.29 (1H, br s Trp IndNH), 8.62 (1H, t, $J = 5.1$ Hz, NHCO), 9.04 (1H, d, $J = 6.9$ Hz, NHCO) 11.32 and 11.57 (2H, 2 × br s, NHCOBu^t). ^{13}C NMR ($CDCl_3$) δ: 28.28, 28.31, 28.44, 28.51 (4 × OBu^t), 28.96 (Trp β-CH_2), 43.25 and 44.88 (2 × Ar CH_2), 55.10 (Trp α-CH), 79.28, 79.84, 83.46 and 83.71 (4 × OBu^t), 110.59–137.71 (aromatics), 152.90 and 153.59 (2 × OCON) 155.61 and 156.73 (2 × OCONH), 163.47 and 163.73 (2 × *C*N) 170.70 (*C*O). Anal. Calcd for $C_{41}H_{58}N_8O_9$: C, 61.02; H, 7.24; N, 13.89. Found: C, 61.02; H, 7.23; N, 13.90.

*N-amidino-L-tryptophan-4-((N-amidino)aminomethyl)benzylamide (**2**)*

Deprotection of **2c** (417 mg, 0.52 mmol) was carried out as described for **1a**, yielding **2** (320 mg, 98%). ^{1}H NMR (DMSO-d_6) δ: 3.02 (1H, dd, $J = 7.8$, 15.0 Hz, Trp β-CH_A), 3.23 (1H, dd, $J = 4.8$, 14.4 Hz, Trp β-CH_B), 4.25 (2H, d, $J = 5.4$ Hz, Ar CH_2), 4.30 (2H, d, $J = 6.0$ Hz, Ar CH_2), 4.45 (1H, m, Trp α-CH), 6.92–7.67 (17H, m, 5H Trp IndH, 4H ArH, and 8H NH), 7.78 (1H, d, $J = 8.70$ Hz, NHCN), 8.12 (1H, t, $J = 6.3$ Hz, NHCN), 8.66 (1H, t, $J = 5.6$ Hz, NHCO), 10.94 (1H, br s Trp IndNH). ^{13}C NMR (DMSO-d_6) δ: 28.77 (Trp β-CH_2), 42.77 and 44.32 (2 × Ar CH_2), 55.69 (Trp α-CH), 109.13–138.82 (aromatics), 157.20, 157.58 (2 × *C*N), 170.70 (*C*O). Anal. Calcd for $C_{25}H_{28}F_6N_8O_5$: C, 47.32; H, 4.45; N, 17.66. Found: C, 47.33; H, 4.46; N, 17.69. MS (ESI): *m/z* 204.2 $[M + 2H]^{++}$.

*Boc-L-phenylalanine-4-((Boc)aminomethyl)benzylamide (**3a**)*

Coupling of 4-(*tert*-butoxycarbonyl-aminomethyl)benzylamine (891 mg, 3.77 mmol) with Boc-L-phenylalanine (1.00 g, 3.77 mmol) was performed as described for compound **1a**. The crude product obtained was purified by column chromatography using

$CHCl_3$/MeOH (98 : 2) as eluant to give **3a** (1.68 g, 98%). $R_f = 0.69$, $CHCl_3$/MeOH (98 : 2). ^1H NMR ($CDCl_3$) δ: 1.32 and 1.43 (18H, 2 × s, OBu^t), 3.04 (1H, d, $J = 7.7$ Hz, Phe β-CH_2), 4.23 (1H, d, $J = 6.9$ Hz, $ArCH_2$), 4.29 (2H, d, $J = 6.0$ Hz, $ArCH_2$), 4.34 (1H, m, Phe α-CH), 4.94 (1H, t, $J = 6.9$ Hz, N*H*COBu^t), 5.19 (1H, d, $J = 7.9$ Hz, N*H*COBu^t), 6.33 (1H, t, $J = 6.0$ Hz, N*H*CO), 7.00–7.24 (5H, m, 5H Phe Ar, and 4H ArH). ^{13}C NMR ($CDCl_3$) δ: 28.48 and 28.64 (2 × OBu^t), 38.53 (Phe β-CH_2), 43.29 and 44.50 (2 × Ar CH_2), 56.20 (Phe α-CH), 79.71 and 80.37 (2 × OBu^t), 127.11–138.45 (aromatics), 155.65 and 156.13 (2 × OCONH), 171.40 (*C*O). Anal. Calcd for $C_{27}H_{37}N_3O_5$: C, 67.06; H, 7.71; N, 8.69. Found: C, 67.10; H, 7.69; N, 10.75.

*N,N′-bis(Boc)amidino-L-phenylalanine-4-((N,N′-bis(Boc)amidino) aminomethyl)benzylamide (**3c**)*

Deprotection of **3a** (843 mg, 1.65 mmol) was performed as described for **1a** to give **3b** (843 mg, quantitative). The product was used without further purification.

Guanylation of **3b** (800 g, 1.56 mmol) was carried out according to the general procedure. The crude product was purified by column chromatography eluting with CH_2Cl_2/EtOAc (9 : 1) yielding **3c** (831 mg, 69%). $R_f = 0.91$, CH_2Cl_2/EtOAc (3 : 1). ^1H NMR ($CDCl_3$) δ: 1.41, 1.47, 1.49 and 1.51 (36H, 4 × s, OBu^t), 3.14 (1H, dd, $J = 7.5$, 13.8 Hz, Phe β-CH_A), 3.19 (1H, dd, $J = 7.5$, 13.8 Hz, Phe β-CH_B), 4.27 (1H, dd, $J = 5.4$, 15.3 Hz, Ar CH_A), 4.41 (1H, dd, $J = 6.0$, 15.3 Hz, Ar CH_B), 4.58 (2H, d, $J = 5.1$ Hz, Ar CH_2), 4.74 (1H, m, Phe α-CH), 6.85 (1H, t, $J = 5.5$ Hz, NHCO), 7.08–7.24 (9H, m, 5H Phe ArH, and 4H ArH), 8.57 (1H, br s, NHCO), 8.80 (1H, d, $J = 7.2$ Hz, NHCO) 11.28 and 11.53 (2H, 2 × br s, NHCOBu^t).^{13}C NMR ($CDCl_3$) δ: 28.22, 28.26, 28.40, 28.52 (4 × OBu^t), 37.67 (Phe β-CH_2), 43.40 and 44.89 (2 × Ar CH_2), 56.17 (Phe α-CH), 79.59, 79.65, 83.43, and 83.72 (4 × OBu^t), 127.13–137.48 (aromatics), 152.90 and 153.39 (2 × OCON), 156.10 and 156.31 (2 × OCONH), 163.15 and 163.80 (2 × *C*N) 170.46 (*C*O). Anal. Calcd for $C_{39}H_{57}N_7O_9$: C, 61.00; H, 7.48; N, 12.77. Found: C, 61.02; H, 7.51; N, 12.71.

*N-amidino-L-phenylalanine-4-((N-amidino)aminomethyl) benzylamide (**3**)*

Deprotection of **3c** (500 mg, 0.65 mmol) was carried out as described for **1a**, yielding **3** (572 mg, 98%). ^1H NMR (DMSO-d_6) δ: 2.84 (1H, dd, $J = 5.5$, 13.7 Hz, Phe β-CH_A), 3.09 (1H, dd, $J = 5.4$, 13.5 Hz, Phe β-CH_B), 4.19 (1H, dd, $J = 5.6$, 13.9 Hz, Ar CH_A), 4.26–4.32 (3H, m, 1H Ar CH_B, and 2H Ar CH_2), 4.43 (1H, m, Phe α-CH), 4.81 (4H, br s, NH), 7.09–7.42 (13H, m, 5H Phe ArH, 4H ArH, and 4H NH), 7.88 (1H, d, $J = 9.0$ Hz, NHCN), 8.16 (1H, t, $J = 6.0$ Hz, NHCN), 8.69 (1H, t, $J = 5.7$ Hz, NHCO). ^{13}C NMR (DMSO-d_6) δ: 38.53 (Phe β-CH_2), 42.67 and 44.27 (2 × Ar CH_2), 56.25 (Phe α-CH), 127.85–138.74 (aromatics), 157.21 and 157.61 (2 × *C*N), 169.85 (*C*O). Anal. Calcd for $C_{23}H_{27}F_6N_7O_5$: C, 46.39; H, 4.57; N, 16.46. Found: C, 46.35; H, 4.49; N, 16.45. MS (ESI): *m/z* 184.7 $[M + 2H]^{++}$.

*Boc-L-tyrosine-4-((Boc)aminomethyl)benzylamide (**4a**)*

Coupling of 4-(*tert*-butoxycarbonyl-aminomethyl)benzylamine (891 mg, 3.77 mmol) with Boc-L-phenylalanine (1.06 g, 3.77 mmol) was performed as described for compound **1a**. The crude product obtained was purified by column chromatography using $CHCl_3$/MeOH (95 : 5) as eluant to give **4a** (1.84 g, 99%). $R_f = 0.57$, $CHCl_3$/MeOH (95 : 5). ^1H NMR ($CDCl_3$) δ: 1.31 and 1.35 (18H, 2 × s, OBu^t), 2.48 (1H, dd, $J = 5.4$, 13.8 Hz, Tyr β-CH_A), 2.83 (1H, dd, $J = 5.5$, 14.0 Hz, Tyr β-CH_B), 4.05 (3H, m, 2H $ArCH_2$, 1H Tyr α-CH), 4.23 (2H, d, $J = 7.6$ Hz, $ArCH_2$), 6.64 (2H, d, $J = 8.8$ Hz, Tyr ArH), 6.89 (1H, d, $J = 8.9$ Hz, N*H*COBu^t), 7.01 (2H, d, $J = 9.5$ Hz, Tyr ArH), 7.06–7.18 (4H, m, ArH), 7.34 (1H, t, $J = 5.9$ Hz, N*H*COBu^t), 8.33 (1H, t, $J = 5.5$ Hz, N*H*CO) 9.17 (1H, s, Tyr OH). ^{13}C NMR ($CDCl_3$) δ: 28.84 and 28.92 (2 × OBu^t), 37.36 (Tyr β-CH_2), 42.41 and 43.77 (2 × Ar CH_2), 56.99 (Tyr α-CH), 79.40 and 78.61 (2 × OBu^t), 115.73–139.27 (aromatics), 155.96 and 156.42 (2 × OCONH), 172.51 (*C*O). Anal. Calcd for $C_{27}H_{37}N_3O_6$: C, 64.91; H, 7.46; N, 8.41. Found: C, 64.91; H, 7.47; N, 8.43.

*N,N′-bis(Boc)amidino-L-tyrosine-4-((N,N′-bis(Boc)amidino)aminomethyl) benzylamide (**4c**)*

Deprotection of **4a** (950 mg, 1.84 mmol) was performed as described for **1a** to give **4b** (968 mg, quantitative). The product was used without further purification.

Guanylation of **4b** (900 mg, 1.71 mmol) was carried out according to the general procedure. The crude product was purified by column chromatography eluting with CH_2Cl_2/EtOAc (9 : 1) yielding **4c** (964 mg, 72%). $R_f = 0.71$, CH_2Cl_2/EtOAc (3 : 1). ^1H NMR ($CDCl_3$) δ: 1.42, 1.45, 1.49, and 1.50 (36H, 4 × s, OBu^t), 2.82 (1H, dd, $J = 6.2$, 13.2 Hz, Tyr β-CH_A), 3.10 (1H, dd, $J = 5.7$, 13.8 Hz, Tyr β-CH_B), 3.94 (1H, dd, $J = 4.2$, 14.1 Hz, Ar CH_A), 4.47–4.70 (4H, m, 1H Ar CH_B, 1H Tyr α-CH, 2H Ar CH_2), 6.10 (1H, t, $J = 5.9$ Hz, NHCO), 6.44 (2H, d, $J = 8.7$ Hz, Tyr ArH), 6.87–6.92 (4H, m, ArH), 7.11 (2H, d, $J = 8.4$ Hz, Tyr ArH), 8.76 (1H, t, $J = 7.4$ Hz, NHCO), 8.92 (1H, d, $J = 7.8$ Hz, NHCO) 11.35 and 11.61 (2H, 2 × br s, NHCOBu^t).^{13}C NMR ($CDCl_3$) δ: 28.27, 28.32, 28.34, 28.46 (4 × OBu^t), 37.79 (Tyr β-CH_2), 43.06 and 44.40 (2 × Ar CH_2), 56.34 (Tyr α-CH), 79.65, 80.16, 83.63 and 83.92 (4 × OBu^t), 115.54–137.07 (aromatics), 152.94 and 153.58 (2 × OCON) 155.78 and 156.72 (2 × OCONH), 163.35 and 163.46 (2 × *C*N) 169.89 (*C*O). Anal. Calcd for $C_{39}H_{57}N_7O_{10}$: C, 59.75; H, 7.33; N, 12.51. Found: C,59.74; H, 7.28; N, 12.52.

*N-amidino-L-tyrosine-4-((N-amidino)aminomethyl)benzylamide (**4**)*

Deprotection of **4c** (500 mg, 0.64 mmol) was carried out as described for **1a**, yielding **4** (566 mg, 97%). ^1H NMR (DMSO-d_6) δ: 2.74 (1H, dd, $J = 7.2$, 12.1 Hz, Tyr β-CH_A), 2.95(1H, dd, $J = 4.8$, 12.5 Hz, Tyr β-CH_B), 4.18–4.67(9H, m, 1H Tyr α-CH, 4H Ar CH_2, 4H NH),6.63 (2H, d, $J = 8.7$ Hz, Tyr ArH), 6.98 (2H, d, $J = 8.9$ Hz, Tyr ArH), 7.09–7.62(8H, m, 4H ArH, and 4H NH), 7.81(1H, d, $J = 8.3$ Hz, NHCN), 8.18 (1H, t, $J = 5.4$ Hz, NHCN), 8.64 (1H, t, $J = 6.0$ Hz, NHCO). ^{13}C NMR (DMSO-d_6) δ: 38.23 (Tyr β-CH_2),42.86 and 43.90 (2 × Ar CH_2),56.57 (Tyr α-CH), 115.18–138.7 (aromatics),157.63, and 157.98 (2 × *C*N), 169.24 (*C*O). Anal. Calcd for $C_{23}H_{27}F_6N_7O_6$: C, 45.18; H, 4.45; N, 16.03. Found: C, 45.21; H, 4.46; N, 16.02. MS (ESI): *m/z* 192.4 $[M + 2H]^{++}$.

*3,5-bis((Boc)amino)benzoyl-L-tryptophyl-L-tryptophan methyl ester (**5a**)*

To a stirred solution of 3,5-bis((Boc)amino)benzoic acid (544 mg, 1.54 mmol) in dry DMF (15 ml), HOBt (254 mg, 1.88 mmol), HBTU (647 mg, 1.70 mmol), DIPEA (0.59 ml, 3.39 mmol), L-tryptophyl-L-tryptophan methyl ester TFA salt (800 mg, 1.54 mmol), and DIPEA (0.27 ml, 1.54 mmol) were added at 0 °C under N_2. After 10 min at 0 °C and 2.5 h at room temperature, the reaction mixture was diluted with EtOAc and washed twice with 5% citric acid, saturated aqueous $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and evaporated under vacuum. The crude product was purified by column chromatography eluting with CH_2Cl_2/EtOAc (5 : 1 to 1 : 1) to give **5a** (610 mg, 54%). $R_f = 0.73$, CH_2Cl_2/EtOAc (1 : 1). ^1H NMR ($CDCl_3$) δ: 1.53 (18H, s, OBu^t), 3.15–3.23 (3H, m, Trp β-CH_2 and Trp β-CH_A), 3.36 (1H, dd, $J = 6.6$, 15.0 Hz, Trp β-CH_B), 3.62 (3H, s, OMe), 4.83 (1H, m, Trp α-CH), 4.97 (1H, m,

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Trp α-CH), 6.66 (1H, d, $J = 7.8$ Hz, NHCO), 6.74–7.65 (14H, m, 5H Trp IndH, 5H Trp IndH, and 3H ArH, 1H NHCO), 8.29 (1H, br s Trp IndNH), 9.12 (2H, br s NHCOBu^t). ^{13}C NMR ($CDCl_3$) δ: 26.55 and 27.75 (2 × Trp β-CH_2), 28.57 (2 × OBu^t), 52.66 and 52.84 (2 × Trp α-CH), 54.16 (OCH_3), 81.14 (2 × OBu^t), 108.81–139.46 (aromatics), 153.05 (2 × OCONH), 167.56, 171.67, and 172.46 (3 × CO). Anal. Calcd for $C_{40}H_{46}N_6O_8$: C, 65.03; H, 6.28; N, 11.37. Found: C, 65.02; H, 6.27; N, 11.35.

*3,5-bis((N,N′-bis(Boc)amidino)amino)benzoyl-L-tryptophyl-L-tryptophan methyl ester (**5c**)*

Deprotection of **5a** (210 mg, 0.28 mmol) was carried out as described for **1a**, yielding **5b** (218 mg, 90%). The product was used without further purification.

Guanylation of **5b** (197 mg, 0.26 mmol) was carried out according to the general procedure. The crude product was purified by column chromatography eluting with CH_2Cl_2/EtOAc (5 : 1 to 3 : 1), yielding **5c** (113 mg, 42.32%). $R_f = 0.63$, CH_2Cl_2/EtOAc (3 : 1). ^{1}H NMR ($CDCl_3$) δ: 1.43 and 1.56 (36H, 2 × s, OBu^t), 3.10 (1H, dd, $J = 7.2$, 15.3 Hz, Trp β-CH_A), 3.28 (3H, m, Trp β-CH_2 and Trp β-CH_B), 3.71 (3H, s, OMe), 4.75–4.86 (2H, m, 2 × Trp α-CH), 6.46 (1H, d, $J = 6.6$ Hz, NHCO), 6.73–7.87 (14H, m, 1H NHCO, 5H Trp IndH, 5H Trp IndH, and 3H ArH), 8.13 and 9.05 (2H, 2 × br s, Trp IndNH), 10.36 and 11.65 (4H, 2 × br s, NHCOBu^t). ^{13}C NMR ($CDCl_3$) δ: 26.34 and 27.46 (2 × Trp β-CH_2), 28.21 and 28.31 (2 × OBu^t), 52.47 and 52.64 (2 × Trp α-CH), 53.56 (OMe), 80.51 and 84.42 (2 × OBu^t), 108.46–136.36 (aromatics), 153.45 (2 × OCON), 154.31 (2 × OCONH), 163.37 (CN), 166.33, 171.11, and 172.52 (3 × CO). Anal. Calcd for $C_{52}H_{66}N_{10}O_{12}$: C, 61.04; H, 6.50; N, 13.69. Found: C, 61.02; H, 6.51; N, 13.70.

*3,5-Bis(N-amidino)aminobenzoyl-L-tryptophyl-L-tryptophan methyl ester (**5**)*

To a stirred solution of **5c** (108 mg, 0.10 mmol) in dry CH_2Cl_2 (4 ml), TFA (4 ml) was added under N_2. After 1.5 h, the solution was evaporated to dryness and the residue was repeatedly evaporated with ether to give **5** (85 mg, 96%). The product was used without further purification. ^{1}H NMR (DMSO-d_6) δ: 3.04–3.22 (4H, m, 2 × Trp β-CH_2), 3.54 (3H, s, OMe), 4.56 and 4.85 (2H, m, 2 × Trp α-CH), 6.94–7.71 (21H, m, 5H Trp IndH, 5H Trp IndH, 3H ArH, 8H NH_2), 8.64–8.71 (2H, m, 2 × NHCO), 10.14 (2H, s, 2 × NHCN) 10.79 and 10.90 (2H, 2 × br s, Trp IndNH). ^{13}C NMR (DMSO-d_6) δ: 27.62 and 28.36 (2 × Trp β-CH_2), 52.54 (OMe), 54.02 and 54.53 (2 × Trp α-CH), 109.97–137.43 (aromatics), 156.78 (2 × CN), 165.24, 172.57, and 172.90 (3 × CO). Anal. Calcd for $C_{36}H_{36}F_6N_{10}O_8$: C, 50.83; H, 4.27; N, 16.46. Found: C, 50.83; H, 4.25; N, 16.48. MS (ESI): *m/z* 312.2 $[M + 2H]^{++}$.

*3,5-Bis((Boc)amino)benzoyl-L-tryptophan methyl ester (**6a**)*

Coupling of 3,5-bis((Boc)amino)benzoic acid (806 mg, 2.29 mmol) with L-tryptophan methyl ester hydrochloride (583 mg, 2.29 mmol) was performed as described for compound **5a**. The crude product obtained was purified by column chromatography using $CHCl_3$/MeOH (99 : 1) as eluant to give **6a** (1.14 g, 90%). $R_f = 0.63$, $CHCl_3$/MeOH (95 : 5). ^{1}H NMR ($CDCl_3$) δ: 1.50 (18H, s, OBu^t), 3.41 (2H, d, $J = 5.4$ Hz, Trp β-CH_2), 3.68 (3H, s, OMe), 5.06 (1H, m, Trp α-CH), 6.75 (1H, d, $J = 8.1$ Hz, NHCO), 7.07–7.56 (8H, m, 5H Trp IndH, and 3H ArH), 7.77 (1H, NHCOBu^t), 8.31 (1H, br s, Trp IndNH). ^{13}C NMR ($CDCl_3$) δ: 27.82 (Trp β-CH_2), 28.54 (OBu^t), 52.71 (OMe), 53.29 (Trp α-CH), 81.00 (2 × OBu^t), 109.82–139.73 (aromatics), 152.93 (2 × OCONH), 166.35, and 172.45 (2 × CO). Anal. Calcd for $C_{29}H_{36}N_4O_7$: C, 63.03; H, 6.57; N, 10.14. Found: C, 63.00; H, 6.58; N, 10.13.

*3,5-Bis((N,N′-bis(Boc)amidino)amino)benzoyl-L-tryptophan methyl ester (**6c**)*

Deprotection of **6a** (1.00 g, 1.81 mmol) was carried out as described for **1a**, yielding **6b** (1.03 g, 97%). The product was used without further purification.

Guanylation of **6b** (500 mg, 0.86 mmol) was carried out according to the general procedure. The crude product was purified by column chromatography eluting with CH_2Cl_2/EtOAc (9 : 1) yielding **6c** (385 mg, 53%). $R_f = 0.57$, CH_2Cl_2/EtOAc (9 : 1). ^{1}H NMR ($CDCl_3$) δ:1.46 and 1.51 (36H, 2 × s, OBu^t), 3.42 (2H, m, Trp β-CH_2), 3.68 (3H, s, OMe), 5.04 (1H, m, Trp α-CH), 6.80 (1H, d, $J = 7.80$ Hz, NHCO), 7.06–8.14 (8H, m, 5H Trp IndH and 3H ArH), 8.21 (1H, br s, Trp IndNH), 10.47 (2H, br s, NHCN), 11.54 (2H, br s, NHC OBu^t). ^{13}C NMR ($CDCl_3$) δ: 28.21 (Trp β-CH_2), 28.26 and 28.35 (2 × OBu^t), 52.64 (OMe), 53.39 (Trp α-CH), 80.29 and 84.25 (2 × OBu^t), 110.18-137.81 (Aromatics), 153.33 (2 × OCON), 153.87 (2 × OCONH), 163.35 (2 × CN), 166.45 and 172.45 (2 × CO). Anal. Calcd for $C_{41}H_{56}N_8O_{11}$: C, 58.84; H, 6.74; N, 13.39. Found: C, 58.85; H, 6.76; N, 13.37.

*3,5-Bis(N-amidino)aminobenzoyl-L-tryptophan methyl ester (**6**)*

Deprotection of **6c** (300 mg, 0.36 mmol) was performed as described for **5c** to give **6** (234 mg, 98%). ^{1}H NMR (DMSO-d_6) δ: 3.24 (2H, m, Trp β-CH_2), 3.61 (3H, s, OMe), 4.72 (1H, m, Trp α-CH), 6.97–7.71 (16H, m, 5H Trp IndH and 3H ArH, 8 NH), 8.97 (1H, d, $J = 6.90$ Hz, NHCO), 10.16 (2H, br s, NHCN), 10.85 (1H, br s, Trp IndNH). ^{13}C NMR (DMSO-d_6) δ: 29.31 (Trp β-CH_2), 53.65 (OMe), 55.08 (Trp α-CH), 111.68–138.81 (aromatics), 157.03 (2 × CN), 165.35 and 172.92 (2 × CO). Anal. Calcd for $C_{25}H_{26}F_6N_8O_7$: C, 45.19; H, 3.94; N, 16.86. Found: C, 45.20; H, 3.93; N, 16.84. MS (ESI): *m/z* 219.2 $[M + 2H]^{++}$.

*3,5-Bis((Boc)amino)benzoyl-L-phenylalanine methyl ester (**7a**)*

Coupling of 3,5-bis((Boc)amino)benzoic acid (700 mg, 1.99 mmol) with L-phenylalanine methyl ester hydrochloride (428 mg, 1.99 mmol) was performed as described for compound **5a**. The crude product obtained was purified by column chromatography using $CHCl_3$/MeOH (99 : 1) as eluant to give **7a** (850 mg, 83%). $R_f = 0.65$, $CHCl_3$/MeOH (98 : 2). ^{1}H NMR ($CDCl_3$) δ: 1.50 (18H, s, OBu^t), 3.21 (2H, d, $J = 5.7$ Hz, Phe β-CH_2), 3.72 (3H, s, OMe), 5.03 (1H, m, Phe α-CH), 6.63 (1H, d, $J = 7.8$ Hz, NHCO), 6.65 (2H, s, NHCOBu^t), 7.14–7.33 (5H, m, 5H Phe ArH), 7.38 (2H, d, $J = 1.8$ Hz, ArH), 7.74 (1H, t, $J = 1.8$ Hz, ArH). ^{13}C NMR ($CDCl_3$) δ: 28.49 (OBu^t), 37.29 (Phe β-CH_2), 52.68 (OMe), 54.24 (Phe α-CH), 81.11 (2 × OBu^t), 112.24–139.65 (aromatics), 153.18 (2 × OCONH), 167.45 and 172.48 (2 × CO). Anal. Calcd for $C_{27}H_{35}N_3O_7$: C, 63.14; H, 6.87; N, 8.18. Found: C, 63.15; H, 6.89; N, 8.15.

*3,5-Bis((N,N′-bis(Boc)amidino)amino)benzoyl-L-phenylalanine methyl ester (**7c**)*

Deprotection of **7a** (700 mg, 1.36 mmol) was carried out as described for **1a**, yielding **7b** (722 mg, 98%). The product was used without further purification.

Guanylation of **7b** (500 mg, 0.92 mmol) was carried out according to the general procedure. The crude product was purified by column chromatography eluting with CH_2Cl_2/EtOAc (98 : 2) yielding **7c** (551 mg, 75%). $R_f = 0.61$, CH_2Cl_2/EtOAc (98 : 2). ^{1}H NMR ($CDCl_3$) δ:1.49 and 1.53 (36H, 2 × s, OBu^t), 3.15 (2H, dd,

$J = 6.3$, 13.8 Hz, Phe β-CH_A), 3.25 (2H, dd, $J = 5.7$, 13.5 Hz, Phe β-CH_B), 3.72 (3H, s, OMe), 5.00 (1H, m, Phe α-CH), 6.80 (1H, d, $J = 7.8$ Hz, NHCO), 7.21–7.31 (5H, m, Phe ArH), 7.88 (2H, d, $J = 1.8$ Hz, ArH), 8.18 (1H, t, $J = 1.8$, ArH), 10.52 (2H, br s, NHCN), 11.56 (2H, br s, NHC OBu^t). ^{13}C NMR ($CDCl_3$) δ: 28.18 and 28.26 (2 × OBu^t), 36.86 (Phe β-CH_2), 52.54 (OMe), 54.07 (Phe α-CH), 80.25 and 84.36 (2 × OBu^t), 116.69–138.61 (aromatics), 153.34 (2 × OCON), 153.68 (2 × OCONH), 163.21 (2 × *C*N), 166.49 and 172.10 (2 × *C*O). Anal. Calcd for $C_{39}H_{55}N_7O_{11}$: C, 58.71; H, 6.95; N, 12.29. Found: C, 58.68; H, 6.91; N, 12.30.

*3,5-Bis(N-amidino)aminobenzoyl-L-phenylalanine methyl ester (**7**)*

Deprotection of **7c** (300 mg, 0.48 mmol) was performed as described for **5c** to give **7** (297 mg, 99%). ^1H NMR (DMSO-d_6) δ: 3.07 (2H, dd, $J = 3.6$, 10.2 Hz Phe β-CH_A), 3.17 (2H, dd, $J = 4.8$, 13.8 Hz Phe β-CH_B), 3.63 (3H, s, OMe), 4.68 (1H, m, Phe α-CH), 7.17–7.29 (5H, m, Phe ArH), 7.35 (1H, t, $J = 2.0$ Hz, ArH), 7.55 (2H, d, $J = 2.1$ Hz, ArH), 7.75 (8H, br s, NH), 9.00 (1H, d, $J = 7.8$ Hz, NHCO), 10.16 (2H, br s, NHCN). ^{13}C NMR (DMSO-d_6) δ: 36.28 (Phe β-CH_2), 52.54 (OMe), 55.18 (Phe α-CH), 117.6–138.61 (aromatics), 156.13 (2 × *C*N), 165.21 and 172.80 (2 × *C*O). Anal. Calcd for $C_{23}H_{25}F_6N_7O_7$: C, 44.17; H, 4.03; N, 15.68. Found: C, 44.15; H, 4.05; N, 15.70. MS (ESI): *m/z* 199.6 $[M + 2H]^{++}$.

*3,5-Bis((Boc)amino)benzoyl-L-tyrosine methyl ester (**8a**)*

Coupling of 3,5-bis((Boc)amino)benzoic acid (700 mg, 1.99 mmol) with L-tyrosine methyl ester (388 mg, 1.99 mmol) was performed as described for compound **5a**. The crude product obtained was purified by column chromatography using CH_2Cl_2/EtOAc (3 : 1) as eluant to give **8a** (894 mg, 85%). $R_f = 0.47$, CH_2Cl_2/EtOAc (3 : 1). ^1H NMR ($CDCl_3$) δ: 1.45 (18H, s, OBu^t), 3.01 (2H, dd, $J = 6.3$, 13.8 Hz, Tyr β-CH_A), 3.06 (2H, dd, $J = 5.4$, 14.0 Hz, Tyr β-CH_B), 3.65 (3H, s, OMe), 4.94 (1H, m, Tyr α-CH), 6.64 (2H, d, $J = 8.1$ Hz, Tyr ArH), 6.92 (3H, m, 2H Tyr ArH, and 1H NHCO), 7.08 (2H, s, $NHCOBu^t$), 7.32 (2H, d, $J = 2.0$ Hz, ArH), 7.76 (1H, t, $J = 2.0$ Hz, ArH). ^{13}C NMR ($CDCl_3$) δ: 28.51 (OBu^t), 37.41 (Tyr β-CH2), 52.68 (OMe), 54.98 (Tyr α-CH), 81.00 (2 × OBu^t), 115.12–138.84 (aromatics), 153.45 (2 × OCONH), 168.25 and 172.32 (2 × *C*O). Anal. Calcd for $C_{27}H_{35}N_3O_8$: C, 61.23; H, 6.66; N, 7.93. Found: C, 61.23; H, 6.67; N, 7.93.

*3,5-Bis((N,N′-bis(Boc)amidino)amino)benzoyl-L-tyrosine methyl ester (**8c**)*

Deprotection of **8a** (700 mg, 1.32 mmol) was carried out as described for **1a**, yielding **8b** (714 mg, 97%). The product was used without further purification.

Guanylation of **8** (500 mg, 0.90 mmol) was carried out according to the general procedure. The crude product was purified by column chromatography eluting with CH_2Cl_2/EtOAc (98 : 2) yielding **8c** (601 mg, 82%). $R_f = 0.41$, CH_2Cl_2/EtOAc (98 : 2). ^1H NMR ($CDCl_3$) δ:1.50 and 1.52 (36H, 2 × s, OBu^t), 3.03 (2H, dd, $J = 5.5$, 13.8 Hz, Tyr β-CH_A), 3.15 (2H, dd, $J = 5.3$, 13.5 Hz, Tyr β-CH_B), 3.70 (3H, s, OMe), 4.96 (1H, m, Tyr α-CH), 6.68 (2H, d, $J = 8.4$ Hz, Tyr ArH), 6.86 (1H, d, $J = 7.9$ Hz, NHCO), 7.04 (2H, d, $J = 8.4$ Hz, Tyr ArH), 7.83 (2H, d, $J = 1.8$ Hz, ArH), 8.14 (1H, t, $J = 1.8$ Hz, ArH), 10.49 (2H, br s, NHCN), 11.52 (2H, br s, NHC OBu^t). ^{13}C NMR ($CDCl_3$) δ: 28.20 and 28.27 (2 × OBu^t), 36.86 (Phe β-CH_2), 52.61 (OMe), 54.56 (Phe α-CH), 80.15 and 84.21 (2 × OBu^t), 115.08–138.61 (aromatics), 154.04 (2 × OCON), 154.37 (2 × OCONH), 164.72 (2 × *C*N), 166.21 and 172.35 (2 × *C*O). Anal. Calcd for $C_{39}H_{55}N_7O_{12}$: C, 57.55; H, 6.81; N, 12.05. Found: C, 57.58; H, 6.80; N, 12.07.

*3,5-Bis(N-amidino)aminobenzoyl-L-tyrosine methyl ester (**8**)*

Deprotection of **8c** (300 mg, 0.37 mmol) was performed as described for **5c** to give **8** (232 mg, 98%). ^1H NMR (DMSO-d_6) δ: 2.95 (2H, dd, $J = 5.8$, 13.2 Hz Tyr β-CH_A), 3.03 (2H, dd, $J = 5.4$, 14.1 Hz Tyr β-CH_B), 3.60 (3H, s, OMe), 4.58 (1H, m, Tyr α-CH), 6.63 (2H, d, $J = 8.4$ Hz, Tyr ArH), 7.04 (2H, d, $J = 8.7$ Hz, Tyr ArH), 7.34 (1H, t, $J = 1.8$ Hz, ArH), 7.56 (1H, t, $J = 1.8$ Hz, ArH), 7.74 (9H, br s, NH), 8.94 (1H, d, $J = 7.8$ Hz, NHCO), 10.22 (2H, br s, NHCN). ^{13}C NMR (DMSO-d_6) δ: 36.19 (Tyr β-CH_2), 52.68 (OMe), 55.36 (Tyr α-CH), 115.20–137.48 (aromatics), 156.64 (2 × *C*N), 165.41 and 172.79 (2 × *C*O). Anal. Calcd for $C_{23}H_{25}F_6N_7O_8$: C, 43.06; H, 3.93; N, 15.28. Found: C, 43.08; H, 3.91; N, 15.27. MS (ESI): *m/z* 207.5 $[M + 2H]^{++}$.

Hydrophobicity

All SAMPs derivatives **1–8** were analyzed by RP-HPLC using a solvent system of H_2O/CH_3CN (0.1% TFA) in the form of a linear gradient from 10 to 80% of CH_3CN in 30 min and a flow rate of 1 ml/min.

Antimicrobial Activity

Two Gram-positive (*S. aureus* ATCC 29213 and *S. epidermidis* ATCC 35984) and two Gram-negative (*E. coli* ATCC 8739 and *K. pneumoniae* 5F) strains, and one fungal strain (*C. albicans* ATCC 10231) – all biofilm producers – were used for the detection of antibacterial activity of the SAMPs derivatives **1–8**.

The peptidomimetic effect on planktonic cells was evaluated by MIC and MBC using the broth microdilution method according to CLSI guidelines [31]. Bacterial suspensions, grown in Mueller–Hinton broth at logarithmic phase, were incubated on microtiter plates at a concentration of 5×10^5 CFU/ml, with several peptide dilutions – 1, 2.5, 5, 7.5, 10, 15.6, 25, 31.3, 50, 62.5, 75, 100, 125, 150, 200, 250, and 300 μg/ml – for 24 h at 37 °C. The MIC was defined as the lowest concentration of peptide giving a complete inhibition of visible growth in comparison with a peptide-free control well, and the MBC was determined as the lowest concentration at which no bacterial growth occurred on Mueller–Hinton agar plates. The MIC detection of *C. albicans* ATCC 10231 was performed in Mueller–Hinton broth for an incubation of 48 h at 25 °C. Data were obtained from at least three independent experiments performed in duplicate. The susceptibility of the bacterial strains against ciprofloxacin was used as an internal standard during MIC determinations. Another complete experiment was conducted by using buffered peptone water – in the same experimental conditions – to compare the MIC and MBC results obtained with Mueller–Hinton broth.

The efficacy on established biofilm was evaluated by determining BIC and BEC according to the method described by Johnson *et al.* [32] with some modifications. Bacterial suspensions, grown in tryptic soy broth supplemented with 0.5% (v/v) glucose at logarithmic phase, were incubated on flat-bottomed microtiter plates at a concentration of 5×10^5 CFU/ml. After 24 h of incubation at 37 °C, the planktonic cells were gently removed and the wells were washed with sterile phosphate-buffered saline (PBS) pH 7.3 and filled with different peptide dilutions ranging from the MIC values to a maximum concentration of 1200 μg/ml. The OD_{600} was

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measured at time 0 and after incubation for 24 h at 37 °C. The BIC was determined as the lowest concentration of the peptide inhibiting growth in the supernatant fluid, confirmed by no increase in optical density compared with the initial reading. The BEC was determined as the lowest concentration at which no bacterial growth occurred on tryptic soy agar plates. Data were obtained from at least three independent experiments performed in duplicate.

Hemolytic Activity

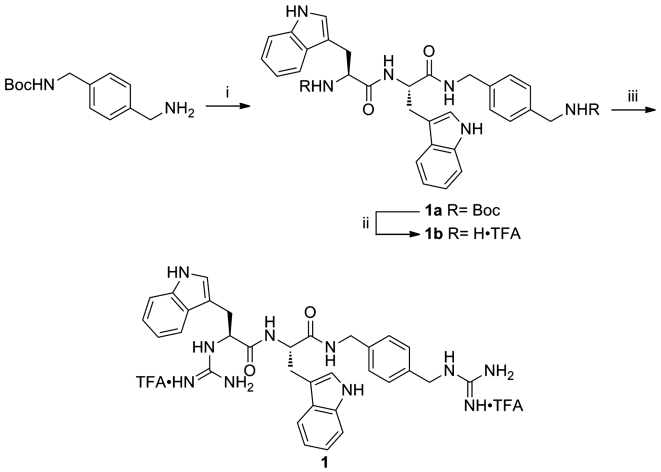
Hemolytic activity of the reference compound was not tested because it is known in literature [17]. Hemolytic activity of the SAMPs derivatives **1–8** was tested against human red blood cells (h-RBCs). Fresh human blood, collected with EDTA, was centrifuged at 3000 rpm for 5 min then washed three times with PBS pH 7.3. Red blood cells were diluted to 4% in PBS and incubated with different peptide dilutions ranging from 1.25 to 200 μg/ml for SAMPs derivatives **1–2** and **5–6** and from 1.25 to 800 μg/ml for **3–4** and **7–8**. After 1 h of incubation at 37 °C, the suspensions were sedimented by centrifugation and the release of hemoglobin was determined by absorbance measurement at 405 nm and compared with a 0% hemolysis control (PBS) and a 100% hemolysis control (PBS with 1% v/v Triton X-100). The percentage of hemolysis was calculated using the following equation:

$$\text{Hemolysis}(\%) = [(\text{OD}_{405}\text{sample} - \text{OD}_{405}0\%\ \text{lysis control})/ (\text{OD}_{405}100\%\ \text{lysis control} - \text{OD}_{405}0\%\ \text{lysis control})] \times 100.$$

Results and Discussion

Synthesis

The SAMPs derivatives **1–8** reported in Figure 1 were synthesized according to the strategies shown in Schemes 1–4. The coupling



Scheme 1. Reagents and condition: (i) Boc-L-tryptophan-L-tryptophan, HBTU, HOBt, DIPEA, DMF, 0 °C, 10 min, and room temperature, 2.5 h; (ii) TFA/CH_2Cl_2 (1 : 10), room temperature, 1.5 h; (iii) 3,4-dimethyl-1H-pyrazole-1-carboximidamide nitrate salt, TEA, THF, reflux, 16 h.

reactions were performed using the HBTU/HOBt method to give the Boc-protected peptidomimetics **1a–8a**. After the removal of Boc-protecting groups, the introduction of guanidine moiety in compound **1b** to obtain **1**, was carried out using 3,4-dimethyl-1*H*-pyrazole-1-carboximidamidenitrate salt as guanylation reagent [33]. Unfortunately, the use of this strategy in the synthesis of the SAMPs derivatives **2–8** resulted disappointing, giving low yields and difficulties in the isolation and purification of the products. Thus, for the peptidomimetics **2–8** an alternative guanylation strategy was employed. The guanidine moiety was added in the compounds **2b-8b** as Boc-protected functionality by reaction with *N,N′*-bis(Boc)-*S*-methyl-isothiourea in the presence of mercury chloride [34]. After the cleavage of the Boc-protecting group using TFA/CH_2Cl_2, the final products were obtained in good yields as trifluoacetate salts.

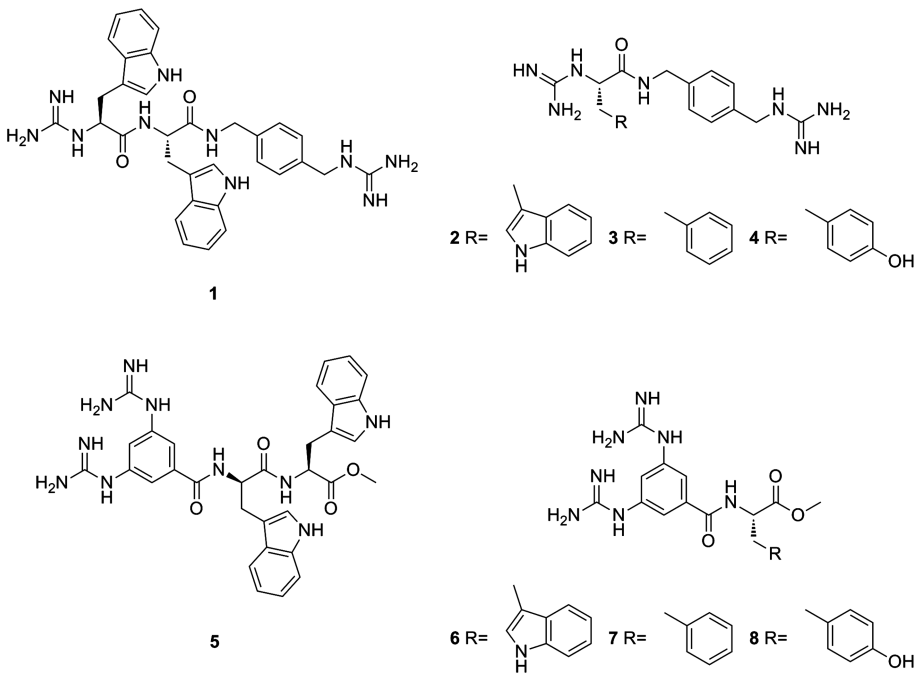
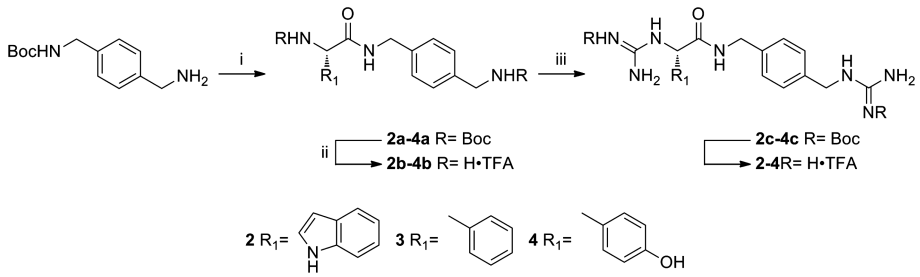
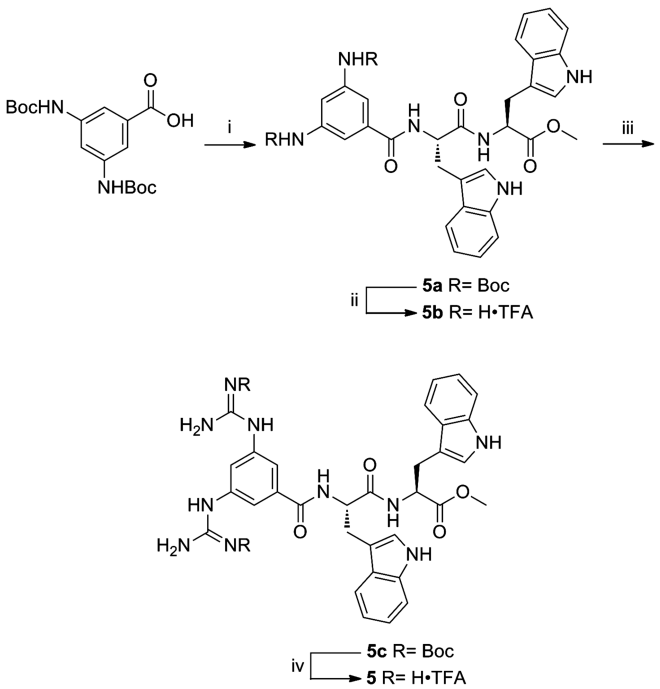


Figure 1. Structures of synthesized SAMPs derivatives **1–8**.



Scheme 2. Reagents and conditions: (i) Boc-L-tryptophan, HBTU, HOBt, DIPEA, DMF, 0 °C, 10 min, and room temperature, 2.5 h; (ii) TFA/CH_2Cl_2 (1 : 10), room temperature, 1.5 h; (iii) *N,N′*-bis(*tert*-butoxycarbonyl)-*S*-methyl-isothiourea, $HgCl_2$, TEA, DMF, 0 °C, 3 h; (iv) TFA/CH_2Cl_2 (1 : 10), room temperature, 1.5 h.



Scheme 3. Reagents and conditions: (i) L-tryptophyl-L-tryptophan methyl ester, HBTU, HOBt, DIPEA, DMF, 0 °C, 10 min, and room temperature, 2.5 h; (ii) TFA/CH_2Cl_2 (1 : 10), room temperature, 1.5 h; (iii) *N,N′*-bis(*tert*butoxycarbonyl)-*S*-methyl-isothiourea, $HgCl_2$, TEA, DMF, 0 °C, 3 h; (iv) TFA/CH_2Cl_2 (1 : 1), room temperature, 1.5 h.

Hydrophobic Parameters

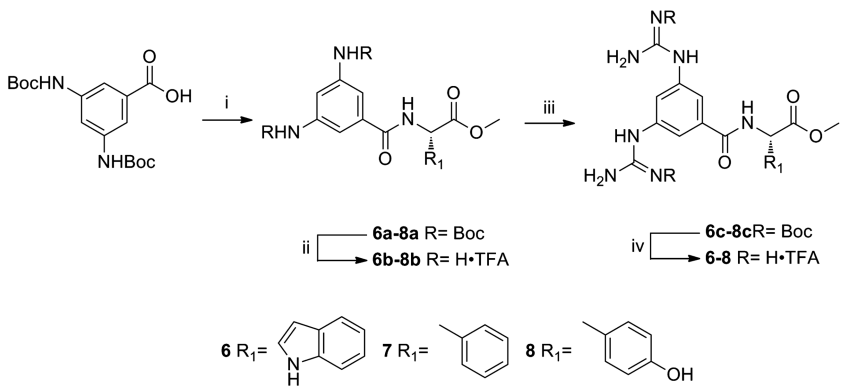
In order to investigate the correlation between hydrophobicity on antimicrobial activity, the overall hydrophobicity was evaluated by measuring the retention time (t_R) of each individual peptidomimetic using RP-HPLC. The t_R values, determined for all synthesized SAMPs derivatives **1–8**, were in the range 11.87–14.56 min as shown in Table 1. The highest values (14.69 and 14.56 min) were observed for the reference compound and peptidomimetic **5**, respectively, showing a high affinity to the HPLC hydrophobic phase. All other synthesized analogues showed reduced retention times, except for SAMPs derivatives **1** and **7** (13.34 min) (Table 1). By evaluating these data, as we expected, the replacement of the tryptophan residue with less lipophilic amino acids, such as phenylalanine or tyrosine, reflected on the total hydrophobicity of the synthesized peptidomimetics.

A clear correlation between retention time on the C_{18} column (i.e. affinity for the hydrophobic column surface) and antibacterial activity was not found, except for peptidomimetic **5**.

Antimicrobial Activity

The antimicrobial activity of the SAMPs derivatives **1–8** was evaluated *in vitro* against two reference Gram-positive strains (*S. aureus* and *S. epidermidis*), two reference Gram-negative strains (*E. coli* and *K. pneumoniae*), and one fungal strain (*C. albicans*) (Table 2a).

We synthesized and tested the reference compound Arg-Trp-OBzl and our SAMPs derivatives **1–8** on different panel of reference bacteria respect to that used by Strom *et al.* [17]. The MIC values



Scheme 4. Reagents and conditions: (i) L-tryptophan methyl ester, HBTU, HOBt, DIPEA, DMF, 0 °C, 10 min, and room temperature, 2.5 h; (ii) TFA/CH_2Cl_2 (1 : 10), room temperature, 1.5 h; (iii) *N,N′*-bis(*tert*-butoxycarbonyl)-*S*-methyl-isothiourea, $HgCl_2$, TEA, DMF, 0 °C, 3 h; (iv) TFA/CH_2Cl_2 (1 : 1), room temperature, 1.5 h.

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Table 1. Hydrophobicity of SAMPs derivatives **1–8** measured as retention time (t_R) on C_{18} column with solvent system of acetonitrile/water in the form of a linear gradient starting from 10 to 80% of CH_3CN in 30 min

SAMPs derivatives	MW	t_R (min)
Arg-Trp-OBzl	523.46	14.69
1	820.29	13.34
2	634.53	12.78
3	595.49	12.13
4	611.49	11.93
5	850.72	14.56
6	664.51	12.96
7	625.48	12.34
8	641.48	11.87

against Gram-positive species ranged from 7.5 to 200 μg/ml, except for peptidomimetic **4** with MIC values >300. The SAMPs derivatives **5** and **6** revealed the most antibacterial activity – among the tested peptidomimetics – both against *S. aureus* ATCC 29213 (10 μg/ml) and *S. epidermidis* ATCC 35984 (7.5 μg/ml). SAMPs derivatives **1** and **2** were moderately active; in particular, peptidomimetic **2** showed MIC values of 25 μg/ml against both *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 35984. However, our most active SAMPs derivatives (**1–2** and **5–6**) showed better MIC values with respect to the reference compound (75 μg/ml). Other tested peptidomimetics showed no valuable antimicrobial activity against Gram-positive bacteria (Table 2a). The MIC values, recorded in SAMPs derivatives **5**, **1**, and **3** against *S. aureus* ATCC 29213, and **7** against *S. epidermidis* ATCC 35984 (Table 2a), corresponded to the bactericidal values.

By evaluating the biological results, the order of activity against Gram positive bacteria can be rationalized on the basis of the different structural features between the SAMPs derivatives **1–4** with respect to **5–8**, probably due to the spatial disposition of the cationic groups in the two series of analogues. In the SAMPs derivatives **1–4**, the positively charged moieties are at the opposite sides of the molecule, whereas in peptidomimetics **5–8**, they are closer in space. It could be interesting to note that the spatial disposition in SAMPs derivatives **1–4** could affect the capacity of the molecules to effectively assume the amphipathic conformation with separate hydrophobic and hydrophilic portions, as required to exert antimicrobial activity [35,36]. The introduction of arginine mimetics in all SAMPs derivatives **1–8** as cationic residue in place of lysine was based on the hypothesis that the guanidine group of arginine would interact more strongly with the negatively charged phospholipids of the bacterial cell membrane by forming both electrostatic and hydrogen-bonding interactions [37]. It is important to note that the replacement of the tryptophan residue by phenylalanine or tyrosine does significantly alter the antibacterial activity (SAMPs derivatives **3–4** and **7–8**). These data were not surprising because tyrosine and phenylalanine were less effective in providing bulk and lipophilicity than tryptophan, thus lowering antibacterial activity [38,39].

The SAMPs derivatives **2**, **5**, and **6** revealed the same low efficacy against the tested Gram-negative bacteria and *C. albicans* (Table 2a). In particular, *K. pneumoniae* 5F was inhibited and killed by using peptidomimetic **2** at 31.3 μg/ml, whereas peptidomimetic **5** was active against *E. coli* ATCC 8739 at 25 μg/ml. *C. albicans* ATCC 10231 was inhibited and killed at 50 and 62.5 μg/ml by using SAMPs derivatives **2** and **5**, respectively. As explained by Den Hertog *et al.* [40], the candidacidal effect of SAMPs derivatives **2** and **5** could be related to the impairment of the functional and structural integrity of cell membrane.

The difference of susceptibility between Gram-positive and Gram-negative bacteria was not investigated. Considering the lower activity against Gram-negative bacteria, with respect to Gram-positive species, the effect of SAMPs derivatives on established biofilms was evaluated only against *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 35984. The most active SAMPs derivatives (**1–2** and **5–6**), against the tested Gram-positive bacteria, were chosen to evaluate their capability to disrupt the mature biofilms (Table 2b). Results showed BIC values ranging from fourfold to 20-fold higher than the corresponding MIC values, whereas BECs were never under 800 μg/ml for all peptidomimetics tested. Thus, all synthesized SAMPs derivatives **1–2** and **5–6** showed an appreciable inhibiting activity on established Gram-positive biofilms (BIC < 200 μg/ml), i.e. peptidomimetic **1** (100 μg/ml) but a lower capacity to eradicate the biofilms (BEC > 800). The relatively low BIC values probably reflect the ability of the compounds to act on slow-growing or non-growing bacteria [9], whereas the high BEC values are likely due to the difficulties of peptidomimetics to diffuse in the extracellular biofilm polymers (EPS). In fact, the polysaccharide intercellular adhesin, a positively charged homopolymer of beta-1,6 linked to *N*-acetylglucosamine residues, is considered to play a critical role in the development and maturation of *Staphylococcus* biofilms [41]. This cell surface-associated EPS, being positively charged, acts as an intercellular adhesin and binds the negatively charged bacterial cells together via electrostatic interactions, resulting in an increased cellular accumulation [42].

Hemolytic Activity

All analyzed peptidomimetics did not produce human blood hemolysis at their MIC values. In particular, all SAMPs derivatives never expressed hemolytic activity higher than 33% at their maximum concentration tested, except for peptidomimetic **5**, which displayed a cytotoxic effect showing hemolytic activity over 50% at 100 μg/ml.

The dose–response curves for the hemolytic activity of the SAMPs derivatives were shown in Figure 2. A weak linear trend between hemolytic activity and lipophilicity was found, and peptidomimetic **5** – the most hydrophobic one – being the most hemolytic as well. On the other hand, there is not a clear correlation between antimicrobial potency and hemolytic activity for the SAMPs derivatives **1**, **2**, and **6**.

Conclusion

In conclusion, novel antimicrobial peptidomimetics containing the 1-(4-(aminomethyl)benzyl)guanidine, or the 3,5-diguanidino benzoic acid, and aromatic α-guanidino amino acids have been synthesized.

In the present study, all the synthesized peptidomimetics fulfilled the pharmacophore model of short AMPs with antimicrobial activity by having a net charge of +2 and an amphipathic structure. It was observed that the presence of tryptophan moieties favored the antibacterial activity especially against Gram-positive bacterial strains. On the contrary, compounds bearing phenylalanine or tyrosine as bulky groups resulted in a considerable loss of the antibacterial action (SAMPs derivatives

Table 2. Antimicrobial activity of SAMPs derivatives **1–8**, expressed in μg/ml (μM) against planktonic and sessile bacterial strains

(a) Planktonic bacterial strains

SAMPs derivatives	*S. aureus* ATCC 29213		*S. epidermidis* ATCC 35984		*E. coli* ATCC 8739		*K. pneumoniae* 5F		*C. albicans* ATCC 10231	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Arg-Trp-OBzl	75 (143)	75 (143)	150 (287)	150 (287)	300 (573)	> 300 (573)	> 300 (573)	> 300 (573)	300 (573)	300 (573)
1	50 (61)	50 (61)	25 (31)	75 (91)	150 (183)	200 (244)	150 (183)	150 (183)	200 (244)	250 (305)
2	25 (39)	50 (79)	25 (39)	50 (79)	75 (118)	150 (236)	31.3 (49)	31.3 (49)	50 (79)	50 (79)
3	200 (336)	200 (336)	125 (210)	150 (252)	300 (504)	300 (504)	300 (504)	300 (504)	> 300 (504)	> 300 (504)
4	> 300 (491)	> 300 (491)	> 300 (491)	> 300 (491)	> 300 (491)	> 300 (491)	> 300 (491)	> 300 (491)	> 300 (491)	> 300 (491)
5	10 (12)	10 (12)	7.5 (9)	10 (12)	25 (29)	50 (59)	62.5 (74)	100 (118)	62.5 (74)	62.5 (74)
6	10 (15)	25 (38)	7.5 (11)	15 (23)	50 (75)	100 (151)	75 (113)	75 (113)	100 (151)	100 (151)
7	75 (120)	125 (200)	75 (120)	75 (120)	150 (240)	150 (240)	150 (240)	300 (480)	200 (320)	200 (320)
8	50 (78)	75 (117)	50 (78)	75 (117)	150 (234)	150 (234)	250 (390)	250 (390)	200 (312)	250 (390)

(b) Sessile bacterial strains

SAMPs derivatives	*S. aureus* ATCC 29213		*S. epidermidis* ATCC 35984	
	BIC	BEC	BIC	BEC
Arg-Trp-OBzl	400 (764)	800 (1528)	400 (764)	800 (1528)
1	100 (122)	> 1200 (1463)	200 (244)	1200 (1463)
2	200 (315)	1200 (1891)	100 (158)	1200 (1891)
5	100 (118)	1200 (1411)	100 (118)	800 (940)
6	100 (151)	800 (1204)	200 (301)	800 (1204)

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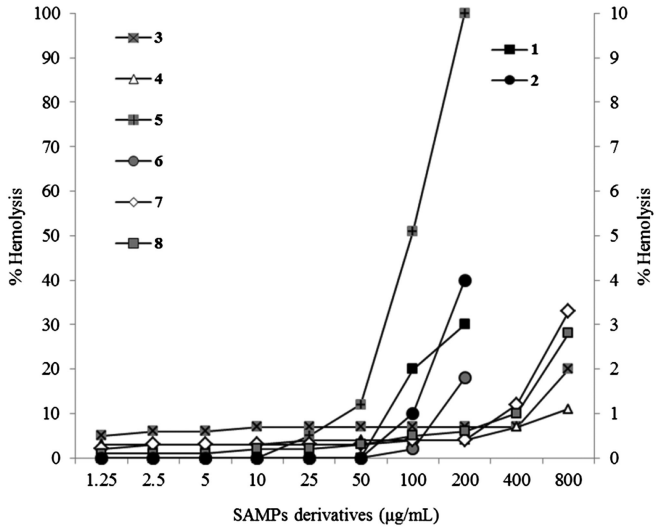

Figure 2. Dose–response curve of hemolytic activity of SAMPs derivatives **1–8** against h-RBCs. Peptidomimetics **1–2** refer to *y*-axis on the right whereas peptidomimetics **3–8** refer to the *y*-axis on the left.

3–4 and **7–8**) against both Gram-positive and Gram-negative bacterial strains. In this study, an appreciable antifungal activity was observed only for SAMPs derivatives **2** and **5**.

In light of the results presented in this work and taking into account that this is preliminary studies, we can conclude that the synthesized SAMPs derivatives **1–2** and **6** showed a promising antimicrobial activity both in planktonic and sessile growth mode. Further studies are needed to well investigate their potential antimicrobial activity against a wide panel of Gram-positive strains.

Acknowledgements

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Conflict of Interest

The authors have declared no conflict of interest.

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