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Chemoenzymatic Synthesis and Antimicrobial and Haemolytic Activities of Amphiphilic Bis(phenylacetylarginine) Derivatives

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Novel bis(N$^{\alpha}$-phenylacetyl-L-arginine)-α,ω-alkanediamide dihydrochloride (bis(PhAcArg)) derivatives with antimicrobial activity were designed and synthesised by a chemoenzymatic strategy. The new structures consist of two N$^{\alpha}$-phenylacetyl-L-arginine moieties connected by an alkanediamine spacer chain of 6, 8, 10, 12, and 14 methylene units through amide bonds. The key step in the chemoenzymatic strategy is the double aminolysis of the N$^{\alpha}$-phenylacetyl-L-arginine methyl ester by the corresponding α,ω-alkanediamine catalyzed by papain in ethanolic media. The compounds synthesised were tested as antimicrobials against 15 bacterial and 8 fungal species. The antimicrobial activity and selectivity depend strongly on the spacer chain length. The bis(PhAcArg) derivative with the spacer chain of 12 methylene groups gave the lowest MIC values against Gram-positive bacteria, whereas that with 14 methylene units was the best against Gram-negative bacteria. Interestingly, these novel compounds showed enhanced antibacterial activity relative to the lead compound, bis(N$^{\alpha}$-caproyl-L-arginine)-1,3-propanediamide dihydrochloride ($C_3(CA)_2$), and moderate antifungal activity. Moreover, tests of haemolytic activity toward human erythrocytes revealed that haemolysis increases with spacer chain length. Importantly, the compounds were classified as not irritating to eyes, with the exception of the compound with the spacer chain of 14 methylene groups, which was a slight eye irritant.

Introduction

The synthesis, physicochemistry, and biological activity of arginine fatty acid conjugates have been the focus of our attention during the last decade.[1–3] Arginine fatty acid conjugates are a class of amphiphilic compounds that possess excellent surface and interface activity, rich self-assembly behaviour, a low toxicity profile, high biodegradability, and broad antimicrobial activity.[4,5] These exceptional characteristics make them candidates of choice as preservatives and antiseptics in pharmaceutical, food, and dermatological formulations. Among the arginine-based surfactants synthesised by our research group, bis(*N*$^{\alpha}$-caproyl-L-arginine)-1,3-propanediamide dihydrochloride ($C_3(CA)_2$), a novel dimeric (that is, double-chain/double-polar-head compound), showed the lowest minimum inhibitory concentration (MIC) values against a broad spectrum of Gram-positive and Gram-negative bacteria.[6,7]

Biguanides are powerful antiseptics widely employed in many pharmaceutical and personal-care formulations. Chlorhexidine (CHX) is the most representative commercial biguanide compound. It possesses high efficacy against both Gram-positive and Gram-negative bacteria and low toxicity.[8,9] Interestingly, the MIC values of CHX are about 2- to 48-fold lower, depending on the microorganism, than those of $C_3(CA)_2$.[7] In general, microbicides lack target specificity, although in the case of biguanides such as CHX and cationic surfactants including $C_3(CA)_2$, the main target appears to be the cytoplasmic bacterial membrane.[7,8,10] Moreover, both biguanides and cationic surfactants share similar mechanisms of action.[11] Thus, structural factors and an appropriate hydrophobic–hydrophilic balance are mostly responsible for the various antimicrobial efficiencies observed. To improve the antimicrobial potency of arginine–lipid conjugates, we sought to design new arginine derivatives, namely bis(PhAcArg)s, analogues of $C_3(CA)_2$.

$C_3(CA)_2$ and CHX are both dicationic, and their positive charges are connected through a hydrocarbon spacer chain (Figure 1). As $C_3(CA)_2$ already has good antibacterial activity, we intended to keep structural changes in the molecule to a minimum. Hence, with the CHX structure in mind, we thought of substituting the two *N*$^{\alpha}$-caproyl groups of $C_3(CA)_2$ with two *N*$^{\alpha}$-phenylacetyl residues and to vary the length of the spacer alkyl chain (see Figure 1). The spacer chain length modulates the hydrophobic–hydrophilic balance, which influences the interfacial properties and therefore, the antimicrobial activity of

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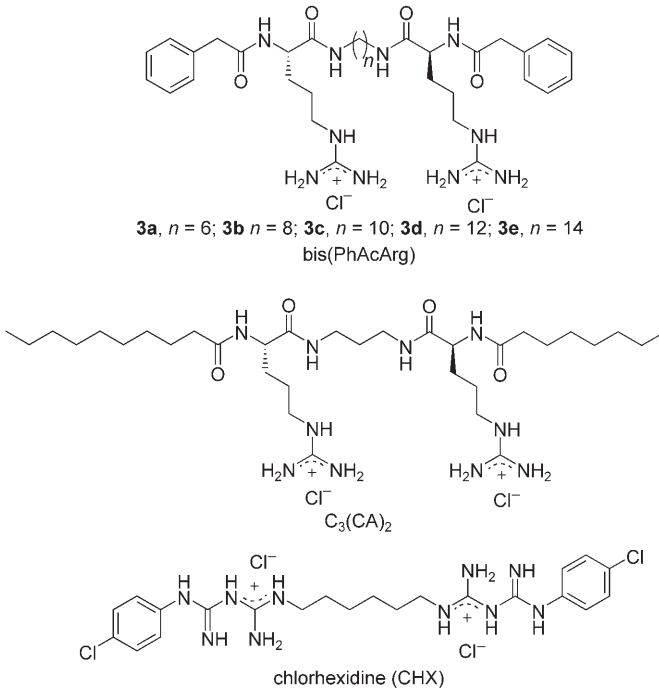


Figure 1. Structures of bis(PhAcArg), $C_3(CA)_2$, and CHX.

the molecule.[12] Based on previous work[1,13] with dimeric arginine derivatives, alkanediamine spacers of 6, 8, 10, 12, and 14 methylene groups were chosen as the most appropriate.

Herein we report the chemoenzymatic synthesis of these new bis(PhAcArg) derivatives and their antimicrobial activity against 15 bacterial and 8 fungal species. Moreover, to assess cytotoxicity and the potential for acute eye irritation, we determined their haemolytic and protein denaturation effects on erythrocytes and compared those with the effects observed in the presence of CHX by using a quick in vitro screening test.

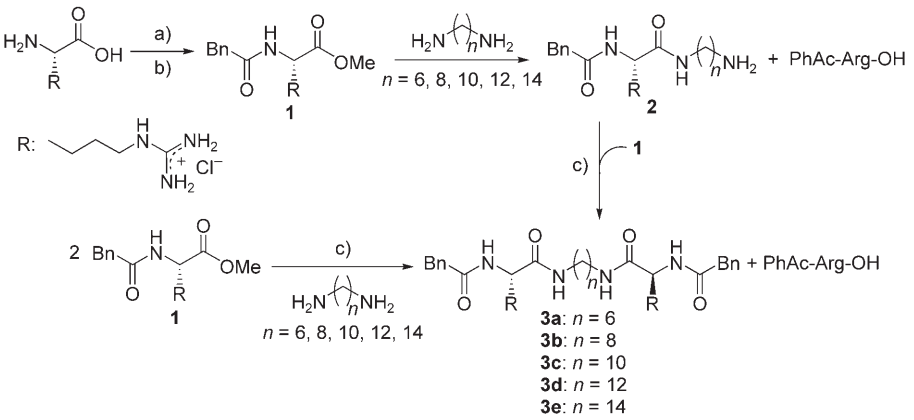
Results and Discussion

Chemoenzymatic synthesis of bis(PhAcArg) derivatives

Based on our previous work in the chemoenzymatic preparation of arginine-based gemini surfactants,[2] the synthesis of the new bis(PhAcArg) amphiphiles was initially planned in four steps (Scheme 1).

The first two steps, esterification of the α-carboxyl group of the arginine moiety (98%) and acylation of the α-amine with phenylacetyl chloride (73%) gave access to intermediate **1**. The third step was the aminolysis of the α-methyl carboxylate group of **1** by one of the amino groups of the α,ω-alkanediamine. This reaction took place without any catalyst in good yield (Table 1) by using 5–7 equiv diamine at temperatures above the melting point of the given diamine, which acted as solvent and reagent. Derivatives **3a**–**3e** were then obtained by papain-catalysed amidation of **1** by **2** in ethanol/boric acid–borate buffer (0.1 M, pH 8.2, 99.5:0.5) in good yield (Table 2). The final purification steps required to afford highly pure products lead to moderate overall yields. The enzymatic amide bond formation failed for the reaction between **1** and α,ω-tetradecyldiamine. Excess diamine from the previous step that had not been eliminated by simple washings appears to be responsible for the low conversion. In fact, it had been observed that the diamines had a negative effect on papain activity by either acting as an inhibitor or by modifying the enzyme ionization state owing to the basicity of the product.[2]

To avoid the presence of excess diamine, the formation of bis(PhAcArg) in a one-pot enzymatic reaction was undertaken, starting from the diamine and 2 equiv compound **1**. Interestingly, the new synthetic enzymatic scheme furnished bis(PhAcArg) in three



Scheme 1. Chemoenzymatic synthesis of bis(PhAcArg) derivatives: a) $SOCl_2$/MeOH; b) BnCOCl; c) Papain onto celite, EtOH/boric acid–borate buffer (99.5:0.5). Bn = benzyl.

Table 1. Aminolysis of **1** by α,ω-alkanediamines of various length.

$H_2N(CH_2)_nNH_2$	T [°C]	PhAc-Arg-OH[a] [%]	Product **2** [%][a,b]	Product **3** [%][a]
$n=6$	50	6	90	6
$n=8$	55	9	85	9
$n=10$	66	7	88	7
$n=12$	72	6	87	6
$n=14$	100	8	87	8

[a] Molar percent conversion into the corresponding product with respect to PhAc-Arg-OMe (compound **1**) measured by HPLC of the crude reaction mixture using purified standards; reaction time: 2 h. [b] Isolated yields were not calculated, as a simple workup was performed to remove excess diamine; therefore, the final product contained PhAc-Arg-OH and product **3**.

Table 2. Papain-catalyzed amide bond formation between **1** and **2**.

Product	Conversion [%][a]	PhAc-Arg-OH [%][a]	Isolated Yield [%]
3a	48	38	25
3b	48	29	21
3c	54	34	27
3d	58	25	26
3e	27	65	nr[b]

[a] Molar percent conversion into the corresponding product with respect to PhAc-Arg-OMe (compound **1**) measured by HPLC of the crude reaction mixture using purified standards; reaction time: 72 h; 1.5 equiv **2** per mol PhAc-Arg-OMe. [b] No reaction.

steps at room temperature and with reaction conversions similar to those obtained by the procedure described above (Table 3). Most importantly, this strategy allowed us to prepare the bis(PhAcArg) **3e** with α,ω-tetradecyldiamine in isolated yields similar to those obtained with shorter spacer chain lengths.

Antibacterial activity

Minimum inhibitory concentration (MIC) values for **3a**–**e** along with those for $C_3(CA)_2$ and CHX are summarised in Table 4. To compare compounds with different molecular weights precisely, MIC values are expressed in μM instead of the typical mg L^{-1}. For the novel bis(PhAcArg) series **3**, there is a clear effect of the spacer chain length on antimicrobial property. Overall, the lowest MIC values against Gram-positive bacteria were observed with **3d** (12 methylene groups in the spacer chain), whereas **3e** (14 methylene groups) showed the most potent inhibition of growth toward Gram-negative bacteria.

Table 3. Papain-catalyzed synthesis of **3** in one-pot reaction.

Product	PhAc-Arg-OH [%][a]	Product **2** [%][a]	Product **3** [%][a]
3a	13	16	55
3b	11	19	59
3c	11	17	55
3d	13	19	59
3e	10	20	37

[a] Molar percent conversion into the corresponding product with respect to PhAc-Arg-OMe (compound **1**) measured by HPLC of the crude reaction mixture using purified standards; reaction time: 176 h; 1.1 equiv α,ω-alkanediamine per mol PhAc-Arg-OMe.

Gram-negative are generally more resistant to antimicrobial agents than are Gram-positive bacteria. This can be explained by the different cell envelope structure of the two bacterial types. Gram-negative bacteria possess an outer membrane composed mainly of lipopolysaccharides and porins which restrict the entrance of biocides and amphiphilic compounds.[14,15] The perturbation of this outer membrane requires a fine tuning of the hydrophobic–hydrophilic balance of the microbicide molecule.[4] Gram-positive bacteria possess a thick, rigid, and highly porous cell wall of peptidoglycans. Thus, small hydrophilic molecules such as penicillin can move through it without difficulty, allowing easy penetration of compounds into the cell.[14] Moreover, bacteria often possess efflux proteins located in the cytoplasm membrane. These serve as a protective mechanism against antimicrobial activity by pumping antimicrobial molecules out of the cell.[16]

Table 4. MIC values (μM) for compounds **3a**–**e**, $C_3(CA)_2$, and CHX against bacteria.[a]

Species	**3a**	**3b**	**3c**	**3d**	**3e**	$C_3(CA)_2$	CHX
			Gram-positive				
Bacillus cereus var. mycoides	>323	>307	78	18	18	21	2
Staphylococcus epidermidis	>323	19	39	2	36	10	2
Bacillus subtilis	>323	154	39	2	36	21	2
Staphylococcus aureus	>323	77	19	5	36	3	1
Micrococcus luteus	>323	154	19	18	71	21	2
Enterococcus hirae	>323	>307	156	10	36	10	3
Mycobacterium phlei	>323	77	19	nd	nd	21	nd
Mycobacterium smegmatis	nd	nd	nd	nd	36	10	3
			Gram-negative				
Bordetella bronchiseptica	>323	154	39	37	36	5	2
Pseudomonas aeruginosa	>323	>307	>311	149	71	42	14
Salmonella typhimurium	>323	>307	>311	75	36	42	7
Enterobacter aerogenes	>323	>307	>311	149	18	42	14
Escherichia coli	>323	154	156	37	18	10	3
Klebsiella pneumoniae	>323	>307	>311	149	18	21	7
Serratia marcescens	>323	>307	>311	>298	>286	>333	7

[a] MIC: the lowest concentration of compound required for the inhibition of growth of test strains; nd = not determined.

Compared with $C_3(CA)_2$, compounds **3d** and **3e** have enhanced activity against Gram-positive and Gram-negative bacteria, respectively. The different antimicrobial efficiency of these compounds can be attributed to the combination of several physicochemical parameters: hydrophobicity, adsorption, aqueous solubility, and transport in the test medium. As a measure of hydrophobicity, we estimated the log octanol–water partition coefficient (log *P*) of the compounds by using KowWin, software that is based on the atom/fragment contribution method.[17] The estimated values are as follows: **3a** 0.80, **3b** 1.78, **3c** 2.76, **3d** 3.74, **3e** 4.73, $C_3(CA)_2$ 3.77, and CHX 4.85. It is important to stress that these are estimated rather than experimental values. As expected, log *P* increased with increasing spacer chain length. Interestingly, the antimicrobial activity showed good correlation with log *P* for the bis(PhAcArg)s, in good agreement with QSAR studies of biguanide biocides.[12,18] The most active compounds, including CHX, have an estimated log *P* value in the range between 3.74 and 4.85. It has been observed that the spacer chain length modulates the lipophilicity of the molecule, but is not a key structur-

al parameter for antimicrobial activity.[12] The optimal length of the spacer chain depends on the structure and nature of the polar head groups as well as on the presence of other alkyl chains in the molecule.[1,6,19] Therefore, antimicrobial activity cannot be determined by any given individual structural moiety alone. It is the right combination of positive charges and hydrophobic groups that provide the adequate hydrophilic–lipophilic balance.[20]

Apart from high activity (that is, high preservation capacity), a balance between antimicrobial activity on one hand, and low toxicity and efficient biodegradability on the other is always pursued. Hence, the relatively low activity against Gram-negative bacteria may facilitate the subsequent biodegradability of these compounds, thus decreasing their environmental impact.

Antifungal activity

The MIC values for compounds **3a**–**e**, $C_3(CA)_2$, and CHX against two yeast species and six molds are shown in Table 5. Similarly to the antibacterial activity, the results for the novel bis-(PhAcArg) series **3** against fungi indicate that there is a clear effect of the spacer chain length on antimicrobial properties. Antifungal capacity increases with increasing spacer chain length; compounds **3a** and **3b** did not show antimicrobial activity in the concentration range tested; an increase in spacer chain length did increase the antifungal effect (compounds **3c**–**e**). However, $C_3(CA)_2$ and CHX are more potent than compounds **3.** These results suggest again that the amphipathicity is the key parameter for activity.

Compounds **3c**–**e**, $C_3(CA)_2$, and CHX showed both antibacterial and antifungal activity. Fungi are eukaryotic organisms, and in many instances, antibacterial agents have no effect on them. An exception to this are the cationic biocides, the primary target of which are the bacterial cytoplasmic and fungal plasma membranes.[21]

The mechanism of action of some antifungal compounds is related to the inhibition of ergosterol biosynthesis, among other metabolic pathways.[22] However, the mode of action of amphiphilic fungicides is still poorly understood. Some amphiphilic fungicides such as amphotericin B and nystatin bind to ergosterol which disrupts membrane function and increases permeability, thus causing cell lysis.[22] Pentamidine analogues are dicationic aromatic compounds with antifungal activity equal or greater than that of fluconazole and amphotericin B.[23] The mitochondrion appears to be the primary cellular target of these compounds.[24]

Therefore, the mode of antifungal action of **3c**–**e** could be mostly through a perturbation of the plasma membrane, likely by binding to ergosterol, among other things. Furthermore, a potential effect on mitochondrial processes might be also considered; as this action requires the uptake of the compound through the membrane, an accurate lipophilic–hydrophilic balance of the molecule is also required.

Haemolytic effect and potential ocular irritation

In many cases, antimicrobial agents that kill or inhibit the growth of microbial cells can also be cytotoxic to others such as red blood cells. The determination of haemolytic action is a good way to discriminate cytotoxic from non-cytotoxic compounds and also to asses the potential for acute eye irritation.[25] Compounds **3a**, **3b**, and **3c** showed low haemolytic activity at the highest concentration tested and did not induce haemoglobin denaturation. Compounds **3d**, **3e**, and CHX showed haemolytic activity, and the results of haemolysis obtained at different concentrations are presented in a dose–response curve (Figure 2).

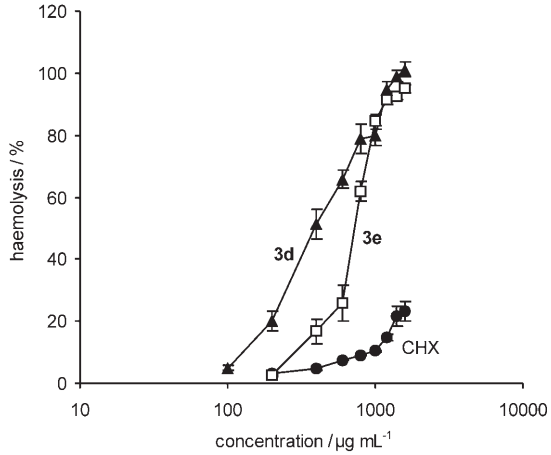


Figure 2. Haemolysis induced by **3d** (▲), **3e** (□), and CHX (●). Values are expressed as the mean ± SD of three experiments.

Table 5. MIC values (μM) for compounds **3a**–**e**, $C_3(CA)_2$, and CHX against fungi.

Fungi	3a	3b	3c	3d	3e	$C_3(CA)_2$	CHX
			Yeast				
Candida albicans	>323	>319	312	149	143	166	55
Saccharomyces cerevisiae	>323	>319	156	149	143	166	28
			Moulds				
Aspergillus repens	>323	319	78	19	36	5	7
Aspergillus niger	>323	>319	312	75	72	10	55
Penicillium chrysogenum	>323	>319	312	75	143	83	28
Cladosporium cladosporoides	>323	160	78	37	18	5	7
Trychophyton mentagrophytes	>323	>319	312	149	72	21	14
Penicillium funiculosum	>323	160	78	19	18	21	7

The values of HC_{50} for **3d**, **3e**, and CHX are presented in Table 6 along with the denaturation index (DI) and the lysis/denaturation ratio (L/D). From the L/D data, CHX and **3d** can be considered as nonirritant to the eyes, whereas **3e** was a slight irritant. Interestingly, CHX, **3d**, and **3e** were lesser irritants than $C_3(CA)_2$, which was found to be a moderate irritant and more haemolytic.[26] In the pres-

Table 6. Haemolytic data of **3d**, **3e**, and CHX determined with human erythrocytes.

Parameter	**3d**	**3e**	CHX
HC_{50} [$\mu g\,mL^{-1}$][a]	1002 ± 81	366 ± 4	2525 ± 270
DI[b]	0	8 ± 1	0
L/D[c]	∞	46	∞
Classification	Nonirritant	Slight irritant	Nonirritant

[a] Concentration giving 50% haemolysis; values expressed as the mean $\pm$SD. [b] Denaturation index. [c] Lysis/denaturation ratio.

ent study, CHX showed an HC_{50} value higher than that previously reported for which rabbit blood cells were used.[27] This could be attributed to both the different erythrocyte sources (human versus rabbit blood cells) and the different methodologies employed.[28] Human erythrocytes have been proven to be more resistant than those from other species.[29]

When the compounds were added to the erythrocyte suspension in aqueous medium, they could first distribute between the erythrocyte membrane and the solution by adsorption until equilibrium is reached. The interaction between the compound and erythrocyte membrane at sublytic concentration might be governed by the partition of the compound between the aqueous medium and the membrane. This partitioning is closely related to both the hydrophobicity of the compound and the ionic interactions present. Haemolysis probably begins when the erythrocyte membranes are saturated with the given compound. Herein, a good correlation (that is, exponential relationship) was observed between the haemolysis induced by compounds **3a**–**e** at 1000 $\mu g\,mL^{-1}$ and the number of methylene units in the spacer chain (Figure 3). Various examples of the relationship between haemolysis and alkyl chain length of surfactants have been published.[26,30] Overall, the longer the alkyl chain length, the greater the haemolytic activity within a family of structurally related compounds.

Despite the fact that the log*P* values of CHX, **3e**, and $C_3(CA)_2$ are similar, CHX showed lower haemolytic activity. It was observed that the interfacial activity of CHX was much lower than that of $C_3(CA)_2$; therefore, this may also be an important parameter to consider.[7] Again, the right combination of more than one parameter may explain the overall activity of the compounds.

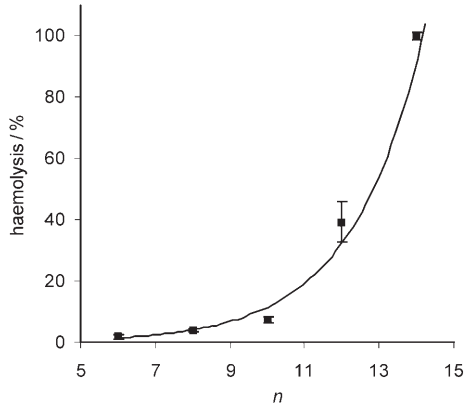


Figure 3. Haemolytic activity of **3a**–**e** at 1000 $\mu g\,mL^{-1}$ as a function of the number of methylene units (*n*) on the spacer chain. Results are expressed as mean $\pm$SD of three experiments; $R^2 = 0.986$ for the fitted curve.

Conclusions

In conclusion, the synthesis of novel bis(N^{α}-phenylacetyl-L-arginine)-α,ω-alkanediamide dihydrochloride (bis(PhAcArg)) derivatives can be carried out by an effective chemoenzymatic strategy in good reaction conversions. The key step in the synthesis is the papain-catalysed amide bond formation between the N^{α}-phenylacetyl arginine methyl ester and the corresponding α,ω-alkanediamine.

Replacement of the N^{α}-caproyl groups in $C_3(CA)_2$ by N^{α}-phenylacetyl moieties lead to novel bis(PhAcArg) compounds with enhanced antibacterial activity but lower antifungal activity than that of either $C_3(CA)_2$ or CHX. The cytoplasmic bacterial and plasma fungal membranes appear to be the target of these new arginine conjugates. The results suggest that the lipophilic–hydrophilic balance is crucial for antimicrobial activity and selectivity. Finally, the products showed low haemolytic activity against human erythrocytes and therefore low potential for ocular irritation and cytotoxicity.

Experimental Section

Chemicals and reagents: Chlorhexidine dihydrochloride, phenylacetyl chloride, oxalyl chloride and 1,12-dodecanedicarboxylic acid were obtained from Aldrich. Arginine hydrochloride, lithium sodium hydride, 1,10-diaminodecane, 1,12-diaminododecane, 1,4-dithio-D,L-threitol (DTT), and celite 545 (particle size, 26 μm; mean pore diameter, 17000 nm; specific surface area (BET method), 2.19 $m^2\,g^{-1}$) were obtained from Fluka. Papain (EC 3.4.22.2) from *Carica papaya* crude powder was obtained from Sigma (1.7 $U\,mg^{-1}$ protein; one unit (U) causes the hydrolysis of 1.0 $mmol\,min^{-1}$ benzyl-L-arginine ethyl ester (BAEE) at pH 6.2, 25 °C). 1,6-Diaminohexane and 1,8-diaminooctane were purchased from Merck. Ammonia was obtained from Praxair. bis(N^{α}-Caproyl-L-arginine)-1,3-propanediamide dihydrochloride ($C_3(CA)_2$) was synthesised in our laboratory by following previously described procedures.[2] Tetrahydrofuran and dichloromethane were distilled over sodium and CaH_2, respectively, just before use. All other solvents and reagents were of analytical grade and were used without further purification. Human blood from healthy volunteers was obtained from the Blood Bank of the Hospital Clinic (Barcelona, Spain).

Instruments: ^{1}H NMR (500 MHz) spectra of compounds were recorded with a Varian Unity-500 spectrometer, and ^{13}C NMR (100 MHz) spectra, with a Varian Unity-400. IR spectra of compounds in KBr tablets were recorded with a Nicolet Avatar 360 FTIR instrument. Elemental analyses were performed by Servei de Microanàlisi Elemental at Instituto de Investigaciones Químicas y Ambientales (IIQAB-CSIC). Specific rotations were measured with a Perkin–Elmer Model 341 polarimeter. Mass spectrometric data were obtained using MALDI-TOF MS with a VOYAGER-DE-RP at the University of Barcelona. The melting point of 1,14-diaminotetradecane was determined with a Kofler apparatus.

Enzyme immobilization: Papain was immobilized by deposition onto celite 545. The procedure was the following: papain (500 mg)

and DTT (250 mg) were dissolved in boric acid–borate buffer (0.1 M, pH 8.2, 5 mL, 0.5% *v/v*). This solution was thoroughly mixed with celite 545 (5 g) and dried under vacuum (80 μbar) until constant weight was reached.

HPLC analysis: The amounts of acyl donor, product, and hydrolyzed acyl donor produced in the reactions were measured by HPLC (Merck-Hitachi, Licrograph) analysis using a Licrosphere 100 CN (propylcyano) column (5 μm, 250×4 mm). The solvent system used was: solvent A, trifluoroacetic acid (TFA) 0.1% (*v/v*) in H_2O, and solvent B, H_2O/ACN 1:4, TFA 0.095% (*v/v*); flow rate 1 mL min^{-1}; detection at $\lambda = 215$ nm. Samples (50 μL) were withdrawn from the reaction medium at various times (0–96 h) depending on the synthesis and were diluted with EtOH (800 μL).

Preparation of 1,14-diaminotetradecane: 1,12-Dodecanedicarboxylic acid (5.6 g, 21.8 mmol) and DMF (1.68 mL, 21.8 mmol) were dissolved in hexanes (350 mL), and the mixture was cooled at 4 °C. To this solution, oxalyl chloride (0.2 mol) was added dropwise over a period of 30 min, and then the mixture was allowed to react at room temperature overnight. The reaction mixture started as a white suspension and ended as a transparent solution with brown oily precipitated drops on the reactor wall. The solvent was evaporated under vacuum, and the residue was dissolved in freshly distilled CH_2Cl_2. This solution was saturated by NH_3 at 4 °C, and a white precipitate formed immediately. The reaction mixture was left to proceed at room temperature overnight. The solvent and excess NH_3 were then removed under vacuum. The solid obtained was washed with cold water and cold EtOAc and finally dried under vacuum to afford 1,12-dodecanediamide as a brown solid (6.4 g, 98% yield); ^{1}H NMR (500 MHz, [D$_6$]DMSO, 40 °C): $\delta = 7.16$ (s, 2H), 6.57 (s, 2H), 2.09–1.92 (m, 4H), 1.58–1.38 (m, 4H), 1.24 ppm (s, 16H); ^{13}C NMR (100 MHz, [D$_6$]DMSO, 40 °C): $\delta = 174.14$, 34.95, 28.78, 28.73, 28.58, 28.51, 24.90 ppm; IR (KBr): $\tilde{\nu} = 3387$, 3189, 2923, 2848, 1650, 1416, 1337, 1281, 1230, 1179, 1122, 799, 646 cm^{-1}.

The 1,12-dodecanediamide thus obtained (2.5 g, 9.73 mmol) was added to a solution of $LiAlH_4$ (1.5 g, 40 mmol) in freshly distilled THF (0.5 L) at 4 °C. The reaction proceeded overnight at reflux to favour the solubility of the diamide. The reaction was stopped by slow and careful addition of cold water. The solid obtained was filtered off, and the filtrate was dried under vacuum to afford 1,14-diaminotetradecane as a white solid (1.6 g, 70% yield); ^{1}H NMR (500 MHz, [D$_6$]DMSO, 40 °C): $\delta = 1.35$–1.28 (m, 4H), 1.24 ppm (s, 24H); ^{13}C NMR (100 MHz, [D$_6$]DMSO, 40 °C): $\delta = 41.52$, 33.29, 28.86, 28.81, 28.79, 26.24 ppm; IR (KBr): $\tilde{\nu} = 3335$, 3251, 2923, 2846, 1603, 1461, 1319, 1062, 1004, 920, 714 cm^{-1}.

Preparation of N^{α}-phenylacetyl-L-arginine methyl ester hydrochloride (PhAc-Arg-OMe·HCl) (1): L-Arginine hydrochloride (20.0 g, 94.9 mmol) was suspended in MeOH (0.5 L) and cooled to ≈ −40 °C. $SOCl_2$ (50 mL, 0.69 mol) was added dropwise to this suspension over a period of one hour. Afterward the mixture was stirred at room temperature for 48 h to obtain a clear solution. The solvent, excess $SOCl_2$, and HCl generated were then removed under vacuum by repeated addition of EtOH until a solid residue was formed. The residue was filtered, washed with Et_2O, and dried to afford L-arginine methyl ester dihydrochloride as a white solid (24.2 g, 98% yield). The L-arginine methyl ester hydrochloride thus obtained (10.0 g, 38.29 mmol) was dissolved with ACN/H_2O (15:1). $NaHCO_3$ (6.4 g, 76.2 mmol) and Na_2CO_3 (10.1 g, 95.3 mmol) were added to this solution. The mixture was cooled with ice water, and phenylacetyl chloride (5.6 mL, 42.12 mmol) was added dropwise. The reaction medium was stirred overnight at room temperature. The resulting mixture was filtered, acidified to pH 1–2, and the solvent was removed under vacuum. The residue was dissolved in water (80 mL) and washed with EtOAc (3×100 mL), and the aqueous phase was lyophilized. The solid thus obtained was suspended in cool EtOH to precipitate the salts, which were filtered out. This desalting process was repeated three times. Finally, the ethanol was evaporated under vacuum, the residue was dissolved in water and lyophilized to afford the title compound as a white solid (9.83 g, 75% yield).

Preparation of bis(N^{α}-phenylacetyl-L-arginine)-α,ω-dialkylamide dihydrocloride (3): *a) Two-step procedure; step 1: Preparation of N^{α}-phenylacetyl-L-arginine(ω-aminoalkyl)amide monohydrochloride derivatives (PhAc-Arg-NH-(CH$_2$)$_n$-NH$_2$·HCl)* ***2***: PhAc-Arg-OMe·HCl **1** (4.0 g, 11.67 mmol) was mixed with the corresponding α,ω-diaminoalkane (81.70 mmol). The mixture was heated at different temperatures depending on the diamine: 50 °C for 1,6-diaminohexane, 55 °C for 1,8-diaminooctane, 66 °C for 1,10-diaminodecane, 72 °C for 1,12-diaminododecane, and 100 °C for 1,14-diaminotetradecane to obtain a homogeneous mixture after few minutes. After 1.5 h, the reactions were quenched by adding EtO_2 (25 mL for **2a**), ACN/EtO_2 (1:1, 25 mL for **2b**), or ACN/EtO_2 (2:1, 25 mL for **2c** and **2d**); for compound **2e**, see text. The resulting mixture was stirred, sonicated at room temperature, and finally cooled at −40 °C. The precipitate thus obtained was collected and washed with EtO_2 (2× 25 mL). Finally, the solid was dried under vacuum to yield the title compound. These intermediate products were identified by MS: **2a**, *m/z* [M^++1]: 391; **2b**, *m/z* [M^++1]: 419; **2c**, *m/z* [M^++1]: 447; and **2d**, *m/z* [M^++1]: 475.

a) Step 2: *Preparation of bis(PhAcArg) (**3**)*: The corresponding compounds **2** (13.30 mmol) and **1** (3.04 g, 8.87 mmol) were dissolved in EtOH (150 mL) free of O_2 and dried over molecular sieves (3 Å). Aqueous boric acid–borate buffer (0.1 M, pH 8.2, 0.75 mL, 0.5% *v/v*) was added To this solution. The mixture was stirred and sonicated thoroughly to obtain a homogeneous solution. Then, the immobilized papain preparation (17 g) was added. The reaction mixture was placed in a reciprocal shaker (125 rpm) at 25 °C under argon atmosphere. After 72 h, MeOH (100 mL) was added, and the immobilized preparation was filtered off and washed with MeOH (3× 100 mL). The organic phases were pooled, and the solvent was evaporated under vacuum. The residue was purified first by ion-exchange chromatography and then by preparative HPLC as described below.

b) One-pot procedure: PhAc-Arg-OMe·HCl (24 mg, 0.07 mmol) and the corresponding α,ω-alkanediamine (0.04 mmol) were dissolved in EtOH (1 mL) free of O_2 and dried over molecular sieves (3 Å). Boric acid–borate buffer (0.1 M, pH 8.2, 5 μL, 0.5% *v/v*) was added to this solution. The immobilized papain preparation (100 mg) was then added, and the reaction mixture was placed in a reciprocal shaker (125 rpm) at 25 °C under argon atmosphere. After 72 h, the reaction was stopped, worked up as previously described (see above), and the residue was purified first by ion-exchange chromatography and then by preparative HPLC as described below.

Preparative ion-exchange chromatography: Purification by preparative ion-exchange chromatography was performed as follows. The crude product was loaded onto a preparative column (285 cm^3) filled with MacroPrep High S, 50 μm stationary phase. The flow rate was 50–80 mL min^{-1}. Impurities were first eluted using NaCl (40 mM) in boric acid–borate (10 mM, pH 9.5) aqueous buffer/ethanol 40:60. The products, bis(PhAcArg), were finally eluted with NaCl (0.5 M) in H_2O/EtOH 40:60. Analysis of the fractions was carried out by HPLC under conditions used for monitor-

ing the reaction. The fractions with the reaction product were further purified by preparative HPLC.

Preparative HPLC: Purification by preparative HPLC was performed as follows. The crude products were loaded onto a preparative PrepPak (Waters) column (47×300 mm) filled with Delta-Pak C4, 300 Å, 15 μm stationary phase. Products were eluted using ACN gradients in 0.1% aqueous TFA: 20–36% in 30 min for **3a**, 15–30% in 30 min for **3b**, 19–34% in 30 min for **3c**, 23–38% in 30 min for **3d** and 16–32% in 30 min for **3e.** The flow rate was 80 mL min^{-1}, and the products were detected at $\lambda = 254$ nm. Analysis of the fractions was carried out by analytical HPLC under isocratic conditions: 41% solvent B for **3a**, 45% B for **3b**, 49% B for **3c**, 53% B for **3d**, and 59% B for **3e**. The pure fractions were pooled and lyophilized in the presence of aqueous HCl (37%, 2 equiv) to obtain the products in hydrochloride form.

Bis(N^{α}-phenylacetyl-L-arginine)-1,6-hexanediamide dihydrochloride (3a): The title compound was prepared by following the general methodology described above (a) (1.05 g, 13% isolated yield), 98% purity by HPLC: gradient elution 10–70% B in 30 min, retention factor (k') = 6.94; $[\alpha]_D^{20} = -25.3$ ($c = 1.0$ in methanol); IR (KBr): $\tilde{\nu}$ = 3258, 3165, 2930, 2853, 1650, 1537, 1445, 1347, 1250, 1168, 702 cm^{-1}; ^{1}H NMR (500 MHz, [D$_4$]CH$_3$OH, 22 °C): $\delta = 7.36$–7.17 (m, 10H), 4.31 (dd, J = 8.46, 5.58 Hz, 2H), 3.60 (m, 4H), 3.16 (m, 8H), 1.93–1.51 (m, 8H), 1.45 (s, 4H), 1.28 ppm (s, 4H); ^{13}C NMR (75 MHz, [D$_4$]CH$_3$OH, 22 °C): δ = 174.22, 173.85, 158.53, 136.83, 130.24, 129.63, 127.99, 54.71, 43.56, 41.92, 40.16, 30.33, 30.12, 27.28, 26.47 ppm; elemental analysis calcd (%) for $C_{34}H_{54}Cl_2N_{10}O_4 \cdot 3H_2O$ (791.8): C 51.57, H 7.64, N 17.69, found: C 51.56, H 7.56, N 17.56.

Bis(N^{α}-phenylacetyl-L-arginine)-1,8-octanediamide dihydrochloride (3b): The title compound was prepared following the general methodology described above (a) (1.37 g, 21% isolated yield), 99% purity by HPLC: gradient elution 10–70% B in 30 min, k' = 8.5; $[\alpha]_D^{20} = -23.0$ ($c = 1.0$ in methanol); IR (KBr): $\tilde{\nu}$ = 3268, 3155, 2930, 2853, 1650, 1537, 1455, 1358, 1250, 1163, 697 cm^{-1}; ^{1}H NMR (500 MHz, CD$_3$OD): $\delta = 7.34$–7.20 (m, 10H), 4.31 (m, 2H), 3.59 (m, 4H), 3.16 (s, 8H), 1.83 (s, 2H), 1.74–1.50 (m, 6H), 1.46 (s, 4H), 1.32–1.25 ppm (m, 8H); ^{13}C NMR (101 MHz, CD$_3$OD): δ = 174.17, 173.70, 158.56, 136.87, 130.21, 129.66, 128.03, 54.48, 43.63, 41.96, 40.42, 30.40, 30.32, 30.21, 27.78, 26.44 ppm); elemental analysis calcd (%) for $C_{36}H_{58}Cl_2N_{10}O_4 \cdot 2H_2O$ (801.9): C 53.92, H 7.79, N 17.47, found: C 54.14, H 7.83, N, 17.63.

Bis(N^{α}-phenylacetyl-L-arginine)-1,10-decanediamide dihydrochloride (3c): The title compound was prepared following the general methodology described above (a) (1.61 g, 27% isolated yield), 98% purity by HPLC: gradient elution 10–70% B in 30 min, k' = 10.0; $[\alpha]_D^{20} = -22.4$ ($c = 1.0$ in methanol); IR (KBr): $\tilde{\nu}$ = 3268, 3176, 2925, 2848, 1650, 1537, 1455, 1358, 1245, 1163, 692 cm^{-1}; ^{1}H NMR (300 MHz, CD$_3$OD): $\delta = 7.34$–7.19 (m, 10H), 4.31 (dd, J = 8.64, 5.55 Hz, 2H), 3.59 (m, 4H), 3.16 (m, 8H), 1.95–1.36 (m, 12H), 1.36–1.16 ppm (m, 12H); ^{13}C NMR (75 MHz, CD$_3$OD): δ = 174.17, 173.72, 158.54, 136.86, 130.21, 129.63, 127.99, 54.52, 43.63, 41.94, 40.46, 30.57, 30.36, 27.91, 26.42 ppm; elemental analysis calcd (%) for $C_{38}H_{62}Cl_2N_{10}O_4 \cdot 3/2H_2O$ (820.9): C 55.60, H 7.98, N 17.06, found: C 55.41, H 8.12, N 16.80.

Bis(N^{α}-phenylacetyl-L-arginine)-1,12-dodecanediamide dihydrochloride (3d): The title compound was prepared following the general methodology described above (a) (1.45 g, 26% isolated yield), 97% purity by HPLC: gradient elution 10–70% B in 30 min, k' = 11.2; $[\alpha]_D^{20} = -21.2$ ($c = 1.0$ in methanol); IR (KBr): $\tilde{\nu}$ = 3278, 3170, 2920, 2848, 1639, 1537, 1455, 1358, 1250, 1163, 692 cm^{-1}; ^{1}H NMR (300 MHz, CD$_3$OD): $\delta = 7.37$–7.18 (m, 10H), 4.39–4.24 (m, 2H), 3.59 (m, 4H), 3.16 (m, 8H), 1.94–1.37 (m, 12H), 1.36–1.20 ppm (m, 16H); ^{13}C NMR (75 MHz, CD$_3$OD): δ = 174.17, 173.81, 158.56, 136.87, 130.21, 129.64, 128.00, 54.53, 43.69, 43.65, 41.95, 40.61, 40.48, 30.72, 30.67, 30.42, 30.38, 27.96, 26.41 ppm; elemental analysis calcd (%) for $C_{40}H_{66}Cl_2N_{10}O_4 \cdot 2H_2O$ (858.0): C 56.00, H 8.22, N 16.33, found: C 56,31, H 8,39, N 16,38.

Bis(N^{α}-phenylacetyl-L-arginine)-1,14-tetradecanediamide dihydrochloride (3e): The title compound was prepared following the general methodology described above (b) (1.80 g, 26% isolated yield), 98% purity by HPLC: gradient elution 10–70% B in 40 min, k' = 12.4; $[\alpha]_D^{20} = -22.0$ ($c = 1.0$ in methanol); IR (KBr): $\tilde{\nu}$ = 3283, 3165, 2920, 2848, 1644, 1537, 1455, 1358, 1250, 1158, 718 cm^{-1}; ^{1}H NMR (500 MHz, CD$_3$OD): $\delta = 7.35$–7.20 (m, 10H), 4.31 (dd, J = 8.46, 5.70 Hz, 2H), 3.59 (m, 4H), 3.16 (m, 8H), 1.83 (m, 2H), 1.74–1.50 (m, 6H), 1.50–1.41 (m, 4H), 1.29 ppm (s, 20H); ^{13}C NMR (101 MHz, CD$_3$OD): δ = 174.20, 173.68, 158.55, 136.84, 130.19, 129.65, 128.02, 54.43, 43.65, 41.94, 40.49, 30.80, 30.77, 30.71, 30.45, 30.37, 27.97, 26.39 ppm; elemental analysis calcd (%) for $C_{42}H_{70}Cl_2N_{10}O_4 \cdot 5/2H_2O$ (895.0): C 56.36, H 8.45, N 15.65, found: C 56.52, H 8.43, N 15.63.

Minimum inhibitory concentration (MIC): The MIC values of **3a**–**e**, $C_3(CA)_2$, and CHX, were determined in vitro by using a broth microdilution assay.[31] Muller Hinton broth (Scharlau Chemie, Barcelona, Spain) was used for the antibacterial test, and Sabouraud liquid medium (ADSA Micro, Barcelona, Spain) was used for the antifungal test. Serial dilutions of compounds between 256 and 0.25 mg L^{-1} final concentration in the corresponding liquid medium were dispensed into 96-well polystyrene microtitre plates (Nunc, Roskilde, Denmark). The corresponding dilutions were inoculated with a suspension of the test organism on the corresponding liquid medium to a final concentration of $\approx 10^4$ CFU mL^{-1} (CFU = colony-forming unit). MIC is defined as the lowest concentration of antimicrobial agent that visibly inhibits the development of bacterial or fungal growth after 24 h at 37 °C and 48–72 h at 30 °C, respectively. Experiments were conducted in duplicate.

Microorganisms: The microorganisms used were: Gram-positive bacteria (eight): *Bacillus cereus var. mycoides* ATCC 11778, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC *6538*, *Micrococcus luteus* ATCC 9341, *Enterococcus hirae* ATCC 10541, *Mycobacterium phlei* ATCC 41423, and *Mycobacterium smegmatis* ATCC 3017. Gram-negative bacteria (seven): *Bordetella bronchiseptica* ATCC 4617, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella typhimurium* ATCC 14028, *Enterobacter aerogenes* ATCC 13048, *Escherichia coli* ATCC 8739, *Klebsiella pneumoniae* ATCC 4352, and *Serratia marcescens* ATCC 274. Fungi: Yeasts (two): *Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae* ATCC 9763; Moulds (six): *Aspergillus repens* ATCC 28604, *Aspergillus niger* ATCC 16404, *Penicillium chrysogenum* ATCC 9480, *Cladosporium cladosporoides* ATCC 16022, *Trychophyton mentagrophytes* ATCC 18748, and *Penicillium funiculosum* CECT 2914.

Haemolysis assays: Erythrocytes were washed three times in isotonic phosphate saline buffer (PBS, pH 7.4): Na_2HPO_4 (22.2 mM), KH_2PO_4 (5.6 mM), NaCl (123.3 mM), and glucose (10.0 mM). The cells (8×10^9 cell mL^{-1}) were then suspended in isotonic saline solution (NaCl 0.9%). Different volumes (10–80 μL) of stock solutions of compounds (20 mg mL^{-1}) were mixed with PBS in polystyrene tubes to a final volume of 1 mL; the final concentrations of products ranged from 200 to 1600 μg mL^{-1}. Aliquots of erythrocyte suspension (25 μL) were added to these solutions, and the mixtures were incubated for 10 min with constant shaking at room temperature. After incubation, the tubes were centrifuged at 1500 *g* over

5 min. The percent haemolysis was then determined by comparing the absorbance ($\lambda = 540$ nm) of the supernatant with that of control samples totally haemolysed with distilled water.[25] The dose–response curve was determined from the haemolysis results, and the concentration that induces the haemolysis of 50% of the cells (HC_{50}) was calculated.

The potential ocular irritation of the compounds was studied with a method based on the haemolysis test (cell lysis) and the damage caused to the cellular proteins by the compound (denaturation). The irritation index was determined according to the lysis/denaturation ratio (L/D) obtained by dividing the HC_{50} value (in $\mu g\,mL^{-1}$) by the denaturation index (DI). The DI of each compound was measured by comparing the haemoglobin denaturation (D) induced by the compound and by sodium dodecyl sulphate (SDS) as positive control. Haemoglobin denaturation was determined after inducing haemolysis by adding SDS ($10\,mg\,mL^{-1}$) to the erythrocytes and measuring the absorption ratio of the supernatant at $\lambda =$ 575 nm and $\lambda = 540$ nm. The resulting L/D ratio was used instead of the ocular irritancy score in the acute phase of in vivo evaluation. The compounds can be classified according to this L/D ratio as: nonirritant: >100, slight irritant: >10, moderate irritant: >1, irritant: >0.1, and very irritant: <0.1.[25]

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