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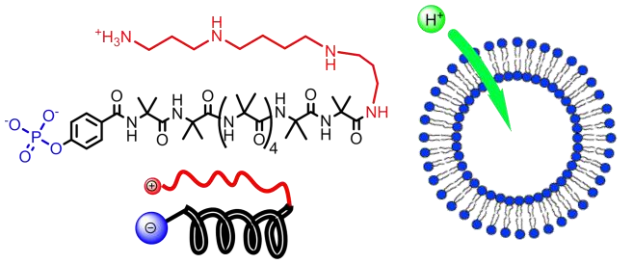

New ionophores based on a hydrophobic helical Aib-peptide shows high activity on model membranes and an attracting antimicrobial activity against a broad spectrum of microorganisms.

Bioorganic & Medicinal Chemistry
journal homepage: www.elsevier.com

Helical peptide–polyamine and –polyether conjugates as synthetic ionophores

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ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
Synthetic ionophores
Helical peptide
Liposome
Membrane permeability
Antimicrobial activity

ABSTRACT

Two new synthetic ionophores in which the hydrophobic portion is represented by a short helical Aib-peptide (Aib = α-amino-isobutyric acid) and the hydrophilic one is a poly-amino (**1a**) or a polyether (**1b**) chain have been prepared. The two conjugates show a high ionophoric activity in phospholipid membranes being able to efficiently dissipate a pH gradient and, in the case of **1b**, to transport Na^+ across the membrane. Bioactivity evaluation of the two conjugates shows that **1a** has a moderate antimicrobial activity against a broad spectrum of microorganisms and it is able to permeabilize the inner and the outer membrane of *E. coli* cells.

1. Introduction

Common feature found in several natural ionophores is the presence of a lipophilic, usually rigid, backbone connected to a hydrophilic subunit. Two cases in point are constituted by amphotericin B (AmB) and squalamine (Figure 1). The first one[1] is a macrocyclic compound in which the hydrophobic portion is a conjugate polyene and the hydrophilic one is a polyol. Squalamine, on the contrary, is a sterol-spermidine conjugate that has been isolated from tissues of the dogfish shark, *Squalus acanthias*, in 1993 (Figure 1).[2] Both compounds show remarkable biological activity, in particular as antifungal (AmB)[3] and antibacterial (squalamine)[4] agents, which is thought to be related to their ability to perturb the cellular membrane permeability.[5,6,7] While the macrocyclic nature of AmB ensures a stable facially amphipathic structure that is responsible of its activity, the active conformation of squalamine is still matter of controversy. Following the analysis proposed by Regen,[8,9] its key functional elements are *i)* the long and rigid hydrophobic steroid nucleus; *ii)* the flexible hydrophilic spermidine chain terminating with a positively charged amino group; *iii)* the anionic polar head group. The arrangement of these three units favors the formation of a pseudomacrocyclic structure, like the one shown in Figure 1, possibly stabilized by a salt bridge between the terminal primary ammonium group and the sulphate moiety, which is located at the opposite end of the molecule. In this conformation the pseudomacrocycle is characterized by a facially amphipathic structure analogous to that of AmB and it may insert in the membrane altering its permeability. Mimics of such molecules (particularly squalamine) have been reported[10] with interesting ionophoric activity and in some cases powerful antibiotic activity similar to that of the natural compound.[11] In all cases, however, the hydrophobic element so far exploited has been a steroid backbone.

Following our interest in synthetic ionophores[12] and in particular in the use of peptide foldamers as structural/functional design elements,[13] we reasoned that the rigid hydrophobic backbone may be substituted by a lipophilic peptide in its helical conformation with advantages in term of synthetic accessibility, control of the size and insertion of functional group along the peptide backbone. We focused on an acyclic compound (as squalamine) and designed compounds **1a,b** (Figure 1). In these molecules the hydrophobic portion is constituted by a homo-octapeptide made by the α,α-disubstituted amino acid Aib (α-amino-isobutyric acid). The peptide was then modified on the *N*-terminus with a phosphate moiety linked through a phenyl spacer as an anionic head group and on the *C*-terminus with a spermine chain (**1a**) or with a short polyether chain ending with an ammine group (**1b**). The 8-mer Aib sequence was chosen because it is well known to assume a stable 3_{10}-helical conformation[14] in apolar solvents,[15] that is maintained in membrane–like environments.[16] Furthermore, in view of the ionophoric ability of peptaibols,[17] short helical peptides rich in Aib amino acids, this structural feature could add an extra activity to the system. Due to the gem-α-dimethyl substituents, the helix is hydrophobic thus representing, in our design, a pseudo-rigid linear hydrophobic element that may well insert in the phospholipid membrane. In this conformation the length of the hydrophobic portion of compounds **1**, comprising the peptide and the N-terminal phenyl

substituent, is about 19 Å, which is sufficient to span the hydrocarbon region of a phospholipid monolayer (i.e. one leaflet of a membrane). Therefore, we hypothesized that **1** could bind to the membrane by anchoring the phosphate head group at the surface and inserting the hydrophobic peptide parallel to the fatty acid hydrocarbon chains. The hydrophilic spermine or polyether chain, which is attached to the rigid peptide at the opposite side of the phosphate head group, might then extend along the peptide toward the membrane surface forming an amphipathic pseudo-macrocyclic structure or might extend in the opposite direction reaching the membrane surface on the other side. This is possible since **1a,b** are acyclic molecules, at variance with AmB and in analogy to squalamine. At any rate, in both cases the membrane should be destabilized resulting in increased ion permeability. In this work we report on the synthesis and characterization of the new squalamine mimics, on the investigation of their ion transport properties across phospholipid membranes as well as *in vitro* data on their antimicrobic activity.

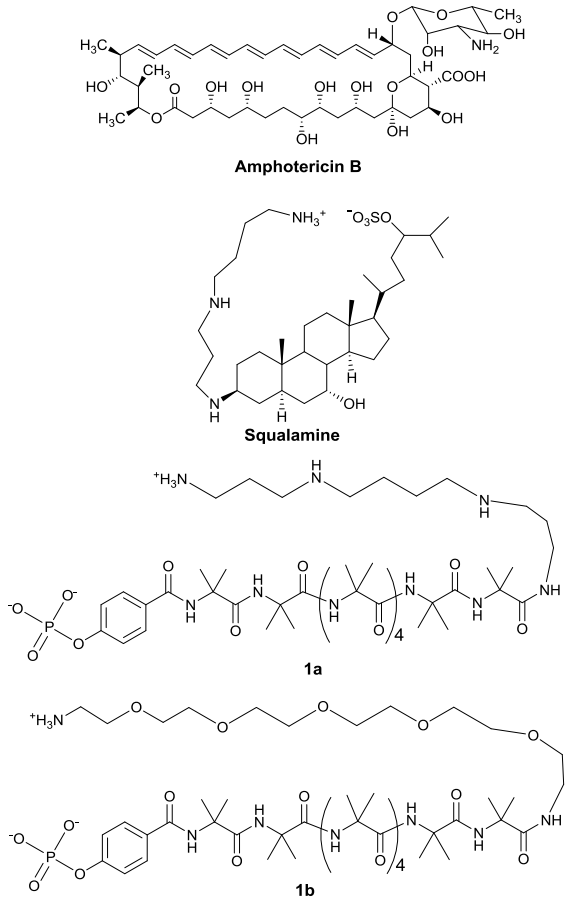
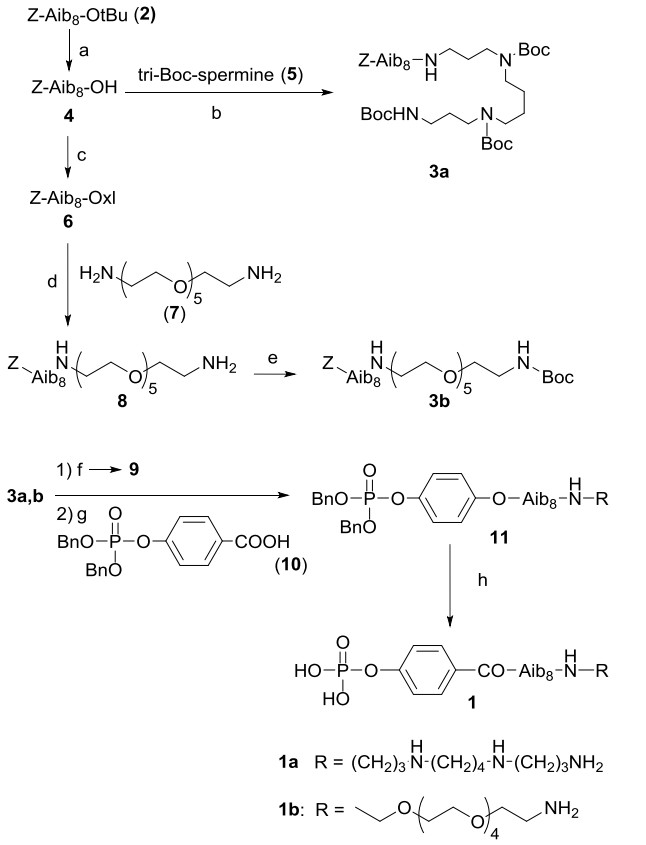


Figure 1. Chemical structures of amphotericin B, squalamine, and of the synthetic ionophores (**1a** and **1b**) investigated in this work.

2. Result and Discussion

2.1 Chemistry

Conjugates **1** were prepared starting from the Z-$(Aib)_8$-OtBu peptide (Scheme 1, tBu = *tert*-butyl, Z = benzyloxycarbonyl) that was synthesized by conventional solution peptide chemistry following the procedure reported by Toniolo et al.[18] Deprotection of the *C*-terminus with trifluoroacetic acid (TFA) and coupling with tri-Boc-spermine in presence of (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (Py-BOP) at room temperature afforded the intermediate **3a**. This was deprotected at the *N*-terminus by catalytic hydrogenation and coupled with 4-[[bis(phenylmethoxy)phosphinyl]oxy]benzoic acid (**10**) using *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) and 1-hydroxybenzotriazole (HOBt) as condensing agents. Full removal of the Boc from the amino groups and of the benzyl esters from the phosphate moiety with TFA afforded **1a** as a trifluoroacetate salt.



Scheme 1: a) TFA, CH_2Cl_2, r.t., 4 h, quant.; b) Py-BOP, *N*,*N*-diisopropylethylamine (DIEA), CH_2Cl_2, r.t., 24 h, 58%; c) $(CH_3CO)_2O$, reflux, 3 h, 97%; d) Triethylamine (TEA), CH_3CN, r.t, 24 h, 77%; e) $(Boc)_2O$, CH_2Cl_2, r.t., 3 h, 45%; f) H_2, Pd/C, CH_3OH, r.t., 24h, quant.; g) EDC, HOBt, TEA, CH_2Cl_2, r.t., 24 h; h) TFA, CH_2Cl_2, r.t., 24h, quant.

Intermediate **3b** was obtained by a slightly different procedure in which the Z-$(Aib)_8$-OH was first transformed into the corresponding 5(4H)-oxazolone by reaction with acetic anhydride. Reaction of the oxazolone with pentaoxaheptadecane-1,17-diamine followed by Boc-protection of the free terminal primary amine afforded intermediate **3b**. This was then subjected to the same steps as **3a** giving **1b** as a trifluoroacetate salt, after final treatment with TFA. Compounds **1a,b** have been fully characterized by ^1H and ^{13}C NMR and MS analysis

2.2 Ion transport across lipid membranes

The ability of conjugates **1** to permeabilize a phospholipid model membrane was evaluated with two different assays using large unilamellar vesicles (100 nm diameter) with a 95:5 egg phosphatidylcholine (PC) and egg phosphatidylglycerol (PG) lipid composition. In the first assay the transport of Na^+ from outside to inside the membrane was evaluated using $^{23}Na^+$ NMR and a shift reagent ($DyCl_3$–tripolyphosphate) to differentiate the population of Na^+ inside the liposome water pool from bulk Na^+. In the experiment, a gradient of Na^+ is built up by addition of NaCl after vesicle formation (NaCl 100 mM outside, 0 mM inside). Since the shift reagent is confined in the external bulk aqueous phase, the Na^+ entering the vesicular compartment appears as a separate (unshifted) resonance and integration of internal Na^+ signal, as a function of time, yields the kinetics

profiles shown in Figure 2a. Conjugate **1b** is highly effective in promoting the transport of Na^+ across the phospholipid membrane and its activity is even higher than that recorded for AmB taken as a positive reference compound. On the contrary, **1a** is almost inactive and after more than 13 h the amount of Na^+ entering the internal vesicular compartment is only slightly higher than in the absence of any additive. The inability of spermine functionalized ionophores to promote cation transport has been previously observed,[19] and it was attributed to the inability of the positively charged polyamine chain to stabilize the cation during the crossing of the membrane. Fitting of the kinetics traces recorded for different concentrations of **1b** afforded the first-order rate constants (k_{Na}^+, min^{-1}) for the transport process that are reported in Figure 2b. The profile shows a linear increase of activity with the concentration of **1b**, indicating that the species responsible for the transport process is monomeric, at least in the concentration range explored.

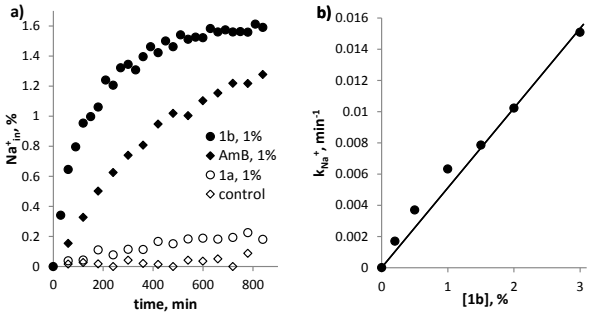


Figure 2. a) Kinetics profiles for the entry of Na^+ into 95:5 egg PC/PG vesicles containing **1a**, **1b**, AmB (1 %) and without additives (control) at 25 °C. b) Dependence of the observed rate constant for the transport process (k_{Na}^+, min^{-1}) vs. concentration of **1b**, (see Supplementary Material for the original kinetics traces). The concentration of ionophores is given in percent with respect to the total concentration of lipids (10 mM).

In a second assay we evaluated the ability of the ionophores to dissipate a pH gradient across the membrane exploiting the response of the intravesicular pH-sensitive fluorophore 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS).[20] In this experiment a 0.6 units pH gradient is established, by external addition of NaOH, across the membrane of vesicles prepared in HEPES buffer at pH 7.0 containing 100 mM NaCl (HEPES = 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid). The increase of the HPTS fluorescence emission in response to the applied transmembrane pH-gradient indicates basification of the inner water pool that may derive either from H^+ efflux or OH^- influx.

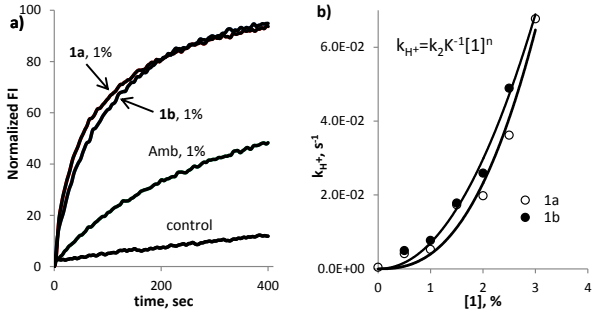


Figure 3. a) Normalized fluorescence change in HPTS emission as a function of time after base pulse to 95:5 PC/PG vesicles containing **1a**, **1b**, AmB (1 %) and without additives (control) at 25 °C. b) Dependence of the observed rate constant for the transport process (k_H^+, s^{-1}) vs concentration of compounds **1** (see Supplementary Material for the original kinetics traces). The curves correspond to the fitting of the data with the Regen's equation reported in the graph. The concentration of ionophores is given in percent with respect to the total concentration of lipids (0.17 mM).

Figure 3a shows the kinetics profiles obtained in the presence of conjugates **1** and AmB in the HPTS assay. The two conjugates are considerably effective in promoting the pH gradient collapse showing higher activity with respect to AmB. In this assay **1a** and **1b** show a very similar activity as well as a similar dependence of the transport rate from the concentration of ionophore (Figure 3b). However, in this case the profiles show an upward curvature and interpolation of the experimental points with the equation proposed by Regen[9] gives n = 2.5 and 2.1 for **1a** and **1b**, respectively, suggesting that the species responsible for the ionophoric effect is an aggregate formed by approximately two peptide monomers.

Taken together, these data show that the two peptide conjugates have a high ionophoric activity in model membranes. As reported above, it is reasonable to assume that compounds **1** insert in the membrane with the phosphate head group anchored to the surface while the hydrophobic peptide aligns with the fatty acid hydrocarbon chains. Here the hydrophilic chain perturbs the membrane creating defects that allow the transit of the ions. The different behaviour observed in the Na^+ transport and in the pH discharge experiment is probably related to the different requirements of the two transport processes. Indeed, while Na^+ has to be stabilized by specific interactions with the ionophore during the crossing of the membrane, the pH discharge may occur by a hydrogen-bonded chain mechanism in which the proton hops from one molecule of water to the next along a water molecules transmembrane chain (Grotthuss' mechanism).[21] In this case, a direct interaction with the ion is not required and the ionophore accelerates the transport process by stabilizing the transmembrane water row. It is tempting to suggest that the polyether chain shuttles Na^+ from the surface of the external leaflet of the liposomal membrane to the internal water pool. This would explain the requirement of monomeric **1b** molecule, while in the case of the pH discharge the stabilization of the water channel requires the alignment of two ionophore molecules to fully span the membrane. Moreover, the inability to transport Na^+ associated with potent pH discharge activity suggests that **1a** forms anion selective pores in which the transport of H^+ (or OH^-) is counterbalanced by the transport of chloride in analogy with the anion selective transport mechanism described for squalamine mimics bearing polyamine chains.[19a,22] In any case, control experiments with the relatively large calcein dye indicate that, whatever the mechanism, the membrane defects formed by conjugates **1** are not large enough to allow the transport of calcein and that the ionophores are not able to cause lysis of the liposomes (see Supplementary Material).

2.3 Biological activity

The antibiotic activity of **1a** and **1b** was screened against representative bacteria and fungi and the results are shown in Table 1, along with data that have been previously reported for squalamine.[2] It is significant that conjugate **1a** shows an interesting antimicrobial activity against a broad spectrum of microorganism, mimicking that reported for squalamine, although at a lower level of potency. The inactivity of compound **1b** confirms the observation of Regen[8] that the polyamine chain plays an important role for the biological activity of these compounds. This suggests that a strict correlation between ionophoric activity in model membranes and antimicrobial properties is not possible, probably due to a more favorable interaction of the more positively charged **1a** with the cell membrane.

Table 1. *In vitro* antimicrobial activity of peptide conjugates towards fungal or bacterial strains

Conjugate	Minimum inhibitory concentration (MIC) in μM					
	C. neoformans[a]	*C. albicans*[a]	*S. aureus 16*[a,b]	*S. aureus 34*[a]	*P. aeruginosa ATCC 27853*	*E. coli ATCC 25922*
squalamine	-	4-8	-	1-2	4-8	1-2
1a	16	32	32	16	64	32
1b	> 128	> 128	> 128	> 128	> 128	> 128

[a] Clinical isolate. [b] Methicillin-resistant (MRSA) strain.

To study the interaction of the conjugates with the bacterial membranes, we performed a permeabilization assay with the *E. coli* ML-35 pYC strain, which constitutively expresses both a cytoplasmic β-galactosidase and a periplasmic β-lactamase. A damage to the outer membrane allows CENTA, a chromogenic substrate for the lactamase, to enter the periplasmic space, where its hydrolysis determines an increased absorption at 405 nm. On the other hand, a damage to the cytoplasmic membrane allows 2-nitrophenyl β-D-galactopyranoside (Gal-ONp), a chromogenic substrate for the galactosidase, to reach this enzyme, also increasing absorption at 405 nm. The results of these tests are reported in Figure 4 and clearly show that **1a** efficiently permeabilizes both the inner and the outer membrane of *E. coli* cells. Interestingly, **1b**, even though tested at a higher concentration, is poorly active suggesting that the antimicrobial activity of **1a** is correlated to its ability to permeabilize the bacterial membranes.

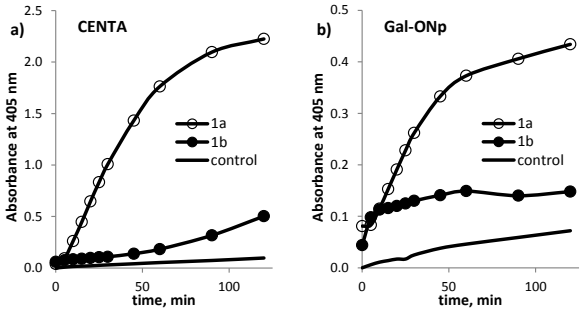


Figure 4. Permeabilization of the outer (left panel) or inner (right panel) membranes of *E. coli* cells determined by following the hydrolysis of the impermeable chromogenic substrates CENTA or Gal-ONp in the presence of **1a** (64 μM), **1b** (256 μM) and without additives (control).

3. Conclusions

In conclusion, we have reported a new approach to the design of synthetic ionophoric molecules using a helical peptide as a rigid lipophilic moiety. It was based on an Aib-peptide that assumes in membranes a stable 3_{10}-helical conformation allowing a favorable interaction of the ionophore with the phospholipid bilayer. The two conjugates show a high ionophoric activity being able to efficiently dissipate a pH gradient and, in the case of **1b**, to transport Na^+ across the membrane. Moreover, **1a** shows a moderate antimicrobial activity against a broad spectrum of microorganisms. Although a direct correlation between ionophoric activity in model membranes and biological activity is complex to demonstrate,[23] the ability of **1a** to permeabilize the bacterial membranes suggests that ion transport might play a significant role in the mechanism of action of this compound in cells. The use of a peptide moiety in the design of acyclic ionophores introduces interesting mutation possibilities such as, for example, an easy control of its length or the insertion along the sequence of functional residues. Studies are in progress to evaluate these possibilities and to better define the mechanism of action of this new class of biological active synthetic ionophores.

4. Experimental section

4.1 General methods

All commercially available reagents were purchased from *Aldrich*, *Fluka* and *Strem Chemicals* and used without purification unless otherwise mentioned. Solvents were purchased from *Aldrich*, *VWR*, *Fluka* and *Riedel*, and deuterated solvents from *Cambridge Isotope Laboratories* and *Aldrich*. Reactions were routinely monitored by thin-layer chromatography (TLC) on silica gel (precoated F_{254} *Merck* plates). Chromatography was performed on *Merck* silica gel 60F-254 (230÷400 Mesh) and the solvents employed were of analytical grade. NMR spectra were recorded on a Jeol 400 spectrometer (operating at 400 MHz for proton and at 100 MHz for carbon) or on Jeol X-270 (operating at 270 MHz for proton and at 67.8 MHz for carbon). Chemical shifts (δ) are reported in ppm using the solvent residual signal as an internal reference. Coupling constants (*J*) are quoted in Hz. Electrospray mass spectra were recorded at 5600 eV with a *Perkin Elmer* API I spectrometer or on a *Perspective Biosystems* Mariner ESI-TOF spectrometer, after dissolving the compounds in CH_3OH. Infrared spectra (IR) were measured on a Jasco FT/IR-200 instrument. Melting points (m.p.) were measured with a Büchi SHP-20 apparatus and are not corrected. Z-Aib$_8$-OtBu (**2**),[16,18] (N^1,N^4,N^9-tri-tert-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane[24] (**5**, triboc-spermine), 3,6,9,12,15-pentaoxaheptadecane-1,17-diamine[25] (**7**) 4-[[bis(phenylmethoxy)phosphinyl]oxy]-benzoic acid[26] (**10**) were synthesized as reported.

4.2 Synthesis of compounds 1a and 1b.

***N^1*-Z-Aib$_8$-(N^4,N^9,N^{13}-tri-*tert*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane (3a).** 500 mg of Z-Aib$_8$-OtBu (MW: 889.09; 0.56 mmol) were dissolved in 10 mL of a 1:1 CH_2Cl_2/TFA mixture and stirred at room temperature for 4 h monitoring the reaction by TLC ($CHCl_3$/CH_3OH, 9/1 v/v). The solvent was removed and the residue co-evaporated several times with diethyl ether (Et_2O), until all the TFA traces have been removed, affording Z-(Aib)$_8$-OH (**4**) in quantitative yield (466 mg, MW: 832.98; 0.56 mmol), R_f: 0.4 ($CHCl_3$/CH_3OH; 9/1). The acid thus obtained was suspended in 15 mL of anhydrous CH_2Cl_2 and 0.291 g of Py-BOP (MW: 520.39; 0.56 mmol), 0.309 g of triboc-spermine (**5**, MW: 502.69; 0.616 mmol), and 0.18 mL of DIEA (MW: 129.24; 1.04 mmol) were added. The reaction mixture was stirred at r.t. for 24 hours monitoring the reaction by TLC ($CHCl_3$/CH_3OH; 95/5). The solvent was then evaporated and the residue dissolved in ethyl acetate (EtOAc) (200 mL) and washed with $KHSO_4$ 5% (2 x 100 mL), H_2O (2 x 100 mL) and $NaHCO_3$ 5% (2 x 100 mL). The organic phase was dried over anhydrous Na_2SO_4, the solvent removed and the crude product was purified by column chromatography (silica, toluene/EtOH from 95/5 to 91/9) affording 0.422 g of N^1-Z-Aib$_8$-(N^4,N^9,N^{13}-tri-

tert-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane (**3a**) (MW: 1317.66; 0.32 mmol) as a white solid. Yield = 58 %. **R_f**: 0.6 ($CHCl_3/CH_3OH$, 9/1); 0.3 (toluene/EtOH, 7/1). **^{1}H-NMR** ($CDCl_3$, 400 MHz): δ 7.58 (m, 5H, NH); 7.41 (m, 3H, NH); 7.28 (m, 5H, Ph); 6.55 (s, 1H, NH); 5.95 (s, 1H, NH); 5.02 (s, 2H, Ph*CH_2*O); 3.07 (m, 12H, CH_2 (1, 3, 5, 8, 10, 12)); 1.70 (m, 2H, CH_2 (2)); 1.40 (m, 81H: 48H CH_3 (Aib), 27H tBu, 6H CH_2 (6, 7, 11)). **ESI-MS** (CH_3OH): m/z 1318.7 ($M+H^+$), 1340 ($M+Na^+$), 1355.9 ($M+K^+$), 678.5 ($M+H+K^+$)/2

***N^1*-Z-Aib$_8$-1,17-diamine-3,6,9,12,15-pentaoxaheptadecane (8).** Z-$(Aib)_8$-OH (**4,** 0.466 g, MW: 832.98; 0.56 mmol) was dissolved in 4 mL of freshly distilled acetic anhydride and refluxed for 3 h monitoring the reaction by TLC ($CHCl_3/CH_3OH$, 9/1 v/v). The solvent was removed and the residue co-evaporated several times with toluene, until all the Ac_2O traces have been removed, affording of Z-$(Aib)_8$-Oxl (**6**) with 97 % yield (0.442 g, MW: 814.97; 0.54 mmol), R_f: 0.7 ($CHCl_3/CH_3OH$; 9/1). The obtained oxazolone (0.256 g, MW: 814.97; 0.31 mmol) was then dissolved in 15 mL of dry CH_3CN and a solution of 3,6,9,12,15-pentaoxaheptadecane-1,17-diamine x 2 HCl (**7**, 0.220 g, MW: 353.28; 0.62 mmol) and TEA (0.172 mL, MW: 101.19; 1.24 mmol) in 15 mL of dry CH_3CN was added. The reaction mixture was kept under stirring at r.t. for 12 h monitoring the reaction by TLC ($CHCl_3/CH_3OH$; 9/1). The solvent was then removed and the crude product was purified by column chromatography (silica, $CHCl_3/CH_3OH$ from 100/0 to 85/15) affording 0.263 g of *N^1*-Z-Aib$_8$-1,17-diamine-3,6,9,12,15-pentaoxaheptadecane (**8**) (MW: 1095.33; 0.24 mmol). Yield = 77 %. **R_f**: 0.2 ($CHCl_3/CH_3OH$; 9/1). **^{1}H-NMR** ($CDCl_3$, 400 MHz): δ 8.15 (s, 1H, NH); 7.76 (s, 1H, NH); 7.71 (s, 2H, 2NH); 7.59 (brt, 1H, NH); 7.50 (s, 1H, NH); 7.46 (s, 1H, NH); 7.44 (s, 1H, NH); 7.37 (s, 1H, NH); 7.27 (m, 5H, Ph); 5.10 (s, 2H, Ph*CH_2*O); 3.64 (m, 20H, CH_2(2,4,5,7,8,10,11,13,14,16)); 3.51 (m, 2H, CH_2(1)); 3.11 (m, 2H, CH_2(17)); 1.50-1.25 (m, 48H, CH_3(Aib)). **ESI-MS** (CH_3OH): m/z 1096.4 ($M+H^+$).

***N^1*-Z-Aib$_8$-(*N^{17}*-*tert*-butoxycarbonyl)-1,17-diamine-3,6,9,12,15-pentaoxaheptadecane (3b).** The amine **8** (206 mg, MW: 1095.33; 0.19 mmol) was dissolved in 10 mL of dry CH_2Cl_2 and 0.264 mL of TEA (MW: 101.19; 0.19 mmol) and 41 mg of $(Boc)_2O$ (MW: 218.25; 0.19 mmol) were added. The reaction mixture was stirred at r.t. for 3 hours monitoring the reaction by TLC ($CHCl_3/CH_3OH$; 9/1). The solvent was then evaporated and the residue dissolved in EtOAc (50 mL) and washed with $KHSO_4$ 5% (2 x 30 mL), H_2O (1 x 30 mL) and $NaHCO_3$ 5% (2 x 30 mL). The organic phase was dried over anhydrous Na_2SO_4, the solvent removed and the crude product was purified by column chromatography (silica, $CHCl_3/CH_3OH$ from 100/0 to 90/10) affording 103 mg of *N^1*-Z-Aib$_8$-(*N^{17}*-*tert*-butoxycarbonyl)-1,17-diamine-3,6,9,12,15-pentaoxaheptadecane (**3b**) (MW: 1195.44; 0.087 mmol). Yield = 45 %. **R_f**: 0.5 ($CHCl_3/CH_3OH$, 9/1). **^{1}H-NMR** ($CDCl_3$, 400 MHz): δ 7.71 (m, 4H, 4NH); 7.53 (br, 1H, NH); 7.49 (s, 1H, NH); 7.43 (s, 1H, NH); 7.32 (m, 6H: 5H Ph, NH); 7.21 (s, 1H, NH); 6.92 (s, 1H, NH); 5.08 (s, 2H, Ph*CH_2*O); 3.56 (m, 20H, CH_2 (2,4,5,7,8,10,11,13,14,16)); 3.41 (m, 2H, CH_2(1)); 3,28 (m, 2H, CH_2(17)); 1.51-1.23 (m, 57H: 9H, tBu; 48H, CH_3 (Aib)). **ESI-MS** (CH_3OH): m/z 1196 ($M+H^+$), 1217.9 ($M+Na^+$), 1233.8 ($M+K^+$).

General procedure for the preparation of the peptide conjugates 1. To a solution of **3** (0.087 mmol) in 10 ml of CH_3OH, 10 mg of 5% Pd/C were added. The flask was evacuated and flushed with hydrogen three times. The reaction mixture was stirred vigorously, under hydrogen atmosphere, overnight. Then, it was filtered through a short pad of Celite® and the solvent removed to afford the Z-deprotected **9** with quantitative yield. **9a**: (MW: 1183.52) R_f: 0.4 ($CHCl_3/CH_3OH$; 9/1). **9b**: (MW: 1061.31) R_f: 0.3 ($CHCl_3/CH_3OH$; 9/1).

14 mg of HOBt (MW: 135.12; 0.104 mmol) and 17 mg of EDC (MW: 191.70; 0.087 mmol) were added to a solution of 35 mg of **10** (MW: 398.35; 0.087 mmol) in 10 mL of dry CH_2Cl_2 cooled at 0°C and kept under stirring. After ten minutes, the above obtained *N*-terminus deprotected peptide **9** (0.087 mmol) and 0.14 mL of TEA dissolved in 5 mL of anhydrous CH_2Cl_2 were added to the reaction mixture. The reaction mixture was stirred at r.t. for 24 h monitoring the reaction by TLC ($CHCl_3/CH_3OH$; 95/5). The solvent was then evaporated and the residue dissolved in EtOAc (50 mL) and washed with $KHSO_4$ 5% (2 x 30 mL), H_2O (1 x 30 mL) and $NaHCO_3$ 5% (2 x 30 mL). The organic phase was dried over anhydrous Na_2SO_4, the solvent removed and the crude product was purified by column chromatography (silica, $CHCl_3/CH_3OH$ from 100/0 to 90/10) affording the protected peptide conjugates **11**.

***N^1*-[4'-[[Bis(phenylmethoxy)phosphinyl]oxy]-benzoyl-Aib$_8$-(*N^4*,*N^9*,*N^{13}*-tri-*tert*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane (11a).** 68 mg, yield 50 % (MW: 1563.85; 0.044 mmol). R_f: 0.4 ($CHCl_3/CH_3OH$; 95/5). **^{1}H-NMR** ($CDCl_3$; 400 MHz): δ 7.80 (m, 3H: Ar (2',6'), NH); 7.69 (m, 4H, NH); 7.47 (brt, 1H, NH); 7.35 (m, 11H: 10H Bn, 1H, NH); 7.17 (m, 3H: Ar (3',5'), NH); 7.03 (s, 1H, NH); 6.52 (s, 1H, NH); 5,13 (d, 4H, J = 9 Hz, Ph*CH_2*O); 3.14 (m, 12H, CH_2 (1, 3, 5, 8, 10, 12)); 1.79 (m, 2H, CH_2 (2)), 1.48 (m, 81H: 48H CH_3 (Aib), 27H tBu, 6H CH_2 (6, 7, 11)). **ESI-MS** (CH_3OH): m/z 1564.8 ($M+H^+$), 1586.7 ($M+Na^+$), 1602.6 ($M+K^+$), 793.8 ($M+H^+Na^+$)/2, 801.9 ($M+H+K^+$)/2.

***N^1*-[4'-[[Bis(phenylmethoxy)phosphinyl]oxy]-benzoyl-Aib$_8$-(*N^{17}*-*tert*-butoxycarbonyl)-1,17-diamine-3,6,9,12,15-pentaoxaheptadecane (11b).** 41 mg, yield 36 % (MW: 1441.64; 0.028 mmol). R_f: 0.6 ($CHCl_3/CH_3OH$; 9/1). **^{1}H-NMR** ($CDCl_3$; 400 MHz): δ 8.14 (m, 1H, NH); 7.88 (d, 2H, J = 8 Hz, Ar (2',6'); 7.81 (br, 2H, NH); 7.67 (m, 3H, NH); 7.43 (m, 2H, NH); 7.24 (m, 11H: 10H Bn, 1H NH); 7.14 (d, 2H, J = 8 Hz, Ar (3',5'); 7.10 (br, 1H, NH); 5.04 (d, 4H, J = 8 Hz, Ph*CH_2*O); 3.55 (m, 20H, CH_2 (2,4,5,7,8,10,11,13,14,16)); 3.38 (m, 4H, CH_2 (1,17)); 1.44 (m, 57H: 48H, CH_3 (Aib), 9H, tBu). **ESI-MS** (CH_3OH): m/z 1441.7 ($M+H^+$), 1463.9 ($M+Na^+$), 1479.5 ($M+K^+$), 740.9 ($M+H+K^+$)/2.

Full removal of the Boc-protecting groups from the amino groups and of the benzyl esters from the phosphate moiety of the above obtained peptide conjugates **11** was achieved by dissolving the compounds in 3 mL of a 1:1 CH_2Cl_2/TFA mixture which was stirred at room temperature for 12 h, monitoring the reaction by TLC ($CHCl_3/CH_3OH$, 9/1 v/v). The solvent was removed and the residue co-evaporated several times with Et_2O, until all the TFA traces have been removed, affording peptide conjugates **1** in quantitative yield as trifluoroacetate salts.

***N^1*-[4'-[Phosphinyloxy]-benzoyl-Aib$_8$-1,12-diamino-4,9-diazadodecane (1a) x 3 TFA.** 62 mg, (MW: 1425.33; 0.044 mmol). M.p. = 242 °C. R_f: 0 ($CHCl_3/CH_3OH$; 9/1). **^{1}H-NMR** (CD_3OD, 400 MHz) : δ 8.04 (s, 1H, NH); 7.92 (m, 7H, 2H Ar (2',6'), 5H NH); 7.80 (brt, 1H, NH); 7.70 (bs, 1H, NH); 7.30 (d, 3H, J = 8 Hz, 2H Ar (3',5'), 1H, NH); 3.08 (m, 12H, CH_2 (1, 3, 5, 8, 10, 12)); 2.07 (m, 2H, CH_2 (2)); 1.93 (m, 2H, CH_2 (11)); 1.81 (m, 4H, CH_2 (6, 7)); 1.48 (m, 48 H, CH_3 (Aib)). **^{13}C-NMR** (CD_3OD): 178.9, 178.3, 178.2, 177.4, 177.3, 177.2, 177.0, 169.2, 157.6, 130.3, 130.0, 120.0, 58.2, 58.1, 58.0, 57.9, 57.8, 57.7, 48.3, 48.2, 46.3, 45.9, 37.8, 37.2, 27.3, 25.5, 25.4, 24.3. **HR-ESI-MS** m/z 1083.6621 [$M+H^+$] (calcd for $C_{49}H_{88}N_{12}O_{13}P$ 1083.6331).

***N*^{1}-[4'-[Phosphinyl]oxy]-benzoyl-Aib$_8$-1,17-diamine-3,6,9,12,15-pentaoxaheptadecane (1b) x TFA.** 36 mg, (MW: 1275.31; 0.028 mmol). M.p. = 230 °C. R_f: 0 ($CHCl_3$/CH_3OH; 9/1). **^{1}H-NMR** (CD_3OD, 400 MHz): δ 7.96 (s, 1H, NH); 7.89 (m, 2H, 2 NH); 7.79 (m, 5H, 2H Ar (2',6'), 3H NH); 7.60 (brt, 1H, NH); 7.56 (brs, 1H, NH); 7.18 (m, 2H, Ar (3',5')); 6.80 (s, 1H, NH); 3.64 (t, 2H, J = 5 Hz, CH_2 (2)); 3.53 (m, 18H, CH_2 (4,5,7,8,10,11,13,14,16)); 3.25 (t, 2H, J 5 Hz, CH_2(1)); 3.03 (brt, 2H, CH_2 (17)); 1.34 (m, 48H, -CH_3 (Aib)). **^{13}C-NMR** (CD_3OD/$CDCl_3$ 9:1): 178.0, 177.9, 177.7, 177.6, 177.5, 177.2, 177.0, 176.9, 169.2, 157.6, 130.0, 129.8, 120.9, 71.4, 71.3, 71.2, 71.1, 71.0, 70.8, 70.7, 70.6, 68.0, 58.3, 58.2, 57.9, 57.8, 57.7, 57.6, 57.5, 40.6, 40.5, 26.2, 25.9, 25.5, 25.3, 25.2, 25.1. **HR-ESI-MS** m/z 1161.5982 [M+H$^+$] (calcd for $C_{51}H_{90}N_{10}O_{18}P$ 1161.6172).

4.3 Ionophoric Activity

General Procedures. Egg yolk L-α-phosphatidyl-DL-glycerol sodium salt (EYPG, 20 mg/mL chloroform solution) was purchased from Avanti Polar Lipids; egg yolk phosphatidylcholine (EYPC, 100 mg/mL chloroform solution), calcein and 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) were from Sigma; Triton® X-100 and HEPES buffer were from Fluka; all salts were of the best grade available from Aldrich and were used without further purification. Size exclusion chromatography (SEC) was performed using Sephadex™ G-75 or pre-packed columns Sephadex™ G-25 M (PD-10) from Amersham Biosciences. Liposome were prepared by extrusion using a 10 mL Lipex™ Thermobarrel EXTRUDER (Northern Lipids Inc.) connected to a thermostatic bath (25°C). The 100 μm policarbonate membranes are Nucleopore Track-Etch Membranes from Whatman. Fluorescence spectra were recorded on a Varian Cary Eclipse fluorescence spectrophotometer, ^{23}Na-NMR-spectra on Jeol GX-270 spectrometer (270 MHz). All experiments were conducted at 25°C. The ionophores concentration is given in percent with respect to the total concentration of lipid. Stock solutions of ionophores were prepared in CH_3OH. Control experiments showed that the amount of CH_3OH added to the vesicular suspension in the different experiments (maximum amount 1.6 % in volume) did not affect the permeability of the membrane

^{23}Na-NMR Transport Assays. A mixture of 150 μL of EYPC chloroform solution (100 mg/mL, 20 μmol), 40 μL of EYPG chloroform solution (20 mg/mL, 1 μmol) and an aliquot of CH_3OH solution of the desired ionophore was first evaporated with Ar-flux to form a thin film and then dried under high vacuum for 3h. The lipid film was hydrated in 1 mL of LiCl solution (100 mM in H_2O/D_2O 90:10) for 30 minutes at 40°C. The lipid suspension was submitted to 5 freeze-thaw cycles (-196°C/40°C) using liquid nitrogen and a thermostatic bath, and then extruded under nitrogen pressure (15 bar) at room temperature (10 extrusions through a 0.1 μm polycarbonate membrane). 350 μL of the lipid dispersion and 350 μL of a *shift reagent* solution (4 mM $DyCl_3 \cdot 6H_2O$, 12 mM $Na_5P_3O_{10}$, 40 mM NaCl in H_2O/D_2O 90:10) were mixed in a 5 mm NMR-tube. During the experiment the spectra were recorded every 30 minutes over 14 h (accumulation *in continuo*), the areas of the signals were calculated with Jeol Delta software. The % of Na^+ inside liposome was calculated respect to the signal of Na^+ outside (liposome entrapped volume 2 %). The same experiment has been repeated adding an aliquot of CH_3OH solution of ionophore to the preformed vesicle (single side addition) with identical results.

HPTS assay. A mixture of 150 μL of EYPC chloroform solution (100 mg/mL, 20 μmol) and 40 μL of EYPG chloroform solution (20 mg/mL, 1 μmol) was first evaporated with Ar-flux to form a thin film and then dried under high vacuum for 3 h. The lipid cake was hydrated in 1.5 mL of 0.1 mM HPTS solution (HEPES 25 mM, 100 mM NaCl, pH 7) for 30 min at 40 °C. The lipid suspension was submitted to 5 freeze-thaw cycles (–196 °C/40 °C) using liquid nitrogen and a thermostatic bath, and then extruded under nitrogen pressure (15 bar) at room temperature (10 extrusions through a 0.1 μm polycarbonate membrane). The LUV suspension was separated from extravesicular dye by size exclusion chromatography (SEC) (stationary phase: pre-packed column *Sephadex*™ G-25, mobile phase: HEPES buffer) and diluted with HEPES buffer to give a stock solution with a lipid concentration of 5 mM (assuming 100% of lipids were incorporated into liposomes). 104 μL of the lipid suspension were placed in a fluorimetric cell, diluted to 3040 μL with the same buffer solution used for the liposome preparation and kept under gentle stirring. The total lipid concentration in the fluorimetric cell was 0.17 mM. An aliquot of solution of the ionophore in CH_3OH (5-30 μL of the appropriate mother solution in order to obtain the desired $mol_{compound}/mol_{lipid}$ ratio) was then added to the lipid suspension and the cell was incubated at 25 °C for 20 min. After incubation the time course of the fluorescence emission was recorded for 100 s (λ_{ex1} = 460 nm, λ_{ex2} = 403 nm, λ_{em}= 510 nm) and then 50 μL of 0.5 M NaOH were rapidly added through an injector port and the fluorescence emission was recorded for 400 s. Maximal changes in dye emission were obtained by final lysis of the liposomes with detergent (40 μL of 5% aqueous Triton® X-100). The data set consists of emission intensities at 510 nm modulated by alternating excitation at 403 nm and 460 nm on a 0.5 + 0.5 s cycle. The concentration of the conjugate base form of HPTS is related to the emission intensity at 510 nm during the period when the dye is excited at 460 nm (E_{460}) while the concentration of the protonated form is related to the emission intensity at 510 nm during the period when the dye is excited at 403 nm (E_{403}). Fluorescence time course were normalized using the following equation, where the subscript 0, ∞ and *t* denote the emission ratio before the base pulse, after detergent lysis, and at an intermediate time, respectively.

$$FI = \frac{\left(\frac{E_{403}}{E_{460}}\right)_t - \left(\frac{E_{403}}{E_{460}}\right)_0}{\left(\frac{E_{403}}{E_{460}}\right)_\infty - \left(\frac{E_{403}}{E_{460}}\right)_0} \times 100$$

4.4 Antimicrobial Activity

The antimicrobial activities of compounds **1a** and **1b** was evaluated by the broth microdilution susceptibility test performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI), to determine the minimum inhibitory concentration (MIC) values, as previously described.[27,28] Two-fold serial dilutions of each compound were prepared in 96-well polypropylene microtiter plates (Sarstedt, Germany) in 5% Tryptic Soy Broth (TSB, Difco) in 10 mM Na-phosphate buffer, pH 7.4, to a final volume of 50 μl. Each series included a well without addition of any compound, as a growth control. A total of 50 μl of the adjusted inoculum, diluted in 5% TSB, was then added to each well to obtain a final concentration of approximately $5x10^5$ cells/ml for bacteria, and $1x10^5$ cells/ml for fungi. The microplates were incubated at 37°C for 24h for bacteria, or at 30°C for 48h for fungi. The MIC value was taken as the lowest concentration of compounds resulting in the complete inhibition of visible growth after incubation. Data are the mean of at least three independent determinations performed in duplicate with values differing by one dilution at the most.

4.5 Bacterial membrane permeabilization assays

The permeabilization of the outer membrane of *E. coli* by the compounds was evaluated by following the unmasking of the periplasmic hydrolytic enzyme β-lactamase, using extracellular CENTA® as substrate,[29] whilst that of the cytoplasmic membrane by unmasking cytoplasmic β-galactosidase activity using extracellular 2-nitrophenyl-β-D-galactopyranoside (Gal-ONp) as substrate, as described previously.[30,31] The hydrolysis of both chromogenic substrates was monitored at 405 nm on a microplate reader. The β-galactosidase constitutive, lactose-permease deficient *E. coli* ML-35 pYC strain was used (~10^7 cells/ml in 10 mM phoshate buffer, pH 7.4, containing 100 mM NaCl and 5 mM glucose), in the presence of 64 μM compound **1a** or 256 μM compound **1b**, and 50 μM CENTA (for the evaluation of the outer membrane integrity) or 1.5 mM Gal-ONp (for the evaluation of the inner membrane integrity).

Acknowledgments

This work was supported by Fondazione Beneficentia Stiftung, MIUR-PRIN 2010JMAZML and UNITS-FRA_2013 projects.

Supplementary Material

NMR spectra of compounds **1** and kinetics data for the ionophoric activity.

References and notes

1. Medoff, G.; Brajtburg, J.; Kobayashi, G. S.; Bolard, J. *Annu. Rev. Pharmacol. Toxicol.* **1983**, *23*, 303.
2. Moore, K. S.; Wehrli, S.; Roder, H.; Rogers, M.; Jr. Forrest, J. N.; McCrimmon, S.; Zasloff, M. *Proc. Natl. Acad. Sci. U.S.A.*, **1993**, *90*, 1354.
3. Mora-Duarte, J.; Betts, R.; Rotstein, C.; Colombo, A. L.; Thompson-Moya, L.; Smietana, J.; Lupinacci, R.; Sable, C.; Kartsonis, N.; Perfect, J. *N. Engl. J. Med.* **2002**; *347*, 2020
4. Alhanout, K.; Rolain, K. M.; Brunel, J. M. *Curr. Med. Chem.*, **2010**, ***17***, 3909-3917.
5. Bolard, J. *Biochim. Biophys. Acta* **1986**, *864*, 257.
6. Selinsky, B. S.; Zhou, Z.; Fojtik, K. G.; Jones, S. R.; Dollahon, N. R.; Shinnar, A. E. *Biochim. Biophys. Acta* **1998**, *1370*, 218.
7. Alhanout, K.; Malesinki, S.; Vidal, N.; Peyrot, V.; Rolain, J. M.; Brunel, J. M. *J Antimicrob. Chemother.* **2010**, *65*, 1688.
8. Sadownik, A.; Deng, G.; Janout, V.; Regen, S. L.; Bernard, E. M.; Kikuchi, K.; Armstrong, D. *J. Am. Chem. Soc.* **1995**, *117*, 6138.
9. Merritt, M.; Lanier, M.; Deng, G.; Regen, S. L. *J. Am. Chem. Soc.* **1998**, *120*, 8494.
10. Brunel, J. M.; Letourneux, Y. *Eur. J. Org. Chem.* **2003**, 3897.
11. Chen, W. H.; Wennersten, C.; Jr. Moellering, R. C.; Regen, S. L. *Chem. Biodivers.* **2013**, *10*, 385.
12. (a) De Riccardis, F.; Izzo, I.; Montesarchio, D.; Tecilla, P. *Acc. Chem. Res.* **2013**, *46*, 2781; (b) Montesarchio, D.; Coppola, C.; Boccalon, M.; Tecilla, P. *Carbohydr. Res.* **2012**, *356*, 62.
13. (a) Scrimin, P.; Veronese, A.; Tecilla, P.; Tonellato, U.; Monaco, V.; Formaggio, F.; Crisma, M.; Toniolo, C. *J. Am. Chem. Soc.* **1996**, *118*, 2505; (b) Licini, G.; Prins, L. J.; Scrimin, P. *Eur. J. Org. Chem.* **2005**, 969.
14. Crisma, M.; De Zotti, M.; .Formaggio F.; Peggion, C.; Moretto, A.; Toniolo, C. *J. Pept. Sci.* **2015**, *21*, 148.
15. (a) Toniolo, C.; Crisma, M.; Formaggio F.; Peggion, C. *Biopolymers (Pept. Sci.)* **2001**, *60*, 396; (b) Bellanda, M.; Mammi, S.; Geremia, S.; Demitri, N.; Randaccio, L.; Broxterman, Q. B.; Kaptein, B.; Pengo, P.; Pasquato, L.; Scrimin, P. *Chem. Eur. J.* **2007**, *13*, 407.
16. Ceccacci, F.; Mancini, G.; Rossi, P.; Scrimin, P.; Sorrenti, A.; Tecilla, P. *Chem. Commun.* **2013**, *49*, 10133.
17. Toniolo, C.; Brueckner, H. (Eds), Peptaibiotics Wiley-VCH, **2009**.
18. Toniolo, C.; Bonora, G. M.; Barone, V.; Bavoso, A.; Benedetti, E.; Di Blasio, B.; Grimaldi, P.; Lelj, F.; Pavone, V.; Pedone, C. *Macromolecules* **1985**, *18*, 895.
19. a) Otto, S.; Osifchin, M.; Regen, S. L. *J. Am. Chem. Soc.* **1999**, *121*, 7276; b) Izzo, I.; Licen, S.; Maulucci, N.; Autore, G.; Marzocco, S.; Tecilla, P.; De Riccardis, F. *Chem. Commun.* **2008**, 2986.
20. Sakai, N.; Matile, S. *J. Phys. Org. Chem.* **2006**, *19*, 452.
21. Tuckerman, M. E.; Marx, D.; Parrinello, M. *Nature* **2002**, *417*, 925.
22. a) Jiang, C.; Lee, E. R.; Lane, M. B.; Xiao, Y.-F.; Harris, D. J.; Cheng, S. H. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2001**, *281*, L1164-L1172; b) Lu, Y.-M.; Deng, L.-Q.; Chen, W. H. *RSC Adv.* **2014**, *4*, 43444.
23. Alfonso, I.; Quesada, R. *Chem. Sci.* **2013**, *4*, 3009.
24. Geall, A. J.; Blagbrough, I. S. *Tetrahedron* **2000**, *56*, 2449.
25. Jahromi, A. H.; Fu, Y.; Miller, K. A.; Nguyen, L.; Luu, L. M.; Baranger, A. M.; Zimmerman, S. C. *J. Med. Chem.* **2013**, *56*, 9471.
26. a) Metcalf III, C. A.; Eyermann, C. J.; Bohacek, R. S.; Haraldson, C. A.; Varkhedkar, V. M.; Lynch, B. A.; Bartlett, C.; Violette, S. M.; Sawyer, T. K. *J. Comb. Chem.* **2000**, *2*, 305; b) Silverberg, L. J.; Dillon, J. L.; Vemishetti, P. *Tetrahedron Lett.* **1996**, *37*, 771.
27. Benincasa, M.; Scocchi, M.; Podda, E.; Skerlavaj, B.; Dolzani, L.; Gennaro, R. *Peptides* **2004**, *25*, 2055.
28. Benincasa, M.; Pacor, S.; Wu, W.; Prato, M.; Bianco, A.; Gennaro, R. *ACS Nano* **2011**, *5*, 199.
29. Jones, R. N.; Wilson, H. W.; Novick Jr., W. J.; Barry, A. L.; Thornsberry, C. *J. Clin. Microbiol.* **1982**, *15*, 954.
30. Tiozzo, E.; Rocco, G.; Tossi, A.; Romeo, D. *Biochem. Biophys. Res. Commun.* **1998**, *249*, 202.
31. Zelezetsky, I.; Pacor, S.; Pag, U.; Papo, N.; Shai, Y.; Sahl, H. G.; Tossi, A. *Biochem. J.* **2005**, *390*, 177.