Research Article

Journal of PeptideScience

Received: 18 October 2014 Revised: 11 February 2015 Accepted: 18 February 2015 Published online in Wiley Online Library: 25 March 2015

(wileyonlinelibrary.com) DOI 10.1002/psc.2771

Antimicrobial benzodiazepine-based short cationic peptidomimetics

Galina M. Zats,[a,b] Marina Kovaliov,[a,b] Amnon Albeck[a]* and Shimon Shatzmiller[b]

Antimicrobial peptides (AMPs) appear to be good candidates for the development of new antibiotic drugs. We describe here the synthesis of peptidomimetic compounds that are based on a benzodiazepine scaffold flanked with positively charged and hydrophobic amino acids. These compounds mimic the essential properties of cationic AMPs. The new design possesses the benzodiazepine scaffold that is comprised of two glycine amino acids and which confers flexibility and aromatic hydrophobic 'back', and two arms used for further synthesis on solid phase for incorporation of charged and hydrophobic amino acids. This approach allowed us a better understanding of the influence of these features on the antimicrobial activity and selectivity. A novel compound was discovered which has MICs of 12.5 μg/ml against Staphylococcus aureus and 25 μg/ml against Escherichia coli, similar to the well-known antimicrobial peptide MSI-78. In contrast to MSI-78, the above mentioned compound has lower lytic effect against mammalian red blood cells. These peptidomimetic compounds will pave the way for future design of potent synthetic mimics of AMPs for therapeutic and biomedical applications. Copyright © 2015 European Peptide Society and John Wiley & Sons, Ltd.

Additional supporting information may be found in the online version of this article at the publisher's website.

Keywords: antimicrobial peptide; benzodiazepine scaffold; cationic peptides; drug design; peptidomimetics

Introduction

Antimicrobial peptides (AMPs) play an essential protective role in the immune system in various kingdoms of living organisms such as animals, fungi, bacteria, and amphibians [1–5]. They share several common features, i.e. positive charge domains (contain lysines (Lys) and arginines (Arg)), hydrophobic amino acids (AAs) [valine (Val) and phenylalanine (Phe) and amphipathic structures [6,7]. Most native AMPs have no cytolytic activity against normal mammalian cells at their minimal antimicrobial inhibitory concentrations (MICs) [8]. The advantage of AMPs over conventional antibiotics is their operating mechanism, which refers to the interaction between the bacteria's membrane and the peptide, resulting in the disintegration of the cell wall [9–11]. This fact makes it much more difficult for the microorganisms to develop resistance to AMPs. However, natural AMPs have high molecular weight, low stability in the blood serum, low selectivity, and high synthetic cost [12–15]. For these reasons, the pharmaceutical industry shows interest in synthetic mimics of antimicrobial peptides (SMAMPs). For a number of years, several research groups, including ours, have focused on the development of very short SMAMPs [14–20]. These studies have shown that the presence of two cationic units and a minimum of one lipohylic and bulky unit is required for antibacterial activity [21–28].

We used benzodiazepine (BDZ) derivatives, which received significant attention, as their core is indeed a 'privileged scaffold' found in active compounds against a variety of target types [29,30]. BDZ are known as the core structure of pharmaceutically significant agents, are widely used as enzyme inhibitors [31,32], possess antiproliferative properties on tumor cell [29], and have been proven to be efficient peptidomimetics [33]. Moreover, BDZ was reported as an antimicrobial compound against gram-positive and gram-negative bacteria [34–36].

Herein, we present the design and synthesis of a series of antibacterial compounds, SMAMPs, that are based on short cationic antibacterial peptides that flank a BDZ scaffold, providing drug-like properties.

Results and Discussions

Design

The design strategy was based on modification of AA sequences as well as their chemical nature. Furthermore, the AA themselves were replaced by a BDZ scaffold. In these series of SMAMPs, hydrophobic groups, the BDZ scaffold, and a positive charge were all present (Figure 1). These aspects have been suggested to be the minimum requirement for antimicrobial activity [14,22]. To evaluate the effect of different non-polar and polar groups on biological properties, the AAs were varied. Lys and Histidine (His) represent basic, naturally occurring AAs. Isoleucine (Ile), leucine (Leu), valine (Val), and Phe were included to establish the effect of hydrophobicity. Cysteine was used as a polar uncharged AA, containing a thiol

* *Correspondence to: Amnon Albeck, Department of Chemistry, Bar-Ilan University, Ramat Gan 52900, Israel. E-mail: amnon.albeck@biu.ac.il*

a *Department of Chemistry, Bar-Ilan University, Ramat Gan, 52900, Israel*

b *Department of Biological Chemistry, Ariel University, Ariel, 40700, Israel*

Abbreviations: *AA, amino acid; AMPs, antimicrobial peptides; BDZ, benzodiazepine scaffold; Boc, t-butyloxycarbonyl; tBu, tert butyl; DCM, dichloromethane; DIEA, N,N-diisopropylethylamine; DMF, dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; MIC, minimum inhibitory concentration; NMP, N-methyl-2-pyrrolidone; SMAMPS, synthetic mimics of antimicrobial peptides; SPPS, solid-phase peptide synthesis; TIPS, triisopropyl silane.*

Copyright © 2015 European Peptide Society and John Wiley & Sons, Ltd.

Journal of PeptideScience

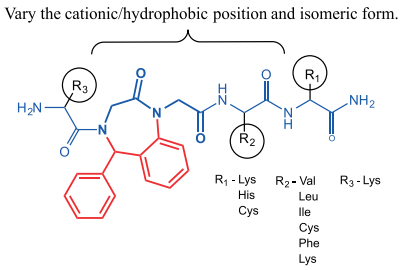


Figure 1. Design and structure of SMAMs.

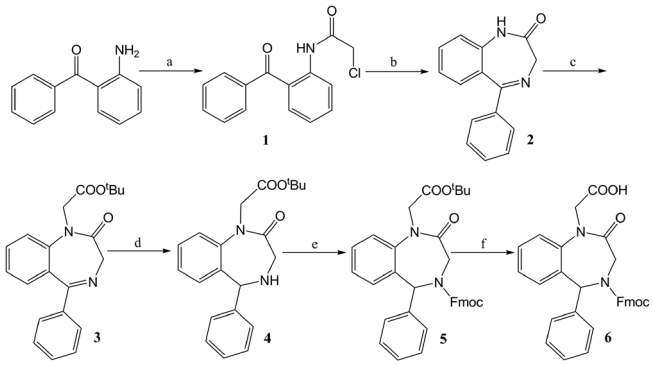
functionality, which was reported as an important determinant of antibiotic activity against bacteria [37–40]. The C terminal was blocked as an amide to prevent metabolic degradation.

The BDZ scaffold was used throughout this study because of its special features: (a) a wide range of biological activities displayed by BDZ-derived compounds, including the antimicrobial field; (b) a very effective platform for the development of new biological compounds; (c) the molecule contains two arms for further modifications and the scaffold is a mimic of two AAs. Figure 1 shows the design of a BDZ scaffold. This structure contains a glycyl–glycine dipeptide mimic that confers some flexibility, an aromatic hydrophobic 'back', and two arms used to incorporate the charged and hydrophobic AAs. With this aim, nine SMAMP compounds based on the BDZ scaffold were prepared.

Each compound was designed to test various effects: compounds **P7** and **P8** and the compounds **P3** and **P5**, which contain the same AA sequence but of different stereochemical configurations, may provide correlation between the absolute configuration of AAs and biological activity. Moreover, the previous sequences, which do not include a hydrophobic AA, examine the importance of the hydrophobicity effect. Compounds **P4** and **P9**, with His and Lys, respectively, examine the effect of the identity of the positively charged AA. Compounds **P2** and **P6**, consist of the same AAs but in deferent order, shed light on the importance of the location of the positively charged AA along the peptide sequence. Compounds **P9**, **P2**, and **P7,** each including the same order of the positively charged AAs but various types of hydrophobic AAs, examine the effect of the hydrophobic AAs on biological activity. Finally, **P1**, which consists of only two AAs, positive and hydrophobic, examines the importance of the peptide general charge as well as the location of the charged AA.

(a) Synthesis. The synthesis of the new peptidomimetic compounds consists of two parts: (a) a multi-step synthesis of the BDZ scaffold and (b) solid-phase peptide synthesis (SPPS) of the BDZ containing peptides. The scaffold itself contained two functional groups for the attachment of the AAs.

Benzodiazepine synthesis: The synthesis of the BDZ scaffold was achieved by six steps, as shown in Scheme 1. Aminobenzophenone was acylated with chloroacetyl chloride, affording 2-chloroacetamido-benzophenone **1** as previously reported [41]. It was then followed by modification of the known hexamethylenetetramine-based cyclization reaction, developed by Blazevic and Kajfez, resulting in **2** [42]. The final scaffold construction steps were designed to include two glycine AAs and two functional groups for the attachment of the flanking AAs. Thus, the first arm for contact was installed by alkylation of BDZ **2** with *t*-butyl bromoacetate to generate **3** in 98% yield after crystallization [41,43]. The second arm for contact was installed by imine reduction with sodium cyanoborohydride [44], which

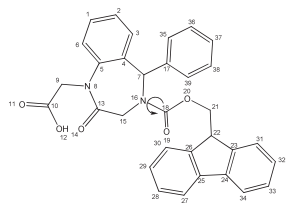


Scheme 1. Reagents and conditions: (a) chloroacetyl chloride, toluene, 85% yield; (b) ammonium acetate, hexamethylenetetramine, EtOH, 95% yield; (c) *t*-butyl bromoacetate, NMP, $CsCO_3$, 98% yield; (d) $NaCNBH_3$, AcOH, 98% yield; (e) CH_2Cl_2, DIEA, Fmoc chloride, 62% yield; and (f) 4 N HCl/dioxane, 78% yield.

yielded a racemic mixture **4**, which was subsequently protected with Fmoc-Cl to provide compound **5** as a racemic mixture. Finally, hydrolysis of ester **5** yielded the desired product **6** as a racemic mixture, which was subjected to subsequent SPPS. In order to study the structure of the final protected monomer **6**, several NMR experiments were performed (see Scheme 1). These experiments revealed two rotamers in 55 to 45% ratio, representing $G° \cong 15.9 \pm 0.2$ kJ (Table 1) [45,46].

SPPS: The syntheses of all the SMAMPs were carried out according to Scheme 2, applying a manual solid-phase method

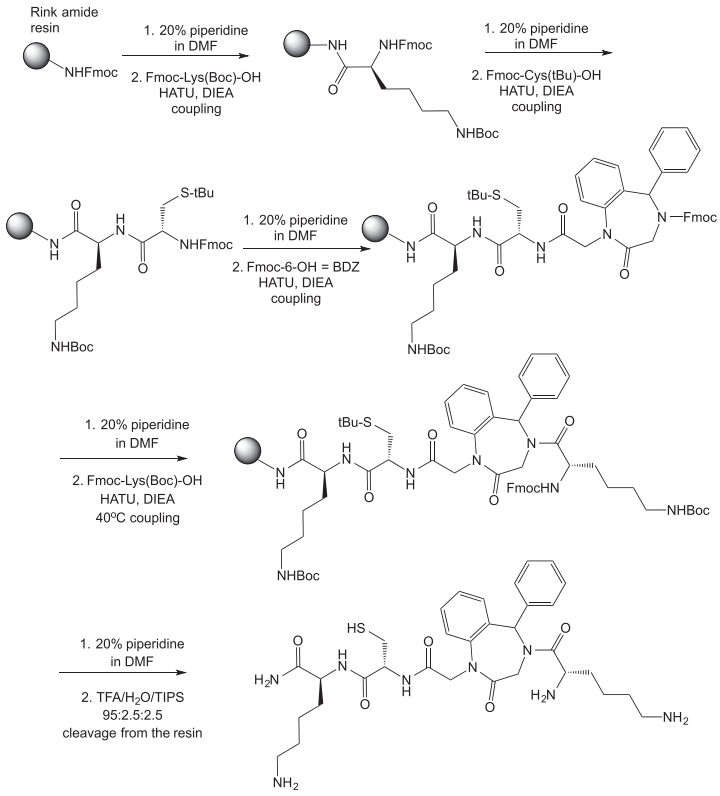
Table 1. ^{1}H NMR and ^{13}C NMR data of the BDZ scaffold (BDZ) **6**



Atom No.	^{1}H-NMR (DMSO, δ, ppm)		^{13}C NMR (DMSO, δ, ppm)	
	Major[a]	Minor[b]	Major[a]	Minor[b]
4	—	—	131.9	132.8
5	—	—	141.3	141.3
7	6.29	6.18	61.7	62.1
9	~3.43	~3.43	49.9	49.9
10	—	—	170.1	170.1
13	—	—	165.6	165.1
15	3.52; 4.55	3.55; 4.39	47.6	47.5
18	—	—	153.9	154.3
17	—	—	140.2	140.5
21	4.41; 4.59	4.25; 4.49	67.4	67.9
22	4.38	4.20	46.8	46.6
23,26	—	—	143.7	143.7
24,25	—	—	140.7	140.7

[a]Major rotamer 55%.
[b]Minor rotamer 45%.
[c]Major and minor may be altered. Assignment of the aromatic protons (7.0–8.0 ppm) and carbons (120–124 ppm) is too complex to analyze.

 Copyright © 2015 European Peptide Society and John Wiley & Sons, Ltd.



Scheme 2. Solid-phase synthesis of the SMAMP **P2**.

on Rink-amide resin using Fmoc strategy [21,27]. After the introduction of the first two AAs, the BDZ scaffold Fmoc-**6**-OH was coupled through its acidic arm. Fmoc deprotection (20% piperidine/DMF), followed by coupling of the next *N*-protected AA (at 40 °C using HATU, which suites coupling to secondary amines) and finally deprotection and cleavage provided the BDZ scaffold-based pentapeptide mimetics. In most cases, the last coupling was repeated twice to give the final SMAMPs **P(1-9)**. The overall yield of the peptidomimetic compounds was 16–29%, in 90–95% purity.

(b) Antimicrobial activity. The antimicrobial activity of the peptidomimetic compounds against bacteria was tested by two methods: the disk diffusion method and the dilution method to determine MIC [47], summarized in Table 2. Both methods showed similar results, although the first method was less effective to SMAMPs because of the mechanism of action of anti-bacterial peptides [10]. Thus, they may have suffered diffusion limitation, which prevented the compounds from reaching a critical concentration of activity (Figure 2). In general, all compounds shown in Table 2 have MICs comparable to the magainin analogue MSI-78, an antimicrobial R-helical peptide with peptide sequence G-I-G-K-F-L-K-K-A-K-K-F-G-K-A-F-V-K-I-L-K-K-NH_2. They show better activity against gram-positive (Staphylococcus aureus) than gram-negative bacteria (Escherichia coli) [48].

Among the peptidomimetic compounds prepared for this study, peptide **P2** exhibited the highest activity against both gram-positive and gram-negative bacteria strains with MIC values of 12 and 25 μg/ml respectively. The nine sequences showed the following trend: **P1** with just one basic residue and **P4**, containing one His and one Lys as positively charged AAs, had no antibacterial activity. In the contrary, the **P2**, **P7,** and **P9** compounds, which contain two Lys in a sequence similar to that of **P4**, inhibited the growth of gram-positive and gram-negative bacteria. However, sequences **P3** and **P5**, which also contain two charged Lys residues, showed no activity. This probably stems from the total length of the peptide, which is one AA shorter, and from the distance between the two charged AAs. Our results indicate that the minimum length required for antibacterial activity is five AAs (considering the BDZ to mimic the Gly–Gly dipeptide). Comparison of peptides **P2** and **P6**, which share the same total length and AA composition, shows that the positions of the polar uncharged AA and/or the distance between the positively charged residues influence the antimicrobial activity. Because of the antibacterial properties of thiol, the presence of Cys in **P2** appears fundamental, but also its position, considering that **P6** did not display any antibacterial activity. Sequences **P3**, **P5**, **P7**, and **P8** are pairs of enantiomers that show very similar activity against both types of bacteria, indicating the lack of stereoselective interactions with the biological target. To conclude, a hydrophobic AA, in particular, a polar uncharged AA such as Cys, in the fourth position (after the Gly–Gly dipeptide of the BDZ) increases the antibacterial activity, if the SMAMPs include two charged Lys residues. Moreover, variability in the hydrophobic AA in the fourth position does not influence the antibacterial activity.

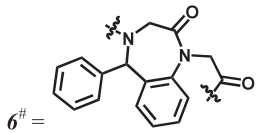
One of the major disadvantages associated with AMPs is that they are prone to protease degradation. This is also reflected in the loss of activity in the presence of blood plasma. [49] We tested the antimicrobial activity of our most active compound **P2** against *S. aureus* in 50% blood plasma, and no loss of activity

Copyright © 2015 European Peptide Society and John Wiley & Sons, Ltd.

Journal of PeptideScience

Table 2. Antimicrobial activity against gram-negative and gram-positive bacteria and hemolytic activity of the BDZ peptidomimetic compounds

Compound	SMAMPs	MIC (μg/ml)		Hemolysis (%)	HC_{50} (μg/ml)
		E. coli	*S. aureus**		
P1	**6**$^{\#}$-Ile-Lys-NH_2	>200	>200	1.8	>1000
P2	Lys-**6**$^{\#}$-Cys-Lys-NH_2	25	12.5	1.2	>1000
P3	D-Lys-**6**$^{\#}$-D-Lys-NH_2	>200	>200	2.3	>1000
P4	Lys-**6**$^{\#}$-Val-His-NH_2	>200	>200	1.2	>1000
P5	Lys-**6**$^{\#}$-Lys-NH_2	>200	>200	2.2	>1000
P6	Lys-**6**$^{\#}$-Lys-Cys-NH_2	>200	100	1.2	>1000
P7	Lys -**6**$^{\#}$-Phe-Lys-NH_2	100	100	2.2	>1000
P8	D-Lys -**6**$^{\#}$-D Phe-D-Lys-NH_2	100	100	2.0	>1000
P9	Lys -**6**$^{\#}$-Leu-Lys-NH_2	100	100	2.1	>1000
MSI-78	—	16-32[a]	8–16[a]	ND[c]	120[b]
vancomycin	—	ND[c]	0.9[c]	ND[c]	ND[c]



$6^{\#}$ =

*Methicillin-resistant *S. aureus*.
[a]Literature values obtained from reference [48].
[b]Literature values obtained from reference [50].
[c]Literature values obtained from reference [15].
[d]ND stands for 'not determined'.

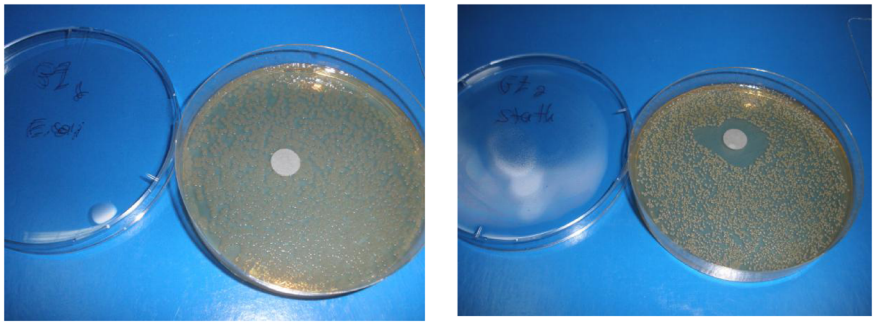


Figure 2. Results of the disk diffusion assay. Left: No antibacterial activity of **P1** against *E. coli*. Right: the most active **P2** against methicillin-resistant *S. aureus*.

(12.5 μg/ml) was observed following 3 h preincubation, which is a physiologically relevant time frame. However slight loss of activity was observed after 6 h (25 μg/ml). (Figure S7 in the supporting information).

Hemolytic Activity

In order to evaluate the cytotoxicity of these compounds toward mammalian cells, the ability to induce lysis in human erythrocytes was measured. None of the peptidomimetics displayed significant hemolytic activity (<3%) at concentrations up to 1000 μg/ml. It is important to mention that the **P2** lead compound shows antimicrobial values similar to the well-known AMP, MSI-78, but it displays a higher MIC value than vancomycin [15]. **P2** does not display any significant hemolytic activity (1.2%) at concentrations up to 1000 μg/ml, which is in a sharp contrast to MSI-78. [50] Indeed, the HC_{50} (μg/ml) of **P2** was less toxic toward mammalian cells compared with recently published SMAMPs [14,15].

The main goal of this manuscript was to report the design and synthesis of a series of peptidomimetic antibacterial compounds that were based on short cationic antibacterial peptides, flanking a BDZ scaffold, in order to provide drug-like properties. The concept of using short peptides with cationic AAs is not new nor is the BDZ scaffold, but we thought that the combination of the two would provide these compounds with drug-like properties. In addition, these antibacterial peptidomimetic compounds could solve almost all problems of AMPs, which suffer from high molecular weight, low stability, poor selectivity, high synthetic cost, and toxicity. We believe that our lead compound, **P2**, and its future improved and modified forms should be tested *in vivo* for antimicrobial activity. Currently, we are developing

 Copyright © 2015 European Peptide Society and John Wiley & Sons, Ltd.

SAMP compounds with fluorescent markers for the detection and study of *in vivo* blood brain barrier penetration and antibacterial activity. These studies will be reported in due course.

Conclusions

We have reported here the synthesis of a new BDZ scaffold suitably designed for incorporation into peptidomimetic compounds by SPPS. This work combines both solution and solid-phase synthesis. The BDZ scaffold was selected because of its biological properties and its structure, with a carboxylate and Fmoc-protected amine arms (which mimic the C-termini and N-termini of a dipeptide) for linking. Although the exact conformational aspects responsible for the activity of SMAMPs are not known, all these compounds resemble AMPs in terms of their charge and amphiphilicity. Among the nine prepared compounds, **P2** (Lys–BDZ–Cys–Lys–NH_2) showed the best antibacterial activity with MIC values of 12.5 μg/ml against *S. aureus* and 25 μg/ml against *E. coli*. The other SMAMPs showed MIC values of 100 μg/ml and higher. All the SMAMPs demonstrated no direct lytic effect against mammalian red blood cells. Admittedly, further investigations are necessary to evaluate the importance of the molecular size and the number of positive charges before these new mimetic peptide compounds can be transformed into drugs useful for systemic application. In particular, the required studies include synthesis of additional peptidomometic compounds with modification of additional AAs, study of the mechanism of action of these compounds, and most importantly *in vivo* studies. We believe that these relatively small and easy to prepare molecules can serve as a basis for further refinement toward lead compounds for novel antibacterial agents, and these preliminary but important studies could guide and encourage further studies in the field.

Materials and Methods

Solution Synthesis

N-(2-benzoylphenyl)-2-chloroacetamide (1)

A solution of chloroacetyl chloride (8.4 ml, 11.93 g, 1.06 eq) in toluene (20 ml) was added dropwise at 10 ºC during 30 min to a solution of 2-amino-benzophenone (20 g, 0.1 mol) in toluene (200 ml). The reaction mixture was then stirred at room temperature for 3 h. The resulting mixture was evaporated to dryness. The crude product was crystallized by stirring in 96% ethanol (100 ml) at room temperature for 20 h. The crystals were separated by filtration, washed with 96% ethanol (3 × 10 ml), and dried at 50 °C for 20 h to yield **1** (23.6 g, 85% yield) as colorless crystals. HPLC: λ_{260}-99% purity. M.p: 120 °C. ^{1}H-NMR (300 MHz $CDCl_3$): δ 4.20 (s, 2H, CH_2), 7.37–7.41 (m, 8H, Ar), 8.61 (d, J = 7.8 Hz, 1H, Ar) 11.62 (s, 1H, NH) MS ES^+: *m/z* 274 (MH^+).

5-Phenyl-1H-benzo[e][1,4]diazepin-2(3H)-one (2)

Hexamethylenetetramine (14.8 g, 2.2 eq) and ammonium acetate (8.22 g, 2.2 eq) were added to a solution of N-(2-benzoylphenyl)-2-chloroacetamide **1** (18.6 g, 0.079 mol) in 96% ethanol (300 ml). The reaction mixture was stirred at reflux temperature for 6 h. Then the reaction mixture was evaporated to dryness. Distilled water (200 ml) was added, and the resulting suspension was stirred at 60 °C for 0.5 h. The suspension was cooled to 15 °C and filtered. The crude product was dried at 105 °C for 5 h. It was then suspended in toluene (50 ml) at 70 °C for 0.5 h and then cooled to 10 °C, and the obtained crystals were filtered and washed with cold toluene (3 × 10 ml). The purified product was dried at 105 °C for 5 h to yield **2** (15.3 g, 95% yield) as colorless crystals. HPLC: λ_{260}-96.8% purity. M.p: >210 °C ^{1}H-NMR (300 MHz $CDCl_3$): δ 4.12 (s, 2H, CH_2), 7.17–7.62 (m, 9H, Ar) 10.53 (s, 1H, NH). IR (KBr): ν 3057, 2900, 2252, 1669, 1584, 1482, 1433, 1380, 1242, 1217, 1178 cm^{-1}. MS ES^+: *m/z* 234 (MH^+).

Tert-butyl 2-(2-oxo-5-phenyl-2,3-dihydro-1H-benzo[e] [1,4] diazepin-1-yl) acetate (3)

A round bottomed flask, equipped with a magnetic stirring bar and nitrogen inlet, was sequentially charged with 5-phenyl-1H-benzo[e] [1,4]diazepin-2(3H)-one **2** (5.7 g, 0.02 mol) NMP (16.2 ml), tert-butyl bromoacetate (2.62 ml, 1.42 eq), and cesium carbonate (5.2 g, 1.42 eq). After stirring overnight at ambient temperature, the reaction mixture was diluted with water (100 ml) and extracted with EtOAc (3 × 30 ml). The combined organic phase was washed with water (4 × 15 ml) and brine (10 ml), dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. This material was crystallized from hexanes/ethyl acetate to provide **3** (3.59 g, 98% yield) as a white crystalline solid. HPLC: λ_{260}-99.8% purity M.p: >200 °C. ^{1}H-NMR (300 MHz $CDCl_3$): δ 1.45 (s, 9H, *t*-Bu), 3.85 (d, J = 11.9 Hz, 1H, CH_2), 4.21 (d, J = 17.8 Hz, 1H, CH_2), 4.48 (d, J = 11.9 Hz, 1H, CH_2), 4.53 (d, J = 17.8 Hz, 1H, CH_2), 7.21–7.64 (m, 9H, Ar). IR (KBr): ν 3330, 3057, 1669, 1584, 1482, 1493, 1380, 1249, 1217, 1121 cm^{-1}.

Tert-butyl 2-(2-oxo-5-phenyl-2,3,4,5-tetrahydro-1H-benzo[e] [1,4] diaze pin-1-yl) acetate (4)

Tert-butyl 2-(2-oxo-5-phenyl-2,3-dihydro-1H-benzo[e] [1,4] diazepin-1-yl) acetate **3** (3.54 g, 0.01 mol) was dissolved in acetic acid (195 ml) and cooled to −10 °C. Sodium cyanoborohydride (1.25 g, 2 eq) was added to the yellow solution all at once. After stirring for 15 min at 10 °C, the mixture was diluted with H_2O (200 ml), made basic with saturated Na_2CO_3 (aq.), and extracted with EtOAc (2 × 480 ml). The combined organic phase was washed with brine, dried over $MgSO_4$, filtered, and evaporated to dryness in vacuo. The residue was chromatographed (7 : 3 petroleum ether: EtOAc) to provide **4** (3.5 g, 98% yield) as white crystalline solid. HPLC: λ_{260}-98.8% purity. M.p: >200 °C. ^{1}H-NMR (300 MHz $CDCl_3$): δ 1.48 (s, 9H, *t*-Bu), 3.40 (d, J = 19.1 Hz, 1H, CH_2), 3.46 (d, J = 19.1 Hz, 1H, CH_2), 4.37 (d, J = 17.9 Hz, 1H, CH_2), 4.47 (d, J = 17.9 Hz, 1H, CH_2) 5.70 (s, 1H), 6.67 (d, J = 7.8 Hz, 1H, Ar), 7.01–7.60 (m, 8H, Ar). ^{13}C NMR ($CDCl_3$): δ 28.3, 50.0, 50.8, 59.4, 82.4, 121.3, 126.9, 128.5, 128.6, 128.9, 129.6, 134.7, 141.0, 142.7, 168.1, 171.1. IR (KBr): ν 3257, 3057, 1868, 1686, 1610, 1517, 1479, 1367, 1318, 1334, 1023 cm^{-1}.

(9H-Fluoren-9-yl)methyl-1-(2-tert-butoxy-2-oxoethyl)-2-oxo-5-phenyl-2,3-dihydro-1H benzo[e] [1,4] diazepine-4(5H)-carboxylate (5)

tert-Butyl 2-(2-oxo-5-phenyl-2,3,4,5-tetrahydro-1H-benzo[e] [1,4] diazepin-1-yl) acetate **4** (2.55 g, 7.28 mmol) was dissolved in DCM (40 ml) and FmocCl (2.56 g, 7.28 mmol) and DIEA (14 ml, 8 eq) were added at 0 °C. After 30 min, the cold bath was removed and the solution was stirred overnight. The reaction mixture was diluted with citric acid (10%). The organic extracts were washed with water (4 × 15 ml) and brine (10 ml), dried over $MgSO_4$, and evaporated to dryness. The crude product was crystallized from EtOAc/hexane to give white crystalline solid (2.59 g, 62% yield). HPLC: λ_{260}-100% purity. M.p: >200 °C. ^{1}H-NMR (300 MHz $CDCl_3$): Two rotamers (55 : 45 ratio): (major) δ 1.48 (s, 9H, *t*-Bu), 3.46 (d, J = 16.2 Hz, 1H, CH_2CO_2H), 3.70 (d, J = 16.2 Hz, 1H, CH_2CO_2H), 4.05; 4.63, (m, 3H, 2 × CH_2), 4.76 (d, J = 16.2 Hz, 1H, CH_2), 6.15 (s, 1H, CH), 6.96 (d, J = 8.5 Hz, 1H, Ar), 7.15–7.79 (16H, Ar). (minor) δ 1.48 (s, 9H, *t*-Bu), 3.04 (d, J = 16.2 Hz,

Copyright © 2015 European Peptide Society and John Wiley & Sons, Ltd.

Journal of PeptideScience

1H, CH_2CO_2), 3.63 (d, J = 16.2 Hz, 1H, CH_2CO_2), 4.05–4.63, (m, 3H, 2 × CH_2), 4.71 (d, J = 16.2 Hz, 1H, CH_2), 6.40 (s, 1H, CH), 6.96 (d, J = 8.5 Hz, 1H, Ar), 7.15–7.79 (16H, Ar) ^{13}C NMR ($CDCl_3$): (major + minor) δ 28.2, 48.3, 50.6, 51.2, 62.5, 65.4, 82.5, 120.3, 124.9, 125.4, 127.2, 127.4, 127.8, 128.6, 130.5, 131.7, 132.8, 141.5, 141.7, 142.0, 143.8, 144.0, 155.3, 167.6, 168.5. MS ES$^+$: *m/z* 575 (MH^+).

2-(4(((H-Fluoren-9-yl)methoxy)carbonyl)-2-oxo-5-phenyl-2,3,4,5-tetrahydro-1Hbenzo[e][1,4] diazepin-1-yl) acetic acid (6)

HCl (20 ml, 4 N in dioxane) was added to a solution of 9H-zfluoren-9-yl)methyl 1-(2-tert-butoxy-2-oxoethyl)-2-oxo-5-phenyl 2,3-dihydro-1H-benzo[e][1,4]diazepine-4(5H)-carboxylate **5** (1 g, 1.74 mmol) in dioxane (30 ml) at 4 °C. After 30 min, the cold bath was removed and the solution was stirred overnight. The organic layer was evaporated to dryness, and the crude product was crystallized from EtOAc/hexane to give **6** as white crystalline solid (78% yield). HPLC: λ_{260}-93% purity. M.p: >200 °C. ^{1}H-NMR (700 MHz DMSO): Two rotomers (55 : 45 ratio): (major) δ 3.43 (2H, CH_2CO_2H), 4.55;3.52 (2H, CH_2), 4.59;4.41 (2H, CO_2CH_2), 4.36 (1H, CH), 6.29 (1H, CH), 7.0–8.0 (16H, Ar). (minor) δ 3.43 (2H, CH_2CO_2H), 4.39;3.55, (2H, CH_2), 4.49;4.25, (2H, CO_2CH_2), 4.20 (1H, CH), 6.18 (1H, CH), 7.0–8.0 (16H, Ar). ^{13}C NMR ($CDCl_3$): (major) δ 46.8, 47.5, 61.7, 67.4, 131.9, 140.2, 140.7, 141.3, 143.7, 165.6, 170.1. (minor) δ 46.6, 47.4, 49.7, 62.1, 67.9, 132.3, 140.5, 140.7, 141.3, 143.3, 165.1, 170.1. M.S ES$^+$: *m/z* 519 (MH^+), 541 (MNa^+), 557 (MK^+).

General Procedure for SPPS

Rink amide resin (0.4 mmol) was shaken with DCM : NMP 1 : 1 (3 ml) for 24 h. in a Merrifield flask. Solvent was filtrated under vacuum, and the resin was treated with 20% piperidine in DMF (*v/v*) to deprotect the amino groups (2 × 10 min). Then the solid was washed five times with NMP and three times with DCM. Positive Kaiser test indicated successful removal of the Fmoc protecting group. A solution of absolute DMF (3 ml), HATU (4 eq), Fmoc-AA (0.4 mmol), and DIEA (3 eq) was added to the resin, and the mixture was agitated for 3 h. During the coupling to the secondary amine of the BDZ scaffold, the agitating was performed for 2 h at 40 °C and the next AA was added twice. At the end of the peptide synthesis, the peptide-loaded resin was dried in vacuum for 2 h. The peptide was then cleaved by addition of a solution of TFA: H_2O: TIS (95 : 2.5 : 2.5, 4 ml), which turned the solid and the solution deep red. After agitating for 2 h, the solution was pressed out of the Merrifield flask, vacuum filtered, and evaporated under nitrogen. The resulting highly viscous liquid/oil was precipitated from cold diethyl ether (6 ml) and lyophilized. The SMAMPs were finally purified by solid-phase extraction pack (RP-18), first washed with water and then extracted with acetonitrile. Their purity was determined by HPLC (RP-18, CH_3CN/0.1% TFA (aq.), 2 : 1).

BDZ-Ile-Lys-NH_2 (P1). HPLC: λ_{260} -95% purity. M.S ES$^+$: *m/z* 537.3 (MH^+).
Lys-BDZ-Cys-Lys-NH_2 (P2). HPLC: λ_{260}-95% purity. M.S ES$^+$: *m/z* 655.3 (MH^+).
D-Lys-BDZ-D-Lys-NH_2 (P3). HPLC: λ_{260}-93% purity. M.S ES$^+$: *m/z* 575.4 (MNa^+).
Lys-BDZ-Val-His-NH_2 (P4). HPLC: λ_{260}-90% purity. M.S ES$^+$: *m/z* 660.1 (MH^+).
Lys-BDZ-Lys-NH_2 (P5). HPLC: λ_{260}-95% purity. M.S ES$^+$: *m/z* 552.3 (MH^+).
Lys-BDZ-Lys-Cys-NH_2 (P6). HPLC: λ_{260}-95% purity. M.S ES$^+$: *m/z* 655.3 (MH^+).
Lys-BDZ-Phe-Lys-NH_2 (P7). HPLC: λ_{260}-95% purity. M.S ES$^+$: *m/z* 699.1 (MH^+).
D-Lys-BDZ-D-Phe-D-Lys-NH_2 (P8). HPLC: λ_{260}-92% purity. M.S ES$^+$: *m/z* 699.1 (MH^+).
Lys-BDZ-Leu-Lys-NH_2 (P9). HPLC: λ_{260}-95% purity. M.S ES$^+$: *m/z* 665.3 (MH^+).

Antimicrobial Activity

The peptidomimetic compounds (**P1–P9**) were subjected to MIC testing, identifying the lowest concentration of antimicrobial compounds that inhibit the growth of a microorganism after 24 h incubation at 37 °C. Microplate autoreader E1309 Bio-tek Instrument was used to measure absorption at 690 nm. MIC values for gram-positive (methicillin-resistant *S. aureus*) and gram negative (*E. coli*) bacteria were determined. The concentrations of the antimicrobial peptidomimetics in the microplates were 400, 200, 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.2, 0.1, and 0.05 μg/ml. The MIC values and errors are reported as averages and standard errors of mean of tree independent experiments (each experiment was performed in triplicates) respectively. The error of the experiments is less than 5%.

Antimicrobial Activity in Plasma

Fresh human blood cells were centrifuged at 3000 rpm for 5 min. The plasma, which separated from hRBCs, was collected. **P2** was dissolved in water and then diluted twofold into plasma solution so that final concentration of the compound was 800 μg/ml. This sample was preincubated for 0, 3, or 6 h. Then the MIC values for methicillin-resistant *S. aureus* were determinate as mentioned before in antimicrobial assay.

Hemolytic Activity

The hemolytic activity of each peptidomimetic compound was tested against human red blood cells (hRBCs), obtained from healthy volunteers. Fresh heparinized hRBCs were rinsed three times with phosphate buffered saline (PBS) buffer (35 mM, 150 mM NaCl, pH 7.4) by centrifugation (10 min, 1500 rpm), followed by resuspension and dilution in PBS (10% hematocrit). Various concentrations of the peptidomimetics were then dissolved in PBS buffer and added to the hRBC solution, yielding a final erythrocyte concentration of 1% *v/v*. The suspensions were incubated under agitation for 1 h at 37 °C, followed by centrifugation (5 min, 4000 rpm). The release of hemoglobin was monitored by measuring the absorbance of the supernatant at 450 nm. Negative controls for zero hemolysis and positive controls (100% hemolysis) consisted of hRBC suspended in PBS and Triton 1%, respectively. The degree of hemolysis is defined as the ratio of the optical density (OD) of the peptidomimetic sample relative to the OD of the difference between the positive and negative controls for hemolysis.

Acknowledgements

Our special thanks go to Dr. Hugo Gottlieb for the NMR determination of the final BDZ scaffold **6**.

References

1 Brown KL, Hancock RE. Cationic host defense (antimicrobial) peptides. *Curr. Opin. Immunol.* 2006; **18**: 24–30.
2 Nakatsuji T, Gallo RL. Antimicrobial peptides: old molecules with new ideas. *J. Invest. Dermatol.* 2012; **132**: 887–895.

 Copyright © 2015 European Peptide Society and John Wiley & Sons, Ltd.

3 Fjell CD, Hiss JA, Hancock RE Schneider G. Designing antimicrobial peptides: form follows function. *Nat. Rev. Drug Discov.* 2011; **11**: 37–51.
4 Jenssen H, Hamill P Hancock RE. Peptide antimicrobial agents. *Clin. Microbiol. Rev.* 2006; **19**: 491–511.
5 Cochrane SA, Lohans CT, Brandelli JR, Mulvey G, Armstrong GD Vederas JC. Synthesis and structure–activity relationship studies of N-terminal analogues of the antimicrobial peptide tridecaptin A1. *J. Med. Chem.* 2014; **57**: 1127–1131.
6 Toke O. Antimicrobial peptides: new candidates in the fight against bacterial infections. *Biopolymers* 2005; **80**: 717–735.
7 Siano A, Húmpola MV, de Oliveira E, Albericio F, Simonetta AC, Lajmanovich R Tonarelli GG. Antimicrobial peptides from skin secretions of hypsiboas pulchellus (Anura: Hylidae). *J. Nat. Prod.* 2014; **77**: 831–841.
8 Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature* 2002; **415**: 389–395.
9 Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat. Rev. Microbiol.* 2005; **3**: 238–250.
10 Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. *Pharmacol. Rev.* 2003; **55**: 27–55.
11 Giuliani A, Pirri G Nicoletto S. Antimicrobial peptides: an overview of a promising class of therapeutics. *Cent. Eur. J. Biol.* 2007; **2**: 1–33.
12 Koczulla AR, Bals R. Antimicrobial peptides: current status and therapeutic potential. *Drugs* 2003; **63**: 389–406.
13 Marr AK, Gooderham WJ Hancock RE. Antibacterial peptides for therapeutic use: obstacles and realistic outlook. *Curr. Opin. Pharmacol.* 2006; **6**: 468–472.
14 Thaker HD, Sgolastra F, Clements D, Scott RW Tew GN. Synthetic mimics of antimicrobial peptides from triaryl scaffolds. *J. Med. Chem.* 2011; **54**: 2241–2254.
15 Ghosh C, Manjunath GB, Akkapeddi P, Yarlagadda V, Hoque J, Uppu DS, Konai MM Haldar J. Small molecular antibacterial peptoid mimics: the simpler the better!. *J. Med. Chem.* 2014; **57**: 1428–1436.
16 Karstad R, Isaksen G, Wynendaele E, Guttormsen Y, De Spiegeleer B, Brandsdal BO, Svendsen JS Svenson J. Targeting the S1 and S3 subsite of trypsin with unnatural cationic amino acids generates antimicrobial peptides with potential for oral administration. *J. Med. Chem.* 2012; **55**: 6294–6305.
17 Dehsorkhi A, Castelletto V Hamley IW. Self-assembling amphiphilic peptides. *J. Pept. Sci.* 2014; **20**: 453–467.
18 Domingues TM, Buri MV, Daffre S, Campana PT, Riske KA Miranda A. Structure-activity relationship of Trp-containing analogs of the antimicrobial peptide gomesin. *J. Pept. Sci.* 2014; **20**: 421–428.
19 Zhou C, Wang M, Zou K, Chen J, Zhu Y Du J. Antibacterial polypeptide-grafted chitosan-based nanocapsules as an 'armed' carrier of anticancer and antiepileptic drugs. *ACS Macro Lett.* 2013; **2**: 1021–1025.
20 Baldassarre L, Pinnen F, Cornacchia C, Fornasari E, Cellini L, Baffoni M Cacciatore I. Synthesis of short cationic antimicrobial peptidomimetics containing arginine analogues. *J. Pept. Sci.* 2012; **18**: 567–578.
21 Guo C, Pan L, Xiao S, Chen H Jiang Z. Short simple linear peptides mimic antimicrobial complex cyclodecapeptides based on the putative pharmacophore. *Med. Chem.* 2014; **4**: 233–329.
22 Haug BE, Stensen W, Stiberg T Svendsen JS. Bulky nonproteinogenic amino acids permit the design of very small and effective cationic antibacterial peptides. *J. Med. Chem.* 2004; **47**: 4159–4162.
23 Saravanan R, Li X, Lim K, Mohanram H, Peng L, Mishra B, Basu A, Lee JM, Bhattacharjya S Leong SS. Design of short membrane selective antimicrobial peptides containing tryptophan and arginine residues for improved activity, salt-resistance, and biocompatibility. *Biotechnol. Bioeng.* 2014; **111**: 37–49.
24 Faccone D, Veliz O, Corso A, Noguera M, Martinez M, Payes C, Semorile L Maffia PC. Antimicrobial activity of de novo designed cationic peptides against multi-resistant clinical isolates. *Eur. J. Med. Chem.* 2014; **71**: 31–35.
25 Isaksson J, Brandsdal BO, Engqvist M, Flaten GE, Svendsen JSM Stensen W. A synthetic antimicrobial peptidomimetic (LTX 109): stereochemical impact on membrane disruption. *J. Med. Chem.* 2011; **54**: 5786–5795.
26 Mahindra A, Bagra N, Wangoo N, Khan SI, Jacob MR Jain R. Discovery of short peptides exhibiting high potency against Cryptococcus neoformans. *ACS Med. Chem. Lett.* 2014; **5**: 315–320.
27 Albada HB, Prochnow P, Bobersky S, Langklotz S, Bandow JE Metzler-Nolte N. Short antibacterial peptides with significantly reduced hemolytic activity can be identified by a systematic L-to-D exchange scan of their amino acid residues. *ACS Comb. Sci.* 2013; **15**: 585–592.
28 Sivertsen A, Isaksson J, Leiros H-K, Svenson J, Svendsen J-S Brandsdal B. Synthetic cationic antimicrobial peptides bind with their hydrophobic parts to drug site II of human serum albumin. *BMC Struct. Biol.* 2014; **14**: 1–14.
29 Mwande-Maguene G, Jakhlal J, Lekana-Douki J-B, Mouray E, Bousquet T, Pellegrini S, Grellier P, Ndouo FST, Lebibi J Pelinski L. One-pot microwave-assisted synthesis and antimalarial activity of ferrocenyl benzodiazepines. *New J. Chem.* 2011; **35**: 2412–2415.
30 Horton DA, Bourne GT Smythe ML. The combinatorial synthesis of bicyclic privileged structures or privileged substructures. *Chem. Rev.* 2003; **103**: 893–930.
31 Carter MC, Alber DG, Baxter RC, Bithell SK, Budworth J, Chubb A, Cockerill GS, Dowdell VC, Henderson EA, Keegan SJ, Kelsey RD, Lockyer MJ, Stables JN, Wilson LJ Powell KL. 1,4-Benzodiazepines as inhibitors of respiratory syncytial virus. *J. Med. Chem.* 2006; **49**: 2311–2319.
32 Anzini M, Braile C, Valenti S, Cappelli A, Vomero S, Marinelli L, Limongelli V, Novellino E, Betti L, Giannaccini G, Lucacchini A, Ghelardini C, Norcini M, Makovec F, Giorgi G Ian Fryer R. Ethyl 8-fluoro-6-(3-nitrophenyl)-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate as novel, highly potent, and safe antianxiety agent. *J. Med. Chem.* 2008; **51**: 4730–4743.
33 Ettari R, Nizi E, Di Francesco ME, Micale N, Grasso S, Zappalà M, Vičík R Schirmeister T. Nonpeptidic vinyl and allyl phosphonates as falcipain-2 inhibitors. *ChemMedChem* 2008; **3**: 1030–1033.
34 Kavali JR, Badami BV. 1,5-Benzodiazepine derivatives of 3-arylsydnones: synthesis and antimicrobial activity of 3-aryl-4-[2′-aryl-2′,4′,6′,7′-tetrahydro-(1′H)-1′,5′-benzodiazepine-4′-yl]sydnones. *Il Farmaco* 2000; **55**: 406–409.
35 Singla RK, Kumar A, Khan S, Shrivastava R, Bhat GV Jagani H. Evaluation of antimicrobial activity of 3-(4-1H-Indol-3-yl)-(2,3-dihydro-1H-benzo[b] diazepin-2-yl)-2H-chromen-2-one. *Indo-Global J. Pharm. Sci.* 2011; **1**: 127–133.
36 Joshi RKY, Synthesis C. Spectral studies and biological activity of 3H-1, 5-benzodiazepine derivatives. *ARKIVOC* 2007; **xiii**: 142–149.
37 Goswami M, Jawali N. N-acetylcysteine-mediated modulation of bacterial antibiotic susceptibility. *Antimicrob. Agents Chemother.* 2010; **54**: 3529–3530.
38 Silverstein KA, Moskal WA, Jr, Wu HC, Underwood BA, Graham MA, Town CD VandenBosch KA. Small cysteine-rich peptides resembling antimicrobial peptides have been under-predicted in plants. *Plant J.* 2007; **51**: 262–280.
39 Klüver E, Schulz-Maronde S, Scheid S, Meyer B, Forssmann W-G Adermann K. Structure–activity relation of human β-defensin 3: influence of disulfide bonds and cysteine substitution on antimicrobial activity and cytotoxicity†. *Biochemistry* 2005; **44**: 9804–9816.
40 Silveira LFM, Baca P, Arias-Moliz MT, Rodriguez-Archilla A Ferrer-Luque CM. Antimicrobial activity of alexidine alone and associated with N-acetylcysteine against Enterococcus faecalis biofilm. *In J. Oral Sci* 2013; **5**: 146–149.
41 Selnick HG, Liverton NJ, Baldwin JJ, Butcher JW, Claremon DA, Elliott JM, Freidinger RM, King SA, Libby BE, McIntyre CJ, Pribush DA, Remy DC, Smith GR, Tebben AJ, Jurkiewicz NK, Lynch JJ, Salata JJ, Sanguinetti MC, Siegl PK, Slaughter DE Vyas K. Class III antiarrhythmic activity in vivo by selective blockade of the slowly activating cardiac delayed rectifier potassium current IKs by (R)-2-(2,4-trifluoromethyl)-N-[2-oxo-5-phenyl-1-(2,2,2-trifluoroethyl)-2, 3-dihydro-1H-benzo[e][1,4]diazepin-3-yl]acetamide. *J. Med. Chem.* 1997; **40**: 3865–3868.
42 Cepanec I, Litvić M Pogorelić I. Efficient synthesis of 3-hydroxy-1,4-benzodiazepines oxazepam and lorazepam by new acetoxylation reaction of 3-position of 1,4-benzodiazepine ring. *Org. Process Res. Dev.* 2006; **10**: 1192–1198.
43 Kazmierski WM., Ed. *Peptidomimetics Protocols.* Springer, Berlin, 1999.
44 Evans BE, Rittle KE, Bock MG, DiPardo RM, Freidinger RM, Whitter WL, Gould NP, Lundell GF Homnick CF. Design of nonpeptidal ligands for a peptide receptor: cholecystokinin antagonists. *J. Med. Chem.* 1987; **30**: 1229–1239.
45 Tezer N. Conformation and tautomerizm of the 2-methyl-4-pyridin-2′-yl-1,5-benzodiazepine molecule. An ab initio study. *J. Mol. Model.* 2008; **14**: 11–20.
46 Cortes CE, Becerra LMI, Osornio PYM, Díaz TE Jankowski K. 2D NMR analysis of highly restricted internal rotation in 2-methylthio-3H-4-p-bromophenyl)-7-[(ortho- and para-substituted)-phenylthio]-1,5-benzo

 Copyright © 2015 European Peptide Society and John Wiley & Sons, Ltd.

diazepines. *Spectrochim. Acta A Mol. Biomol. Spectrosc.* 2000; **56**: 1661–1673.
47 Andrews JM. Determination of minimum inhibitory concentrations. *J. Antimicrob. Chemother.* 2001; **48**: 5–16.
48 Ge Y, MacDonald DL, Holroyd KJ, Thornsberry C, Wexler H Zasloff M. In vitro antibacterial properties of pexiganan, an analog of magainin. *Antimicrob. Agents Chemother.* 1999; **43**: 782–788.
49 Radzishevsky IS, Rotem S, Bourdetsky D, Navon-Venezia S, Carmeli Y Mor A. Improved antimicrobial peptides based on acyl-lysine oligomers. *Nat. Biotech.* 2007; **25**: 657–659.
50 Liu D, Choi S, Chen B, Doerksen RJ, Clements DJ, Winkler JD, Klein ML DeGrado WF. Nontoxic membrane-active antimicrobial arylamide oligomers. *Angew. Chem. Int. Ed.* 2004; **43**: 1158–1162.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's website.

 Copyright © 2015 European Peptide Society and John Wiley & Sons, Ltd.