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Biscarbamate analogues of the chemotactic tripeptide fMLF–OMe

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Abstract

Based on the sequence of the prototypical chemotactic tripeptide HCO–Met–Leu–Phe–OH (fMLF) and by taking into account the versatility shown by its *N*-terminal carbamate analogues, the new biscarbamates MeOCO–Met–Leu–gPhe–COOMe (**2**) and Boc–Met–Leu–gPhe–COOMe (**4**) were synthesized. These two new ligands are characterized by the presence of a *gem*-diamino residue (gPhe) replacing the *C*-terminal Phe and a carbamate functionality positioned at both the ends of the molecule. The activity of the two new compounds has been determined on human neutrophils and compared to that shown by the corresponding *N*-terminal monocarbamates MeOCO–Met–Leu–Phe–OMe (**1**) and Boc–Met–Leu–Phe–OMe (**3**).

Keywords: Biscarbamates; Carbamates; Chemotactic peptides; Human neutrophils

1. Introduction

Neutrophils are involved in host defense mechanisms against bacterial infections and play a crucial role in inflammatory processes. Their activation depends upon different extracellular signals among which those mediated by chemotactic *N*-formylated peptides of bacterial origin [1] which are also related to *N*-terminal fragments of specific mitochondrial proteins [2]. The chemotactic peptides exert their effects by interacting with specific receptors located on the neutrophil plasma membrane [3–5]. The most intensively studied member of the *N*-formylated chemotactic peptides is the tripeptide *N*-formyl-L-methionyl-L-leucyl-L-penylalanine (for–Met–Leu–Phe; fMLF) together with its methyl ester fMLF–OMe [6,7].

Based on the sequence of the prototypical model fMLF a variety of chemical modifications have been performed in order to obtain more potent and selective ligands and to study structure–activity relationships. It is now well established that the *N*-formylation is not an absolute requirement for potent agonist activity and that other *N*-terminal functionalities, different for size, flexibility, and nature of electrostatic interactions, can lead to derivatives capable of efficiently binding to the receptor [8–10]. An interesting class of *N*-terminal modified tripeptides, based on the MLF sequence, is that of the *N*-alkoxycarbonyl derivatives (amino-terminal carbamates) [6,11,12] recently studied in detail by Higgins et al. [9]. This functionality maintains the receptor binding affinity and can impart agonist or antagonist activity to the tripeptide depending on the nature of the alkyl group. In particular, it has been proven that *N*-terminal carbamates with unbranched small alkyl groups generate agonists whereas those with branched and sterically demanding substituents result in antagonist peptides. Furthermore, in addition to the property of modulating the agonist versus antagonist activity the *N*-terminal carbamate functionalization leads to ligands which are significantly and specifically influenced by the nature of the *C*-terminal modification in a manner which is not encountered in the case of the classic *N*-formyl–tripeptides. An example is represented by the relative potency of *C*-terminal methyl

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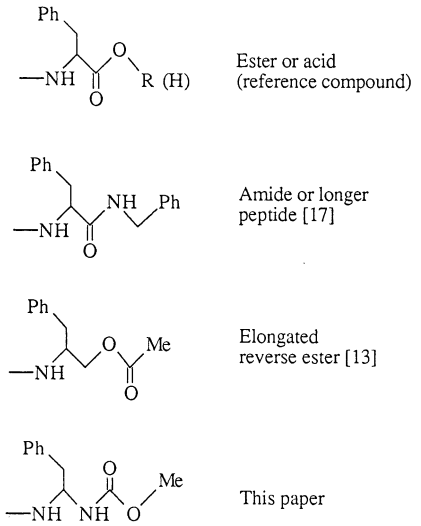
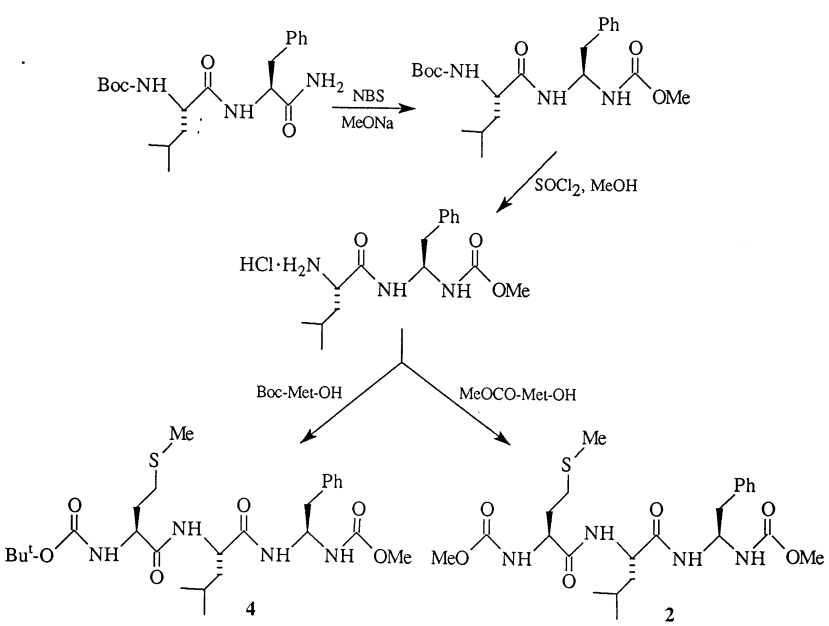


Fig. 1. Functionalities at the *C*-terminal Phe carboxyl group of MLF based tripeptides analogues discussed in the text.

esters compared to their corresponding acids; this is found to be specific for each pair of carbamates and an ester can be more or less active than the corresponding acid depending upon the nature of the *N*-terminal carbamate [9]. Thus, in addition to the classic and well known *N*-formyl tripeptides, the *N*-alkoxycarbonyl derivatives of the reference tripeptide Met–Leu–Phe represent versatile lead compounds for designing new ligands and for structure activity studies.

As prosecution of our previous studies in the field [13–16] and by taking into account the tendency of the *N*-terminal carbamates to be highly influenced by the nature of the *C*-terminal functionalization, it seemed interesting to examine the activity of a new type of tripeptide ligands possessing the carbamate functionality both at the *N*-terminal and *C*-terminal position. These biscarbamate analogues should represent efficient ligands since the *C*-terminal carbamate group maintains, as compared with acids and esters, the carbonylic oxygen and contains in addition a NH group. This observation is in accordance with results of early studies on chemotactic tripeptides, which put in evidence that a crucial requirement for the activity was, in addition to the *N*-terminal formylation, the presence at the third position of a carbonylic oxygen as H-bond acceptor; furthermore, the presence of an additional NH group, as in the case of tetrapeptides or tripeptide amides of a primary amine as benzylamine, led to derivatives more potent than the parent [17]. These data suggest critical interactions of both the *N*- and *C*-terminal end of the tripeptide ligand with the corresponding receptor areas and a significant role of H-bonding interactions in correspondence of the *C*-terminal residue. Fig. 1 reports a series of *C*-terminal functionalizations which have been found compatible with the activity, together with the here adopted carbamate modification; it is worth noting that these new ligands, based on the prototypical MLF sequence, represent the first examples of analogues in which the carbamate functionality has been introduced at both ends of the peptide molecule.



Scheme 1.

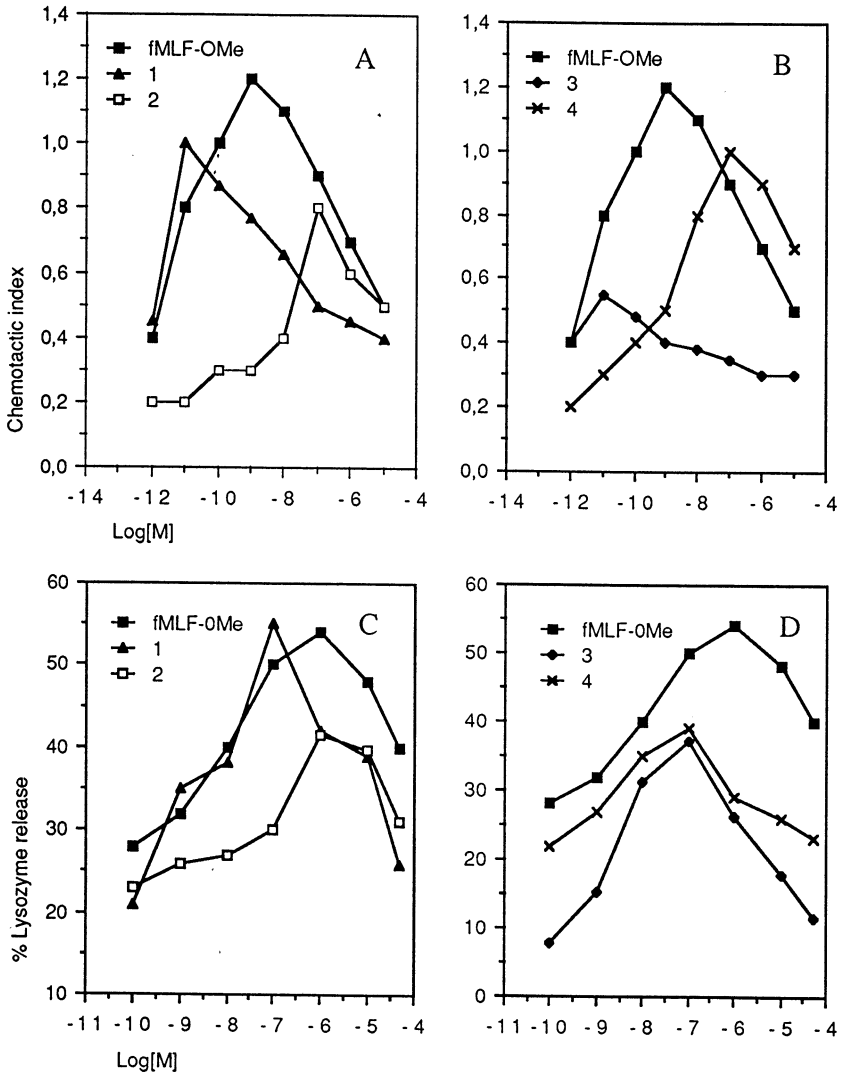


Fig. 2. Biological activity of fMLF–OMe, methyl carbamate analogues **1**, **2**, and Boc derivatives **3**, **4** towards human neutrophils. (A and B) Chemotactic activity. (C and D) Release of neutrophil granule enzymes evaluated by determining lysozyme activity.

2. Chemistry

The two biscarbamates MeOCO–Met–Leu–gPhe–COOMe (**2**) and Boc–Met–Leu–gPhe–COOMe (**4**) were synthesized according to Scheme 1. The transformation of Boc–Leu–Phe–NH_2 into the compound containing the *gem*-diamino analogue of the Phe residue, namely Boc–Leu–gPhe–COOMe, was performed by adopting a modified procedure of the Hofmann rearrangement. Deprotection of the intermediate biscarbamate with $SOCl_2$/MeOH afforded the key derivative HCl·Leu–gPhe–COOMe. Coupling of this carbamate with MeOCO–Met–OH or Boc–Met–OH to give the biscarbamates MeOCO–Met–Leu–gPhe–COOMe (**2**) and Boc–Met–Leu–gPhe–COOMe (**4**), respectively, was performed by the mixed anhydride method with isobutyl chloroformate. The activity of **2** and **4** was compared to that of the corresponding and already known *N*-terminal monocarbamate methyl esters MeOCO–Met–Leu–Phe–OMe (**1**) and NBoc–Met–Leu–Phe–OMe (**3**).

3. Biological results

The biological activity of the carbamate-modified ligands **1**–**4** has been determined on human neutrophils and compared with that of the standard agonist tripeptide fMLF–OMe; directed migration (chemotaxis), superoxide anion production, and lysozyme release have been measured. All the tested derivatives were found unable to elicit superoxide anion production. As shown in Fig. 2(A), the methyl carbamates **1** is significantly more active, as chemoattractant, than the dimethoxycarbonyl derivative **2** containing the gPhe residue. In particular the fMLF–OMe analogue **1** is, although less

potent, more efficient than the parent, being maximally effective at the concentration of 10^{-11} M. As compared with the methyl carbamate **1**, the corresponding Boc derivative **3** exhibits a lower chemotactic activity (Fig. 2(A) and (B)); on the contrary, the biscarbamate Boc–Met–Leu–gPhe–COOMe (**4**) shows an activity peak higher than that exhibited by the corresponding dimethoxycarbonyl derivative **2** at concentration of 10^{-7} M. When tested as secretagogue agent, the tripeptide **1** shows, at low concentration (10^{-7} M), an activity higher than the parent (Fig. 2(C)); a significantly lower potency is found in the case of the three other methyl carbamates **2**, **3**, and **4**. The Boc derivatives **3**–**4** show a homogeneous dose dependent behavior, and a similar activity as degranulation inducers (Fig. 2(D)). Finally, no substantial change in lysozyme release was detected after the substitution in **3** of the Phe with gPhe residue.

4. Discussion

An analysis of the reported biological results indicates that the introduction of a *C*-terminal carbamate function into the *N*-methoxycarbonyl derivative **1**, obtained by replacing of the Phe with the gPhe residue, causes a significant decrease of the directed migration activity and lysozyme release of the resulting modified derivative **2**. A different behaviour is exhibited by the *N*-*t*-butoxycarbonyl derivative **3**; in this case, an increase of chemotactic activity, although observed at concentration 10^{-7} M, is shown by the corresponding biscarbamate **4**. Thus, the same *C*-terminal modification is beneficial or detrimental depending upon the nature of the *N*-terminal protecting group. This result corresponds to the observation recently made by Higgins et al. [9] when examining a series of different amino-terminal monocarbamate analogues of fMLF.

The results reported in Fig. 2(A) and (B) show that the methoxycarbonyl derivative **1** exhibits chemotactic activity on human neutrophils whereas the bulky and branched analogue **3** is practically inactive. Whereas these findings could be anticipated on the basis of literature data, it is worth noting that the chemotactic activity of these compounds has not been reported before.

Synthesis of analogues of bioactive peptides, obtained by adopting the here reported approach of a symmetrical functionalization at both ends of the molecule, is now being further examined in our laboratories.

5. Experimental

5.1. Chemistry

Melting points were determined with a Kofler hot stage apparatus and are uncorrected. Optical rotations were taken at 20°C with a Schmidt–Haensch Polartronic D polarimeter (1 dm cell). IR spectra were recorded employing a Perkin–Elmer 983 spectrophotometer. ^{1}H NMR spectra were recorded on a Bruker AM 200 spectrometer with Me_4Si as internal standard. Thin-layer (TLC) and preparative chromatographies (PLC) were performed on silica gel Merck 60 F_{254} plates. The drying agent was sodium sulfate. Boc–Met–OH (Fluka Chemie AG, Switzerland) was employed without purification. MeOCO–Met–OH [9], MeOCO–Met–Leu–Phe–OMe [9], Boc–Leu–Phe–NH_2 [18], Boc–Met–Leu–Phe–OMe [19], and parent fMLF–OMe [19] were prepared as described elsewhere. Elemental analyses were performed in the laboratories of the Servizio Microanalisi del CNR, Area della Ricerca di Roma, Montelibretti, Italy and were within $\pm 0.4\%$ theoretical values. The abbreviations used are as follows: Boc, *t*-butoxycarbonyl; DMF, *N*,*N*-dimethylformamide; gPhe, *gem*-diamino analog of phenylalanine; KRPG, Krebs–Ringer-phosphate containing 0.1% w/v glucose (pH 7.4); NBS, *N*-bromosuccinimide; NMM, *N*-methylmorpholine.

5.1.1. Boc–Leu–gPhe–COOMe

Following the procedure of Huang and Keillor [20], to a solution of MeONa, prepared by the addition of Na (0.485 g, 21 mmol) to dry methanol (24 ml), Boc–Leu–Phe–NH_2 (0.612 g, 1.62 mmol) and NBS (96.9%; 0.299 g, 1.62 mmol) were added and the reaction mixture was heated to reflux. After time intervals of 3 and 6 min, additional portions of NBS (96.9%; 0.149 g, 0.81mmol) were added. After refluxing for a total of 10 min, the solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate, washing with brine to neutrality. The organic phase was dried and evaporated to give an oily residue which was purified by PLC [CH_2Cl_2/EtOAc (95:5) as eluant], affording homogeneous title compound (0.319 g, 48%) as an oil. $[\alpha]_D = -28°$ ($c = 2.0$, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3422, 2956, 2933, 1690, 1495, 1162. ^{1}H NMR ($CDCl_3$): δ (ppm) 0.83 [6H, m, $(CH_3)_2CH$], 1.20–1.70 [12H, m, $C(CH_3)_3$ (s at 1.40) superimposed on Leu β-CH_2 and γ-CH], 2.90 (2H, m, gPhe β-CH_2), 3.27 (3H, s, $COOCH_3$), 4.01 (1H, m, Leu α-CH), 4.97 (1H, d, $J = 8.2$ Hz, Leu NH), 5.34 (1H, m, gPhe α-CH), 6.51 (1H, d, $J = 9.5$ Hz, gPhe NH–CO), 7.10–7.42 (5H, m, aromatic). Two isomers have been detected and the signals of the major component are reported. *Anal.* (C, H, N) for $C_{21}H_{33}N_3O_5$.

5.1.2. Boc–Met–Leu–gPhe–COOMe (**4**)

Thionyl chloride (0.05 ml, 0.633 mmol) was added dropwise to a solution of the above biscarbamate (0.258 g, 0.633 mmol) in dry methanol (1.3 ml), cooled at −15°C. After stirring at −15°C for 30 min and at 45°C for 2 and a half hours, the solution was evapo-

rated under vacuum to give HCl·H–Leu–gPhe–COOMe as a foam (0.212 g). This salt was used without further purification. Isobutyl chloroformate (98%, 0.084 ml, 0.633 mmol) was added at −15°C to a stirred solution of Boc–Met–OH (0.158 g, 0.633 mmol) and NMM (0.084 ml, 0.76 mmol) in dry CH_2Cl_2 (3 ml). The temperature was kept at −15°C for 10 min, then a solution of the above prepared HCl·H–Leu–gPhe–COOMe and NMM (0.07 ml, 0.633 mmol) in dry CH_2Cl_2 (2.3 ml) was added. The mixture, stirred at −15°C for 15 min and at room temperature for 1 day, was then evaporated under vacuum. The residue was dissolved in ethyl acetate and washed with 2 N HCl, brine, saturated aqueous $NaHCO_3$, and brine. The organic phase was dried and evaporated to give a residue (0.333 g), which was purified by PLC [CH_2Cl_2/EtOAc (7:3) as eluant], affording pure Boc–Met–Leu–gPhe–COOMe (**4**) (0.145 g, 42%). M.p. 151–152°C (EtOAc/*n*-hexane). $[\alpha]_D = -35°$ ($c = 1.0$, $CHCl_3$). IR (KBr) cm^{-1}: 3314, 2957, 2931, 1690, 1646, 1527, 1170. ^1H NMR ($CDCl_3$): δ (ppm) 0.81 [6H, m, $(CH_3)_2CH$], 1.15–1.60 [12H, m, $C(CH_3)_3$ (s at 1.44) superimposed on Leu β-CH_2 and γ-CH], 1.75–2.02 (2H, m, Met β-CH_2), 2.08 (3H, s, S-CH_3), 2.52 (2H, t, $J = 7.1$ Hz, CH_2–S), 2.87 and 3.03 (2H, A and B of an ABX, $J = 5.8$, 7.0, and 14.0 Hz, gPhe β-CH_2), 3.29 (3H, s, $COOCH_3$), 4.31 (1H, m, Met α-CH), 4.38 (1H, m, Leu α-CH), 5.35 (1H, m, gPhe α-CH), 5.53 (1H, d, $J = 7.1$ Hz, Met NH), 6.96 (2H, apparent d, Leu NH and gPhe NH), 7.12–7.37 (5H, m, aromatic). *Anal.* (C, H, N) for $C_{26}H_{42}N_4O_6S$.

5.1.3. MeOCO–Met–Leu–gPhe–COOMe (2)

Following the procedures above described for the synthesis of Boc–Met–Leu–gPhe–COOMe, Boc–Leu–gPhe–COOMe (0.319 g, 0.782 mmol) and an equimolar amount of MeOCO–Met–OH (0.162 g) gave, after final PLC, pure MeOCO–Met–Leu–gPhe–COOMe (**2**) (0.154 g, 40%). M.p. 162–163°C (EtOAc/*n*-hexane). $[\alpha]_D = -39°$ ($c = 1.0$, $CHCl_3$). IR (KBr) cm^{-1}: 3295, 2955, 2930, 1694, 1642, 1533. ^1H NMR $(CD_3)_2SO$: δ (ppm) 0.74 and 0.79 [6H, two d, $J = 6$ Hz, $(CH_3)_2CH$], 1.09–1.40 [3H, m, Leu β-CH_2 and γ-CH], 1.62–1.90 (2H, m, Met β-CH_2), 2.01 (3H, s, S–CH_3), 2.42 (2H, t, $J = 7.7$ Hz, CH_2–S), 2.75 and 2.93 (2H, A and B of an ABX, $J = 4.8$, 8.6, and 14.0 Hz, gPhe β-CH_2), 3.15 (3H, s, gPhe NH–$COOCH_3$), 3.52 (3H, s, Met NH–$COOCH_3$), 4.06 (1H, m, Met α-CH), 4.18 (1H, m, Leu α-CH), 5.08 (1H, m, gPhe α-CH), 7.10–7.42 [6H, m, aromatic and Met NH (d, $J = 8.0$ Hz)], 7.94 (1H, d, $J = 7.6$ Hz, Leu NH), 8.43 (1H, d, $J = 9.3$ Hz, gPhe NH). *Anal.* (C, H, N) for $C_{23}H_{36}N_4O_6S$.

5.2. Biological assay

5.2.1. Peptides

Stock solutions, 10^{-2} M of fMLF–OMe and peptide analogues, were prepared in dimethyl sulphoxide and diluted in KRPG, before use. At the concentration used, dimethyl sulphoxide did not interfere with any of the biological assays performed.

5.2.2. Cell preparation

Cells were obtained from the blood of healthy subjects, and human peripheral blood neutrophils were purified employing the standard techniques of dextran (Pharmacia, Uppsala, Sweden) sedimentation, centrifugation on Ficoll–Paque (Pharmacia), and hypotonic lysis of contaminating red cells. The cells were washed twice and resuspended in KRPG, at a final concentration of 50×10^6 cells/ml and kept at room temperature until used. The percentage of neutrophils was 98–100% pure and ≥99% viable, as determined by the Trypan blue exclusion test.

5.2.3. Random locomotion

Random locomotion was performed with 48-well microchemotaxis chamber (Bio Probe, Milan, Italy) and the migration into the filter was evaluated by the method of leading-front [21]. The actual control random movement is 32 μm ± 3 SE of ten separate experiments performed in duplicate.

5.2.4. Chemotaxis

In order to study the potential chemotactic activity, each peptide was added to the lower compartment of the chemotaxis chamber. Peptides were diluted from a stock solution with KRPG containing 1 mg/ml of bovine serum albumin (Orha Behringwerke, Germany) and used at concentrations ranging from 10^{-12} to 10^{-5} M. Data were expressed in terms of chemotactic index, which is the ratio: (migration toward test attractant minus migration toward the buffer)/migration toward the buffer; the values are the mean of six separate experiments performed in duplicate. Standard errors are in the 0.02–0.09 chemotactic index range.

5.2.5. Superoxide anion (O_2^-) production

The superoxide anion was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome *c* (Sigma, St. Louis, MO, USA) modified for microplate-based assays. Tests were carried out in a final volume of 200 μl containing 4×10^5 neutrophils, 100 nmol cytochrome *c* (Sigma) and KRPG. At zero time, different amounts ($10^{-9} - 2 \times 10^{-5}$ M) of each peptide were added and the plates were incubated into a microplate reader (Ceres 900, Bio-TeK Instruments, Inc.) with the compartment temperature set at 37°C. Absorbance was recorded at wavelengths of 550 and 465 nm. Differences in absorbance at the two wavelengths were used to calculate nmol of O_2^- produced using an absorptivity for cytochrome *c* of 15.5 mM^{-1}/cm. Neutrophils were incubated with 5 μg/ml cytochalasin B (Sigma) for 5 min prior to activation by peptides.

5.2.6. Enzyme assay

The release of neutrophil granule enzymes was evaluated by determination of lysozyme activity, modified for microplate-based assays. Cells, 3×10^6/well, were first incubated in triplicate wells of microplates with 5 μg/ml cytochalasin B at 37°C for 15 min and then in the presence of each peptide in a final concentration of $10^{-10} - 2 \times 10^{-5}$ M for a further 15 min. The plates were then centrifuged at $400 \times g$ for 5 min and the lysozyme was quantified nephelometrically by the rate of lysis of cell wall suspension of *Micrococcus lysodeikticus*. The reaction rate was measured using a microplate reader at 465 nm. Enzyme release was expressed as a net percentage of total enzyme content released by 0.1% Triton X-100. Total enzyme activity was 85 ± 1 μg/1×10^7 cells/min.

5.2.7. Statistical analysis

The non-parametric Wilcoxon test was used in the statistical evaluation of differences between groups.

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