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Chemotactic peptides: fMLF-OMe analogues incorporating proline–methionine chimeras as N-terminal residue

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Abstract—The new fMLF analogues **1**–**4**, incorporating chimeric *S*-proline–methionine residues (namely the homochiral *cis*-4(*S*)-methylthio-(*S*)-proline (**10**) and the heterochiral *trans*-4(*R*)-methylthio-(*S*)-proline) (**17**) in place of the native *S*-methionine, have been prepared; their solution conformation and activity as agonists or antagonists of formylpeptide receptors have been studied. In addition to peptides **1**–**4**, which maintain the Met γ-thiomethyl-ether function, the analogues Boc-PLF-OMe (**18**) and For-PLF-OMe (**19**) devoid, as compared with **1**–**4**, of position 1 side chain, have been synthesized and their activity examined.

1. Introduction

Neutrophils, which are activated by the interaction of their plasma membrane receptors with chemoattractants, represent the first line of host defense against bacterial and fungal infections.[1,2] Chemoattractants bind to G-protein coupled receptors and trigger chemotaxis as well as a subsequent cascade of biochemical events such as lysosomal enzyme release and superoxide anion production.[3–5] Since the discovery of *N*-formyl-methionine-containing peptides as potent agonists at the neutrophil chemotactic receptors, the hydrophobic *N*-formyl tripeptide For-Met-Leu-Phe-OH (fMLF) and its methyl ester (fMLF-OMe) have been chosen as the reference molecules for a systematic research on biochemical mechanism and structure–activity relationships.[6,7] Results of these studies firmly establish the key role of the sulfur-containing side chain of the N-terminal methionine residue for both receptor recognition and optimal activity of *N*-formylpeptide ligands. Thus, although different alterations at the central Leu and C-terminal Phe residues of the reference tripeptide are tolerated, replacement or alteration at the N-terminal Met side chain causes, with very rare exceptions,[8,9] significant to very high decrease of the chemotactic activity.[7,10] To explain this point it has been proposed that a discrete area of positive charge is present, around the relatively electron-rich sulfur atom, in the hydrophobic pocket of the receptor devoted to the accommodation of the Met side chain.[7] This conclusion, when considered together with the well-established critical role for binding and activity exerted by the chemical nature and overall size of the group attached to the Met NH_2,[11–15] clearly suggests the importance of the receptor area specifically structured to accommodate the N-terminal moiety of the chemotactic tripeptides. In correspondence of this region, a still not well understood interplay between the sulfurated Met side chain and the N-protecting group concurs to determine an environment which is critical for the overall fit of the tripeptide into the

Abbreviations: Boc_2O, di-*tert*-butyl dicarbonate; $\Delta\delta$, ^1H NMR chemical shift difference (ppm) exhibited by the NH protons in $CDCl_3$ solution containing 10% DMSO-d_6 and in neat $CDCl_3$; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; EEDQ, ethyl 2-ethoxy-1,2-dihydro-1-quinolinecarboxylate; EtOAc, ethyl acetate; OMe, methoxy; *i*-BuOCOCl, isobutyl chloroformate; MsCl, methanesulfonyl chloride; NMM, *N*-methylmorpholine; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

Keywords: Chemotaxis; Conformation; Formylpeptides; Human neutrophils; Proline–methionine chimeras.

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receptor pocket.[14] Thus, it is not surprising that several fMLF analogues, modified at the N-terminal protecting group and/or at the Met side chain, have been synthesized and used in an effort to gather information on the structure of the receptor–ligand complex in correspondence of the N-terminus. However, an examination of the literature reveals that, among the several synthetic fMLF analogues modified at the N-terminal residue, those which maintain intact the critical γ-thiomethylether side chain are surprisingly scarce. In Figure 1, the structures of the so far used analogues of the methionine residue, which exhibit this structural feature and have been used to synthesize fMLF analogues, are shown.[16–18]

Based on the above-reported literature data and as prosecution of our previous studies in this field,[18,19] it seemed interesting to examine new fMLF analogues incorporating N-terminal residues in which the native Met side chain is maintained although conformationally restricted around the critical C^{α}-C^{β} (χ_1) and/or C^{β}-C^{γ} (χ_2) torsion angles. These compounds appear in fact well suited to give information on the role of the spatial orientation of the Met side chain on binding and activity. To the best of our knowledge, the utilization of chemoattractant ligands containing residues of this type (e.g., 2,3-didehydro-Met, 2,3-cyclopropane-Met, proline–methionine chimeras) has not been described so far. Here, we report studies on this topic and describe the results obtained by examining the new fMLF analogues **1**–**4** incorporating chimeric L-proline–methionine residues (namely the homochiral *cis*-4(*S*)-methylthio-(*S*)-proline (**10**) and the heterochiral *trans*-4(*R*)-methylthio-(*S*)-proline) (**17**), in place of the native *S*(L)-methionine. These new ligands maintain the γ-thiomethyl-ether functionality and this is covalently bound, with opposite spatial orientation, at the quite rigid pyrrolidine skeleton which fixes the χ_1 and χ_2 torsion angles of the residue. In this same context and to evaluate the role on the activity possibly exerted by the presence of the N-terminal unsubstituted Pro pyrrolidine ring, we have synthesized and examined, in addition to the tripeptides **1**–**4**, the *N*-Boc and *N*-formyl derivatives (**18** and **19**, respectively) of the tripeptide H-Pro-Leu-Phe-OMe.

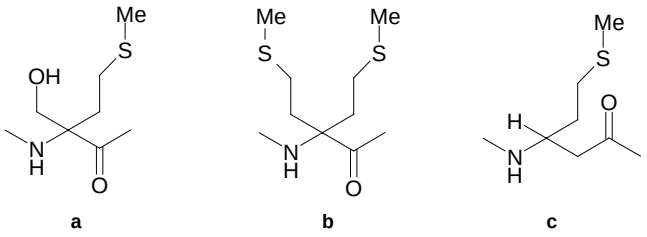


Figure 1. Amino acid residues, containing the γ-methylthio functionality, used to replace the native methionine in synthetic fMLF analogues: (a) C^{α}-hydroxymethyl methionine (HmMet);[17] (b) 2-[2′-(methylthio)ethyl]methionine (BisMet);[16] (c) β^3-HMet.[18]

2. Chemistry

A literature examination shows that different approaches have been followed to obtain *cis*- and *trans*-4-methylthio-proline derivatives.[20–23] To synthesize the still unknown stereoisomers *N*-Boc-*cis*-4(*S*)-methylthio-(*S*)-proline (**10**) and *N*-Boc-*trans*-4(*R*)-methylthio-(*S*)-proline (**17**), we found it convenient, in terms of both overall yields and control of the stereochemistry at the C-4 carbon atom, to follow the strategy reported in Schemes 1 and 2, respectively. Thus, the *N*-Boc-*cis*-analogue **10** has been obtained starting from the commercially available 4-hydroxy-*trans*-Pro (**5**) by treatment of the corresponding *N*-Boc-*trans*-4-mesylate **8** with potassium thioacetate followed by mild hydrolysis of the thioester **9**, alkylation of the thiol group and subsequent hydrolysis to give **10**. The *N*-Boc derivative **11** of 4-hydroxy-*trans*-Pro (**5**) has been used to synthesize the *N*-Boc-*trans*-analogue **17** (Scheme 2). In this case, two successive configurational inversions of the chiral centre at position 4 have been performed; the first inversion takes place with the intermediacy of the 4-oxo-analogue **12** which, through stereoselective reduction with $NaBH_4$,[24] provides the *N*-Boc-(2S,4S)-*cis*-isomer **13**. The second inversion derives from the nucleophilic substitution with potassium thioacetate already applied in Scheme 1.

Coupling of H-Leu-Phe-OMe with the *N*-Boc-*cis*-(**10**) or *N*-Boc-*trans*-4-methylthio-(*S*)-proline (**17**), to give the *N*-Boc-protected tripeptides **1** and **3**, respectively, was performed by the mixed anhydride method with isobutyl chloroformate (Scheme 3). Formylation of Boc-tripeptide methyl esters to afford *N*-formyl-tripeptides **2** and **4** was accomplished in one step by following the procedure of Lajoie and Kraus.[25]

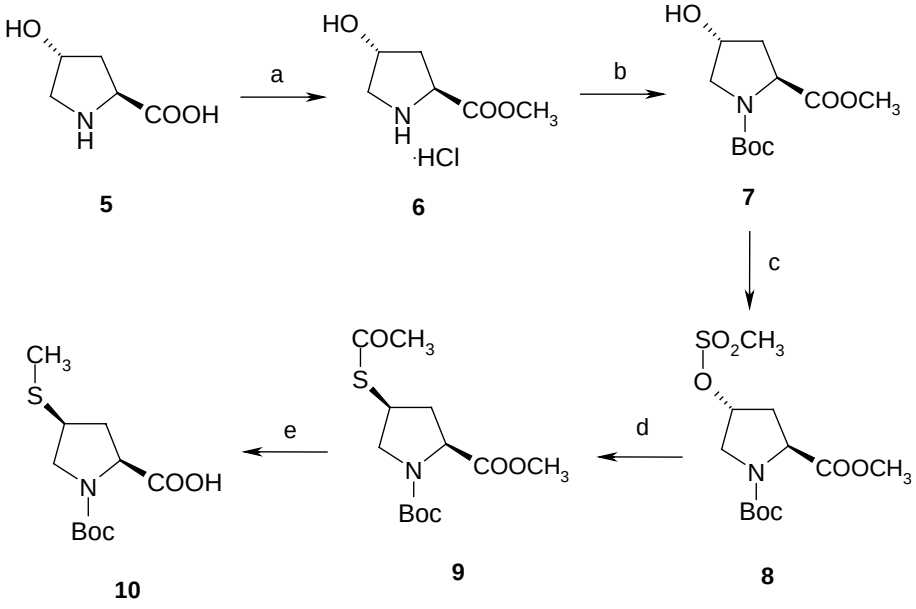
3. Synthesis

For synthesis refer Schemes 1–3.

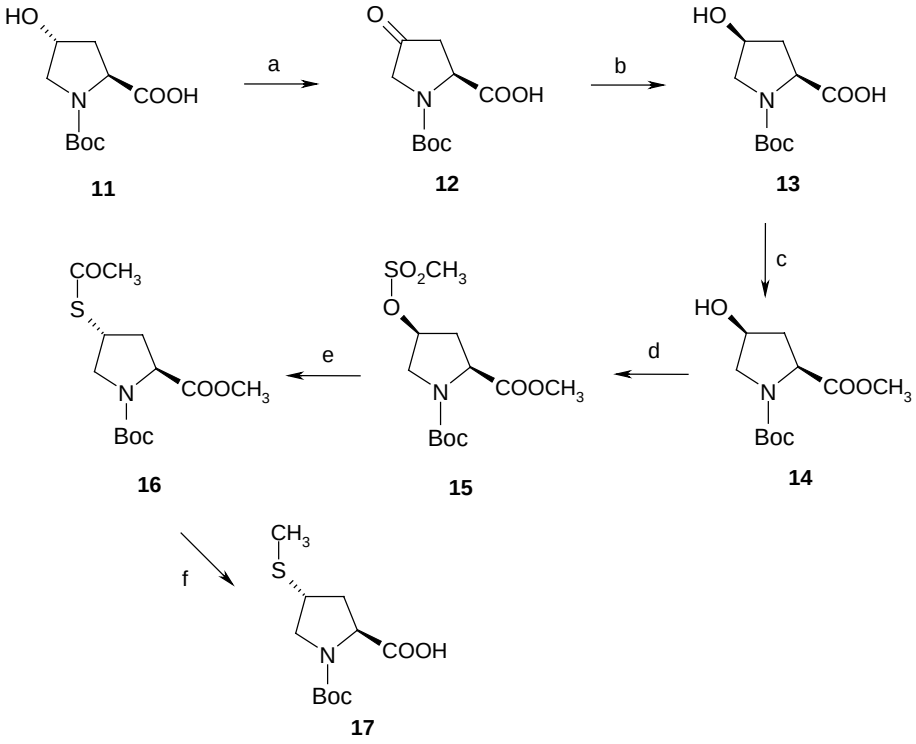
4. ^{1}H NMR studies

With the aim to probe whether the different spatial arrangement of the proline–SMe group influences the conformational preferences adopted in $CDCl_3$ solution by the diastereisomeric N-protected tripeptides containing *cis*- or *trans*-4-mercaptomethyl-proline, a ^{1}H NMR study on HCO-*cis*-(**2**) and HCO-*trans*-4-mercaptomethyl-Pro-Leu-Phe-OMe (**4**) was undertaken. Table 1 reports the observed nuclear Overhauser effects (NOEs) in the spectra of the two tripeptides. For both these diastereoisomeric compounds the proton at the Pro C^5 carbon atom, which is *cis* to the Pro $C^{\alpha}H$, has been termed H_A and that *trans* to the same proton has been termed H_B; thus, due to the different stereochemistry of the two tripeptides at the Pro C^4 (γ-position), the proton H_A is *trans* to the –SMe in the tripeptide **2** and *cis* to the same group in the tripeptide **4**.

Except for the absence of the intraresidue Pro $C^{\alpha}H\cdots$ Pro C^4H NOE, characteristic of the *cis* arrangement of

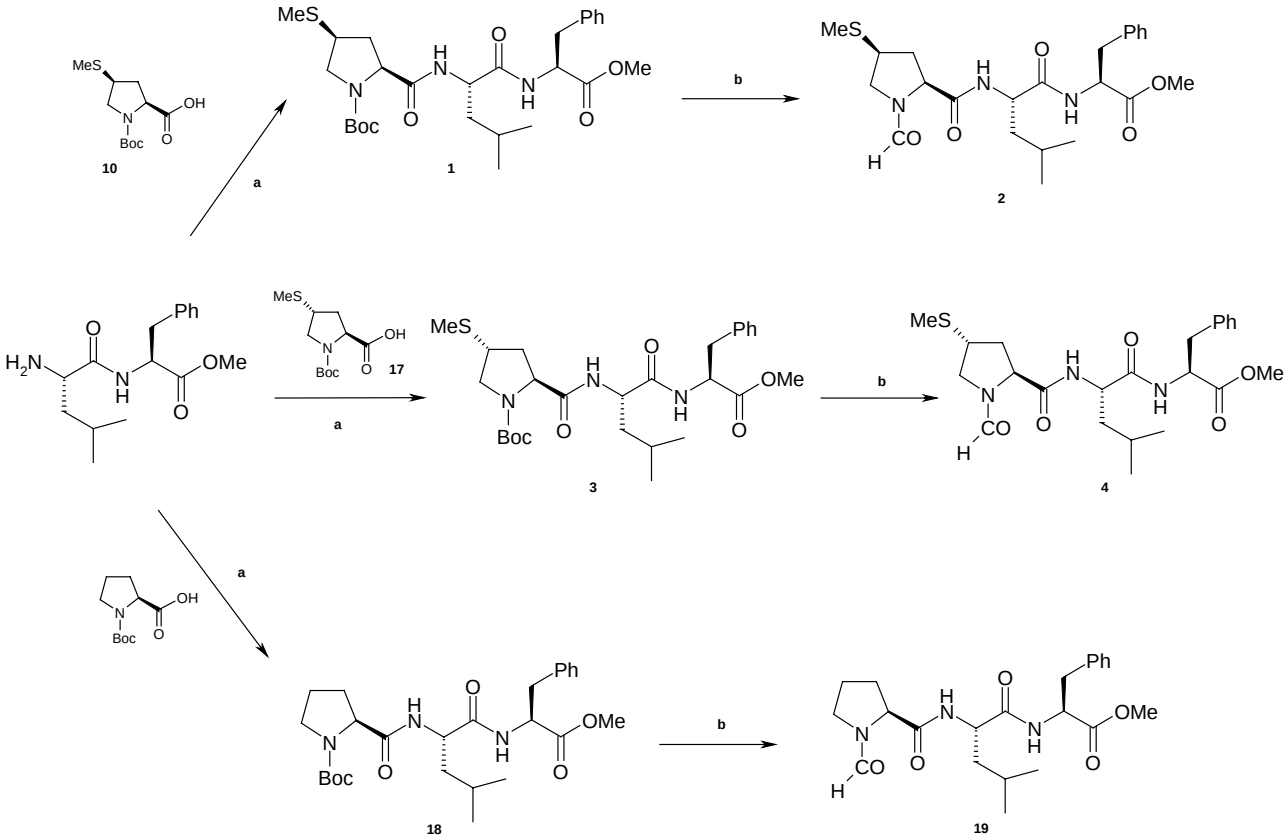


Scheme 1. Reagents and conditions: (a) $SOCl_2$/MeOH 2 h at rt, then 4 h at 45 °C; (b) Boc_2O, dioxane/Na_2CO_3; (c) MsCl, TEA, CH_2Cl_2, 20 min at 0 °C; (d) $KSCOCH_3$/DMF, 4 h at 65 °C; (e) 1 N NaOH and MeO_2SO_2, 30 min at rt, then 1 N NaOH, 3 h at rt.



Scheme 2. Reagents and conditions: (a) CrO_3/Py, 30 min at 0 °C, then 90 min at rt; (b) $NaBH_4/H_2O$, MeOH, 1 h at 0 °C and 15 h at 4 °C; (c) diazomethane/MeOH; (d) MsCl, TEA, CH_2Cl_2, 20 min at 0 °C; (e) $KSCOCH_3$, DMF, 4 h at 65 °C; (f) 1 N NaOH and $(MeO)_2SO_2$, 30 min at rt, then 1 N NaOH 3 mmol, 3 h at rt.

these two protons in the *cis*-4-mercaptomethyl-Pro residue, the NOESY spectrum of the tripeptide **4**, containing the *trans*-4-mercaptomethyl-Pro residue, is very similar to that of the diastereoisomeric compound **2**. However, while in the tripeptide **2** the H-CO proton is NOE related to the Pro C^5H_A which is *trans* to the –SMe and resonates at lower field (H_A 3.97 δ; H_B 3.37 δ), in diastereoisomer **4** an analogous NOE is exhibited by the proton on the Pro C^5 which is *cis* to the –SMe and resonates at higher field (H_A 3.53 δ; H_B 3.80 δ). This effect is caused by the different arrangement of the 4-mercaptomethyl group in the two diastereisomers and indicates that a shielding effect is exerted by the –SMe group on the adjacent cisoidal proton. The assignment



Scheme 3. Synthesis of *N*-protected tripeptides **1**–**4** and **18**–**19**. Reagents: (a) *i*-BuOCOCl, NMM; (b) HCOOH, EEDQ.

Table 1. Observed nuclear Overhauser effects (NOEs) in the NOESY spectra of HCO-*cis*-4-mercaptomethyl-Pro-Leu-Phe-OMe (**2**) and HCO-*trans*-4-mercaptomethyl-Pro-Leu-Phe-OMe (**4**)

NOEs	Compounds[a]	
	2	**4**
Pro $C^{\alpha}H \cdots$ Leu NH	s	s
Pro $C^{5}H_{A} \cdots$ H-CO	m	m
Pro $C^{\alpha}H \cdots$ Pro $C^{4}H$	w	—
Pro $C^{5}H_{A} \cdots$ Pro $C^{4}H$	m	—
Pro C^{5} $H_{B} \cdots$ Pro $C^{4}H$	—	m
Leu $C^{\alpha}H \cdots$ Leu NH	m	m
Leu $C^{\alpha}H \cdots$ Phe NH	s	s
Leu NH $\cdots$ Phe NH	w	w
Phe $C^{\alpha}H \cdots$ Phe NH	m	m
Pro $C^{5}H_{A} \cdots$ Pro SCH_3	—	w

[a] s, strong; m, medium; w, weak.

of the configuration to the Pro $C^{5}H_{A}$ proton, involved in the NOE with H-CO, was made, in the case of the tripeptide **2** (see Fig. 3), on the basis of its spatial connectivity with the $C^{4}H$ whose stereochemistry is known. Analogously, in the case of the tripeptide **4**, the $C^{5}H_{A}$ configuration has been deduced by the lack of connectivity of this proton with the $C^{4}H$ and the contemporary presence of the spatial connectivities $C^{5}H_{B}/C^{4}H$ and $C^{5}H_{A}$/SMe (see Table 1).

Particularly useful in defining the preferred backbone conformation of **2** and **4** are the two inter-residue NOEs Pro $C^{\alpha}H \cdots$Leu NH and Leu $C^{\alpha}H \cdots$Phe NH. These successive NOEs ($C_{i}^{\alpha}H \cdots N_{i+1}H$) are in fact indicative of an extended backbone conformation or, as recently reported, of a double γ-turn (C_7 structures) stabilized by two consecutive 1 ← 3 H-bonds.[26]

Further information concerning the involvement of the two NH groups of the tripeptides **2** and **4** in intramolecular hydrogen bonds was obtained by evaluating the chemical shift solvent dependence in $CDCl_3$-$(CD_3)_2SO$ mixtures. Also in this case (see Fig. 2) the shifts of NH signals of tripeptide **2**, caused by the increasing concentration of the DMSO-d_6, are similar to those observed for the diastereoisomeric derivative **4**. The Leu NH appears strongly solvent shielded ($\Delta\delta = 0.24$ and 0.25 ppm in the case of **2** and **4**, respectively) and a low solvent accessibility is also shown by the Phe NH ($\Delta\delta = 0.47$ and 0.48 ppm in the case of **2** and **4**, respectively). These data indicate that Leu NH and, to a less extent, the Phe NH groups are presumably involved in intramolecular hydrogen bonds.

Both the above-reported NOEs and titration data suggest that the two diastereoisomeric formyltripeptides adopt in $CDCl_3$ a preferred conformation characterized by a system of two consecutive γ-turns centred at the first two residues (Fig. 3). The occurrence of the C_7 structure centred on the N-terminal residue is in accordance with the observation of the H-CO$\cdots$Pro $C^{5}H_{A}$NOE connectivity (Fig. 3). The lower solvent shielding of the Phe NH, as compared with the Leu-NH, may be ascribed to the presence of a certain

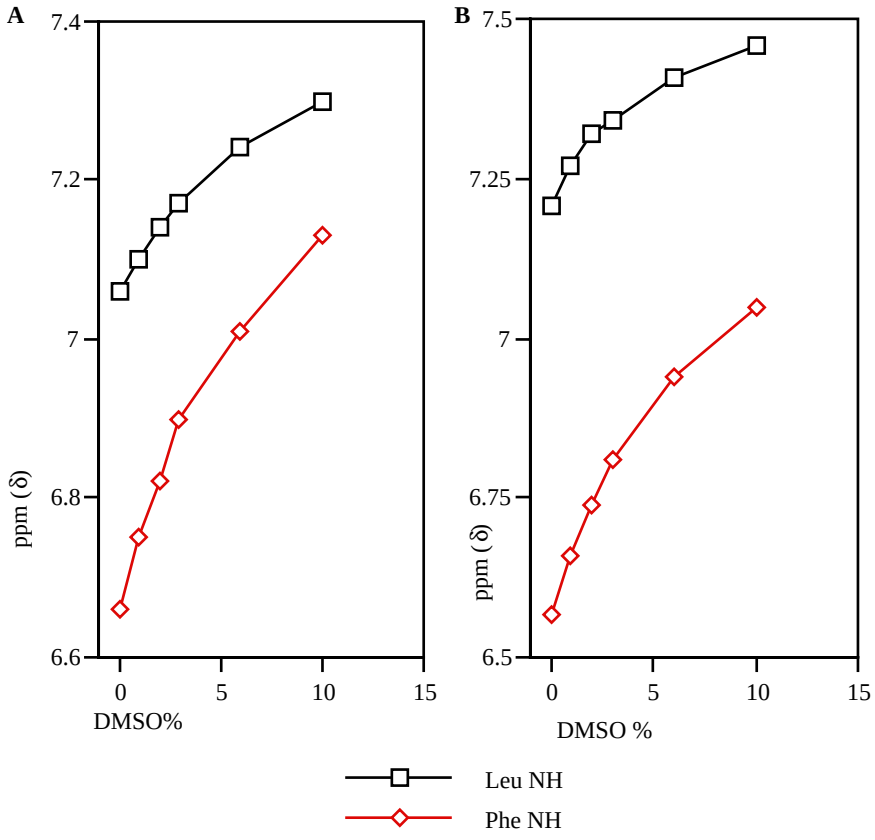


Figure 2. Plots of NH proton chemical shifts in the ^{1}H NMR spectra of dipeptide derivatives **2** (A) and **4** (B) as a function of increasing amounts of DMSO-d_6 (v/v) added to the $CDCl_3$ solution (peptide concentration 10 mM).

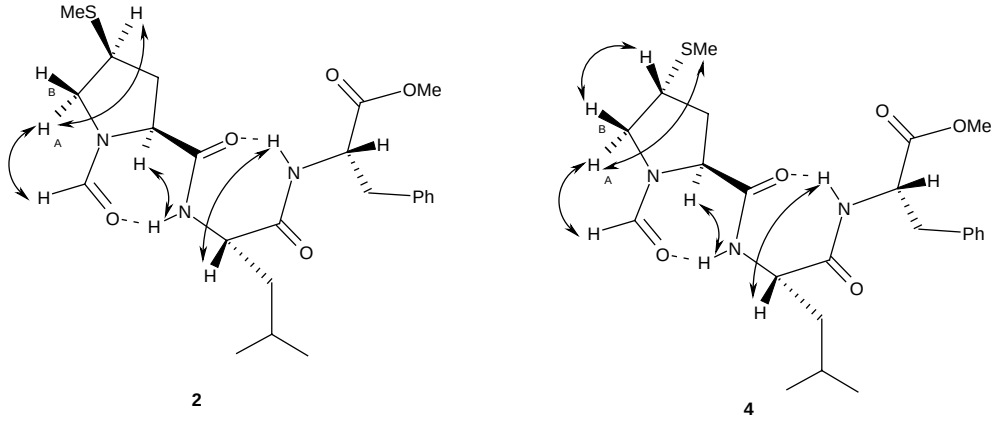


Figure 3. The double γ-turn conformation of the tripeptides **2** and **4** containing *cis* and *trans*-4-mercaptomethyl-L-proline, respectively. Bold arrows indicate the diagnostic NOEs, dotted lines the intramolecular 1 ← 3 H-bonds.

population of conformers in which the C-terminal part of the backbone maintains an extended conformation. Finally, the lack of a strong NOE between the Leu and Phe NH groups rules out the occurrence of any significant population of conformers adopting β-turn folding.

5. Biological activity

Biological activity of the new analogues **1**–**4** and **18**–**19** has been determined on human neutrophils and compared with that of the reference ligand fMLF-OMe. Directed migration (chemotaxis), superoxide anion production, and lysozyme release have been measured.

In Figure 4, the chemotactic activity of the modified tripeptides **1**–**4** is reported. It can be seen that while the *N*-formyl tripeptides **2** and **4** are practically inactive, both the two *N*-Boc derivatives **1** and **3** show significant activity; in particular, the diastereomer **1**, containing N-terminal *cis*-4-mercaptomethyl-proline, exhibits the highest value. These findings are unexpected since the

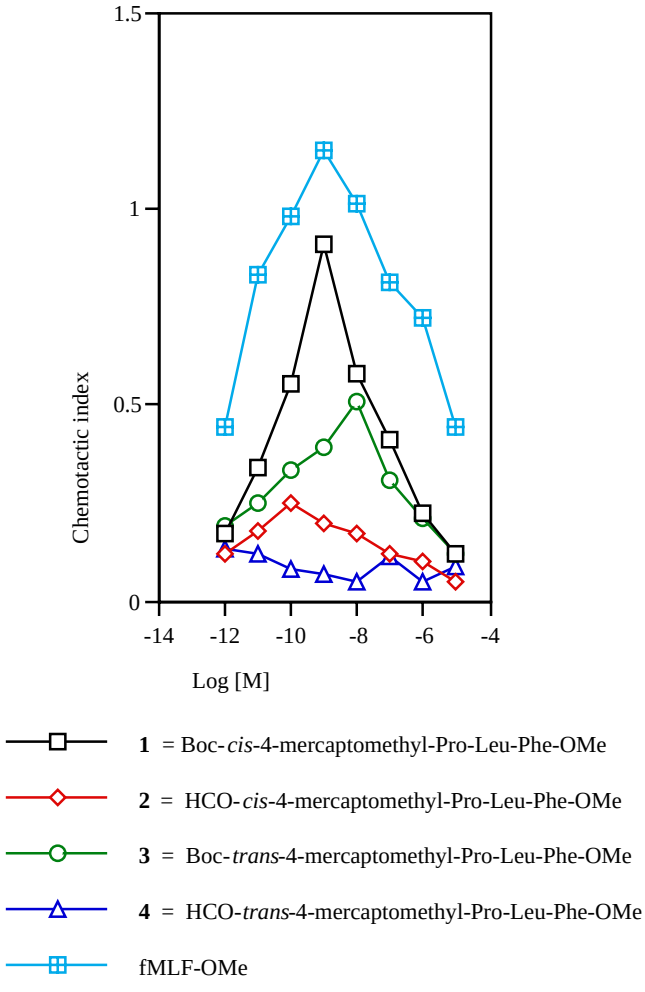


Figure 4. Chemotactic activity of tripeptide derivatives **1**–**4**.

so far available literature data indicate that the fMLF analogues containing branched and bulky carbamate functions bound at the Met amino group behave as antagonists and show a very weak agonistic activity.[13] Thus, to the best of our knowledge, compounds **1** and **3** represent the first examples of *N*-Boc fMLF tripeptides which are more active as chemoattractants than the corresponding *N*-formyl derivatives. Compounds **1**–**4** are completely inactive in superoxide anion production and the same behaviour is observed for the lysozyme release, except for a weak activity shown by the two *N*-formyl derivatives **2** and **4**.

Figure 7 shows the three functional activities on human neutrophils exhibited by the tripeptides *N*-Boc-Pro-Leu-Phe-OMe (**18**) and *N*-For-Pro-Leu-Phe-OMe (**19**). Concerning these tripeptides, here examined so as to evaluate the effect of the unsubstituted Pro pyrrolidine ring on the activity, it can be recalled that the two ligands *N*-Boc-Pro-Leu-Phe-OH and *N*-For-Pro-Leu-Phe-OH were synthesized several years ago, together with several related fMLF analogues, during a research centred on tripeptides potentially active as chemoattractants towards bull spermatozoa.[27] In such a biological test, the presence of the *N*-formyl group was found to be essential, while all the *N*-Boc derivatives were found to be inactive, including the ligand-containing N-terminal Boc-Met (i.e. Boc-MLF-OH). Furthermore, among the tested *N*-formyl derivatives, For-Met-Leu-Phe-OH was found to be highly active and the replacement of the native N-terminal Met or central Leu with Pro (to give the ligands *N*-For-Pro-Leu-Phe-OH and *N*-For-Met-Pro-Phe-OH, respectively) led to highly active chemoattractants, particularly in the case of the analogue with Pro as central residue. In the present work, however, where the activity is measured towards human neutrophil receptors,[2b] the Pro-containing tripeptides **18** and **19** show only a very weak activity as chemoattractants (Fig. 7A) and secretagogue agents (Fig. 7C) and are completely inactive, as already found in the case of the 4-methylthio Pro derivatives **1**–**4**, in superoxide anion production.

The *N*-formyl derivatives **2** and **4**, which have been found to be inactive as chemoattractants, have been examined as antagonists by measuring their ability to inhibit the effect stimulated by an optimal dose of fMLF. The influence of increasing concentrations of these two compounds on the chemotaxis induced by 10 nM fMLF is shown in Fig. 5A. A relevant and similar inhibition is observed for both the models with the derivative **4**, containing the *trans*-4-mercaptomethyl-proline residue, being more potent at higher concentration.

The antagonism towards the superoxide anion production exhibited by compounds **1**–**4** is reported in Fig. 5B; the *N*-Boc derivatives **1** and **3** are, as expected, more efficient antagonists than the corresponding *N*-formyl derivatives and exert a statistically significant reduction ($p < 0.05$) on the activity induced by 1 μM fMLF starting from 10^{-7} M; the inhibition increases with the concentration up to 52%. As shown in Fig. 5C, the outcomes of the inhibitory action of compounds **1**–**4** on the lysozyme release are very similar to those observed in the case of the superoxide anion production.

Receptor binding experiments have been carried out on derivatives **1** and **2**. Figure 6 shows the [^{3}H]fMLF displacements caused by increasing concentrations of the new analogues **1** and **2** and of the reference tripeptide fMLF-OMe. The order of potency in [^{3}H]fMLF displacement assays for the tested peptides is: fMLF-OMe > **1** > **2**. Thus, fMLF-OMe is the most potent peptide, with affinity in the nanomolar range (60 nM), while **1** and **2** display affinities in the micromolar range. Up to the concentration of 1 μM, as shown in Figure 4, the affinity values of compounds of **1** and **2** are low. Furthermore, while the formyl derivative **2** maintained low affinity at a concentration of 10 μM, the Boc derivative **1** exhibited remarkable affinity at the same concentration.

As previously observed,[28] an agonist capable of activating all the biological functions (full agonist) of human neutrophils is very effective in displacing the labelled peptide from its binding site, while a selective chemoattractant is much less efficacious;[29] this is the consequence of the existence at least of two isoforms of the

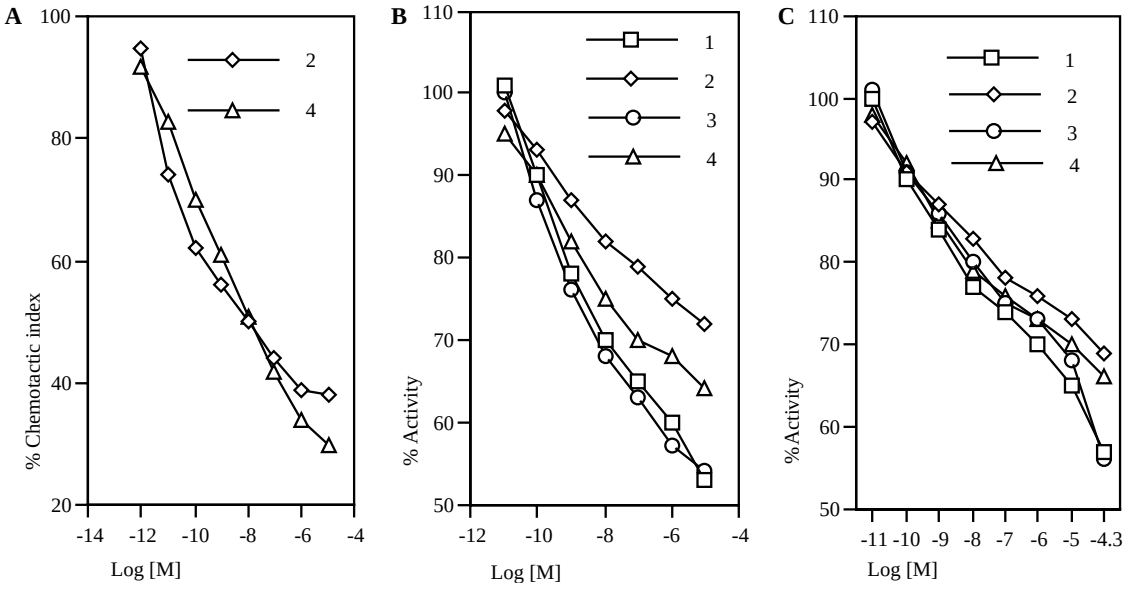


Figure 5. Effect of *N*-protected tripeptides **1**–**4** on the neutrophil activities triggered by fMLF-OMe. (A) Chemotactic activity; (B) superoxide anion production; (C) release of neutrophil granule enzymes evaluated by determining the lysozyme activity.

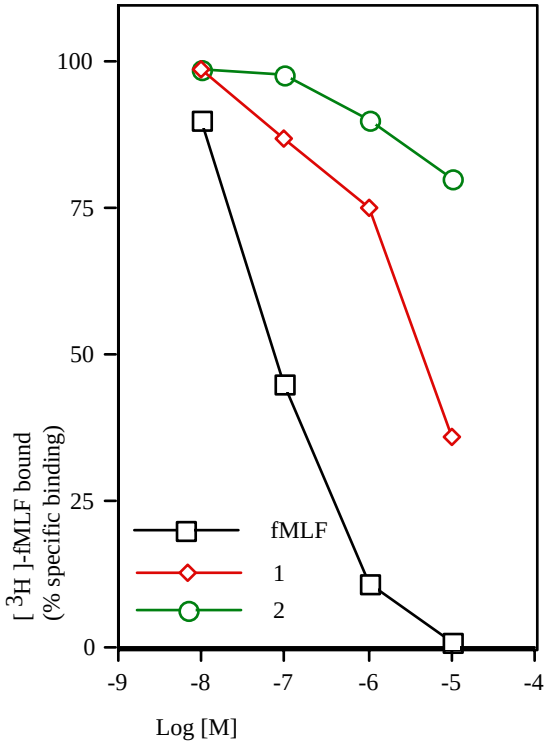


Figure 6. Competition curves of specific [^{3}H]fMLF binding to human neutrophils of compound **1** and **2**. Curves are representative of a single experiment taken from a series of three independent experiments. Nonspecific binding was determined in the presence of 100 μM fMLF.

formylpeptide receptor: a high affinity state, which controls the chemoattractant activity, and a low affinity state, which is involved in the release of lysozyme and superoxide anion production. The present finding is in accordance with this hypothesis and indicates that only a compound that exhibits superoxide anion production and lysozyme release can displace a high concentration of radiolabelled compound. The strong binding observed at 10^{-5} M for compound **1**, which is a selective agonist for chemotaxis but an antagonist for superoxide anion production and lysozyme release, is in large part due to its binding as an antagonist to the low affinity isoform of formylpeptide receptor. In accordance with this, the chemotactic agonist **1** shows an activity peak at the concentration of 10^{-9} M (see Fig. 4), while a consistent displacement of [^{3}H]fMLF occurs only at a higher concentration when the same tripeptide acts as antagonist towards superoxide anion production and lysozyme release.

6. Conclusion

In the present paper, we have examined fMLF analogues **1**–**4**, which combine the presence at position 1 of a strong conformational restriction involving the native side chain with N-terminal protecting groups of different nature and size. As previously mentioned, these two features are critical for the activity and only few papers deal with fMLF analogues modified at the Met residue which maintain intact the native γ-thiomethylether function.[16–18] Our solution conformational analysis indicates that these compounds are significantly folded and the biological results reported in Fig. 4 show that the presence of the bulky N-terminal Boc substituent, when combined with the cisoidal disposition of the two substituents on the pyrrolidine ring (i.e., presence of the homochiral *N*-Boc *cis*-4(*S*)-methylthio-(*S*)-proline), leads to the tripeptide **1**, which exhibits the highest chemotactic activity (CI = 0.9 at 10^{-9} M) among the examined compounds. The inversion of chirality at position 4 as in **3** (i.e., presence of the heterochiral *N*-Boc *trans*-4(*R*)-methylthio-(*S*)-proline residue) as well as the replacement of the bulky *N*-Boc with the small *N*-For group as in **2** (i.e., presence of the homochiral *N*-

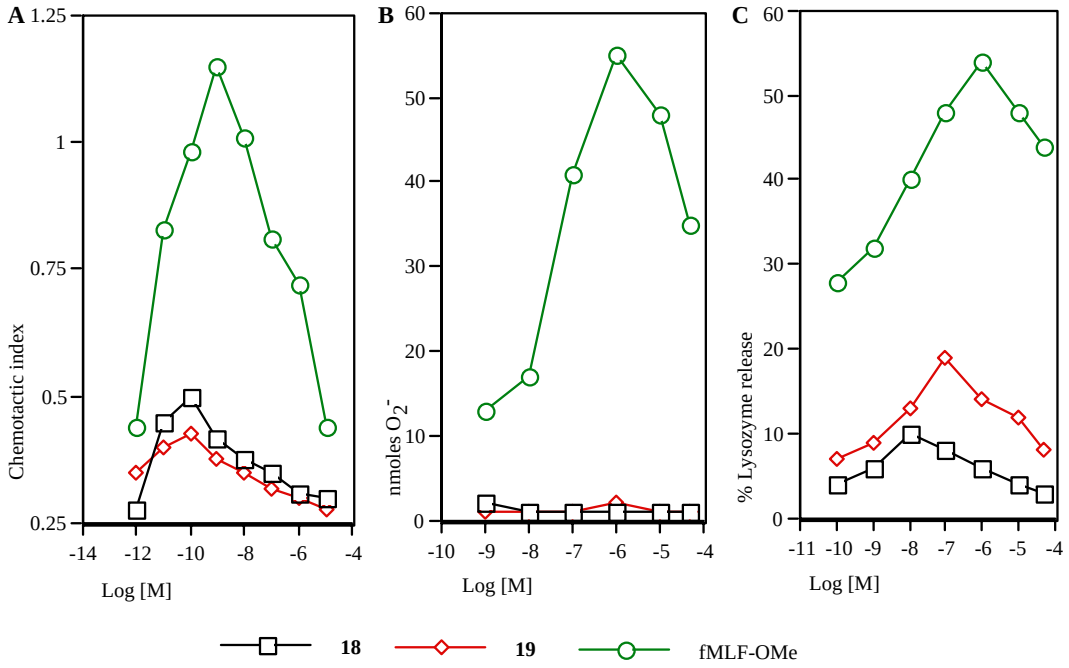


Figure 7. Biological activity of tripeptides **18** and **19**. (A) Chemotactic activity; (B) superoxide anion production; (C) release of neutrophil granule enzymes evaluated by determining the lysozyme activity.

For *cis*-4(*S*)-methylthio-(*S*)-proline residue) lead to a progressive diminution of the activity (CI = 0.5 at 10^{-8} M for **3** and 0.25 at 10^{-10} M for **2**). Finally, the introduction of both the heterochiral *trans*-4(*R*)-methylthio-(*S*)-proline residue and the *N*-For-protecting group, as in model **4**, gives rise to a ligand completely inactive as the chemoattractant. In summary, the 4(*R*) absolute configuration and the consequent transoidal orientation of the thiomethyl group with respect to the *S*-proline carboxamide is detrimental for the activity of both the *N*-Boc (on passing from **1** to **2**) and *N*-formyl derivatives (on passing from **2** to **4**). In this context, the unexpected higher chemotactic activity found in the case of the *N*-Boc **1** and **3** as compared with **2** and **4** confirms the critical role of the receptor pocket which accommodates the N-terminal moiety of the chemotactic formyltripeptides. In particular, the here performed study of fMLF analogues characterized by new structural modifications at the Met residue suggests that a predetermined and blocked spatial orientation of the native side chain at position 1 can profoundly alter the usual overall accommodation in the hydrophobic pockets of the tripeptide backbone with particular reference to the groups bound at the N-terminus moiety. On the other hand, the low activity of the two models **18** and **19** containing the unsubstituted proline ring clearly confirms the importance of the native Met side chain positioned at the N-terminus. A final consideration concerns the selectivity towards the three tested functional activities: all the derivatives (**1**–**4**, **18**, and **19**) are inactive in superoxide anion production and only a very weak activity as secretagogue agents has been observed for the formyl derivatives **2** and **4**. Thus, the *N*-Boc tripeptide **1**, which shows the highest activity as chemoattractant is, together with the other *N*-Boc derivative **3**, a pure chemoattractant being completely inactive for both superoxide anion production and lysozyme release.

In conclusion, the here described fMLF analogues provide new information on structure–activity relationships concerning chemotactic peptides and formylpeptide receptors, and represent valuable tools to shed light on a crucial point: how the overall fit into the receptor pocket can be influenced by structural modifications performed at the N-terminal Met residue. Additional studies are planned to further explore this topic.

7. Experimental

7.1. General

Compounds **5** and **11** were purchased from Fluka (Switzerland). TLC and PLC were performed on Merck 60 F_{254} silica gel plates. Column chromatography was carried out using Merck 60 silica gel (230–400 mesh). Elemental analyses (C, H, and N), were within ±0.4% of theory. Optical rotations were taken at 20 °C with a Schmidt–Haensch Polartronic D polarimeter in a 1 dm cell. IR spectra were recorded employing a Perkin-Elmer 1600 FTIR spectrophotometer. ^{1}H NMR spectra were determined on a Bruker 400 MHz instrument (δ expressed in ppm).

7.2. Chemistry

7.2.1. Boc-*trans*-4-hydroxy-L-proline methyl ester (7). Thionyl chloride (1.2 mL, 16.7 mmol) was added dropwise to a stirred solution of *trans*-4-hydroxy-L-proline (**5**) (2 g, 15.2 mmol) and anhydrous methanol (16 mL)

at 0 °C. The resultant solution was stirred at rt for 2 h and then heated at 45 °C for 4 h. Ten milliliters of methanol was added to the mixture and then evaporated under reduced pressure. This procedure was repeated four times to afford *trans*-4-hydroxyproline methyl ester hydrochloride (**6**). The crude salt **6** (2.70 g, 15.2 mmol) was dissolved in *p*-dioxane/H_2O 2:1 (45 mL). A solution of 1 M Na_2CO_3 (15 mL) was added to the mixture under stirring at 0 °C. Boc_2O (3.6 g, 16.5 mmol) was added after 40 min and the mixture was stirred at rt for 16 h, then, the two phases were separated. The aqueous phase was extracted with diethyl ether and then acidified with 10% $KHSO_4$ to pH 3. The aqueous layer was extracted with EtOAc. The combined organic layers were dried (Na_2SO_4) and concentrated to afford 3.7 g (99%) of pure **7** as a viscous oil: $[\alpha]_D^{20} = -65$ (*c* 0.60, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.42 (9H, s, Boc), 2.05–2.31 (2H, m, Pro C^3H_2), 3.48–3.67 (2H, m, Pro C^5H_2), 3.75 (3H, s, $COOCH_3$), 4.39–4.51 (2H, m, Pro αCH and Pro C^4H). Anal. Calcd for $C_{11}H_{19}NO_5$: C, 53.87; H, 7.81; N, 5.71. Found: C, 53.77; H, 7.80; N, 5.73.

7.2.2. *N*-Boc-*trans*-4-mesyloxy-L-proline methyl ester (8). To a solution of the methyl ester **7** (1.7 g, 6.93 mmol) and TEA (1.16 mL) in dry CH_2Cl_2 (9 mL), MsCl (0.65 mL, 8.34 mmol) was added in one portion. The resulting mixture was stirred for 20 min at rt. Then the mixture was diluted with CH_2Cl_2 and washed with 1 N HCl, $NaHCO_3$ ss and brine. The organic layer was dried (Na_2SO_4) and concentrated to yield 2.2 g (96%) of pure **8** as a pale-yellow oil: $[\alpha]_D^{25} = -52$ (*c* 1.6, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.41 (9H, s, Boc), 2.21–2.67 (2H, m, Pro C^3H_2), 2.98 (3H, s, CH_3SO_2), 3.70–3.85 (5H, m, Pro C^5H_2 and $COOCH_3$), 4.44 (1H, m, Pro αCH), 5.24 (1H, m, Pro C^4H). Anal. Calcd for $C_{12}H_{21}NO_7S$: C, 44.57; H, 6.55; N, 4.33; S, 9.92. Found: C, 44.67; H, 6.58; N, 4.32; S, 9.90.

7.2.3. *N*-Boc-*cis*-4-acetylthio-L-proline methyl ester (9). A solution of **8** (0.450 g, 1.40 mmol) and potassium thioacetate (0.207 g, 1.82 mmol) in dry DMF (4.5 mL) was stirred at 65 °C for 4 h. Then the reaction mixture was diluted with EtOAc (100 mL), and washed with ice-cooled brine (100 mL), and 1 N HCl to adjust the aqueous layer at pH 4 was added. The organic layer was successively washed with another portion of saturated brine, dried (Na_2SO_4) and evaporated under reduced pressure. The oily residue was purified by silica gel chromatography (1:40, *n*-hexane/EtOAc 4:1 as eluant) to give **9** as an orange-colored oil (0.170 g, 40%): $[\alpha]_D^{20} = -43.3$ (*c* 1.15, $CHCl_3$), ^1H NMR ($CDCl_3$) δ 1.44 (9H, s, Boc), 1.98 and 2.75 (2H, 2 m, Pro C^3H_2), 2.34 (s, 3H, CH_3CO); 3.36 and 3.99 (3H, 2 m, Pro C^5H_2 and Pro C^4H), 3.76 (3H, s, $COOCH_3$), 4.40 (1H, m, Pro αCH). Anal. Calcd for $C_{13}H_{21}NO_5S$: C, 51.47; H, 6.98; N, 4.62; S, 10.57. Found: C, 51.41; H, 6.99; N, 4.61; S, 10.59.

7.2.4. *N*-Boc-*cis*-4-methylthio-L-proline (10). A solution of **9** (1.1 g, 3.6 mmol) in methanol (19 mL) was treated with 1 N NaOH (3.9 mL) and dimethyl sulfate (0.39 mL) at rt. After stirring the mixture for 30 min, an additional 1 N NaOH (7.9 mL) portion was added and the reaction mixture was kept at the same conditions for additional 3 h. Then, volatiles were removed under reduced pressure, and the residue was partitioned between 10% $KHSO_4$ and EtOAc. The combined organic layers were washed with brine, dried (Na_2SO_4) and concentrated under reduced pressure to give 0.880 g of crude product as orange oil. Purification by flash chromatography (1:40, 2% acetic acid in EtOAc/*n*-hexane 1:4) gave 0.460 g product **10** (49%) as a pale-yellow oil which solidified on cooling as an amorphous solid; $[\alpha]_D^{20} = -43$ (*c* 0.43, DMF); ^1H NMR ($CDCl_3$) δ 1.46 (9H, s, Boc), 2.10–2.63 (5H, m, CH_3S and Pro C^3H_2), 3.20–4.93 (5H, m, Pro C^5H_2, Pro C^4H and Pro αCH). Anal. Calcd for $C_{11}H_{19}NO_4S$: C, 50.56; H, 7.33; N, 5.36; S, 12.47. Found: C, 50.43; H, 7.31; N, 5.35; S, 12.39.

7.2.5. *N*-Boc-4-oxo-L-proline (12). To a mixture of pyridine (4.2 mL) and dry CH_2Cl_2 (9.6 mL) cooled at 0 °C, CrO_3 (2.53 g) was added. Stirring was continued at 0 °C for 30 min, and then the mixture was allowed to warm at room temperature over a period of 30 min. A solution of *N*-Boc-4-*trans*-hydroxy-L-proline (**11**) (1 g, 4.32 mmol) in CH_2Cl_2 (15 mL) was added over 5 min, and stirring was continued for 1 h at room temperature. The mixture was filtered and evaporated under reduced pressure. The residue was taken up in diethyl ether and washed with 5% citric acid and brine and then dried (Na_2SO_4). The organic layer was filtered and evaporated under reduced pressure to give crude compound **12**, (0.353 g, 35%) as a pale-yellow solid. $[\alpha]_D + 18$ (*c* 0.49, acetone); ^1H NMR ($CDCl_3$) δ 1.45 (9H, s, Boc), 2.75 (2H, m, Pro C^3H_2), 3.50–3.90 (2H, m, Pro C^5H_2), 4.75 (1H, m, Pro αCH). Anal. Calcd for $C_{10}H_{15}NO_5$: C, 52.40; H, 6.60; N, 6.11. Found: C, 52.33; H, 6.61; N, 6.10.

7.2.6. *N*-Boc-*cis*-4-hydroxy-L-proline (13). *N*-Boc-4-oxo-L-proline (**12**) (0,330 g, 1.47 mmol) was dissolved in methanol (6.46 mL) and the reaction flask was cooled to 0 °C. A solution of $NaBH_4$ (0.180 g, 4.85 mmol) in water (0.80 mL) was added dropwise to the flask. After 10 min, the reaction was allowed to stand at 4 °C for 15 h and concentrated under reduced pressure. The residue was diluted with H_2O, acidified to pH 3 with 10% citric acid and extracted with EtOAc. The organic layer was washed with brine and dried (Na_2SO_4) and concentrated to yield 0.330 g of pure derivative **13** (97%) as a foam: $[\alpha]_D$ −39 (*c* 0.66 MeOH); ^1H NMR ($CDCl_3$) δ 1.45 (9H, s, Boc), 2.19–2.59 (2H, m, Pro C^3H_2), 3.44–3.56 (2H, m, Pro C^5H_2), 4.53–4.65 (2H, m, Pro αCH and Pro C^4H). Anal. Calcd for $C_{10}H_{17}NO_5$: C, 51.94; H, 7.41; N, 6.06. Found: C, 52.89; H, 7.43; N, 6.02.

7.2.7. *N*-Boc-*cis*-4-hydroxy-L-proline methyl ester (14). To a solution of *N*-Boc-*cis*-4-hydroxy-L-proline (**13**) (0.378 g, 1.63 mmol) in methanol (5.45 mL) was added an ethereal diazomethane solution until a yellow colour persisted. The mixture was concentrated and the residue was purified by silica gel chromatography [light petroleum (40–60 °C bp fraction)/EtOAc 1:1] to give compound **14** as a foam (0.250 g, 63%): $[\alpha]_D$ −63.9 (*c* 2.3,

EtOH); ^{1}H NMR ($CDCl_3$) δ 1.45 (9H, s, Boc), 2.07–2.49 (2H, m, Pro C^3H_2), 3.52–3.65 (2H, m, Pro C^5H_2), 3.77 (3H, s, $COOCH_3$), 4.27–4.40 (2H, m, Pro αCH and Pro C^4H). Anal. Calcd for $C_{11}H_{19}NO_5$: C, 53.87; H, 7.81; N, 5.71. Found: C, 53.90; H, 7.80; N, 5.74.

7.2.8. *N*-Boc-*cis*-4-mesyloxy-L-proline methyl ester (15). To a solution of *N*-Boc-*cis*-4-hydroxy-L-proline methyl ester (**14**) (0.070 g, 0.286 mmol) and TEA (0.048 mL) in dry CH_2Cl_2 (5 mL) cooled in ice bath was added MsCl (0.027 mL, 0.343 mmol) and the resulting mixture was stirred for 20 min. The solution was diluted with CH_2Cl_2 and washed with 1 N HCl, Na_2CO_3 ss and brine. The organic layer was dried (Na_2SO_4) and concentrated to yield 0.090 g (97%) of pure **15** as a pale-yellow oil that solidified in the freezer: ^{1}H NMR ($CDCl_3$) δ 1.44 (9H, s, Boc), 2.48–2.60 (2H, m, Pro C^3H_2), 3.03 (3H, s, CH_3SO_2), 3.75 and 4.45 (2H, m, Pro C^5H_2), 3.77 (3H, s, $COOCH_3$), 4.44 and 5.29 (2H, m, Pro αCH and Pro C^4H). Anal. Calcd for $C_{12}H_{21}NO_7S$: C, 44.57; H, 6.55; N, 4.33; S, 9.92. Found: C, 44.55; H, 6.52; N, 4.31; S, 9.91.

7.2.9. *N*-Boc-*trans*-4-(acetylthio)-L-proline methyl ester (16). A solution of **15** (0.578 g, 1.79 mmol) and potassium thioacetate (0.263 g, 2.3 mmol) in DMF (7.8 mL) was stirred at 68 °C for 4 h. 1 N HCl was added to adjust the aqueous layer to pH 4. EtOAc (100 mL) was added and the solution was washed with ice-cooled brine (100 mL). The organic layer was separated and successively washed with another portion of saturated brine, dried over Na_2SO_4 and evaporated under reduced pressure. The residue was purified by silica gel chromatography (*n*-hexane/EtOAc 4:1, as eluant) to give pure **16** as an orange-coloured oil (0.450 g, 83%): $[\alpha]_D^{20} = -33$ (*c* 0.9, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.41 (9H, s, Boc), 2.10 and 2.47 (2H, m, Pro C^3H_2), 2.34 (s, 3H, CH_3CO); 3.31–4.10 (3H, m, Pro C^5H_2 and Pro C^4H), 3.75 (3H, s, $COOCH_3$), 4.40 (1H, m, Pro αCH). Anal. Calcd for $C_{13}H_{21}NO_5S$: C, 51.47; H, 6.98; N, 4.62; O, 26.37; S, 10.57. Found: C, 51.39; H, 6.90; N, 4.60; O, 26.40; S, 10.55.

7.2.10. *N*-Boc-*trans*-4-(methylthio)-L-proline (17). A solution of **16** (0.440 g, 1.45 mmol) in methanol (7.6 mL) was treated successively with 1 N NaOH (1.6 mL) and dimethyl sulfate (0.150 mL, 1.6 mmol) at rt. After stirring for 30 min, an additional 1 N NaOH (3.15 mL) portion was added and the reaction mixture was stirred for 3.5 h at rt. Volatiles were removed under reduced pressure, and the residue was partitioned between 10% $KHSO_4$ and EtOAc. The combined organic layers were washed with brine, dried (Na_2SO_4) and concentrated under reduced pressure to give 0.360 g of crude product **7** as an orange oil. Purification of the crude residue by flash chromatography (2% acetic acid in EtOAc/*n*-hexane 1:4) gave 0.330 g of pure **17** (87%) as a pale-yellow oil; $[\alpha]_D^{20} = 37.2$ (*c* 0.46, DMF); ^{1}H NMR ($CDCl_3$) δ 1.42 (9H, s, Boc), 2.15 and 2.55 (5H, m, CH_3SO_2 and Pro C^3H_2), 3.23–4.47 (4H, m, Pro C^5H_2, Pro C^4H, and Pro αCH). Anal. Calcd for $C_{11}H_{19}NO_4S$: C, 50.56; H, 7.33; N, 5.36; S, 12.27. Found: C, 50.40; H, 7.35; N, 5.32; S, 12.28.

7.2.11. HCl·HLeu-Phe-OMe. Thionyl chloride (0.078 mL, 1.06 mmol) was added to a solution of Boc-Leu-Phe-OMe (0.4 g, 1.02 mmol) in dry methanol (2.5 mL) and cooled at −15 °C. After stirring at −15 °C for 40 min and at 45 °C for 3 h, the solution was evaporated under reduced pressure to give the title hydrochloride as a foam. This was used in the next step without further purification.

7.2.12. *N*-Boc-*cis*-4-methylthio-Pro-Leu-Phe-OMe (1). *i*-BuOCOCl (0.065 mL, 0.5 mmol) was added at −15 °C to a stirred solution of **10** (0.130 g, 0.5 mmol) and NMM (0.042 mL, 0.6 mmol) in dry CH_2Cl_2 (4 mL). The temperature was maintained at −15 °C for 10 min, and HCl·HLeu-Phe-OMe (0.5 mmol), NMM (0.042 mL, 0.6 mmol) and dry CH_2Cl_2 (4 mL) were added. The mixture was stirred at −15 °C for 15 min and then allowed to warm to rt. Dry DMF (10 drops) was added and the solution was stirred overnight. EtOAc was added and the organic layer was washed with 1 N HCl, brine, saturated aqueous $NaHCO_3$ and brine. The organic phase was dried (Na_2SO_4) and evaporated under reduced pressure to give crude solid tripeptide **1**, which was purified by silica gel chromatography (CH_2Cl_2/EtOAc 4:1 and 1:1 as eluants) to give pure tripeptide **1** as an amorphous white solid (0.210 g, 78%), mp 153–154.5 °C; $[\alpha]_D$ −37 (*c* 1.0, $CHCl_3$); IR (KBr) 3290, 1705, 1660, 1549, 1402 cm^{-1}, ^{1}H NMR ($CDCl_3$) δ 0.91 [6H, m, $CH(CH_3)_2$], 1.44–1.74 [3H, m, $CH_2CH(CH_3)_2$], 1.48 [9H, s, $C(CH_3)_3$], 2.14 (3H, s, SCH_3), 2.45 (2H, br, Pro C^3H_2), 3.04–3.35 (4H, m, Pro C^4H, 1H of Pro C^5H_2 and Phe βCH$_2$), 3.71 (3H, s, $COOCH_3$), 3.85 (1H, br, 1H of Pro C^5H_2), 4.29 (1H, m, Pro αCH), 4.40 (1H, m, Leu αCH), 4.82 (1H, m, Phe αCH), 6.78–7.35 (7H, m aromatic, Leu and Phe NH). Anal. Calcd for $C_{27}H_{41}N_3O_6S$: C, 60.54; H, 7.71; N, 7.84; S, 5.99. Found: C, 60.59; H, 7.72; N, 7.80; S, 6.01.

7.2.13. *N*-For-4-*cis*-methylthio-Pro-Leu-Phe-OMe (2). The *N*-Boc-tripeptide (**1**) (0.093 g, 0.174 mmol) was dissolved in formic acid (1 mL) and the mixture was stirred at room temperature for 24 h. After removal of the excess of formic acid under reduced pressure, the residue was dissolved in 1 mL of dry chloroform. EEDQ (0.052 g, 0.208 mmol) was added and the solution was stirred at room temperature for 24 h. Evaporation under reduced pressure afforded a crude residue, which was purified by PLC (chloroform/EtOAc 3:1, as eluant) to give 0.080 mg (99%) of pure *N*-For-*cis*-4-methylthio-Pro-Leu-Phe-OMe (**2**) as a foam; $[\alpha]_D$ −55.5 (*c* 0.83, $CHCl_3$), IR (KBr) 3297, 1746, 1654, 1542, 1383 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.88–0.91 [6H, two d, J = 6.3 Hz, $CH(CH_3)_2$], 1.42–1.75 [3H, m, CH_2 ($CH_3)_2$], 2.18 (3H, s, SCH_3), 2.46 (2H, m, Pro C^3H_2), 3.05–3.25 (3H, m, Phe βCH$_2$ and Pro C^4H), 3.37 and 3.97 (2H, two m, Pro C^5H_2), 3.73 (3H, s, $COOCH_3$), 4.36 (1H, m, Leu αCH), 4.46 (1H, t, J = 8.1 Hz, Pro αCH), 4.84 (1H, m, Phe αCH), 6.68 (1H, d, J = 7.7 Hz Phe NH), 7.06 (1H, d, J = 7.7 Leu NH), 7.13 (5H, m, aromatic), 8.28 (1H, s, HCO). Anal. Calcd for $C_{23}H_{33}N_3O_5S$: C, 59.59; H, 7.17; N, 9.06; S, 6.92. Found: C, 59.40; H, 7.18; N, 9.03; S, 6.90.

7.2.14. *N*-Boc-*trans*-4-methylthio-Pro-Leu-Phe-OMe (3). *i*-BuOCOCl (0.079 mL, 0.61 mmol) was added at −15 °C to a stirred solution of *N*-Boc-4-*trans*-methylthio-Pro-OH (**17**) (0.160 g, 0.61 mmol) and NMM (0.074 mL, 0.67 mmol) in dry CH_2Cl_2 (5 mL). The temperature was maintained at −15 °C for 10 min, and HCl·HLeu-Phe-OMe (0.61 mmol), NMM (0.074 mL, 0.67 mmol) and dry CH_2Cl_2 (7 mL) were added. The mixture was stirred at −15 °C for 15 min and then allowed to warm to rt. Dry DMF (0.5 mL) was added and stirring was continued overnight. EtOAc was added and the organic layer was washed with 1 N HCl, brine, and saturated aqueous $NaHCO_3$ and brine. The organic phase was dried and evaporated under reduced pressure to give crude solid tripeptide **3**, which was purified by silica gel chromatography ($CHCl_3$/MeOH 98:2) to give the title compound (0.280 g, 85%) as a colorless oil which solidified on cooling as an amorphous solid: $[\alpha]_D$ −50 (*c* 0.9, $CHCl_3$); IR (KBr) 3296, 1743, 1705, 1659, 1553, 1395 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.86–0.93 [6H, m, $CH(CH_3)_2$], 1.45–1.75 [3H, m, CH_2 $CH(CH_3)_2$], 1.48 [9H, s, $C(CH_3)_3$], 1.87 and 2.66 (2H, two m, Pro C^3H_2), 2.16 (3H, s, SCH_3), 3.09 and 3.16 (2H, AB part of an ABX, J = 6.3, 5.2 and 14 Hz, Phe βCH_2), 3.26 and 3.68 (2H, two m, Pro C^5H_2), 3.38 (1H, m, Pro C^4H), 3.73 (3H, s, $COOCH_3$), 4.34 (2H, m, Leu αCH and Pro αCH), 4.83 (1H, m, Phe αCH), 6.67 (1H, Br, Phe NH), 7.10–7.36 (6H, m, aromatic and Leu NH). Anal. Calcd for $C_{27}H_{41}N_3O_6S$: C, 60.54; H, 7.71; N, 7.84; S, 5.99. Found: C, 60.55; H, 7.74; N, 7.82; S, 5.97.

7.2.15. *N*-For-*trans*-4-methylthio-Pro-Leu-Phe-OMe (4). *N*-Boc-*trans*-4-methylthio-Pro-Leu-Phe-OMe (**3**) (0.180 g, 0.33 mmol) was dissolved in formic acid (2 mL) and the mixture was stirred at room temperature for 24 h. After removal of the excess of formic acid under reduced pressure, the residue was dissolved in 2 mL of dry chloroform. EEDQ (0.103 g, 0.41 mmol) was added and the solution was stirred at room temperature for 24 h. Evaporation under reduced pressure afforded a crude residue, which was purified by PLC (chloroform/MeOH 9:1, as eluant) to give 0.099 g (65%) of pure **4** as a foam; $[\alpha]_D$ −40.4 (*c* 1.26, $CHCl_3$), IR (KBr) 3298, 1745, 1654, 1541, 1383 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.87–0.91 [6H, two d, J = 6.3 Hz, $CH(CH_3)_2$], 1.40–1.75 [3H, m, $CH_2CH(CH_3)_2$], 1.99 and 2.73 (2H, two m, Pro C^3H_2), 2.17 (3H, s, SCH_3), 3.10 and 3.18 (2H, A and B of an ABX, J = 5.7, 6.2 and 14 Hz, Phe βCH_2), 3.40–3.56 (2H, m, 1H of Pro C^5H_2 and Pro C^4H), 3.73 (3H, s, $COOCH_3$), 3.81 (1H, m, 1H of Pro C^5H_2), 4.32 (1H, m, Leu αCH); 4.52 (1H, m, Pro αCH), 4.85 (1H m, Phe αCH), 6.60 (1H, d, J = 7.7 Hz, Phe NH), 7.05–7.40 (6H, m, aromatic and Leu NH), 8.27 (1H, s, HCO). The values of major conformer are given. Anal. Calcd for $C_{23}H_{33}N_3O_5S$: C, 59.59; H, 7.17; N, 9.06; S, 6.92. Found: C, 59.63; H, 7.18; N, 9.04; S, 6.90.

7.2.16. *N*-Boc-Pro-Leu-Phe-OMe (18). *i*-BuOCOCl (0.065 mL, 0.5 mmol) was added at −15 °C to a stirred solution of Boc-Pro-OH (0.130 g, 0.5 mmol) and NMM (0.042 mL, 0.6 mmol) in dry CH_2Cl_2 (4 mL). The temperature was maintained at −15 °C for 10 min, and HCl·HLeu-Phe-OMe (0.5 mmol), NMM (0.042 mL, 0.6 mmol) and dry CH_2Cl_2 (4 mL) were added. The mixture was stirred at −15 °C for 15 min and then allowed to warm to rt. Dry DMF (10 drops) was added and the solution was stirred overnight. EtOAc was added in excess and the organic layer was washed with 1 N HCl, brine, and saturated aqueous $NaHCO_3$ and brine. The organic phase was dried (Na_2SO_4) and evaporated under reduced pressure to give crude solid tripeptide **18**, which was purified by silica gel chromatography (CH_2Cl_2/EtOAc 4:1 and 1:1 as eluants) to give pure tripeptide **18** as an oil (0.210 g, 80%): IR ($CHCl_3$, 10 mmol) 3418, 1742, 1678, 1230, 778 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.89 [6H, m, $CH(CH_3)_2$], 1.40–1.70 [12H, m, $CH_2CH(CH_3)_2$ and $C(CH_3)_3$, s at 1.45], 1.87–2.30 (4H, br, Pro C^3H_2, Pro C^4H_2), 3.08 (2H, m, Phe βCH_2), 3.38 (2H, m, Pro C^5H_2), 3.68 (3H, s, $COOCH_3$), 4.22 (1H, br, Pro αCH), 4.34 (1H, br, Leu αCH), 4.81 (1H, m, Phe αCH), 6.73–7.35 (7H, m, aromatic, Leu and Phe NH). Anal. Calcd for $C_{26}H_{39}N_3O_6$: C, 63.78; H, 8.03; N, 8.58. Found: C, 63.79; H, 8.01; N, 8.60.

7.2.17. *N*-For-Pro-Leu-Phe-OMe (19). The *N*-Boc-tripeptide (**18**) (0.093 g, 0.174 mmol) was dissolved in formic acid (1 mL) and the mixture was stirred at room temperature for 24 h. After removal of the excess formic acid under reduced pressure, the residue was dissolved in 1 mL of dry chloroform. EEDQ 97% (0.052 g, 0.208 mmol) was added and the solution was stirred at room temperature for 24 h. Evaporation under reduce pressure afforded a crude residue, which was purified by PLC (chloroform/EtOAc 3:1, as eluant) to give 0.080 g (90%) of pure *N*-For-Pro-Leu-Phe-OMe (**19**) as a foam; IR ($CHCl_3$, 10 mM) 3421, 1742, 1672, 1509 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.86–0.94 [6H, m, $CH(CH_3)_2$], 1.40–1.68 [7H, m, $CH_2CH(CH_3)_2$ and Pro C^3H_2, Pro C^4 H_2], 3.08 (2H, m, Phe βCH_2), 3.56 (2H, m, Pro C^5H_2), 3.72 (3H, s, $COOCH_3$), 4.32 (1H, br, Pro αCH), 4.35 (1H, br, Leu αCH), 4.85 (1H, m Phe αCH), 6.67–7.35 (7H, m aromatic, Leu and Phe NH), 8.30 (1H, s, HCO). Anal. Calcd for $C_{22}H_{31}N_3O_5$: C, 63.29; H, 7.48; N, 10.06. Found: C, 63.18; H, 7.44; N, 10.02.

7.3. Biological assays

7.3.1. Cell preparation. Cells were obtained from the blood of healthy subjects, and human peripheral blood neutrophils were purified by using the standard techniques of dextran (Pharmacia, Uppsala, Sweden) sedimentation, centrifugation on Ficoll–Paque (Pharmacia), and hypotonic lysis of contaminating red cells. Cells were washed twice and resuspended in Krebs–Ringer phosphate (KRPG), pH 7.4, at a final concentration of 50×10^6 cells/mL and kept at room temperature until used. Neutrophils were 98–100% viable, as determined using the Trypan blue exclusion test. The study was approved by the local Ethics Committee, and informed consent was obtained from all participants (see Fig. 7).

7.3.2. Random locomotion. Random locomotion was performed with 48-well microchemotaxis chamber (Bio

Probe, Milan, Italy) and migration into the filter was evaluated by the leading-front method.[21] The actual control random movement is 35 ± 3 μm SE of 10 separate experiments performed in duplicate.

7.3.3. Chemotaxis. 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 (BSA; Orha Behringwerke, Germany) and used at concentrations ranging from 10^{-12} to 10^{-5} M. Data were expressed in terms of chemotactic index (CI), which is the ratio: (migration towards test attractant minus migration towards the buffer)/migration towards the buffer; the values are means of six separate experiments performed in duplicate. Standard errors are in the 0.02–0.09 CI range.

7.3.4. Superoxide anion (O_2^-) production. This anion was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome *c* (Sigma, USA) modified for microplate-based assays. Tests were carried out in a final volume of 200 mL containing 4×10^5 neutrophils, 100 nmoles cytochrome *c* and KRPG. At zero time different amounts (10^{-10}–8×10^{-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 550 and 468 nm. The difference in absorbance at the two wavelengths was used to calculate nmoles of O_2^- produced using an absorptivity for cytochrome *c* of 18.5 mM^{-1} cm^{-1}. Neutrophils were incubated with 5 μg/mL cytochalasin B (Sigma) for 5 min prior to activation by peptides. Results were expressed as net nmoles of O_2^- per 1×10^6 cells per 5 min and are means of six separate experiments performed in duplicate. Standard errors are in 0.1–4 nmoles O_2^- range.

7.3.5. 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 at a final concentration of 10^{-10}–8×10^{-5} M for a further 15 min. The plates were then centrifuged at 400*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 mg per 1×10^7 cells/min. The values are means of five separate experiments done in duplicate. Standard errors are in the range 1–6%.

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