Chemotactic Peptide Analogues

Centrally Constrained Chemotactic *N*-Formyltripeptides: Synthesis, Conformation, and Activity of Two New Analogues

Giampiero Pagani Zecchini[a], Mario Paglialunga Paradisi[a], Ines Torrini[a], Gino Lucente[a]*, Gaia Mastropietro[a], Maurizio Paci[b], and Susanna Spisani[c]

[a] Dipartimento di Studi Farmaceutici and Centro di Studio per la Chimica del Farmaco del CNR, Università "La Sapienza", 00185 Roma, Italy

[b] Dipartimento di Scienze e Tecnologie Chimiche, Università di Tor Vergata, 00173 Roma, Italy

[c] Dipartimento di Biochimica e Biologia Molecolare, Università di Ferrara, 44100 Ferrara, Italy

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Summary

The role exercised by the central residue of the chemotactic *N*-formyltripeptide HCO-Met-Leu-Phe-OMe (fMLP-OMe) in controlling both the backbone conformation and the biochemical activity is the subject of recent interest. Here, two new centrally constrained fMLP-OMe analogues, namely HCO-Met-azaPro-Phe-OMe (**4**) and HCO-Met-(γ-lactam)-Phe-OMe (**6**) have been synthesized and their $CDCl_3$ solution conformation and activity have been studied. The azapeptide **4** adopts β-folded conformation with the azaPro residue at the i+2 position and an intramolecular H-bond involving the formylic oxygen and the Phe NH. The γ-lactam tripeptide **6** prefers a semi-extended backbone conformation. When tested on human neutrophils both the new models were found practically devoid of biological activity. The role exerted by the NH groups as well as by the conformational preferences is discussed.

Introduction

In the field of the structure-activity relationships concerning chemotactic *N*-formyltripeptides we reported previously several studies on the chemical modification of the prototypical chemotactic ligand HCO-Met-Leu-Phe-OMe (fMLP-OMe)[1–4]. Attention was recently focused by us, as well as by other groups, on the conformational and biochemical consequences due to the structural alterations introduced at the central residue[5–14]. The reason for this interest resides in the pronounced influence which the nature of this residue can exert on the overall conformation of the tripeptide backbone as well as the rather low receptor specificity corresponding to this position as compared with that exhibited at the *N*- and *C*-terminal residues. Furthermore, the importance of the central NH group for a favourable interaction with the appropriate receptor area has been recently evidenced[7,15]. In accordance with this observation it has been found that fMLP-OMe analogues lacking the H-bond donor group at the Met-Leu junction are devoid of activity. On the other hand, some simple tripeptide models, strictly related to the lead structure of fMLP-OMe and possessing the central NH, are found to be practically inactive[4,5,15]. Thus, chemical modifications at the central residue can modulate the activity through a rather complex interplay of structural and conformational effects.

In this context we wish to report the results concerning synthesis, conformational analysis, and activity of the two new fMLP-OMe analogues **4** and **6**. In both the models a local conformational constraint has been introduced by replacing the native central leucine with a five membered cyclic system. Model **4** is devoid of the central NH and contains the achiral azaPro residue which is a strong β-turn inducer at the Xaa-azaPro sequence[16–18]. In the model **6** the backbone constraint is due to the bridging of the central α-carbon atom, through a γ-lactam ring, with the subsequent NH group. As a consequence the tripeptide **6** maintains the central NH and is devoid of the *C*-terminal NH. Two γ-lactam-containing chemotactic *N*-formylpeptides have been previously reported, namely HCO-Met-(γ-lactam)-Leu-Phe-Phe-OH[13] and HCO-Met-(γ-lactam)-Leu-Phe-OMe[14]. It is interesting to note that in both compounds the γ-lactam residue does not replace the native leucine but is inserted, as a conformationally constrained spacer, between the Met and Leu residues. No data on the conformational preferences exhibited by these two compounds are at present available.

Results and Discussion

Chemistry

The synthesis of the *N*-formyl derivative **4** was performed according to Scheme 1.

Deprotection of 1-benzyloxycarbonyl-2-*t*-butoxycarbonylpyrazolidine[19] by treatment with trifluoroacetic acid (TFA), followed by coupling with Boc-Met-OH, carboxy-activated with bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BOP-Cl), gave Boc-Met-azaPro-OBzl (**1**). A two step activating transprotection (catalytic transfer hydrogenation in the presence of Pd/C and 1,4-cyclohexadiene[20] followed by acylation with *p*-nitrophenyl chloroformate) afforded the *p*-nitrophenyl ester **2**. Reaction with H-Phe-OMe in the presence of 4-(dimethylamino)pyridine gave the Boc-protected tripeptide **3** which was then transformed into the *N*-formyltripeptide **4** by the usual treatment with formic acid followed by ethyl 2-ethoxy-1,2-dihydroquinolinecarboxylate (EEDQ).

The synthesis of the *N*-formyl derivative **6** was performed according to Scheme 2 by following the stereospecific cycli-

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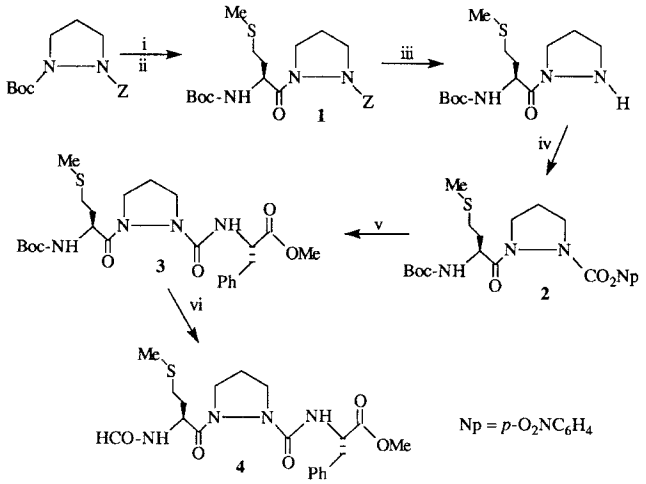
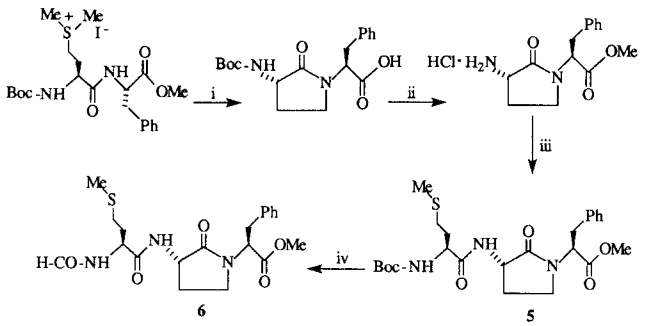


Scheme 1. i: TFA, $CHCl_3$; ii: Boc-Met-OH, BOP-Cl, TEA; iii: 1,4-cyclohexadiene, 10% Pd/C; iv: *p*-nitrophenyl chloroformate, TEA; v: H-Phe-OMe, 4-(dimethylamino)pyridine; vi: HCOOH, EEDQ.

zation of Freidinger[21]. According to this method the lactam ring closure from Boc-Met-Phe-OMe methylsulphonium iodide is accompanied by partial (12–15%) racemization at the Phe α-carbon atom[21]. The major component **5** of the reaction maintains, at the two chiral centres, the absolute configuration of the starting Boc-dipeptide methyl ester[21–23]. In the present istance the intermediate **5** and its minor epimeric isomer have been accurately isolated (see Experimental Part) in relative amount (73 and 12% yields) exactly corresponding to that found by Freidinger *et al.* in their pioneering work[21]. The major component **5** was then used for the formylation reaction to afford the *N*-formylpeptide **6**.



Scheme 2. i: NaH, DMF-CH_2Cl_2; ii: $SOCl_2$, MeOH; iii: Boc-Met-OH, *i*-BuOCOCl, NMM; iv: HCOOH, EEDQ.

Solution Conformation

The ^1H NMR of the formyltripeptide **4** has been performed in $CDCl_3$ solution. The characteristic HCO proton resonance occurs as a singlet at 8.18 δ. The Phe NH doublet ($J = 8$ Hz), which resonates at 7.53 δ, has been identified by spin decoupling experiments; the Met NH appears as an unresolved signal at higher field (δ = 6.57). The lack of both α-CH and NH protons, at the central residue, strongly limits the information which can be obtained from NMR data on the preferred conformation adopted by **4** in $CDCl_3$. The remarkable difference (Δδ = 1.17 and 1.53 ppm) between the chemical shifts of the geminal protons at both β- and δ-CH_2 groups of the azaPro residue is an interesting feature of the spectrum of **4**. This resonance pattern is related to the diamagnetic anisotropic effect exerted by the two carbonyl groups bonded at the nitrogen atoms of the pyrazolidine ring[17,24] and indicates that one of the two protons of each CH_2NCO group is nearly coplanar with the adjacent carbonyl; as a consequence the two carbonyl groups point in opposite directions.

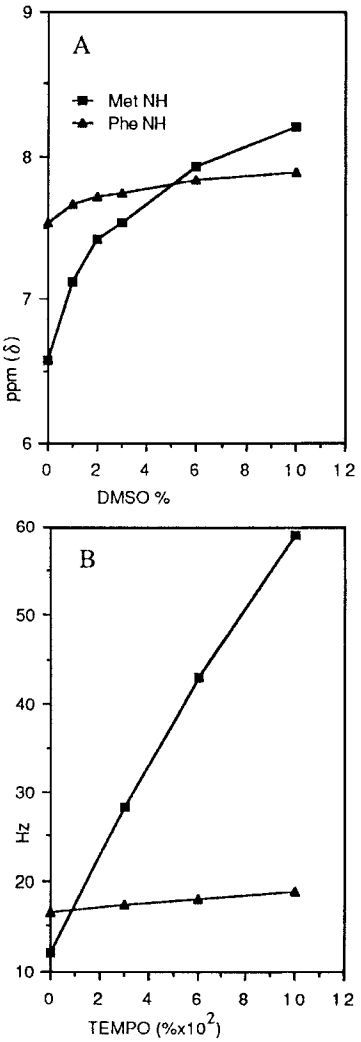


Figure 1. Delineation of hydrogen-bonded NH groups in formyltripeptide **4**. (A) Chemical shift dependence of the NH resonances as a function of the [D_6]DMSO concentration (% *v/v*) in $CDCl_3$ solution. Peptide concentration 10 mM. (B) Line broadening of NH resonances with increasing concentration of radical TEMPO in $CDCl_3$. Peptide concentration 10 mM.

The involvement of the NH groups in intramolecular H-bonds was evaluated on the basis of the chemical shift solvent dependence in $CDCl_3$-[D_6]DMSO mixtures (Figure 1A) and paramagnetic radical induced line broadening[25] (Figure 1B). In the solvent titration experiment the Met NH resonance moves downfield with increasing concentration of [D_6]DMSO (Δδ = 1.62), while the Phe NH is scarcely affected by the change of the solvent composition (Δδ = 0.35). Addition of the paramagnetic radical probe 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) to the $CDCl_3$ solution of **4** causes a drastic broadening of the Met NH signal, whereas the Phe NH is practically unaffected (Figure 1B). These

Table 1. Observed Nuclear Overhauser effects (NOEs) in the ROESY spectra[a,b] of **4** and **6**.

Compound **4**		Compound **6**	
HCO····Met NH	m	HCO····Met NH	s
Phe NH····Met CH^{α}	m	Phe CH^{α}····Aromatic	s
Phe NH····Phe CH^{α}	w	Phe CH^{α}····Phe CH_2^{β}	s
Phe NH····Phe CH_2^{β}	m	Met NH····Met CH^{α}	w
Phe CH^{α}····Phe CH_2^{β}	s	Met CH^{α}····Met CH_2^{β}	s
Met NH····Met CH_2^{β}	m	Met CH^{α}····Met CH_2^{γ}	m
Met NH····Met CH_2^{γ}	w	γ-lactam NH····Met CH^{α}	s
Met CH^{α}····Met CH_2^{β}	s	γ-lactam CH^{β}····γ-lactam $CH^{\beta'}$	s
Met CH^{α}····Met CH_2^{γ}	m	γ-lactam CH^{γ}····γ-lactam $CH^{\gamma'}$	s
azaPro CH^{β}····azaPro $CH^{\beta'}$	s	γ-lactam CH^{α}····γ-lactam CH^{β}	s
azaPro CH^{δ}····azaPro $CH^{\delta'}$	s	γ-lactam CH^{α}····γ-lactam $CH^{\beta'}$	w
azaPro CH_2^{β}····azaPro CH_2^{γ}	s	γ-lactam CH^{β}····γ-lactam $CH^{\gamma'}$	s
azaPro CH_2^{δ}····azaPro CH_2^{γ}	s	γ-lactam CH^{γ}····γ-lactam $CH^{\beta'}$	s
		γ-lactam CH^{α}····γ-lactam NH	w

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

[b] β′, γ′, and δ′ refer to the methylenic proton which resonates to higher field.

results indicate that the Phe NH is solvent inaccessible and presumably involved in an intramolecular hydrogen bond.

Solution IR spectra have been examined in order to confirm the presence of intramolecularly H-bonded NH groups. Figure 2 shows the N-H stretching region of the IR spectrum of **4** in $CHCl_3$. Two bands are observed at 3430 and 3306 cm^{-1}, corresponding to free (3430 cm^{-1}) and H-bonded (3306 cm^{-1}) NH groups. The ratio between the intensity of the free and H-bonded absorptions varies only slightly over the range 10–0.3 mM. Thus, peptide self-association occurs, if any, at a limited extent under these experimental conditions and the band at 3306 cm^{-1} can be assigned to intramolecularly H-bonded Phe NH group.

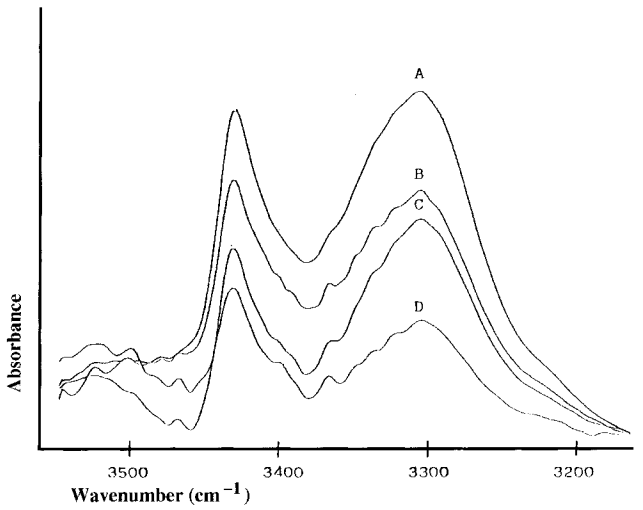


Figure 2. The IR absorption spectra (NH stretching bands) of **4** in chloroform at various concentrations: (A) 10 mM, (B) 5 mM, (C) 1 mM, and (D) 0.3 mM. 0.5 mm cells were used for spectra A and B, 2 mm and 3mm cells for spectra C and D, respectively.

Nuclear Overhauser effect (NOE) experiments have been performed to provide additional information on the preferred solution conformation of **4** and the estimates of the cross peak relative intensities are summarized in Table 1, on an arbitrary

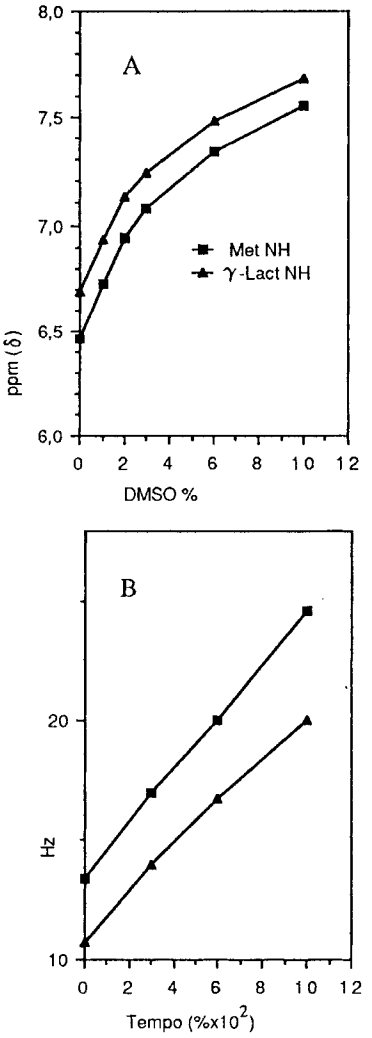


Figure 3. Delineation of hydrogen-bonded NH groups in formyl derivative **6**. (A) Chemical shift dependence of the NH resonances as a function of the [D_6]DMSO concentration (% *v/v*) in $CDCl_3$ solution. Peptide concentration 10 mM. (B) Line broadening of NH resonances with increasing concentration of radical TEMPO in $CDCl_3$. Peptide concentration 10 mM.

scale. The interresidue NOE HCO···Met NH, which is consistent with the *cis* configuration of the two H atoms of the formamido group, as always found in the crystal structures of *N*-formylpeptides[8], occurs with medium intensity. The medium interresidue NOE observed in the ROESY spectrum of **4** concerns the spatial proximity of Phe NH with Met α-CH, and clearly indicates a folding of the peptide backbone at the central azaPro residue. This finding, together with the low solvent accessibility shown by the Phe NH, suggests that, in agreement with recent literature data concerning crystal and solution conformation of azaPro containing peptides[16–18], the formyltripeptide **4** adopts a β-turn structure with the azaPro residue at the i+2 corner position and supported by an intramolecular 4→1 H-bond between the Phe NH and the formylic oxygen.

An analogous investigation of the preferred solution conformation in $CDCl_3$ has been performed on the γ-lactam-containing analogue **6**. In the ^1H NMR spectrum the H-CO resonance occurs as a singlet at 8.18 δ. The Met and γ-lactam NH resonate as doublets at 6.46 δ (J = 8.3 Hz) and 6.69 δ (J = 6.8 Hz), respectively. The [D_6]DMSO titration results (Figure 3A) clearly indicate that the two groups show a very similar accessibility to the solvent. Addition of TEMPO to the $CDCl_3$ solution of **6** causes a significant broadening of both the NH signals (Figure 3B), thus both the NH are not involved in intramolecular hydrogen bonds. The solvent-exposed nature exhibited by γ-lactam NH group rules out the existence of any significant population of folded γ-turn conformations stabilized by intramolecular 3→1 H-bond involving the formylic carbonyl. On the other hand, the lack of the Phe NH excludes the participation of the HCO to H-bond stabilized β-turns and this is in accordance with the high value (8.3 Hz) observed for the Met $J_{NH\text{-}CH\alpha}$.

IR spectra in the NH stretching region (Figure 4), performed in $CHCl_3$ solution, show a strong band centered at 3414 cm^{-1} attributable to free NH groups; no significant lower absorptions are observed, in accordance with the absence of intramolecularly H-bonded structures evidenced by the NMR study. This behaviour is completely different from that exhibited by the related tripeptide **4** which shows a strong band at 3306 cm^{-1} corresponding to intramolecularly H-bonded structures.

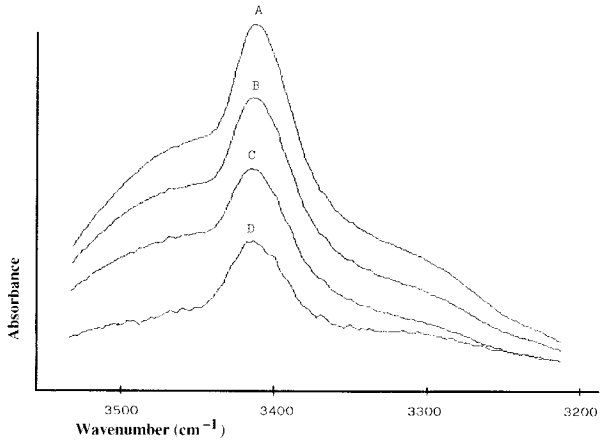


Figure 4. The IR absorption spectra (NH stretching bands) of **6** in chloroform at various concentrations: (A) 10 mM, (B) 5 mM, (C) 1 mM, and (D) 0.3 mM. 0.5 mm cells were used for spectra A and B, 2 mm and 3mm cells for spectra C and D, respectively.

The NOEs observed in the ROESY spectrum of **6** are reported in Table 1. The usual interresidue HCO···Met NH NOE, which is indicative of the *cis* configuration of the two H atoms of the formamido group, is accompanied by a strong interresidue NOE indicating the connectivity of the γ-lactam NH with the Met CH^{α}. The observation of this NOE which is of the $C^{\alpha}_{i}H\cdots N_{i+1}H$ type, taken together with the free accessibility to the solvent shown by the NH groups and the Met and γ-lactam $J_{NH\text{-}CH\alpha}$ values (8.3 and 6.8 Hz, respectively), suggests that the peptide **6** adopts in $CDCl_3$ a semi-extended conformation.

Biological Activity

The new analogues **4** and **6** were evaluated for their potential ability to activate chemotaxis, to stimulate superoxide anion production, and to induce lysozyme release from granules of human neutrophils. Their activity was compared to that of the parent tripeptide fMLP-OMe. Both compounds **4** and **6** are unable to elicit any of the tested biological activities; the observed slight chemotactic activity exhibited by **4** (Figure 5) is not statistically significant ($p > 0.05$).

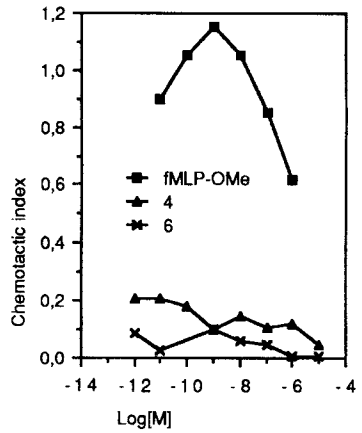


Figure 5. Chemotactic activity of **4**, **6**, and fMLP-OMe toward human neutrophils.

Conclusion

The above reported conformational analysis indicates that the fMLP-OMe analogues **4** and **6**, both conformationally restricted at the central position through short range cyclization, adopt in $CDCl_3$ solution different conformations. In accordance with literature findings[16–18] the azaPro residue present in the tripeptide **4** induces an H-bond stabilized β-turn structure with the aza-residue positioned at the right corner (i+2) of the turn. In the case of the tripeptide **6**, on the other hand, the γ-lactam ring favours a semi-extended backbone conformation. It should be noted here that the γ-lactam modification, developed by Freidinger *et al.*[21], has been introduced as a strategy to stabilize folded β-turn conformations with the γ-lactam occupying the left-corner (i+1) position of the turn[26]. Subsequent studies evidenced that γ-lactams do not favour β-turns unless appropriately modified (i.e. presence of a bulky substituent on the amide nitrogen[27]). In the

present case a H-bonded β-bend incorporating the γ-lactam moiety cannot be formed because of the lack of the appropriate H-bonding donor. It is interesting to note that an extended backbone conformation adopted in the crystal by an *N*-acyl tripeptide containing a central γ-lactam ring has been previously reported by Valle *et al.*[28,29].

The lack of biological activity shown by the tripeptide **4** parallels that already evidenced in the case of the *N*-formyltripeptide *incorporating proline*[6] *or N-methyl-leucine*[15] *at* the central position. Although the conformational preferences of the *N*-methyl-leucyl derivative have not been studied, a comparison between the biological activity and the conformation inside a series of centrally substituted fMLP-OMe analogues has been recently made by Dugas *et al.*[6] while describing the properties of the new ligand HCO-Met-Pro-Phe-OMe. This compound adopts a folded conformation different from that here evidenced for the analogue **4** and shows no chemotactic activity[6,7]. By taking into account these results as well as the high activity shown by fMLP-OMe analogues which are folded in β-turn structures[11], the most reasonable explanation for the lack of activity of the tripeptide **4** is the absence of the H-bonding donor in the amino acid residue at the central position.

In the case of the tripeptide **6**, incorporating the γ-lactam ring and maintaining the central H-bonding donor NH group, the intrinsic nature of the residue replacing the leucine, rather than the absence of the NH at the *C*-terminal phenylalanine, seems responsible for the observed inactivity. This interpretation is supported by previous findings[1,30] showing that at the level of the *C*-terminal fragment the key binding interaction with the receptor is exerted by an H-bond acceptor carbonyl group. The activity shown by the previously studied tetrapeptide HCO-Met-(γ-lactam)-Leu-Phe-OMe[14] and pentapeptide HCO-Met-(γ-lactam)-Leu-Phe-Phe-OH[13], both maintaining the leucine side chain and the *C*-terminal phenylalanine, also supports this explanation.

The lack of biological activity here evidenced for the two fMLP-OMe analogues **4** and **6** confirms the primary role exerted by the central NH as H-bond donor as well as by the nature of the central side chain in order to establish efficient interactions with the receptor. The conformational restrictions induced by the two cyclic residues seem, in the case of the examined models, subordinate to the H-bonding involving the central NH and the *C*-terminal carbonyl, as well as to the hydrophobic interactions established by the central side chain.

Acknowledgements

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Experimental Part

Melting points: Kofler hot stage apparatus, uncorrected.– Optical rotations: Schmidt-Haensch Polartronic D polarimeter, 1 dm cell (in $CHCl_3$, unless otherwise noted), 20 °C.– IR spectra ($CHCl_3$): Perkin-Elmer 983 and Perkin-Elmer 16FPC FT-IR (using 0.5, 2, and 3 mm CsI cells) spectrophotometers.– ^{1}H NMR spectra ($CDCl_3$): Varian EM-390 and XL-300 spectrometers, TMS as int. stand.– A Bruker AM 400 spectrometer operating at 400.13 MHz was used to run two dimensional (2D) spectra. 2D NMR experiments were performed in phase sensitive mode with TPPI phase cycling using 2K of memory for 512 increments. The number of scans was optimized to obtain a satisfactory signal to noise ratio. NOE dipolar correlated 2D spectra in the rotating frame were obtained using the ROESY pulse sequence[31]. The mixing times for the magnetization exchange were 0.3 s. Data were processed on a microVAX II computer system using the program TRITON, written by Boelens and Vuister, University of Utrecht, The Netherlands (courtesy of Prof. R. Kaptein). The FIDs were weighted in both dimensions by a sine-bell apodization function typically shifted by π/3 degrees. The final 2D NMR spectra consisted of 1024 × 1024 data point matrices with a digital resolution of 5 Hz/point. Baseline correction was performed in both dimensions with a 4 term polynomial fit.– Column chromatographies: Merck silica gel 60 (230–400 mesh) (1:30).– TLC and PLC: silica gel Merck 60 F_{254} plates.– Drying agent: sodium sulphate.– Parent fMLP-OMe was prepared according to ref.[32]. – Elemental analyses: Servizio Microanalisi del CNR, Area della Ricerca di Roma, Montelibretti, Italy.

Benzyloxycarbonylpyrazolidine trifluoroacetic salt

1-Benzyloxycarbonyl-2-*t*-butoxycarbonylpyrazolidine[19] (0.391 g, 1.276 mmol) was dissolved in a mixture of TFA (0.8 ml) and dry chloroform (2 ml) and stirred at room temperature for 5 h. The organic solvent was removed under vacuum to afford oily title compound which was used without further purification.

Boc-Met-azaPro-OBzl (**1**)

BOP-Cl (0.3 g, 1.177 mmol) and dry triethylamine (TEA) (0.165 ml, 1.177 mmol) were added at 0 °C to a stirred solution of Boc-Met-OH (0.245 g, 0.981 mmol) in dry tetrahydrofuran (THF) (5 ml). After stirring at 0 °C for 5 min, a solution of the above described TFA salt in dry THF (3 ml) and *N,N'*-dimethylformamide (DMF) (0.5 ml), neutralized with TEA, was added. A further amount of TEA (0.15 ml, 1.08 mmol) was added and the mixture was stirred at 0 °C for 1 h and at room temperature overnight. Ethyl acetate was added in excess and the organic layers were washed with 2N HCl, brine, saturated aqueous $NaHCO_3$ and brine. The organic solution was dried and evaporated under reduced pressure to give an oily residue (0.487 g). Purification on PLC (CH_2Cl_2-EtOAc 8:2) yielded Boc-Met-azaPro-OBzl (**1**) as an homogeneous oil (0.351 g, 82%).– $[\alpha]_D = -1.5°$ (*c* 2.0).– IR: 3431; 1722; 1711; 1665; 1496 cm^{-1}.– ^{1}H NMR: δ = 1.37 [s, 9H, $C(CH_3)_3$], 1.65–2.15 [m, 7H, Met β-CH_2, azaPro γ-CH_2, and S-CH_3 (s at δ 2.00)], 2.47 (t, *J* = 7.5 Hz, 2H, CH_2-S), 3.21 and 4.11 (two broad signals, 4H, azaPro β- and δ-CH_2), 4.79 (m, 1H, Met α-CH), 5.19 (s, 2H, Ph-CH_2-O-CO), 5.25 (broad signal, 1H, NH-CO-O), 7.40 (s, 5H, aromatic).

Boc-Met-azaPro-ONp (**2**)

Following a known procedure[20], the benzyloxycarbonyl group of compound **1** was removed by catalytic transfer hydrogenation under nitrogen atmosphere at 35–40 °C. To a solution of **1** (0.465 g, 1.062 mmol) in absolute ethanol (4.3 ml), 10% Pd/C (0.465 g) and 1,4-cyclohexadiene 98% (1.02 ml, 10.62 mmol) were added. After stirring for 30 min, additional portion of catalyst (0.465 g) and diene (1.02 ml) were added. The mixture was stirred for 30 min and then the catalyst was removed through Celite. The solvent was evaporated under reduced pressure and the oily residue was partitioned between ethyl acetate and 2N HCl. The aqueous phase was alkalinized at 0 °C with 2N NaOH and extracted with ethyl acetate. The organic layers were washed with water, dried and evaporated to afford pure oily 1-Boc-methionylpyrazolidine (0.252 g, 78%).– $[\alpha]_D = +29°$ (*c* 1.0).– IR: 3427; 1704; 1641; 1499; 1483 cm^{-1}.– ^{1}H NMR: δ = 1.41[s, 9H, $C(CH_3)_3$], 1.79–2.26 [m, 7H, Met β-CH_2, azaPro γ-CH_2, and S-CH_3 (s at δ 2.06)], 2.55 (t, *J* = 7.5 Hz, 2H, CH_2-S), 3.01 and 3.54 (two m, 4H, azaPro β- and δ-CH_2), 5.02 (m, 1H, Met α-CH), 5.41 (d, *J* = 9.5 Hz, 1H, NH-CO-O).

This unstable intermediate was immediately *N*-protected according to following procedure: to a stirred solution of the above cited pyrazolidine (0.093 g, 0.305 mmol) in ethyl acetate (1.2 ml) cooled at 0 °C, dry TEA (0.4 mmol) and *p*-nitrophenyl chloroformate (0.074 g, 0.366 mmol) in ethyl acetate (0.6 ml) were added. After 5 min at 0 °C, the suspension was stirred at 50 °C for 20 h. Ethyl acetate was added and the organic solution was washed with satd. aqueous Na_2CO_3 and water, dried and evaporated. The

resulting oil (0.173 g) was chromatographed on PLC (CH_2Cl_2-EtOAc 8:2) to give the title *p*-nitrophenyl derivative **2** as a foam (0.117 g, 82%).– $[\alpha]_D$ = +13° (*c* 1.0).– IR: 3431; 1745; 1705; 1677; 1526; 1492; 1346 cm^{-1}.– ^1H NMR: δ = 1.41 [s, 9H, $C(CH_3)_3$], 1.83–2.38 (m, 7H, Met β-CH_2, azaPro γ-CH_2, and S-CH_3), 2.55 (broad signal, 2H, CH_2-S), 3.50 and 4.07 (two broad signals, 4H, azaPro β- and δ-CH_2), 4.97 (broad signal, 1H, Met α-CH), 5.23 (broad signal, 1H, NH-CO-O), 7.50 and 8.32 (two d, *J* = 9 Hz, 4H, aromatic).

Boc-Met-azaPro-Phe-OMe (**3**)

To a stirred solution of **2** (0.202 g, 0.432 mmol) in dry DMF (2.2 ml) 4-(dimethylamino)pyridine (0.014 g, 0.108 mmol), phenylalanine methyl ester hydrochloride (0.094 g, 0.432 mmol), and TEA (0.06 ml, 0.432 mmol) were added. After stirring at 40 °C for 24 h, ethyl acetate was added in excess and the organic phase was washed with 2N HCl, water, satd. aqueous Na_2CO_3, and water, dried and evaporated. The resulting oil (0.216 g) was chromatographed on PLC (CH_2Cl_2-EtOAc 8:2) to give starting material (34%) and the required azaPro-containing tripeptide **3** as a foam (0.117 g, 53%).– $[\alpha]_D$ = +8° (*c* 1.8, DMF).– IR: 3437; 3313; 1743; 1693; 1672; 1494 cm^{-1}.– ^1H NMR: δ = 1.45 [s, 9H, $C(CH_3)_3$], 1.83–2.38 (m, 4H, Met β-CH_2 and azaPro γ-CH_2), 2.09 (s, 3H, S-CH_3), 2.41–2.88 (two m, 4H, CH_2-S, 1 H of azaPro β-CH_2, and 1 H of azaPro δ-CH_2), 3.04 and 3.28 (A and B of an ABX, *J* = 5, 11, and 14 Hz, 2H, Phe β-CH_2), 3.72 (s, 3H, $COOCH_3$), 3.97 and 4.33 (two m, 2H, 1 H of azaPro β-CH_2, 1 H of azaPro δ-CH_2), 4.52 (m, 1H, Phe α-CH), 4.70 (m, 1H, Met α-CH), 5.21 (d, *J* = 6.5 Hz, 1H, NH-CO-O), 7.15–7.31 (m, 5H, aromatic), 7.31 (d, *J* = 7 Hz, 1H, Phe NH).

Another attempt, carried out for two days, did not improve the yield of **3**.

HCO-Met-azaPro-Phe-OMe (**4**)

Boc-Met-azaPro-Phe-OMe (0.14 g, 0.275 mmol) was dissolved in HCOOH (1.7 ml) and the mixture was stirred at room temperature for one day. After removal of the excess of formic acid under vacuum, the residue was dissolved in dry $CHCl_3$ (1.7 ml) and EEDQ 97% (0.084 g, 0.33 mmol) was added. The solution was stirred at room temperature for 24 h. Evaporation under reduced pressure afforded the crude oily residue (0.218 g) which was purified on PLC (CH_2Cl_2-EtOAc 1:1) to give the title formylderivative as an homogeneous oil (0.11 g, 92%).– $[\alpha]_D$ = –19° (*c* 1.0).– IR: 3429; 3311; 1743; 1672; 1497 cm^{-1}.– ^1H NMR: δ = 1.65–2.03 (m, 4H, Met β-CH_2 and azaPro γ-CH_2), 2.10 (s, 3H, S-CH_3), 2.57 (m, 2H, CH_2-S), 2.79 (m, 2H, 1 H of azaPro β-CH_2 and 1 H of azaPro δ-CH_2), 3.07 and 3.27 (A and B of an ABX, *J* = 4.5, 10.5, and 14 Hz, 2H, Phe β-CH_2), 3.73 (s, 3H, $COOCH_3$), 3.96 and 4.32 (two m, 2H, 1 H of azaPro β-CH_2, 1 H of azaPro δ-CH_2), 4.58 (m, 1H, Phe α-CH), 4.96 (m, 1H, Met α-CH), 6.57 (broad signal, 1H, Met NH), 7.12–7.39 (m, 5H, aromatic), 7.53 (d, *J* = 8 Hz, 1H, Phe NH), 8.18 (s, 1H, H-CO).

Boc-Met-(γ-lactam)-Phe-OMe (**5**)

The mixture (12–15% epimerization) of *t*-butoxycarbonylamino-γ-lactam-bridged dipeptide acids (0.627 g, 1.8 mmol), obtained by treatment of Boc-Met-Phe-OMe methylsulphonium iodide with NaH[21], was dissolved in dry methanol (2 ml) . The solution was cooled at –15 °C and thionyl chloride (0.16 ml) was added. After stirring at –15 °C for 30 min and at 45 °C for 4 h, the solvent was evaporated under reduced pressure and the residue was dried under vacuum. The obtained methyl ester hydrochloride mixture was used without further purification. Isobutyl chloroformate 95% (0.25 ml, 1.8 mmol) was added at –15 °C to a solution of Boc-Met-OH (0.449 g, 1.8 mmol) and *N*-methylmorpholine (NMM) (0.24 ml, 2.16 mmol) in dry dichloromethane (8.6 ml). The temperature was kept at –15 °C for 10 min, and then a solution of the above hydrochlorides and NMM (0.2 ml, 1.8 mmol) in dry dichloromethane (6.5 ml) was added. After stirring at –15 °C for 15 min and at room temperature for one day, the solvent was removed under reduced pressure. The residue was partitioned between ethyl acetate and 5% aqueous $KHSO_4$, and the organic layers were washed with water, saturated aqueous $NaHCO_3$, and water, and then dried and evaporated. The oily residue (0.875 g) was purified by chromatography on a silica gel column eluting with dichloromethane and dichloromethane-ethyl acetate (8:2 and 7:3). Further purification by PLC (dichloromethane-ethyl acetate 1:1) afforded the homochiral Boc-protected derivative **5** (0.649 g, 73%), as an homogeneous oil.– $[\alpha]_D$ –23° (*c* 1.0).– IR : 3427; 2982; 2921; 1740; 1703; 1678; 1495; 1169 cm^{-1}.– ^1H NMR: δ = 1.43 [s, 9H, $C(CH_3)_3$], 1.78 (m, 1H, 1 H of γ-lactam CH_2-CH_2-N), 1.83–2.11 (m, 2H, Met β-CH_2), 2.09 (s, 3H, S-CH_3), 2.51–2.62 [m, 3H, CH_2-S (t at 2.57, *J* = 7.3 Hz) superimposed on 1 H of γ-lactam CH_2-CH_2-N], 2.99 (A of an ABX, *J* = 11.3 and 14.7 Hz, 1H, 1 H of Phe β-CH_2), 3.24 (m, 1H, 1 H of γ-lactam CH_2-CH_2-N), 3.35–3.49 (m, 2H, 1 H of Phe β-CH_2 and 1 H of γ-lactam CH_2-CH_2-N), 3.75 (s, 3H, $COOCH_3$), 4.20 (m, 1H, γ-lactam C*H*-CH_2), 4.30 (m, 1H, Met α-CH), 5.09 (dd, *J* = 11.3 and 5.3 Hz, 1H, Phe α-CH), 5.31 (d, *J* = 8.5 Hz, 1H, NH-CO-O), 6.83 (d, *J* = 5 Hz, 1H, γ-lactam NH), 7.15–7.34 (m, 5H, aromatic).

PLC purification afforded also a more polar epimer (0.104 g, 12%) , as an homogeneous oil.– $[\alpha]_D$ +4° (*c* 1.0).– IR : 3426; 2980; 2923; 1741;1703; 1678; 1494; 1166 cm^{-1}.– ^1H NMR: δ = 1.43 [s, 9H, $C(CH_3)_3$], 1.54 (m, 1H, 1 H of γ-lactam CH_2-CH_2-N), 1.82–2.11 (m, 2H, Met β-CH_2), 2.09 (s, 3H, S-CH_3), 2.50–2.62 [m, 3H, CH_2-S (t at 2.58, *J* = 7.3 Hz) superimposed on 1 H of γ-lactam CH_2-CH_2-N], 3.04 (A of an ABX, *J* = 10.8 and 14.5 Hz, 1H, 1 H of Phe β-CH_2), 3.27–3.42 (m, 3H, 1 H of Phe β-CH_2 and γ-lactam CH_2-CH_2-N), 3.73 (3H, s, $COOCH_3$), 4.23 (m, 1H, Met α-CH), 4.42 (m, 1H, γ-lactam C*H*-CH_2), 5.06 (dd, *J* = 10.8 and 5.8 Hz, 1H, Phe α-CH), 5.27 (d, *J* = 8 Hz, 1H, NH-CO-O), 6.49 (d, *J* = 6.5 Hz, 1H, γ-lactam NH), 7.17–7.36 (m, 5H, aromatic).

HCO-Met-(γ-lactam)-Phe-OMe (**6**)

Boc-Met-(γ-lactam)-Phe-OMe (**5**) (0.242 g, 0.491 mmol) was treated with HCOOH and EEDQ following the procedure described above for compound **3**. Usual work up afforded an oily residue (0.277 g) which was purified on PLC (CH_2Cl_2-EtOAc 7:3) to give the title formyl derivative (0.169 g, 82%), mp 135 °C (dichloromethane-*n*-hexane).– $[\alpha]_D$ –34° (*c* 1.0).– IR : 3415; 2982; 2923; 1742; 1693; 1673; 1495; 1434 cm^{-1}.– ^1H NMR: δ = 1.81 (m, 1H, 1 H of γ-lactam CH_2-CH_2-N), 1.93–2.11 (m, 2H, Met β-CH_2), 2.10 (s, 3H, S-CH_3), 2.49–2.64 [m, 3H, CH_2-S (t at 2.58, *J* = 7.3 Hz) partially superimposed on 1 H of γ-lactam CH_2-CH_2-N], 2.99 (A of an ABX, *J* = 11.3 and 14.7 Hz, 1H, 1 H of Phe β-CH_2), 3.25 (m, 1H, 1 H of γ-lactam CH_2-CH_2-N), 3.34–3.48 (m, 2H, 1 H of Phe β-CH_2 and 1 H of γ-lactam CH_2-CH_2-N), 3.76 (s, 3H, $COOCH_3$), 4.24 (m, 1H, γ-lactam C*H*-CH_2), 4.72 (m, 1H, Met α-CH), 5.06 (dd, *J* = 11.3 and 5.3 Hz, 1H, Phe α-CH), 6.46 (d, *J* = 8.3 Hz, 1H, Met NH), 6.69 (d, *J* = 6.8 Hz, 1H, γ-lactam NH), 7.14–7.36 (m, 5H, aromatic), 8.18 (s, 1H, HCO). –Anal. ($C_{20}H_{27}N_3O_5S$) C, H, N.

Biological Assay

Cells

Human peripheral blood neutrophils were purified employing the standard techniques of dextran (Pharmacia) sedimentation, centrifugation on Ficoll-Paque (Pharmacia), and hypotonic lysis of red cells. The cells were washed twice and resuspended in KRPG (Krebs-Ringer phosphate containing 0.1% w/v glucose, pH 7.4) at a concentration of 50×10^6 cells/ml. Neutrophils were 98–100% pure.

Random Locomotion

Random locomotion was performed with 48-well microchemotaxis chamber (Bio Probe, Italy) and the migration into the filter was evaluated by the method of leading-front[33].The actual control random movement is 32 μm ± 3 SE of ten separate experiments done in duplicate.

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 (10^{-2} M in DMSO) with KRPG containing 1 mg/ml of bovine serum albumin (Orha Behringwerke, FRG) and used at concentrations ranging from 10^{-12} M 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 done in duplicate. Standard errors are in the 0.02–0.09 chemotactic index range.

Superoxide Anion (O_2^-) Production

O_2^- release was monitored continuously in a thermostatted spectrophotometer as superoxide dismutase-inhibitable reduction of ferricytochrome c (Sigma, USA), as described elsewhere[34]. At zero time, different amounts (10^{-9} – 2×10^{-5} M) of each peptide were added and absorbance change accompanying cytochrome c reduction was monitored at 550 nm. 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 $O_2^-/2\times10^6$ cells/5 min and are the mean of six separate experiments done in duplicate. Standard errors are in 0.1–4 nmoles O_2^- range.

Enzyme Assay

Release of neutrophil granule enzymes was evaluated by determining lysozyme activity[34]; this was quantified nephelometrically by the rate of lysis of cell wall suspension of *Micrococcus lysodeikticus* (Sigma). 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. To study the degranulation-inducing activity of each peptide, neutrophils were first incubated with cytochalasin B for 15 min at 37 °C and then in the presence of each peptide in a final concentration of 10^{-9} – 2×10^{-5} M for a further 15 min. The values are the mean of five separate experiments done in duplicate. Standard errors are in 1–6% range.

Statistical Analysis

The nonparametric Wilcoxon test was used in the statistical evaluation of differences between groups.

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