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Probing Structural Requirements of fMLP Receptor: On the Size of the Hydrophobic Pocket Corresponding to Residue 2 of the Tripeptide

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Abstract: The conformationally constrained f-L-Met-Ac$_n$c-L-Phe-OMe (n = 4,9–12) tripeptides, analogues of the chemoattractant f-L-Met-L-Leu-L-Phe-OH, were synthesized in solution by classical methods and fully characterized. These compounds and the published f-L-Met-Xxx-L-Phe-OMe (Xxx = Aib and Ac$_n$c where n = 3, 5–8) analogues were compared to determine the combined effect of backbone preferred conformation and side-chain bulkiness at position 2 on the relation of 3D-structure to biological activity. A conformational study of all the analogues was performed in solution by FT-IR absorption and ^{1}H-NMR techniques. In parallel, each peptide was tested for its ability to induce chemotaxis, superoxide anion production and lysozyme secretion from human neutrophils. The biological and conformational data are discussed in relation to the proposed model of the chemotactic receptor on neutrophils, in particular of the hydrophobic pocket accommodating residue 2 of the tripeptide.

Keywords: 1-aminocycloalkane-1-carboxylic acids; chemotactic peptides; $C^{\alpha,\alpha}$-dialkylated glycines; peptide conformation; β-turn

INTRODUCTION

The discovery that *N*-formyl tripeptides are chemoattractants for neutrophils and are capable of inducing lysosomal enzyme release has led to the investigation of the nature of the peptide–receptor interaction (for leading review articles see [1,2]). A number of structure–activity relationship investigations have been undertaken pertaining to the chemical nature of the two terminal groups and the constituent amino acid side chains. It has been determined that: (i) replacement of the *N*-terminal formyl group induces a dramatic loss of activity; (ii) esterification (in particular, methylation) of the *C*-terminal carboxylic acid group does not result in an appreciable reduction of activity; (iii) increasing the side-chain length of the *N*-terminal amino acid increases the activity, with the presence of a sulphur atom enhancing this effect; (iv) the central amino acid should be hydrophobic, with branched aliphatic residues being the most active and the optimal

Abbreviations: Ac$_n$c, 1-aminocycloalkane-1-carboxylic acid (n, number of carbon atoms in the cycloalkane ring); Aib, α-aminoisobutyric acid; Boc, *tert*-butyloxycarbonyl; DMSO, dimethylsulphoxide; EDC, *N*-ethyl,*N*′-[(3-dimethylamino)propyl]-carbodiimide; f, formyl; HOAt, 7-aza-1-hydroxy-1,2,3-benzotriazole; NMM, *N*-methylmorpholine; OMe, methoxy; OSu, 1-oxysuccinimido; TEMPO, 2,2,6,6-tetramethylpiperidinyl-1-oxy; Z, benzyloxycarbonyl.

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activity given by hydrocarbon groups containing at least three carbon atoms; (v) the *C*-terminal amino acid should be aromatic, with Phe being the preferred choice. Thus, f-L-Met-L-Leu-L-Phe-OH (OMe) best represents these criteria and is accepted as the standard for such chemotactic investigations.

Structural and biological studies of f-L-Met-Xxx-L-Phe-OY (where Xxx is a $C^{\alpha,\alpha}$-dialkylated glycine, e.g. Aib or Ac_nc with $n = 3–8$, and Y is H or Me) analogues have shed light on the biologically active (receptor-bound) conformation of the tripeptide [3–14]. In particular, Aib and the Ac_nc residues [15–20] are known to strongly favour intramolecularly H-bonded conformers of the β-turn [21–23] type. The folded analogues have been found to be biologically highly active in the β-glucosaminidase release experiments on rabbit neutrophils, the $[Ac_nc]^2$ ($n = 6–8$) analogues being even more active than the standard [L-Leu]^2 tripeptide. In this series the $[Ac_7c]^2$ and $[Ac_8c]^2$ analogues are equal to or slightly greater in activity than the $[Ac_6c]^2$ tripeptide, the [Aib]^2 and the $[Ac_nc]^2$ ($n = 3–5$) analogues being significantly less active.

The aim of this paper is to describe the results of a systematic, detailed conformational analysis in solution, using FT-IR absorption and ^{1}H NMR techniques, and of a biological study on human neutrophils (using chemotactic activity, superoxide anion production, and release of the granule enzyme lysozyme) of the f-L-Met-Xxx-L-Phe-OMe (Xxx = L-Leu, Aib, Ac_nc) tripeptides where the number of carbon atoms in the cyclic, $C^{\alpha,\alpha}$-dialkylated glycine series is expanded to include all members from 3 to 12 [16–20].

MATERIALS AND METHODS

Synthesis and Characterization of Peptides

Melting points were determined using a Leitz (Wetzlar, Germany) model Laborlux 12 apparatus and are not corrected. Optical rotations were measured using a Perkin-Elmer (Norwalk, CT, USA) model 241 polarimeter equipped with a Haake (Karlsruhe, Germany) model D thermostat. Thin-layer chromatography was performed on Merck (Darmstadt, Germany) Kieselgel $60F_{254}$ precoated plates using the following solvent systems: 1 ($CHCl_3$–EtOH, 9 : 1), 2 (Bu^nOH–AcOH–H_2O, 3 : 1 : 1), 3 (toluene–EtOH 7 : 1). The chromatograms were examined by UV fluorescence or developed by chlorine-starch-potassium iodide or ninhydrin chromatic reaction as appropriate. All the compounds were obtained in a chromatographically homogeneous state. Amino acid analyses were carried out using a C. Erba (Rodano, Milan, Italy) model 3A-30 amino acid analyser. The Aib and Ac_nc colour yields with ninhydrin are 10–20 times lower than those of protein amino acids.

Infrared Absorption

The solid-state infrared absorption spectra (KBr disk technique) were recorded with a Perkin-Elmer model 580 B spectrophotometer equipped with a Perkin-Elmer model 3600 IR data station and a model 660 printer. The solution spectra were obtained using a Perkin-Elmer model 1720 X FT-IR spectrophotometer, nitrogen flushed, equipped with a sample-shuttle device, at 2 cm^{-1} nominal resolution, averaging 100 scans. Cells with path lengths of 0.1, 1.0 and 10 mm (with CaF_2 windows) were used. Spectrograde deuterochloroform (99.8%, d) was purchased from Merck (Darmstadt, Germany). Solvent (baseline) spectra were recorded under the same conditions.

^{1}H-NMR

The ^{1}H-NMR spectra were recorded with a Bruker (Karlsruhe, Germany) model AM 400 spectrometer. Measurements were carried out in deuterochloroform (99.96% d; Aldrich, Milwaukee, WI, USA) and deuterated dimethylsulphoxide (DMSO) (99.96% d_6; Stohler, Waltham, MA, USA) with tetramethylsilane as the internal standard. The free radical 2,2,6,6-tetramethylpiperidinyl-1-oxy (TEMPO) was purchased from Sigma (St Louis, MO, USA).

Preparation of Human Neutrophils

Cells were obtained from the blood of healthy subjects. Neutrophils were purified employing the standard techniques of dextran sedimentation, centrifugation on Ficoll-Paque and hypotonic lysis of contaminating red cells. The cells were washed twice, resuspended in Krebs-Ringer phosphate containing 0.1% w/v glucose (KRPG), pH 7.4, 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 Trypan blue exclusion test.

Random Locomotion

Random locomotion was evaluated using a 48-well microchemotaxis chamber, by estimating the

distance in micrometers which the leading front of the cell migrated, using the method of Zigmond and Hirsch [24] after 90 min incubation at 37 °C. A 3 μm pore-size filter separated upper and lower compartments. The actual control for random locomotion was 40 ± 4 μm SE (standard error) from 15 separate experiments.

Chemotaxis

This assay was performed by adding each peptide 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 and used at concentrations ranging from 10^{-12} to 10^{-5} M. Data were expressed in terms of chemotactic index (C.I.) which is the ratio: migration towards test attractant minus migration towards the buffer/migration towards the buffer.

Superoxide Anion (O_2^-) Production

This assay was performed by the superoxide dismutase-inhibitable reduction of ferricytochrome c [11] modified for microplate-based assays [25]. The tests were carried out in a final volume of 200 μl containing 4×10^5 neutrophils, 100 nmol of cytochrome c and KRPG. At zero time, different amounts (10^{-10}–5×10^{-5} M) of each peptide were added and the plates were incubated into a microplate reader model Ceres 900 (Bio-Tek Instruments) with the compartment T set at 37 °C. Absorbance was recorded at wavelengths of 550 and 468 nm. Differences in absorbance at the two wavelengths were used to calculate the net nmol of O_2^- produced, using a molar extinction coefficient for cytochrome c of 15.5 mM^{-1} cm^{-1}. Neutrophils were preincubated with 5 μg/ml of cytochalasin B for 5 min prior to activation by peptides.

Granule Enzyme Assay

Release of neutrophil granule enzymes was evaluated by determining lysozyme activity [11] modified for microplate-based assays [25]. Cells were incubated in microplate wells in the presence of each peptide in a final concentration of 10^{-10}–5×10^{-5} M for 15 min at 37 °C. The plates were then centrifuged for 5 min at 400× g and lysozyme was quantified nephelometrically by the rate of lysis of a cell wall suspension of *Micrococcus lysodeikticus*. Neutrophils were preincubated with 5 μg/ml of cytochalasin B for 5 min at 37 °C prior to activation by peptides. The reaction rate was measured with a microplate reader at 465 nm. The enzyme was expressed as the 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.

Statistical Analysis

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

RESULTS AND DISCUSSION

Peptide Synthesis

The Ac_nc (n = 4,9-12) α-amino acid hydrochlorides were prepared by treatment of the corresponding cycloalkanone with sodium cyanide, acetic acid and excess of ammonia, and subsequent acid hydrolysis (HCl/HCOOH at 0°–20 °C) of the α-aminonitrile intermediate (Strecker synthesis). A more drastic acid hydrolysis (6 N HCl, under reflux) of the Ac_nc amide hydrochloride afforded the free amino acid.

The Z-protected Ac_nc derivatives were obtained either by reacting the free amino acid with Z-Cl in an acetone — water (pH 10.9) mixture or, more conveniently, with Z-OSu in acetonitrile in the presence of tetramethylammonium hydroxide.

The Z-Ac_nc-L-Phe-OMe dipeptides and Boc-L-Met-Ac_nc-L-Phe-OMe tripeptides were synthesized (45%–90% yield) using EDC in the presence of HOAt as the hydroxylamine-based additive [26]. Removal of the Z N^{α}-protecting group was performed by catalytic hydrogenation and of the Boc group by acidolysis with HCl/CH_3OH. The formyl group was introduced via the HCOOH/EDC method.

The physical properties and analytical data for the newly synthesized, chromatographically homogeneous f-L-Met-Xxx-L-Phe-OMe analogues and their peptide intermediates are listed in Table 1. All compounds were also characterized by ^{1}H-NMR and the N^{α}-formylated tripeptides by amino acid analysis as well (data not reported).

Solution Conformational Analysis

The conformational preferences of the f-L-Met-Xxx-L-Phe-OMe (Xxx = Aib, Ac_nc with n = 3–12) analogues were determined in a structure-supporting solvent ($CDCl_3$) by FT-IR absorption and ^{1}H-NMR in comparison with those of the parent tripeptide (Xxx = L – Leu) [6].

Figure 1 shows, as a representative example, the FT-IR absorption spectrum of the $[Ac_{10}c]^2$ analogue

Table 1 Physical Properties and Analytical Data for the Newly Synthesized f-L-Met-Xxx-L-Phe-OMe Analogues and their Peptide Intermediates

Compound	Melting point (°C)	Recrystallization solvent[a]	$[\alpha]_D^{20}$ (°)[b]	TLC			IR[c] (cm^{-1})
				R_{FI}	R_{FII}	R_{FIII}	
Z-Ac_4c-L-Phe-OMe	105–106	EtOAc/LP	−1.3; +3.8[d]	0.95	0.95	0.50	3303, 1747, 1683, 1653, 1543, 1520
Boc-L-Met-Ac_4c-L-Phe-OMe	132–134	EtOAc/LP	−16.6	0.95	0.95	0.45	3399, 3375, 3282, 1728, 1693, 1678, 1650, 1524
f-L-Met-Ac_4c-L-Phe-OMe	135–136	EtOAc/LP	−12.0[e]	0.85	0.95	0.40	3364, 3343, 3282, 1724, 1679, 1643, 1630, 1525
Z-Ac_9c-L-Phe-OMe	140–141	EtOAc/LP	−14.9	0.95	0.95	0.60	3410, 3279, 1721, 1648, 1542
Boc-L-Met-Ac_9c-L-Phe-OMe	157–158	EtOAc/LP[f]	−25.9	0.95	0.95	0.50	3422, 3288, 1741, 1708, 1680, 1653, 1517
f-L-Met-Ac_9c-L-Phe-OMe	132–133	EtOAc/LP	−16.3[e]	0.90	0.85	0.40	3286, 1741, 1658, 1525
Z-Ac_{10}c-L-Phe-OMe	143–144	EtOAc/LP	−3.4	0.95	0.85	0.70	3277, 1738, 1719, 1644, 1582, 1542
Boc-L-Met-Ac_{10}c-L-Phe-OMe	171–173	EtOAc/LP	−15.3	0.95	0.85	0.60	3400, 3277, 1739, 1719, 1644, 1541
f-L-Met-Ac_{10}c-L-Phe-OMe	134–136	EtOAc/LP	−17.0	0.95	0.75	0.45	3294, 1740, 1657, 1524
Z-Ac_{11}c-L-Phe-OMe	140–141	EtOAc/LP	−2.9; −8.5[d]	0.95	0.85	0.55	3391, 3290, 1739, 1719, 1644, 1582, 1541
Boc-L-Met-Ac_{11}c-L-Phe-OMe	170–171	EtOAc/LP	−14.6	0.95	0.85	0.30	3415, 3338, 1743, 1706, 1675, 1654, 1519
f-L-Met-Ac_{11}c-L-Phe-OMe	136–138	EtOAc/LP	−9.4	0.85	0.80	0.15	3278, 1740, 1657, 1527
Z-Ac_{12}c-L-Phe-OMe	162–163	EtOAc	−6.8	0.95	0.85	0.70	3391, 3299, 1741, 1721, 1643, 1541
Boc-L-Met-Ac_{12}c-L-Phe-OMe	184–185	EtOAc	−34.5	0.95	0.85	0.55	3368, 1748, 1699, 1678, 1658, 1518
f-L-Met-Ac_{12}c-L-Phe-OMe	172–173	$CHCl_3$/DE	−22.2	0.80	0.80	0.40	3295, 1741, 1660, 1532

[a] EtOAc, ethyl acetate; LP, light petroleum; DE, diethyl ether.
[b] $c = 0.5$, methanol.
[c] The IR absorption spectra were obtained in KBr pellets (only significant bands in the 3500–3200 and 1800–1500 cm^{-1} regions are reported).
[d] $[\alpha]_{436}^{20}$.
[e] $c = 0.1$, methanol.
[f] Purified by flash chromatography using a silica-gel column.

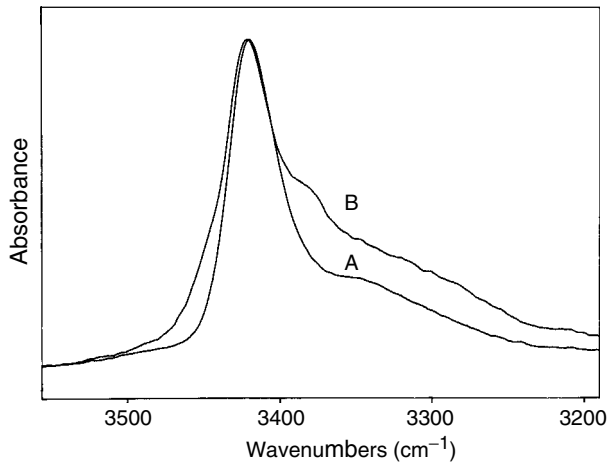

Figure 1 FT-IR absorption spectra (N–H stretching region) of f-L-Met-L-Leu-L-Phe-OMe (A) and f-L-Met-Ac_{10}c-L-Phe-OMe (B) in $CDCl_3$ solution (peptide concentration 1 mM).

compared with that of the [L-Leu]^2 prototype at 1 mM concentration. In each spectrum a strong band at about 3425 cm^{-1} is clearly observed, corresponding to the stretching mode of free (solvated) N–H groups [27]. A less intense band at approximately 3375 cm^{-1} in the [Ac_{10}c]^2 tripeptide and a shoulder at about 3340 cm^{-1} in the [L-Leu]^2 tripeptide, both assigned to H-bonded N–H groups [27], are also seen. The intensity of the 3340 cm^{-1} band of the [L-Leu]^2 tripeptide is strongly enhanced with increasing concentration, but the medium-intensity 3375 cm^{-1} band of the [Ac_{10}c]^2 tripeptide is concentration-insensitive. Therefore, we assign the band at 3340 cm^{-1} to *inter*molecularly H-bonded N–H groups, while the band at 3375 cm^{-1} to *intra*molecularly H-bonded N–H groups. These FT-IR absorption results strongly suggest that in f-L-Met-Ac_{10}c-L-Phe-OMe, in contrast to the [L-Leu]^2 parent tripeptide, a folded conformation, stabilized by an intramolecular C=O···H–N H-bond, is significantly populated in $CDCl_3$ solution. The spectra of the [Aib]^2 and the other [Ac_nc]^2 analogues closely reflect that of the [Ac_{10}c]^2 analogue.

The assignment of the three NH protons in the ^{1}H-NMR spectrum of f-L-Met-Ac_{10}c-L-Phe-OMe is unequivocal. The NH proton signal of the C^α-tetrasubstituted α-amino acid Ac_{10}c was unambiguously recognized as the only singlet NH resonance, while the L-Met and L-Phe signals were assigned via ROESY and TOCSY bidimensional NMR experiments. The involvement of the *C*-terminal NH group (Phe) in intramolecular H-bonding at 1 mM concentration was determined on the basis of the modest variation in chemical shift experienced upon addition of the strong H-bonding acceptor solvent DMSO to the $CDCl_3$ solution [28,29] and of the lack of line broadening in the presence of the paramagnetic, free radical nitroxide TEMPO [30] (Figure 2). Conversely, the behaviour of the NH proton resonances of the L-Met and Ac_{10}c residues are in favour of the conclusion that these NH groups are solvent accessible. At higher concentrations a limited amount of self-association takes place, as indicated by the small variation (to lower fields) of the chemical shifts of the NH protons, particularly of those residues (Met and Ac_{10}c) not involved in intramolecular H-bonding (data not shown). It is worth recalling that all three NH proton

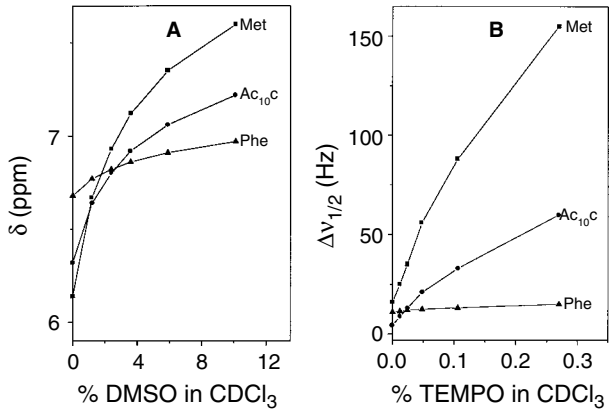

Figure 2 (A) Plot of NH proton chemical shifts in the ^{1}H-NMR spectrum of f-L-Met-Ac_{10}c-L-Phe-OMe as a function of increasing percentages of DMSO (v/v) added to the $CDCl_3$ solution. (B) Plot of bandwidths of the NH proton signals in the ^{1}H-NMR spectrum of the same peptide as a function of increasing percentages of TEMPO (w/v) added to the $CDCl_3$ solution (peptide concentration 1 mM).

chemical shifts of the parent tripeptide f-L-Met-L-Leu-L-Phe-OMe exhibit pronounced concentration effects in $CDCl_3$ solution, moving sharply downfield [31]. All these conclusions fit nicely with those extracted from the IR absorption analysis (discussed above).

The spectroscopic evidence thus indicates that a significant population of species characterized by the Phe NH group in an intramolecular H-bond is present in the conformational equilibrium mixture of the $[Ac_{10}c]^2$ tripeptide analogue in $CDCl_3$ solution. The known stereochemical bias of the Aib and Ac_nc residues for values of the ϕ,ψ backbone torsion angles in the helical region of the conformational space [$\phi = \pm 60^\circ(\pm 20^\circ)$, $\psi = \pm 30^\circ(\pm 20^\circ)$] [15–20] would then support an overwhelming occurrence of β-turn conformers.

However, both type-I (or the related type-III) and type-II β-turns are sterochemically acceptable for an -L-Met-Xxx- (Xxx = Aib or Ac_nc) sequence, due to the helicogenic and achiral characteristics of these $C^{\alpha,\alpha}$-symmetrically disubstituted glycines. To discriminate between these possibilities an NOE investigation was undertaken. The interproton distance C^{α}_{i+1}–H···N_{i+2}–H in an ideal type-II β-turn is estimated to be 2.1 Å, while it is ca. 3.5 Å in a type-I (or III) β-turn. Therefore, an intense NOE of this type is expected only in the type-II β-turn conformation. Indeed, in the f-L-Met-$Ac_{10}c$-L-Phe-OMe tripeptide a significant NOE is observed between the Met C^{α}–H and $Ac_{10}c$ N–H proton resonances. At variance with f-L-Met-L-Leu-L-Phe-OMe [31] closely similar NMR results have also been found for all of the N^{α}-formylated tripeptide methyl esters incorporating in position 2 either an Aib or a different Ac_nc residue.

Taken together, the above findings strongly support the view that in $CDCl_3$ solution the -L-Met-Aib- and -L-Met-Ac_nc- sequences of the formyl chemotactic tripeptide methyl ester analogues favour a type-II β-turn conformation stabilized by an intramolecular H-bond between the formyl C=O and the Phe N–H groups.

Biological Activity

The biological activities of the tripeptides f-L-Met-Xxx-L-Phe-OMe (Xxx = Aib, Ac_nc with n = 3–12) were determined in human neutrophils and compared with that of the parent tripeptide (Xxx = L-Leu). Directed migration (chemotaxis) (Figure 3), superoxide anion production (Figure 4) and lysozyme release (Figure 5) were measured.

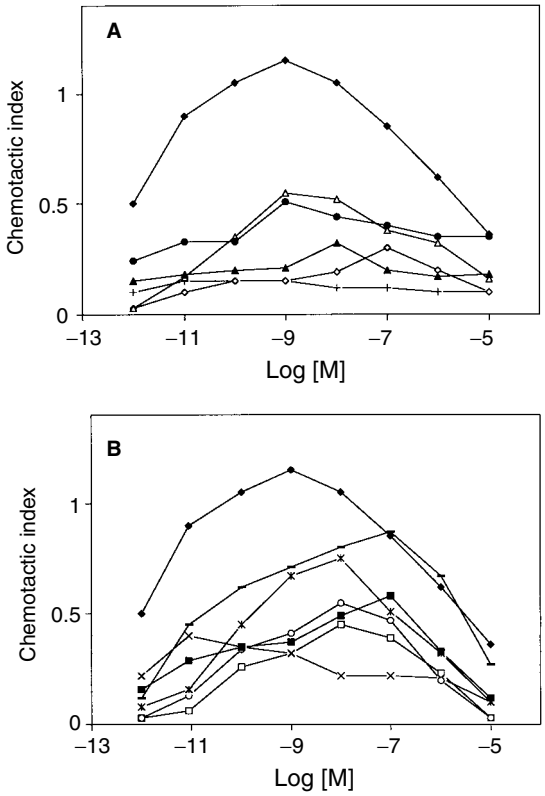

Figure 3 Chemotactic activity of f-L-Met-Xxx-L-Phe-OMe and its analogues towards human neutrophils. The data are the mean of five separate experiments performed in duplicate. SEs are in the 0.01 — 0.09 chemotactic index range. (A) Xxx = L-Leu (♦), Aib (+), Ac_3c (✧), Ac_4c (Δ), Ac_5c (▲), and Ac_6c (●). (B) Xxx = L-Leu (♦), Ac_7c (–), Ac_8c (□), Ac_9c (✖), $Ac_{10}c$ (❍), $Ac_{11}c$ (✳), and $Ac_{12}c$ (■).

As shown in Figure 3 (panel B), the peptides $[Ac_4c]^2$ and $[Ac_6c]^2$ exhibit the same efficiency (peptide concentration at which the maximal activity is observed [32]) as the parent $[L\text{-Leu}]^2$ peptide, being maximally effective at 10^{-9} M, but they are chemotactically significantly less potent (0.55 C.I.). The small C.I. values shown by the $[Aib]^2$, $[Ac_3c]^2$, $[Ac_5c]^2$ analogues are not statistically significant. Both a lower efficiency and potency as chemotaxins, when compared with the $[L\text{-Leu}]^2$ tripeptide, are exhibited by the analogues shown in Figure 3 (panel B). All tripeptides, except the $[Ac_9c]^2$ analogue, require high doses to exert a chemotactic activity, being maximally effective at 10^{-8} M for the $[Ac_8c]^2$, $[Ac_{10}c]^2$ and $[Ac_{11}c]^2$ analogues and at 10^{-7} M for the $[Ac_7c]^2$ and $[Ac_{12}c]^2$ analogues. The activity shown by the $[Ac_9c]^2$ analogue is at the limit of statistical significance. As Figure 3 (panels A and B) illustrate, the chemotactically active peptides

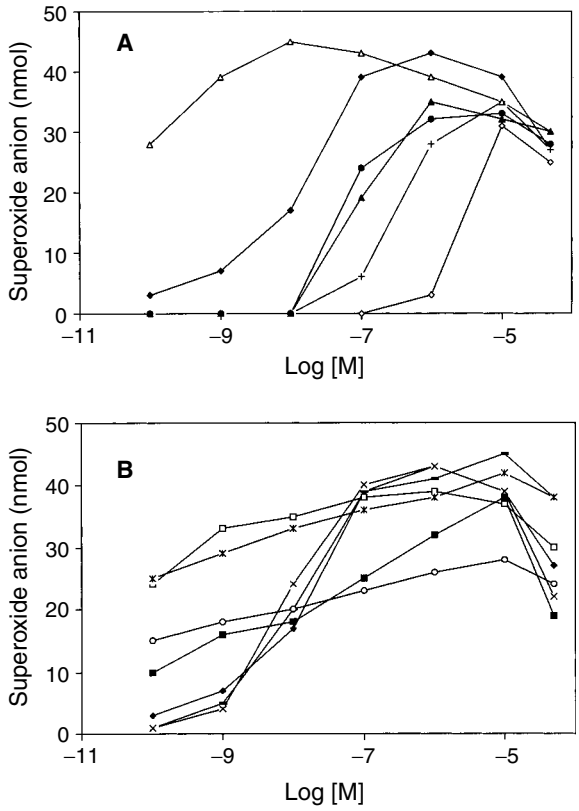

Figure 4 Superoxide anion production of f-L-Met-Xxx-L-Phe-OMe and its analogues towards human neutrophils. The data are the mean of five separate experiments performed in duplicate. SEs are in the 0.1–4 nmol O_2^- range. The symbols for the various peptides are the same as those in Figure 3.

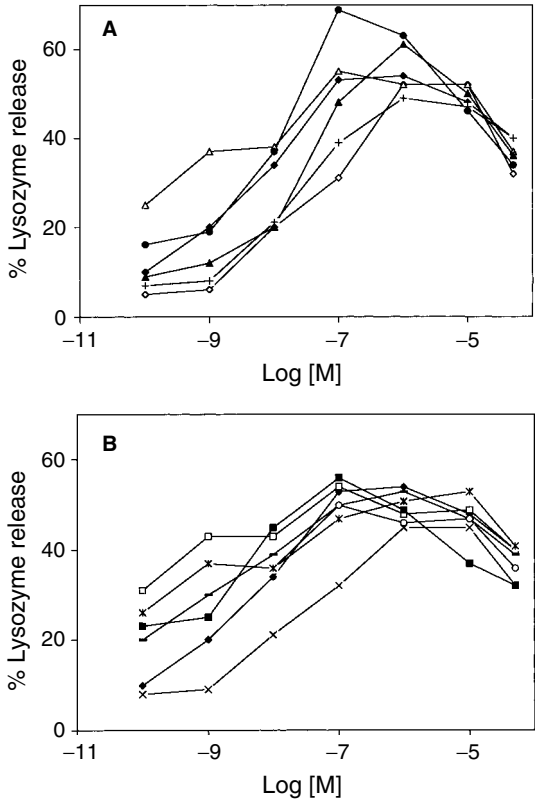

Figure 5 Release of neutrophil granule enzymes evaluated by determining lysozyme activity induced by f-L-Met-Xxx-L-Phe-OMe and its analogues. The data are the mean of five separate experiments performed in duplicate. SEs are in the 1%–6% range. The symbols for the various peptides are the same as those in Figure 3.

show biphasic curves with inhibition at higher concentration, as reported for other chemotactic factors [33].

In the superoxide anion production assays (Figure 4, panel A) the $[Aib]^2$, $[Ac_3c]^2$, $[Ac_5c]^2$ and $[Ac_6c]^2$ tripeptides show a behaviour similar to that of the parent tripeptide at higher concentrations (10^{-5}–5×10^{-5} M). At 10^{-6} M concentration the $[Ac_3c]^2$ analogue is inactive, while the order of potency of the other analogues is: $[L\text{-Leu}]^2 > [Ac_4c]^2 > [Ac_5c]^2 > [Ac_6c]^2 > [Aib]^2$. The $[Ac_4c]^2$ analogue maintains the activity throughout all concentrations tested, having its maximum at 10^{-8} M concentration. As shown in panel B, at the physiological concentrations of 10^{-7}–10^{-5} M, the $[Ac_nc]^2$ ($n = 7$–9, 11 and 12) peptides exhibit a biological activity comparable to that of the parent tripeptide. The $[Ac_{10}c]^2$ analogue is active, even if with a lower potency than those exhibited by the other tripeptides.

As for the enzyme secretagogue activity, all peptides are active in the range of physiological concentrations (10^{-7}–10^{-5} M). In particular, the $[Ac_6c]^2$ analogue (Figure 5, panel A) shows a remarkable activity at 10^{-7} M concentration, significantly higher than that of the parent tripeptide. The $[Ac_nc]^2$ ($n = 4$ in panel A; $n = 7,8,10,12$ in panel B) tripeptides behave similarly to the parent tripeptide. The $[Aib]^2$, $[Ac_3c]^2$ and $[Ac_5c]^2$ analogues (panel A) exert their maximal potency at 10^{-6} M concentration, while the $[Ac_9c]^2$ and $[Ac_{11}c]^2$ analogues at 10^{-5} M concentration.

In conclusion, the results of our biological assays support the view that most of the formyl tripeptide analogues investigated in this work bind to the receptor conformation able to selectively activate the transduction pathways involved in superoxide production and lysozyme release, but the same peptides fulfill some but not all the criteria required

to bind the appropriate area of the receptor responsible for chemotaxis.

CONCLUSIONS

In recent years we and others [6,9,34–38] have shown that the exploitation of the family of cyclic $C^{\alpha,\alpha}$-dialkylated glycines (Ac_nc) to map the binding pocket of protein receptors may be complementary to the classical amino acid residue scan approach. The two concepts differ in the sense that the classical type of scan involves the incorporation of the same residue (e.g. Ala) at different positions of the peptide chain, whereas the Ac_nc scan [19] involves the incorporation of different residues at the same position of the peptide chain. Both types of scan strictly require that the various replacements would not alter the overall conformation of the set of peptides under investigation.

In this work we have expanded the Ac_nc scan approach by use of a complete and larger set of $C^{\alpha,\alpha}$-dialkylated glycines characterized by cycloalkane rings of widely different size, i.e. from cyclopropyl ($n = 3$) to cyclododecyl ($n = 12$) systems. Our solution conformational analysis allows us to conclude that all of the $[Ac_nc]^2$ formyl tripeptide chemoattractants examined are significantly folded in a type-II β-turn conformation. Consequently, it is safe to relate any trend observed in the biological activity of the members of this peptide series to a variation in their side-chain effective volume and hydrophobicity at position 2.

In previous investigations [3–6,9] a series of chemotactic tripeptide analogues characterized by a more limited set of $[Ac_nc]^2$ ($n = 3$–8) replacements provided a hint towards a possible relationship between cycloalkane ring size of the ligand and granule enzyme (β-glucosaminidase) release in rabbit neutrophils, thereby confirming [1,2] the potentially discriminating role of a hydrophobic cavity in this receptor in accommodating the residue 2 side chain of the tripeptide. In contrast, in the present study a marked difference is not observed upon increasing the size of the cycloalkane system at position 2 in any of the three types of assays (chemotactic activity, O_2^- production and lysozyme secretion) performed on human neutrophils. The significance of the discrepancies in the behaviour of the tripeptide ligands towards rabbit and human neutrophils is unclear, but heterogeneity and differences among chemotactic peptide receptors are real possibilities [39–41]. In any case, the availability of high affinity ligands in well defined and distinct assays, such as some of those described in this paper, should provide useful tools in further probing 3D-structural requirements of formyl peptide receptors in different cell lines.

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