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# The Importance of the Peptide Bond at Position 2 in HCO-Met-Leu-Phe-OMe Analogues as shown by Studies on Human Neutrophils

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Abstract: The formylpeptides formyl-methionyl-*N*-methylleucyl-phenylalanine methyl ester [for-Met-(*N*Me)Leu-Phe-OMe] **1**, formyl-methionyl-2-aminotetralin-2-carboxyl-phenylalanine methyl ester [for-Met-Atc-Phe-OMe] **2**, formyl-methionyl-1,2,3,4-tetrahydroisoquinoline-3-carboxyl-phenylalanine methyl ester [for-Met-Tic-Phe-OMe] **3** and formyl-methionyl-2-aminoxy-4-methylvaleryl-phenylalanine methyl ester [for-Met-OLeu-Phe-OMe] **4** were synthesized in order to investigate the role of the amide bond at position 2 on biological activities on human neutrophils. Only analogue **2**, which keeps the NH group at position 2, was found to retain activity though sterically encumbered.

Keywords: *N*-formylmethionyl peptides; human neutrophils; chemotaxis; superoxide anion generation; lysozyme release

## INTRODUCTION

The plasma membranes of human neutrophils possess specific receptors for *N*-formyl chemotactic peptides. These peptides are believed to be structural analogues of bacterial metabolic products and are potent chemoattractants for granulocytes. Binding of peptides to specific receptors, functionally coupled to a G-protein, stimulates chemotaxis, lysosomal enzyme secretion, superoxide anion production and an array of other cellular responses. These functional responses seem to be mediated through the generation of a transduction mechanism that involves the activation of phospholipases C, D and $A_2$ [1–3]. HCO-Met-Leu-Phe-OH (fMLP) is the prototype of the formyl chemotactic peptides, and together with its methyl ester derivative has become the reference peptide for studying leucocyte migration, oxidative metabolism and degranulation. Human fMLP receptors are found to consist of several components, the biggest of which is a glycoprotein of 55–70 kDa molecular weight [4–6]. fMLP receptors on human neutrophils exist in at least two isoforms characterized by different molecular weights and binding affinities [7,8]; different receptor subtypes may activate separate second messenger pathways, and hence specific biological responses [9].

Referring to prototype fMLP, it is ascertained that *N*-formylation is necessary for optimal activity and simulates the situation at the amino end of proteins produced by bacteria such as *E. coli*. Methionine at position 1 is optimal for binding to and activation of the receptor, owing to the free doublet of the sulphur that allows the peptide to link to a positive area on the receptor. Phenylalanine seems to be the most effective amino acid at position 3 [10]. Concerning

Abbreviations: Atc, 2-aminotetralin-2-carboxylic acid; EEDQ, *N*-ethoxy-carbonyl-2-ethoxy-1,2-dihydroquinoline; IBCF, iso-butylchloroformate; *N*(Me)Leu, *N*-methylleucine; NMM, *N*-methylmorpholine; OLeu, L-2-aminoxy-4-methylvaleric acid; SE, standard error; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TMS, tetramethylsilane.

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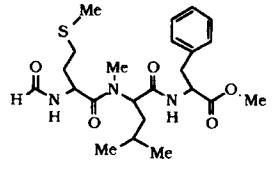
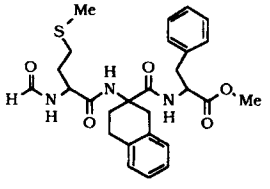
position 2, a huge number of substitutions have been accomplished, introducing both natural and synthetic amino acids [11–15]. The importance of substitutions is linked to the possibility that the peptide chain is properly allocated in the receptor pocket.

While the majority of current studies aim to clarify patterns of both the specific receptor and the chemotactic peptides that have to fit well with it, rather little has been done to clarify the mechanisms of hooking the chemotactic molecules to the receptor. The authors thought it would be interesting to study *N*-formyl tripeptides in which the peptide linkage at position 2 is varied. In a previous work [16] we found that substitution of the amide bond at position 2 with an ester linkage causes loss of all biological activities, and we hypothesized that it can be due to the inability of those peptides to recognize and/or to link to the specific receptor, or to be hydrolysed by a specific peptidase [17, 18]. However, it is not clear so far if the loss of biological response is due to the different nature of the ester bond with respect to the amide bond.

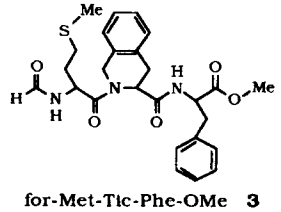
To shed more light on the problem we synthesized (a) compound **1** where the NH group at position 2 is methylated, so depriving the amide linkage of a possible hydrogen bond donor; (b) compound **2** where the Leu residue is substituted by Atc which causes a remarkable stiffening of the backbone but keeps the amide bond intact; (c) compound **3** where the Leu residue is substituted by Tic which is also sterically encumbered and moreover leads to an aprotic amide linkage being the NH group annulated in the isoquinoline ring; and (d) compound **4** where the amide group is substituted by an amidoxy group that keeps the NH protic but influences the electronic distribution and introduces a possible hydrogen bond acceptor.



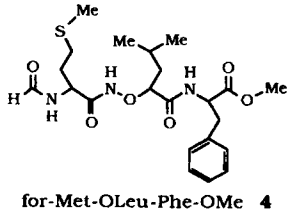
for-Met-(NMe)Leu-Phe-OMe **1**



for-Met-Atc-Phe-OMe **2**



for-Met-Tic-Phe-OMe **3**



for-Met-OLeu-Phe-OMe **4**

## MATERIALS AND METHODS

The $^1$H-NMR spectra were recorded in $CDCl_3$ on a Brucker AC200 spectrometer at 200 MHz. Chemical shifts are expressed as $\delta$ related to the TMS signal.

Melting points were determined on a Reichert-Kofler block and are uncorrected.

Thin layer chromatography was performed on precoated silica gel $F_{254}$ (Merck) with the solvent system: dichloromethane–toluene–methanol (17:1:2).

Satisfactory microanalyses were obtained for all compounds, analytical results being within $\pm 0.4\%$ of the theoretical values.

L-Met, L-Leu, L-Phe and DL-Tic residues were purchased from Fluka as hydrochlorides. L-(NMe)Leu was purchased from Biochim as hydrochloride. The Atc residue was prepared from $\beta$-tetralone-hydantoin and was obtained as a DL mixture [19]. L-OLeu was synthesized according to the literature [20]. The *t*-Boc amino acids were synthesized using di-*tert*-butyl dicarbonate as the di-*tert*-butyloxycarbonylating agent [21]. Removal of the *t*-Boc group was performed by treatment with a 1:1 mixture of TFA-$CHCl_3$. Peptide coupling was achieved (a) by the racemization-free mixed-anhydride method with IBCF [22] and (b) by the DCC–HOBt method [23]. The formyl group was introduced at the level of tripeptide according the EEDQ method [24].

### For-Met-(NMe)Leu-Phe-OMe

The peptide was synthesized following standard procedures in solution (Figure 1). Oil ($R_F$ 0.83). $^1$H-NMR: 0.81 and 0.85 (6H; $2CH_3$; 2d; $J = 7.50$ $H_2$); 1.35–1.75 (5H; $CH_2 + CH_2 + CH$; m); 2.03 (3H; $SCH_3$; s); 2.45 (2H; $CH_2$; m); 2.73 (3H; $NCH_3$; s); 2.94 and 3.13 (2H; $CH_2$; ABX; 2dd); 3.67 (3H; $OCH_3$; s); 4.58 (1H; CH; m); 4.74 (1H; CH; m); 5.00 (1H; CH; m); 6.35 (1H; NH; d; $J = 7.63$ Hz); 6.43 (1H; NH; d; $J = 8.49$ Hz); 6.35 (1H; NH; d; $J = 7.62$ Hz); 6.95–7.34 (5H; $C_6H_5$; m); 8.12 (1H; HCO; s).

### For-Met-Atc-Phe-OMe

The peptide was synthesized following standard procedures in solution (Figure 1). Solid ($R_F$ 0.54). $^1$H-NMR: the diastereomeric mixture shows among other signals 1.95 [2 × (3H; $CH_3$); 2s]; 3.68 and 3.71 [2 × (6H; $2CH_3$); 2s]; 7.92 and 7.96 [2 × (1H; HCO); 2s].

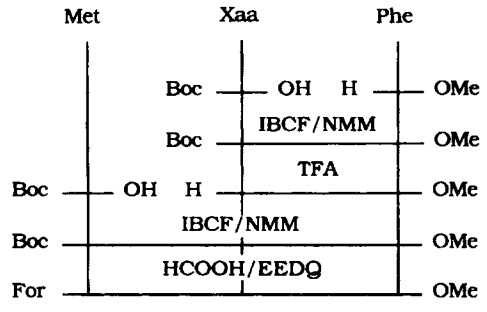



Figure 1 Synthesis of compounds **1** [Xaa = (NMe)Leu], **2** [Xaa = Atc] and **3** [Xaa = Tic].

### For-Met-Tic-Phe-OMe

The peptide was synthesized following standard procedures in solution (Figure 1). Deliquescent solid ($R_F$ 0.58). $^1$H-NMR: the diastereomeric mixture shows among other signals 2.03 and 2.09 [2 × (3H; $CH_3$); 2s]; 3.62 and 3.67 [2 × (6H; $2CH_3$), 2s]; 8.01 and 8.19 [2 × (1H; HCO); 2s].

### For-Met-OLeu-Phe-OMe

The peptide was synthesized following standard procedures in solution (Figure 2). Solid (m.p. 140–142°C; $R_F$ 0.75). $^1$H-NMR: 0.87 and 0.84 (6H; $2CH_3$; 2d; $J$ = 6.50 Hz); 1.44 (2H; $CH_2$; m); 2.35–1.85 (4H; $2CH_2$; m); 2.10 (3H; $SCH_3$; s); 2.53 (2H; $CH_2$; m); 3.22 and 2.97 (2H; $CH_2$; AB of ABX; 2dd; $J_{AB}$ = 13.94 Hz; $J_{AX}$ = 5.25 Hz; $J_{BX}$ = 9.44 Hz); 3.72 (3H; $OCH_3$; s); 4.25–4.21 (1H; CH; m); 4.82–4.58 (2H; 2CH; m); 6.87 (1H; NH; d; $J$ = 8.44 Hz); 7.34–7.20 (5H; $C_6H_5$; m); 7.84 (1H; NH; d; $J$ = 8.16 Hz); 8.14 (1H; HCO; s); 10.15 (1H; NHO; s).

### Human Neutrophils

Human neutrophils were purified using the standard techniques of dextran sedimentation of heparinized blood, followed by centrifugation on Ficoll-Paque and hypotonic lysis of red cells. The cells were washed twice and resuspended in Krebs–Ringer–phosphate containing 0.1% w/vol. glucose (KRPG), pH 7.4. The percentage of neutrophils was 98–100% pure.

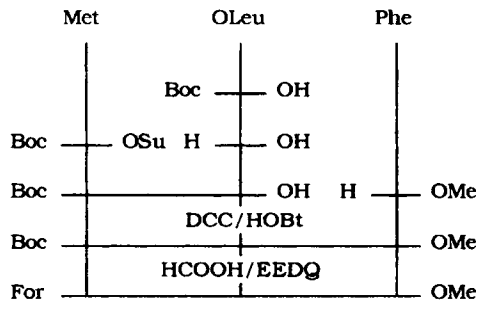



Figure 2 Synthesis of compound **4**.

### Random Locomotion

Random locomotion was performed with a 48-well microchemotaxis chamber and the migration into the filter was evaluated by the leading-front method [25]. The actual control random movement is 32 $\mu$m ± 3 SE of ten separate experiments done in duplicate.

### Chemotaxis

Chemotaxis was studied 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 which is the ratio (migration toward test attractant minus migration toward the buffer)/(migration toward the buffer). The dose–response curves are typical of chemoattractants that rise to a peak and then decline to zero as the concentration of the ligand is increased above its optimum value.

### Superoxide Anion Production

Superoxide anion ($O_2^-$) production was monitored continuously in a thermostatted spectrophotometer as superoxide dismutase-inhibitable reduction of ferricytochrome c, as described elsewhere [26]. At zero time, different amounts ($10^{-8}$–$10^{-5}$ M) of each peptide were added and absorbance change accompanying cytochrome c reduction was monitored at 550 nm. Results were expressed as net nmoles of $O_2^-/2 \times 10^6$ cells/5 min. Neutrophils were incubated with 5 $\mu$g/ml cytochalasin B for 5 min prior to activation by peptides.

### Enzyme Assay

The lysozyme was quantified nephelometrically by the rate of lysis of cell wall suspension of *Micrococcus lysodeikticus*. Enzyme release was expressed as a net percentage of the total enzyme content released by 0.1% Triton X-100. Total enzyme activity was 85 ± 1 $\mu$g/l × $10^7$ cells/min. To study the degranula-

tion-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 $10^{-8}$–$10^{-5}$ M for a further 15 min.

### Statistical Analysis

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

## RESULTS

All the biological results reported have been compared with the parent HCO-Met-Leu-Phe-OMe. Directed migration (chemotaxis), superoxide anion production and lysozyme release have been measured in human neutrophils.

Our data show that Tic, (NMe)Leu and OLeu derivatives are devoid of any biological activity.

Chemotaxic activity for the Atc derivative, as reported in Figure 3, shows a reduction: it is about four times less active than the parent compound in a range of concentrations as low as $10^{-12}$–$10^{-8}$ M. Superoxide anion production triggered by Atc, as shown in Figure 4, is similar ($p = 0.05$) to the parent peptide at physiological concentrations of $10^{-6}$–$10^{-5}$ M. As shown in Figure 5, Atc lysozyme release is lower than the parent compound at all the concentrations tested, thus exhibiting a lower efficacy.

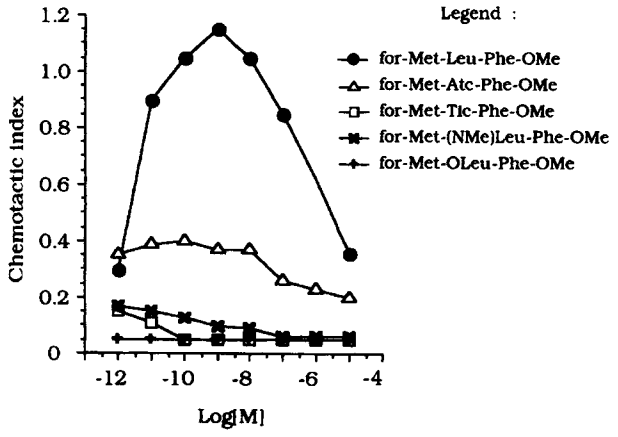



Figure 3 Chemotactic activity of fMLP-OMe and its analogues toward human neutrophils. The points are the mean of five separate experiments done in duplicate. The SE is the 0.02–0.09 chemotactic index range.

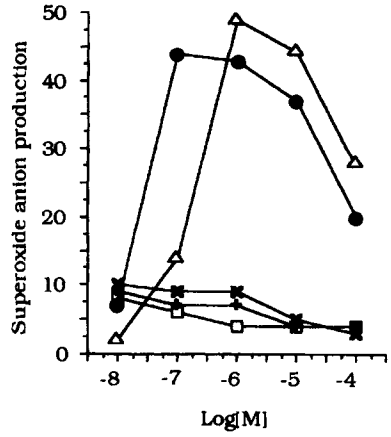



Figure 4 Superoxide anion production of fMLP-OMe and its analogues toward human neutrophils. The points are the mean of five separate experiments done in duplicate. The SE is the 0.1–4 nmol $O_2^-$ range.

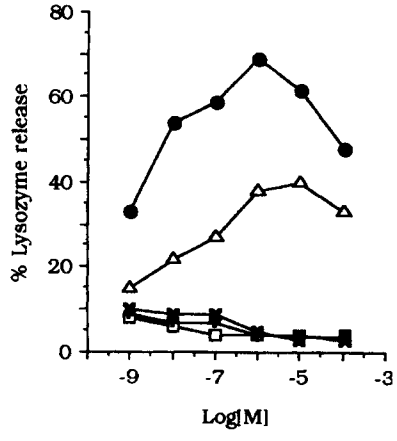



Figure 5 Release of neutrophil granule enzymes evaluated by determining lysozyme activity induced by fMLP-OMe and its analogues. The points are the mean of five separate experiments done in duplicate. The SE is in the 1–6% range.

## DISCUSSION

From our data it clearly emerges that chemotactic peptides need to have a protic amide bond at position 2 to show biological activity. The data give value to our previous hypothesis [16] and are in accordance with Dugas's work, which inserted a Pro residue at position 2, obtaining a tripeptide devoid of biological activity on human neutrophils [15].

Actually, both methylation and annulation of the amide NH deprive the peptides of biological activity (compounds **1** and **3**), while the perturbation of the peptide linkage, caused by insertion of an oxygen atom (compound **4**) which modifies the electronic

feature, makes the peptide inactive although the linkage itself remains protic.

The Atc derivative (compound **2**) possesses a biological activity both as chemotactic agent and in the lysozyme release as well as in the superoxide anion production. Its lower potency can be explained by the steric hindrance that interferes with a correct spatial arrangement, thereby hindering a proper contact with the receptor.

In conclusion we can state that any interference that modifies or perturbs the amide bond at position 2 abolishes all biological activity. At present it is impossible to affirm if this lack of activity is a problem that concerns the linkage of the chemotactic peptide to the receptor or its hydrolysis by the specific membrane enzyme, because both mechanisms are conditioned by the same feature of the molecule, i.e. the proticity of the amide linkage. Clearly, both ester and aprotic amide linkages cannot give a hydrogen bond and in both cases the specific protease can be unsuccessful in breaking the linkage. Further studies are in progress in our laboratory to clarify this feature of the problem.

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