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Synthetic Formyl Tripeptide Chemoattractants: a $C^{\alpha},^{\alpha}$-Dialkylated, Amphiphilic Glycyl Residue at Position 1

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Abstract: The two diastereomeric tripeptides f-(S)-HmMet-Leu-Phe-OMe and f-(R)-HmMet-Leu-Phe-OMe, analogues of the prototypical chemoattractant f-Met-Leu-Phe-OH, were synthesized in solution by classical methods and fully characterized. A conformational study was performed in solution by ^{1}H-NMR. Concomitantly, the two peptides were tested for their ability to induce chemotaxis, superoxide anion production and lysozyme secretion from human neutrophils. The conformational and biological data are discussed with regard to the proposed model of the chemotactic receptor on neutrophils. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: chemotactic peptides; $C^{\alpha\alpha}$-dialkylated glycines; C^{α}-hydroxymethyl methionine; nuclear magnetic resonance; peptide conformation

INTRODUCTION

Chemotaxis, the reaction by which the direction of migration of cells is determined by substances in their environment, is one of the fundamental responses of prokaryotic and eukaryotic cells to external stimuli [1]. Prokaryotic microorganisms such as bacteria use it to search for food and to avoid toxins. In vertebrates, chemotaxis is generally accepted as a major process bringing leukocytes from the blood to the tissue sites of inflammation. The responses of neutrophils to chemotactic factors have been studied intensively. This is partly because of the importance of neutrophils in inflammation, their ability to give different kinds of responses to a single stimulus, and the availability of highly active formyl oligopeptide chemotactic factors of known structure. This use of the formyl peptides stems from the discovery that formyl-methionine oligopeptides are chemolocomotory [2]. This led to a systematic study of the relationship of the structure of N^{α}-formylated peptides to their chemotactic and other biological activities (for leading review articles see [1,3]). These studies culminated in the synthesis and testing of the most active compound,

Abbreviations: AcOH, acetic acid; Boc, *tert*-butyloxycarbonyl; BuOH, butanol; Bz, benzoyl; DIEA, diisopropylethylamine; DMSO, dimethylsulphoxide; EtOAc, ethyl acetate; f, formyl; HmMet, C^{α}-hydroxymethyl methionine; OMe, methoxy; TBTU, N-[(1*H*-benzotriazol-1-yl) (dimethylamino)methylene]-N-methylmethanaminium tetrafluoroborate N-oxide.

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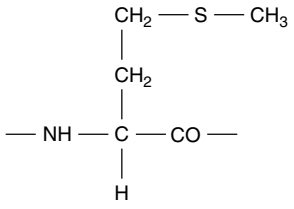
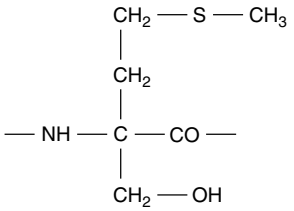
the tripeptide f-Met-Leu-Phe-OH. These peptides activate the neutrophils by interacting with a specific receptor, the formyl peptide receptor, on the neutrophil surface [4–6]. In particular, in the variety of published investigations of the effect of changes of structure of the formyl peptides on their biological activity it was shown that esterification (e.g. methylation) of the *C*-terminal carboxylic group does not result in a reduction of activity and that the sulphur-containing, thioether side chain of Met[1] produces optimal activity. Substitution of Met[1] by α-amino acids with linear aliphatic side chains from Gly to Nle (norleucine), the isostere of Met, induces large increases in activity. However, f-Nle-Leu-Phe-OH is 10 times less active than f-Met-Leu-Phe-OH. In the working model of the portion of the receptor accommodating the tripeptide, five critical areas in the receptor that interact with the peptide were identified. An important area of interaction is the hydrophobic cavity in the receptor occupied by the Met side chain. This cavity is of limited depth: side chains larger than four carbon atoms show no further increase in activity. In addition, a discrete area of positive charge in this cavity was postulated as complementary to the relatively electron-rich sulphur atom of the Met residue.



Met



HmMet

The aim of this article is to discuss the results of a conformational analysis in solution, using ^{1}H-NMR, in relation to those of a biological study on human neutrophils (using chemotactic activity, superoxide anion production and release of the granule enzyme lysozyme) of the two diastereomeric tripeptides f-(S)-HmMet-Leu-Phe-OMe and f-(R)-HmMet-Leu-Phe-OMe characterized by the substitution of Met[1] with the $C^{\alpha,\alpha}$-dialkylated glycyl residue HmMet. This amino acid represents the first truly amphiphilic replacement at position 1 of the formyl tripeptide chemoattractant and, as the other members of its family, induces increased proteolytic stability in the resulting backbone-modified peptide. A preliminary account of part of this work has been reported [7].

MATERIALS AND METHODS

Synthesis and Characterization of Peptides

All solvents were purified by conventional methods. Evaporations were carried out under reduced pressure. Melting points were determined on a capillary melting point apparatus and are uncorrected. ^{1}H-NMR spectra were recorded at 250 MHz on a Bruker Avance model DPX-250 spectrometer. The optical rotations were measured in a 1 dm cell (1 ml) on a Horiba high-speed automatic polarimeter at 589 nm. For thin-layer chromatography 250 nm silica gel GF precoated uniplates (Analtech) were used with the following solvent systems: (A) $CHCl_3$/MeOH/AcOH 90 : 10 : 2, (B) 1-BuOH/AcOH/EtOAc/H_2O 1:1:1:1, (C) EtOAc/hexane 1 : 1, (D) $CHCl_3$/MeOH/ 95 : 5, (E) CH_2Cl_2/MeOH/H_2O 14 : 6 : 1. The chromatograms were visualized with chlorine followed by starch/KI or with ninhydrin. HPLC was performed on a LDC/Milton-Roy analytical instrument using a Vydac C_{18} (0.46 $\times$ 25 cm) column: flow rate 1.0 ml/min, detection at 220 nm, and eluants (A) 0.05% trifluoroacetic acid in water and (B) 0.038% trifluoroacetic acid in acetonitrile/H_2O 90 : 10 with a gradient application.

^{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 DMSO (99.96% d_6; Acros Organics, Geel, Belgium) with tetramethylsilane as the internal standard.

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 $\times$ 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 that the leading front of the cell migrated, using the method of Zigmond and Hirsch [8] 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 of the migration towards the test attractant minus the 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 [9] modified for microplate-based assays [10]. 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 [9] modified for microplate-based assays [10]. Cells were incubated in microplate wells in the presence of each peptide to a final concentration of 10^{-10}–5×10^{-5} M for 15 min at 37 °C. Then, the plates were centrifuged for 5 min at $400 \times g$ 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. Enzyme activity 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

Chemistry

HmMet was readily prepared as a racemic product by a general procedure developed in the Lodz laboratory [11], involving selective C^{α}-hydroxymethylation of the 5(4*H*)-oxazolone derived from (R,S)-Met. Dehydration of Bz-(R,S)-Met-OH to the corresponding 5(4*H*)-oxazolone, followed by treatment with aqueous formaldehyde in the presence of pyridine, yielded the six-membered ring lactone 5-benzoylamino-5-methylthioethyl-4-oxo-1,3-dioxane. Ring opening of this lactone under controlled alkaline conditions resulted in the formation of Bz-HmMet-OH with an overall yield of 58% for the four step synthesis. Resolution of this N^{α}-blocked HmMet derivative into its enantiomers was achieved by fractional crystallization of the diastereomeric salts formed with (–) quinine. By this procedure both enantiomers of Bz-HmMet-OH could be isolated. In the last step they were separately hydrolysed under acidic conditions to afford free (+) and (–) H-HmMet-OH of 99% enantiomeric purity. The absolute configuration of (+) H-HmMet-OH was elucidated to be (R) by x-ray diffraction analysis of its N^{α}-benzoyl derivative [12].

The N^{α}-Boc-protected HmMet enantiomers were obtained in 60% yield by reacting the free amino acid with $(Boc)_2O$ [13] in an aqueous–dioxane mixture under alkaline conditions. The Boc-Leu-Phe-OMe

dipeptide [14] and the diastereomeric Boc-(S)-HmMet-Leu-Phe-OMe and Boc-(R)-HmMet-Leu-Phe-OMe tripeptides were synthesized (58%–90% yield) using the TBTU carboxyl activation method [15] in methylene chloride in the presence of a tertiary amine (DIEA). Removal of the Boc protection was achieved by mild acidolysis (either by treatment with HCl/MeOH at room temperature or with 85% aqueous HCOOH). The formyl group was introduced at the *N*-terminus of the N^{α}-deprotected diastereomeric tripeptides again by use of the TBTU/DIEA procedure (56%–70% yield).

The physical properties and analytical data for the newly synthesized, chromatographically homogeneous, HmMet analogues of the formyl tripeptide chemoattractant and their intermediates are listed in Table 1. All compounds were also characterized by ^{1}H- and ^{13}C-NMR (data not reported).

Solution Conformational Analysis

The preferred conformation of the diastereomeric tripeptides f-(S)-HmMet-Leu-Phe-OMe and f-(R)-HmMet-Leu-Phe-OMe was determined in a structure-supporting solvent ($CDCl_3$) by ^{1}H-NMR and compared with that of the parent chemoattractant f-Met-Leu-Phe-OMe.

The assignment of the three NH protons in the ^{1}H-NMR spectra of the two HmMet tripeptides is unequivocal. The NH proton signal of the C^{α}-tetrasubstituted α-amino acid (HmMet) was unambiguously recognized as the only singlet NH proton resonance, while the Leu and Phe NH proton signals were assigned via ROESY and TOCSY bidimensional NMR experiments. The delineation of inaccessible (or intramolecularly H-bonded) NH groups in the two tripeptides by ^{1}H-NMR was performed using solvent dependence of NH proton chemical shifts, by adding increasing amounts of the strong H-bonding acceptor solvent DMSO [16,17] to the $CDCl_3$ solution (Figure 1). It appears that for both peptides the chemical shifts of all three NH protons are quite sensitive to the addition of DMSO. Interestingly, in both peptides the sensitivity of the Phe N(3)H proton was the highest observed, thereby excluding the occurrence of a significant population of β-turn [18–20] conformers in the solution equilibrium mixtures. It is worth noting that β-turn formation is a rather common feature for short peptide sequences involving a C^{α}-tetrasubstituted α-amino acid [21]. It is also pertinent to recall here that all three NH proton chemical shifts of the parent tripeptide show pronounced solvent (DMSO) dependencies in $CDCl_3$ solution [22].

In summary, our ^{1}H-NMR data on the two diastereomeric HmMet tripeptides tend to exclude the occurrence of folded conformations to any significant extent in $CDCl_3$ solution, but rather they favour the conclusion that these compounds adopt an extended disposition in analogy to the conformational preference of f-Met-Leu-Phe-OMe.

Biological Activity

The biological activities of the diastereomeric tripeptides f-(S)-HmMet-Leu-Phe-OMe and f-(R)-Hm

Table 1 Physical Properties and Analytical Data for the Newly Synthesized Formyl Tripeptide Chemoattractants and their Intermediates

Compound	Melting point (°C)	$[\alpha]_D^{20}$ (°)	TLC				
			R_FA	R_FB	R_FC	R_FD	R_FE
Boc-(S)-HmMet-OH	97–99	−10.6[a]	0.41	—	0.32	—	0.72
Boc-(R)-HmMet-OH	97–99	+10.6[a]	0.42	—	0.32	—	0.72
Boc-(S)-HmMet-Leu-Phe-OMe	153–155	−17.2[a]	0.59	—	0.41	0.42	—
Boc-(R)-HmMet-Leu-Phe-OMe	144–146	−25.6[a]	0.59	—	0.41	0.42	—
HCOOH·H-(S)-HmMet-Leu-Phe-OMe	oil	—	0.25	0.74	—	—	0.81
HCOOH·H-(R)-HmMet-Leu-Phe-OMe	oil	—	0.25	0.74	—	—	0.80
f-(S)-HmMet-Leu-Phe-OMe	109–110	−20.6[b]	0.42	—	0.14	0.26	—
f-(R)-HmMet-Leu-Phe-OMe	119–121	−15.8[c]	0.42	—	0.15	0.27	—

[a] $c = 0.50$, MeOH.
[b] $c = 0.36$, MeOH.
[c] $c = 0.39$, MeOH.

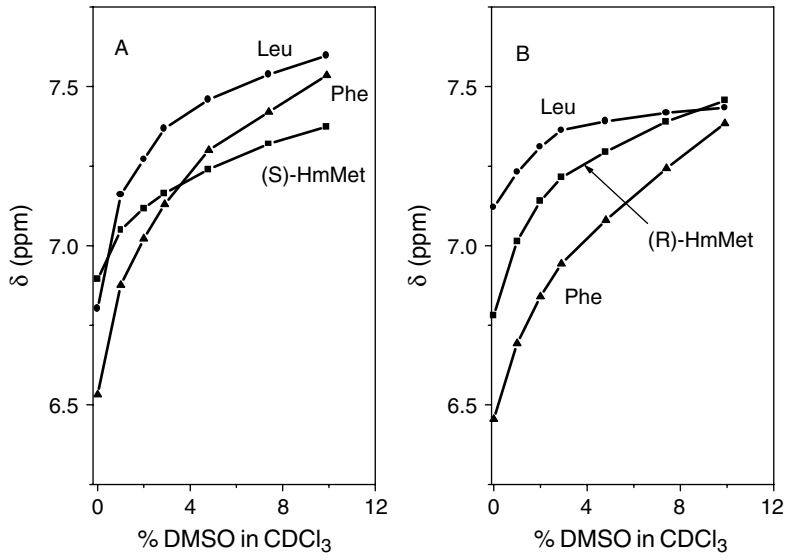


Figure 1 Plots of NH proton chemical shifts in the ^{1}H-NMR spectra of f-(S)-HmMet-Leu-Phe-OMe and f-(R)-HmMet-Leu-Phe-OMe (B) as a function of increasing percentages of DMSO (v/v) added to the $CDCl_3$ solution (peptide concentration 1 mM).

Met-Leu-Phe-OMe were determined in human neutrophils and compared with those of the parent tripeptide f-Met-Leu-Phe-OMe. Directed migration (chemotaxis), superoxide anion production and lysozyme release were measured.

As illustrated in Figure 2, the (S)HmMet tripeptide was chemotactically active, although less potent than f-Met-Leu-Phe-OMe, and exhibited even a higher efficiency (peptide concentration at which the maximal activity was observed) [23], being maximally effective at 10^{-11} M. Conversely, the small

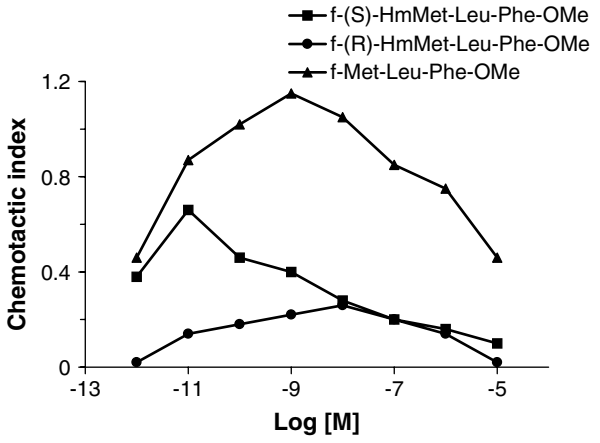


Figure 2 Chemotactic activity of f-Met-Leu-Phe-OMe and its two HmMet diastereomeric analogues at position 1 towards human neutrophils. The data are the mean of five separate experiments performed in duplicate. SEs are in the 0.01–0.09 chemotactic index range.

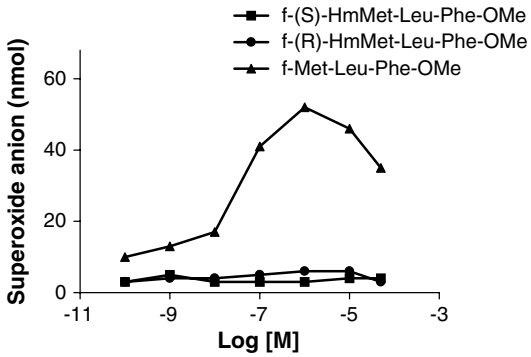


Figure 3 Superoxide anion production of f-Met-Leu-Phe -OMe and its two HmMet diastereomeric analogues at position 1 towards human neutrophils. The data are the mean of five separate experiments performed in duplicate. SEs are in the 0.1–4 nmol O_2^- range. The symbols for the three peptides are the same as those in Figure 2.

chemotactic index value shown by the (R)-HmMet tripeptide was not statistically significant. In the superoxide anion production assays (Figure 3) both peptides were inactive. As for the enzyme secretagogue activity (Figure 4), the two HmMet tripeptides showed a similar behaviour, i.e. a modest potency, significantly lower than that of the prototypical tripeptide.

CONCLUSIONS

A number of papers have described the results of conformational and biological studies of f-Met-Leu-

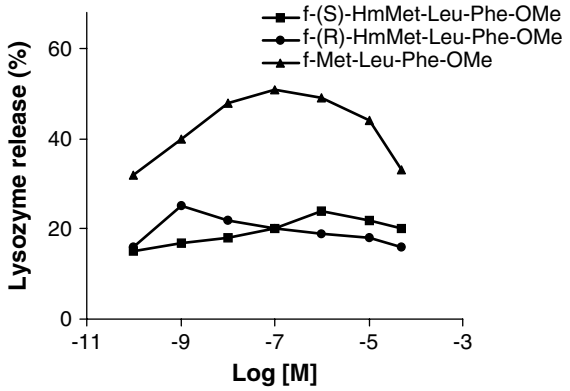


Figure 4 Release of neutrophil granule enzyme evaluated by determining lysozyme activity induced by f-Met-Leu-Phe-OMe and its two HmMet diastereomeric analogues at position 1. The data are the mean of five separate experiments performed in duplicate. SEs are in the 1%–6% range. The symbols for the three peptides are the same as those in Figure 2.

Phe-OMe analogues at position 1 [1,3,9,24–31]. In this framework this article is the second that discusses data on analogues that contain a Met[1] α-amino acid replacement with two linear side chains bound at the C^{α}-atom. The first contribution [9] dealt with an achiral residue, 2-[2′-(methylthio)ethyl]methionine, with two -$(CH_2)_2$-S-CH_3 thioether side chains. This tripeptide adopts an extended conformation and was inactive in all tested assays. Of the two, truly amphiphilic, HmMet-based tripeptides reported in this work, only the (S)-diastereomer exhibited a significant biological activity, i.e. it remarkably stimulated the directed mobility of human neutrophils, even at very low concentrations. However, it appears that both diastereomeric tripeptides are essentially extended in solution. Interestingly, the biological responses of the (S)-HmMet tripeptide strictly parallel those reported for the f-Met-Leu-Phe-OMe analogue characterized at position 1 by a 4-aminotetrahydrothiopyran-4-carboxylic acid, the only other analogue in this position based on a conformationally restricted, $C^{\alpha,\alpha}$-dialkylated glycyl residue suitable to mimic the *N*-terminal Met residue (however, the two side chains of this achiral α-amino acid are cyclized to a six-membered ring system) [24,25].

It has been ascertained that the N-formyl peptide receptor in human neutrophils is a glycosylated protein which can be resolved in two isoforms of different molecular weight and binding affinity [4–6]. Also, two different signal transduction systems for chemoattractants in neutrophils have been invoked: one which provides for locomotion, the other for superoxide generation and general enzyme release [32]. From the results obtained in this work it is safe to conclude that only the (S)-HmMet analogue at position 1 of the formyl tripeptide is able to bind effectively to the receptor responsible for the selective activation of the pathway involved in the chemotactic response, whereas none of the two HmMet diastereomeric tripeptides interacts appropriately with the receptor able to activate the pathway leading to superoxide production and lysozyme release.

Acknowledgements

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