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Review

Structure–activity relationship of for-L-Met L-Leu-L-Phe-OMe analogues in human neutrophils

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Abstract

Neutrophils constitute the first line of defence against bacterial invasion. They migrate to infected tissues along a concentration gradient of chemoattractant molecules, the most important of which is for-Met-Leu-Phe-OH (fMLP). Different responses arise from formylpeptides binding to different isoforms of the specific receptor. The aim of the studies reported herein was to clarify (i) the role of fMLP-OMe amide bonds in receptor–ligand cross-linking, (ii) the nature of the group occupying the N- and C-terminal positions, (iii) the features peculiar to the Met, Leu, and Phe receptor pockets, and (iv) the features which determine the specific neutrophil response.

Keywords: Structure–activity relationship; fMLP binding; *N*-Formylpeptides; Human neutrophils; Chemotaxis; Superoxide anion production

1. Introduction

In spite of the numerous studies into the biological responses of human neutrophils arising from their interaction with the chemotactic formyl-L-Met-L-Leu-L-Phe-OMe (fMLP-OMe) [1–9], nobody has yet clarified which chemical mechanisms are involved

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in the binding of this ligand to its receptor. The receptor has been studied in order to understand its position in the membrane and the steric characteristics which allow the best biological responses [10–14], but not to establish how the ligand hooks into its receptor pocket.

Moreover, the consequences arising from introducing unconventional residues in place of Met, Leu, and Phe amino acids have still to be verified. Previous studies have indicated that the Met residue may be fundamental for good biological activity, due to its side-chain electron-rich sulphur atom which allows the peptide to interact with a restricted positive area on the receptor. The Leu residue has always been substituted with bulky hydrophobic amino acids, since the receptor pocket, in which the second residue is located, was thought to be a hydrophobic area [15–17]. Studies have also determined that the C-terminal amino acid should be aromatic, Phe being the preferred choice [18,19,2].

The aim of the research reported here was twofold: to clarify the mechanisms whereby the formyl-L-Met-L-Leu-L-Phe-OMe ligand hooks the specific receptor on human neutrophils and to verify the biological consequences of introducing unusual amino acids in place of the Met, Leu, and Phe residues. We demonstrated that: (i) the amidic bonds are involved, although with varying importance, in the binding of formylpeptides to the receptor; (ii) the ability to activate human neutrophils response is not strictly due to the presence of the formyl group; (iii) the Met pocket is narrow in dimension, and carries a positive charge located at a well defined distance from the peptide backbone; it is oriented in a specific position, surrounding most of the internally located side-chain; (iv) the Met residue is essential for chemotaxis, but specific substitutions can introduce selectivity towards killing mechanisms, which can be strongly stimulated; (v) hydrophobic residues at position 2 are decisive solely for chemotaxis, which is depressed by introducing hydrophilic amino acids, while both superoxide anion production and lysosomal lytic enzymes are strongly and selectively stimulated; (vi) hydrophobicity is not a mandatory feature for the third residue, and (vii) the requirement of a C-terminal carboxylic group, either free or esterified, to trigger a biological response in human neutrophils.

The studies herein reported allow us to modulate the neutrophil biological responses in two ways:

(1) variation of the amidic bonds between amino acids allows us to modulate the intensity of the biological response from zero to maximum;
(2) variation of the character (more or less hydrophilic or lipophilic) of the residues allows us, in some way, to select either chemotaxis or killing mechanisms, i.e. superoxide anion production or lytic enzyme release.

2. Role of the amide bond in ligand–receptor cross-linking

Several studies have attempted to correlate structure–activity data in order to establish the structural features necessary for biological activity. Optimal agonist potency occurs when (a) the N-terminal group is formylated, (b) the first amino acid is Met, (c) the second amino acid has a bulky hydrophobic side-chain, and (d) the third amino acid is Phe [1–3,19]. A critical interaction of the Phe C=O group with the receptor, possibly via hydrogen bonding, has also been hypothesized [3,20]. Alteration of the C-terminal carboxylic group does not result in any loss of activity [17].

In the receptor pocket: (a) the formyl group is small enough to reach the bottom of the binding cavity through a narrow passage between two Phe side-chains [3,21]; (b) the Met side-chain sulphur atom interacts with positively charged residues at the extracellular boundaries of the second and third transmembrane domains [3,12]; (c) the carboxylic terminus of the molecule, as well as the side-chains of Leu and Phe residues, sweeps back toward the entrance of the binding pocket; (d) the pockets which accommodate Leu and Phe side-chains involve the hydrophobic residues in the first extracellular loop, and in parts of the third, sixth and seventh transmembrane domains [12].

All these data are very useful to explain *where* the ligand sits in the receptor pocket, but they are not able to explain *how* the ligand binds to the receptor after it has penetrated its specific pocket.

It has been generically hypothesized that the formyl group participates as an H-donor in linking to the receptor, and it seems that its role in this interaction cannot be ruled out [3,21,22]. As it is not certain that this single bond can firmly bind the ligand to the formylpeptide receptor, the importance of the amide bonds along the tripeptide backbone was investigated in order to understand whether the proton of the amide bonds participate (and in which role) in linking to the fMLP receptor. To this end, modifications in the amide bonds of the prototype fMLP-OMe peptide seem to be important.

In order to clarify whether this is so, the amide bonds were substituted with ester bonds (depsipeptides), *N*-methylated or N secondary amide bonds to obtain an aprotic linkage. These substitutions, even if they both lead to aprotic bonds, are very different from one another: indeed, a depsipeptide does not have an amidic bond and, for instance, cannot be hydrolysed by enzymes like peptidases, whereas both *N*-alkylated and N secondary amides are always amides, although aprotic, and are therefore subject to normal enzymatic degradation.

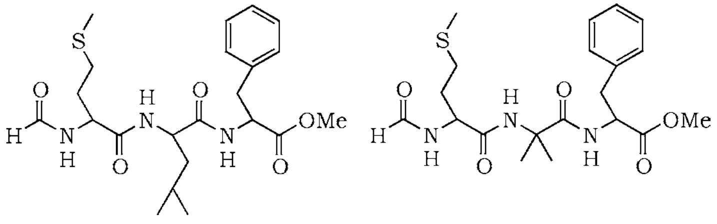
Moreover, none of the aprotic analogues can, in this manner, act as an H bond donor, but only as an H bond acceptor. If these substitutions do not influence biological responses, the amide bond does not take part in ligand–receptor binding. If, instead, the amide bond is involved, some type of variation in biological response occurs.

2.1. The amide bond at position 1

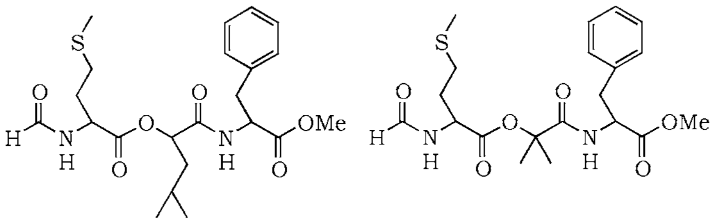
To understand which role the amidic bond at position 1, i.e. between the formyl group and the Met residue, plays in binding to the receptor, this peptide bond was replaced by an ester linkage, thereby leading to the depsipeptide analogue for-Hmb-Leu-Phe-OMe, where Hmb = 2-hydroxy-4(methylthio)butyric acid [23]. The formate ester, in place of the formamido group, maintains the small size but is not able to form a hydrogen bond. This compound shows good activity as a chemotactic agent and triggers superoxide anion production and granule enzyme release even if these require higher doses than the parent fMLP-OMe.

2.2. The amide bond at position 2

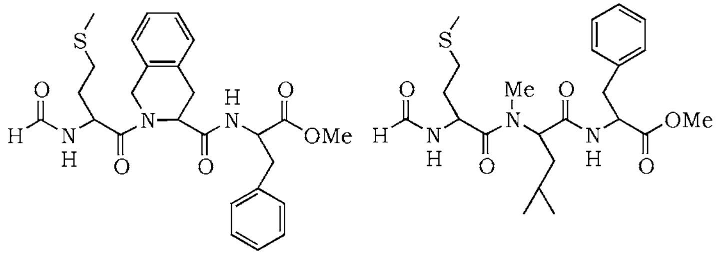
To understand whether or not the amidic bond at position 2 plays a role in binding to the receptor, and, if so, its importance, depsipeptides **3** and **4**, analogues to fMLP-OMe **1** and for-Met-Aib-Phe-OMe **2**, respectively, were synthesized [24] (Fig. 1).



for-L-Met-L-Leu-L-Phe-OMe **1** for-L-Met-Aib-L-Phe-OMe **2**



for-Met-Ψ(COO)Leu-Phe-OMe **3** for-Met-Ψ(COO)Aib-Phe-OMe **4**



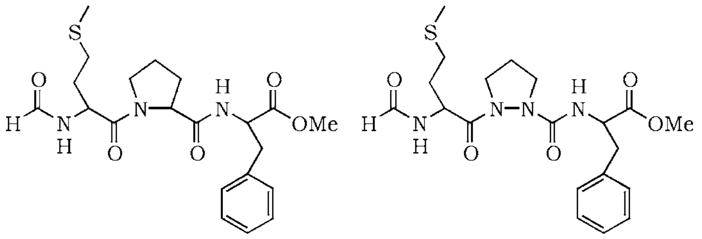
for-Met-Tic-Phe-OMe **5** for-Met-(NMe)Leu-Phe-OMe **6**

Fig. 1. Structure of reference compounds (**1**, **2**), depsi-analogues (**3**, **4**), and aprotic amide derivatives (**5**, **6**).

Substitution of an amide bond with an ester bond between Met and Leu residues leads to peptides which are devoid of chemotactic activity, as well as of superoxide anion production and lysozyme release.

In order to evaluate whether this behaviour is due to the different nature of the ester bond in comparison with the amide bond, or to the aprotic nature of depsipeptides, compounds **5** and **6** were synthesized (Fig. 1). The Tic residue [Tic = 1, 2, 3, 4-tetrahydro isoquinoline-3-carboxylic acid] of for-Met-Tic-Phe-OMe **5** is sterically encumbered and leads to an aprotic amide linkage, as the NH group is annulated in the isoquinoline ring; the NH group at position 2 of for-Met-(NMe)Leu-Phe-OMe **6** is methylated, thus depriving the amide linkage of a possible hydrogen bond donor [25]. Neither of these compounds (which do not possess a hydrogen bond donor group at the Met-Leu junction) is able to elicit any biochemical activity. This lack of activity parallels that already evidenced for *N*-formyl tripeptides incorporating a Pro **7** [26] or an azaPro residue **8** [27] (Fig. 2).

We can reasonably assume that a hydrogen bond is formed between the amide bond at position 2 and the receptor, highlighting the mandatory importance of the protic NH group. Any alteration of this group prevents the formylpeptide from interacting with



for-Met-Pro-Phe-OMe **7**   for-Met-azaPro-Phe-OMe **8**

Fig. 2. Structure of compounds in which the second residue has been substituted with particular amino acids: $[\text{Pro}]^2$ **7**, and aza$[\text{Pro}]^2$ **8**.

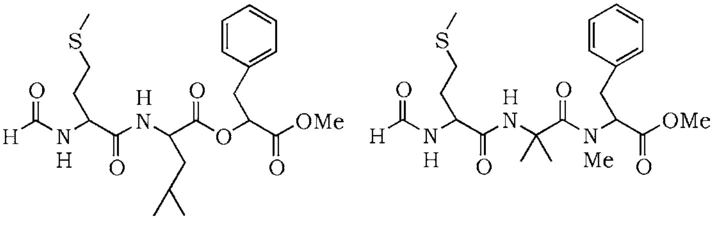
the specific receptor area, thereby impeding any biological responses: clearly, an aprotic bond does not permit the ligand to link and/or to recognise the receptor itself.

2.3. The amide bond at position 3

To understand whether or not the amidic bond at position 3 plays a role in binding to the receptor, and, if so, its importance, we synthesized two different types of analogues: for-Met-Leu-Ψ(COO)Phe-OMe **9** and for-Met-Leu-(NMe)Phe-OMe **10** [28] (Fig. 3).

Both of these analogues, with an aprotic bond at position 3, activate only weak chemotactic activity, while killing mechanisms (i.e. superoxide anion production and lytic lysosomal enzyme release) are triggered to a greater extent than by the prototype fMLP-OMe. This suggests that the amidic bond between the second and third residue is very important for eliciting effective chemotaxis, but not mandatory. As regards killing mechanisms, these can be strongly stimulated, thereby evidencing that the isoforms responsible for chemotaxis and for the killing response are quite different.

In order to understand whether it is possible to overcome the lack of an amide bond proton, we synthesized for-Met-Leu-Ψ(COO)Phe-NHBzl **11** (Fig. 4) which, besides the amide bond substituted by an ester bond, has the C-terminal carboxylic protecting group changed from methyl ester to benzylamide [24]. Our results evidence an interaction (even if not optimal) with the specific receptor, probably through the formation of a hydrogen bond of the benzylamide protecting group, which can clearly substitute the lack at position 3, since a fairly good chemotactic response has been stimulated.



for-Met-Leu-Ψ(COO)Phe-OMe **9**   for-Met-Leu-(NMe)Phe-OMe **10**

Fig. 3. Structure of depsi-analogues (**9**–**10**).

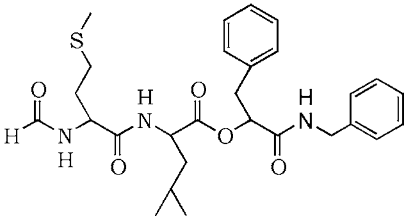


Fig. 4. Structure of depsi-analogue **11**.

These findings strongly suggest that the amide linkage at position 3 attaches to the receptor and elicits chemotaxis, but its role is not as important as the role of the amide at position 2, and, furthermore, it does not affect the response which elicits superoxide anion production and lysozyme release.

2.4. Conclusions

The above analogues are useful tools for demonstrating that it is possible to partially select the receptor isoform by modulating the amide bonds. In fact, the lack of a protic linkage at position 3 does not allow a strong chemotactic response, while it does elicit superoxide anion production, thus stressing that the isoform which triggers superoxide anion production is probably less demanding than the one responsible for chemotactic activity.

From our findings we can conclude that (a) a protic bond at position 2 is mandatory for all human neutrophil biological activities; (b) a protic bond at positions 1 and 3 is not mandatory for elicitation of biological activities; (c) the isoform that triggers the chemotactic response is structurally more exacting than the isoform eliciting superoxide anion production [29].

Hence, we can hypothesize two orders of importance in ligand–receptor binding to stimulate chemotaxis. We can call the link set up by/between the amide group at position 2 and the receptor “primary hooking”, for which the presence of the amide proton is mandatory. This link probably activates the isoform, triggering chemotaxis. At this point, the ligand binds to the receptor with at least one other hydrogen bond, formed using the proton of the amide bond at position 1 and/or 3. This other hydrogen bond can be called “secondary hooking”; its lack allows only a weak specific receptor stimulation. From these remarks, it can be suggested that the ligand approaches the receptor, exhibiting its amide bond at position 2; this is possible whether the molecule is in β-turn or in extended conformation. In the case of a β-turn, the molecule opens, allowing the secondary hooking/s. Evidently, the more the receptor is filled by the ligand, the more chemotaxis is triggered. In contrast, lesser bonds are required in the isoform responsible for triggering superoxide anion production. The “primary hooking” is sufficient to tie the ligand to the receptor, and to full activate the receptor.

3. Substitution of the formyl group

The formyl group on the N-terminal methionine is small enough to reach the bottom of the binding cavity through a narrow passage; it participates as hydrogen-donor in linking to the receptor and it seems that its role in this interaction cannot be ruled out.

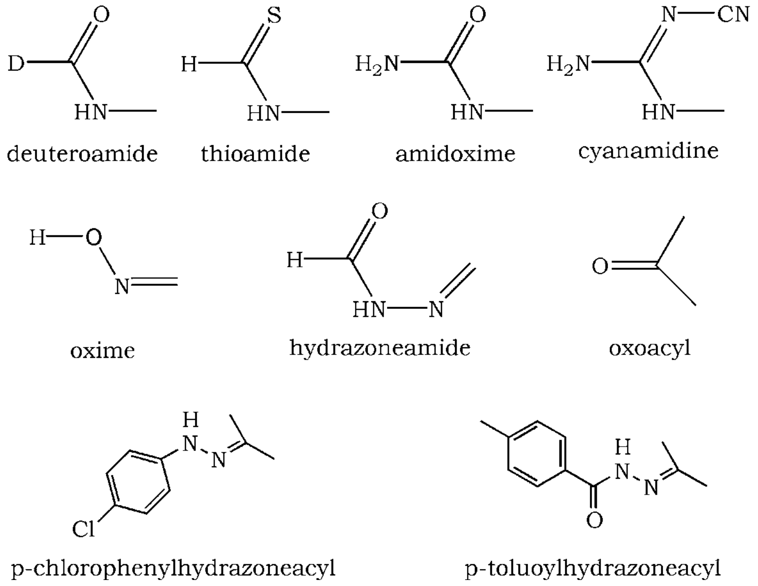
As a consequence, all studies performed are directed, on the one hand, to finding groups small enough to penetrate the specific receptor pocket and, on the other, to finding substitutions which can allow a hydrogen bond in the receptor pocket. Modifications performed on the HCO group are reported in Fig. 5.

Both deuteroformyl and thioamide derivatives show a strong loss of activity, even if they are structurally very similar to the formyl prototype [30–32]. Amidoxime, cyanamidino, and formylhydrazone derivatives have structures that cannot fit the receptor pocket, thereby leading to decreased biological activity [33–35]. The small oxoacyl analogue, which is distinctive for its absence of the hydrogen, is completely devoid of activity [35].

The very hindered aryl and aroyl hydrazone analogues, which differ in both conformational preferences and hydrogen bond forming capability, are totally unable to bind the receptor [36]. The lack of activity of the aryl analogue may be related to the absence of an H-bonding carbonyl acceptor group in the proper position for establishing favourable receptor interactions.

The oximic derivative, small and polar as required for optimal interaction with the specific receptor area, is able to participate as a donor or acceptor group for hydrogen bond interactions, and not unexpectedly shows a high activity peak [35].

In conclusion, we can observe that the biological activity of the oximic derivative seems to support recent observations indicating that the ability to activate the human neutrophil response is not strictly due to the presence of the formyl group [37–40]. In fact, unbranched carbammates (methoxycarbonyl, ethoxycarbonyl, and *n*-butoxycarbonyl) resulted in good activity [38], as well as analogues in which the urea functionality replaces the prototype formamido group [39].



deuteroamide thioamide amidoxime cyanamidine

oxime hydrazoneamide oxoacyl

p-chlorophenylhydrazoneacyl p-toluoylhydrazoneacyl

Fig. 5. Modifications performed on the formyl group.

4. Substitution of Met, Leu, and Phe residues with hydrophilic amino acids

4.1. Variations in the Met residue

With the aim of clarifying the structure–activity relationship peculiar to the Met receptor pocket, a large number of formylpeptides modified at position 1 have been synthesized and biologically tested. These analogues can be grouped into two categories (Fig. 6):

(i) analogues with a residue carrying a sulphur atom on the side-chain, such as *S*-methylcysteine Cys(Me) and ethionine Eth [2], 4-amino-tetrahydrothiopiran-4-carboxylic acid (Thp) [41], methionine sulphoxide Met(O) and methionine sulphone Met(O_2) [42,43], difluoro-Met (DFM) and trifluoro-Met (TFM) [44].

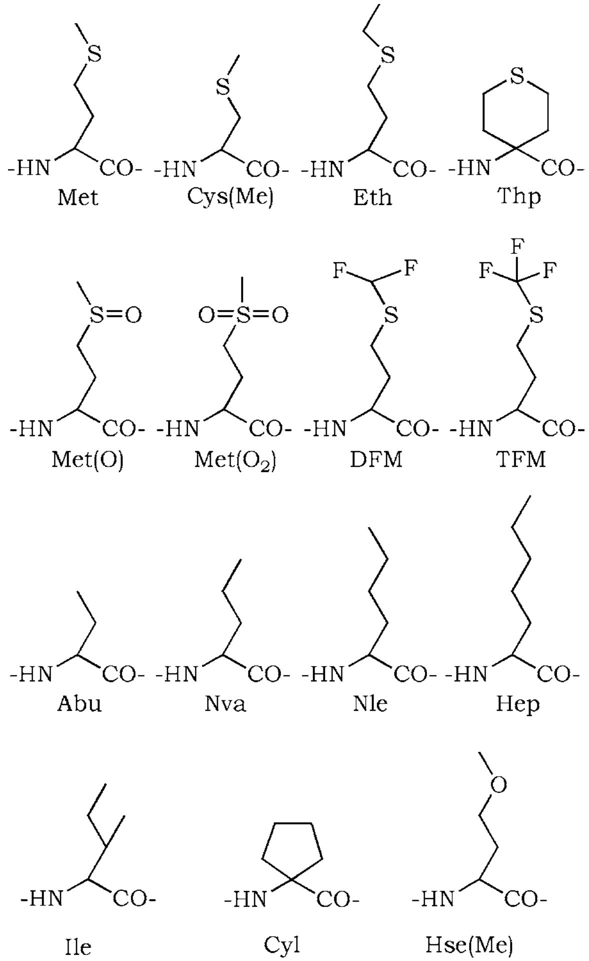


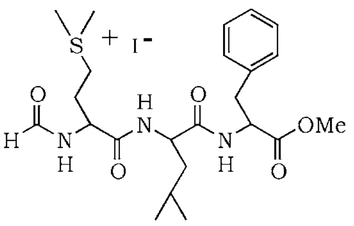
Fig. 6. Amino acids introduced in place of the Met residue.

(ii) Analogues carrying an aliphatic or aromatic side-chain, such as Gly, Ala, Abu [α-aminobutyric acid], Nva, Nle, α-aminoheptanoic Hep, Phe, Ile, Val, Leu, Cyl [cycloleucine] [2], and Hse(Me) [*O*-methylhomoserine] [45].

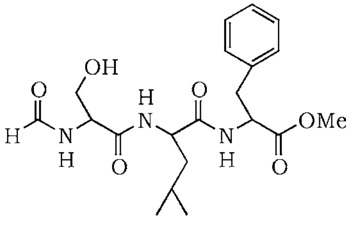
The studies that led to these data were originally directed at finding potent analogues of fMLP-OMe, but they were also useful for setting up a comprehensive picture of the Met receptor pocket. It generally emerges that both electronic and conformational properties play a key role in triggering a potent biological response.

In order to completely characterize the Met receptor pocket, which seems to carry a positive charge, it was necessary to synthesize fMLP-OMe analogues carrying (i) a different charge typology, (ii) a partially positive charge at different distances from the peptide backbone, and (iii) a charge which faces the receptor wall pocket in different positions.

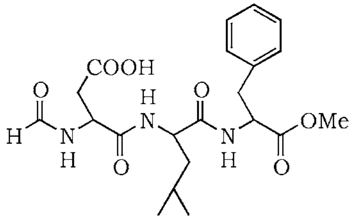
The HCO-Met-Leu-Phe-OMe methylsulphonium iodide (fMLP-OMe·MSI) analogue **12** (Fig. 7) carries a definite separate negative charge arising from salification [46]. Not surprisingly, chemotaxis is strongly reduced while superoxide anion production shows a potency comparable to that of the reference fMLP-OMe. Thus, chemotaxis is not consistent with a sharp positive charge, probably due to a repellent interaction with the specific receptor pocket. Superoxide anion production is not sensitive to this type of variation.



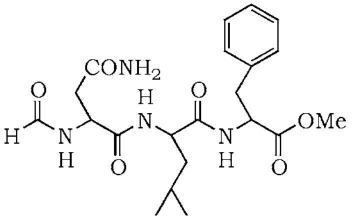
fMLP-OMe.MSI **12**



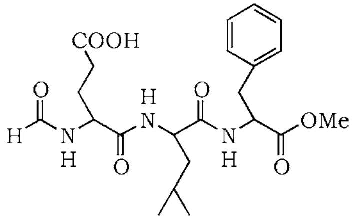
for-Ser-Leu-Phe-OMe **13**



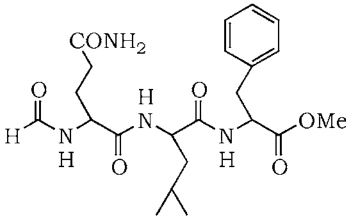
for-Asp-Leu-Phe-OMe **14**



for-Asn-Leu-Phe-OMe **15**



for-Glu-Leu-Phe-OMe **16**



for-Gln-Leu-Phe-OMe **17**

Fig. 7. Structure of compounds (**12**–**17**) substituted at [Met[1]].

To determine the exact position of the positive charge in the pocket, we synthesized and biologically tested analogues carrying a residue with a partially negative charge in different areas at position 1. These analogues carry a negative charge as far as one methylene (for-Ser-Leu-Phe-OMe **13**), one ethylene (for-Asp-Leu-Phe-OMe **14** and for-Asn-Leu-Phe-OMe **15**), and one propylene (for-Glu-Leu-Phe-OMe **16** and for-Gln-Leu-Phe-OMe **17**) from the backbone [47,48] (Fig. 7). These substitutions should allow us to clarify whether the charge is localized in a restricted area or broadly distributed inside the pocket. A loss of chemotactic activity was observed with all these analogues, and was especially dramatic for Asp and Glu. As far as lysozyme release activity and superoxide anion production are concerned, only Gln was potent. Consequently, it is clear that the ligand must have a charge—which is negative and unchanged with respect to the Met residue—and must be located two methylenes after the backbone; moreover, any variation in the intensity and position of the charge gives rise to ligand–receptor interactions which are imperfect or unsuitable for eliciting chemotactic activity.

Some amino acid residues, maintaining the γ-methylthio functionality, have been used to replace the native methionine (Fig. 8).

Two diastereomeric tripeptides for-(*S*)-HmMet-Leu-Phe-OMe and for-(*R*)-HmMet-Leu-Phe-OMe, in which the Met residue was substituted with the C^{α}-hydroxymethyl methionine HmMet, were synthesized. The results evidenced that only the (*S*)-HmMet analogue at position 1 of the formyl tripeptide is able to bind effectively to the receptor responsible for selective activation of the pathway involved in the chemotactic response, whereas neither of the two HmMet diastereometric tripeptides interacts appropriately with the receptor able to activate the pathway leading to superoxide anion production and lysozyme release [49].

The 2-[2′-(methylthio)ethyl]methionine (Dmt) residue was introduced in place of methionine, and proved to be practically inactive in all tested assays [50]. The β^3-HMet-Leu-Phe-OMe analogue exhibits less chemoattractant activity than the parent tripeptide, whereas it has been found to be highly active as a superoxide anion producer [51]. One of the most recent studies incorporates a chimeric *cis*-4(*S*) and *trans*-4(*R*)-methylthioproline [52]. Biological assays show its activity ranging from very week to null, thereby suggesting that a predetermined and blocked spatial orientation of the native side-chain at position 1 can profoundly alter the usual overall accommodation in the hydrophobic pockets of the tripeptide backbone with particular reference to the groups bound at the N-terminus moiety.

As the last step, we synthesized for-D-Met-L-Leu-L-Phe-OMe with Met in the D configuration [53]. All biological responses resulted markedly lowered with respect to the proto-

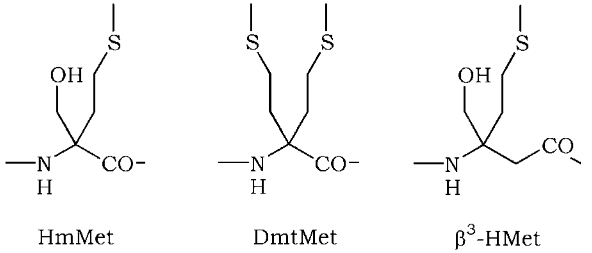


Fig. 8. Sulphurated analogues of Met.

type values at physiological concentrations. Chemotaxic potency was reduced from half to a third, and superoxide anion production showed lower potency. From these data we may deduce that the positive charge within the Met receptor pocket does not completely surround the sulphur of the Met side-chain. Indeed, this loss of activity could imply the presence of a repulsive interaction along the side-chain.

4.1.1. Conclusions

These results evidence the extreme difficulty in finding Met substitutions which are small enough to reach the bottom of the specific pocket, and, at the same time, have a suitably located charge on the side-chain.

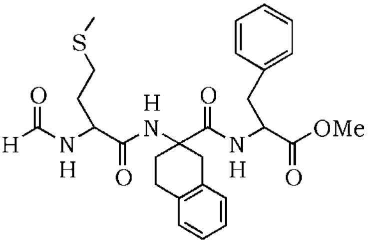
The Met pocket cannot bear any shift in the negative charge on the side-chain of the first residue. As frequently found, this role is more important for chemotaxis than for superoxide anion production, which is much less demanding.

4.2. Variations in the Leu residue with hydrophobic residues

It is commonly considered as established that the Leu side-chain penetrates into a hydrophobic pocket which comprises hydrophobic transmembrane domain residues [12,54,55] in the first extracellular loop and in parts of the third, sixth, and seventh. As a result, all subsequent research has been focussed on sterically hindered hydrophobic residues.

In order to understand whether, as in the case of compound **5**, steric hindrance can play a negative role in the biological response, the tripeptide for-Met-Atc-Phe-OMe **18** (Atc = 2-aminotetralin-2-carboxylic acid) (Fig. 9) has been synthesized and evaluated on human neutrophils [25]. This compound, being protic, retains some activity, despite having a lower potency than that of the control. The explanation for this can be found in its steric hindrance, which interferes with the proper spatial arrangement, thereby hindering contact with the receptor.

Research aimed at understanding the best steric hindrance of the residue at position 2, started by introducing the branched Aib (amino-isobutyric acid) and cyclo Ac*n*c residues [4,5]. $[\text{Aib}]^2$ and $[\text{Ac}_n\text{c}]^2$ have been exhaustively investigated, both biologically [4,7] and structurally, shedding light on the best ligand–receptor relationship [7,17,50,56–60]. $[\text{Aib}]^2$ and all $C^{\alpha,\alpha}$ dialkylated glycine analogues $[\text{Ac}_n\text{c}]^2$, characterized by cycloalkane rings of different sizes (*n* from 3 to 12), show, in human neutrophils, a comparable ability as chemotactic agents and in triggering both superoxide anion production and lysozyme



for-Met-Atc-Phe-OMe **18**

Fig. 9. Structure of $[\text{Atc}]^2$ compound **18**.

secretion, which consequently do not depend upon the ring size or steric hindrance [61]. It is worth stressing that in rabbit neutrophils, this ability, however, decreases with residue size.

With the same aim, conformationally restricted tripeptides, α-methylated at position 2 have been synthesized. [L-(α-Me)Val]^2, [D-(α-Me)Val]^2, [L-(α-Me)Leu]^2, and [L-(α-Me)Leu]^2 show enhancement in activity due to an increase in the bulkiness of the substitute, and the biological response is different for diastereomeric analogues [59].

One of the most favourable substitutions of Leu at position 2 originates from the introduction of the dipropylglycine (Dpg) residue to give the for-Met-Dpg-Phe-OMe tripeptide. In fact this analogue, with its extended β-sheet structure, shows one of the highest activity in human peripheral blood neutrophils, being 8/16 times more active than the parent fMLP-OMe [17].

Another favourable substitution results from the introduction of the Tau residue, which exhibits a remarkable activity [62], while $[(CH_2)_n]^2$ ($n = 3$–5) analogues are all less potent in stimulating biological responses [63].

Unusual substitutions have been made too, such as introducing an Adt (4-amino-1,2-dithiolane-4 carboxylic acid) [64] or a γ-lactam residue [65]. The biological activity of [Adt]^2 is very low and that of the [γ-lactam]^2 is practically absent.

4.2.1. Substitution of the Leu residue with hydrophilic residues

As we thought it would be interesting to verify whether there is incompatibility with the hydrophilic nature of the second residue, and whether steric hindrance can overcome this possible incompatibility, we synthesized for-Met-Ser-Phe-OMe **19**, for-Met-Cys-Phe-OMe **20**, for-Met-Lys-Phe-OMe **21**, and for-Met-Tyr-Phe-OMe **22** (Fig. 10) [66].

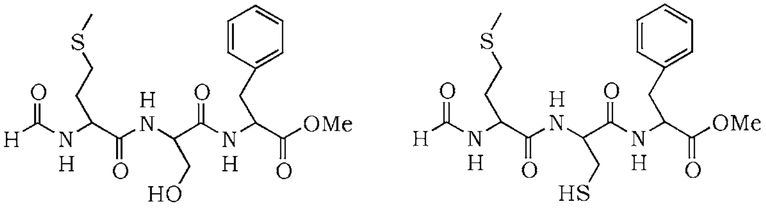
Our results evidenced a fascinating picture: on one hand, chemotaxis is lowered by all of the analogues, whether with a bulky or small side-chain; on the other hand, superoxide anion production is strongly improved at physiological concentrations (10^{-6} M) if the side-chain is sterically encumbered. In other words, while chemotaxis is strongly dependent on the hydrophilic or hydrophobic nature of the side-chain, superoxide anion production can be optimally triggered, even in the presence of a hydrophilic side-chain of large dimensions, as in the case of the analogue for-Met-Tyr-Phe-OMe, which associates hydrophilicity with steric hindrance.

4.2.2. Substitution of the Leu residue with bulky protected hydrophilic residues

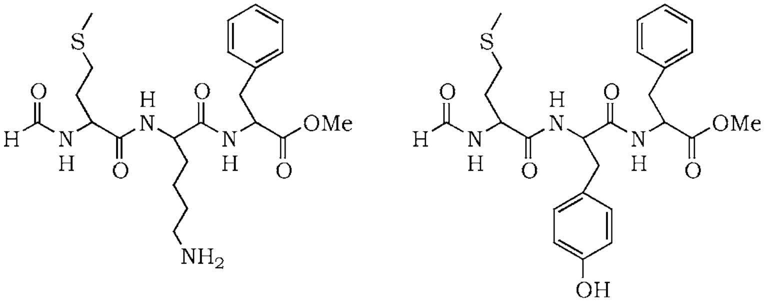
The above interesting results induced us to evaluate the contribution of a bulky hydrophobic protecting group for the functionalized side-chain.

To this end, we synthesized for-Met-Ser(Bzl)-Phe-OMe [Ser(Bzl)^2], for-Met-Cys(Bzl)-Phe-OMe [Cys(Bzl)^2], for-Met-Tyr(Bzl)-Phe-OMe [Tyr(Bzl)^2], and for-Met-Lys(Z)-Phe-OMe [Lys(Z)^2], and the activities of these new compounds were compared to those of the analogues with an unprotected side-chain at position 2 [67].

Our results showed that: (i) substitution of the [Leu]^2 residue in the parent fMLP-OMe with residues carrying side-chain hydrophilic groups leads to weak chemotaxis. This trend cannot be offset by adding a bulky hydrophobic protecting group to the side-chain; (ii) with the exception of the Tyr(Bzl)^{2}analogue, all analogues showed that lysozyme release occurs in a narrow range of potency, which is, in any case, higher than that of the parent, fMLP-OMe. As a consequence, bulky protecting groups render the dimensions of the side-chain unimportant.



for-Met-Ser-Phe-OMe **19** for-Met-Cys-Phe-OMe **20**



for-Met-Lys-Phe-OMe **21** for-Met-Tyr-Phe-OMe **22**

Fig. 10. Structure of compounds (**19**–**22**) substituted at $[Leu]^2$.

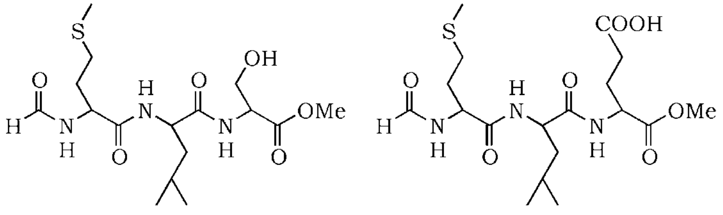
In summary, if compared to the parent fMLP-OMe, analogues with a large hydrophobic protecting group at position 2 are able to induce an appreciable biological selectivity by depressing the chemotactic response and strongly stimulating killing mechanisms. The $Tyr(Bzl)^2$ analogue benefits from the bulky protecting group by stimulating superoxide anion production with a high efficacy and maintaining the same strong potency as that of the side-chain unprotected analogue. Unexpectedly, the analogue with the relatively small $Ser(Bzl)^2$ residue is the most potent and effective degranulating agent.

4.2.3. Conclusions

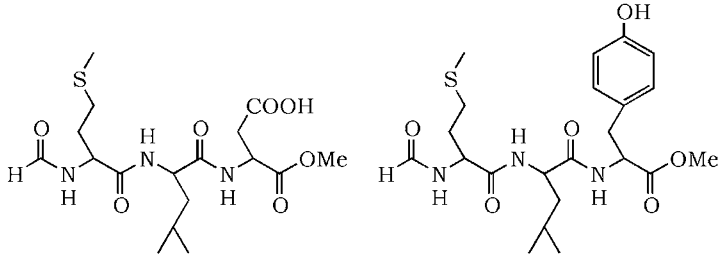
(1) The steric hindrance and hydrophobicity of the second residue is the mandatory for stimulating good chemotaxis;
(2) only properly chosen cycloalkane rings allow a good neutrophil response;
(3) in order to trigger superoxide anion production, hydrophilicity associated to good steric hindrance elicits a highly potent response;
(4) effective hindrance, together with functionalisation of the second residue, could thus afford peptides which selectively improve the biological response.

4.3. Variations in the Phe residue

Like the Leu side-chain, the Phe side-chain also accommodates in a hydrophobic pocket in the same transmembrane domain zone [12]. Hence, in this case too, all subsequent research was focussed on hydrophobic residues, e.g. [68].



for-Met-Leu-Ser-OMe **23** for-Met-Leu-Glu-OMe **24**



for-Met-Leu-Asp-OMe **25** for-Met-Leu-Tyr-OMe **26**

Fig. 11. Structure of compounds (**23**–**26**) substituted at [Phe]^3.

In view of the lower specificity required by the receptor isoform which triggers killer mechanisms, we synthesized for-Met-Leu-Ser-OMe **23**, for-Met-Leu-Glu-OMe **24**, for-Met-Leu-Asp-OMe **25**, and for-Met-Leu-Tyr-OMe **26** (Fig. 11). These compounds were characterized, and their biological activity on human neutrophils was evaluated [69].

Our results unexpectedly indicate that these analogues elicit good chemotaxis, while superoxide anion production is less stimulated, except at the highest physiological concentrations. The order of potency of these analogues is: **25** > **23** > **24** ≈ **26**. These data suggest that the residue at position 3 is not required to be sterically encumbered, and that its hydrophobicity is not a mandatory feature.

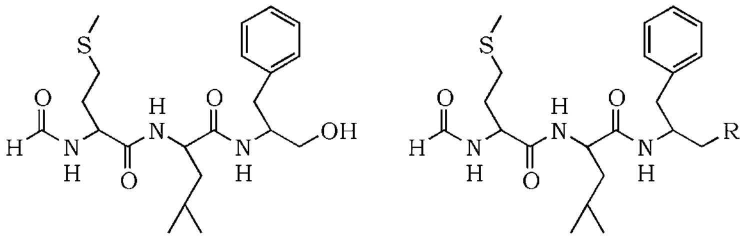
4.3.1. Conclusions

Our findings suggest that the Phe pocket is less exacting than previously supposed [2,3,70–73]. Furthermore, the hydrophilicity of the residue at position 3 negatively affects superoxide anion production, while it is unimportant for chemotaxis.

5. Variation at carboxylic group

A few alterations of the C-terminal function have been made to understand whether and how to change the carboxylic group in the search for an increase in the activity and/or biological selectivity. It has been shown that the ability to activate human neutrophils (using as biological reference the release of β-glucuronidase) is $\text{—CONH}_2<$ $\text{—COOMe}<\text{—COOH}\ll\text{—COOBzl}$ [74].

Phenylalaninol analogues have been introduced at position 3, thereby varying both the third residue and the carboxylic group [75]. The biological response is interesting because, the [Pheol]^3 compound **27** being devoid of any activity, the carboxylic analogues were shown to be able to select killing mechanisms, thus stressing the importance of the presence of the carboxylic group **28** or its methylester **29** (Fig. 12).



[Pheol]^3 **27** [Pheol]^{3}esters (R=COOH **28**; =OMe **29**)

Fig. 12. Modifications in C-terminal moiety (compound **27**–**29**).

5.1. Conclusions

All studies demonstrate the necessity of a carboxylic group, both free and esterified, to trigger a biological response in human neutrophils.

6. More variations

6.1. Cyclic analogues

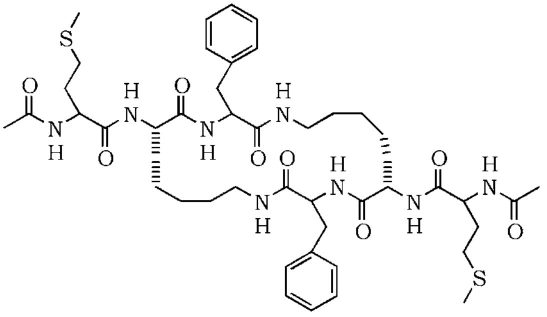
Some studies on the formyl tripeptide structure–activity relationship have been directed toward cyclic analogues, aimed at introducing conformational restrictions into the backbone. Cyclopeptides offer several advantages, among which are reduced conformational heterogeneity, enhanced stability toward enzymatic degradation and the possibility of exerting a certain control on the relative spatial orientation of the side-chains. This latter property is relevant for the design of bioactive ligands in order to improve molecular recognition and, ultimately, ligand–receptor binding affinity and selectivity. We report here two examples of this type of compound which particularly highlight the difficulty of choosing a whole steric cyclo-hindrance which allows good interaction with the specific neutrophil receptor.

The cyclic dimeric compound for-Met-Lys-Phe for-Met-Lys-Phe-**30** (Fig. 13) is a dimeric analogue with a Leu residue at position 2, thereby allowing the formation of an amide bond with the carboxylic group of Phe; that is, **36** contains a 20-membered cyclic moiety in which two equivalent isopeptide bonds are formed between the Lys ε-amino group and the Phe carboxyl [76]. When tested on human neutrophils, this analogue is active as a chemoattractant, secretagogue, and superoxide anion generating agent.

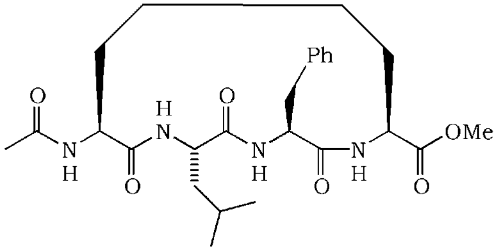
Peptide for-Cys-Leu-Phe-Cys-OMe **37**’ instead, needs four residues to cyclize (Fig. 13), and the resulting disulfide cyclopeptide is 14-membered. These features do not allow correct interaction with the specific receptor, being inactive towards human neutrophils [77].

6.1.1. Conclusions

The different behaviour, in eliciting the biological response by the two cyclic peptides, could be due to the folding of the peptide’s backbone and to the resulting side-chain topology, as proposed by Edmunson and Ely [21].



for-Met-Lys-Phe for-Met-Lys-Phe **30**



for-Cys-Leu-Phe-Cys-OMe **31**

Fig. 13. Cyclic analogues **30–31**.

6.2. Didehydro analogues

The presence of a double bond between C^{α} and C^{β} atoms not only affects the backbone conformation but also restricts the conformational flexibility of the side-chain, thereby influencing the biological activity. DidehydroLeu $[\Delta^{Z}Leu]^{2}$ and Phe $[\Delta^{Z}Phe]^{3}$ have been the most studied derivatives.

6.2.1. Didehydro analogues at position 2

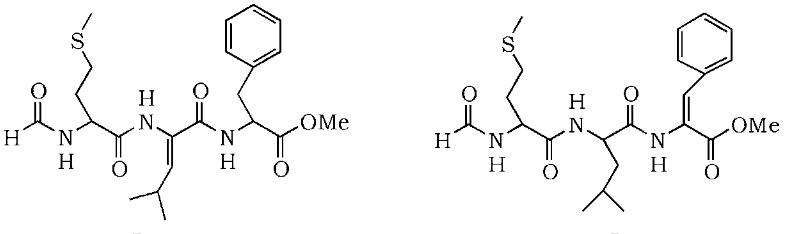
The unsaturated (*Z*)-α,β-didehydroleucine $[\Delta^{Z}Leu]^{2}$ **32** analogue is practically inactive as a chemoattractant but is highly active in triggering killing mechanisms [78–81]. The conformational restriction imposed on the backbone by the presence of the didehydro-residue may be responsible for the observed bioactivity (Fig. 14).

6.2.2. Didehydro analogues at position 3

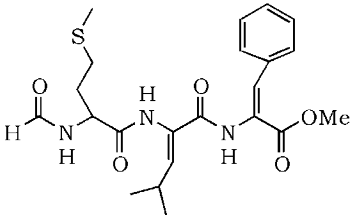
The unsaturated (*Z*)-α,β-didehydrophenylalanine $[\Delta^{Z}Phe]^{3}$**33**, introduced in place of Phe as C-terminal residue, affords an analogue devoid of any biological activity. It has been supposed that an improper orientation of the aromatic ring is responsible for the lack of interaction with the receptor [73].

6.2.3. Didehydro analogues at position 2 and 3

The unusual introduction of two consecutive α,β-didehydro residues bound to the native for-Met gave the for-Met-Δ^{Z}Leu-Δ^{Z} Phe-OMe **34** analogue, which retained all



for-Met-Δ^ZLeu-Phe-OMe **32** for-Met-Leu-Δ^ZPhe-OMe **33**



for-Met-Δ^ZLeu-Δ^ZPhe-OMe **34**

Fig. 14. Dehydro analogues **32**–**34**.

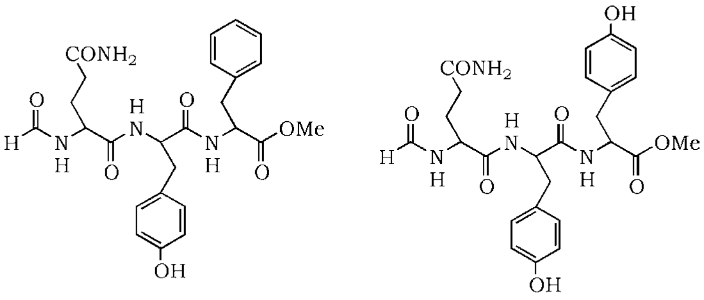
the typical biological activities even if less triggered. It has been suggested that the presence of two consecutive unsaturated residues alter the stereochemistry of the molecule, thereby partially overcoming the unfavourable influence of the presence of a single Δ^Z Phe residue [82].

A similar explanation can account for the activity of the tetrapeptide for-Met-Leu-Δ^Z Phe-Phe-OMe, which can link and stimulate its receptor [83].

6.3. Other approaches

The previous results induced us to study the biological response of human neutrophils to the double-substituted peptide for-Gln-Tyr-Phe-OMe **35** [to understand whether combining these two substitutions (i.e. Gln instead of Met, and Tyr instead of Leu), as each one alone strongly triggers killing mechanisms, would lead to an even stronger response: i.e. if there is a potentiating mechanism arising from targeted multi-substitutions] and the analogue for-Gln-Tyr-Tyr-OMe **36** [introducing a Tyr residue, which has often been demonstrated to be an intriguing variation of the prototype Phe [69], at position 3 of analogue **35**] (Fig. 15). In particular, the analogue **36** shares only the amino-protecting formyl group with the prototype fMLP-OMe. This feature should allow clarification of whether or not the formyl group alone (independently of the three native residues) is able to bind FPR and activate neutrophil responses [84].

Whereas neither compound stimulates chemotaxis, both are able to elicit killing mechanisms. However, compound **35** is able to trigger copious superoxide anion production, while compound **36** elicits only minor superoxide anion production. In binding experiments on formylpeptide receptors, both compounds showed affinity values in the micromolar range. These derivatives demonstrate inability to find a positive contribute from single substitutions.



for-Gln-Tyr-Phe-OMe **35** for-Gln-Tyr-Tyr-OMe **36**

Fig. 15. Structure of compounds **35**–**36** with two and three substitutions.

7. Conclusions

The important points evidenced by this review are:

1. Amide bonds are able to modulate and/or select biological responses.
2. The formyl group is not mandatory for triggering the physiological functions of human neutrophils.
3. Both hydrophilic and hydrophobic residues can fit the specific receptor pockets. In particular, hydrophilic substitutions at position 2 are optimal for triggering killing mechanisms.
4. Cyclic analogues, such as the cyclic dimeric tripeptide, can exhibit good activity and a promising selectivity of action if properly chosen.
5. Didehydro analogues are more or less able to trigger biological responses depending on the position occupied by the didehydro residue.

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