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Further Studies on the Structural Requirements for Synthetic Peptide Chemoattractants†

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ABSTRACT: Thirty small molecular weight peptides related to the chemotactic peptide *N*-formylmethionylleucylphenylalanine (CHO-Met-Leu-Phe-OH) have been prepared by both solid-phase and classical peptide synthesis. Compounds were prepared to investigate the structural requirements in the 1 position (*N*-formylmethionine) and the 3 position (phenylalanine). Each analogue was tested for its ability to induce lysosomal enzyme release from cytochalasin B treated rabbit polymorphonuclear leukocytes in vitro. In addition, some were also tested for their ability to stimulate neutrophil chemotaxis in vitro and for inhibition of specific binding of a ^{3}H-labeled chemotactic peptide, CHO-Nle-Leu-Phe-OH. The results show that the formyl group of CHO-Met-Leu-Phe-OH is essential for good biological activity since N-acetylation, removal of the α-amino group (i.e., desamino), or replacement by an ethyl group results in a drastic loss of chemotactic potency (approximately 5000-fold). In addition, the sulfur-containing side chain of methionine produces optimum activity of the tripeptide. Analogues containing other sulfur amino acids [ethionine, Cys(Me)] were less active, as were a variety of analogues containing linear aliphatic, aromatic, or branched aliphatic side chains at position 1. A limited number of analogues were prepared to probe structure–activity relationships at position 3. The data indicate that the primary sequence, Met-Leu-Phe, generates the most active chemoattractants, although addition of a large, highly charged Lys residue allowed the retention of a large degree of chemotactic activity. A free carboxyl group may be desirable, however, since CHO-Met-Leu-β-phenethylamine (-descarboxy-Phe) is relatively inactive. Finally, several related compounds have been identified which are specific competitive antagonists of CHO-Met-Leu-Phe-OH-induced lysosomal enzyme release and chemotaxis.

Although a large number of partially or totally uncharacterized substances have been reported to be chemotactic for neutrophils (Wilkinson, 1974), a systematic study of the phenomenon at the molecular level was not possible due to the unknown structure of these chemoattractants. In recent years, however, the finding of Schiffmann et al. (1975) that *N*-formylmethionine was chemotactic stimulated the development of a new group of synthetic chemoattractants. These are small molecular weight, N-acylated di-, tri-, and tetrapeptides, the most active of which is *N*-formyl-Met-Leu-Phe-OH [ED_{50} for chemotaxis of (7–9.1) $\times 10^{-11}$ M]. These compounds were also shown to induce the release of lysosomal enzymes (Showell et al., 1976), an effect which was absolutely correlated ($R > 0.95$) with their ability to stimulate chemotaxis. In addition, by the use of a radiolabeled analogue (*N*-formyl-Nle-Leu-[^{3}H]Phe-OH) (Day et al., 1977), specific binding sites on both rabbit (Aswanikumar et al., 1977a) and human (Williams et al., 1977) neutrophils were demonstrated. Later work with rabbit neutrophils has shown that these receptors are in the extracellular membrane and have a dissociation constant ($K_D = 2.9 \times 10^{-9}$ M) for the ^{3}H-labeled chemoattractant virtually identical with the ED_{50} (6.6×10^{-10} M) for the unlabeled chemoattractant (CHO-Nle-Leu-Phe-OH) (Sha'afi et al., 1978).

It was clear from our earlier studies (Showell et al., 1976) that the receptor substance for this group of peptides exhibited striking specificity. For example, in every instance tested, N-formylation of these peptides enhanced chemotactic potency by 1000–20 000-fold. Also, there were marked increases in biological activity in a series of tripeptides when compared to a reference dipeptide, *N*-formyl-Met-Leu-OH. Maximum activity was observed with the acylated tripeptide *N*-formyl-Met-Leu-Phe-OH (ED_{50} for chemotaxis of 7.0×10^{-11} M), although a highly active tetrapeptide, *N*-formyl-$(Met)_4$-OH (ED_{50} for chemotaxis of 3×10^{-10} M), was also identified. In addition to the size and N-acylation requirements, it was also found that methionine at position 1 and phenylalanine at position 3 optimized activity.

In this study we have further evaluated the structure–activity relationship in the highly active CHO-Met-Leu-Phe-OH. This includes an extensive investigation of the role of the *N*-formylmethionine residue. This has resulted in identification of specific antagonists of CHO-Met-Leu-Phe-OH-induced

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Table I: Analytical Data

compd	structure[a]	thin-layer chromatography (R_f) [b] sys A	sys B	sys C	other[c]
III	Ac-Met-Leu-Phe-OH	0.69	0.76	0.57	a, b
IV	desamino-Met-Leu-Phe-OH	0.72	0.80	0.65	a, b
V	2-ethylhexanoyl-Leu-Phe-OH	0.82	0.82	0.78	a, b
VI	CHO-Nle-Leu-Phe-OH	0.79	0.74	0.64	a, b, c, d
VII	Nle-Leu-Phe-OH	0.63	0.39	0.35	a, b, c, d
VIII	desamino-Nle-Leu-Phe-OH	0.74	0.82	0.67	a, b
IX	CHO-Gly-Leu-Phe-OH	0.62	0.62	0.56	a, b
X	CHO-Ala-Leu-Phe-OH	0.67	0.64	0.55	a, b
XI	CHO-Abu-Leu-Phe-OH	0.71	0.70	0.58	a, b
XII	CHO-Nva-Leu-Phe-OH	0.73	0.74	0.62	a, b
XIII	CHO-Hep-Leu-Phe-OH	0.78	0.79	0.67	a, b
XIV	CHO-Cys(Me)-Leu-Phe-OH	0.72	0.74	0.61	a, b
XV	CHO-Eth-Leu-Phe-OH	0.76	0.78	0.64	a, b
XVI	CHO-Phe-Leu-Phe-OH	0.77	0.77	0.65	a, b
XVII	CHO-Ile-Leu-Phe-OH	0.76	0.78	0.66	a, b
XVIII	CHO-Val-Leu-Phe-OH	0.74	0.75	0.63	a, b
XIX	CHO-Leu-Leu-Phe-OH	0.77	0.77	0.65	a, b
XX	CHO-Cyl-Leu-Phe-OH	0.70	0.71	0.60	a, b
XXI	CHO-Met-Ala-Leu-Phe-OH	0.69	0.72	0.58	a, b
XXII	CHO-Met-Leu-Ala-Phe-OH	0.69	0.74	0.57	a, b
XXIII	CHO-Met-Leu-Pea	0.72	0.76	0.79	a, b
XXIV	CHO-Met-Leu-Phe-Lys-OH	0.48	0.24	0.30	a, b, c
XXV	Boc-Met-Leu-Phe-OH	0.72	0.68	0.75	a, c
XXVI	Z-Met-Leu-Phe-OH	0.75	0.84	0.59	a, c
XXVII	MeO-Met-Leu-Phe-OH	0.67	0.65	0.75	a, c
XXVIII	Boc-Met-Leu-Pea	0.75		0.83	a, c
XXIX	Boc-Leu-Phe-OH	0.76	0.82	0.80	a, c
XXX	Boc-Phe-Leu-Phe-OH	0.72	0.80	0.73	a, c
XXXI	Boc-Leu-Phe-Leu-Phe-OH	0.68	0.75	0.65	a, c
XXXII	Boc-Phe-Leu-Phe-Leu-Phe-OH	0.70	0.78	0.68	a, c

[a] Nle = norleucine, Abu = α-aminobutyric acid, Nva = norvaline, Hep = α-aminoheptanoic acid, Cys(Me) = *S*-methylcysteine, Eth = ethionine, Cyl = cycloleucine, Pea = β-phenethylamine, Boc = *tert*-butyloxycarbonyl, Z = carbobenzoxy, and MeO = carbomethoxy. [b] Thin-layer chromatography on silica gel: sys A = 1-butanol–acetic acid–water (4:1:1); sys B = chloroform–methanol–acetic acid–water (60:30:4:1); sys C = benzene–water–acetic acid (9:1:9). [c] a = amino acid analysis, b = high-voltage electrophoresis, c = melting point, and d = elemental analysis.

chemotaxis and lysosomal enzyme release. In addition, analogues have been prepared to further probe the requirements in and around the phenylalanine residue.

Methods

Peptides were synthesized by both solid-phase and classical routes. The former was carried out by a modification (Day & Freer, 1978) of the method described by Stewart & Young (1969). Classical syntheses were carried out by the rapid mixed anhydride method described by Tilak (1970). In both routes the *tert*-butyloxycarbonyl (Boc) group was used to block the α-amino group. These Boc compounds were prepared by the method of Anderson & McGregor (1957). The ε-amino group of lysine was blocked by carbobenzoxylation. The carbobenzoxy and methoxy peptides were prepared by reaction of the free base form of the peptide with benzyl and methyl chloroformate, respectively.

Peptides were purified by either crystallization or countercurrent distribution. Compounds were considered homogeneous when a single spot was observed (100-μg load) in at least two thin-layer chromatography systems using two different methods of detection (i.e., UV, ninhydrin, *o*-tolidine). Similarly, compounds were considered homogeneous when testing by high-voltage electrophoresis at both pH 5 and 1.9 gave similar results. Again at least two detection reagents were used. Finally, compounds were subjected to amino acid analysis using a Beckman Model 119C amino acid analyzer. The analytical data are summarized in Table I.

Each compound was tested for its ability to induce the release of the lysosomal enzymes β-glucuronidase and lysozyme from cytochalasin B treated rabbit neutrophils. The chemoattractant potency of the peptides was evaluated in a modified Boyden chamber assay. Details of these assays have been published previously (Showell et al., 1976). In inhibition studies, the cells were exposed simultaneously to the suspected antagonist and the agonist (i.e., CHO-Met-Leu-Phe-OH).

The binding of peptides to neutrophil receptors was assayed by measuring the extent to which they competed with the binding of a labeled attractant, CHO-Nle-Leu-*p*-[^{3}H]Phe-OH, to whole cells (Aswanikumar et al., 1977a). A 2-mL volume of Gey's balanced salt solution containing a suspension of 4.4 × 10^6 cells, 0.2 μmol of tosyl-L-phenylalanylchloromethane (TPCK), 2.0 pmol (50 000 cpm) of ^{3}H-labeled peptide, and varying concentrations of the competing peptide were incubated at 4 °C for 1 h. The cells were then rapidly filtered onto Whatman glass fiber filters (GF/B) under vacuum and washed rapidly with two 7-mL portions of cold 0.02 M phosphate-buffered saline, pH 7.4 (PBS). The filters were then suspended in 10 mL of Aquasol (New England Nuclear), and scintillation spectrometry was carried out in a Beckman scintillation spectrometer with an efficiency of 40% for tritium. ID_{50} concentrations of peptides were determined from complete displacement curves (done in triplicate) which varied less than 10%.

Results

Role of the Formyl Group. Our previous work with these compounds had clearly demonstrated the importance of N-acylation in enhancing chemotactic potency (Showell et al., 1976) as well as in enhancing specific binding to neutrophils (Aswanikumar et al., 1977a). The striking effect of formylation (i.e., 1000–20 000-fold increase in activity) (compare compounds I and II, Table II) prompted us to design several analogues to determine if there is a unique structural re-

Table II: Modification of the Position 1 α-Amino Group

compd	structure	ED_{50} for lysosomal enzyme release (M)[a]		ED_{50} for chemotaxis (M)[a]
		lysozyme	β-glucuronidase	
I	CHO-Met-Leu-Phe-OH	$(3.2 \pm 0.8) \times 10^{-10}$	$(3.9 \pm 0.6) \times 10^{-10}$	$(9.1 \pm 1.0) \times 10^{-11}$
II	Met-Leu-Phe-OH	$(8.9 \pm 1.4) \times 10^{-7}$	$(9.7 \pm 0.12) \times 10^{-7}$	$(6.7 \pm 1.9) \times 10^{-7}$
III	Ac-Met-Leu-Phe-OH	$(1.4 \pm 0.2) \times 10^{-6}$	$(1.7 \pm 0.3) \times 10^{-6}$	$(2.0 \pm 1.1) \times 10^{-7}$
IV	desamino-Met-Leu-Phe-OH	$(1.1 \pm 0.3) \times 10^{-6}$	$(1.7 \pm 0.3) \times 10^{-6}$	$(2.0 \pm 1.1) \times 10^{-7}$
V	2-ethylhexanoyl-Leu-Phe-OH	$(1.6 \pm 0.6) \times 10^{-6}$	$(1.9 \pm 0.7) \times 10^{-6}$	$(1.9 \pm 0.4) \times 10^{-7}$

[a] ED_{50} = concentration of peptide required to produce 50% of the maximum effect as determined from the concentration effect curve. Each value is the average ± SEM of three to eight determinations done in duplicate.

Table III: Modification of Position 1 (Methionine) Side Chain

compd	structure[a]	ED_{50} for lysosomal enzyme release (M)[b]		ED_{50} for chemotaxis (M)[b]
		lysozyme	β-glucuronidase	
I	CHO-Met-Leu-Phe-OH	$(3.2 \pm 0.8) \times 10^{-10}$	$(3.9 \pm 0.6) \times 10^{-10}$	$(9.1 \pm 1.0) \times 10^{-11}$
VI	CHO-Nle-Leu-Phe-OH	$(1.5 \pm 0.2) \times 10^{-9}$	$(1.9 \pm 0.2) \times 10^{-9}$	$(6.6 \pm 1.2) \times 10^{-10}$
VII	Nle-Leu-Phe-OH	$(1.5 \pm 0.4) \times 10^{-4}$	$(3.1 \pm 0.8) \times 10^{-4}$	$(9.1 \pm 5.9) \times 10^{-6}$
VIII	desamino-Nle-Leu-Phe-OH	$(6.1 \pm 1.1) \times 10^{-6}$	$(1.4 \pm 0.3) \times 10^{-5}$	$(4.6 \pm 2.2) \times 10^{-7}$
IX	CHO-Gly-Leu-Phe-OH	$(7.6 \pm 0.9) \times 10^{-6}$	$(1.0 \pm 0.1) \times 10^{-5}$	$(4.3 \pm 1.1) \times 10^{-6}$
X	CHO-Ala-Leu-Phe-OH	$(3.9 \pm 1.8) \times 10^{-5}$	$(3.7 \pm 1.4) \times 10^{-5}$	$(3.4 \pm 1.6) \times 10^{-6}$
XI	CHO-Abu-Leu-Phe-OH	$(1.8 \pm 0.4) \times 10^{-6}$	$(2.3 \pm 0.6) \times 10^{-6}$	$(3.8 \pm 1.1) \times 10^{-7}$
XII	CHO-Nva-Leu-Phe-OH	$(1.3 \pm 0.3) \times 10^{-8}$	$(1.6 \pm 0.4) \times 10^{-8}$	$(5.8 \pm 2.1) \times 10^{-9}$
XIII	CHO-Hep-Leu-Phe-OH	$(1.7 \pm 0.3) \times 10^{-9}$	$(1.9 \pm 0.3) \times 10^{-9}$	$(3.3 \pm 1.0) \times 10^{-10}$
XIV	CHO-Cys(Me)-Leu-Phe-OH	$(8.5 \pm 2.0) \times 10^{-8}$	$(7.1 \pm 2.0) \times 10^{-8}$	$(2.4 \pm 0.3) \times 10^{-8}$
XV	CHO-Eth-Leu-Phe-OH	$(1.2 \pm 0.3) \times 10^{-9}$	$(1.5 \pm 0.5) \times 10^{-9}$	$(4.3 \pm 1.1) \times 10^{-10}$
XVI	CHO-Phe-Leu-Phe-OH	$(5.7 \pm 1.3) \times 10^{-7}$	$(8.5 \pm 0.2) \times 10^{-7}$	$(1.5 \pm 0.7) \times 10^{-7}$
XVII	CHO-Ile-Leu-Phe-OH	$(1.9 \pm 0.2) \times 10^{-9}$	$(2.2 \pm 0.3) \times 10^{-9}$	$(2.6 \pm 1.0) \times 10^{-10}$
XVIII	CHO-Val-Leu-Phe-OH	$(7.6 \pm 1.9) \times 10^{-8}$	$(7.9 \pm 1.9) \times 10^{-8}$	$(7.2 \pm 1.9) \times 10^{-9}$
XIX	CHO-Leu-Leu-Phe-OH	$(5.0 \pm 1.5) \times 10^{-8}$	$(5.0 \pm 1.2) \times 10^{-8}$	$(1.2 \pm 0.3) \times 10^{-8}$
XX	CHO-Cyl-Leu-Phe-OH	$(3.9 \pm 1.4) \times 10^{-6}$	$(5.4 \pm 2.4) \times 10^{-6}$	$(4.2 \pm 0.8) \times 10^{-7}$

[a] Nle = norleucine, Abu = α-aminobutyric acid, Nva = norvaline, Hep = α-aminoheptanoic acid, Cys(Me) = *S*-methylcysteine, Eth = ethionine, and Cyl = cycloleucine. [b] ED_{50} = concentration of peptide required to produce 50% of the maximum effect as determined by the concentration effect curve. Each value is the average ± SEM of three to eight determinations.

quirement for the formyl group or if its effect is due to simple neutralization of the α-amino function, steric considerations, etc. To this end, three additional analogues (Table II) were prepared in which the α-amino group was either acetylated (compound III), removed (compound IV), or replaced by an ethyl group (compound V). As can be seen, each of these analogues showed a reduction in activity to approximately the same level seen with the unacylated tripeptide (compound II). Indeed each retained less than 0.05% of the activity of CHO-Met-Leu-Phe-OH.

Side-Chain Variations in Position 1. The striking requirement for the formyl group just noted, as well as the structural specificity shown in our previous work (Showell et al., 1976), made it quite probable that relatively minor alterations in the methionine side chain might also produce dramatic changes in biological activity. A number of N^{α}-formylated peptides were synthesized in which the methionine side chain was replaced either by other sulfur-containing side chains or by those containing a variety of aliphatic, aromatic, or branched side chains to probe this possibility. The data are shown in Table III and are graphically shown in Figure 1.

It is interesting that of all of the variations tested here none even approached the potency of the parent compound, CHO-Met-Leu-Phe-OH. For example, the most potent analogue prepared was CHO-Nle-Leu-Phe-OH (VI) and even that had less than 10% of the activity of the methionine compound. Like the parent compound, this also exhibited a stringent requirement for the formyl group as evidenced by the low activity of the unacylated (VII) and desamino (VIII) analogues.

As with the formyl group, the side-chain variations in position 1 also exhibited extreme specificity. For example, total

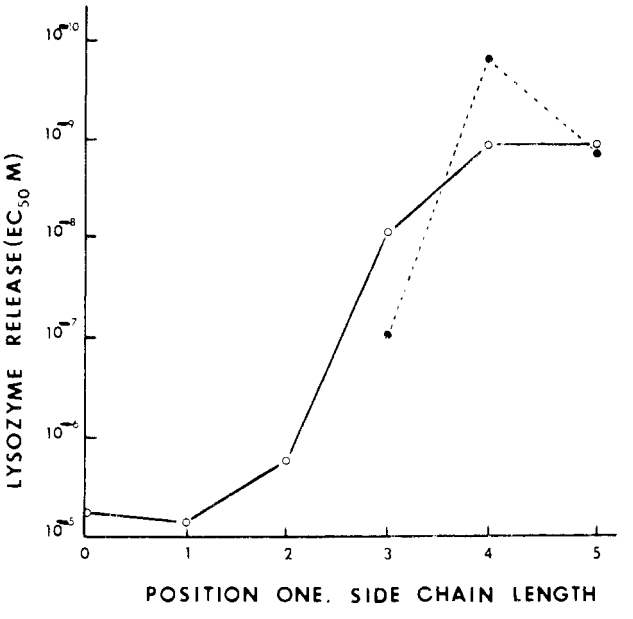

FIGURE 1: Lysozyme release by peptides with varying side-chain length in position 1. (●) Sulfur-containing amino acids (3 = methylcysteine, 4 = methionine, and 5 = ethionine); (○) nonsulfur amino acids (0 = glycine, 1 = alanine, 2 = α-aminobutyric acid, 3 = norvaline, 4 = norleucine, and 5 = α-aminoheptanoic acid). Data are the average of three to eight determinations (SEM is shown in Table III).

elimination of the side chain in position 1 (i.e., CHO-Gly-Leu-Phe-OH, compound IX; Table III) reduces chemotactic potency by nearly 5 orders of magnitude (from 9.1×10^{-11} to 4.3×10^{-6} M). Similarly, replacing methionine by alanine (compound X) also resulted in a severe reduction in activity.

Table IV: Modification of Position 3 Phenylalanine

compd	structure[a]	ED_{50} for lysosomal enzyme release (M)[b]		ED_{50} for chemotaxis (M)[b]
		lysozyme	β-glucuronidase	
I	CHO-Met-Leu-Phe-OH	$(3.2 \pm 0.8) \times 10^{-10}$	$(3.9 \pm 0.6) \times 10^{-10}$	$(9.1 \pm 1.0) \times 10^{-11}$
XXI	CHO-Met-Ala-Leu-Phe-OH	$(4.5 \pm 1.0) \times 10^{-8}$	$(5.7 \pm 1.4) \times 10^{-8}$	$(5.3 \pm 0.9) \times 10^{-9}$
XXII	CHO-Met-Leu-Ala-Phe-OH	$(1.1 \pm 0.5) \times 10^{-8}$	$(1.5 \pm 0.6) \times 10^{-8}$	$(1.9 \pm 0.3) \times 10^{-9}$
XXIII	CHO-Met-Leu-Pea	$(2.0 \pm 0.3) \times 10^{-8}$	$(2.4 \pm 0.4) \times 10^{-8}$	$(1.9 \pm 0.4) \times 10^{-7}$
XXIV	CHO-Met-Leu-Phe-Lys-OH	$(1.7 \pm 0.5) \times 10^{-9}$	$(1.9 \pm 0.3) \times 10^{-9}$	$(7.0 \pm 1.0) \times 10^{-10}$

[a] Pea = β-phenethylamine = descarboxyphenylalanine. [b] ED_{50} = concentration of peptide required to produce 50% of the maximum effect as determined by the concentration effect curve. Each value is the average ± SEM of five to eight determinations.

However, further extensions of the side chain with linear aliphatic groups produced marked increases in activity up to a maximum of 1.5×10^{-9} M for CHO-Nle-Leu-Phe-OH (III) and 1.9×10^{-9} M for CHO-Hep-Leu-Phe-OH (XIII). It is most remarkable that simple extension of the side chain by two carbon atoms produces a 1200-fold increase in activity (compare VI and XI). Although not strictly comparable, the same trend is observed in those residues containing a S atom in their side chain (I, XIV, and XV). Particular note should be made of the 3 orders of magnitude decrease in activity observed when *S*-methylcysteine (XIV) is substituted for methionine (I). This, of course, represents a difference of only one carbon atom. A similar, but not as dramatic, decrease is seen when an additional methylene carbon is added to give the *S*-ethyl compound [i.e., the ethionyl analogue (XV)].

The final group of variations are those containing branched as opposed to linear side chains. These include β- (XVII and XVIII) and γ-branched (XIX) groups as well as aromatic (XVI) and saturated ring systems (XX). Surprisingly, the β branching of isoleucine and valine is quite well tolerated (ED_{50} for lysozyme release of 1.9×10^{-9} and 7.6×10^{-8} M, respectively). This is particularly true of the Ile analogue which is nearly as active as the unbranched Nle compound. Similarly, the Val analogue still retains 20% of the activity of the norvaline compound and is, in fact, twice as active as its sulfur-containing counterpart, CHO-Cys(Me)-Leu-Phe-OH (ED_{50} of 8.5×10^{-8} M). γ-branching, however, is less well tolerated; the Leu analogue retains only 3.3% of the activity (ED_{50} of 5.0×10^{-8} M) of the norleucyl compound and 0.2% of the activity of the methionine-containing peptide (I). Further reductions in activity are observed when either a Phe (XVI) residue or a cycloleucine (XX) residue is substituted for the methionine. The former retains only 0.01% and the latter 0.002% of the activity of CHO-Met-Leu-Phe-OH (I).

Role of Phenylalanine in Position 3. It was apparent from our earlier work that, although the presence of *N*-formylmethionine at the amino terminus was required for optimum activity, almost equally beneficial was the presence of a Phe residue in the third position. Compounds containing alanine "spacers" were prepared [compounds XXI (CHO-Met-Ala-Leu-Phe-OH) and XXII (CHO-Met-Leu-Ala-Phe-OH), Table IV] in order to determine if the relative position of the CHO-Met and Phe, with respect to each other, was critical for good biological activity. Both peptides showed greater than 99% loss in activity compared to CHO-Met-Leu-Phe-OH, indicating that the relative position of the Met and Phe in the peptide chain may well be important. Along these same lines, two analogues were prepared to determine if there exists a requirement for a free α-carboxyl group on the Phe residue. The results suggest that a free carboxyl group is beneficial but that it is not necessary for it to be on the Phe itself for significant activity to occur. For example, CHO-Met-Leu-des-COOH-Phe (XXIII) retains less than 0.05% activity while CHO-Met-Leu-Phe-Lys-OH (XXIV) has ~5% of the activity of CHO-Met-Leu-Phe-OH (I). This relatively high activity seen with the lysine-containing compound is somewhat surprising given the presence of the basic side chain and the generally deleterious effect of charged groups on other compounds tested (Showell et al., 1976).

Table V: Effect of Selected Peptides on Specific Binding[a]

compd[b]	structure[c]	binding inhibn (ID_{50}, M)[d]
I	CHO-Met-Leu-Phe-OH	3.3×10^{-10}
III	Ac-Met-Leu-Phe-OH	4.5×10^{-7}
IV	desamino-Met-Leu-Phe-OH	1.9×10^{-6}
V	2-ethylhexanoyl-Leu-Phe-OH	6.4×10^{-6}
VI	CHO-Nle-Leu-Phe-OH	3.4×10^{-9}
VIII	desamino-Nle-Leu-Phe-OH	6.6×10^{-6}
XI	CHO-Abu-Leu-Phe-OH	3.0×10^{-6}
XV	CHO-Eth-Leu-Phe-OH	1.0×10^{-9}
XVI	CHO-Phe-Leu-Phe-OH	9.5×10^{-7}
XX	CHO-Cyl-Leu-Phe-OH	9.2×10^{-6}
XXI	CHO-Met-Ala-Leu-Phe-OH	4.0×10^{-7}
XXIV	CHO-Met-Leu-Phe-Lys-OH	1.0×10^{-8}

[a] CHO-Nle-Leu-*p*-[^{3}H]Phe-OH was incubated with five concentrations of nonradioactive peptides, and the concentration required to displace 50% of the specific binding was estimated from plots of percent inhibition of specific binding vs. peptide concentration. Values are means of triplicate values varying less than 10%. [b] Numerals refer to compounds listed in Tables I–IV. [c] Nle = norleucine, Abu = α-aminobutyric acid, Eth = ethionine, and Cyl = cycloleucine. [d] ID_{50} = concentration required to displace 50% of specifically bound CHO-Nle-Leu-*p*-[^{3}H]Phe-OH.

Correlation of Binding Inhibition and Biologic Response. We have previously prepared (Day et al., 1977) and used (Aswanikumar et al., 1977a) an intrinsically labeled chemoattractant, CHO-Nle-Leu-*p*-[^{3}H]Phe-OH, to demonstrate the presence of a specific binding site on the neutrophil membrane. Using a limited number of synthetic peptides, we were able to show an excellent correlation between receptor binding, as evidenced by binding inhibition curves, and both chemotaxis and lysosomal enzyme release. These studies have been expanded to include compounds from each of the previous groups. The binding data are included in Table V, and their correlation vs. chemotaxis and lysosomal enzyme release is shown in Figure 2. On inspection it is clear that there is a very strong correlation between the ability of the synthetic peptides to induce a biological response and their ability to compete for the receptor.

Modifications Producing Antagonists. A recent study has shown that a protected pentapeptide, H-L-Phe-D-Leu-L-Phe-D-Leu-L-Phe-OH, related to gramicidin is an excellent chemoattractant when it is formylated ($ED_{50} = 4.0 \times 10^{-9}$ M) and a weak, but specific, antagonist ($ID_{50} = 8.0 \times 10^{-7}$ M) when the α-amino is blocked as in the *tert*-butyloxycarbonyl (Boc) derivative (Aswanikumar et al., 1977b). The difference is, of course, that the Boc derivative is a urethan linkage as opposed to the peptide linkage in the formylated peptide. Following this lead, we have prepared several analogues which

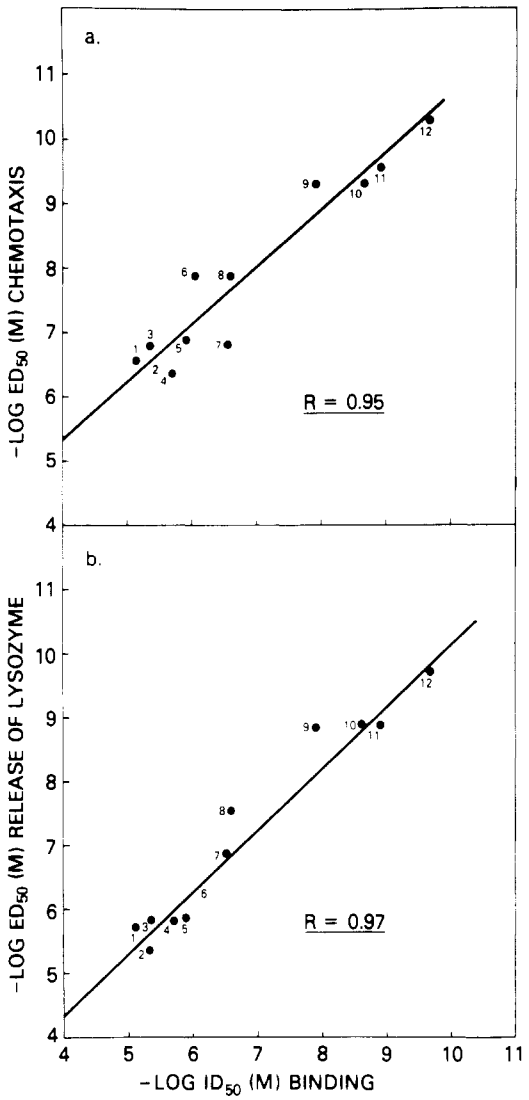

FIGURE 2: Relations between peptide-induced chemotaxis and inhibition of binding (upper) and lysosomal enzyme (lysozyme) release and inhibition of binding (lower). CHO-Cyl-Leu-Phe (1); desamino-Nle-Leu-Phe (2); 2-ethylhexanoyl-Leu-Phe (3); CHO-Abu-Leu-Phe (4); desamino-Met-Leu-Phe (5); CHO-Phe-Leu-Phe (6); Ac-Met-Leu-Phe (7); CHO-Met-Ala-Leu-Phe (8); CHO-Met-Leu-Phe-Lys (9); CHO-Nle-Leu-Phe (10); CHO-Eth-Leu-Phe (11); CHO-Met-Leu-Phe (12). Peptides were prepared and assayed for chemotaxis, lysozyme releasing activity, and binding inhibition as described under Methods. Values are means of triplicate measurements which varied less than 10%. Correlation coefficients (R values) were calculated from the equation $R = [\sum(X_i - \bar{X})(Y_i - \bar{Y})]/[[\sum(X_i - \bar{X})^2][\sum(Y_i - \bar{Y})^2]]^{1/2}$. The straight lines on each graph were drawn according to the method of least squares (Weast & Selby, 1975).

contain urethan type protecting groups at the amino terminus (Table VI). Overall, the results indicate that the simple presence of this type of linkage is not, in itself, sufficient to produce an antagonist. For example, when the Boc group is incorporated into the most active tripeptide sequence (i.e., H-Met-Leu-Phe-OH), a weak antagonist results (XXV) (ID_{50} for lysozyme release of 6.4×10^{-7} M). However, when other urethan blocking groups are used [e.g., carbomethoxy (XXVII) or carbobenzoxy (XXVI)], the result is weak agonist activity. Further modification of XV by removing the α-carboxyl group (XXVIII) did not improve antagonist potency but rather decreased it.

Finally, we have prepared a family of C-terminal homologues (XXIX–XXXII) related to the gramicidin analogue recently reported (Aswanikumar et al., 1977b) to have antagonist activity. In contrast to those previous studies, we have used L-leucine rather than the D isomer. The results indicate that a minimal requirement for inhibition is the tripeptide sequence Boc-Phe-Leu-Phe-OH (XXX). A further increase in inhibitory activity is seen on increasing to the tetrapeptide stage (XXXI), but no significant improvement in activity occurs by increasing the chain length to the full pentapeptide sequence (XXXII). The protected dipeptide Boc-Leu-Phe-OH (XXIX) is virtually inactive.

Table VI: Antagonists of CHO-Met-Leu-Phe-OH-Induced Lysozyme Release

compd	structure[a]	lysozyme release[b] ED_{50} (M)	lysozyme release[b] ID_{50} (M)
XXV	Boc-Met-Leu-Phe-OH		$(6.4 \pm 0.6) \times 10^{-7}$
XXVI	Z-Met-Leu-Phe-OH	$(3.5 \pm 1.3) \times 10^{-7}$	
XXVII	MeO-Met-Leu-Phe-OH	$(2.1 \pm 0.7) \times 10^{-8}$	
XXVIII	Boc-Met-Leu-Pea		$(9.0 \pm 1.7) \times 10^{-6}$
XXIX	Boc-Leu-Phe-OH	inactive	inactive
XXX	Boc-Phe-Leu-Phe-OH		$(5.7 \pm 1.1) \times 10^{-7}$
XXXI	Boc-Leu-Phe-Leu-Phe-OH		$(3.0 \pm 0.5) \times 10^{-7}$
XXXII	Boc-Phe-Leu-Phe-Leu-Phe-OH		$(2.6 \pm 0.6) \times 10^{-7}$

[a] Pea = β-phenethylamine = descarboxyphenylalanine. [b] Concentrations of peptides required to stimulate (ED_{50}) or inhibit (ID_{50}) 50% release of lysozyme. Data are taken from concentration effect curves and are the average of five to eight determinations.

Discussion

The striking feature of this group of compounds is the specificity of the receptor substance controlling the chemotactic response. Indeed, our previous studies (Showell et al., 1976) had indicated that this might be true and, with the aid of an intrinsically labeled peptide (Day et al., 1977), a specific binding site on the membranes of the neutrophil leukocyte was demonstrated (Aswanikumar et al., 1977a; Sha'afi et al., 1978). The binding was saturable and exhibited an almost absolute correlation with chemotaxis and lysosomal enzyme release.

With the studies reported herein, we have confirmed and extended our understanding of the specificity of this receptor by the preparation of a number of analogues with relatively subtle modifications of the *N*-formylmethionine residue of our most active compound, CHO-Met-Leu-Phe-OH (ED_{50} for chemotaxis = 9.1×10^{-11} M).

As shown previously, the formyl group is essential for maximum chemotactic and lysosomal enzyme releasing activity; for example, it increases the activity of Met-Leu-Phe-OH 10000-fold. Its function is not simply to neutralize the charge of the α-amino group because acetylation or complete removal of the amino function increased activity only threefold over the unacylated tripeptide (compare II with III and IV). Similarly, the formyl residue does not serve simply as steric bulk to fill a critical area of the receptor since replacement of CHO-NH- by CH_3CH_2-, as in 2-ethylhexanoyl-Leu-Phe-OH (V), again results in a severe reduction of biological activity. This is also consistent with the low activity of the N-acetylated analogue. With these data in mind, we suggest that there is some unique chemical property of the formyl group responsible for its dramatic effect. Consistent with this is our finding, using NMR spectroscopy, that CHO-Met-

Leu-Phe exhibits a high degree of rigidity in solution (Becker et al., 1979). In addition, the proton of the formyl group (as well as the other amide protons) appears to be readily available for hydrogen bonding, possibly with a compatible area of the receptor. Therefore, it is quite possible that formylation produces this striking enhancement of biological potency via effects on peptide conformation and/or specific receptor interactions.

Specificity is again the key word when considering the structure–activity relationships in the side chain of position 1. In the linear aliphatic series (compounds VI and IX–XIII), norvaline would appear to represent the minimum for good biological activity (i.e., >1% of that of CHO-Met-Leu-Phe-OH). Additional extension by one carbon to CHO-Nle-Leu-Phe-OH (VI) enhances activity approximately 10-fold with a slight reduction occurring with addition of another carbon atom (XIII). A similar but not identical effect is seen in the equivalent series in which the linear side chain contains a sulfur atom substituted for one carbon. In contrast to the linear aliphatics, the difference in activity between the CHO-Met-Leu-Phe-OH (I) and its next lower homologue, CHO-Cys(Me)-Leu-Phe-OH (XIV), is 3 orders of magnitude. This indicates that the simple presence of the sulfur in the side chain is not sufficient to confer maximum biological activity. In fact, the CHO-Cys(Me)-Leu-Phe-OH is 10-fold less active than its aliphatic counterpart, CHO-Nva-Leu-Phe-OH (XII). The opposite is true, however, when one compares CHO-Met-Leu-Phe-OH (I) with CHO-Nle-Leu-Phe-OH (VI) and CHO-Ile-Leu-Phe-OH (XVII). Here the S atom is clearly beneficial, although its mere presence at that position in the side chain is not the entire answer. This is evidenced by the reduction in activity seen with the CHO-Eth-Leu-Phe-OH (XV) in spite of the fact that the S is still δ to the carbonyl carbon.

To summarize this aspect of the work, it is evident that we have demonstrated a remarkable degree of specificity in the area of the receptor interacting with the N terminus of these synthetic chemoattractants. Nevertheless, *N*-formylmethionine remains the residue of choice in this position. This high degree of specificity may in fact extend to the full tripeptide sequence as shown in our earlier studies and confirmed here by insertion of the alanine spacers as in compounds XXI and XXII. These latter analogues indicate that the relationship of the residues to each other within the tripeptide sequence is also critical for good biological activity.

The role of the α-carbonyl group in these peptides is less clear than our understanding of the requirements at the amino terminus. The results are somewhat in opposition, in that removal of the carboxyl (XXIII) reduces activity, and might ordinarily suggest interaction of the carboxylate anion with a cationic site on the receptor. However, addition of a lysine residue (i.e., CHO-Met-Leu-Phe-Lys-OH, XXIV) has relatively little effect. This is further confused by the fact that the lysine side chain is quite basic and would tend to be repelled from a cationic site on the receptor. We can only conclude that, while the presence of a free carboxyl may be beneficial, its relative position is not critical. Of interest is the report of Ho et al. (1978) that the methyl ester of CHO-Met-Leu-Phe-OH is more active in stimulating macrophage chemotaxis than the parent compound, although it is less active against neutrophils. Whether this is true of the compounds we have prepared with modified carboxyl groups is currently under investigation.

A final point worthy of note here is the finding of Wilkinson (1979) that a number of weak agonists showed virtually no structural specificity. Unfortunately, these data were collected at a relatively inaccurate portion of the dose–response curve (i.e., at the maximum response) and there was also no indication if these compounds were inducing true chemotaxis or simply chemokinesis. Also, no data to implicate a specific receptor were presented and therefore a relationship between these data and the more potent peptides is highly questionable.

Another point worthy of discussion is those changes resulting in specific antagonists. Unfortunately, the limited number of compounds prepared does not allow for a mechanistic explanation. It is clear though that a urethan type of linkage to the α-amino group is essential. In addition, the nature of the R group on the urethan is also important. It is not simply the size of the R group since both the carbomethoxy (XXVI) and carbobenzoxy (XXV) compounds are weak agonists. The *tert*-butyloxy derivative (XXIV), on the other hand, is clearly an antagonist. In all cases (agonist and antagonist) the potency of these urethan-protected analogues does not approach that of CHO-Met-Leu-Phe-OH. This may be yet another example of the remarkable specificity of this receptor substance.

Finally, as can be seen on inspection of the data in Tables II–IV, there exists an almost absolute correlation between the ability of this group of compounds to produce chemotaxis and their ability to induce lysosomal enzyme release. In addition, both biological responses clearly result from interaction of these substances with a specific receptor. This is evidenced by a high degree of correlation between binding inhibition and both chemotaxis and lysosomal enzyme release. This is consistent with our previous studies; it is also consistent with the conclusion from these and other studies (O'Flaherty et al., 1978; Becker et al., 1979) that the same receptor is capable of inducing several different biological functions of the neutrophil (Becker, 1976).

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Interpretation of the Resonance Raman Spectrum of Bathorhodopsin Based on Visual Pigment Analogues†

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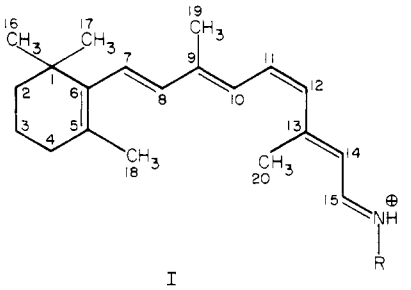
ABSTRACT: Resonance Raman spectra of visual pigment analogues have been used to evaluate various models for the structure of the retinal chromophore in bathorhodopsin. Deuteration or removal of the 18-methyl on the β-ionyl ring or of the 19-methyl on the polyene chain demonstrates that the three intense low wavenumber bands of bathorhodopsin at 853, 875, and 920 cm^{-1} are not due to exomethylene or ring modes. Rather, assignment of these lines to out-of-plane vinyl hydrogen motions on the chain best accounts for the experimental data. Our calculations show that the intensity of these vibrations can be explained by twists of 10–30° about chain single bonds. The 1100–1400-cm^{-1} Raman fingerprint indicates that the configuration of the double bonds is trans. This suggests that the structure of the bathorhodopsin chromophore is twisted all-trans.

Black and white vision in vertebrates is triggered by light-induced changes in the rod cell photoreceptor pigment, rhodopsin. This pigment consists of an 11-*cis*-retinal chromophore (I) bound covalently to a lysine residue of the apo-



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protein opsin by a protonated Schiff base (PSB) linkage.[1] Absorption of a photon by the 11-cis chromophore raises the pigment to a high free energy form which decays thermally via a series of distinct, metastable intermediates to a bleached mixture of *all-trans*-retinal and opsin as depicted in Figure 1 (Yoshizawa & Wald, 1963; Hubbard & Kropf, 1958). The chromophore binding site of opsin can also accommodate the 9-cis isomer of retinal to form isorhodopsin, which bleaches by the same pathway. Thus, the net effect of light absorption on the visual pigment chromophores is to isomerize them from an 11-cis or 9-cis configuration to an all-trans configuration.[2] It is not known precisely when in the bleaching process this isomerization is first achieved, although resonance Raman (RR) evidence indicates that by the time metarhodopsin I is formed the chromophore configuration is relaxed all-trans (Doukas et al., 1978a).

The fact that bathorhodopsin is the common photoproduct of both 11-*cis*-rhodopsin and 9-*cis*-isorhodopsin, together with the observed thermal decay of bathorhodopsin to *all-trans*-retinal and opsin, led naturally to the proposal that bathorhodopsin's chromophore configuration is distorted trans (Yoshizawa & Wald, 1963). Recent workers have coined the term "transoid" (Honig et al., 1979) to describe this trans configuration with an unspecified conformation. However, other possible batho structures involving out-of-plane deformations of chain carbons may be accessible to both 11-cis and 9-cis chromophores (Warshel & Deakyne, 1978; Lewis, 1978). Given the paucity of experimental data, a detailed evaluation of even these simplified models has not yet been possible.

Considerable evidence indicates that the formation of bathorhodopsin is, in fact, quite complex. The observed fast rise time of bathorhodopsin (~6 ps; Busch et al., 1972) seems intuitively to be too rapid for the movement of bulky groups involved in isomerization, although this assumption has been challenged (Warshel, 1976; Weiss & Warshel, 1979; Birge & Hubbard, 1980). Furthermore, picosecond absorption studies on rhodopsin equilibrated with D_2O at low temperature have demonstrated a kinetic isotope effect on the formation time of bathorhodopsin (Peters et al., 1977). This indicates that the primary photochemical event involves proton translocation. Reported incorporation of modest but persistent amounts of deuterium into the retinal chromophore of rhodopsin bleached in D_2O tends to support this view (Fransen

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[1] Abbreviations used: RR, resonance Raman; PSB, protonated Schiff base; 18-CD_3, 18-trideuterioretinal; 19-CD_3, 19-trideuterioretinal; HOOP, hydrogen out-of-plane.

[2] We define *configuration* to indicate the cis or trans structure of the double bonds and use *conformation* to refer to the set of rotation angles about single bonds in a given configuration.

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