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Synthesis and biological activities of new *N*-formylated methionyl peptides containing an α-substituted glycine residue

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Summary — Small molecular weight peptides related to the well-known chemotactic peptide *N*-α-formyl-methionyl-leucyl-phenylalanine have been prepared. These compounds were designed to evaluate the requirements in position 3 (phenylalanine). Each analogue containing an α-substituted glycine in position 3 was tested for its ability to induce lysosomal enzyme release from cytochalasin B treated human neutrophils. In addition, each analogue was also tested for its ability to antagonize superoxide generation. The results indicate that the analogue *N*-formyl-methionyl-leucyl-(α-anilino)glycine allyl ester is a potent antagonist of superoxide generation. Its ID_{50}'s suggest that this compound could be very promising in the field of anti-inflammatory drugs. A hypothetical inhibition mechanism is discussed.

***N*-formylmethionyl peptides / superoxide generation / lysosomal enzyme release**

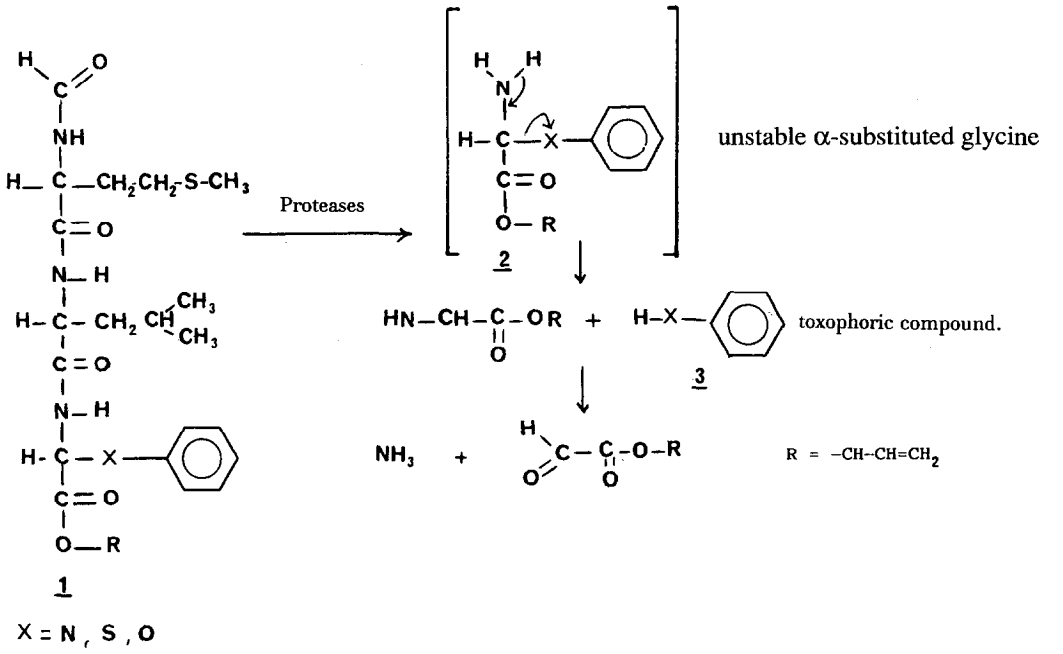
Introduction

N-α-Formyl-methionyl-leucyl-phenylalanine (CHO-Met-Leu-PheOH) is the prototype of a series of small molecular mass oligopeptides which stimulates chemotaxis, lysosomal enzyme secretion and a variety of other cellular responses in mammalian phagocytes [1, 2]. These biological effects are mediated through the interaction of the *N*-formyl-methionine peptide with a specific membrane receptor [3, 4]. All these observations have stimulated considerable interest in developing models for the biologically active conformation of these peptides [5–8]. On the basis of extensive structure–activity studies, in which the various elements of CHO-Met-Leu-PheOH were systematically varied [6, 9], Freer *et al* [10] reported that the chemotactic peptide exists on the receptor in a β-folded sheet; there are at least five critical areas of interaction between the tripeptide and the receptor, and the receptor appears to have sufficient 'room' to accomodate at least a tetrapeptide. It has also been demonstrated that CHO-Met-Leu-PheOH is one of the best biologically active substrates of the membrane-bound enzyme (neutral endopeptidase 24.11, EC 3.4.24.11) which cleaves peptides at the amino side of hydrophobic amino acids, and thereby inactivates a variety of peptide hormones [11]. This observation, associated with the fact that neutrophil degranulation and chemotaxis require the cleavage of chemotactic peptides [12, 13], prompted us to design new analogues of the chemotactic peptide CHO-Met-Leu-PheOH, in which the methylene group of phenylalanine residue was substituted with various heteroatoms such as N, S, or O.

The concept of α-substituted glycine peptide stability and enzyme-mediated breakdown has already been introduced by Kingsbury *et al* [14, 15] in the case of the dipeptide alanyl-α-(phenylthio) glycine. These authors found that the release of thiophenol rapidly followed the entry of the peptide into the cell. The modified peptides possess similar intrinsic chemical properties to those previously described herein (*eg* **1**, scheme 1). The substitution at the methylene group of the Phe residue with a heteroatom (O, S, N), is equivalent to the introduction of an α-substituted glycine residue in place of the corresponding Phe residue. Since glycines, when α-substituted by suitable leaving groups, represent highly reactive species, it was of interest to design these new peptides on the basis of this chemical reactivity.

As has been described for related peptides containing the α-substituted glycine moiety [14, 15] during the internalization process, these modified peptide analogues, stabilized by *N*-acylation of the α-substituted glycine moiety, are cleaved by endopeptidases of neutrophil membranes (scheme 1).

The cleavage of the peptide bond between the Leu and the α-substituted Gly residues leads to unstable



Scheme 1.

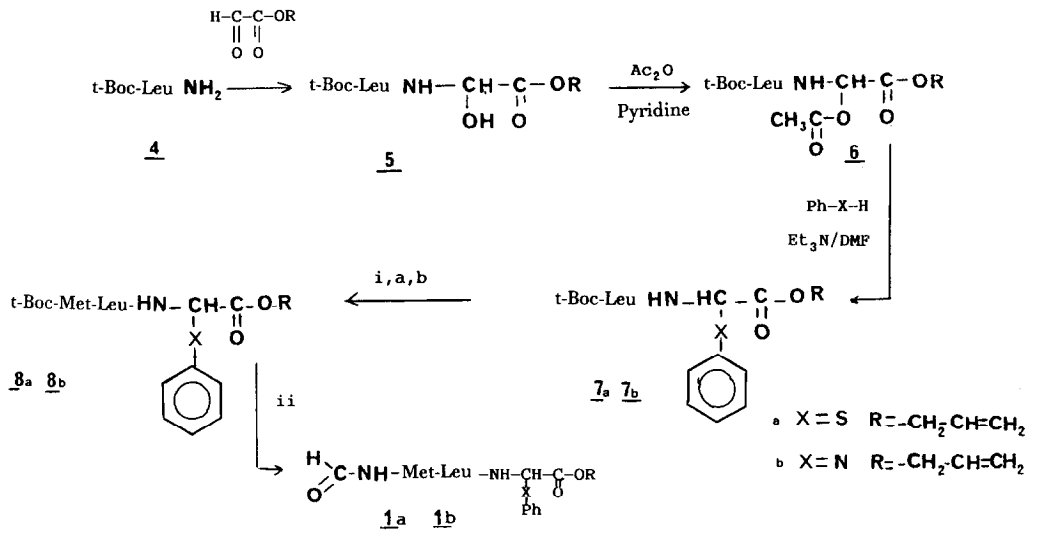
glycine intermediates **2**. Upon intracellular decomposition, the intermediates **2** release toxophoric molecules **3** (*eg* phenol, thiophenol and aniline). The presence of such molecules might be expected to modify the biological responses of neutrophils. The aim of the present work was to study the early molecular events when human neutrophils are activated by these new formylated-methionine peptides analogues. The measured induced-responses were enzyme degranulation (exocytosis) and superoxide generation. These two biological responses are usually considered to be the basic criteria for a drug to be active in inflammatory processes [16].

Chemistry

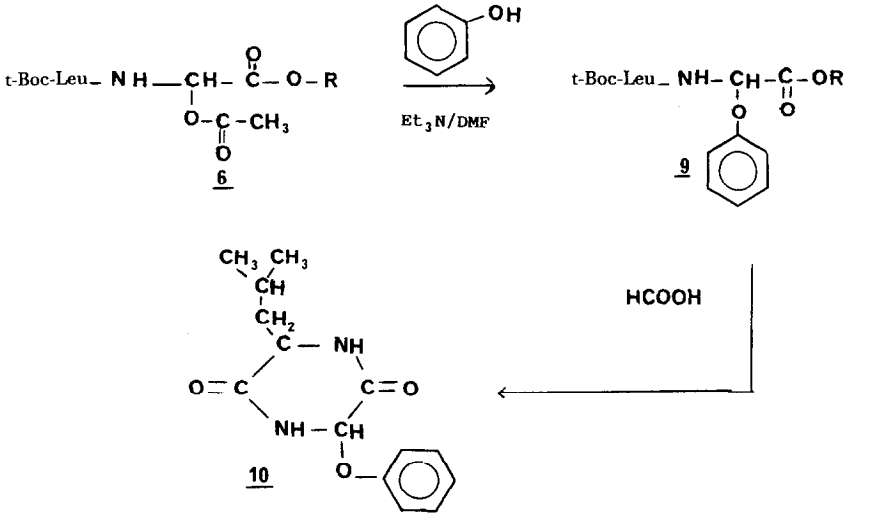
The synthesis of these new peptides requires the preparation of the key intermediates **7a** and **7b**. For this purpose we applied a strategy initiated by Kingsbury *et al* [14, 15]. Scheme 2 summarizes the synthetic route.

N-t-butoxycarbonyl-(L)-leucine amide **4** condensed with allyl glyoxylate leads to the dipeptide **5**. At this step, it should be noted that the key peptide **5** was obtained as a mixture of diastereoisomers. Attempts to separate these 2 isomers by HPLC were unsuccessful. Consequently compounds **1a**, **1b**, **8a** and **8b** were obtained as a mixture of diastereoisomers. Subsequent treatment of **5** with acetic anhydride in pyridine gives the corresponding peptide acetate **6**. Reaction of **6** with thiophenol or aniline produces respectively the *N-t*-butoxycarbonyl-(L) leucyl-2(thiophenyl)(D,L) glycine allylester **7a** and the *N-t*-butoxycarbonyl-(L) leucyl-2(anilino)(D,L) glycine allylester **7b**. After removal of the protecting groups by formic acid and performing a coupling with *N-t*-Boc-Met using conventional methods [18], the intermediates **8a** and **8b** are obtained. Formylation of the N-terminal methionyl residue is accomplished using 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) in chloroform as already reported [17], leading to the desired final compounds **1a** and **1b**. It must be emphasized that when the above procedure is used to synthesize the corresponding phenoxyglycine allyl ester analogue (X = O) the nucleophilic substitution of the acetate group by a phenoxide ion does not lead to the expected phenoxy analogue but the diketopiperazine **10**, resulting from an internal cyclization, as shown in scheme 3.

This result was somewhat surprising since this cyclization reaction does not occur with *S*-phenyl and *N*-phenyl groups, even if the reaction conditions (solvent, temperature) are changed. This result could be attributed to the electronegativity of the phenol moiety. However, a more detailed study should be undertaken in order to provide a more complete explanation (*eg* phenyl group substitution with electron withdrawing groups).



Scheme 2. (i) Reagents: a) formic acid, b) *t*-Boc-Met, c) dicyclohexylcarbodiimide (DCC), d) 1-hydroxybenzotriazole (HOBT) using DMF as solvent; (ii) deprotection of *t*-Boc protecting group with formic acid and formylation using 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) in $CHCl_3$ [17].



Scheme 3.

Results and discussion

New peptide analogue-induced granule enzyme release

When neutrophils are incubated in an appropriate medium in the presence of various peptide inducers, enzymes from the granules and lysosomes are released in the incubation medium. Using a specific assay described in the *Experimental protocols*, it is possible to measure the total activity of the enzyme release. The lysosyme-induced activities of the peptides were determined from dose–response curves as ED_{50} values.

The ED_{50} value represents the concentration causing 50% of the maximal release of lysosomal enzymes. In order to compare the results obtained, the differences in the responsiveness of the cell samples were taken into account as follows. The ED_{50} values of the standard peptides CHO-Met-Leu-PheOCH$_3$ **11**

and CHO-Met-Leu-PheOCH_2-CH=CH_2 **12** were determined and the activity of each other peptide under investigation expressed relatively to these corresponding standard peptides.

As reported in table I, it can be seen that the enzyme release induced by the peptide analogues is under the control of different structural parameters which are: i) the structure of the ester group on the terminal carboxylic residue; ii) the nature of the chemical function present on the terminal amino group; and iii) the structure of the α-substitutent group linked to the terminal glycine residue. As already reported by Belleau *et al* [19], the overall tolerance for structural modifications of the receptor binding site for the terminal carboxylic group is very substantial. This observation led us to test as standard peptides the methyl and allyl esters **11** and **12** of the corresponding tripeptide CHO-Met-Leu-PheOH.

Being 18 times more potent than the methyl ester **11**, the allyl analogue **12** was selected as the standard compound in this study. In addition, our results confirm previous observations [10] that the presence of a formyl group is required on the *N*-methionine residue in order to induce cell degranulation. Indeed, similar to the protected *N-t*-Boc-Met-Leu-Phe-*O*-allyl, the new analogues **8a** and **8b** were totally inactive. More surprising is the granule enzyme release induced by compounds **1a** and **1b**. Although their structure includes the allyl ester group and the *N*-formyl-Met terminal residue required for an optimal activity, these compounds behave as very poor degranulation inducers. The simple replacement of the methylene group of the Phe residue by S or N atoms severely affects the degranulation process. This result suggests that the lack of biological activity of compounds **8a** and **8b** can be attributed to the absence of the *N*-formyl group on the Met residue rather than to the presence of the heteroatoms N, or S. Since the receptor for formyl-Met peptides is known to be a hydrophobic entity [20], it is reasonable to assume that compounds **1a** and **1b** with a lower hydrophobicity than that of the corresponding standard compound **12** would interact less tightly with the receptor. However, the drastic difference in the biological activity of compounds **1a** and **12** may not be solely accounted for on the basis of their respective affinity for the receptor.

Table I. Effect of new peptide analogues on release of lysosomal enzymes from human neutrophils.

Compounds	R_2	X	R_1	ED_{50} *(M)* [a]	*Relative potency %* [b]
11	Formyl	CH_2	Methyl	$5.0 \pm 1.5\ 10^{-8}$	–
12	Formyl	CH_2	Allyl	$9.0 \pm 1.8\ 10^{-9}$	100
13	*t*-Boc	CH_2	Allyl	Inactive	–
8a	*t*-Boc	S	Allyl	Inactive	–
8b	*t*-Boc	N	Allyl	Inactive	–
1a	Formyl	S	Allyl	$2.4 \pm 0.6\ 10^{-6}$	0.38
1b	Formyl	N	Allyl	$5.2 \pm 1.2\ 10^{-8}$	17.5

[a] ED_{50} values represent the concentration of peptide analogues causing 50% of the maximal release of lysosomal release, as determined by concentration effect curve. Each value is the average SEM of 3 to 8 determinations; [b] CHO-Met-Leu-Phe-*O*-allyl **12** is the reference compound, used to calculate the relative potency of the other analogues.

New peptide analogue-induced superoxide generation

Superoxide radical generation by human neutrophil is known to be induced by various peptides including pepstatin and CHO-Met-Leu-PheOH [21], as well as by other compounds such as phorbol myristate acetate (PMA). In preliminary experiments, compounds **8a**, **8b**, **1a** and **1b** revealed no enhancement effect on superoxide production by human neutrophils. In contrast, the standard analogue CHO-Met-Leu-Phe-*O*-allyl **12** was found to be active in this process. Its potency is similar to that of the parent methyl ester analogue **11** indicating that the presence of the allyl ester group makes no major contribution to the intensity of the stimuli. A point that arouses concern is found in the following experiments. When the neutrophil suspensions in cuvettes are preincubated with various concentrations of analogues **8a**, **8b**, **1a** and **1b** for 5 min at 37°C, prior to the addition of the inducer PMA (2 μg/ml), superoxide production was inhibited in a dose-dependent fashion. The results are expressed as ID_{50} values corresponding to the peptide concentration causing 50% of inhibition relative to the response obtained in the absence of inhibitor. The relative inhibitory effects reported in table II are **1b** > **8b** > **1a** > **8a** > Cbz-Phe-Met-OH.

In the present study, Cbz-Phe-Met-OH (carbobenzoxy-phenylalanine-methionine), a well-known inhibitor for the binding of synthetic chemotactic peptides to the receptor [22], is used as the standard inhibitor. As shown in table II, compounds **8b** and **1b** are very potent inhibitors of neutrophil superoxide generation, the *N*-formyl analogue **1b** being 120 times more potent than the standard inhibitor Cbz-Phe-Met-OH. In contrast, the corresponding sulfur analogues **1a** and **8a** are comparatively weaker inhibitors than **1b** and **8b** but are also more potent than Cbz-Phe-Met-OH. Since enzymatically active serine proteases [23] of the cell membrane are essential for initiation and maintenance of O_2 production by human neutrophils in response to a stimulus, 2 hypotheses may be put forward to explain the observed inhibitory effect: a) either the peptide analogous **8a**, **8b**, **1a** and **1b** are inhibitors of the proteases involved in the O_2 production process; b) or these new analogues following their binding to specific receptors located

Table II. Inhibition of superoxide radical generation from human neutrophils by new peptides analogues. Standard peptide or new peptide analogues **8a**, **8b**, **1a** and **1b** or an equal volume of buffer were preincubated with human neutrophils for 5 min at 37°C before adding the inducer phorbol myristate acetate (2 μg/ml). The medium composition and the assay of superoxide radical generation are described in the *Experimental protocols*.

Compounds	R_2	X	ID_{50} *(M)* [b]	*Relative potency %* [a]
CBz-Phe-Met-OH[a]	–	–	$1.2 \pm 0.4\ 10^{-5}$	100
8a	*t*-Boc	S	$6.0 \pm 2.0\ 10^{-5}$	200
8b	*t*-Boc	N	$2.2 \pm 0.6\ 10^{-7}$	5450
1a	Formyl	S	$4.0 \pm 1.6\ 10^{-6}$	300
1b	Formyl	N	$1.3 \pm 0.3\ 10^{-7}$	12000
12	Formyl	CH_2	Inactive	–

[a]Cbz-Phe-Met-OH is the reference compound used to calculate the relative potency of the other analogues; [b]ID_{50} values correspond to the peptide concentration causing 50% of inhibition relatively to the response obtained in the absence of inhibitor. Values are means ± SE of at least 4 separate experiments. [c]This peptide is found to be an inducer of superoxide production, as potent as the corresponding methylester (CHP-Met-Leu-PheOCH$_3$).

on the cell surface membrane may behave as good substrates able to be cleaved by membrane proteases. The cleavage of these compounds leads to the expulsion of aniline or thiophenol, and these toxophoric molecules may inhibit the proteases.

In order to test the validity of such a hypothesis, we attempted to measure spectrophotometrically the release of thiophenol using Ellman's reagent. For this purpose a neutrophil suspension was incubated with the thiophenoxy peptide **1a**. However, thiophenol release was not detected. This result should be viewed with caution since the peptidase activity in human blood is normally very low [23], but can be higher with neutrophils from donors who smoke. Moreover, when various concentrations of exogenous thiophenol were incubated in neutrophil suspension no modification was found on lysosomal release or superoxide production.

From the present results, it is difficult to ascertain the mechanism by which the α-substituted glycine peptide analogues inhibit the generation of superoxide by neutrophils. Indeed inasfar as the mixture of diastereoisomers represented by compounds **1a** and **1b** has not been separated and tested as pure isomers, it is difficult to support with enough evidence any hypothesis. However, considering that the proteases involved in the O_2 production by human neutrophils resemble enzymes such as chymotrypsin [24], it would be of interest to test these optically active isomers on the chymotrypsin activity. In any case, the present results clearly demonstrate that the replacement of the benzyl group of Phe residue by a thiophenoxy or anilino group in the chemotactic peptide CHO-Met-Leu-PheOH induces drastic changes of the biological responses mediated by such a type of chemotactic peptide, in particular lysosome release and superoxide generation. The fact that compound **1b** is 10^4 more potent, *in vitro*, than the standard antagonist Cbz-Phe-Met-OH also suggests that such an antagonist may provide a new and unique approach to anti-inflammatory therapy.

Experimental protocols

Chemistry

Analytical methods

Proton magnetic spectra were recorded on either Varian XL 200 or XL 300 spectrometers. Chemical shifts are reported as δ values in parts per million downfield from internal tetramethylsilane. IR spectra were recorded on a Perkin–Elmer model 297 spectrophotometer. Mass spectra were obtained from HP5984 or LKB 9000 mass spectrometers. Melting points were determined in closed capillary tubes on a Buchi SMP-20 apparatus and are uncorrected. Analytical thin layer chromatography was carried out on aluminium-based sheets precoated with Kieselgel 60 F_{254}, 0.2 mm thick (Merck Co, Darmstadt, Germany). Column and flash chromatography were performed with Merck silica gel (230–400 mesh). Elemental analyses were performed at Guelph Laboratories Guelph, Ontario.

Reagents

Reagents grade solvents were used, unless otherwise specified. THF and diethylether were distilled from sodium. Triethylamine was purified by distillation from barium oxide. Other solvents were dried by standing over molecular sieves. All amino acids starting material used were of the L-configuration and used as supplied from the manufacturers (Aldrich, Sigma and Bachem or Chemalog) for the Boc-amino acids. 2-Ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) (Aldrich) was recrystallized from ether. 1-Hydroxybenzotriazole hydrate (HOBT) and *N,N'*-dicyclohexylcarbodiimide (DCC) were purchased from Aldrich. Allylglyoxylate was a gift from Dr B Belleau (Mc Gill University).

N-t-Butoxycarbonyl (L) leucyl-(D,L) 2-hydroxy glycine allyl ester **5**

Allylglyoxylate (1.66 g, 13 mM) was added to a stirred suspension of *N-t*-butoxy-carbonyl-leucine amide (2.18 g, 11 mM) in CH_2Cl_2 (30 ml), and the reaction mixture was stirred at room temperature until all of the starting Boc-leucine amide was reacted as indicated by TLC (silica gel; 5% CH_3OH in CH_2Cl_2). After 72 h, the reaction mixture was chilled (0–5°C) for 5 h and the resulting solid was collected, washed with a small amount of CH_2Cl_2 and purified by flash column chromatography with 2% methanol in CH_2Cl_2, leading to the desired material (1.20 g; 40%). R_f = 0.4 $CHCl_3$-CH_3OH [9.5:0.5]. ^1H NMR ($CDCl_3$) δ 0.88 (m, 6H), 1.41 (s, 9H), 1.51 (m, 2H), 4.20 (m, 1H), 4.4 (broad s, 1NH), 4.65 (dd, 2H), 4.95 (broad s, 1NH), 5.42 (m, 2H), 5.55 (m, 1H), 5.8 (m, 1H), 7.5 (1 OH); mass spectrum (70 eV) *m/e* 332 MH^+. Anal $C_{15}H_{28}N_2O_6$ (C, H, N).

N-t-Butoxycarbonyl-(L) leucyl-(D,L)-2-acetoxyglycine allyl ester ***6***
A stirred suspension of **5** (560 mg, 1.68 mM) in Ac_2O (8.5 ml) was cooled to approximately 0°C and pyridine (6 ml) was added. The solution became homogeneous and the reaction mixture was held at this temperature for 24 h and concentrated. The residue was dissolved in EtOAc and washed with H_2O (2 x 10 ml), 5% $NaHCO_3$ (3 x 8 ml) and dried ($MgSO_4$). Filtration, concentration of the filtrate gave 580 mg (92%). R_f = 0.6 $CHCl_3$-CH_3OH [9.5:0.5]. ^1H NMR $CDCl_3$ δ 0.92 (m, 6H), 1.42 (s, 9H), 1.65 (m, 2H), 2.20 (s, 3H), 4.25 (broad, NH), 4.70 (dd, 2H), 4.82 (t, 1H), 5.4 (m, 2H), 5.90 (m, 1H), 6.45 (dd, 1H), 7.5 (broad, NH); mass spectrum (70 eV) *m/e* 376 MH^+. Anal $C_{17}H_{30}N_2O_7$ (C, H, N).

N-t-Butoxycarbonyl-(L)-leucyl-2-thiophenoxy-(D,L) glycine allyl ester ***7a***
Acetate **6** (570 mg, 1.52 mM), thiophenol (168 mg, 1.52 mM), and triethylamine (155 mg, 1.52 mM) were combined in dry DMF (2 ml) and stirred for 20 h. The solvent was removed by evaporation *in vacuo*, and the residue was diluted with water (10 ml), extracted with EtOAc, dried (Na_2SO_4), and evaporated *in vacuo*. The crude product was purified by silica gel flash column chromatography with EtOAc-CH_2Cl_2 [1:1] mixture; yield 585 mg (90%). R_f = 0.3 CH_2Cl_2. ^1H NMR ($CDCl_3$) δ 0.94 (m, 6H), 1.40 (s, 9H), 1.52 (m, broad, 2H), 4.15 (m, 1H), 4.60 (dd, 2H), 4.75 (m, 1H), 5.42 (m, 2H), 5.85 (m, 1H), 6.9 (broad, 1NH), 7.25–7.6 (5H aromatics); mass spectrum (70 eV) *m/e* 437 MH^+. Anal $C_{22}H_{32}N_2O_5S$ (C, H, N).

N-t-Butoxycarbonyl-(L)-methionyl-(L)-leucyl-2-thiophenoxy-(D,L) glycine allyl ester ***8a***
7a (580 mg, 1.33 mM) was dissolved in formic acid 98% (3 ml) and stirred at room temperature for 2 h. Excess acid was removed *in vacuo* at 30°C. The resulting formate salt was then neutralized by the addition of $NaHCO_3$ 5% (10 ml) and extracted with CH_2Cl_2 (2 x 15 ml) and dried ($MgSO_4$).
To a solution of *t*-Boc-methionine (331 mg, 1.33 mM) and 5-hydroxybenzotriazole (HOBT) (198 mg, 1.47 mM) and DMF (3 ml) at 0°C was added dicyclohexylcarbodimide (DCC) (289 mg, 1.40 mM) and the mixture stirred at 0°C for 1 h. After this time, the solution of deprotected peptide previously cooled at 0°C was added and the resulting mixture stirred at 0°C for 1 h, and at room temperature for 15 h. The solvents were evaporated *in vacuo* and the residue suspended in EtOAc and filtered. The filtrate was washed with 5% citric acid solution (10 ml), and 5% $NaHCO_3$ solution (2 x 8 ml). After drying ($MgSO_4$) and evaporation, the resulting solid was purified by silica gel flash column chromatography with 9/1 CH_2Cl_2-EtOAc: yield 602 mg (81%). R_f = 0.30 CH_2Cl_2-EtOAc [9:1], mp = 88–89°C. ^1H NMR ($CDCl_3$) δ 0.88 (m, 6H), 1.45 (s, 9H), 1.52–1.70 (m, 3H), 1.83–2.07 (m, 2H), 2.09 (s, 3H), 2.54 (t, 2H), 4.20 (m, 1H), 4.38 (m, 1H), 4.6 (m, 2H), 5.20 (m, 1H), 5.35 (m, 2H), 5.70 (m, 1NH), 5.8 (m, 1H), 6.55 (m, 1H), 6.85 (m, 1NH), 7.25–7.55 (m, 5H aromatics). Mass spectrum (70 eV) *m/e* 568 MH^+. Anal $C_{27}H_{41}N_3O_6S_2$ (C, H, N).

N-t-Butoxycarbonyl-(L)-leucyl-2-phenylamino-(D,L) glycine allyl ester ***7b***
Acetate **6** (400 mg, 1.08 mM), aniline (101 mg, 1.08 mM) and Et_3N (110 mg, 1.08 mM) were combined in dry DMF (2 ml) and stirred for 24 h. The solvent was removed by evaporation *in vacuo*, and the residue was diluted with water (8 ml), extracted with EtOAc (15 ml) and dried ($MgSO_4$). The crude product was purified by silica gel flash column chromatography with CH_2Cl_2-EtOAc mixture [2:1]: yield 322 mg (71%). R_f = 0.6 CH_2Cl_2-EtOAc [8:2]. ^1H NMR ($CDCl_3$) δ 0.90 (m, 6H), 1.42 (s, 9H), 1.52–1.70 (m, 2H + 1H), 4.10 (m, 1H), 4.65 (dd, 2H), 4.72 (broad, 1H), 5.32 (m, 2H), 5.8–6.0 (m, 1H + 1NH), 6.66–6.7 (m, 1NH), 6.8 and 7.1–7.4 (m, 5H aromatics). Mass spectrum (70 eV) *m/e* 420 MH^+. Anal $C_{22}H_{33}N_3O_5$ (C, H, N).

N-t-Butoxycarbonyl-(L)-methionyl-(L)-leucyl-2-phenylamino-(D,L) glycine allyl ester ***8b***
Compound **7b** (315 mg, 0.75 mM) was dissolved in formic acid 98% (2 ml) and stirred at room temperature for 2 h. Excess acid was removed *in vacuo* at 30°C. The resulting formate salt was then neutralized by the addition of $NaHCO_3$ 5% (8 ml) and extracted with CH_2Cl_2 (2 x 10 ml) and dried ($MgSO_4$). To a solution of *t*-Boc-methionine (188 mg, 0.75 mM) and HOBT (112 mg, 0.83 mM) and DMF (2 ml) at 0°C was added dicyclohexylcarbodimide (DCC) (171.2 mg, 0.83 mM) and the mixture stirred at 0°C for 1 h. After this time, the solution of deprotected peptide, previously cooled at 0°C was added and the resulting mixture stirred at 0°C for 1 h, and at room temperature for 15 h. The solvents were evaporated *in vacuo* and the residue suspended in EtOAc and filtered. The filtrate was washed with 5% citric acid solution (10 ml) and 5% $NaHCO_3$ solution (2 x 8 ml). After drying ($MgSO_4$) and evaporation, the resulting solid was purified by silica gel flash column chromatography with CH_2Cl_2-MeOH [8:1], yield 210 mg (41%). R_f: 0.39 CH_2Cl_2-CH_3OH [8:1], yield 210 mg (41%). R_f = 0.39 CH_2Cl_2-CH_3OH [8:1] mp = 93–94°C. ^1H NMR ($CDCl_3$) δ 0.92 (m, 6H), 1.48 (s, 9H), 1.52–1.70 (m, 3H), 1.83–2.09 (m, 2H), 2.15 (s, 3H), 2.56 (t, 2H), 4.20 (m, 1H), 4.50 (m, 1H), 4.67 (m, 2H), 5.35 (m, 1H), 5.85 (m, NH), 6.0 (m, 1H), 6.72 (m, 1NH), 7.08 (m, 1NH), 6.5–7.4 (m, 5H aromatics). Mass spectrum (70 eV) 550 MH^+. Anal $C_{27}H_{42}N_4O_6S$ (C, H, N).

N-Formyl-(L)-methionyl-(L)-leucyl-2-thiophenoxy-(D,L) glycine allyl ester ***1a***
Compound **8a** (322 mg, 0.568 mM) was dissolved in formic acid 98% (5 ml) for 3 h. Evaporation of formic acid *in vacuo* gave an oil which was dissolved in $CHCl_3$ (15 ml). EEDQ (351 mg, 1.42 mM) was then added. The reaction mixture was stirred at room temperature for 4 h. The solvent was evaporated *in vacuo*. The residue was dissolved in EtOAc (40 ml) washed with 5% citric acid solution (2 x 15 ml) and 5% $NaHCO_3$ solution (2 x 15 ml). The organic layer was dried ($MgSO_4$) and filtered. Evaporation of solvent gave a crude solid, which was purified by silica gel flash column chromatography with pure EtOAC. Yield 252 mg (90%) R_f = 0.15 CH_2Cl_2-EtOAc [4:1]. mp = 108–109°C. ^1H NMR ($CDCl_3$) δ: 0.93 (m, 6H), 1.57 (m, 4H), 2.05 (m, 2H), 2.10 (s, 3H), 2.52 (m, 2H), 4.48 (m, 1H), 4.60 (m, 1H), 4.62 (m, 2H), 4.72 (m, 1H), 5.32 (m, 2H), 5.72 (m, 1NH), 5.85 (M, 1H), 6.65 (m, 1NH), 6.78 (m, 1NH), 7.3–7.5 (m, 5H aromatics), 8.24 (d, 1H). Mass spectrum (70 eV) *m/e* 496 MH^+. Anal $C_{23}H_{33}N_3O_5S_2$ (C, H, N).

N-Formyl-(L)-methionyl-(L)-leucyl-2-phenylamino-(D,L) glycine allyl ester ***1b***
Compound **8b** (57 mg, 0.103 mM) was dissolved in formic acid 98% (1 ml) for 3 h. Evaporation of formic acid *in vacuo* gave an oil which was dissolved in $CHCl_3$ (5 ml). EEDQ (48 mg, 0.19 mM) was then added. The reaction mixture was stirred at room temperature for 4 h. The solvent was evaporated *in vacuo*. The residue was dissolved in EtOAc (20 ml), washed with 5% citric acid solution (2 x 8 ml) and 5% $NaHCO_3$ solution (2 x 7 ml). The organic layer was dried ($MgSO_4$) and filtered. Evaporation of solvent gave a crude solid, which was purified by silica gel flash column chromatography with

CH_2Cl_2-CH_3OH [9:1], yield 18 mg (50%). R_f = 0.13 CH_2Cl_2-CH_3OH [9:1], mp = 78–79°C, ^1H NMR ($CDCl_3$) δ 0.95 (m, 6H), 1.65–12.78 (m, 4H), 2.05 (m, 2H), 2.08 (s, 3H), 2.54 (m, 2H), 4.47 (m, 1H), 4.62 (m, 1H), 4.70 (m, 2H), 5.02 (m, 1H), 5.25 (m, 2H), 5.85 (m, 1H), 6.12 (m, 1NH), 7.2–7.62 (m, 5H aromatics), 7.72 (m, 1NH), 8.20 (m, 1NH), 8.42 (m, 1H). Mass spectrum (70 eV) *m/e* 479 MH^+. Anal $C_{23}H_{34}N_4O_5S$ (C, H, N).

N-t-Butoxycarbonyl-(L)-leucyl-2-phenoxy-(D,L) glycine allyl ester ***9***

Acetate **6** (355 mg, 0.95 mM), phenol (90 mg, 0.95 mM) and triethylamine (96 mg, 0.95 mM) were combined in dry DMF (2 ml) and stirred for 20 h. The solvent was removed by evaporation *in vacuo*, and the residue was diluted with water (8 ml), extracted with EtOAc (15 ml) and dried ($MgSO_4$). After evaporation of the solvent, the crude product was purified by silica gel flash column chromatography with CH_2Cl_2-EtOAc [9:1] mixture: yield 120 mg (40%). R_f = 0.62, CH_2Cl_2-EtOAc [9:1]. ^1H NMR ($CDCl_3$) δ 0.95 (m, 6H), 1.42 (s, 9H), 1.65 (m, 2H), 4.2 (m, 1H), 4.75 (dd, 2H), 4.80 (m, 1H), 5.4 (m, 2H), 5.85 (m, 1H), 6.40 (m, 1NH), 7.1–7.4 (m, 5H aromatics). Mass spectrum (70 eV) *m/e* 409 MH^+. Anal $C_{21}H_{32}N_2O_6$ (C, H, N).

3-Isobutyl-6-phenoxy-2,5-di-oxo piperazine ***10***

Compound **9** (105 mg, 0.25 mM) was dissolved in formic acid 98% (2 ml) and stirred at room temperature for 2 h. Excess acid was removed *in vacuo* at 30°C. The resulting formate salt is then neutralized by the addition of $NaHCO_3$ 5% (8 ml) and extracted with CH_2Cl_2 (2 x 10 ml) and dried ($MgSO_4$). Yield 40 mg (65%). R_f = 0.1 CH_2Cl_2-EtOAc [7:2], mp = 209°C ^1H NMR ($CDCl_3$) δ 0.98 (m, 6H), 1.8 (m, 3H), 4.00 (m, 1H), 5.49 (d, 1H), 7.2–7.35 (m, 5H aromatics), 7.4 (s, 1NH), 7.67 (s, 1NH). Mass spectrum (70 eV) 251 MH^+. Anal $C_{13}H_{18}N_2O_3$ (C, H, N).

Bioassay methods

Lysosyme release assay

Blood from healthy human donors was drawn by venipuncture into 10-ml vacutainer tubes with 143 USP units sodium heparin and was used within 2 h of collection. The blood in 3–3.5 ml aliquots was layered over 3-ml of mono-poly resolving medium, in 16 x 102 mm posy-allomer tubes. The tubes were centrifuged at 300 *g* for 30 min in a swinging bucket rotor at room temperature. From this point on, all manipulations were carried out at 4°C unless specified otherwise. The polymorphonuclear (PNM) layer was drawn off with a Pasteur pipette and washed once in Gey's solution with 0.2% bovine serum albumin (BSA). This was centrifuged at 275 *g* for 10 min and resuspended for 30 min in erythrocyte lysing solution (8.29 g/l NH_4Cl, 0.37 g/l Na_2EDTA, 1 g/l $KHCO_3$), centrifuged for 10 min, and washed twice with Gey's BSA. The cells were resuspended with Gey's BSA to 10^7/ml and used within 1 h. The cell suspension was preincubated with cytochalasin B (5 μg/ml) at 37°C for 5 min. Reaction mixtures contained 100 μl of the treated cell suspension and 100 μl of the appropriate compounds, in sterile 5-ml polypropylene tubes. Controls were run daily containing treated cells and Gey's BSA. Total lysozyme release was obtained by incubating treated cells with 1% Triton X-100 in 0.067 M phosphate buffer pH 6.25. Lysozyme was measured in supernatants of reaction mixtures after 15 min of incubation at 37°C. The assays for lysozyme were conducted at room temperature and all runs were performed 3 times in duplicate. The substrate used was freeze-dried *Micrococcus lysodeikticus*, which was diluted in 0.067 M phosphate buffer pH 6.25 (10 mg/100 ml) to give an absorbance at 450 nm between 0.6 and 0.7 OD units, against a buffer reference. The solution was discarded after use. The reaction mixture consisted of: 0.9 ml of substrate, 0.05 ml 1% Triton X-100, and 0.05 ml enzyme supernatant or standard. In a 1.5-ml quartz cuvette with a light path of 1 cm. The cuvette was stoppered and inverted 2–3 times and the change in absorbance was read immediately for 3 min. Three concentrations of egg white lyzosyme standard solution were run daily to ensure that the standard curve did not vary greatly from day to day. The lysozyme-inducing activity for each peptide was obtained from the dose–response curve as its ED_{50}, the molar concentration of peptide causing 50% of the maximal release of lysozyme. In order to correct for changes in the responsiveness of different cells, the dose response curve of the standard peptide f-Met-Leu-PheOH (Sigma Chem Co) was also measured so that the activity or potency relative to the standard could also be calculated.

Inhibition of superoxide O_2^- production by intact human neutrophils

The purification of neutrophils from freshly drawn whole blood involves 3 basic steps: dextran sedimentation, lysis of contaminating red blood cells, and Ficoll-hypaque density gradient centrifugation to separate neutrophils from platelets and mononuclear cells. This purification was performed according to a standard procedure described in [25].

O_2^- was assayed by the reduction of ferricytochrome *c*. A volume of 0.7 ml of neutrophil suspension (1–5 10^6 cells/ml) was placed in each of 2 siliconized test tubes. 10 μl superoxide dismutase (3 mg/ml bovine erythrocyte) was added to one of the test tubes, and 10 μl of water to the other. After 2 min incubation at 37°C, 0.05 ml cytochrome *c* (horse heart cytochrome *c*, Sigma type III) followed directly by 0.75 ml of phorbol myristate acetate (2 μl/ml). After 15 min incubation at 37°C, the reaction was stopped by placing the tubes in melting ice, and the cells were removed by centrifugation at 1 500 *g* for 5 min at 4°C. Finally, cytochrome *c* reduction was measured in a double beam spectrophotometer (Kontron 940) by scanning between 530 and 570 nm, using the dismutase-free supernatant as the sample and the dismutase-containing supernatant as the reference.

The assay to measure the peptide inhibition effect involved the preincubation of various inhibitor concentrations for 5 min before adding, cytochrome *c* and the stimulating factor phorbol myristate. The superoxide generation inhibition was estimated from conventional dose–response curves, and the results expressed as ID_{50} values (50% of maximum superoxide O_2^- production).

Any changes in the responsiveness were corrected by constructing dose–response curves from parallel experiments using Cbz-Phe-MetOH as the appropriate standard inhibitor so that the relative analogue potencies were all quantitatively meaningful.

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