Development of Highly Potent and Selective Phosphinic Peptide Inhibitors of Zinc Endopeptidase 24-15 Using Combinatorial Chemistry*

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Jiří Jiráček‡, Athanasios Yiotakis§, Bruno Vincenti, Alain Lecq§, Anna Nicolaou, Frédéric Chelcret*, and Vincent Diveči

From the Département d’Ingénierie et d’Études des Protéines, DSV, CE-Saclay, 91191 Gif/Yvette Cedex, France, the Department of Organic Chemistry, Laboratory of Organic Chemistry, University of Athens, Panepistimiopolis, Zografou, Athens 15771, Greece, and the Institute de Pharmacologie Moléculaire et Cellulaire, Université de Nice Sophia Antipolis, Sophia Antipolis 06560 Valbonne, France

Several hundred phosphinic peptides having the general formula Z-(L,D)Phe(PO2CH2)(L,D)Xaa-Yaa-Zaa, where Xaa = Gly or Ala and Yaa and Zaa represent 20 different amino acids, have been synthesized by the combinatorial chemistry approach. Peptide mixtures or individual peptides were evaluated for their ability to inhibit the rat brain zinc endopeptidases 24-15 and 24-16. Numerous phosphinic peptides of this series act as potent (Ki in the nanomolar range) mixed inhibitors of these two peptidases. However, our systematic and comparative strategy led us to delineate the residues located in P_2 and P_3 positions of the inhibitors that are preferred by these two peptidases. Thus, endopeptidase 24-15 exhibits a marked preference for inhibitors containing a basic residue (Arg or Lys) in the P_2 position, while 24-16 prefers a proline in this position. The P_2 position has less influence on the inhibitory potency and selectivity, both peptidases preferring a hydrophobic residue at this position. On the basis of these observations, we have prepared highly potent and selective inhibitors of endopeptidase 24-15. The Z-(L,D)Phe(PO2CH2)(L,D)Ala-Arg-Met compound (mixture of the four diastereoisomers) displays a Ki value of 70 pm for endopeptidase 24-15. The most selective inhibitor of endopeptidase 24-15 in this series, Z-(L,D)Phe(PO2CH2)(L,D)Ala-Arg-Phe, exhibits a Ki value of 0.160 nm and is more than 3 orders of magnitude less potent toward endopeptidase 24-16 (Ki = 530 nm). Furthermore, at 1 μM this selective inhibitor is unable to affect the activity of several other zinc peptidases, namely endopeptidase 24-11, angiotensin-converting enzyme, aminopeptidase M, leucine aminopeptidase, and carboxypeptidases A and B. Therefore, Z-(L,D)Phe(PO2CH2)(L,D)Ala-Arg-Phe can be considered as the most potent and specific inhibitor of endopeptidase 24-15 developed to date. This new inhibitor should be useful in assessing the contribution of this proteolytic activity in the physiological inactivation of neuropeptides known to be hydrolyzed, at least in vitro, by endopeptidase 24-15. Our study also demonstrates that the combinatorial chemistry approach leading to the development of phosphinic peptide libraries is a powerful strategy for discovering highly potent and selective inhibitors of zinc metalloproteases and should find a broader application in studies of this important class of enzymes.

The endopeptidase 24-15 (EC 3.4.24.15) belongs to the zinc metallopeptidase family (1) and resembles a peptidase previously purified from rabbit brain by Camargo et al. (2). Later, endopeptidase 24-15 was named thimet oligopeptidase with respect to the thiol and metal dependence of its catalytic activity (3–5). Molecular cloning of the cDNA of endopeptidase 24-15 revealed a HEXXXH motif, which characterizes peptidases belonging to this family, and a cysteine residue, lying on the C-terminal side of the second histidine of the zinc binding motif (6). This cysteine residue has been proposed to be responsible for activation of the enzyme by 2-mercaptoethanol or dithiothreitol, as well as for inhibition of the enzyme activity by thiol reactive reagents (6).

24-15 displays several biochemical and physicochemical properties (for a review, see Ref. 7) in common with another zinc-containing metallopeptidase, endopeptidase 24-16 (EC 3.4.24.16) (8). Interestingly, these two peptidases have the ability to hydrolyze numerous bioactive or synthetic peptides at the same cleavage site, suggesting that they have a closely related active site (7–9).

We previously reported that phosphodiepryl03, a phosphonamide peptide, acts as a potent mixed inhibitor of 24-15 and 24-16, with K values in the nanomolar range (10). This inhibitor was shown to be unable to block the activity of several other zinc peptidases such as endopeptidase 24-11, angiotensin-converting enzyme, aminopeptidase M, leucine aminopeptidase, and carboxypeptidases A and B. We particularly studied the effect of this inhibitor in vivo on the neuropeptide catabolism, since we previously established that 24-15 and 24-16 participated in vitro in the inactivation of this neuropeptide (11, 12). We established that phosphodiepryl03 prevented neuropeptide degradation in vivo in vascularly perfused dog ileum (13). More recently, several phosphodiepryl03 analogues were also proved to be potent but still acting as mixed inhibitors of

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§ To whom correspondence should be addressed. Tel.: 33-1-69083585; Fax: 33-1-60089071.

1 The abbreviations used are: 24-15, thimet oligopeptidase (EC 3.4.24.15); 24-16, neuropepsin (EC 3.4.24.16); 24-11, nephrypsin (EC 3.4.24.11); S– indicates that the peptide bond has been modified, and the formula of the group that has replaced this peptide bond is in parentheses; CPP-AAF-pAB, N-(1-[R,S]-carboxy-3-phenylpropyl)-Ala-Ala-Phe-p-aminobenzoyl; Z, benzoyloxycarbonyl; Fmoc, N-(9-fluorenyle)methoxycarbonyl; Mcc, 7-methoxycoumarin-3-carboxyl; Lys(Dnp), N°-(2,4-dinitrophenyl)-lysine; Tbu, tertiary-butyl; Boc, t-butoxycarbonyl; Trt, triphenylmethyl; Pmc, 2,2,5,7,8-pentamethyldi-hexyl-6-sulfonyl; Ad, adamantyl; HPLC, high performance liquid chromatography; Nle, N-aminoisocapric acid.
24-15 and 24-16 (14). The most potent analogue of this series drastically potentiated neurotensin-induced antinociceptive effects in hot-plate-tested mice after i.c.v. administration and enhanced the neurotensin-induced contraction of isolated longitudinal smooth muscle from guinea pig ileum (14). However, the delineation of the respective contribution of these enzymes in the neurotensin degradation will depend on the development of highly selective inhibitors able to discriminate between 24-15 and 24-16. To this end, a systematic approach was thus devised to find such potent and selective inhibitors. Peptides containing a phosphinic bond (PO2CH2) instead of a phosphoamide bond (PO2NH) were selected because the former are more chemically stable than the latter. Furthermore, with bacterial collagenase, a zinc metalloprotease, we recently demonstrated that the phosphinic peptide inhibitors have nearly the same potency as the corresponding parent phosphonamide peptide inhibitors (15).

In this paper, we demonstrate that the synthesis, by combinatorial chemistry, of several hundred different phosphinic peptides having the general formula Z-L,L,Phe(PO2CH2)_nXaa-Yaa-Zaa has led to the discovery of both highly potent and selective inhibitors of 24-15.

### MATERIALS AND METHODS

**Diisopropyl fluorophosphatetreated carboxypeptidases A and B, leucine aminopeptidase, and angiotensin-converting enzyme were from Sigma. Endopeptidase 24.11 was purified and kindly provided by Drs. P. Crine and G. Boileau (Département de Biochimie, Université de Montréal, Canada). All the Fmoc-amino acid derivatives, the Mcc-Pro-Leu-Gly-Pro(Lys(Dnp)) synthetic substrate and the 2-chlorotrityl resin were from Novabiochem.**

**Purification of 24-15 and 24-16**

The rat brain endopeptidases 24-15 and 24-16 were purified as previously described (8, 16).

**Enzyme Assays and Inhibition Studies**

24-15 and 24-16 Assays—Unless otherwise noted, all assays were performed at 25°C in 5 mM tricine/NaOH buffer, pH 7.5, containing 0.1 mM dithiothreitol. Under these conditions, the enzymes retain their activity for 24 h. Endopeptidase activities were assayed with Mcc-Pro-Leu-Gly-Pro(Lys(Dnp)) substrate as previously described (17, 18). Continuous assays were performed by recording the hydrolysis of this quenched substrate with a fluorimeter, setting excitation and emission wavelengths at 347 and 405 nm, respectively. In typical experiments, a cuvette containing 0.9 ml of buffer, 9 μM of substrate was brought to thermal equilibrium in a jacketed holder in a constant temperature water bath and then placed in a Gilson system equipped with a variable wavelength detector. Peptide purities were checked by TLC, analytical HPLC (Vydac 218TP104 column), and mass spectrometry.

**RESULTS**

We previously reported the ability of a phosphonamide peptide, phenylglyoxylid-PO2NH-Gly-Pro-Nle (phosphodieryl03), to act as a potent mixed inhibitor of both 24-16 and 24-15 (10). The structure of this inhibitor, which is thought to encompass the S2 to S3 subsites of the catalytic site of these enzymes, was therefore used as the starting point of a strategy aimed at developing fully specific blockers of 24-15. Preliminary experiments examined the putative influence of 1) substitution of the phosphonamide surrogate by a phosphinic group; 2) stereochemical modifications introduced at the P1, P2, and P3 positions.

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The influence of the P3 different amino acids, was performed. Each peptide was purified by checking systematically the role played by the terminal carboxylate (compounds 2 and 6). Based on these preliminary observations, the search for new compounds was undertaken by synthesizing several phosphinic peptides of the general formula Z-(L,D)Phe(PO2CH2)Gly-Yaa-Zaa9. The preference for 24-15 and 24-16 of the inhibitors toward both peptidases is shown in Fig. 3. Two inhibitors, containing a phenylalanine or a methionine in the P3 position, are identified as rather selective compounds for 24-15, with selectivity factors of 410 and 380, respectively. From this diagram, it appears that aromatic, hydrophobic, and basic side chains in the P3 positions also confer some selectivity for 24-15 to the phosphonic peptide, while the presence of a small, acidic amino acid or proline residue reduces the inhibitor selectivity. Furthermore, from Table II, it can be seen that the two selective phosphonic peptides, with a Phe or Met residue in the P3 position, are also potent inhibitors of 24-15. From the potency point of view, 24-15 seems to prefer a linear hydrophobic side chain, with a clear preference for the Met residue in the P3 position of the inhibitors.

Influence of the P2 Positions in Phosphinic Peptide Mixtures

Having the General Formula Z-(L,D)Phe(PO2CH2)Gly-Yaa-Zaa9—Previous inhibitor studies (25), as well as recent results in our laboratory (14), have shown that an alanine residue in the P1 position of the inhibitor slightly increases the potency of the inhibitors, as compared to the compounds containing a glycine in this position. Accordingly, phosphinic peptide mixtures containing an alanine in the P2 position were synthesized to investigate the effect of this residue both on inhibitor potency and selectivity. Based on the results observed in this study, four peptide mixtures containing, respectively, a Pro, Lys, Arg, or Nle residue in the P2 position were prepared. The IC50 of these four mixtures toward endopeptidases 24-15 and 24-16, as well as their selectivity factors for these peptidases, are reported in Table III. The presence of a Pro or Nle residue in the P2 position does not lead to selective inhibitors, while a basic residue in the same position provides potent and selective inhibitors. Remarkably, as compared to the results reported in Fig. 2, with an Ala in the P2 position, the inhibitor mixture containing an Arg in the P2 position appears more selective than that one with a lysine in this position. This striking observation led us to synthesize several phosphonic peptides containing an alanine in the P2 position and a Lys or Arg residue in the P3 position. The data in Table IV confirm that the presence of arginine, as compared with lysine, influences the selectivity of the inhibitors and provides with Z-(L,D)Phe(PO2CH2)Gly-Lys-Arg-Phe a compound that is 3300 times more potent toward 24-15 than toward 24-16. As reported for the phosphonamide peptides (10, 13), it is worth noting that this phosphonic peptide inhibitor at 1 μM is unable to block the activity of several zinc peptidases, like endopeptidase 24-11, angiotensin-converting enzyme, aminopeptidase M, leucine aminopeptidase, and carboxypeptidases A and B (data not shown). Comparison of the results in Fig. 2 and Table IV highlights the role played by the residue in the P3 position in the inhibitor selectivity. However, either with a Gly or Ala

![Diagram showing the influence of amino acids on inhibitor potency and selectivity.](image)

**Table I**

| Inhibitors         | K_{24-15} | K_{24-16} | Selectivity |
|--------------------|-----------|-----------|-------------|
| Z-L,D-Phe(PO2NH)Gly-Pro-Nle (1) | 3 | 5 | 1.7 |
| Z-L,D-Phe(PO2CH2)Gly-Pro-Nle (2) | 10 | 12 | 1.2 |
| Z-L,D-Phe(PO2CH2)Gly-Pro-Nle (3) | 300 | 320 | 1.6 |
| Z-L,D-Phe(PO2CH2)Gly-Pro-Nle (4) | 3400 | 7700 | 2.3 |
| Z-L,D-Phe(PO2CH2)Gly-Pro-Nle (5) | 4800 | 2900 | 0.6 |
| Z-L,D-Phe(PO2CH2)Gly-Pro-Nle-OMe (6) | 830 | 750 | 0.9 |

1 Peptide mixtures are prepared by combining twenty different Zaa9-R samples, where Zaa9 represented all the natural amino acids (Lys is replaced by Nle). The Zaa9 concentration is the same in each sample.

2 Coupling of a unique amino acid to each portion

3 Coupling of the phosphonic dipeptide

4 Determination of the potency of each sample

![Preparation of phosphinic peptide mixtures of general formula Z-(L,D)Phe(PO2CH2)Gly-Yaa-Zaa9 by combinatorial chemistry.](image)
residue in the P$_1$ position, it can be seen that the preference of 24-15 in the P$_3$ position is still for methionine.

**DISCUSSION**

The development of phosphinic peptide chemistry by solid phase synthesis, in conjunction with the combinatorial chemistry approach (26, 27), makes it possible to prepare rapidly a huge number of different phosphinic peptides. All these phosphinic peptides, as good analogues of the substrates of zinc metalloproteases in the transition state, are expected to be potent inhibitors of this enzyme family. In fact, as demonstrated in this study, but also in previous work (15), phosphinic peptides provided that they contain the right amino acid sequence, which ensures an optimal recognition of these inhibitors by the target zinc metalloprotease, are highly potent inhibitors of this class of proteases. In this work, we have identified a phosphinic sample (Z$_{(L,D)}$Phe$_{(PO_2CH_2)}$Ala-Arg-Met, a mixture of four distereoisomers) displaying a K$_i$ value of 70 pM. Thus, the phosphinic peptides of this sample are by far much more potent inhibitors of 24-15 than the previously reported carboxyalkyl (25, 28) or hydroxamate (29) peptide inhibitors, all exhibiting K$_i$ values in the range of 10–100 nM.

The other important aspect of the approach is the possibility of optimizing the inhibitor selectivity by screening the peptide mixtures with different proteases. The power of the approach is well illustrated in this work by the recognition of the clear preference of 24-15 for an arginine or lysine residue in the P$_2$ position of the inhibitor, two residues that are much less tolerated at the same position by 24-16. 24-16 prefers a proline residue in this position than a basic one. Interestingly, the proline residue is also well accommodated by 24-15. This result may explain why, for a long time, it has been stated that 24-15 has a preference for proline in the P$_2$ position (28). This lack of selectivity, displayed by the two peptidases toward the recognition of peptide inhibitor with proline in the P$_2$ position,
Inhibition constants of phosphinic peptides having the general formula $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)Gly-Arg-Zaa for endopeptidases 24–15 and 24–16

| Inhibitors –P$_1$– | P$_1$ –P$_2$ – P$_3$ – | $K_{i,j}^{24-15}$ | $K_{i,j}^{24-16}$ |
|-----------------|-----------------|----------------|----------------|
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)Gly-Arg-Zaa | 0.35 | 135 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)Gly-Arg-Met | 1.6 | 215 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)Gly-Arg-Nle | 2.2 | 200 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)Gly-Arg-Ala | 2.7 | 1300 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)Gly-Arg-Tyr | 3.6 | 600 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)Gly-Arg-Leu | 5.0 | 845 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)Gly-Arg-Gln | 6.0 | 680 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)Gly-Arg-Val | 9.5 | 1150 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)Gly-Arg-Ile | 9.5 | 580 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)Gly-Arg-Trp | 13 | 2650 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)Gly-Arg-Gly | 14 | 1700 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)Gly-Arg-Arg | 14 | 2050 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)Gly-Arg-Asn | 20 | 1450 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)Gly-Arg-His | 23 | 2950 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)Gly-Arg-Ser | 29 | 2500 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)Gly-Arg-Thr | 35 | 3600 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)Gly-Arg-Lys | 140 | 2600 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)Gly-Arg-Glu | 160 | 8000 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)Gly-Arg-Asp | 350 | 20300 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)Gly-Arg-Pro | 3500 | 35000 |

Potency and selectivity of phosphinic peptides having the general formula $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)$_2$Gly-Arg-Zaa towards the endopeptidases 24–15 and 24–16, when the Yaa’ position is occupied by a lysine or an arginine

| Inhibitors –P$_1$– | P$_1$ –P$_2$ – P$_3$ – | $K_{i,j}^{24-15}$ | $K_{i,j}^{24-16}$ |
|-----------------|-----------------|----------------|----------------|
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)$_2$Gly-Arg-Zaa | 0.12 | 230 | 1920 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)$_2$Gly-Arg-Met | 0.63 | 165 | 260 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)$_2$Gly-Arg-Nle | 0.85 | 440 | 520 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)$_2$Gly-Arg-Leu | 1.0 | 1650 | 1650 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)$_2$Gly-Arg-Gln | 1.8 | 900 | 500 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)$_2$Gly-Arg-Val | 2.3 | 1150 | 500 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)$_2$Gly-Arg-Ile | 0.07 | 93 | 1330 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)$_2$Gly-Arg-Arg | 0.10 | 100 | 1000 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)$_2$Gly-Arg-Asn | 0.15 | 88 | 590 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)$_2$Gly-Arg-His | 0.16 | 530 | 3300 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)$_2$Gly-Arg-Ser | 0.45 | 840 | 1870 |
| $Z_{i,j}^\alpha$Phe(PO$_2$CH$_2$)$_2$Gly-Arg-Trp | 0.45 | 550 | 1220 |

Inhibition of 24-15 by the Cpp-AAF-pAB inhibitor triggers an increased recovery of lutecinizing hormone-releasing hormone in vivo (31) and potentiated the lutecinizing hormone-releasing hormone-induced release of plasmatic lutecinizing hormone and follicle-stimulating hormone (32). Subsequent studies reported the increased antidepressive properties of dynorphin A$_{1-8}$ and leucine enkephalin-Gly-Gly-Leu after administration of this inhibitor (33). Finally, intravenous infusion of Cpp-AAF-pAB rapidly slowed down the arterial pressure of normotensive rats (34), indicating that 24-15 could play a role in the control of the pressor response in mammals. However, these data are still controversial since it was recently reported that Cpp-AAF-pAB could undergo proteolytic cleavage (35–37). This produces a catabolite that potently inhibits angiotensin-converting enzyme, whose role in blood pressure response has been well documented. Therefore, the potent and selective 24-15 inhibitors reported here represent novel tools for reexamining the real contribution of this enzyme in the control of the above physiological processes. These molecules should also help us to establish the relative contribution of 24-15 and 24-16 in vivo in the neurotensin degradation (13, 14).

This new series of inhibitors might also constitute valuable probes for evaluating the degree of similarity between 24-15 and the family of proteins that exhibit a high percentage of sequence identity with the 24-15 (38, 39). In this connection, it will be of interest to determine the ability of the present inhibitors to block the peptidase activity of porcine-soluble angioten-
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...binding protein, a protein which was recently shown to share a 65% sequence identity with porcine 24-15 (40). To our knowledge, we study the first example of the development of phosphinic peptide libraries and their successful use for discovering potent and selective inhibitors of zinc metalloproteases. Recently, a synthetic procedure for developing peptide libraries containing a phosphonate group (PO2O) was published (41), but no results were given for the potency of these phosphonate peptides as zinc metalloprotease inhibitors. However, for bacterial collagenase and mammalian 24-15 and 24-16, it should be mentioned that phosphonate peptides have been published (41), but no results were given for the potency of these phosphonate peptides as zinc metalloprotease inhibitors.

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