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Identification of ACE pharmacophore in the phosphonopeptide metabolite K-26

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Abstract

The naturally occurring phosphonotripeptide K-26 is a potent angiotensin converting enzyme (ACE) inhibitor containing an α -amino phosphonic acid analog of tyrosine. Previous studies have demonstrated that canonical peptide analogues of K-26 are micromolar inhibitors of ACE. To ascertain the structure-activity relationships in this class of ACE inhibitory natural products, K-26 and eight analogues were chemically synthesized and evaluated. Phosphonyl substitution was found to be the critical determinant of activity, resulting in a 1500-fold increase in ACE inhibition versus carboxyl analogues. Secondly, the absolute configuration of the terminal α -amino phosphonate and *N*-acetylation were found to significantly modulate ACE inhibitory activity.

Phosphonic acids are important pharmacophores of significant relevance to human health. Under physiological conditions, the phosphonate moiety ($R-PO_3H_2$, $pK_a \sim 2.5, 8.0^1$) can serve as an isosteric replacement for phosphate or carboxylate functional groups, which are ubiquitous ligands in the active sites of many enzymes. The carbon-phosphorus bond renders phosphonates hydrolytically stable relative to their phosphate congeners and the mode of action of phosphonates is often either through competitive interaction with substrate binding regions or as an analog of tetrahedral transition states. As a result, synthetic phosphonates find real world application in medicine as antibiotics, antivirals, antiosteoclastics and as environmentally benign herbicides.² The phosphonate moiety is also well represented in biologically active natural products and can be found in such diverse molecular entities as bialaphos (**5**; antifungal, antibiotic, herbicide), fosfomycin (**4**; antibiotic), fosmidomycin (**3**, antimalarial), and K-26 (**1**; ACE inhibitor)(Figure 1).²

K-26 was initially discovered via ACE bioactivity guided fractionation of extracts of a soil dwelling prokaryote, "actinomycete strain K-26".^{3–5} It has been reported to possess ACE inhibitory activity comparable to the widely prescribed antihypertensive drug Captopril in both *in vitro* assays and in intravenously administered animal models. NMR, mass spectrometry, degradation and synthetic studies have demonstrated that K-26 is comprised of *N*-acetylated L -isoleucine, L -tyrosine, and the nonproteinogenic amino acid, (*R*)-1-amino-2-(4-hydroxyphenyl)ethylphosphonic acid (AHEP, **2**). This "phosphonotyrosine" functional group is shared among several ACE inhibitor peptide analogues of K-26 produced by various *Streptosporangium*⁶ and *Actinomadura*⁷ species. The stereochemistry of AHEP has been established by x-ray analysis of the ethyl ester of K-26.

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Supplementary Data Additional information and data concerning synthesis and spectroscopic characterization of synthesized compounds and ACE inhibition assay can be found in the online version.

Synthetic ACE inhibitors are widely prescribed for cardiovascular diseases, including high blood pressure, heart failure, heart attack and kidney failure and have combined annual sales in excess of six billion dollars.⁸ ACE has a critical role in the regulation of blood pressure by catalyzing the hydrolytic cleavage of His-Leu dipeptide from decapeptide angiotensin I to yield angiotensin II, a potent vasoconstrictor. ACE also cleaves bradykinin resulting in antagonistic effects to angiotensin II. Typical ACE inhibitors consist of peptide or peptidomimetic structures incorporating a functional group that interacts with the active site zinc, such as a thiol, a carboxylate, a phosphinic acid (Figure 2).

It has been previously suggested that the phosphonate moiety of K-26 may interact with the active site zinc atom and contribute to the potent inhibitory activity of this class of compounds.⁴ However, small proteinogenic amino acid containing di- and tri- peptides have also been reported to possess significant ACE activity. For instance L-Ile-L-Tyr and other analogs of K-26 have been reported to be low micromolar inhibitors of mammalian ACE⁹ and it has remained unclear how the ACE activity of K-26 is modulated by the substitution of carboxyl group functionality and *N*-acetylation. To identify the structural determinants of ACE activity in the class of chiral α -amino phosphonyl natural products we have synthesized K-26 and several peptide analogues (Table 1).

Production levels of K-26 in the producing organism are low (est. < 10 μ g/L in our hands), necessitating the development of a synthetic route for K-26 and analogues. AHEP was synthesized as previously described^{10–14} with minor modifications. Commercially available 4-benzyloxyphenylacetyl chloride (**9**) was reacted with triethyl phosphite in an Arbuzov type reaction to yield the corresponding α -keto phosphonate (not isolated). Subsequent conversion to the oxime (**10**) with hydroxylamine and reduction with zinc/formic acid resulted in racemic AHEP diethyl ester (**11**). Standard peptide coupling conditions¹⁵ were employed in coupling the free base of AHEP diethyl ester to Ac-Ile-Tyr(Bzl)-COOH (**12**). In a two-step deprotection process, benzyl groups were removed from the resultant tripeptide by catalytic hydrogenation and ethyl groups were removed by reaction with iodotrimethylsilane. This synthetic route resulted in the preparation of a mixture of two major diastereomers of K-26 (**1a** and **1b**), which were readily separated via C18 chromatography using a gradient of 5 – 40% acetonitrile containing 0.1% trifluoroacetic acid. Stereochemistry was assigned by comparison of chemical shift values and NOESY cross-peak correlations to peptide analogue Ac-Ile-Tyr-Tyr (**15a**) and Ac-Ile-Tyr-*D*-Tyr (**15b**). Des-acetyl K-26 (**14a,b**) was synthesized in an analogous fashion to K-26 with the exception that Cbz-Ile-Tyr(Bzl)-COOH was coupled to AHEP in place of Ac-Ile-Tyr(Bzl)-COOH. Stereochemical assignments of the two des-acetyl K-26 diastereomers (**14a** and **14b**) were also established based on chemical shift values and NOESY correlations in comparison to synthetic Ile-Tyr-Tyr (**16a**) and Ile-Tyr-*D*-Tyr (**16b**). Of note, while the structure of K-26 has been previously unambiguously determined, this work provides the first high-resolution NMR spectroscopic data for this series of compounds.

The method outlined by Holmquist and coworkers¹⁶ for measuring ACE activity was adapted for assay in 96-well format (see supplementary material). In short, ACE activity was extracted from rabbit lung acetone powder by soaking with 100 mM borate buffer followed by ultracentrifugation.¹⁷ Furanacryloyl-L-phenylalanyl-glycylglycine (FAPGG) was used as the chromogenic substrate for measuring initial velocities. The progress of the reaction can be monitored in a continuous fashion based on the hypsochromic shift of the absorption spectra that occurs upon hydrolysis of FAPGG to FAP and GG. In a typical reaction, ACE extract was preincubated with a range of inhibitor concentrations in the responsive concentration region for five minutes followed by the addition of a 1 mM solution of FAPGG. The rate of FAPGG hydrolysis was obtained by measuring the change in absorbance at 340 nm versus time. IC₅₀ values were obtained by fitting triplicate measurements to a sigmoidal dose response curve. As a benchmark, the IC₅₀ of Captopril (**6**) was determined in parallel to ensure the accuracy

of the assay. The IC₅₀ values of the peptide inhibitors and Captopril are summarized in Table 1.

Measurement of ACE inhibition by the natural tripeptide analog of K-26, L-Ile-L-Tyr-L-Tyr (**16a**), confirmed the previously reported micromolar range activities of related small natural peptides. *N*-Acetylation of this compound to (**15a**) improved its activity by approximately ten fold. However, the potentiating effect of phosphonyl substitution in this compound class was more pronounced. K-26 (**1a**) was found to be 1500-fold more potent than Ac-Ile-Tyr-Tyr (**15a**). Indeed, all analogues containing AHEP were found to be markedly improved inhibitors relative to their carboxylate congeners.

Other general trends emerge from these data. Separation of the synthetic diastereomers permitted the evaluation of the effect of stereochemistry of AHEP on ACE inhibition. We observed that the cost of inverting the stereochemistry of the phosphonate from the natural configuration (*R*-AHEP) was an approximately ten-fold decrease in activity and similar trends for acetylated and des-acetyl analogues in all series were observed. Additionally, alkylation of the phosphonate group (ethyl esters) decreased activity over 25,000-fold, as demonstrated by the comparison of **1a** and **1c**. The influence of *N*-acetylation on ACE activity in K-26 was also consistent, with an across the board improvement of 10–20 fold for most acetylated analogues.

These data provide an additional basis for understanding the improvement in ACE inhibition by K-26 versus the carboxylate analogues. The ACE potentiating effects of phosphonate substitution may originate from a stronger ionic interaction of the phosphonate centered anion with the enzyme, either through side chain interactions or by direct interaction with Zn²⁺ ligand. Previous X-ray structural studies⁵ have also suggested that phosphonyl substitution may limit the conformational flexibility of the peptide backbone relative to the carboxyl analog. This notion is supported by the observation of weak long range coupling between the γ -methyl of isoleucine (0.5 ppm) and the AHEP phenyl ring (6.7 ppm) in K-26 NOESY experiments. No such distal interactions were observed in Ac-Ile-Tyr-Tyr.

The isolation of a mammalian ACE modulating substance from a bacterium may appear somewhat surprising. Recently however, bacterial ACE homologues have been identified in *Xanthomonas*¹⁸, suggesting that the bacterial homologues of eukaryotic ACE may play important roles in bacterial physiology. While the natural target of these terminal phosphonopeptides remains unidentified, the potent inhibitory activity of this entire class of compounds towards ACE suggests the possibility that these compounds may target analogous metalloproteases, either endogenously or metalloproteases of consequence in the ecological niche of the producing organisms. Indeed, the specific functionalities that distinguish K-26 from its natural peptide congener, *N*-acetylation and α -phosphonylation, are shown here to direct markedly improved activity against the zinc metalloprotease ACE. It is therefore possible that the natural target of K-26 is a catalytically similar metalloprotease and these results suggest that the nature's strategy of C-terminal phosphonyl substitution may find meritorious application in the discovery of new C-terminal phosphonate inhibitors.

Recent studies^{13, 19} have determined that the biosynthesis of K-26 is distinct from most other C-P bond containing metabolites^{20, 21} in that the precursor for AHEP "phosphonotyrosine" is derived from tyrosine, instead of phosphoenolpyruvate. Labeling studies have demonstrated that AHEP is a discrete amino acid precursor of K-26 in the producing organism and that the transformation of tyrosine to AHEP occurs with retention of stereochemical configuration at the α -carbon of tyrosine and retention of nitrogen. The demonstration of the potency of the phosphonate pharmacophore in this class of compounds underlines the utility of further studies aimed at identifying the biosynthetic gene(s) required for assembly of α -aminophosphonates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Bioactive natural C-P containing metabolites.

Scheme 1.

Synthesis of two diastereomers of K-26. Reagents and conditions: (a) $\text{P}(\text{OEt})_3$, THF, $0 \rightarrow 70^\circ\text{C}$; (b) $\text{NH}_2\text{OH}\cdot\text{HCl}$, pyridine, ethanol, 25°C ; (c) Zn/HCOOH , 25°C ; (d) $\text{EDC}\cdot\text{HCl}$, HOBt , 2,4,6-trimethylpyridine, DMF, 25°C ; (e) H_2 , Pd/C , 25°C ; (f) TMSI , thioanisole, MeCN, 0°C .

Table 1

Measured IC₅₀ values of K-26 and analogs

compound	IC ₅₀ (nM)
 1a , (K-26) Ac-L-Ile-L-Tyr-R-AHEP 1b , Ac-L-Ile-L-Tyr-S-AHEP 1c , Ac-L-Ile-L-Tyr-R-AHEP(OEt) ₂	 14.4 139 3.83 × 10 ⁵
 14a , L-Ile-L-Tyr-R-AHEP 14b , L-Ile-L-Tyr-S-AHEP	 234.3 5.46 × 10 ⁴
 15a , Ac-L-Ile-L-Tyr-L-Tyr 15b , Ac-L-Ile-L-Tyr-D-Tyr	 2.1 × 10 ⁴ 2.0 × 10 ⁶
 16a , L-Ile-L-Tyr-L-Tyr 16b , L-Ile-L-Tyr-D-Tyr	 2.36 × 10 ⁵ > 10 ⁶
 captopril	7.7