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A new colorimetric assay for methionyl aminopeptidases: Examination of the binding of a new class of pseudopeptide analog inhibitors

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Abstract

A direct and convenient spectrophotometric assay has been developed for [methionine aminopeptidases](#) (MetAPs). The method employs the [hydrolysis](#) of a substrate that is a methionyl analogue of *p*-nitroaniline (I-Met-*p*-NA), which releases the chromogenic product *p*-nitroaniline. This chromogenic product can be monitored continuously using a [UV–Vis](#) spectrophotometer set at 405 nm. The assay was tested with the type I MetAP from [Escherichia coli](#) (*Ec*MetAP-I) and the type II MetAP from [Pyrococcus furiosus](#) (*Pf*MetAP-II). Using I-Met-*p*-NA, the [kinetic](#) constants k_{cat} and K_{m} were determined for *Ec*MetAP-I and *Pf*MetAP-II and were compared with those obtained with a “standard” [high-performance liquid chromatography](#) (HPLC) discontinuous assay. The assay has also been used to determine the [temperature dependence](#) of the kinetic constant k_{cat} for *Pf*MetAP-II as well as to screen two novel pseudopeptide inhibitors of MetAPs. The results demonstrate that I-Met-*p*-NA provides a fast, convenient, and effective substrate for both type I and type II MetAPs and that this substrate can be used to quickly screen inhibitors of MetAPs.

Keywords

Methionine aminopeptidases, Spectrophotometric assay, Enzyme inhibition

Introduction

[Angiogenesis](#) is a crucial step in the pathogenesis of numerous [human](#) diseases, including diabetic retinopathy, rheumatoid arthritis, and cancer [1], [2], [3], [4]. It has been shown that malignant tumors cannot grow and metastasize without the formation of new blood vessels that provide [oxygen](#), [nutrients](#), and various [growth factors](#). In addition, tumors use newly formed blood vessels to disseminate invasive tumor cells. Thus, the [inhibition](#) of angiogenesis is a promising strategy for the treatment of cancer. TNP-470, a synthetic derivative of a [fungal metabolite fumagillin](#), was one of the first antiangiogenesis agents to enter clinical trials [5]. The activity of this [sesquiterpene](#) epoxide-containing [molecule](#) was evaluated for the treatment of a variety of cancers such as Kaposi’s sarcoma, cervical cancer, brain cancer, and renal cell carcinoma [6]. Based on fumagillin [affinity chromatography](#) and mass spectrometry, [methionine aminopeptidases](#) (MetAPs) [1] were identified as the molecular targets of fumagillin and TNP-470 [7], [8]. Furthermore, *in vivo* studies showed that one of the observed effects of inhibiting MetAPs by antiangiogenesis agents is the failure to expose [glycine residues](#) at the *N* termini of certain signaling [proteins](#) involved in [cell cycle regulation](#), which prevents myristoylation [9]. Therefore, MetAPs are an extremely promising target for the design of a novel class of antiangiogenesis [drugs](#), some of which currently are under clinical investigation [5], [10], [11], [12], [13], [14], [15], [16], [17], [18].

MetAPs are capable of the hydrolytic removal of an *N*-terminal [methionine](#) residue from nascent [polypeptide](#) chains [19], [20], [21], [22]. Because the [biosynthesis](#) of all eukaryotic [proteins](#) present in the cytosol starts with the initiator [methionine](#), MetAPs play a central [role](#) in [protein synthesis](#) [23]. The structure of mature *N* termini plays important roles in *N*-directed [degradation](#) pathways and in targeting [cellular membranes](#) [24], [25]. Therefore, MetAPs are one of the key cellular enzymes involved in [protein maturation](#). MetAPs are organized into two classes (types I and II) based on the absence or presence of an extra 62-amino acid sequence (of unknown function) inserted near the [catalytic domain](#) of type II enzymes. The type I MetAPs from [Escherichia coli](#) (*Ec*MetAP-I), [Staphylococcus aureus](#) (*Sa*MetAP-I), [Thermotoga](#) maritime (*Tm*MetAP-I), and [Homo sapiens](#)

(HsMetAP-I), and the type II MetAPs from *Homo sapiens* (HsMetAP-II) and *Pyrococcus furiosus* (PfMetAP-II), have been characterized crystallographically [26], [27], [28], [29], [30], [31], [32]. All six display a novel “pita bread” fold with an internal pseudo-twofold symmetry that structurally relates the first and second halves of the polypeptide chain to each other. Each half contains an antiparallel β -pleated sheet flanked by two helical segments and a C-terminal loop. Both domains contribute conserved residues to the metallo-active site. In all five structures, a bis(μ -carboxylato)(μ -aquo/hydroxo)dicobalt(II) core is observed with an additional carboxylate residue at each [metal](#) site and a single [histidine](#) bound to Co1.

A major issue that has plagued research [efforts](#) on MetAPs is the lack of a fast and simple spectrophotometric assay. To date, the most widely used assay involves determining the amount of product (I-Met) formed by [high-performance liquid chromatography](#) (HPLC) [33], [34], [35]. Even though this assay is very accurate, it is very time-consuming and expensive to run. To develop a simple, inexpensive, and fast continuous assay for MetAPs, here we report the monitoring of [amide](#) bond cleavage by both type I and type II MetAPs using a methionyl analogue of *p*-nitroaniline (I-Met-*p*-NA). Proof of concept that this assay is functional is shown by comparison of [kinetic data](#) obtained with I-Met-*p*-NA with the [peptide](#) substrate MGMM using a discontinuous HPLC assay. We also use I-Met-*p*-NA to investigate the potency of two new MetAP inhibitors.

Materials and methods

Protein expression, purification, and metal activation

EcMetAP-I and *PfMetAP-II* were purified as described previously [12], [33]. Purified *EcMetAP-I* and *PfMetAP-II* exhibited single bands on [sodium dodecyl sulfate–polyacrylamide gel electrophoresis](#) (SDS–PAGE) at M_r 29,630 and M_r 32,850, respectively. [Protein](#) concentrations for *EcMetAP-I* and *PfMetAP-II* were estimated from the absorbance at 280 nm using extinction coefficients of 16,445 and 21,650 $M^{-1} cm^{-1}$, respectively [12], [33], [35]. Apo-*EcMetAP-I* was washed free of [methionine](#) using Chelex-100-treated methionine-free [buffer](#) (25 mM Hepes [pH 7.5] and 150 mM KCl) and was concentrated by [microfiltration](#) using a Centicon-10 (Amicon, Beverly, MA, USA) prior to all [kinetic](#) assays. Individual aliquots of apo-*EcMetAP-I* were routinely stored at $-80^\circ C$ or in [liquid nitrogen](#) until needed. Apo-*EcMetAP-I* and apo-*PfMetAP-II* [samples](#) used in catalytic measurements were made rigorously anaerobic prior to incubation with 3 eq of Co(II) ($CoCl_2$, $\geq 99.999\%$, Strem Chemicals, Newburyport, MA, USA) for approximately 30 min at $20-25^\circ C$. Co(II)-containing samples were handled throughout in an anaerobic glove box ($Ar/5\% H_2$, ≤ 1 ppm O_2 , Coy Laboratories, Ann Arbor, MI, USA) until frozen. Electronic [absorption](#) measurements were performed on a Shimadzu UV-3101PC spectrophotometer.

Discontinuous enzymatic assay of MetAPs

All enzymatic assays were performed under strict anaerobic conditions in an [inert atmosphere](#) glove box (Coy Laboratories) with a dry bath incubator to maintain the [temperature](#). [Catalytic activities](#) were determined with an [error](#) of $\pm 5\%$ using the substrate MGMM in 25 mM Chelex-100-treated methionine-free Hepes buffer (pH 7.5) containing 150 mM KCl. The amount of product (I-Met) formation was determined by HPLC (Shimadzu LC-10A class-VP5). A typical assay involved the addition of 8 μl of metal-loaded WT *EcMetAP-I* enzyme to a 32- μl substrate–buffer mixture at $30^\circ C$ for 1 min. The reaction was quenched by the addition of 40 μl of a 1% [trifluoroacetic acid](#) (TFA) [solution](#). [Elution](#) of the product was monitored at 215 nm following separation on a C8 HPLC [column](#) (Phenomenex Luna, 5 μm , 4.6×25 cm), as described previously [33], [34], [35].

Synthesis of I-MetStaCys and I-MetStaVal

I-MetStaCys and I-MetStaVal were synthesized by [solid-phase synthesis](#) on Rink [Amide](#) AM resin using an *N*-Boc-protected [statine](#) derivative of I-Met, (3*S*,4*S*)-4-*tert*-butoxycarbonylamino-3-hydroxy-6-methylsulfanyl-hexanoic acid, which was prepared according to Jouin and co-workers [36]. This synton (1.4 eq) was attached to resin-bound Val or Cys(Trt) (1 eq) using *N,N'*-diisopropylcarbodiimide (3 eq) and *N*-hydroxybenzotriazole (5 eq) as coupling agents in a mixture of *N*-methylpyrrolidinone and [dichloromethane](#) overnight. Pseudopeptides were

cleaved off the resin with a mixture of 97% TFA, 1% ethanedithiol, 1% trisopropylsilane, and 1% water. The inhibitors were purified using HPLC and characterized by mass spectrometry.

Results and discussion

Several discontinuous assays have been reported, with the most used ones being (i) HPLC detection of the reaction product I-Met, (ii) amino acid [oxidase](#) together with horseradish [peroxidase](#) and monitoring of the absorbance at 440 nm [24], [37], [38], and (iii) reaction of I-Met with a chromogenic or fluorogenic tag and analysis of the derivatives by HPLC or by [capillary electrophoresis](#) [39], [40]. Although these assays are indeed functional, several problems exist for all of them (e.g., long [incubation times](#), time-consuming, labor-intensive, expensive, significant background noise). Recently, two complementary, continuous MetAP assays were reported [41]. In the first, a [thioester](#) substrate was used; on exposure of the free [thiol](#) group by the action of MetAP, the amount of free [thiol](#) was quantified by 5,5'-dithio-bis(2-nitrobenzoic) acid (DTNB) [41]. Although a creative approach to assay MetAPs, DTNB is known to inactivate *EcMetAP-I* (and likely all type I MetAPs) by modifying [active site cysteine residues](#) [42]. Alternatively, a coupled spectrophotometric assay in which the [dipeptide](#) substrate Met-Pro-*p*-NA was employed was reported [41]. In this assay, the [N-terminal methionine residue](#) was removed by MetAP, followed by cleavage of I-Pro by a prolyl [aminopeptidase](#), releasing *p*-nitroaniline [41]. Although this assay is relatively simple to use, one major problem involves [cross-reactivity of potential](#) MetAP inhibitors when attempting to obtain K_i values. To overcome problems with the currently available assay methods for MetAPs, we have examined [amide](#) bond cleavage by both type I and type II MetAPs using a methionyl analogue of *p*-nitroaniline (I-Met-*p*-NA).

Recently, it was shown that *HsMetAP-II* could use substrates smaller than [dipeptides](#), such as I-methionine-7-amido-4-methylcoumarin (I-Met-AMC) and I-Met-*p*-NA, whereas *EcMetAP-I* was shown to [hydrolyze](#) I-Met from I-Met-AMC [43]. However, it was reported previously that [yeast](#) MetAP-I was unable to cleave I-Met-*p*-NA [44]. Even so, we hypothesized that both type I and type II MetAPs could hydrolyze I-Met-*p*-NA, contrary to previous reports [24]. Therefore, the hydrolytic activity of Co(II)-reconstituted *EcMetAP-I* and *PfMetAP-II* was examined with I-Met-*p*-NA as the substrate. A typical reaction of 1, 4, or 12 μM *PfMetAP-II* or *EcMetAP-I* with I-Met-*p*-NA was carried out at 30 °C in a [quartz](#) cuvette containing 25 mM Hepes [buffer](#) at [pH](#) 7.5 with 150 mM KCl and monitoring the absorbance at 405 nm ($\epsilon = 10,800 \text{ M}^{-1} \text{ cm}^{-1}$) for 10 min. The rate of background [hydrolysis](#) of I-Met-*p*-NA was measured by monitoring the formation of *p*-nitroaniline continuously at 405 nm and was subtracted. Interestingly, the absorbance at 405 nm in the presence of *PfMetAP-II* or *EcMetAP-I* increased linearly with time (Fig. 1), and the amount of product released was linearly dependent on the concentration of the enzyme (Fig. 2). As a control, I-Ala-*p*-nitroaniline and I-Leu-*p*-nitroaniline were examined, and (as expected) neither *PfMetAP-II* nor *EcMetAP-I* could use these substrates. These data clearly show that I-Met-*p*-NA can function as a substrate for both type I and type II MetAPs.

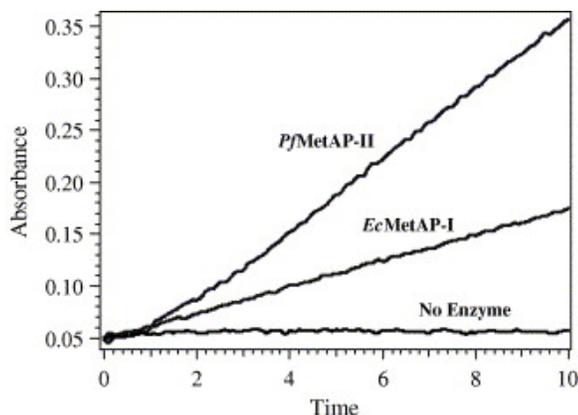


Fig. 1. Time course for the [hydrolysis](#) of I-Met-*p*-NA (1 mM) by *EcMetAP-I* (4 μM) and *PfMetAP-II* (1 μM) in 25 mM Hepes [buffer](#) ([pH](#) 7.5) and 150 mM KCl.

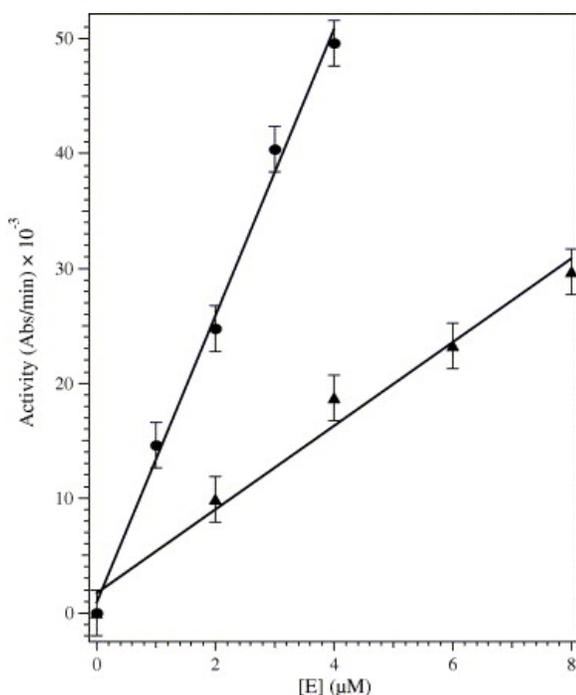


Fig. 2. Effect of *EcMetAP-I* (solid triangles) and *PflMetAP-II* (solid circles) concentration on the [hydrolysis](#) of l-Met-*p*-NA (1 mM) in 25 mM Hepes [buffer](#) (pH 7.5) and 150 mM KCl.

Both *PflMetAP-II* and *EcMetAP-I* exhibited saturation [kinetics](#) when l-Met-*p*-NA was used as a substrate ([Fig. 3](#)). Activity assays using l-Met-*p*-NA were performed in triplicate and revealed that Co(II)-loaded *PflMetAP-II* is more active than Co(II)-loaded *EcMetAP-I* ([Table 1](#)). K_m and k_{cat} values were obtained by nonlinear fitting of the data to the Michaelis–Menten equation. For *PflMetAP-II*, the k_{cat} value observed for l-Met-*p*-NA at 30 °C was 0.057 s^{-1} ($K_m = 0.59 \text{ mM}$), providing a [catalytic efficiency](#) of $97.0 \text{ M}^{-1} \text{ s}^{-1}$, whereas *EcMetAP-I* exhibited a k_{cat} value of 0.006 s^{-1} ($K_m = 0.44 \text{ mM}$), providing a catalytic efficiency of $13.7 \text{ M}^{-1} \text{ s}^{-1}$. For comparison purposes, the [kinetic parameters](#) for Co(II)-loaded *EcMetAP-I* and Co(II)-loaded *PflMetAP-II* were determined using MGMM as the substrate ([Table 1](#)) [[35](#)]. Activity assays were performed in triplicate for 8–15 concentrations of MGMM (0–12 mM), and the product (l-Met) was quantified by a discontinuous HPLC assay [[35](#)]. K_m and k_{cat} values were obtained by nonlinear fitting of the data to the Michaelis–Menten equation. For *PflMetAP-II*, the k_{cat} value for MGMM at 30 °C was 188 s^{-1} ($K_m = 5.1 \text{ mM}$), providing a catalytic efficiency of $37,000 \text{ M}^{-1} \text{ s}^{-1}$, whereas *EcMetAP-I* exhibited a k_{cat} value of 18.3 s^{-1} ($K_m = 3.0 \text{ mM}$), providing a catalytic efficiency of $6100 \text{ M}^{-1} \text{ s}^{-1}$. Therefore, both *EcMetAP-I* and *PflMetAP-II* are much poorer [catalysts](#) for the cleavage of l-Met from l-Met-*p*-NA by approximately 440 and 380 times, respectively. Even though the catalytic efficiencies were small, this assay was found to be very sensitive and reproducible because l-Met-*p*-NA binds more tightly to both type I and type II MetAPs and the highly chromogenic product that can be monitored continuously at 405 nm. Therefore, the simplicity of a direct spectrophotometric assay suggests that l-Met-*p*-NA is the substrate of choice for analyzing MetAP activity.

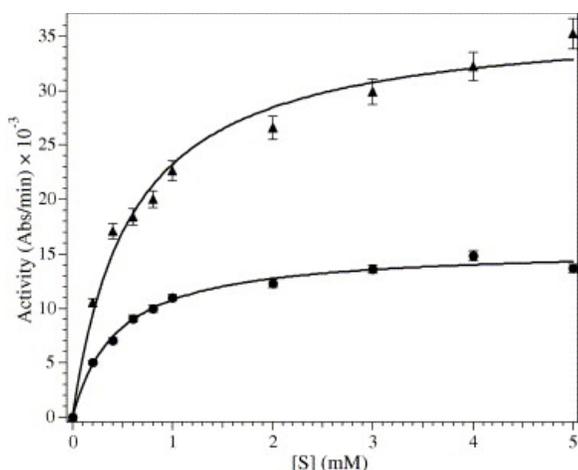


Fig. 3. Michaelis–Menten plot for the hydrolysis of I-Met-*p*-NA by *EcMetAP-I* (solid circles) and *PfMetAP-II* (solid triangles) in 25 mM Hepes buffer (pH 7.5) and 150 mM KCl.

Table 1. Kinetic constants for Co(II)-loaded *EcMetAP-I* and *PfMetAP-II* using MGMM and I-Met-*p*-NA in 25 mM Hepes buffer at 30 °C (pH 7.5) with 150 mM KCl

	Kinetic constants	I-Met- <i>p</i> -NA	MGMM
<i>EcMetAP-I</i>	K_m (mM)	0.44 ± 0.03	3.0 ± 0.1
	k_{cat} (s^{-1})	0.006	18.3
	k_{cat}/K_m ($M^{-1} s^{-1}$)	13.7	6100
	SA (U/mg)	1.3×10^{-2}	36.0
<i>PfMetAP-II</i>	K_m (mM)	0.59 ± 0.07	5.1 ± 0.3
	k_{cat} (s^{-1})	0.057	188
	k_{cat}/K_m ($M^{-1} s^{-1}$)	97.0	37,000
	SA (U/mg)	1.1×10^{-1}	340

It was reported previously [35] that the optimal activity for *PfMetAP-II* toward MGMM occurred at 85 °C in 25 mM Hepes (pH 7.5) and 150 mM KCl buffer. Therefore, the hydrolysis of I-Met-*p*-NA was examined in triplicate between 25 and 90 °C at a substrate concentration of 5 mM. The calculated specific activity values were plotted as a function of temperature between 25 and 90 °C. The specific activity values for I-Met-*p*-NA hydrolysis catalyzed by Co(II)-loaded *PfMetAP-II* were found to increase with increasing temperature. In a simple rapid equilibrium, $V_{max}/[E] = k_p$, the first-order rate constant. Because the enzyme concentration was not altered over the course of the experiment, an Arrhenius plot could be constructed by plotting $\ln(k_{cat})$ versus $1/T$ (Fig. 4). The linearity of the Arrhenius plot indicates that the rate-limiting step does not change as a function of temperature [45]. From the slope of the line, the activation energy, E_a , for temperatures between 296 and 358 K was calculated to be 54 kJ/mol for Co(II)-loaded *PfMetAP-II* but was 13.3 kJ/mol using MGMM as the substrate [35]. These data compare well with the E_a values reported for the aminopeptidase from *Aeromonas proteolytica* (36.5 kJ/mol), which has a similar activation energy to pronase and both thermolysin and carboxypeptidase A [46], [47], [48]. The fact that the activation energy is approximately fourfold higher for the hydrolysis of I-Met-*p*-NA by Co(II)-loaded *PfMetAP-II* is constant with the observed decrease in k_{cat} when I-Met-*p*-NA is used as a substrate.

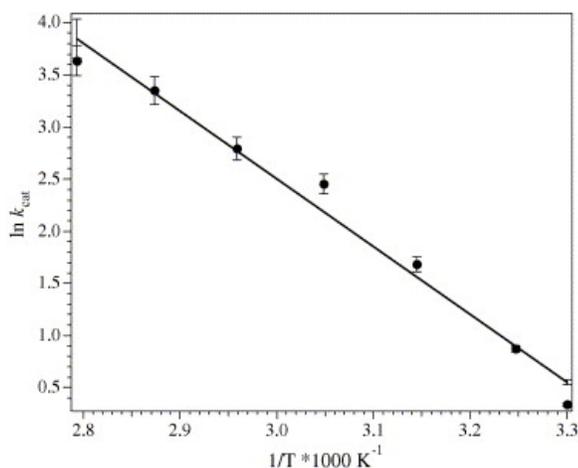


Fig. 4. Arrhenius plot of $\ln(k_{cat})$ versus $1/T$ for Co(II)-loaded *PfMetAP-II* using l-Met-*p*-NA as the substrate in 25 mM Hepes buffer (pH 7.5) and 150 mM KCl.

Because the slope of an Arrhenius plot is equal to $-E_{a1}/R$, where $R = 8.3145 \text{ JK}^{-1} \text{ mol}^{-1}$, thermodynamic parameters for the hydrolysis of l-Met-*p*-NA by Co(II)-loaded *PfMetAP-II* were calculated by the following relations: $\Delta G^\ddagger = -RT \ln(k_{cat}h/k_B T)$, $\Delta H^\ddagger = E_a - RT$, $\Delta S^\ddagger = (\Delta H^\ddagger - \Delta G^\ddagger) / T$, where R , h , and k_B are the gas, Boltzmann, and Planck constants, respectively (Table 2). The enthalpy of activation calculated over the temperature range 25–90 °C is 47.5 kJ/mol for Co(II)-loaded *PfMetAP-II*, whereas the entropy of activation was found to be -79.9 J/mol/K at 25 °C. The positive enthalpy, which is approximately four times larger than the enthalpy observed using MGMM as the substrate, is indicative of a conformation change on substrate binding likely due to the energy of bond formation and breaking during nucleophilic attack on the scissile carbonyl carbon of the substrate [35]. On the other hand, the large negative entropy value, which is similar in magnitude to the enthalpy observed using MGMM as the substrate (-119.7 J/mol/K), suggests that some molecular motions are lost on ES^\ddagger complex formation, possibly due to hydrogen bond formation between catalytically important amino acids and the substrate [35]. All of these factors contribute to the large positive free energy of activation (71.3 kJ/mol), which is nearly double the value observed using MGMM as the substrate [35].

Table 2. Thermodynamic parameters for hydrolysis of l-Met-*p*-NA by Co(II)-loaded *PfMetAP-II* in 25 mM Hepes buffer (pH 7.5) and 150 mM KCl

	E_a (kJ/mol)	ΔH^\ddagger (kJ/mol)	ΔG^\ddagger (kJ/mol)	ΔS^\ddagger (J/mol/K)
l-Met- <i>p</i> -NA	54.0	47.5	71.3	-79.9

Finally, we have designed and synthesized several pseudopeptides that have nonhydrolyzable moiety that mimics the transition state of the substrate in the active site of MetAPs that represent a new class of MetAP inhibitors. These molecules are based on a statine derivative of l-Met with a Cys (l-MetStaCys, **1**) or a Val (l-MetStaVal, **2**) attached to the C terminus of the statine synthon (Fig. 5). Both **1** and **2** were tested for their ability to inhibit Co(II)-loaded *EcMetAP-I* and *PfMetAP-II* (Fig. 5) using l-Met-*p*-NA as the substrate. The reaction rates of Co(II)-loaded *EcMetAP-I* and *PfMetAP-II* in the presence of 0–1 mM of **1** or **2** were monitored continuously at 405 nm, and the initial rates were calculated. The IC_{50} values for **1** and **2** were obtained from plots of the ratio of the initial rates with and without the inhibitor against inhibitor concentrations (Fig. 6). The IC_{50} values for **1** inhibiting Co(II)-loaded *EcMetAP-I* and *PfMetAP-II* were found to be 2.5 ± 0.1 and $1.7 \pm 0.2 \mu\text{M}$, respectively, whereas **2** exhibited IC_{50} values of 4.3 ± 0.4 and $2.0 \pm 0.1 \mu\text{M}$ for Co(II)-loaded *EcMetAP-I* and *PfMetAP-II*, respectively. The IC_{50} values observed for **1** and **2** binding to *PfMetAP-II* using l-Met-*p*-NA as the substrate compare well with IC_{50} values (1.5 ± 0.1 and $2.0 \pm 0.1 \mu\text{M}$, respectively) obtained for the Mn(II)-loaded *PfMetAP-II* using the discontinuous HPLC assay that uses MGMM as the substrate. These data indicate that using l-Met-*p*-NA as a substrate for type I and type II MetAPs provides a fast, convenient, and direct spectrophotometric assay

with which to screen potential inhibitors of MetAPs. Moreover, these data indicate that the l-MetStaCys and l-MetStaVal are good inhibitors of both type I and type II MetAPs, suggesting that the l-MetSta scaffolding provides an excellent base for the synthesis of new MetAP inhibitors.

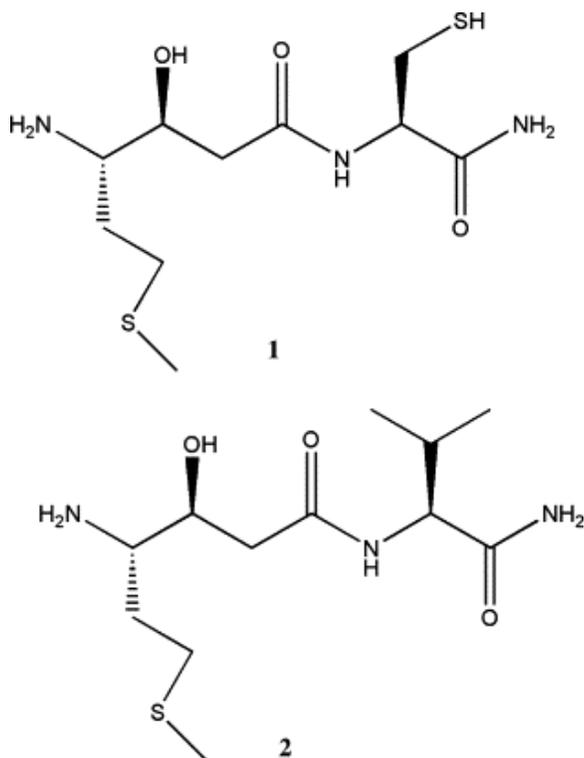


Fig. 5. Chemical structures of the l-MetStaX (X = Cys, **1**, and Val, **2**) pseudopeptide MetAP inhibitors.

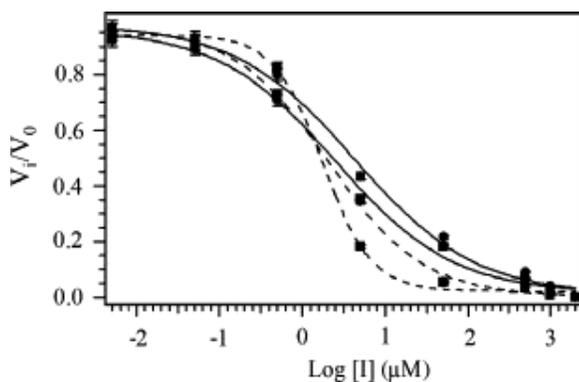


Fig. 6. Dose-dependent curves for the [inhibition](#) of *EcMetAP-I* (solid lines) and *PfMetAP-II* (dashed lines) by **1** (circles) and **2** (squares) at 30 °C in 25 mM HEPES [buffer \(pH 7.5\)](#) and 150 mM KCl.

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1 *Abbreviations used:* MetAP, methionine aminopeptidase; *EcMetAP-I*, type I MetAP from *Escherichia coli*; *SaMetAP-I*, type I MetAP from *Staphylococcus aureus*; *TmMetAP-I*, type I MetAP from *Thermotoga maritima*; *HsMetAP-I*, type I MetAP from *Homo sapiens*; *HsMetAP-II*, type II MetAP from *Homo sapiens*; *PfMetAP-II*, type II MetAP from *Pyrococcus furiosus*; HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; DTNB, 5,5'-dithio-bis(2-nitrobenzoic) acid; I-Met-AMC, I-methionine-7-amido-4-methylcoumarin.