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Solvent-Assisted Slow Conversion of a Dithiazole Derivative Produces a Competitive Inhibitor of Peptide Deformylase

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SUMMARY

Due to its potential as an antibiotic target, *E. coli* peptide deformylase (PDF_{EC}) serves as a model enzyme system for inhibitor design. While investigating the structure-functional and inhibitory features of this enzyme, we unexpectedly discovered that 2-amino-5-mercapto-1,3,4-thiadiazole (AMT) served as a slow-binding inhibitor of PDF_{EC} when the above compound was dissolved only in dimethylformamide (DMF), but not in any other solvent, and allowed to age. The time dependent inhibitory potency of the DMF-dissolved AMT was correlated with the broadening of the inhibitor's 295 nm spectral band toward the visible region, concomitant with the increase in the mass of the parent compound by about 2-fold. These data led to the suggestion that DMF facilitated the slow dimerization of AMT (via the formation of a disulfide bond), and that the dimeric form of AMT served as an inhibitor for PDF_{EC}. The latter is not caused by the simple oxidation of sulfhydryl groups by oxidizing agents such as H₂O₂. Newly synthesized dimeric/dithiolated form of AMT ("bis-AMT") exhibited similar spectral and inhibitory features as given by the parent compound when incubated with DMF. The computergraphic modeling data revealed that bis-AMT could be reliably accommodated within the active site pocket of PDF_{EC}, and the above enzyme-ligand interaction involves coordination with the enzyme resident Ni²⁺ cofactor. The mechanism of the DMF-assisted activation of AMT (generating bis-AMT), the overall microscopic pathway for the slow-binding inhibition of PDF_{EC} by bis-AMT, and the potential of bis-AMT to serve as a new class of antibiotic agent are presented.

Keywords

Peptide Deformylase; Inhibitor; Slow inhibition; Thiadizaole

INTRODUCTION

It has been well-established that in prokaryotes as well as in certain organelles of the eukaryotic system, the initiation of protein synthesis occurs via incorporation of a formylated methionine residue (fMet) at the N-terminus of the nascent polypeptide chain [1–3]. For many proteins in

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the prokaryotic system, the ^FMet residue must be removed to elicit the desired physiological functions via a co-translational process referred to as the N-terminal methionine excision (NME) pathway [4–6]. The NME pathway involves two enzymes: peptide deformylase (PDF) and methionine aminopeptidase (MetAP), and these enzymes sequentially remove the N-terminal formyl group and the Met residue, respectively [7]. Since ^FMet is not utilized in the synthetic pathway of cytosolic proteins in eukaryotes, the absence of PDF in the physiological milieu of higher organisms has been widely justified. However, in recent years, eukaryotic homologues of PDF have been discovered in the chloroplasts and mitochondria of plants and animals including humans [3,8–11]. Nevertheless, the physiological role of the human PDF homolog (which is translated in the cytoplasm and exported to the mitochondrion) is questionable in the light of the fact that proteins synthesized within mitochondria have been shown to retain their N-formyl groups [12,13]. This feature has led to the suggestion that this enzyme is no more than a vestigial remnant of its ancestral counterpart with no or very little physiological function in normal human tissues [14].

Since the discovery of PDF in *E. coli* cells [15], it has been realized that the genomes of all bacterial species harbor at least one putative PDF gene [16,17], and the expression of the enzyme has been shown to maintain the viability of many pathogenic bacteria [18–21]. This coupled with the fact that PDF is non-essential in normal human cells (but may be essential in malignant human cells) [11], is the basis for which the bacterial enzyme has been considered as a prototypical target for designing novel antibiotic agents [22]. In the development of PDF inhibitors as therapeutic agents, most research groups have used actinonin, a natural product inhibitor of the enzyme [23], as a template for the design of inhibitory pseudopeptides. However, due to poor pharmacokinetic profiles, actinonin [24] and many of its derivative compounds [25] do not serve as potent antibiotics under *in vivo* conditions. This is not surprising [26] since several environmental variables of the physiological milieu alter the efficacies of inhibitors for their putative target sites [27]. Irrespectively, a few actinonin analogues have been recently found to exhibit promising results in model animal systems, and they are currently under different phases of clinical trials [22].

In light of the structural-functional studies, it has been deduced that actinonin harbors a hydroxamate group that directly interacts with the active-site resident metal ion as well as a methionine-like sidechain at the P₁' position that binds at the S₁' subsite of the enzyme [28] (A diagram depicting the PDF_{Ec} subsites in relation to the enzyme's substrate is depicted in Figure 6 in the Discussion [17,29–32]). These structural moieties play an important role in the binding of the inhibitor by PDF, which has been shown to exhibit a binding affinity in the nanomolar range for various PDF isozymes [23,33,34]. Kinetic studies of the PDF-actinonin interaction led to the suggestion that the actinonin mediated inhibition of PDF occurs via the slow-binding mode, and the overall inhibition pathway conforms to the two-step binding mechanism [33]. As elaborated subsequently, the second step drives the overall equilibrium to yield the stable (isomerized) form of the PDF-actinonin complex, resulting in greatly enhancing the binding affinity of the enzyme-inhibitor complex and eliciting a potent inhibitory profile.

In screening enzyme inhibitors, it is customary to dissolve putative compounds in DMSO or DMF without considering whether or not such solvents would have any effect on the structural integrity of the parent compounds. Although the above solvents are fairly non-reactive (particularly on the time scale of the screening process), they can react with parent compounds and modify them under prolonged storage condition. For example, DMF has been known to react with small molecules such as carboxylic acid esters [35] as well as phosphorus oxychloride, which is commonly exploited in the Vilsmeier-Haack reaction [36]. In addition, the use of DMF as a solvent has been shown to facilitate the formation of alkanesulfonothioates from alkanesulfinyl chlorides [37]. As will be elaborated in the following sections, we unexpectedly discovered that the solution of 2-amino-5-mercapto-1,3,4-thiadiazole (AMT) in

DMF (but not in any other solvent) served as a slow-binding inhibitor of PDF_{Ec} upon aging. This communication elaborates on the plausible mechanism by which DMF activates AMT, and the resultant (dithiolated “bis-AMT” derivative) serves as a slow binding inhibitor of PDF_{Ec}.

EXPERIMENTAL PROCEDURES

MATERIALS

The commercially available (high purity grade) preparation of 2-amino-5-mercapto-1,3,4-thiadiazole (AMT) was purchased from TCI America, Portland, Oregon. All other reagents were of analytical reagent grade.

METHODS

Protein purification—The full-length, Ni²⁺-substituted form of *E. coli* peptide deformylase (PDF_{Ec}) was purified as described previously [38,39], and the enzyme was judged to be homogeneous via SDS-PAGE analysis. The protein concentration of the purified PDF_{Ec} was determined via the Bradford method [40] using BSA as a standard and a correction factor of 0.56 as described by Rajagopalan *et al* [41].

Activity measurements—The activity of the recombinant PDF_{Ec} was measured via the *Aeromonas* aminopeptidase-coupled reaction using formyl-methionine-leucine-*p*-nitroanilide (^FMet-Leu-*p*-nitroanilide) as substrate [42]. The inhibition studies were performed on either a SpectraMax[®] Plus (Molecular Devices) absorption microplate reader or a Lambda 3B (Perkin-Elmer) spectrophotometer. Initial screening of AMT in the various solvents was performed using 50 mM potassium phosphate buffer, pH 7.0, containing 10 – 100 nM PDF_{Ec}, 0.1 mg/mL BSA, 0.5 units coupling enzyme and 5 – 10% solvent (v/v). The enzyme reaction was initiated by addition of saturating concentrations of ^FMet-Leu-*p*-nitroanilide (200 – 300 μM) and the absorption was monitored for 10 min at 405 nm. The enzymatic assays for the slow-binding inhibition of PDF_{Ec} by DMF-activated AMT and freshly synthesized bis-AMT were performed in the above buffer using 250 – 500 μM substrate, 1 – 2.5 units of *Aeromonas* aminopeptidase, 0.1 mg/mL BSA and 7.5 – 12.5% DMF or DMSO (v/v). During these experiments, the concentration of inhibitor (20 – 1800 μM) was maintained to be much higher than the concentration of the enzyme (3.5 – 6 nM). The reactions were performed in duplicate and were monitored for 90 min at 405 nm after initiation by addition of PDF_{Ec}. Analysis of the resulting activity traces was according to the method of Sculley *et al.* [43]:

$$\text{Abs}_{405} = v_1 \times t + [(v_0 - v_1)/k_{\text{obs}}] \times (1 - e^{-k_{\text{obs}} \times t}) + \text{offset} \quad \text{Eq. 1}$$

where v_0 and v_1 are the initial and final steady-state rates, respectively, and k_{obs} is the observed first-order rate constant for the transition from v_0 to v_1 in the presence of the inhibitor.

To determine the mechanism by which bis-AMT served as a slow-binding inhibitor, plots of k_{obs} as a function of inhibitor concentration were generated. Based on the hyperbolic dependence of k_{obs} on the inhibitor concentration, a two step binding mechanism conceived [43–44].

The experimental data were analyzed by the following equations:

$$k_{obs} = k_4 + k_3 \times [I]/(K_i + [I]) \quad \text{Eq. 2}$$

$$k_{obs} = k_3 \times \{K_i^*/(K_i - K_i^*) + [I]/(K_i + [I])\} \quad \text{Eq. 3}$$

As per the above models, the binding of bis-AMT to PDF first results in the formation of PDF-bis-AMT collision complex (with a dissociation constant of K_i) followed by a slow isomerization process with forward and reverse rate constants being k_3 and k_4 . Note that Eq. 3 was produced via rearrangement of Eq. 2 using the following relationship which describes the resultant effective inhibition constant: $K_i^* = K_i \times k_3/(k_3 + k_4)$.

To directly determine the magnitude of K_i^* for the DMF-activated AMT(bis-AMT), PDF_{Ec} (140 nM) was incubated overnight at 4 °C in the presence of 0.1 mg/mL BSA, 25% DMF (v/v) and increasing concentrations of the inhibitor (0 – 30 μM). Two independent incubations were performed for each inhibitor concentration. The following day, assays of PDF_{Ec} activity were performed as described above in the presence of the inhibitor at concentrations identical to that maintained in the overnight incubation. The reactions were started by the addition of aliquots of PDF_{Ec} from the overnight incubated mixture and the reaction progress was monitored for 60 min at 405 nm in the microplate reader.

To test for competitive inhibition of PDF_{Ec} by the DMF-activated AMT, the steady-state velocity of the PDF_{Ec}-catalyzed reaction was determined in the presence of 100, 250 or 500 μM inhibitor and increasing concentrations (25 – 300 μM) of substrate. The reaction mixtures were as described above and the reaction progress was monitored continuously for 1 hour at 405 nm after addition of PDF_{Ec} and the k_{obs} obtained via Eq. 1. Plots of the dependence of k_{obs} on substrate concentration were analyzed by Eq. 4 as described by Tian and Tsou [44].

$$k_{obs} = k_0/(1 + [S]/K_m) \quad \text{Eq. 4}$$

where k_0 is the intrinsic rate constant for the inhibition of the enzyme in the absence of substrate and the K_m is the apparent binding affinity of substrate for the enzyme. Additionally, inhibition data were analyzed via double-reciprocal plots using increasing concentrations of substrate (10 – 100 μM) and the DMF-activated AMT (0 – 100 μM). The reaction was started by the addition of 22 nM PDF_{Ec} and the initial rate (v) was calculated from the initial 60 seconds of the reaction. The resulting data were plotted as $1/v$ versus $1/[S]$ and analyzed by simultaneous linear regression analysis (Origin® 7.0 software) according to the competitive binding equation:

$$1/v = 1/V_{max} + 1/[S]^*(K_m^*(1 + [I]/K_i))/V_{max} \quad \text{Eq. 5}$$

where K_m is the Michaelis constant, V_{max} is the maximal steady-state rate, and the K_i is the apparent inhibition constant.

The time dependent activation of AMT as a PDF_{Ec} inhibitor was ascertained by dissolving the compound in 100% DMF at a concentration of 20 mM at time zero (performed in duplicate). The stocks, along with a buffer blank and a previously activated 20 mM stock of AMT in DMF, were then diluted to 500 μ M in 45 mM phosphate buffer, pH 7.0 containing 10% glycerol (v/v), 0.1 mg/mL BSA, 300 μ M substrate, 1 unit coupling enzyme and a final concentration of 5% DMSO or DMF (v/v). The reactions were started by addition of PDF_{Ec} and were monitored at 405 nm for 1 hour. This procedure was repeated at various time intervals over the course of one week.

UV/Vis spectroscopy—All UV/Vis absorption spectra were measured on a Molecular Devices SpectraMax[®] Plus microplate reader using either a quartz cuvette (1 cm path length) or a polystyrene microplate (path length approximately 0.3 cm). To compare the spectra of inactive AMT and bis-AMT, 200 μ L of each (250 μ M in 100% DMSO) was prepared and their spectra were obtained after subtraction of a solvent background.

To determine the time dependent changes in the absorption of AMT, the absorption spectrum of the inhibitor (543 μ M) was measured in the microplate reader in 45 mM phosphate buffer, pH 7.0 containing 10% glycerol (final DMF concentration = 5%). The absorption values at 350 nm were plotted as a function of time or the corresponding k_{obs} value obtained at the same time point.

ESI-MS analysis—To determine the time dependent structural changes of active form of AMT, AMT samples were dissolved in 100% DMF (final concentration = 20 mM) at various time points over the course of two weeks. The samples were then diluted in dd-H₂O (0.1% HOAc) and immediately loaded into Agilent[®] MSD Trap SL mass spectrometer with a flow rate of 0.3 mL/hr using a syringe pump (KDS 100, DK Scientific Inc.). MS settings for both full MS (m/z 50 to m/z 600) and MS² scans were: capillary voltage, -4500 V; nebulizer press, 15 psi; dry gas follow rate, 5 L/min; and dry temp 325 °C.

Synthesis of bis-AMT—To directly synthesize the disulfide form of AMT (i.e., bis-AMT), the synthetic protocol employed was that described by Mathes and Beber [45]. Briefly, AMT (500 mg) was placed in a glass test tube to which was added 1.67 mL ddH₂O while stirring to form a thick suspension. At this point, 180 mg NaOH was added and the mixture was stirred for 60 min at 4 °C. A solution of 539 mg of ammonium persulfate in 1.67 mL ddH₂O was prepared and added to the reaction in 200 μ L increments. The resultant mixture was stirred for 30 min at 4 °C during which a bright yellow precipitate was formed. The precipitate was separated by filtration, washed by approximately 500 mL ddH₂O and dried under vacuum for 2 hours. The dried powder that remained was recrystallized using a mixed-solvent system of 40% ddH₂O in DMSO (v/v). The recrystallized powder was washed with ddH₂O and air dried prior to use.

Molecular Modeling—Docking of bis-AMT to the X-ray crystal structure of PDF_{Ec} (PDB ID: 1G2A) [28] was performed on a Microsoft Windows[®]-based molecular modeling workstation utilizing Autodock 4.0 [46,47]. The parameters for the active site Ni²⁺ were set as follows: $r = 1.170$ Å, $q = +2.0$ and van der Waals well depth of 0.100 kcal mol⁻¹ [48]. A grid of 50 × 50 × 50 points was employed with a spacing of 0.375 Å centered on the active site of the enzyme. Using the Lamarckian genetic algorithm (LGA) as the search engine, 30 independent runs with a maximum number of 27,000 LGA operations were performed on a population size of 150 individuals. The maximum number of energy evaluations was set to 2,500,000 with a step size of 1.0 Å. All other parameters were left as the Autodock default

values. The structure of bis-AMT was prepared and energy minimized in ChemBioDraw® 11 using the MM2 force field prior to docking. Structural depictions of the docked complexes were produced using the UCSF Chimera modeling package [49] from the Resource for Biocomputing, Visualization, and Informatics (University of California, San Francisco; supported by NIH P41 RR-01081).

RESULTS

Discovery of 2-amino-5-mercapto-1,3,4-thiadiazole as a slow-binding inhibitor of *E. coli* PDF

While undertaking kinetic studies with *E. coli* peptide deformylase (PDF_{Ec}), we accidentally discovered that 2-amino-5-mercapto-1,3,4-thiadiazole (AMT; Figure 1A) serves as an inhibitor of the enzyme when the latter was dissolved in DMF and the resultant solution was stored for several days. No inhibitory effect of AMT was observed when this solution was freshly prepared in DMF or when the compound was dissolved in any other solvent and allowed to age at 25 °C for any length of time (Table 1). This unexpected observation led to the suggestion that DMF somehow caused slow “activation” of AMT, and the activated product served as an inhibitor of PDF_{Ec}. To ascertain the mechanistic basis of the DMF-assisted activation of AMT, we determined the time courses of the PDF_{Ec}-catalyzed reaction as a function of the incubation time of AMT in DMF (Figure 2, Panel A). Note that when AMT was incubated in DMF only for 30 min (open circles), the initial rate of the enzyme catalyzed reaction was barely lower than that of the control (solid circles). On the other hand, when the above preparation was allowed to age for 7 days, the initial rate of the enzyme catalysis decreased by about 50%. To our further interest, the overall reaction progress curve exhibited a downward curvature, as is apparent by the open triangles in Figure 2A. This has been in marked contrast to the linear increase in the reaction progress curve when AMT was dissolved in other solvents (Table 1) or briefly incubated in DMF. The downward curvature in the steady-state reaction profile was suggestive of the fact that the DMF-modified AMT served as a slow-binding inhibitor of the enzyme, a feature similar to that observed for the binding of actinonin (one of the highly potent inhibitors of the enzyme) [33] as well as other inhibitors of PDF [50–56].

Microscopic pathway for the slow-binding inhibition of PDF_{Ec}

We investigated the effect of AMT (prepared in DMF and stored for an extended period of time) on the steady-state kinetic profile (monitored at 405 nm) for the PDF_{Ec}-catalyzed reaction. These experiments were performed under the pseudo first-order condition for both substrate and AMT. As shown in the inset of Figure 2, Panel B, as the concentration of AMT increases the initial rate of the enzyme catalyzed reaction decreases, and the overall reaction profile exhibits rapidly attaining downward curvature. These observations led to the suggestion that the DMF-activated AMT served both as a conventional (i.e., the rate of inhibition occurred on the same time scale as the catalytic event) as well as the slow-binding inhibitor of the enzyme [43].

The latter feature is clearly apparent by the fact that the increasing concentration of the DMF-activated AMT enhances the rate of transition from the first (initial) steady-state to the second (final) steady-state. The main panel of Figure 2 (Panel B) shows the dependence of k_{obs} on the AMT concentration. Note the hyperbolic dependence of k_{obs} on AMT concentration, which supports the two-step (as opposed to one step) model of slow-binding inhibition (see Discussion). The data of Figure 2, Panel B was analyzed by the model of Eq. 2 (dotted line), with k_3 and k_4 being equal to 6.4 and $1.3 \times 10^{-4} \text{ sec}^{-1}$, respectively, and K_i equal to 190 μM . It should be emphasized that since the magnitude of k_4 is obtained from the extrapolation of the hyperbolic curve of the figure to zero concentration of the inhibitor, it is not very reliable. To circumvent this, recourse was made to analyze the data of Figure 2, Panel B by Eq. 3 (solid line) by fixing the magnitude of K_i^* as being equal to 3.7 μM . This latter value was obtained

by directly measuring the steady-state velocity of the PDF_{Ec}-catalyzed reaction at equilibrium (achieved after overnight incubation of different concentrations of AMT with DMF) as function of the activated concentration of AMT (data not shown). The analysis of the data provided values of k_3 and K_i of $7.3 \times 10^{-4} \text{ sec}^{-1}$ and $129 \mu\text{M}$, respectively, from which k_4 could be derived (via the relationship $K_i^* = K_i \times k_3 / (k_3 + k_4)$) to be $2.2 \times 10^{-5} \text{ sec}^{-1}$.

To ascertain the effect of substrate (⁵Met-Leu-*p*-nitroanilide) on the PDF_{Ec}-catalyzed inhibition by AMT [57], we examined the effect of increasing concentrations of substrate on k_{obs} for the AMT dependent inhibition of the enzyme (Figure 2, Panel C). Note that as the concentration of substrate increases, k_{obs} decreases in a hyperbolic manner, consistent with the competitive mode of binding of the inhibitor with respect to the substrate. The analysis of the data by Eq. 4 yielded the magnitudes of k_0 and K_m as being equal to 0.0027 sec^{-1} and $83 \mu\text{M}$, respectively. Moreover, the inset of Figure 2, Panel C shows the double-reciprocal plot for the initial velocity (v_0) of the PDF_{Ec}-catalyzed reaction in the presence of increasing concentrations of DMF-activated AMT at changing fixed concentrations of the substrate. The figure reveals intersecting linear-regression lines at the Y-axis, which is indicative of a competitive mode of inhibition, and global analysis of the data by Eq. 5 yielded the K_m , V_{max} , and K_i values to be $52 \mu\text{M}$, $0.65 \mu\text{M sec}^{-1}$, $170 \mu\text{M}$, respectively. .

Electronic spectral changes of AMT upon incubation in DMF

We noted that the freshly prepared solution of AMT in DMF was initially colorless, but its color changed to pale yellow upon aging. This observation was suggestive of the fact that the electronic structure of AMT underwent slow changes in the DMF solution. We purported to investigate whether the time dependent changes in the electronic structure of the DMF-dissolved AMT were related to its increased inhibitory potency for PDF_{Ec}.

Figure 3, Panel A shows the absorption spectrum of AMT as a function of different time of its incubation in DMF. Note that at zero time of incubation, the absorption spectrum of AMT was characterized by a peak at 295 nm. As the incubation time increased, the absorption peak at 295 nm started to decrease in intensity concomitant with the red shift of the absorption profile to 350 nm region. Panel B of Figure 3 depicts the increase in the absorption of the inhibitor as function of incubation time in DMF. Evidently, AMT underwent slow structural modification (see below) in DMF solution. Moreover, to our utmost interest, the increase in absorption of AMT at 350 nm was mirrored by the enhancement in inhibitory potency (given by k_{obs}) of the compound toward PDF_{Ec} as a function of the aging time of the compound in DMF solution (Figure 3, Panel C). The similar time dependent profiles supported the premise that the increase in the inhibitory potency of AMT and the changes in its electronic structure were due to the formation of the modified form of the compound (see below). This is further evident by the fact that the observed rate constant for the slow inhibition (k_{obs}) of the enzyme was apparently linearly correlated with the absorption of AMT at its corresponding time point (Figure 3, Panel D). Clearly, the DMF-modified form of AMT, *but not its native (unmodified) form*, served as the slow-binding inhibitor of PDF_{Ec}.

Nature of the structural modification of AMT upon incubation in DMF

To ascertain as to the nature of structural modification of AMT upon incubation in DMF, we subjected the above solution to ESI-MS analysis. Figure 4, Panel A shows the full MS spectrum of AMT after incubation in DMF for 14 days. The minor peaks of m/z 74 and m/z 147 appear to be the protonated form of DMF ($M_{\text{DMF}} + \text{H}^+$) and the protonated DMF dimer ($2M_{\text{DMF}} + \text{H}^+$), while the peaks of m/z 96 and m/z 169 corresponded to cationized DMF ($M_{\text{DMF}} + \text{Na}^+$) and cationized DMF dimer ($2M_{\text{DMF}} + \text{Na}^+$), respectively. On the other hand, the major peak of m/z 265 corresponded to the dimeric form of AMT with an added proton ($264 + 1 = 265$).

This was confirmed by the observation of fragmentation ion of m/z 134 ($M_{\text{AMT}} + 1$) in MS^2 analysis of m/z 265, as depicted in the inset of Figure 4, Panel A.

Based on the initial ESI-MS based assessment of the dimerization of AMT upon incubation in DMF ($m/z = 265$), we conjectured that the intensity of the above peak would increase as a function of the incubation time. To substantiate or refute the above possibility, we performed the ESI-MS experiment with AMT samples incubated for different times in DMF, and determined the intensity of the m/z 265 peak as a function of the incubation time. As shown in Figure 4, Panel A, there is no m/z 265 peak when AMT was freshly dissolved in DMF (i.e., at zero incubation time). However, as the incubation time increased, the intensity of the above peak started to increase in a linear fashion (Figure 4, Panel B). This trend has been similar to the increase in absorption of AMT solution in DMF at 350 nm (Figure 3, Panel B) coupled with the increase in the magnitude of k_{obs} for the slow-binding inhibition of the enzyme (Figure 3, Panel C). Hence, the AMT was slowly converted to a dimeric form (bis-AMT) upon incubation with DMF, which showed an increase in absorbance at 350 nm, and was responsible for the inhibition of the enzyme.

We further considered the possibility whether or not the DMF assisted formation of bis-AMT was due to simple oxidation of sulfhydryl group by oxidizing agent present in the organic solvent. To probe this, we incubated AMT with 0.06% H_2O_2 in buffer and measured the inhibitory potency of this reaction mixture for the enzyme (data not shown). As no inhibition of PDF_{Ec} was observed by the reaction mixture containing H_2O_2 , it appeared evident that H_2O_2 did not promote dimerization/dithiolation of AMT to produce bis-AMT and elicit its inhibitory feature. This coupled with the fact that no other organic solvent (i.e., other than DMF; Table 1) promoted the formation of bis-AMT, we surmise that the latter species is not produced by contamination of some unforeseen oxidizing agent (e.g., peroxides) in the DMF solution.

Confirmation of bis-AMT being the inhibitory species for PDF_{Ec}

In pursuit of unraveling the molecular nature of the putative dimeric form of AMT which was responsible for inhibiting PDF_{Ec} , we surmised that DMF might promote the formation of the disulfide bond between the two AMT moieties to generate bis-AMT. The latter compound (shown in Panel B of Figure 1) would exhibit a mass of 264 daltons, which is in excellent agreement with the observed m/z ratio of 265 ($M_{\text{bis-AMT}} + 1$) obtained from the ESI-MS analysis of the DMF-activated inhibitor (Figure 4, Panel A).

To confirm that the dithiolated bis-AMT was the inhibitory species, we synthesized the latter compound as described by Mathes and Beber [45]. A visual inspection of the crystalline form of the freshly synthesized bis-AMT *vis a vis* its monomeric counterpart (i.e., parent AMT) revealed that the former was bright yellow in color as compared to the pale yellow color of the parent compound. This observation was reminiscent of the color change of AMT upon incubation with DMF (see Figure 3).

Figure 5, Panel A shows the comparative absorption spectra of AMT and newly synthesized bis-AMT (each at a concentration of 250 μM in DMSO). The solid and dashed lines are the spectra of AMT and bis-AMT, respectively. Although the absorption spectra of both these compounds are different in DMSO as compared to that observed when the activated compound was diluted in aqueous solution (containing 5% DMF; Figure 3, Panel A), it is evident that the spectral band of bis-AMT is considerably broad around the 400 nm region and is slightly red-shifted as compared to its monomeric counterpart. These spectral features are qualitatively similar to those observed during the course of the DMF-assisted transformation of AMT to bis-AMT (Figure 3, Panel A).

Finally, we evaluated the efficiency of newly synthesized bis-AMT in inhibiting PDF_{Ec}. This was achieved by measuring the activity of PDF_{Ec} in the presence of increasing concentrations of bis-AMT. Note that the stock of bis-AMT was prepared in DMSO, which did not activate AMT to serve as the PDF_{Ec} inhibitor. The steady-state reaction profiles of PDF_{Ec} in the presence of increasing concentrations of bis-AMT were similar to those observed for the DMF-activated AMT (data not shown), suggesting that bis-AMT rather than its parent compound (AMT) served as the slow-binding inhibitor of PDF_{Ec}. The k_{obs} values derived from such plots (by analyzing the raw data by Eq. 1) exhibited a hyperbolic profile (Figure 5, Panel B), which is similar to the data of Figure 2, Panel B. The analysis of the data yielded the magnitudes of k_3 and k_4 being equal to 8.0×10^{-4} and $3.6 \times 10^{-6} \text{ sec}^{-1}$, respectively, and the K_i value of 490 μM . Using these values, a K_i^* of 2.2 μM was obtained via the relationship $K_i^* = K_i \times k_3 / (k_3 + k_4)$, which is in excellent agreement with the K_i^* of 3.7 μM determined directly for the DMF-activated AMT at equilibrium.

DISCUSSION

The experimental data presented in the preceding sections reveals, for the first time (to the best of our knowledge), that 2-amino-5-mercapto-1,3,4-thiadiazole (AMT) is able to serve as a competitive, slow-binding inhibitor of *E. coli* peptide deformylase (PDF_{Ec}) when it is “activated” by DMF as a solvent. The DMF-mediated activation involves the dimerization of two AMT molecules via the dithiol linkage forming bis-AMT, which serves as a slow-binding inhibitor of the enzyme. This conclusion has been corroborated by the fact that directly synthesized bis-AMT exhibits similar spectroscopic and inhibitory features as those obtained with the DMF-incubated parent compound (AMT).

Since the activation/dimerization of AMT in DMF was extremely slow, it appeared likely that some intermediary species would predominate during the course of the overall process. However, this expectation was not supported by the ESI-MS profiles (Figure 4). This coupled with the fact that the activated form of AMT is the dimer of the parent compound leads to the suggestion that DMF, at the best, functions as a slow catalyst. A tentative sequence of steps involved in the overall dimerization reaction is in Scheme 1. With literature precedents [32–34], we believe DMF promotes initial formylation of AMT, which serves as a site for bridging the two AMT moieties and juxtaposing their thiol groups. These thiol groups are slowly oxidized to produce the dithiolated form of AMT (bis-AMT) with concomitant loss of formic acid. We are currently in the process of probing the existence of putative intermediates as well as the products, and we will report these findings subsequently.

Our experimental data clearly reveal that bis-AMT serves as a slow-binding inhibitor of PDF_{Ec} and that the overall microscopic pathway conforms to the two (instead of one) step binding process. Whereas the latter case is essentially a simple one-step binding mechanism (with a kinetically slow rate of binding defined by the magnitudes of the microscopic rate constants, k_1 and k_2), the two-step mechanism represents the condition where the slow-binding process is biphasic in nature and consists of a relatively rapid collision phase ($E + I \rightleftharpoons EI$) followed by the kinetically slow isomerization phase ($EI \rightleftharpoons EI^*$) [43]. Note that given the magnitudes of K_i and K_i^* as being equal to 129 and 3.7 μM , respectively, it is significant that the slow-binding step enhances the apparent binding affinity of AMT for the enzyme by about 30 fold.

It should be pointed out that the two-step binding of inhibitors to various PDF isozymes is fairly common. In fact, the most prominent inhibitor of PDF, actinonin, has been reported to bind via a two-step mechanism which has been characterized for the *S. aureus* enzyme [33]. The binding of actinonin to Ni²⁺-substituted *S. aureus* PDF (PDF_{Sa}) features microscopic rate constants for the EI to EI^{*} conversion, k_3 and k_4 , of 0.033 and $\leq 9.8 \times 10^{-6} \text{ sec}^{-1}$, respectively

[33]. When compared to the values obtained herein for bis-AMT (8.0×10^{-4} and $3.6 \times 10^{-6} \text{ sec}^{-1}$ for k_3 and k_4 , respectively) it is apparent that whereas the value of k_4 is of the same order of magnitude, the value of k_3 is 40 fold higher for the PDF_{Sa}-actinonin interaction. This explains the slower onset of full inhibition (viz. isomerization phase) in the case of bis-AMT, which features a $t_{1/2}$ of approximately 14 minutes at saturating concentrations of the inhibitor. As a result of the relatively small value of k_3 for the binding of bis-AMT to PDF_{Ec}, the isomerization phase does not pull the overall equilibrium from the collision complex ($E + I \rightleftharpoons EI$) to the isomerized complex ($EI \rightleftharpoons EI^*$). Van Aller *et al.* have reported that the difference in binding energy ($\Delta\Delta G^\circ$) between the two phases is equal to $-4.5 \text{ kcal mol}^{-1}$ at 25°C [33]. Using a standard state of 1.0 M, a $\Delta\Delta G^\circ$ of $-3.2 \text{ kcal mol}^{-1}$ was obtained for bis-AMT upon isomerization via the relationship, $\Delta\Delta G^\circ = RT \ln K_i^*/K_i$. It should be noted that although the reported $\Delta\Delta G^\circ$ for actinonin is $1.3 \text{ kcal mol}^{-1}$ lower than that for bis-AMT, in the latter case, the isomerization phase is responsible for more than 40% of the total $-7.5 \text{ kcal mol}^{-1}$ of binding energy (based on an average K_i^* of $3.0 \mu\text{M}$) as opposed to approximately 30% for actinonin. This demonstrates that the inhibition of PDF_{Ec} by bis-AMT is strongly dependent upon the isomerization phase for the overall energy of the binding interaction.

The origin of the two-step slow-binding mechanism for enzyme-inhibitor interactions is generally attributed to an initial binding event followed by a conformational change in the structure of the enzyme-inhibitor complex which gives rise to the isomerization phase [58–60]. This is equivalent to the induced-fit model of protein-ligand binding, with the distinction that the adjustment of the protein conformation takes place secondary to the initial binding event. However, it has been indicated in the case of actinonin that structural changes which are responsible for the conversion from EI to EI^* are not evident upon comparison between the bound and free forms of PDF [32,33]. This observation, coupled with the recent delineation of at least two coexisting conformational states of PDF_{Ec} (both of which bind actinonin) [34] prompts the question as to whether the slow-binding inhibition of PDF by compounds such as bis-AMT is truly associated with a major conformational change of the enzyme-inhibitor complex or subtle (albeit slow) adjustment in the protein-ligand structure. Revelation of these features must await further structural-functional investigations of the PDF_{Ec} in the presence of bis-AMT.

In agreement with all other PDF inhibitors characterized [25], the data of Figure 2 demonstrates that the inhibition of PDF_{Ec} by bis-AMT is competitive in nature. As the majority of PDF inhibitors are peptides or pseudo-peptides that mimic the natural ^FMet-peptide substrates, much of the binding energy for these inhibitors is proposed to arise due to enzyme-inhibitor contacts that are similar to those of the native substrates [25]. However, as bis-AMT is structurally dissimilar from most other PDF inhibitors, it was unclear as to how it served as a competitive inhibitor of the enzyme. To elucidate this feature, we performed molecular modeling studies for docking bis-AMT to the active site of PDF_{Ec} (PDB ID: 1G2A) [28] via Autodock 4 (see Experimental section). Panel A of Figure 6 depicts the stereo view of the best-docked structure of bis-AMT at the active site of the enzyme. A casual perusal of the docked structure of Figure 6, Panel A reveals several interesting features of the enzyme-inhibitor complex. The most notable structural feature is the orientation of bis-AMT with respect to the active site Ni^{2+} . The metal ion is coordinated to the 2-amino group as well as the adjacent ring nitrogen of the thiadiazole ring at distances of 2.55 and 2.81 Å, respectively. These distances are slightly longer than those observed for the hydroxamate of actinonin complex, viz., 2.20 and 2.30 Å, respectively [28]. This is not surprising since bis-AMT is a weaker inhibitor for PDF_{Ec} than actinonin [23,34]. In addition to the coordination bonds between the active site resident metal ion and bis-AMT, several potential hydrogen bonds (depicted in cyan in Figure 6) were observed between the inhibitor and active site residues which are in close proximity to the active site of the compound. Specifically, putative hydrogen bonds were indicated between the metal-coordinating 2-amino group of the inhibitor and the side-chain oxygens of Glu133 and

Gln50, as well as between the 2-amino group of the distal (solvent exposed) thiadiazole ring and the backbone oxygen of Glu87. The latter hydrogen bonding interaction is important for the bis-AMT interaction, as this represents the only hydrogen bond specific to the dimeric form of the inhibitor versus the AMT monomer. Moreover, the hydrogen bonds involving Glu133 and Gln50 are of particular interest, as these residues are highly conserved amongst PDF isozymes due to their essential function in catalysis [17]. Both residues have been postulated to stabilize the ^FMet-peptide substrates of PDF through the formation of intermolecular hydrogen bonds (see Figure 6, Panel B), with Glu133 also functioning as a proton shuttle [61]. In this regard, it may be concluded that bis-AMT is able to make similar contacts with the enzyme site as those made by the substrate upon binding (Figure 6, Panel C).

It should be further emphasized that although bis-AMT is observed to make van der Waals contacts with both the S₂' and S₃' subsites of PDF_{Ec} [30–32], notably absent in the structure of Figure 6 are contacts between the inhibitor and the S₁' subsite of the enzyme. (For clarity, refer to the schematic diagram representing the residues that comprise the various subsites of PDF_{Ec} provided in Figure 6, Panels B and C.) The latter subsite is largely hydrophobic in nature and represents the pocket where the methionine sidechain of the substrate resides upon binding to the enzyme. As PDF is highly specific for N-formyl methionine substrates, the S₁'-methionine interaction has been postulated to be important in substrate recognition and binding [17,29–32], and consequently the majority of PDF inhibitors exploit this interaction through the use of methionine-like sidechains at their P₁' positions [25]. Since bis-AMT does not feature such a structural moiety, it is not surprising that only limited contact between the inhibitor and the S₁' subsite is present in the docked structure of bis-AMT (Figure 6, Panels A and C). Hence, it is likely that the lower affinity of PDF_{Ec} for bis-AMT versus other inhibitors is a consequence of the lack of interaction between the inhibitor and the enzyme at this position. It is therefore possible that the binding affinity of bis-AMT for the enzyme site may be enhanced significantly through the modification of the bis-AMT structure such that the inhibitor features a hydrophobic moiety in a position where it may interact with the S₁' subsite of PDF_{Ec}.

ABBREVIATIONS

PDF	peptide deformylase
Ec	<i>E. coli</i>
AMT	2-amino-5-mercapto-1,3,4-thiadiazole
DMF	N,N'-dimethylformamide
NME	N-terminal methionine excision
^F Met	formyl-methionine
DMSO	dimethylsulfoxide
BSA	bovine serum albumin
HOAc	acetic acid
ESI	electrospray ionization
Sa	<i>S. aureus</i>

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REFERENCES

1. Marcker K, Sanger F. N-Formyl-Methionyl-S-RNA. *J. Mol. Biol* 1964;8:835–840. [PubMed: 14187409]
2. Meinnel T, Mechulam Y, Blanquet S. Methionine as translation start signal: a review of the enzymes of the pathway in *Escherichia coli*. *Biochimie* 1993;75:1061–1075. [PubMed: 8199241]
3. Giglione C, Meinnel T. Organellar peptide deformylases: universality of the N-terminal methionine cleavage mechanism. *Trends Plant Sci* 2001;6:566–572. [PubMed: 11738381]
4. Giglione C, Vallon O, Meinnel T. Control of protein life-span by N-terminal methionine excision. *EMBO J* 2003;22:13–23. [PubMed: 12505980]
5. Bingel-Erlenmeyer R, Kohler R, Kramer G, Sandikci A, Antolić S, Maier T, Schaffitzel C, Wiedmann B, Bukau B, Ban N. A peptide deformylase-ribosome complex reveals mechanism of nascent chain processing. *Nature* 2008;452:108–111. [PubMed: 18288106]
6. Giglione C, Fieulaine S, Meinnel T. Cotranslational processing mechanisms: towards a dynamic 3D model. *Trends Biochem. Sci* 2009;34:417–426. [PubMed: 19647435]
7. Giglione C, Boularot A, Meinnel T. Protein N-terminal methionine excision. *Cell. Mol. Life Sci* 2004;61:1455–1474. [PubMed: 15197470]
8. Giglione C, Meinnel T. Peptide deformylase as an emerging target for antiparasitic agents. *Expert Opin. Ther. Targets* 2001;5:41–57. [PubMed: 15992167]
9. Serero A, Giglione C, Sardini A, Martinez-Sanz J, Meinnel T. An unusual peptide deformylase features in the human mitochondrial N-terminal methionine excision pathway. *J. Biol. Chem* 2003;278:52953–52963. [PubMed: 14532271]
10. Lee MD, Antczak C, Li Y, Sirotnak FM, Bornmann WG, Scheinberg DA. A new human peptide deformylase inhibitable by actinonin. *Biochem. Biophys. Res. Commun* 2003;312:309–315. [PubMed: 14637138]
11. Lee MD, She Y, Soskis MJ, Borella CP, Gardner JR, Hayes PA, Dy BM, Heaney ML, Philips MR, Bornmann WG, Sirotnak FM, Scheinberg DA. Human mitochondrial peptide deformylase, a new anticancer target of actinonin-based antibiotics. *J. Clin. Invest* 2004;114:1107–1116. [PubMed: 15489958]
12. Von Jagow G, Schagger H, Engel WD, Machleidt W, Machleidt I, Kolb HJ. Beef heart complex III: Isolation and characterization of cytochrome B. *FEBS Lett* 1978;91:121–125. [PubMed: 668902]
13. Tanaka M, Yasunobu KT, Wei YH, King TE. The complete amino acid sequence of bovine heart cytochrome oxidase subunit VI. *J. Biol. Chem* 1981;256:4832–4837. [PubMed: 6262305]
14. Nguyen KT, Hu X, Colton C, Chakrabarti R, Zhu MX, Pei D. Characterization of a human peptide deformylase: implications for antibacterial drug design. *Biochemistry* 2003;42:9952–9958. [PubMed: 12924944]
15. Adams JM. On the release of the formyl group from nascent protein. *J. Mol. Biol* 1968;33:571–589. [PubMed: 4973445]
16. Yuan Z, White RJ. The evolution of peptide deformylase as a target: contribution of biochemistry, genetics and genomics. *Biochem. Pharmacol* 2006;71:1042–1047. [PubMed: 16289392]
17. Giglione C, Pierre M, Meinnel T. Peptide deformylase as a target for new generation, broad spectrum antimicrobial agents. *Mol. Microbiol* 2000;36:1197–1205. [PubMed: 10931273]
18. Meinnel T, Blanquet S. Characterization of the *Thermus thermophilus* locus encoding peptide deformylase and methionyl-tRNA(fMet) formyltransferase. *J. Bacteriol* 1994;176:7387–7390. [PubMed: 7961514]
19. Mazel D, Pochet S, Marlière P. Genetic characterization of polypeptide deformylase, a distinctive enzyme of eubacterial translation. *EMBO J* 1994;13:914–923. [PubMed: 8112305]
20. Bandow JE, Becher D, Büttner K, Hochgräfe F, Freiberg C, Brötz H, Hecker M. The role of peptide deformylase in protein biosynthesis: a proteomic study. *Proteomics* 2003;3:299–306. [PubMed: 12627383]

21. Wang W, White R, Yuan Z. Proteomic study of peptide deformylase inhibition in *Streptococcus pneumoniae* and *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2006;50:1656–1663. [PubMed: 16641432]
22. Guay DRP. Drug forecast - the peptide deformylase inhibitors as antibacterial agents. *Ther. Clin. Risk Manag* 2007;3:513–525. [PubMed: 18472972]
23. Chen DZ, Patel DV, Hackbarth CJ, Wang W, Dreyer G, Young DC, Margolis PS, Wu C, Ni ZJ, Trias J, White RJ, Yuan Z. Actinonin, a naturally occurring antibacterial agent, is a potent deformylase inhibitor. *Biochemistry* 2000;39:1256–1262. [PubMed: 10684604]
24. Broughton BJ, Chaplen P, Freeman WA, Warren PJ, Wooldridge KR, Wright DE. Studies concerning the antibiotic actinonin. Part VIII. Structure-activity relationships in the actinonin series. *J. Chem. Soc. Perkin* 1975;1:857–860.
25. Jain R, Chen D, White RJ, Patel DV, Yuan Z. Bacterial Peptide deformylase inhibitors: a new class of antibacterial agents. *Curr. Med. Chem* 2005;12:1607–1621. [PubMed: 16022661]
26. Kola I, Landis J. Can the pharmaceutical industry reduce attrition rates? *Nat. Rev. Drug Discov* 2004;3:711–715. [PubMed: 15286737]
27. Caldwell J, Gardner I, Swales N. An introduction to drug disposition: the basic principles of absorption, distribution, metabolism, and excretion. *Toxicol. Pathol* 1995;23:102–114. [PubMed: 7569663]
28. Clements JM, Beckett RP, Brown A, Catlin G, Lobell M, Palan S, Thomas W, Whittaker M, Wood S, Salama S, Baker PJ, Rodgers HF, Barynin V, Rice DW, Hunter MG. Antibiotic activity and characterization of BB-3497, a novel peptide deformylase inhibitor. *Antimicrob. Agents Chemother* 2001;45:563–570. [PubMed: 11158755]
29. Becker A, Schlichting I, Kabsch W, Groche D, Schultz S, Wagner AF. Iron center, substrate recognition and mechanism of peptide deformylase. *Nat. Struct. Biol* 1998;5:1053–1058. [PubMed: 9846875]
30. Hao B, Gong W, Rajagopalan PT, Zhou Y, Pei D, Chan MK. Structural basis for the design of antibiotics targeting peptide deformylase. *Biochemistry* 1999;38:4712–4719. [PubMed: 10200158]
31. Ragusa S, Mouchet P, Lazennec C, Dive V, Meinnel T. Substrate recognition and selectivity of peptide deformylase. Similarities and differences with metzincins and thermolysin. *J. Mol. Biol* 1999;289:1445–1457. [PubMed: 10373378]
32. Guilloteau J, Mathieu M, Giglione C, Blanc V, Dupuy A, Chevrier M, Gil P, Famechon A, Meinnel T, Mikol V. The crystal structures of four peptide deformylases bound to the antibiotic actinonin reveal two distinct types: a platform for the structure-based design of antibacterial agents. *J. Mol. Biol* 2002;320:951–962. [PubMed: 12126617]
33. Van Aller GS, Nandigama R, Petit CM, DeWolf WEJ, Quinn CJ, Aubart KM, Zalacain M, Christensen SB, Copeland RA, Lai Z. Mechanism of time-dependent inhibition of polypeptide deformylase by actinonin. *Biochemistry* 2005;44:253–260. [PubMed: 15628866]
34. Berg AK, Srivastava DK. Delineation of alternative conformational states in *Escherichia coli* peptide deformylase via thermodynamic studies for the binding of actinonin. *Biochemistry* 2009;48:1584–1594. [PubMed: 19191548]
35. Malkhasian AYS, Finch ME, Nikolovski B, Menon A, Kucera BE, Chavez FA. N,N'-dimethylformamide-derived products from catalytic oxidation of 3-hydroxyflavone. *Inorg. Chem* 2007;46:2950–2952. [PubMed: 17378555]
36. Vilsmeier A, Haack A. Über die einwirkung von halogenphosphor auf alkylformanilide. Eine neue methode zur darstellung sekundärer und tertiärer p-alkylamino-benzaldehyde. *Chem. Ber* 1927;60:119–122.
37. Freeman F, Keindl M. A facile synthesis of s-(1-chloroalkyl) alkanesulfonothioates. *Synthesis* 1984;6:500–502.
38. Berg AK, Manokaran S, Eiler D, Kooren J, Mallik S, Srivastava DK. Energetic rationale for an unexpected and abrupt reversal of guanidinium chloride-induced unfolding of peptide deformylase. *Protein Sci* 2008;17:11–15. [PubMed: 18042674]
39. Ragusa S, Blanquet S, Meinnel T. Control of peptide deformylase activity by metal cations. *J Mol Biol* 1998;280:515–523. [PubMed: 9665853]

40. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem* 1976;72:248–254. [PubMed: 942051]
41. Rajagopalan PT, Datta A, Pei D. Purification, characterization, and inhibition of peptide deformylase from *Escherichia coli*. *Biochemistry* 1997;36:13910–13918. [PubMed: 9374870]
42. Wei Y, Pei D. Continuous spectrophotometric assay of peptide deformylase. *Anal. Biochem* 1997;250:29–34. [PubMed: 9234895]
43. Sculley MJ, Morrison JF, Cleland WW. Slow-binding inhibition: the general case. *Biochim. Biophys. Acta* 1996;1298:78–86. [PubMed: 8948491]
44. Tian WX, Tsou CL. Determination of the rate constant of enzyme modification by measuring the substrate reaction in the presence of the modifier. *Biochemistry* 1982;21:1028–1032. [PubMed: 7074045]
45. Mathes RA, Beber AJ. A synthesis of 2-thiazolethiol and its disulfide. *J. Amer. Chem. Soc* 1948;70:1451–1452. [PubMed: 18915762]
46. Morris G, Goodsell D, Halliday R, Huey R, Hart W, Belew A, Olson A. Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *Comp. Chem* 1998;19:1639–1662.
47. Huey R, Morris GM, Olson AJ, Goodsell DS. A semiempirical free energy force field with charge-based desolvation. *J. Comput. Chem* 2007;28:1145–1152. [PubMed: 17274016]
48. Musiani F, Arnofi E, Casadio R, Ciurli S. Structure-based computational study of the catalytic and inhibition mechanisms of urease. *J. Biol. Inorg. Chem* 2001;6:300–314. [PubMed: 11315566]
49. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. UCSF Chimera—a visualization system for exploratory research and analysis. *J. Comput. Chem* 2004;25:1605–1612. [PubMed: 15264254]
50. Chu M, Mierzwa R, He L, Xu L, Gentile G, Terracciano J, Patel M, Miesel L, Bohanon S, Kravec C, Cramer C, Fischman TO, Hruza A, Ramanathan L, Shipkova P, Chan TM. Isolation and structure elucidation of two novel deformylase inhibitors produced by *Streptomyces* sp. *Tetrahed. Lett* 2001;42:3549–3551.
51. Grant SK, Green BG, Kozarich JW. Inhibition and structure-activity studies of methionine hydroxamic acid derivatives with bacterial peptide deformylase. *Bioorg. Chem* 2001;29:211–222. [PubMed: 16256693]
52. Madison V, Duca J, Bennett F, Bohanon S, Cooper A, Chu M, Desai J, Girijavallabhan V, Hare R, Hruza A, Hendrata S, Huang Y, Kravec C, Malcolm B, McCormick J, Miesel L, Ramanathan L, Reichert P, Saksena A, Wang J, Weber PC, Zhu H, Fischmann T. Binding affinities and geometries of various metal ligands in peptide deformylase inhibitors. *Biophys. Chem* 2002;101–102:239–247.
53. Nguyen KT, Hu X, Pei D. Slow-binding inhibition of peptide deformylase by cyclic peptidomimetics as revealed by a new spectrophotometric assay. *Bioorg. Chem* 2004;32:178–191. [PubMed: 15110195]
54. Hu X, Nguyen KT, Jiang VC, Lofland D, Moser HE, Pei D. Macrocyclic inhibitors for peptide deformylase: a structure-activity relationship study of the ring size. *J. Med. Chem* 2004;47:4941–4949. [PubMed: 15369398]
55. Boularot A, Giglione C, Petit S, Duroc Y, de Sousa R Alves, Larue V, Cresteil T, Dardel F, Artaud I, Meinnel T. Discovery and refinement of a new structural class of potent peptide deformylase inhibitors. *J. Med. Chem* 2007;50:10–20. [PubMed: 17201406]
56. Shen G, Zhu J, Simpson AM, Pei D. Design and synthesis of macrocyclic peptidyl hydroxamates as peptide deformylase inhibitors. *Bioorg. Med. Chem. Lett* 2008;18:3060–3063. [PubMed: 18093832]
57. Segel, IH. *Enzyme kinetics: behavior and analysis of rapid equilibrium and steady-state enzyme systems*. New York: John Wiley and Sons, Inc.; 1975.
58. Bernhard SA. The coupling of conformational changes to function in proteins. *Stud. Org. Chem* 1982;10:237–252.
59. Johnson JK, Wang ZX, Srivastava DK. Mechanistic investigation of medium-chain fatty acyl-CoA dehydrogenase utilizing 3-indolepropionyl/acryloyl-CoA as chromophoric substrate analogues. *Biochemistry* 1992;31:10564–10575. [PubMed: 1309181]

60. Johnson JK, Srivastava DK. Detection and identification of a chromophoric intermediate during the medium-chain fatty acyl-CoA dehydrogenase-catalyzed reaction via rapid-scanning UV/visible spectroscopy. *Biochemistry* 1993;32:8004–8013. [PubMed: 8267794]
61. Rajagopalan PT, Grimme S, Pei D. Characterization of cobalt(II)-substituted peptide deformylase: function of the metal ion and the catalytic residue Glu-133. *Biochemistry* 2000;39:779–790. [PubMed: 10651644]

A **B**

Figure 1.
Chemical structures of 2-amino-5-mercapto-1,3,4-thiadiazole (AMT) and bis-AMT in Panels A and B, respectively.

Figure 2.

Steady-state and transient kinetic analysis of the inhibition of PDF_{Ec} activity by AMT in the presence of fixed and varied concentrations of substrate. Panel A depicts the inhibition of PDF_{Ec} activity by 500 μM AMT as a function of increasing incubation time of the inhibitor in DMF. The reaction progress curves of the PDF_{Ec}-catalyzed reaction are shown in the absence (solid circles) and presence of 500 μM AMT incubated in DMF for 30 min (open circles) and 7 days (open triangles). The solid lines represent the linear fit of the initial portion of the reaction progress curves, whereas the dashed line represents the best fit of the data by Eq. 1. Panel B shows the dependence of k_{obs} (open circles) on the concentration of DMF-activated AMT and represent the average k_{obs} value from two independent assays. The dashed line represents the

best fit of the data by Eq. 2, which provided values of 6.4×10^{-4} and $1.3 \times 10^{-4} \text{ sec}^{-1}$ for k_3 and k_4 , respectively, and a K_i value of $190 \mu\text{M}$. The solid line represents the best fit of the data by Eq. 3 (with the value for K_i^* fixed at $3.7 \mu\text{M}$) and provided values of 7.3×10^{-4} and $2.2 \times 10^{-5} \text{ sec}^{-1}$ for k_3 and k_4 , respectively, as well as a K_i value of $129 \mu\text{M}$. The inset of panel B shows the reaction progress curves for the PDF_{EC}-catalyzed reaction in the presence of $150 \mu\text{M}$ (open circles), $300 \mu\text{M}$ (solid squares), $600 \mu\text{M}$ (open squares) and $1200 \mu\text{M}$ (solid triangles) inhibitor with the solid lines depicting the best fit of the data by Eq. 1. Panel C shows the dependence of k_{obs} for the PDF_{EC}-catalyzed reaction in the presence of $250 \mu\text{M}$ DMF-activated AMT as a function of ^FMet-Leu-*p*-nitroanilide concentration. The solid line indicates the best fit of the data by Eq. 4 which provided a value of 0.0027 sec^{-1} for k_0 and a K_m of $83 \mu\text{M}$. The double-reciprocal plot for the PDF_{EC}-catalyzed reaction is given in the inset of panel C at concentrations of 0, 30, 50 and $100 \mu\text{M}$ AMT (open squares, solid squares, open triangles and solid circles, respectively). The solid lines of the inset represent the simultaneous fit of all the datasets by Eq. 5, which provided values of V_{max} and K_m of $0.65 \mu\text{M sec}^{-1}$ and $54 \mu\text{M}$, respectively, as well as a K_i of $170 \mu\text{M}$.

Figure 3.

Time dependent changes in the UV/Vis absorption spectrum and k_{obs} of AMT as a function of incubation time in DMF. Panel A shows the spectrum of AMT in 45 mM phosphate buffer, pH 7.0 containing 10% glycerol (final DMF concentration = 5%) after different times of incubation in DMF, with the arrows indicating the time dependent changes in the spectrum. Panel B depicts the dependence of the absorption at 350 nm as a function of incubation time in DMF and represents the average of two independent measurements. Panel C shows is the dependence of the k_{obs} value for the inhibition of PDF_{Ec} by 500 μM AMT (calculated from the average of two independent measurements at corresponding time-points) as a function of incubation time in DMF. Panel D depicts the correlation between the absorption at 350 nm and the k_{obs} at corresponding time-points.

Figure 4.

Mass spectral analysis of AMT upon incubation in DMF. Panel A shows the mass spectrum of AMT (in units of ion current) after 15 days incubation in DMF, with the mass to charge ratio (m/z) indicated by the numbers located above each peak. The inset of Panel A indicates the fragmentation spectrum of the m/z 265 species determined via MS^2 analysis. Panel B shows the intensity of the m/z 265 peak as a function of incubation time of AMT in DMF.

Figure 5.

UV/Vis absorption spectrum and inhibition properties of bis-AMT. Panel A shows the overlay of the monomeric and disulfide forms of AMT at 250 μM in DMSO (solid and dashed lines, respectively). Panel B depicts the transient-kinetic analysis of the slow-binding inhibition of PDF_{Ec} by bis-AMT, where the open circles depict the dependence of k_{obs} on the concentration of inhibitor (as determined by the best fit of the activity traces by Eq. 1). The solid line represents the best fit of the data by Eq. 2, which provided values of 8.0×10^{-4} and $3.6 \times 10^{-6} \text{ sec}^{-1}$ for k_3 and k_4 , respectively, and a K_i value of 490 μM .

Figure 6.

Representations of bis-AMT and ^FMet-Leu-*p*-nitroanilide in complex with the active site of PDF_{Ec}. Panel A depicts the stereo representation of bis-AMT docked to the active site of PDF_{Ec}. Docking of bis-AMT to the structure of the Ni²⁺-substituted form of PDF_{Ec} (PDB ID: 1G2A) [28] was performed on a Microsoft Windows®-based molecular modeling workstation using Autodock 4.0 [46,47]. The figure was produced using the UCSF Chimera modeling package [49] and shows the best-docked (lowest energy) structure of bis-AMT (van der Waals surface shown in grid format) as well as active site residues of PDF_{Ec} which were located within 3.5 Å of the inhibitor. The purple lines indicate coordination bonds whereas the cyan lines indicate putative hydrogen bonds. Panels B and C depict the schematic representation of the putative "subsites" and relevant hydrogen bonding interactions within the active site pocket of PDF_{Ec} with either ^FMet-Leu-*p*-nitroanilide or bis-AMT bound (Panels B and C, respectively) [17,29–32]. In both panels, the bound ligand is shown in black, with hydrogen bonds given by thin dashed lines and coordination bonds given by wide dashed lines.

Scheme 1.

Proposed mechanism for the DMF-mediated activation of AMT to bis-AMT.

Table 1Inhibition of PDF_{Ec} by AMT when dissolved in selected solvents^a

Solvent	Initial rate (mOD min ⁻¹)	% of control
Control (no inhibitor or solvent)	16.9	100
50 mM potassium phosphate, pH 7.0	17.5	103
Ethanol	16.9	100
Acetonitrile	16.3	96
Dimethylsulfoxide	15.9	94
Dimethylformamide (immediately)	17.0	101
Dimethylformamide (1 week post)	7.9	47

^a All inhibitors were tested at a concentration of 500 μ M after more than 2 days incubation in their respective solvent unless stated otherwise. Note that no change was observed at any time of AMT incubation in any solvent except DMF.