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Phosphonamidate and Phosphothioate Dipeptides as Potential Inhibitors of VanX

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Abstract—In an effort to prepare novel inhibitors of VanX, *N*-[(1-aminoethyl)hydroxyphosphinyl]-D-alanine **1** and *S*-[(aminoethyl)hydroxyphosphinyl]-thiolacetic acid **2** were synthesized and evaluated as inhibitors of VanX. Phosphonamidate **1** was shown to be a partial competitive inhibitor of VanX with a K_i of 36±3 μM, and phosphothioate **2** was shown not to inhibit VanX.

VanX is a Zn(II) metalloenzyme that is required for high-level vancomycin resistance in bacteria. The intracellular role of VanX is to hydrolyze vancomycin-binding D-Ala-D-Ala dipeptides that are used to synthesize the normal bacterial peptidoglycan layer.[1] Vancomycin-resistant bacteria have acquired the ability to produce D-Ala-D-lactate depsipeptides which can be integrated into the peptidoglycan layer; however, these depsipeptides bind vancomycin 10^3 weaker than their dipeptide counterparts.[2] Reynolds et al. reported that *Enterococcus faecium* strains containing functional VanX produced vancomycin-suspectible D-Ala-D-Ala containing precursors to vancomycin-resistant D-Ala-D-lactate containing precursors in a ratio of 1:49.[3] The removal of VanX activity changes this ratio to 1:1. Clearly, VanX is an excellent target for the generation of inhibitors, which could be used in combination with vancomycin, to combat vancomycin resistance.

Towards this goal, Walsh and co-workers have examined the inhibition properties of a number of phosphonate and phosphinate analogues of D-Ala-D-Ala as well as of several dithiol compounds.[1,4,5] These analogues were shown to inhibit VanX with K_i values ranging from 90 nM to 300 μM and exhibit competitive, mixed, and slow-binding modes of inhibition. The subsequent crystal structure[6] with bound D-Ala-D-Ala, a phosphonate analogue, and a phosphinate analogue demonstrated that VanX has a small, selective active site binding pocket and that small changes in inhibitor structure can affect K_i values by as much as 10^3, mirroring the exquisite selectivity of VanX for D-Ala-D-Ala over D-Ala-D-lactate.

Previously, Bartlett and co-workers have synthesized a number of phosphorus-containing peptide analogues as inhibitors of hydrolytic enzymes,[7–10] including thermolysin which is a Zn(II)-requiring peptidase. Several studies using thermolysin indicated that phosphonamidate analogues of the peptides bound >800 times stronger than the corresponding phosphonate analogue, due to the fact that the phosphonamidate could form one additional H-bond to the enzyme.[11,12]

To probe whether the phosphonamidate analogue of D-Ala-D-Ala had a similar increase in binding affinity to VanX, *N*-[(1-aminoethyl)hydroxyphosphinyl]-D-alanine **1** and *O*-[(1-aminoethyl)hydroxyphosphinyl]-D-lactic acid **3** were synthesized according to Scheme 1. Diphenyl 1-(phenylmethoxycarbonylamino)ethyl phosphonate **7** was synthesized using triphenyl phosphite **5**, benzyl carbamate **6**, and aldehyde **4** according to the method of Oleksyszyn[13] and converted by ester exchange in methanol to the dimethyl ester **8**. The monomethyl ester **9** was obtained by partial base-catalyzed hydrolysis of dimethyl ester **8** and was subsequently converted by treatment with thionyl chloride in dichloromethane to chlorophosphonate **10**. This strategy revealed the key intermediate chlorophosphonate **10** could be coupled with D-lactic acid methyl ester or D-alanine methyl ester and converted using previously published procedures[8,10,14] to afford the phosphonate **3** and phosphonamidate **1**. Similar synthetic Schemes are thoroughly precedented in the literature.[7–10,14] At pH values ≥7.0,

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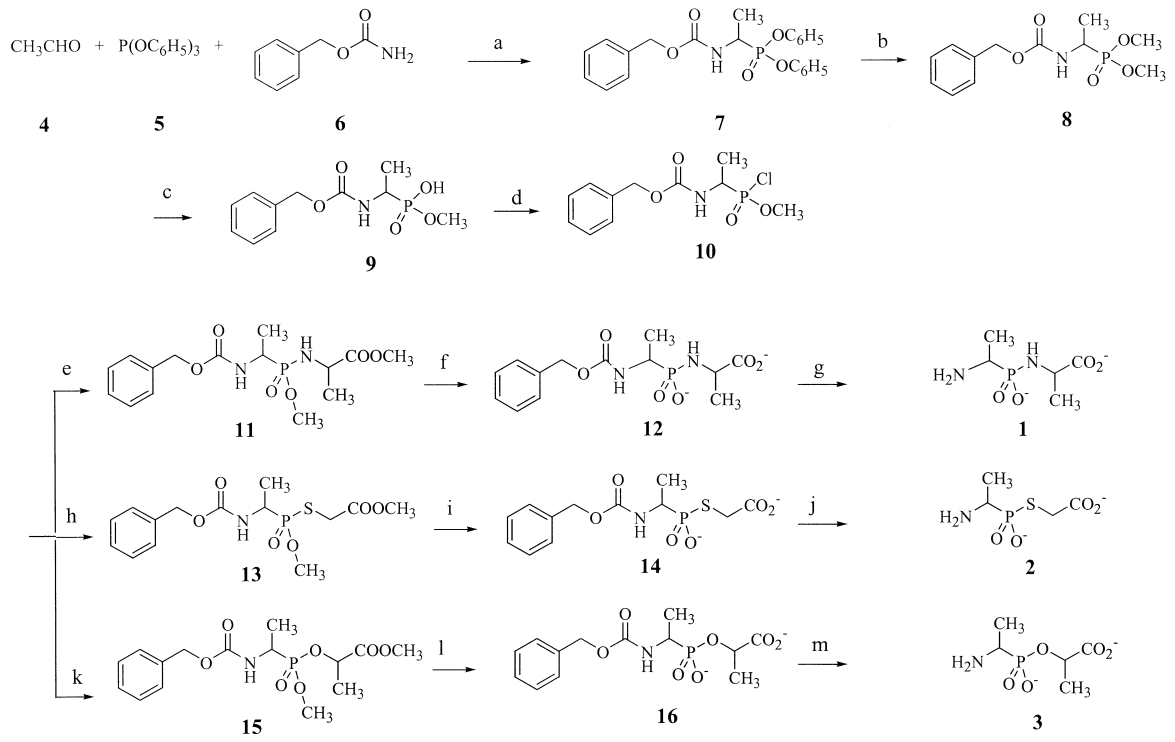

Scheme 1. Reagents and conditions: (a) AcOH, rt–85 °C, 3 h (60%); (b) CH_3ONa, MeOH, NaOH, rt, 2 h (72%); (c) MeOH, NaOH, concentrated HCl, rt, 12 h (86%); (d) CH_2Cl_2, $SOCl_2$, rt, 4 h; (e) $CHCl_3$, triethylamine, D-alanine methyl ester hydrochloride, rt, 10 days (46%); (f) CH_3CN, LiOH, TBK, MeOH, rt, 14 h; (g) 5% Pd/C, H_2O, H_2, rt, 2 h; (h) $CHCl_3$, triethylamine, methyl thioglycolate, rt, 10 days (23%); (i) HMPA, *n*-C_3H_7SLi, $CHCl_3$, TBK, MeOH, rt, 2 h (60%); (j) MeOH, 5% Pd/C, H_2, rt, 2h (69%); (k) CH_2Cl_2, triethylamine, D-lactic acid methyl ester, 1 N HCl, 5% $NaHCO_3$, rt, 5 days (53%); (l) hexamethylphosphoramide (HMPA), *n*-C_3H_7SLi, $CHCl_3$, triethylammonium bicarbonate (TBK), rt, 3 h (60%); (m) 5% Pd/C, MeOH, H_2, rt, 3

phosphonamidate **1** is stable for over a week at 4 °C, in agreement with previous studies by Bartlett [10]

To assess the inhibition properties of these compounds, steady-state kinetic studies were conducted using recombinant VanX and the continuous assay for VanX activity recently reported by our lab.[15] In excellent agreement with Walsh and co-workers,[1] the phosphonate **3** is a competitive inhibitor of VanX with a K_i of 400 ± 8 μM at pH 7.0. The phosphonamidate **1** yielded Lineweaver–Burk plots (Fig. 1) indicating competitive inhibition; however, slope replots of the inhibition data were hyperbolic suggesting a partial competitive mode of inhibition for the inhibitor (Fig. 2).[16] By using a method described by Webb, a K_i value of 36 ± 3 μM was calculated for **1**, which is a factor of 10 lower than the K_i value for phosphonate **3**.

Previously, Walsh and co-workers reported that D-3-[(1-aminoethyl)-phosphinyl]-2-methylpropionic acid (APMP) binds as a mixed noncompetitive inhibitor of VanX with a K_{is} of 0.32 μM.[1] Subsequently, Bussiere et al. demonstrated that the weaker binding of the phosphonate **3**, as compared to APMP, to VanX is because the amino terminus of the phosphonate is rotated about the P–C bond and does not make the same binding contacts as D-Ala-D-Ala or APMP.[6] On the other hand, the amino terminus of the phosphinate analogue forms several strong H-bonds with active site residues, explaining its tighter binding to VanX. It is not clear why phosphonate **1** rotates about the P–C bond and assumes a higher

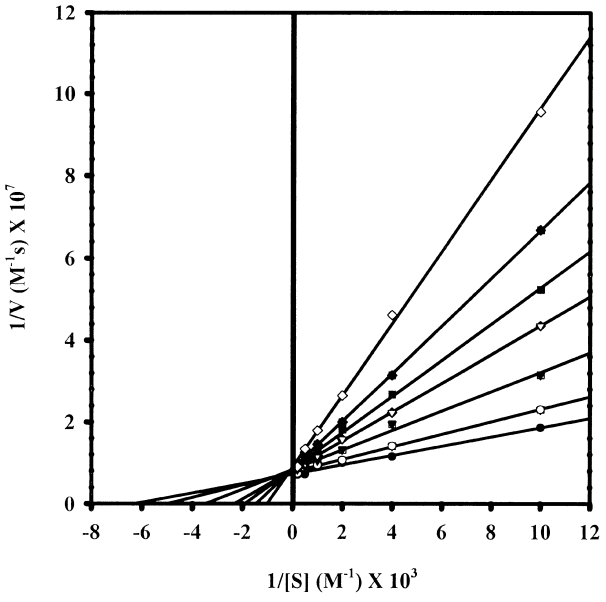

Figure 1. Double-reciprocal plot of VanX inhibition by phosphonamidate **1**.

energy conformation when bound to VanX. It is also not clear why APMP exhibits mixed noncompetitive inhibition[1] since APMP binds in a similar way as D-Ala-D-Ala and no other binding sites remote from the active site were found for APMP.[6] Nonetheless, phosphonamidate **1** apparently binds to VanX in a manner similar

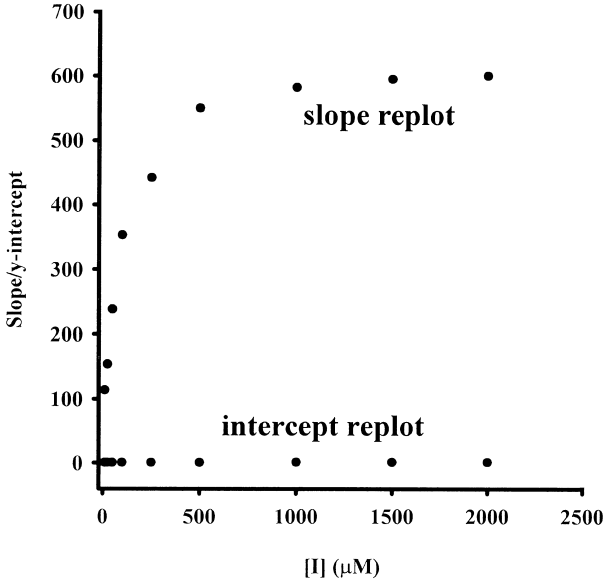

Figure 2. Slope and intercept replots of VanX inhibition by phosphonamidate **1**.

to the phosphonate **3**, and the increased binding affinity of the phosphonamidate is most likely due to the additional H-bond from the N–H of **1** to the enzyme. The crystal structure of VanX demonstrated that the comparable N–H in substrate D-Ala-D-Ala is hydrogen bonded to the backbone carbonyl of Tyr109.[6]

To extend the synthetic route in Scheme 1 to generate additional potential inhibitors of VanX, chlorophosphonate **10** was coupled with methyl mercaptoacetate and deprotected using standard procedures[8,10,14] to yield the phosphothioate **2**. This compound was shown to be stable for over a week at 4 °C and at pH 7.0. Kinetic studies demonstrated that the phosphothioate **2** did not inhibit VanX at concentrations of **2** up to 1 mM, suggesting an important role of the methyl group in substrate/inhibitor binding. This result is in contrast to the crystallographic studies that showed minimal contacts between VanX and the C-terminal methyl on D-Ala-D-Ala, phosphonate **3**, and APMP.[6] We cannot rule out the possibility that the replacement of the methylene in APMP, the amine in **1**, and the oxygen in **3** with sulfur explains the loss of inhibitory behavior by **2**. Attempts to synthesize a phosphothioate analogue containing a C-terminal methyl substituent were unsuccessful.

The phosphorus-containing dipeptide analogues **1**, **2** and **3** were also tested as inhibitors of metallo-β-lactamase L1 from *Stenotrophomonas maltophilia* because these compounds have a similar structure as a part of the putative tetrahedral intermediate formed during β-lactam bond hydrolysis (Fig. 3). However, none of the analogues inhibited L1 at concentrations up to 1 mM at pH 7.0 using nitrocefin as the substrate.[17]

The extremely narrow and selective active site of VanX presents a major obstacle in designing small molecule inhibitors. The results on phosphonamidate **1** suggest

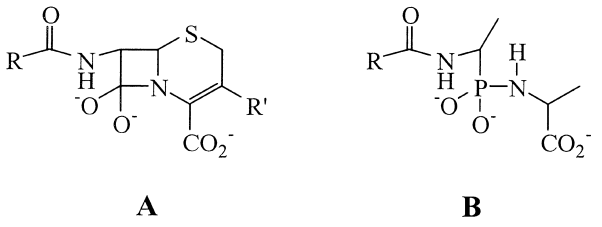

Figure 3. (A) Proposed structure of tetrahedral intermediate of cephalosporin hydrolysis by metallo-β-lactamase L1. (B) structure of phosphonamidate **1**.

that the positioning of a hydrogen in an inhibitor to form a hydrogen bond to the enzyme can result in a 10-fold increase in binding affinity. The ability to control the rotation about the P–C bond in **1** will likely result in further increases in binding affinity and potentially in a clinically-useful inhibitor.

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