Mechanism-Based Inactivation of VanX, a D-Alanyl-D-alanine Dipeptidase Necessary for Vancomycin Resistance†

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ABSTRACT: VanX is a zinc-dependent D-Ala-D-Ala amino dipeptidase required for high-level resistance to vancomycin. The enzyme is also able to process dipeptides with bulky C-terminal amino acids [Wu, Z., Wright, G. D., and Walsh, C. T. (1995) *Biochemistry 34*, 2455−2463]. We took advantage of this observation to design and synthesize the dipeptide-like D-Ala-D-Gly(SΦ*p*-CHF_2)-OH (**7**) as a potential mechanism-based inhibitor. VanX-mediated peptide cleavage generates a highly reactive 4-thioquinone fluoromethide which is able to covalently react with enzyme nucleophilic residues, resulting in irreversible inhibition. Inhibition of VanX by **7** was time-dependent ($K_{irr} = 30 \pm 1$ μM; $k_{inact} = 7.3 \pm 0.3$ min^{-1}) and active site-directed, as deduced from substrate protection experiments. Nucleophilic compounds such as sodium azide, potassium cyanide, and glutathione did not protect the enzyme from inhibition, indicating that the generated nucleophile inactivates VanX before leaving the active site. The failure to reactivate the dead enzyme by gel filtration or pH modification confirmed the covalent nature of the reaction that leads to inactivation. Inactivation was associated with the elimination of fluoride ion as deduced from ^{19}F NMR spectroscopy analysis and with the production of fluorinated thiophenol dimer **12**. These data are consistent with suicide inactivation of VanX by dipeptide **7**. The small size of the VanX active site and the presence of a number of nucleophilic side chains at the opening of the active site gorge [Bussiere, D. E., et al. (1998) *Mol. Cell 2*, 75−84] associated with the high observed partition ratio of 7500 ± 500 suggest that the inhibitor is likely to react at the entrance of the active site cavity.

The emergence of glycopeptide-resistant enterococci is of particular concern since they have become important, both as nosocomial pathogens and as potential reservoirs of resistance genes for other pathogens. Vancomycin and teichoplanin are the last resort antibiotics for treating deep-seated Gram-positive bacterial infections (*1*−*3*). Vancomycin inhibits peptidoglycan (PG)[1] biosynthesis by binding the terminal D-alanyl-D-alanine (D-Ala-D-Ala) dipeptide from pentapeptide precursors of *Enterococcus* bacterial cell walls. To date five types of resistance to glycopeptides have been identified, namely, VanA, VanB, VanC, VanD, and VanE (*4*−*8*). Resistance to vancomycin relies on the exchange of the terminal dipeptide D-Ala-D-Ala from the pentapeptide precursor into either D-Ala-D-lactate (D-Ala-D-Lac) (in the case of VanA, VanB, and VanD) or D-Ala-D-serine (in the case of VanC and VanE). The incorporation of the pentapeptides leads to a drastic drop in the binding affinity of vancomycin for PG precursors, accounting for resistance (*9*). The molecular mechanism of VanA-type resistance has been well characterized (*10*). Transposon Tn*1546* encodes two regulatory proteins (VanS and VanR). VanS controls the level of phosphorylation of VanR in response to the presence of vancomycin in the environment. Phosphorylated VanR activates the transcription of the *vanHAXYZ* operon. VanH is a dehydrogenase that reduces pyruvate to D-lactate, providing the necessary substrate for VanA, a ligase that synthesizes the dipeptide D-Ala-D-Lac. VanX and VanY cleave the D-Ala-D-Ala peptide bond in the free dipeptide and from the pentapeptide, respectively. VanZ is an accessory protein with unknown function in vancomycin resistance.

The essential role of VanX in vancomycin resistance was outlined in 1994 by Reynolds et al. (*11*), who demonstrated that insertional inactivation of *vanX* reestablished vancomycin susceptibility in an *Enterococcus faecalis* derivative. VanX is a Zn-dependent D-D-dipeptidase with a catalytic efficiency 10^6-fold higher for hydrolysis of D-Ala-D-Ala than for hydrolysis of D-Ala-D-Lac. Its role is to deplete the pool of D-Ala-D-Ala synthesized by the Ddl ligase of the host and, to a much lesser extent, by VanA itself (*12*). The VanX

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[1] Abbreviations: CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; D-AAO, D-amino acid oxidase; DAST, diethylaminosulfur trifluoride; FPLC, fast protein liquid chromatography; GSH, glutathione, reduced form; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; IPTG, isopropyl thiogalactopyranoside; LDH, lactate dehydrogenase; MBP, maltose binding protein; NADH, nicotinamide adenine dinucleotide, reduced form; PAGE, polyacrylamide gel electrophoresis; PG, peptidoglycan; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

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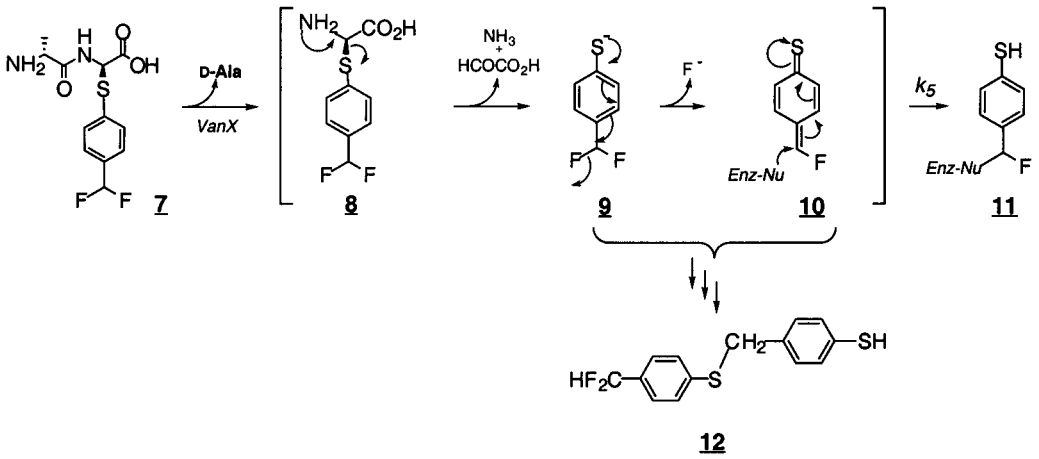
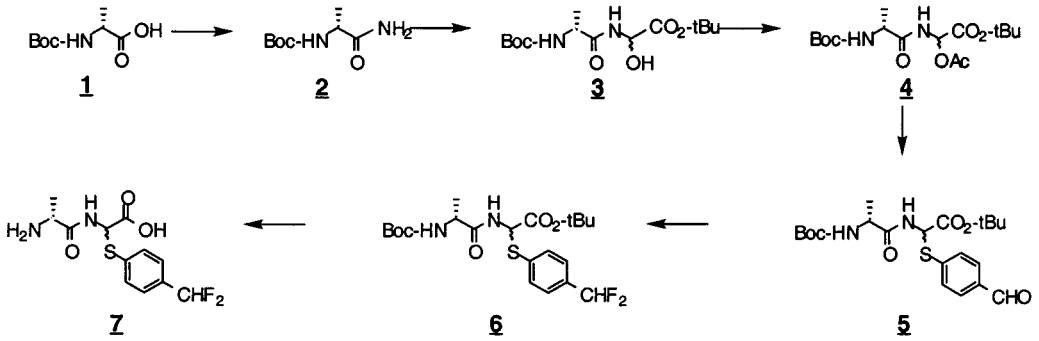
Scheme 1



Scheme 2



structure has been determined by X-ray crystallography (*13*). The active site, which occupies an amphipathic rectangular-shaped cavity of 150 Å^3, has a negative electrostatic potential, whereas the entry ring has a highly polar character. The catalytic Zn ion occupies a central position inside the cavity and is coordinated by His-116, Asp-123, and His-184; the fourth coordination group is a water molecule. In the proposed reaction mechanism, the water molecule displaced by the entry of the substrate is activated by Glu-181 and attacks the zinc-polarized carbonyl, forming a tetrahedral intermediate stabilized by both Zn and Arg-71. Glu-181 donates a proton to the nitrogen of the second alanine residue, which forms a hydrogen bond with Tyr-109 triggering peptide bond cleavage (*13*, *14*).

Attempts at inhibition of VanX have been made. Phosphorus-containing analogues that mimic the tetrahedral intermediate for hydrolysis of D-Ala-D-Ala (*12*, *15*, *16*) and dithiol compounds such as dithiothreitol (*17*) have been found to reversibly inhibit VanX. The small and constricted active site of VanX represents a challenge for the rational design of specific inhibitors (*14*). The VanX active site is highly restrictive for the residue occupying the amine position of the dipeptide; thus, only D-Ala and D-Ser are accepted. In contrast, the active site environment for the second amino acid residue displays fewer steric constraints. The amino acids D-Ala, D-Gly, D-Phe, D-Ser, D-Val, and D-Asp can occupy the carboxyl position of the dipeptide (*12*). In this work, a D-Ala-D-Phe surrogate was designed, synthesized, and evaluated on purified VanX. Enzyme-mediated cleavage of D-Ala-D-Gly(SΦ*p*-CHF_2)-OH generated the metastable 2-*p*-difluoromethylthioglycine, which spontaneously decomposed to afford ammonia, glyoxylic acid, and the electrophile 4-thioquinone fluoromethide. The data are consistent with mechanism-based inhibition of VanX, in agreement with the postulated mechanism (Scheme 1). This report represents the first demonstration of mechanism-based inhibition for VanX.

MATERIALS AND METHODS

Materials. Bacteriological media were purchased from Difco. The expression vector pIADL14 [maltose binding protein−VanX (MBP−VanX)] was a gift from C. T. Walsh (Harvard Medical School, Boston, MA). *Escherichia coli* strain BL21(DE3) and restriction-grade thrombin were obtained from Novagen. The amylose resin was purchased from Biolabs. Superdex-200 and Superose 12 were from Pharmacia. Restriction endonucleases, D-amino acid oxidase (D-AAO), catalase, and NADH were purchased from Boehringer. Lactate dehydrogenase (LDH), HEPES, CHES, and cadmium chloride were obtained from Sigma. D-Ala-D-Ala-OH was ordered from Bachem Feinchemikalien AG. D-Ala was purchased from Jansen Chimica. All other reagents were of the highest quality available.

*Synthesis of D-Ala-D-Gly(SΦp-CHF_2)-OH (**7**)*. Compound **7** was synthesized according to Scheme 2 in a series of six steps from commercial Boc-D-Ala (**1**).

Boc-D-Ala-NH_2 (**2**) (4.7 g, 25 mmol) (classically synthesized from **1** using ethyl chloroformate and ammonia) was mixed with *tert*-butyl glyoxylate (6.5 g, 50 mmol) in 100 mL of THF containing 0.25% APTS and 20 g of molecular sieves (3 Å). The mixture was maintained at 25 °C for 18 h and filtered through Celite. The solid obtained by evaporation of THF was dissolved into CH_2Cl_2. The organic phase was washed three times with water, dried over $MgSO_4$, filtered,

Table 1: Kinetic Parameters of VanX Inhibition[a]

substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	K_{irr} (μM)	k_{inact} (s^{-1})	k_{inact}/K_{irr} ($mM^{-1}\ s^{-1}$)
D-Ala-D-Ala	110 ± 10	41 ± 2	370 ± 20			
7				30 ± 1	0.12 ± 0.005	4.0 ± 0.2

[a] The estimation of the kinetic parameters of unmodified VanX and inactivated VanX were carried out by using the D-AAO−LDH coupled assay as described in Materials and Methods. Data are mean values of three independent experiments.

and evaporated. Purification by flash column chromatography (6:4 ethyl acetate/heptane mixture) afforded Boc-D-Ala-Gly-(OH)-OtBu (**3**) (6.35 g, 80%, mp 162 °C).

Ac_2O (50 mL), **3** (3.2 g, 10 mmol), and 33 mL of pyridine were stirred for 44 h under argon at room temperature. After evaporation and redissolution of the solid in CH_2Cl_2, the organic phase was washed with water, $NaHCO_3$, and water. The resulting product was dried on $MgSO_4$, and Boc-D-Ala-Gly(OAc)-OtBu (**4**) was isolated as a colorless oil (3.54 g, 98%) by flash chromatography (6:4 ethyl acetate/heptane mixture).

Compound **4** (3.78 g, 10.5 mmol), *p*-mercaptobenzaldehyde (1.45 g, 10.5 mmol), 50 mL of anhydrous DMF, and 1.5 mL of Et_3N were stirred under argon for 96 h at room temperature. DMF was evaporated and the solid resuspended in ethyl acetate (160 mL). The organic phase was washed with water and brine and dried on $MgSO_4$. Boc-D-Ala-Gly-(SΦ*p*-CHO)-OtBu (**5**) (3.3 g, 77%) was purified by flash chromatography (2:8 ethyl acetate/heptane mixture, followed by a 4:6 mixture).

To compound **5** (1.5 g, 3.4 mmol) dissolved into anhydrous CH_2Cl_2 (19 mL) under argon was added dropwise 1.5 equiv of DAST at 0 °C. After 26 h at room temperature, the excess of DAST was destroyed with 1 mL of methanol. The solvent was evaporated, and the solid dissolved in 150 mL of CH_2Cl_2 was washed with 5% $NaHCO_3$ and water and dried on $MgSO_4$. Flash chromatography (2:8 ethyl acetate/heptane mixture, followed by a 4:6 mixture) afforded Boc-D-Ala-Gly(SΦ*p*-CHF_2)-OtBu (**6**) (836 mg, 55%).

D-Ala-Gly(SΦ*p*-CHF_2)-OH (**7**) was obtained as a mixture of two diastereoisomers from the deprotection of **6** with TFA in CH_2Cl_2 (1:4) at 4 °C. The two forms (D-D-**7** and D-L-**7**) were separated by reverse phase HPLC (Waters 600).

D-D-**7**: mp 184 °C; ^{1}H NMR (300 MHz, DMSO) δ 9.35 (d, 1H, NH), 8.25 (1H, broad, COOH), 7.75 (d, 2H, ortho position of CHF_2), 7.65 (d, 2H, meta position of CHF_2), 7.15 (t, J = 56.5 Hz, 1H, CHF_2), 5.75 (d, J = 8 Hz, 1H, CH-SΦCHF_2), 4.05 (q, J = 8 Hz 1H, CH-CH_3), 1.45 (d, J = 8 Hz, 3H, CH-CH_3); ^{13}C NMR (75 MHz, DMSO) δ 168.7 (COOH), 168.4 (CONH), 131.6 (2C, meta position of CHF_2), 126.5 (CHF_2), 58.0 (CH-SΦCHF_2), 48.3 (CH-CH_3), 17.5 (CH-CH_3); MS (FAB, thioglycerol + NaCl) *m*/*z* 327 (M + Na), 305 (M + H). This compound is soluble up to 6 mM in water.

D-L-**7**: mp 224 °C; ^{1}H NMR (300 MHz, DMSO) δ 9.35 (d, 1H, NH), 8.25 (1H, broad, COOH), 7.65 (d, 2H, ortho position of CHF_2), 7.6 (d, 2H, meta position of CHF_2), 7.15 (t, J = 56.5 Hz, 1H, CHF_2), 5.85 (d, J = 8 Hz, 1H, CH-SΦCHF_2), 3.95 (q, J = 8 Hz, 1H, CH-CH_3), 1.15 (d, J = 8 Hz, 3H, CH-CH_3); ^{13}C NMR (75 MHz, DMSO) δ 169.8 (COOH), 168.95 (CONH), 132.85 (2C, meta position of CHF_2), 126.8 (CHF_2), 58.6 (CH-SΦCHF_2), 48.7 (CH-CH_3), 17.8 (CH-CH_3); MS (FAB, thioglycerol + NaCl) *m*/*z* 305 (M + H).

VanX Overexpression and Purification. The overexpression and purification of the MBP−VanX fusion protein was performed as described by McCafferty et al. (*14*). The final purification of VanX was modified as follows. MBP−VanX was digested with 1 unit of thrombin/mg of protein in 20 mM Tris-HCl, 150 mM NaCl, and 2.5 mM $CaCl_2$ (pH 7.6) for 8 h at 25 °C. The efficiency of the proteolysis was followed by SDS−PAGE. VanX was purified to homogeneity by gel filtration (Superdex-200, FPLC system) using 50 mM sodium phosphate and 150 mM NaCl (pH 7.6) as the eluent buffer. VanX fractions were pooled, concentrated to 1 mg/mL by ultrafiltration, aliquoted, and frozen in liquid nitrogen for storage at −80 °C. A purified VanX sample (20 μM) was dialyzed overnight at 4 °C against 50 mM Hepes and 100 mM NaCl (pH 7.5) with 1 mM EDTA and submitted to atomic absorption analysis (Varian AA715) for zinc content.

Protein Concentration and SDS−PAGE. The determination of the protein concentration was carried out according to the method of Bradford (*18*) using bovine serum albumin as the standard. For SDS−PAGE analysis, the protein samples were boiled in SDS sample buffer and separated using 12% polyacrylamide resolving gels and 4.5% polyacrylamide stacking gels (*19*).

Enzyme Assays. The formation of D-Ala following VanX digestion was quantified using both the cadmium−ninhydrin assay (*20*) and the D-amino acid oxidase−lactate dehydrogenase (D-AAO−LDH) coupled assay (*21*). Both methods gave similar values of K_m and V_{max} (Table 1). The method of choice was the D-AAO−LDH coupled assay as it allows a continuous monitoring of enzyme activity. Enzyme incubation was carried out in a volume of 1 mL containing 0.1 μg of VanX, 50 mM HEPES, 0.2 mM NADH, 0.05 μg/mL LDH, 0.15 μg/mL D-AAO, 520 units/mL catalase (pH 8) at 37 °C, and various concentrations of D-Ala-D-Ala. D-AAO converts D-alanine into pyruvate, which is reduced by LDH. The consumption of NADH which reflects the formation of lactate produced upon cleavage of the substrate was followed at 340 nm. Hydrolysis of dipeptides was qualitatively detected by cellulose thin-layer chromatography (TLC) using the 12:3:5 *n*BuOH/AcOH/H_2O mixture as the eluent, followed by staining with 2% ninhydrin in ethanol.

Time-Dependent Inhibition of VanX and Determination of Inactivation Constants. To ensure the inhibitor's stability, all the assays regarding VanX inhibition were carried out at pH 7. VanX (172 pmol) was incubated in 400 μL of 50 mM HEPES (pH 7) in the presence of 2 mM D-Ala-D-Ala and various concentrations of **7** (0−4 mM) at 37 °C. At different time intervals, 10 μL aliquots were withdrawn and diluted 100-fold in 1 mL cuvettes containing 50 mM HEPES, 0.2 mM NADH, 0.05 μg/mL LDH, 0.15 μg/mL D-AAO, 520 units/mL catalase, and 1 mM D-Ala-D-Ala (pH 7) at 37 °C. After an initial burst due to the consumption of D-Ala and glyoxylate produced in the incubation mixture, the slope of

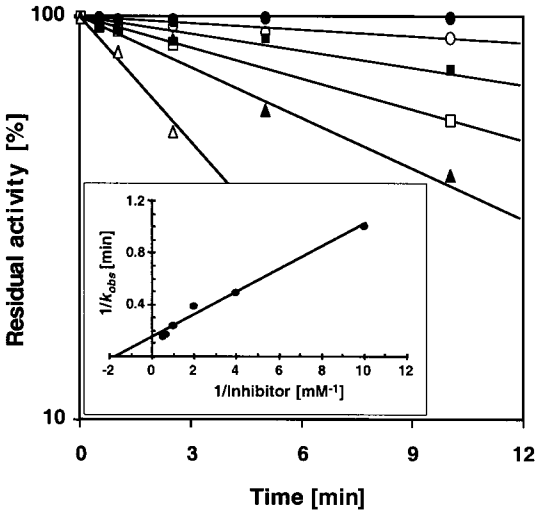


FIGURE 1: Log plot showing time-dependent loss of activity of VanX by D-Ala-D-Gly(SΦ*p*-CHF_2)-OH in (●) 0, (○) 0.1, (■) 0.5, (□) 1, (▲) 2, and (△) 3 mM inhibitor. The inset shows an estimation of inhibition parameters. Double-reciprocal plot of VanX inactivation by **7**. Values of k_{obs} were obtained from the slope of semilogarithmic plots of residual activity (A/A_0) of VanX incubated in the presence of 2 mM D-Ala-D-Ala and variable amounts of **7** vs incubation time.

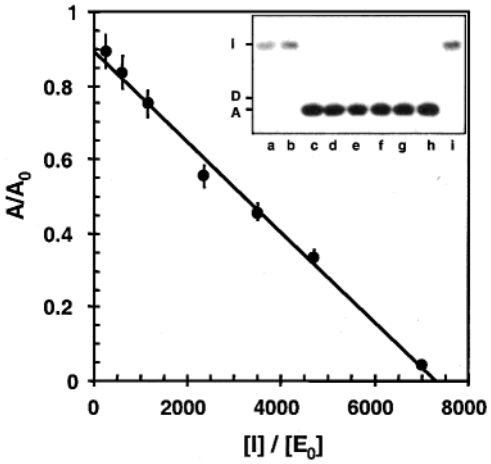


FIGURE 2: Partition ratio determination. Inactivation of VanX with variable amounts of **7** in the presence of 2 mM D-Ala-D-Ala. The plot represents the residual activity vs the ratio [I]/[E_0]. The inset shows the TLC analysis of digestion of **7** by VanX. A marks the position of D-Ala; D marks the position of D-Ala-D-Ala, and I marks the position of **7**: lane a, inhibitor control; and lanes b−h, incubation of VanX (171 pM) with 2 mM **7** at 37 °C. The incubation times were as follows: lane b, 0 min; lane c, 1 min; lane d, 2.5 min; lane e, 5 min; lane f, 10 min; lane g, 20 min; lane h, 30 min; and lane i, inhibitor control after incubation for 30 min at 37 °C. Detection was performed with ninhydrin.

NADH consumption versus time followed at 340 nm reflected the VanX residual activity (1 OD_{340}/min = 160 nmol of product formed/min). Control experiments demonstrated that **7** at 1 mM is not a substrate of the coupling enzymes. Preliminary incubation of each coupling enzyme with 2 mM **7** under the conditions of VanX inactivation did not affect the coupling efficiency, demonstrating that **7** does not inhibit the coupling enzymes. Preincubation of VanX with glyoxylate up to 2 mM under the conditions of the time-dependent inhibition assay did not alter the enzyme activity.

Partition Ratio Determination. VanX (172 pmol) was incubated in 400 μL of 50 mM HEPES (pH 7) at 37 °C in the presence of 2 mM D-Ala-D-Ala and various concentrations of **7** (0−4 mM). After 10 min, a 10 μL aliquot was removed and tested for activity as described above. The partition ratio was obtained by plotting (Figure 2) the remaining activity (A/A_0) against the initial ratio of inhibitor to enzyme ([I]/[E_0]). It was also calculated from the amount of glyoxylic acid produced during the digestion of **7** by VanX using LDH. VanX was incubated with **7** in 50 mM HEPES for 10 min, and the mixture was ultrafiltered (microsep 10 kDa, Pall Filtron). A 50 μL aliquot from the enzyme−inhibitor incubation was diluted into 1 mL of 50 mM HEPES, 0.2 mM NADH, and 0.05 μg/mL LDH (pH 7). The consumption of NADH was followed at 340 nm.

Protection from Inhibition by Substrate and Effects of Nucleophilic Scavengers. VanX (0.43 μM) was incubated in the presence of 1 mM inhibitor and increasing amounts of D-Ala-D-Ala (0−5 mM) in 50 mM HEPES (pH 7). Following a 10 min incubation at 37 °C, a 10 μL aliquot was removed and assessed for activity as described. The effect of exogenous nucleophiles on VanX inhibition was determined by incubating VanX (172 pmol, 0.43 μM) in the presence of 1 mM inhibitor and increasing concentrations of sodium azide (NaN_3, 0−10 mM), potassium cyanide (KCN, 0−25 mM), or reduced glutathione (0−2.5 mM) in 50 mM HEPES (pH 7). After incubation for 10 min at 37 °C, a 10 μL aliquot was removed to measure the remaining enzymatic activity using the D-AAO coupled assay.

pH Reactivation of the Inactivated Enzyme. VanX (172 pmol) was incubated with 2 mM **7** in 400 μL of 50 mM HEPES (pH 7) for 30 min at 37 °C. A 200 μL aliquot was diluted with 200 μL of a buffer solution [either HEPES (pH 7), HEPES (pH 8), CHES (pH 9), or CHES (pH 10)] to a final concentration of 150 mM. The change in pH was controlled. The samples were incubated at 25 °C for 24 h. A 20 μL aliquot was removed at different time points (from 0 to 24 h), diluted 50-fold, and assayed for activity as described. A control sample of VanX in which the enzyme was preincubated without inhibitor was subjected to the same treatment.

UV−Vis Difference Spectra. VanX (50 μg) was incubated with 2 mM **7** in 5 mL of 50 mM HEPES (pH 7) for 30 min at 37 °C. VanX was inactivated to completion according to the D-AAO coupled assay. The solution was clarified by centrifugation, and the supernatant was concentrated by ultrafiltration (macrosep 10 kDa, Pall Filtron) and then passed through a gel filtration column (Superose 12) using 50 mM HEPES and 150 mM NaCl (pH 7) as the eluent. No VanX activity was detected in the recovered enzyme solution. A control sample of VanX was run in the same manner. Under these conditions, the protein was freed of small molecules as controlled in a separate run of a mixture of VanX (20 μg) and D-Ala (0.5 μmol). The latter was followed by the cadmium−ninhydrin assay.

Analysis of the Side Products Formed during VanX Inactivation. VanX (172 pmol) was incubated with 2 mM **7** at 37 °C for 30 min to allow complete inactivation. The cloudy precipitate was collected by centrifugation (10 000 rpm), washed three times with 50 mM HEPES (pH 7), and redissolved in 50 μL of MeOH. HPLC analysis (Waters, Delta Pack 150 mm × 3.9 mm, particle size of 5 μm, 300 Å; eluent A, 20% CH_3CN/80% H_2O; eluent B, CH_3CN)

exhibited a single peak (35 min gradient from 20 to 80% eluent B, isocratic 80% B for 15 min and then to 100% B over the course of 10 min) with a single absorbance at 262 nm. Mass spectrometry analysis (Automass, Thermoquest) using electronic impact yielded the following spectrum: *m*/*z* 281 ($[M - H]^+$, 100%), 247 ($[M - H - H_2S]^+$, 13%), 197 ($[M - H - H_2S - CF_2]^+$, 16%), 160 ($[CHF_2 - C_6H_5 - SH]^+$, 28%), 159 ($[CF_2 - C_6H_5 - SH]^{\bullet +}$, 28%), 69 ($C_5H_9$, 47%).

RESULTS

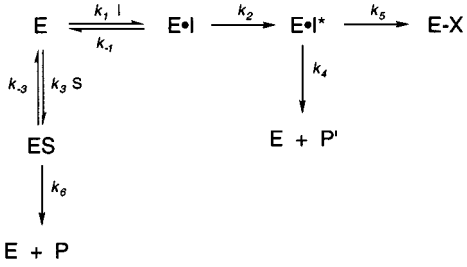
Chemical Synthesis of D*-Ala-*D*-Gly(SΦ*p*-CHF$_2$)-OH (7).* The glyoxylic acid chemistry previously developed in the laboratory (*22*) was used to synthesize the racemic α-alkylthioglycine derivatives shown in Scheme 2. Condensation of Boc-D-alanine amide **2** with glyoxylic acid *tert*-butyl ester occurred in excellent yield to give the pseudo-dipeptide **4**. The nucleophilic displacement of the acetate group of **5** with *p*-mercaptobenzaldehyde provided the intermediate **6**. Fluorination of the aldehyde group with DAST afforded protected **7** with an overall yield of 27% starting from Boc-D-Ala. Deprotection of the carboxyl and amine functions yielded D-Ala-Gly(SΦ*p*-CHF$_2$)-OH as a mixture of diastereoisomers that were separated by HPLC. As previously observed with the corresponding *p*-nitrothiophenyl derivatives (data not shown), only one of the two diastereoisomers was recognized and cleaved by the enzyme. From the reported stereospecificity of VanX, the processed compound was assumed to possess the D,D configuration. The stability of the so-called D-Ala-D-Gly(SΦ*p*-CHF$_2$)-OH **7** (D-D-**7**) was studied as a function of pH and incubation time in 50 mM HEPES (data not shown). From HPLC analysis of the degree of decomposition, the half-life of **7** was determined to be 10 days at pH 7 and 9 h at pH 8 (data not shown). Since the optimal pH for VanX ranges between 7 and 9 (*12*), we selected 7 as the working pH for the inactivation of VanX.

Inhibition of VanX by D*-Ala-*D*-Gly(SΦ*p*-CHF$_2$)-OH (7).* At 37 °C, the inactivation of VanX was a rapid process with >95% inhibition after incubation for 1 min with 2 mM **7**. We chose to add D-Ala-D-Ala to the incubation mixture to diminish the velocity of the enzyme−inhibitor reaction. The incubation of VanX (0.43 μM) with variable amounts of inhibitor **7** (0−4 mM) in the presence of 2 mM D-Ala-D-Ala resulted in time- and concentration-dependent loss of enzyme activity (Figure 1). Pseudo-first-order rate constants of inactivation (k_{obs}) at each concentration of inhibitor were obtained. The data were fitted to eq 1 according to the method of Knight and Waley (*23*):

$$k_{obs} = (k_{inact}[I])/[[I] + K_{irr}(1 + [S]/K_m)] \qquad (1)$$

where k_{obs} is the pseudo-first-order rate constant for inactivation, k_{inact} the maximal rate of inactivation under inhibitor saturation conditions, [I] the concentration of inhibitor at time zero, K_{irr} the irreversible inhibition constant for **7** that is the concentration of inhibitor giving half the maximum rate of inactivation, [S] the concentration of D-Ala-D-Ala, and K_m the Michaelis constant for D-Ala-D-Ala. A double-reciprocal plot of k_{obs} versus inhibitor concentration gave a straight line that intercepted the positive *y*-axis, indicating saturation kinetics (Figure 1, inset). The calculated values for K_{irr} and k_{inact} are 30 ± 1 μM and 7.3 ± 0.3 min^{-1}, respectively.

Scheme 3



When the inactivated enzyme was dialyzed extensively against the reaction buffer, there was no recovery of enzyme activity. The exchange of buffer by ultrafiltration gave similar results. The enzyme controls remained fully active after such treatments, demonstrating that compound **7** irreversibly inactivated VanX.

Protection from Inactivation by the Substrate and Nucleophiles. To determine whether inactivation occurs at the active site, VanX was incubated with **7** in the presence of substrate. The concentration of inhibitor was kept constant (1 mM) while the D-Ala-D-Ala concentration was increased from 0 to 5 mM. The competition between the substrate and inhibitor (Scheme 3) for binding at the active site prevents E·I formation and thereby decreases the rate of inactivation (data not shown). The enzyme activity was similar to that of the control when the concentration of substrate was 5 mM. Thus, the substrate protects VanX from inhibition.

To analyze the role of the reactive species released from the active site during inactivation of VanX, two series of experiments were carried out. First, VanX (172 pmol) was added to 400 μL of 50 mM HEPES and 2 mM **7** (pH 7) at 37 °C. After incubation for 1 min, a second addition of 172 pmol of VanX was made. A third addition of 172 pmol of VanX was made after incubation for an additional 1 min. Aliquots (10 μL) were removed, diluted 100-fold, and assayed for VanX activity using the D-AAO coupled assay. Initially, the residual activity was about 5% of that of VanX control. After the second addition of the enzyme, VanX activity was similar to that of control, and it doubled after the third addition of VanX (data not shown). These results indicate that the reactive species generated upon degradation of **7** by the enzyme were unable to inhibit freshly added VanX.

Second, the influence of exogenous nucleophiles on the inhibition was studied. VanX was incubated with 1 mM **7** in the presence of increasing amounts of NaN_3 (0−10 mM) or KCN (0−25 mM). Control tests showed that higher concentrations of these nucleophiles seriously affected VanX activity. We therefore restricted our analysis to the concentrations mentioned above which did not affect inhibitor integrity as shown by HPLC analysis (data not shown). The residual activity of the treated enzyme was not altered in the presence of sodium azide or potassium cyanide. Similar assays using the nucleophilic GSH up to 2.5 mM also failed to prevent inactivation of VanX.

Determination of the Partition Ratio. The number of molecules of **7** necessary to inactivate VanX was titrated by incubating a fixed amount of enzyme (0.43 μM), 2 mM D-Ala-D-Ala, and variable amounts of **7** (0−4 mM) for 10 min in 50 mM HEPES (pH 7) at 37 °C. VanX was inactivated to completion at an inhibitor concentration of 4

mM (9300 = [I]/[E_0]). The representation of the residual activity (A/A_0) against the [I]/[E_0] ratio gave a partition ratio of 7500 ± 500 (Figure 2). Since D-Ala and glyoxylic acid are simultaneously released upon VanX-mediated cleavage of **7** (Scheme 1), the quantifications of these byproducts are alternative ways of partition ratio determination. The dosage of glyoxylic acid with LDH, and D-Ala with the DAAO−LDH coupled assay provided us with a partition ratio (7450) that was consistent with the previous determination. Control experiments (see Materials and Methods) demonstrated on one hand that 4 mM glyoxylic acid alone did not affect VanX activity and on the other hand that **7** did not interfere with the coupling enzyme (i.e., **7** is neither the substrate nor inhibitor of the coupling enzyme system when tested at 4 mM).

The highly catalytic efficiency of VanX toward the inhibitor is illustrated in the inset of Figure 2. VanX was incubated with 2 mM **7** in 50 mM HEPES (pH 7) at 37 °C, and aliquots (10 μL) were removed at the indicated times and deposed over the aluminum cellulose sheet. Following incubation for 1 min, VanX degraded all detectable **7** by TLC (inset, lane c). At this time, the residual activity of VanX was 5%. The residual activity was about 1% after incubation for 2.5 min and was not detectable afterward. The pseudo-dipeptide **7** did not spontaneously decompose throughout the duration of the experiment (inset, lanes a and i).

pH Reactivation of Inactivated VanX. The covalent nature of the linkage between the electrophile species and the protein was checked after changing the pH of the solution of the inactivated VanX. Equal volumes of 250 mM HEPES (pH 7), HEPES (pH 8), CHES (pH 9), and CHES (pH 10) were added to 200 μL of inactivated VanX in 50 mM HEPES (pH 7). After such additions, the pH values were found to be 7, 7.9, 8.8, and 9.7, respectively. There were slight variations in the activity of unmodified VanX related to the changes in pH, while inactivated VanX showed no recovery in the enzyme activity even after incubation for 24 h at any of the indicated pHs (data not shown).

UV Difference Spectroscopy. To record the absorption spectrum of inactivated VanX, any unbound molecules were removed from the protein by gel filtration. A sample of unmodified VanX was subjected to the same treatment. The presence of the enzyme in both samples was confirmed by SDS−PAGE. A control run of a sample containing VanX and D-Ala demonstrated that the small molecules did not comigrate with the protein. The UV spectrum of inactivated VanX in 50 mM HEPES shows a shift of the absorption peak of 7 nm with respect to that of unmodified VanX (Figure 3). The difference spectrum exhibits two maxima at 264 and 301 nm.

Identification of the Byproducts Formed during Inactivation. The observation that the incubation mixture turned cloudy upon inactivation of VanX by **7** prompted us to study the nature of the precipitate. HPLC analysis (Figure 4) of the solid recovered by centrifugation of the incubation mixture showed the presence of a single product (λ_{max} = 262 nm) which was subjected to mass spectrometry analysis. The *m*/*z* peak at 281 was assigned to compound **12** (Scheme 1) which would easily form the $(M - H)^+$ species stabilized by hyperconjugation. The observed fragmentation (see Materials and Methods) is fully consistent with this assignment.

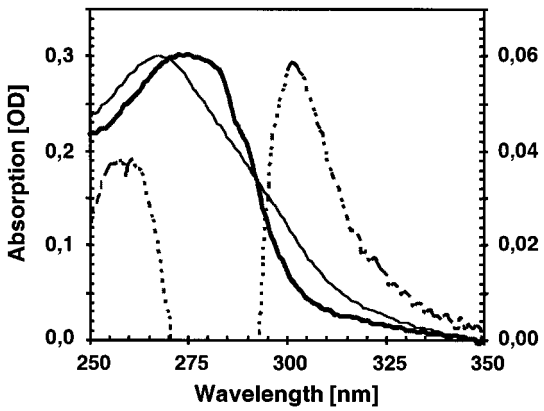


FIGURE 3: UV spectra of unmodified VanX (thick curve) and inhibited VanX (thin curve). Note that the difference spectrum (dotted line) has a different *y*-scale shown on the right.

DISCUSSION

Hydrolysis of D-Ala-D-Ala is the main mechanism by which the synthesis of pentapeptide precursors, the target for glycopeptide action, is prevented in glycopeptide-resistant enterococci (*11*). Selective inhibition of this protein is therefore a reasonable strategy for overcoming resistance. In this context, we designed the pseudo-dipeptide **7** as a potential mechanism-based inhibitor for the D-Ala-D-Ala aminopeptidase VanX. This original compound was synthesized as a mixture of separable diastereoisomers, which failed to give crystals amenable to X-ray structure determination. Identification of the configuration of each of the two derivatives was based on their recognition by VanX, whose active site has been proved to be fairly specific for the D configuration for the second amino acid (*12*). The D,D configuration was therefore attributed to the only isomer which was processed by the enzyme. To be characterized as a mechanism-based inhibitor, **7** needs to satisfy all or most of the following criteria (*24*): (a) time-dependent loss of activity, (b) saturation kinetics, (c) irreversible inhibition, (d) involvement of a catalytic step, (e) stoichiometric attachment of the reactive molecule to the active site, and (f) covalent binding of the active site by the reactive species before leaving it. Each point was analyzed at pH 7 under optimal conditions for enzyme activity and compound stability.

VanX purified as described previously (*12*) exhibited a metal content of 0.75 ± 0.05 Zn/monomer and a catalytic efficiency (Table) similar to that reported recently (*37*). Since the visible-based assay using L-alanine *p*-nitroanilide (*25*) was not available when we initiated this study, VanX activity was followed by quantification of the D-Ala produced during catalysis. We initially compared the discontinuous ninhydrin assay (*12*, *20*) with the UV-coupled assay using D-amino acid oxidase−lactate dehydrogenase (*21*). Both methods gave similar enzyme kinetic parameters. However, the coupled enzyme assay was preferred for monitoring enzyme activity since (i) continuous methods are generally preferred over discontinuous ones (*24*) and (ii) it allows simultaneous visualization of the products resulting from processing of **7**.

Processing of **7** by VanX occurred extremely rapidly at 37 °C, preventing accurate assessment of the time-dependent inhibition. Instead working at lower temperatures, we decided to add the substrate at a fixed concentration as a way of slowing the catalytic cleavage of the inhibitor (*24*,

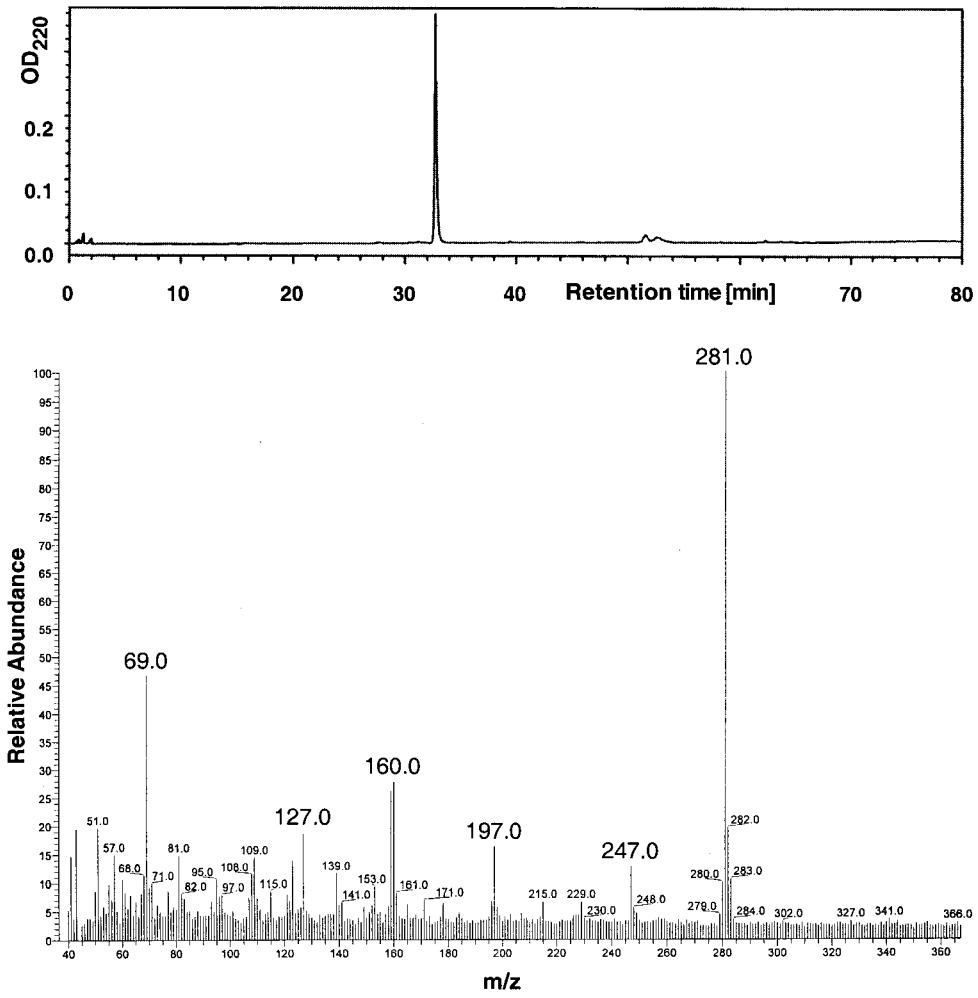


FIGURE 4: Analysis of the precipitate recovered from the inactivation of VanX by 2 mM **7**: (top) reverse phase HPLC profile and (bottom) electron impact mass spectrometry.

26, *27*), since it will compete with **7** for the active site affecting the formation of E·I (Scheme 3). Consequently, the semilogarithmic plot of residual activity versus incubation time (Figure 1) gave an apparent inhibition rate constant for each inhibitor concentration that was fitted according to eq 1. A double-reciprocal plot afforded the apparent inhibition constant value K_{irr} modified by the factor $1 + [S]/K_m$. The rate constant for inactivation k_{inact} $[=k_{+2}k_{+5}/(k_{+2} + k_{+4} + k_{+5})]$ (*23*) was not affected by the presence of the substrate. The positive intercept of the straight line issued from the double-reciprocal plot (Figure 1, inset) with the *y*-axis is an indication that the irreversible inhibition of VanX by **7** displays saturation kinetics at a rate of 7.3 ± 0.3 min^{-1}. The K_{irr} value of 30 ± 1 μM indicates that **7** is better recognized by VanX than D-Ala-D-Ala.

The direct involvement of a catalytic step differentiates a mechanism-based inhibitor from an affinity label. Typically, an affinity label resembles the substrate but is chemically reactive per se and does not need to be modified by the enzyme to become destructive (*28*). The stoichiometric relationship between D-Ala and glyoxylic acid generated upon catalysis supports the hypothesis that the generation of the reactive 4-thioquinone fluoromethide requires the enzymatic hydrolysis of the pseudo-dipeptide **7**. Catalytic hydrolysis of **7** by the enzyme then leads to the formation of D-Ala and the metastable D-Gly(SΦ*p*-CHF_2) (**8**) molecules at the active site (Scheme 1). D-Gly(SΦ*p*-CHF_2) spontaneously breaks down into glyoxylic acid and *p*-difluoromethyl thiophenol (**9**) (Scheme 1). The latter undergoes elimination of a fluoride ion to give a thioquinone fluoromethide (**10**) that will alkylate the enzyme. The elimination of a fluoride ion, crucial for the generation of the electrophilic thioquinone methide **10**, was assessed in the dead enzyme mixture by ^{19}F NMR. The solution exhibited a characteristic singlet of fluoride at δ −117 ppm. Quantification of fluoride using *p*-fluorobenzoic acid (−108 ppm) as an internal standard indicates the liberation of 0.75 ± 0.04 fluorine atom from each inhibitor molecule initially present in solution, thus supporting the requirement of fluorine elimination for suicide inhibition. This value strongly suggests that unmodified **7** should be present in the solution after the enzyme is fully inactivated. Since no trace of starting compound could be detected following incubation for 1 min (see Determination of the Partition Ratio in the Results) with VanX, the unstable intermediates must in part escape the enzyme active site to react on their own.

The number of molecules required to inactivate VanX is a function of the reactivity of the generated electrophile **10**, the rate of diffusion of **8**−**10** from the active site, and the proximity of a nucleophile residue at the active site for covalent bond formation (*24*). Quinone methides are a class of reactive electrophilic compounds that are capable of alkylating proteins and nucleic acids; there are precedents for such quinone methides involved in mechanism-based

inhibition (*29*−*32*). Since processing of **7** is extremely rapid, the limiting step for the inactivation of VanX is not likely to rely on the catalytic efficiency of the peptide bond cleavage by the enzyme, but rather on the formation of the electrophile 4-thioquinone fluoromethide. The possibility that 4-thioquinone fluoromethide might be generated outside the protein or leave it to accumulate in the medium before reentering the active site could be ruled out from the lack of protection against inactivation by potent nucleophile scavengers such as sodium azide, potassium cyanide, and GSH which are known to be the most nucleophilic species that can be used (*33*). The activity of fresh enzyme added to the inhibition mixture after short incubation times indicates that the 4-thioquinone fluoromethide and/or reactive intermediate species leaving the active site were very rapidly destroyed in the assay medium.

In the absence of data concerning the rates of spontaneous breakdown of the α-phenylthioglycine and of fluoride elimination of *p*-difluoromethylthiophenol, it is difficult to know which of the steps leading to protein alkylation is rate-limiting. Along this line, the partition ratio of 7500, i.e., the k_4/k_5 ratio (Scheme 3), might result from a long lifetime of one of the reactive intermediates **8**, **9**, or **10** which would escape the active site before reacting with a nucleophilic residue such as those covering the active site entrance. The cloudy precipitate which formed following inactivation at saturating concentrations of inhibitor was identified as compound **12** resulting from the condensation between intermediates **9** and **10** followed by fluorine elimination and rearomatization. The fragmentation observed by mass spectrometry as well as its absorption maximum at 262 nm (Figure 4), characteristic of aromatic thiols and sulfides, supports such a hypothesis. Taken together, these results argue against a possible nonspecific inactivation by the reactive intermediates in solution.

The failure to reactivate the dead enzyme by changing the pH of the incubation mixture or by exhaustive dialysis is consistent with a covalent modification. Preliminary evidence of the formation of an adduct between a nucleophilic amino acid and the 4-thiofluoroquinone methide is provided by the UV spectrum of VanX. The nature of this adduct between **10** and VanX remains to be established since mass spectrometric analyses have been of little help in its identification. The observed absorption at 301 nm, a wavelength higher than the typical value (∼260 nm) of phenylthiols (*34*) but lower than that reported for a doubly conjugated thioketone (330 nm) (*35*), might reveal further evolution of the initial adduct **11** resulting from nucleophilic 1,6-addition (*36*) on the thioquinone methide **10** formed according to Scheme 1. Since the fitting of **7** deep into the active site is not probable (*13*), the presence of a number of nucleophilic side chains at the opening of the active site would explain the evolution to a conjugated thioketone through reaction of a second enzyme residue on the initial adduct **11**.

Summary. D-Ala-D-Gly(SΦ*p*-CHF_2)-OH is the first mechanism-based inhibitor for VanX reported to date. The high affinity of a protein crucial in vancomycin resistance toward a peptide analogue contrasts, however, with poor catalytic efficiency and high partition ratio. This work demonstrates the possibility of developing selective inhibitors based on the known substrate specificity of the enzyme and should stimulate further research in this direction.

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