Design and Evaluation of Mechanism-Based Inhibitors of D-Alanyl-D-alanine Dipeptidase VanX

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Dedicated to Professor Marc Julia on the occasion of his 80th birthday

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VanX protein is a D-alanyl-D-alanine dipeptidase essential for vancomycin resistance in *Enterococcus*. It is also a key drug target in circumventing clinical glycopeptide antibiotic resistance. The dipeptide-like compound D-Ala-D-Gly(SC_6H_4-*p*-CHF_2) (**1**) was recently reported as the first mechanism-based inhibitor with high affinity for the enzyme but poor inhibitory efficiency (k_{inact}/K_{irr} = 9320 m^{-1} s^{-1}) and a high partition ratio (7600). In order to assess the effects of variations in aromatic substituents on the in vitro inhibition properties of **1**, we have prepared a few derivatives and analyzed them using the alternative substrate D-Ala-D-Gly(SPh)-OH (**15**) designed according to a similar strategy. Whereas moving the electrophilic difluoromethyl group to the *ortho* position slightly improved the inhibition parameters (k_{inact}/K_{irr} = 1140 M^{-1} s^{-1}) with a small decrease in the partition ratio (7200), introduction of a methoxy group in D-Ala-D-*ortho*/*para*-(difluoromethyl)phenylthioglycines resulted in a marked decrease in inhibitory efficiency. These results demonstrate the difficulties in improving the leading compound **1** given the restricted space available at the VanX active site.

Introduction

During the three decades since the introduction of glycopeptide antibiotics into therapy, enterococci have developed an incredible resistance to vancomycin, and this resistance is still growing. Vancomycin acts by binding to the peptidoglycan precursor at the D-Ala-D-Ala terminus, thus blocking cell-wall formation.[1] Acquired resistance in bacteria requires VanX, a dipeptidase that cleaves D-Ala-D-Ala, allowing for the synthesis of D-Ala-D-Lac mediated by the coupled actions of dehydrogenase VanH and ligase VanA.[2] The new D-Ala-D-Lac precursor can then be integrated into the peptidoglycan layer, resulting in a 1000-fold weaker binding of vancomycin compared to D-Ala-D-Ala, the constituent of the wild type. This essential role of VanX was outlined by Reynolds et al.,[3] who demonstrated that insertional inactivation of VanX reestablished vancomycin susceptibility in an *Enterococcus faecalis* resistant strain. VanX has since become a key drug target in circumventing clinical antibiotic resistance.

Phosphorus-containing dipeptide analogs that mimic the tetrahedral intermediate during hydrolysis of D-Ala-D-Ala by VanX have been found to behave as potent, reversible inhibitors of VanX in the micromolar range.[4–6] We recently reported the first mechanism-based inhibitor D-Ala-D-Gly(SC_6H_4-*p*-CHF_2) (**1**) for VanX.[7] As illustrated in Scheme 1, enzyme-mediated cleavage of **1** generates 4-thioquinone fluoromethide, which is able to react covalently with enzyme nucleophilic residues, resulting in irreversible inhibition.

While the affinity of VanX towards **1** is strong, the inactivation efficiency is poor and the partition ratio is high (k_{inact}/K_{irr} = 9320 M^{-1} s^{-1}, partition ratio = 7600). As part of our continuing interest in the search for VanX inhibitors, we have tried to improve the efficiency of this dipeptide surrogate by chemical modification of its aromatic ring. We describe in this paper the syntheses of three novel compounds in which the reactivity of each compound has been tuned by its aromatic substitution. Also included are the evaluation of the effects of these compounds on purified VanX using D-Ala-D-Gly(SPh)-OH as the substrate, as its turnover product can be detected by 5,5′-dithiobis(2′-nitrobenzoic acid) (Ellman's reagent).

Results

Chemical Synthesis of the Pseudodipeptides

The synthesis of α-thio-substituted glycine from the corresponding α-acetoxyglycine was performed using the strategy described by Kingsbury et al.[8] The key step (Scheme 2) in the syntheses of the α-[(difluoromethyl)arylthio]glycines **12**, **13** and **14** resides in the substitution of the acetoxy

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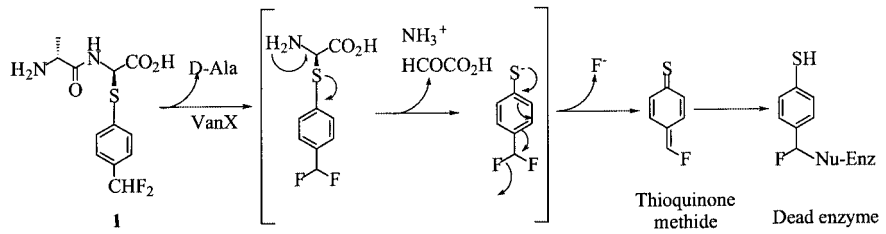
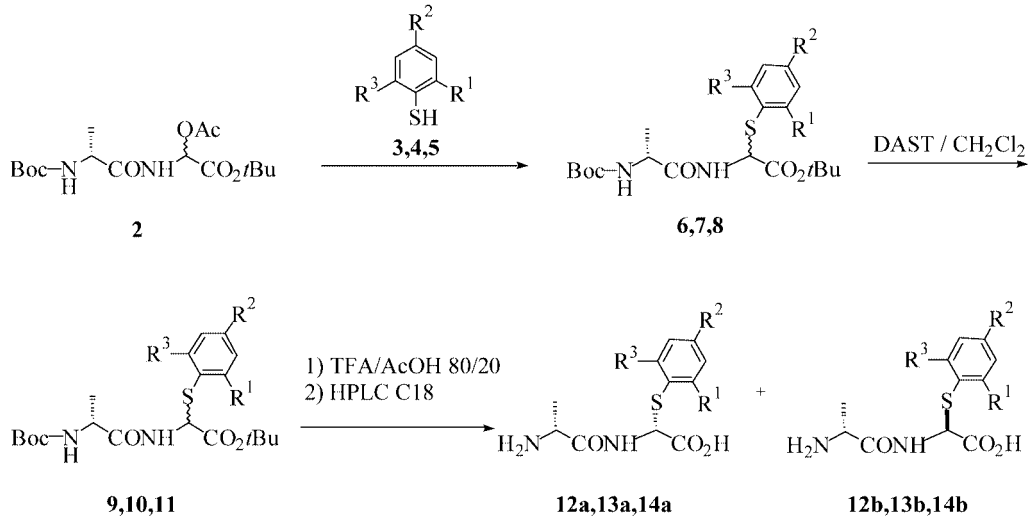


Scheme 1. Principle of mechanism-based inactivation of VanX with compound **1**



	3	4	5	6	7	8	9	10	11	12a	13a	14a	12b	13b	14b
R^1	H	CHO	H	H	CHO	H	H	CHF_2	H	H	CHF_2	H	H	CHF_2	H
R^2	CHO	H	H	CHO	H	H	CHF_2	H	H	CHF_2	H	H	CHF_2	H	H
R^3	MeO	MeO	CHO	MeO	MeO	CHO	MeO	MeO	CHF_2	MeO	MeO	CHF_2	MeO	MeO	CHF_2
Yield (%)				45	55	78	47	70	51	44	43	46	38	35	26

Scheme 2. Synthesis of pseudodipeptides

group of the racemic pseudodipeptide **2**[7] by the corresponding thio-substituted benzaldehydes **3**, **4** and **5**. Compounds **3** and **4** were obtained by the Newman−Kwart rearrangement[11,12] of *O*-dimethylthiocarbamoylated vanillin[9] and *ortho*-vanillin,[10] respectively. 2-Thiosalicylaldehyde (**5**) was conveniently synthesized in 32% yield by formylation of the thiophenol dilithium salt.[13,14] Nucleophilic displacement of the acetate in **2** was accomplished under standard conditions (Et_3N/DMF)[15] to afford **6** and **7** in 45−55% yield.

Unexpectedly, compound **5** reacted differently under these conditions, giving only an unidentified compound in which the aldehyde function has disappeared. However, replacing DMF by $CHCl_3$ resulted in the suppression of this unwanted reaction, leading to the formation of **8** with a 78% yield. Compounds **6**,**7** and **8** were then fluorinated by DAST or deoxo-fluor™, the resulting products of which were deprotected with TFA/AcOH to afford **12**, **13** and **14**, respectively, in 40−60% yield. Subsequent reverse-phase preparative HPLC gave the analytically pure diastereoisomers, each with an overall yield of 10−15% from **2** after lyophilization.

Attempts to determine the absolute configuration of the carbon atom carrying the sulfur-containing side chain of each diastereoisomer of **12**, **13** and **14** by X-ray analysis was hampered by the difficulty in obtaining suitable crystals of these compounds. However, the difference in retention time in HPLC,[8] variations in ^{1}H NMR spectroscopic data[16] and the difference of yields for each stereoisomer,[17] as reported in the literature, have allowed the tentative assignment of the stereochemistry as shown in Scheme 2. Considering the peptide bond as a single bond between the α-carbon atoms, as proposed by Kingsbury et al.,[8] the side chains would have the *trans* and *gauche* relationships in the D,D- and D,L-peptides, respectively. Therefore, the D,L-peptide would exist with both lateral chains located on the

same side of the molecule, causing it to be better absorbed to the RP-18 column than the D,D-peptide, in which the lateral chains are situated *trans* to one another. This would suggest the first eluting isomers to be the D,D-stereoisomers **12a**, **13a** and **14a** (Scheme 2). Various ^{1}H NMR spectroscopic studies of stereoisomeric dipeptides containing one aliphatic and one aromatic residue[16] have shown that the β-protons of the aliphatic residue are more shielded in the D,L- and L,D-dipeptides than in their D,D- or L,L-analogs. In all three dipeptides **12**, **13** and **14** the signals of the methyl protons of the alanine residue of isomer **a** are shifted downfield by 0.32, 0.44 and 0.13 ppm, respectively, with respect to their counterparts in isomer **b**, confirming the assigned configuration. Finally, the difference in isolated yields between isomer **a** (45%) and isomer **b** (ca. 35%) could similarly reflect a preferential cyclization, which is possible only in the **b** isomers due to the position of its side chain.

Biochemical Evaluation of the Effects of Pseudodipeptides 12, 13 and 14 on Purified VanX

Incubation of VanX (ca. 0.5 μM) in the presence of millimolar concentrations of **12a** or **14a** resulted in total disappearance of the compound within minutes. On the other hand, isomers **12b** and **14b** remain unaffected by prolonged incubation with large amounts of the enzyme. On the basis of the reported specificity of VanX for D,D-peptides, it can be concluded that the first eluting isomers **12a** and **14a** most likely have the same D,D-configuration.

Interestingly, none of the isomers of compound **13** were cleaved by VanX.

VanX inhibition by **1** has previously been analyzed by quantification of the residual activity using the coupled actions of D-amino acid oxidase and lactate dehydrogenase in the presence of NADH. In an effort to improve the assay, we have developed a convenient and sensitive method based on the ability of the enzyme to recognize dipeptides containing α-substituted (phenylthio)glycine as the C-terminal amino acid. This assay, which quantifies the released thiophenol using Ellman's reagent, allows continuous monitoring of enzyme activity and is perfectly suited to high-throughput screening of inhibitors (Scheme 3).[18]

Upon incubation of **14a** (2 mM) with VanX, time-dependent inactivation occurred, resulting in greater than 95% inhibition after 1 min. Therefore D-Ala-D-Ala was added to the incubation mixture to reduce the rate of the enzyme-inhibitor reaction. Incubation of VanX with compounds **12a** and **14a** under these conditions exhibited a time-dependent loss of enzyme activity (Figure 1) following first-order kinetics. The data were fitted to Equation (1) according to the method of Knight and Waley,[19] where k_{obs} is a 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 the compound under study or 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. Double-reciprocal plots of k_{obs} versus inhibitor concentrations give straight lines that intercept the positive *y* axis, indicating saturation kinetics (Figure 1). The calculated values of these kinetic parameters are given in Table 1.

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

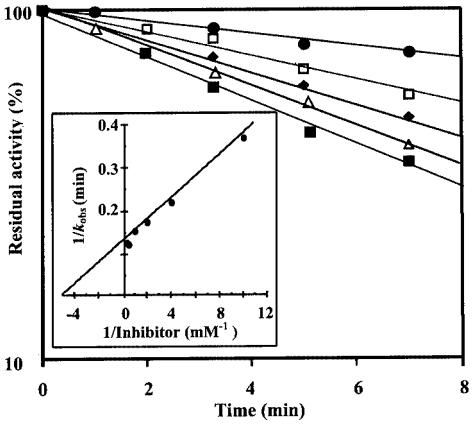
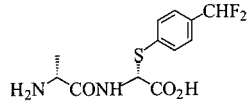
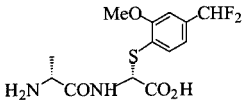
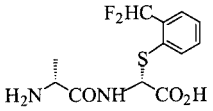
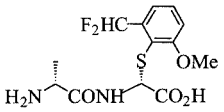


Figure 1. Semi-log plot showing time-dependent loss of activity of VanX by D-Ala-Gly(SC_6H_4-*o*-CHF_2)-OH (**14a**) at 0.1 mM (•), 0.25 mM (□), 1 mM (♦), 2 mM (△), 3mM (■); inset: double-reciprocal plot of VanX inactivation by **14a**; values of k_{obs} were obtained from the slope of semilogarithmic plots of residual activity (A/A_0) of VanX incubated in the presence of 2 mM D-Ala-D-Ala and variable amounts of **14a** vs. incubation time

Table 1. Kinetic parameters of VanX inhibition

 **1** First mechanism-based inhibitor[7]	K_{irr} = 22 ± 1 μM k_{inact} = 12.3 ± 0.8 min^{-1} k_{inact}/K_{irr} = 9320 ± 400 M^{-1} s^{-1} Partition ratio = 7600 ± 450
 **12a**	K_{irr} = 113 ± 10 μM k_{inact} = 7.7 ± 0.5 min^{-1} k_{inact}/K_{irr} = 1140 ± 100 M^{-1} s^{-1} Partition ratio = 9000 ± 600
 **14a**	K_{irr} = 10.7 ± 0.7 μM k_{inact} = 8.2 ± 0.3 min^{-1} k_{inact}/K_{irr} = 12800 ± 350 M^{-1} s^{-1} Partition ratio = 7200 ± 500
 **13a**	Not recognized as a substrate

The number of inhibitor molecules necessary to inactivate VanX was determined by incubating a fixed amount of enzyme (43 pmol) and variable amounts of inhibitors for 7 min in 50 mM HEPES at pH =7 and 37 °C. A plot of the residual activity (A/A_0) against the (I/I_0) ratio (Figure 2) gave the partition ratios, which are listed in Table 1.

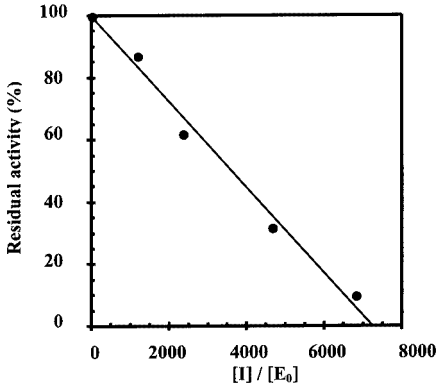


Figure 2. Partition-ratio determination: inactivation of VanX was performed as described in the Expt. Sect. with variable amounts of **14a**; the plot represents the residual activity vs. the ratio [*I*]/[E_0]

The incubation of VanX with compounds **13a** or **13b** did not affect the enzyme activity. The catalytic efficiency of enzyme toward either of these compounds as a substrate was examined by TLC analysis. Even incubation at 37 °C of 2 mM **13a** or **13b** for 1 h with an excess of VanX (concentration 10 times higher than usual conditions) did not allow the detection of D-Ala. These two compounds are, therefore, not recognized by the enzyme as substrates. The inhibition parameters are reported in Table 1.

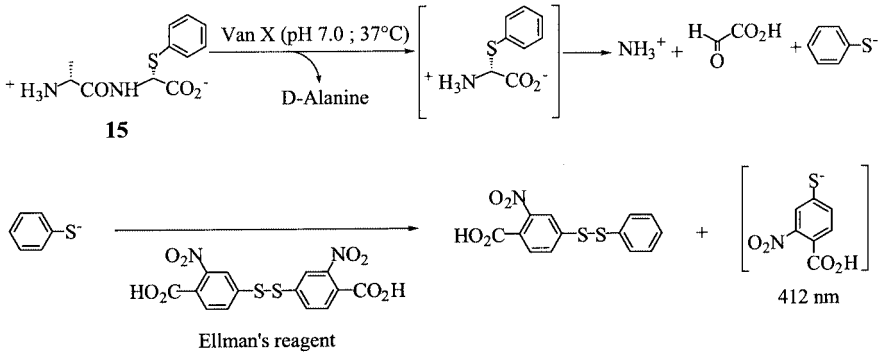
Discussion

In the search for VanX inhibitors, we tried to develop an approach using the enzyme recognition properties for the alternative substrate D-Ala-D-Phe,[4] while also considering the possibility of generating highly reactive 4-thioquinone fluoromethide through the enzyme-catalyzed peptide cleavage (Scheme 1). Because of the less-than-satisfactory performance of the initial mechanism-based inhibitor **1**, improving its efficiency by increasing the reactivity of the intermediate species by changing the fluorine atom's position and/or the substitution of the aromatic ring was conceived and attempted.

Preliminary investigations with the *p*-nitrophenylthio equivalent of **1** showed the marked instability of D-Ala-D-Gly(SC_6H_4-*p*-NO_2) in neutral solutions. Therefore, we excluded the possibility of introducing additional electron-withdrawing groups on the aromatic ring of **1** as a means of facilitating the decomposition of the substituted 2-phenylthioglycine intermediate (Scheme 1). The substitution of the aromatic ring by electron-donating groups could, on the other hand, promote fluoride elimination from the substituted (difluoromethyl)thiophenol. Such an approach has been demonstrated by β-glucosidase inhibition, in which the addition of a methoxy group to the aromatic ring of (difluoromethyl)aryl-β-D-glucosides resulted in an increase of inhibitor efficiency.[20]

The aforementioned instability of D-Ala-D-Gly(SC_6H_4-*p*-NO_2) precluded its utilization as a substrate for VanX. Thus, in our evaluation of enzyme activity, we used D-Ala-D-Gly(SPh)-OH as an alternate VanX substrate. Detection of the thiophenol released by enzyme action was monitored continuously by Ellman's reagent derivatization (Scheme 3).

Analysis of the inhibition data revealed that only compounds **12a** and **14a** affected VanX activity. The introduction of two additional groups in the *ortho* positions of the aromatic ring generates steric constraints in the small-size enzyme active site,[21] resulting in loss of recognition of **13a** by VanX. The change of the difluoromethyl position from *para* to *ortho* (Table 1) resulted in a twofold increase in binding, but a diminished inactivation rate constant without noticeable changes in the partition ratio. The overall efficiency increased 1.4-fold. On the other hand, addition of a methoxy group in the *ortho* position (**12a**) led to a substantial (10-fold) decrease of inactivation efficiency. The effects observed in this case are opposite to those reported for glucosidase inhibition. This difference may be ascribed to the distinctions between our inhibitor activation mode and the one proposed by Danzin's group for β-glucosidase. In the case of β-glucosidase, enzyme-catalyzed hydrolysis of the glucosidic linkage directly generates (difluoromethyl)-phenol, which is assumed to rapidly afford reactive fluorinated quinone methide. The substitutions are therefore be-



Scheme 3. Rationale for VanX assay

lieved to facilitate the formation of thioquinone methide or increase its reactivity towards enzyme nucleophiles. In the VanX case, the formation of *p*-(difluoromethyl)thiophenol requires the initial decomposition of α-(arylthio)glycine derived from the peptide bond cleavage. Although the latter was reported to be unstable, its lifetime obviously affects the rate of formation of *p*-(difluoromethyl)thiophenol, which is the actual inactivating species. However, in the absence of data concerning the rate of spontaneous breakdown of the intermediates and without comparing enzyme active sites, the attribution of the poor catalytic efficiency of **12a** to a slow decomposition of α-(arylthio)glycine can only be considered as speculation.

Conclusion

We have studied the effects of aromatic substitution on the in vitro inhibition properties of the first mechanism-based inhibitor of dipeptidase VanX D-Ala-D-Gly(SC_6H_4-*p*-CHF_2). From this brief study it appears that both the narrowness and the selectivity of the VanX active site is a major obstacle to the design of efficient mechanism-based dipeptide-like inhibitors. Nevertheless, this study is a good example of the complexity of inhibitor design, which will continue to challenge and inspire the ingenuity of bioorganic chemists.

Experimental Section

General Remarks: Where appropriate, reactions were carried out in dried glassware under argon. All solvents were dried by standard methods before use. Dichloromethane was distilled from calcium hydride under argon. Triethylamine was kept dry over sodium under argon; DMF and TMEDA over molecular sieves. 60 SDS silica gel was employed for flash chromatography. ^{1}H and ^{13}C NMR spectra were recorded with Bruker AC 250 or 300 spectrometers. Low-resolution MS (ESI) data were obtained with a Navigator (Thermoquest) instrument and high-resolution mass spectra were recorded by the mass spectroscopic service at the Institut de Chimie Moléculaire (Orsay). HPLC analyses were performed with a Waters 600 E instrument, equipped with a Waters 490 E UV detector, on analytical Delta Pack C18 Milligen (6 μm, pore size 60 Å, 0.8 × 10 cm) and preparative Waters RCM (2.5 × 10 cm) columns. Analytical runs were performed under 30 min gradient conditions from water to acetonitrile (both solvents contained 0.01% TFA). α-Acetoxyglycine (**2**) was synthesized as described earlier.[7] 4-Mercapto-3-methoxybenzaldehyde (**3**),[9,11,12] 2-mercapto-3-methoxybenzaldehyde (**4**),[10−12] and 2-thiosalicylaldehyde (**5**)[13,14] were prepared according to literature methods.

Boc-D-Ala-Gly(SC_6H_4-*o*-OCH_3-*p*-CHO)-O*t*Bu (6): Et_3N (280 μL, 1.99 mmol) and a solution of **3** (481 mg, 2.88 mmol) in DMF were added to **2** (580 mg, 1.6 mmol) in DMF (13 mL). The initial red solution became orange after 1 h. Stirring was continued for 16 h at room temperature. DMF was then evaporated under vacuum and the residue was separated by silica gel chromatography (EtOAc/heptane, 4:6) to give **6** as a colorless viscous liquid (337 mg, 45%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.32 (d, *J* = 7.2 Hz, 1.5 H), 1.34 (d, *J* = 7.2 Hz, 1.5 H), 1.40 (s, 9 H), 1.42 (s, 9 H), 3.91 (s, 1.5 H), 3.97 (s, 1.5 H), 4.19 (m, 1 H), 5.03 (d, *J* = 7.0 Hz, 0.5 H), 5.06 (d, *J* = 7.0 Hz, 1 H), 6.19 (dd, *J* = 9.0, *J* = 2.0 Hz, 1 H), 7.18 (d, *J* = 9.0 Hz, 1 H), 7.33 (s, 1 H), 7.35 (dd, *J* = 7.8, *J* = 1.2 Hz, 1 H), 7.54 (d, *J* = 7.8 Hz, 1 H), 9.86 (s, 0.5 H), 9.90 (s, 0.5 H) ppm. ^{13}C NMR ($CDCl_3$,75 MHz): δ = 18.00, 28.30, 28.70, 55.60, 57.84, 59.41, 83.26, 123.80, 126.96, 134.59, 136.28, 152.37, 154.88, 165.94, 169.21, 191.59 ppm. MS (ESI): *m*/*z* = 469.1 [M + H]$^+$, 491.1 [M + Na]$^+$.

Boc-D-Ala-Gly(SC_6H_4-*o*-OCH_3-*o*-CHO)-O*t*Bu (7): A solution of **4** (2.9 g, 17.5 mmol) in DMF was added to a solution of **2** (3.5 g, 9.71 mmol) in DMF (15 mL) and Et_3N (2.05 mL, 14.6 mmol). The reaction was carried out as described for **6** giving **7** as a viscous liquid (2.5 g, 55%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.27 (d, *J* = 7.1 Hz, 3 H), 1.43 (s, 9 H), 1.42 (s, 9 H), 3.99 (s, 3 H), 4.17 (m, 1 H), 4.89 (m, 1 H), 5.91 (d, *J* = 9.0 Hz, 0.5 H), 5.94 (d, *J* = 9.0 Hz, 0.5 H), 6.81 (d, *J* = 9.0 Hz, 0.5 H), 6.85 (d, *J* = 9.0 Hz, 0.5 H), 7.15 (d, *J* = 8.0 Hz, 1 H), 7.45 (t, *J* = 7.3 Hz, 1 H), 7.54 (d, *J* = 8.0 Hz, 1 H), 10.69 (s, 1 H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 17.88, 27.44, 28.10, 55.14, 56.26, 60.22, 83.10, 115.68, 120.10, 123.10, 130.67, 139.33, 155.19, 160.71, 166.34, 171.74, 192.36 ppm. MS (ESI): *m*/*z* = 491 [M + Na]$^+$, 507 [M + K]$^+$.

Boc-D-Ala-Gly(SC_6H_4-*o*-CHO)-O*t*Bu (8): Compound **5** (844 mg, 6.10 mmol) and Et_3N (780 μL, 5.55 mmol) were added at room temperature to a solution of **2** (2 g, 5.55 mmol) in chloroform (26 mL). After 18 h, the reaction mixture was dried and separated by flash chromatography on silica gel (EtOAc/heptane, 3:7) to give **8** as a white solid (1.61 g, 78%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.29 (d, *J* = 7.1 Hz, 1.5 H), 1.36 (d, *J* = 7.1 Hz, 1.5 H), 1.38 (s, 9 H), 1.42 (s, 9 H), 4.18 (m, 1 H), 4.98 (d, *J* = 7.2 Hz, 0.5 H), 5.06 (d, *J* = 7.2 Hz, 0.5 H), 5.65 (d, *J* = 4.4 Hz, 0.5 H), 5.62 (d, *J* = 4.4 Hz, 0.5 H), 7.22 (br. d, *J* = 7.2 Hz, 1 H), 7.48 (t, *J* = 7.4 Hz, 1 H), 7.58 (dt, *J* = 7.5, *J* = 1.2 Hz, 1 H), 7.74 (d, *J* = 7.2 Hz, 1 H), 7.93 (dd, *J* = 6.4, *J* = 1.3 Hz, 1 H), 10.54 (s, 0.5 H), 10.57 (s, 0.5 H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 17.60, 17.77, 27.74, 28.23, 57.66, 57.75, 83.89, 129.11, 129.23, 129.43, 134.11, 136.77, 166.63, 166.75, 171.80, 191.99, 192.34 ppm. MS (ESI): *m*/*z* = 461.1 [M + Na]$^+$, 477.1 [M + K]$^+$, 899.5 [2 M + Na]$^+$, 915.3 [2 M + K]$^+$.

Boc-D-Ala-Gly(SC_6H_4-*o*-OCH_3-*p*-CHF_2)-O*t*Bu (9): DEOXOFLUOR® (286 μL, 1.55 mmol) was slowly added under argon to a solution of **6** (427 mg, 0.91 mmol) in anhydrous CH_2Cl_2 (5 mL) at 0 °C. The reaction mixture was stirred at room temperature for 12 h, then absolute ethanol was added (1 mL). After 30 min, the solvent was evaporated and the crude residue separated by silica gel chromatography (EtOAc/heptane, 4:6) to give **9** as a white solid (210 mg, 47%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.35 (d, *J* = 3.3 Hz, 1.5 H), 1.37 (d, *J* = 3.3 Hz, 1.5 H), 1.44 (s, 9 H), 1.48 (d, *J* = 0.6 Hz, 9 H), 3.98 (s, 3 H), 4.20 (m, 1 H), 5.04 (m, 1 H), 5.89 (dd, *J* = 9.0, *J* = 4.0 Hz, 1 H), 6.23 (dd, *J* = 9.0, *J* = 4.0 Hz, 1 H), 6.61 (t, *J* = 56.3 Hz, 1 H), 7.01 (s, 1 H), 7.02 (d, *J* = 8.0 Hz, 1 H), 7.53 (d, *J* = 8.0 Hz, 1 H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.18, 27.65, 28.23, 54.92, 56.07, 66.35, 83.11, 107.72, 112.88 (t, *J* = 236 Hz, CHF_2), 114.04, 118.00, 135.85, 159.71, 166.87, 171.64 ppm. MS (ESI): *m*/*z* = 491.1 [M + H]$^+$, 513.0 [M + Na]$^+$, 1003.4 [2 M + Na]$^+$.

Boc-D-Ala-Gly(SC_6H_4-*o*-OCH_3-*o*-CHF_2)-O*t*Bu (10): DEOXOFLUOR® (944 μL, 5.12 mmol) was slowly added under argon to a solution of **7** (1.6 g, 3.42 mmol) in anhydrous CH_2Cl_2 (50 mL) at 0 °C. The solution was stirred for 18 h, then absolute ethanol was added (1 mL) as described for **9**. The reaction solvents were evaporated and the residue separated by silica gel chromatography

(EtOAc/heptane, 3:7). Compound **10** was obtained as a white solid (1.61 g, 70%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.41 (d, *J* = 7.1 Hz, 1.5 H), 1.44 (d, *J* = 7.1 Hz, 1.5 H), 1.50 (s, 9 H), 1.51 (d, *J* = 8.0 Hz, 4.5 H), 1.54 (d, *J* = 8.0 Hz, 4.5 H), 4.04 (s, 3 H), 4.20 (m, 1 H), 4.94 (br. d, *J* = 7.4 Hz, 0.5 H), 5.04 (br. d, *J* = 7.4 Hz, 0.5 H), 6.02 (dd, *J* = 9.0, *J* = 4.0 Hz, 1 H), 7.08 (d, *J* = 8.2 Hz, 1 H), 7.20 (d, *J* = 9.0 Hz, 1 H), 7.23 (t, *J* = 55.2 Hz, 1 H), 7.36 (d, *J* = 8.1 Hz, 1 H), 7.51 (t, *J* = 8.1 Hz, 1 H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 17.97, 27.48, 28.20, 54.83, 56.19, 66.50, 66.64, 83.16, 109.45, 112.60 (t, *J* = 236 Hz, CHF_2), 112.83, 117.88, 130.94, 160.43, 166.52 ppm. MS (ESI): *m*/*z* = 513 [M + Na]$^+$.

Boc-D-Ala-Gly(SPh-*o*-CHF$_2$)-O*t*Bu (11): DAST (1.54 mL, 11.62 mmol) was added under argon to a solution of **8** (3 g, 6.84 mmol) in anhydrous CH_2Cl_2 (100 mL) at 0 °C. After 16 h, the reaction mixture was washed with water until neutral (7 × 50 mL), then dried with $MgSO_4$ and the solvents were evaporated. Separation by silica gel chromatography (EtOAc/heptane, 3:7) gave **11** as a white viscous solid (1.6 g, 51%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.25 (d, *J* = 5.5 Hz, 1.5 H), 1.28 (d, *J* = 5.5 Hz, 1.5 H), 1.37 (s, 9 H), 1.43 (d, *J* = 2.4 Hz, 9 H), 4.16 (m, 1 H), 4.89 (d, *J* = 7.4 Hz, 0.5 H), 4.99 (d, *J* = 7.4 Hz, 0.5 H), 5.54 (d, *J* = 8.4, *J* = 3.1 Hz, 1 H), 5.56 (d, *J* = 8.4, *J* = 3.1 Hz, 1 H), 7.17 (dt, *J* = 55.1, *J* = 1.9 Hz, 1 H), 7.17 (br. s, 1 H), 7.45 (m, 2 H), 7.70 (dt, *J* = 7.0, *J* = 1.4 Hz, 2 H) ppm. ^{13}C NMR ($CDCl_3$,75 MHz): δ = 17.75, 27.68, 28.23, 57.83, 57.92, 83.80, 112.77 (t, *J* = 236 Hz, CHF_2), 126.11, 129.75, 131.02, 136.74, 136.94, 166.80, 166.92, 171.72 ppm. MS (ESI): *m*/*z* = 483.1 [M + Na]$^+$, 499.1 [M + K]$^+$, 943.3 [2 M + Na]$^+$.

D-Ala-Gly(SC$_6$H$_4$-*o*-OCH$_3$-*p*-CHF$_2$)-OH (12a, 12b): TFA (10.7 mL) was added to a stirred solution of **9** (800 mg, 1.63 mmol) in acetic acid (2.7 mL). After 6 h, the solvents were evaporated. The crude residue was redissolved in acetic acid (8 mL) and filtered. The two diastereoisomers were separated by preparative HPLC (water/acetonitrile, 95:5), then lyophilized to give **12a** (239 mg, 44%) and **12b** (207 mg, 38%) as white powders. **1st Eluted Diastereoisomer 12a:** ^{1}H NMR ([D$_6$]DMSO, 250 MHz): δ = 1.29 (d, *J* = 6.5 Hz, 3 H), 3.78 (q, *J* = 6.5 Hz, 1 H), 3.83 (s, 3 H), 5.42 (s, 1 H), 6.96 (t, *J* = 55.8 Hz, 1 H), 7.06 (d, *J* = 7.4 Hz, 1 H), 7.09 (s, 1 H), 7.69 (d, *J* = 7.4 Hz, 1 H), 8.83 (br. s, 1 H) ppm. MS (ESI): *m*/*z* = 334.9 [M + H]$^+$, 356.9 [M + Na]$^+$, 372.7 [M + K]$^+$. **2nd Eluted Diastereoisomer 12b:** ^{1}H NMR ([D$_6$]DMSO, 250 MHz): δ = 0.97 (d, *J* = 7.0 Hz, 3 H), 3.80 (q, *J* = 7.0 Hz, 1 H), 3.84 (s, 3 H), 5.61 (d, *J* = 8.0 Hz, 1 H), 6.95 (t, *J* = 55.8 Hz, 1 H), 7.02 (d, *J* = 7.8 Hz, 1 H), 7.07 (s, 1 H), 7.52 (d, *J* = 7.8 Hz, 1 H), 8.20 (br. s, 2 H), 8.69 (d, *J* = 8.7 Hz, 1 H) ppm. ^{13}C NMR ([D$_6$]DMSO, 75 MHz): δ = 17.40, 47.96, 55.76, 56.94, 107.43, 114.74 (t, *J* = 238 Hz, CHF_2), 117.86, 130.69, 157.03, 168.06, 168.93 ppm. MS (ESI): *m*/*z* = 334.9 [M + H]$^+$, 356.9 [M + Na]$^+$. HRMS (ESI): calcd. for $C_{13}H_{16}F_2N_2NaO_4S$ [M + Na]$^+$ *m*/*z* = 357.06965, found 357.07021.

D-Ala-Gly(SC$_6$H$_4$-*o*-OCH$_3$-*o*-CHF$_2$)-OH (13a, 13b): TFA (28.4 mL) was added to a stirred solution of **10** (2.12 g, 4.32 mmol) in acetic acid (7.1 mL). After 5 h, the solvents were evaporated and the residue redissolved in acetic acid, then the diastereoisomers were separated by preparative HPLC to give **13a** (620 mg, 43%) and **13b** (505 mg, 35%) as white powders after lyophilization. **1st Eluted Diastereoisomer 13a:** ^{1}H NMR ([D$_6$]DMSO, 300 MHz): δ = 1.12 (d, *J* = 6.9 Hz, 3 H), 3.57 (q, *J* = 6.9 Hz, 1 H), 3.86 (s, 3 H), 5.50 (br. s, 1 H), 7.13 (d *J* = 7.5 Hz, 1 H), 7.17 (d, *J* = 5.7 Hz, 1 H), 7.37 (t, *J* = 55.6 Hz, 1 H), 7.44 (t, *J* = 8.0 Hz, 1 H), 8.54 (br. s, 1 H) ppm. ^{13}C NMR ([D$_6$]DMSO, 62.5 MHz): δ = 17.32, 48.08, 56.02, 68.13, 109.56, 113.33 (t, *J* = 236 Hz, CHF_2), 117.56, 130.44, 160.47, 168.70, 168.82 ppm. MS (ESI): *m*/*z* = 334.9 [M + H]$^+$, 356.9 [M + Na]$^+$. HRMS (ESI): calcd. for $C_{13}H_{16}F_2N_2NaO_4S$ [M + Na]$^+$ *m*/*z* = 357.06965, found 357.07022. **2nd Eluted Diastereoisomer 13b:** ^{1}H NMR ([D$_6$]DMSO, 300 MHz): δ = 0.68 (d, *J* = 6.5 Hz, 3 H), 3.59 (q, *J* = 6.5 Hz, 1 H), 3.87 (s, 3 H), 5.89 (d, *J* = 9.7 Hz, 1 H), 7.08 (d, *J* = 8.5 Hz, 1 H), 7.12 (d, *J* = 8.0 Hz, 1 H), 7.34 (t, *J* = 55.6 Hz, 1 H), 7.41 (t, *J* = 8.0 Hz, 1 H), 8.10 (br. s, 1 H), 8.61 (d, *J* = 9.7 Hz, 1 H) ppm. ^{13}C NMR ([D$_6$]DMSO, 75 MHz): δ = 17.11, 47.73, 55.87, 57.81,113.00, 113.29 (t, *J* = 240 Hz, CHF_2), 129.93, 138.53, 160.54, 168.66, 168.37 ppm. MS (ESI): *m*/*z* = 334.9 [M + H]$^+$, 356.9 [M + Na]$^+$, 372.8 [M − H + 2 Na]$^+$. HRMS (ESI): calcd. for $C_{13}H_{16}F_2N_2NaO_4S$ [M + Na]$^+$ *m*/*z* = 357.06965, found 357.07015.

D-Ala-Gly(SC$_6$H$_4$-*o*-CHF$_2$)-OH (14a, 14b): TFA (28 mL) was added to a solution of **11** (1.6 g, 3.47 mmol) in acetic acid (7 mL). After 4 h, the solvents were evaporated and the diastereoisomers purified by preparative HPLC, then lyophilized to give **14a** (486 mg, 46%) and **14b** (275 mg, 26%) as white powders. **1st Eluted Diastereoisomer 14a:** ^{1}H NMR ([D$_6$]DMSO, 300 MHz): δ = 1.28 (d, *J* = 6.8 Hz, 3 H), 3.94 (q, *J* = 6.8 Hz, 1 H), 5.17 (d, *J* = 4.0 Hz, 1 H), 7.32 (t, *J* = 55.3 Hz, 1 H), 7.49 (t, *J* = 6.5 Hz, 1 H), 7.50 (dt, *J* = 6.3, *J* = 2.3 Hz, 1 H), 7.62 (dd, *J* = 6.3, *J* = 2.3 Hz, 1 H), 7.80 (d, *J* = 6.5 Hz, 1 H), 8.25 (br. s, 2 H), 8.90 (d, *J* = 4.0 Hz, 1 H) ppm. ^{13}C NMR ([D$_6$]DMSO, 75 MHz): δ = 18.05, 50.63, 59.76, 115.04 (t, *J* = 234 Hz, CHF_2), 117.53, 119.37, 128.15, 132.40, 133.35, 138.84, 163.82, 171.21, 171.42 ppm. MS (ESI): *m*/*z* = 304.9 [M + H]$^+$, 326.8 [M + Na]$^+$, 342.8 [M + K]$^+$. HRMS (ESI): calcd. for $C_{12}H_{15}F_2N_2O_3S$ [M + H]$^+$ *m*/*z* = 305.0771, found 305.07686. **2nd Eluted Diastereoisomer 14b:** ^{1}H NMR (D_2O, 300 MHz): δ = 1.15 (d, *J* = 7.1 Hz, 3 H), 3.88 (q, *J* = 7.1 Hz, 1 H), 5.75 (s, 1 H), 7.10 (t, *J* = 55.0 Hz, 1 H), 7.47 (m, 2 H), 7.53 (d, *J* = 7.3 Hz, 1 H), 7.62 (d, *J* = 7.2 Hz, 1 H) ppm. ^{13}C NMR (D_2O, 75 MHz): δ = 18.00, 50.70, 59.19, 115.10 (t, *J* = 235 Hz, CHF_2), 128.30, 132.49, 133.44, 138.99, 157.33, 171.57 ppm. MS (ESI): *m*/*z* = 304.9 [M + H]$^+$, 326.9 [M + Na]$^+$, 342.9 [M + K]$^+$.

VanX Preparation: The expression vector pIADL14 (maltose-binding-protein−VanX [MBP-VanX]) was a gift from Prof. C. T. Walsh (Harvard Medical School, Boston, USA). VanX was expressed in BL21(DE3) *Escherichia coli* cells and purified as described earlier.[7]

Inhibition Assays: To ensure the inhibitor's stability, all assays regarding VanX inhibition were carried out at pH = 7. VanX (43 pmol, 1 μg) was incubated in 100 μL of 50 mM HEPES (pH = 7) in the presence of various concentrations of inhibitor and 2 mM D-Ala-D-Ala in the case of **14a** at 37 °C. At different time intervals, 3-μL aliquots were withdrawn and diluted 333-fold in 1-mL cuvettes containing 50 mM HEPES, 2.5 mM Ellman's reagent and 5 mM of D-Ala-D-Gly(SPh)-OH (**15**) as a substrate at 37 °C.[18] After the initial burst due to the presence of 4-thioquinone fluoromethide in the incubation mixture, the slope of thionitrobenzoate production vs. time, reflecting the VanX residual activity, was monitored continuously at 412 nm with a CARY 100 spectrophotometer. The initial rates were calculated for the first 300 s, and absorbance changes were converted into concentration changes using the molar extinction coefficient of liberated nitrothiobenzoic acid [ε(412 nm) = 13600 M^{-1} cm^{-1}].

Partition Ratio Determination: VanX (43 pmol) was incubated in 100 μL of 50 mM HEPES (pH = 7) at 37 °C in the presence of various concentrations of inhibitors (0−3 mM) and 2 mM D-Ala-D-Ala in the case of **14a**. After 10 min, a 3-μL aliquot was removed and tested for activity as described above. The partition ratio was obtained as described earlier.[7]

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