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Inhibition of Dipeptidyl Peptidase IV (DPP IV) by 2-(2-Amino-1-fluoro-propylidene)-cyclopentanecarbonitrile, a Fluoroolefin Containing Peptidomimetic

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Abstract—Novel, potent inhibitors of dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5, CD26), containing the fluoroolefin peptide isostere ψ [CF=C], have been prepared via the intermediacy of the Peterson fluoroolefination reaction. The nitrile containing inhibitors were found to inhibit dipeptidyl peptidase IV competitively with K_i values for the *l*-**3** and *u*-**3** inhibitors of 7.69 and 6.03 μM, respectively. In contrast to earlier reported fluoroolefin containing inhibitors, the nitriles underwent no detectable degradation at pH 7.6 under buffered conditions.

Introduction

Dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5, CD26),[1,2] is an extracellular membrane-bound serine protease expressed on the surface of a variety of cells. Human DPP IV/CD26 is a 766 amino acid protein with a six amino acid cytoplasmic region and a 22-residue hydrophobic transmembrane region. The catalytic site, a Ser, Asp, His triad, is located in the 260 amino acid C-terminal region.[3] Uniquely specific for the cleavage of dipeptides from the N-terminus of polypeptides, DPP IV, sequentially cleaves the peptide bond at the N-terminus of a peptide whenever a proline or alanine residue is present at the penultimate position (scissile residue, P_1-position) of the substrate.

$$+H_3N\text{–}\ P_2\text{–Pro}\downarrow\text{–}P'_1\text{–}P'_2$$

Any native amino acid can occupy the P_2 position (N-terminal from scissile residue) as long as the N-terminal amino function is unprotected and protonated. In the case of proline substrates (Pro in P_1-position), DPP IV has an absolute requirement for the *S*-configuration at both the penultimate and N-terminal amino acid residues[4,5] but when alanine is in the P_1-position), the stereochemical specificity for the N-terminus is lost.

The role of DPP IV in metabolism and regulation via the degradation and post-translational processing of peptides is increasingly appreciated. The natural substrates of DPP IV have been described with the recognition that inhibition of DPP IV has potential therapeutic utility in modulation of glucagon-like peptide processing and in attenuation of the immune response.[6]

DPP IV is recognized to play a role in the activation and proliferation of lymphocytes.[7] As a cell surface protease, the physical regulation of neuropeptides, circulating hormones and chemokines by DPP IV has been reported.[8–10] DPP IV can be found as an ectopeptidase on a variety of lymphocytes[11–13] where it has been suggested to be associated with adenosine deaminase (ADA) and therefore with T-cell recruitment.[14,15] CD26 inhibition has been shown to suppress certain immune responses relative to T-cell function in vitro and in vivo[16,17] among which are Th1 and Th2 cellular responses.[18] The role of DPP IV in glucagon-like peptide (GLP) processing is also well-known.[19–21] Given the importance of regulation of this process, DPP IV inhibition has an obvious application to the treatment of diabetes.[22–24] Additionally DPP IV inhibition has been reported to be effective in controlling melanoma proliferation and the development of arthritis.[25]

With the potential therapeutic utility of DPP IV inhibition clear, it is especially important to develop a family

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of inhibitors with selectivity to engage the desired response selectively.[26] Mechanism-based inhibitors such as a series of *N*-peptidyl-*O*-acylhydroxyl-amines[27] specifically and irreversibly inhibit DPP IV, but with K_i values only in the micromolar range.[28] Azapeptides[29] and amino-acid amides of the pyrrolidide[30] and thiazolidide[31] types are effective inhibitors, but highly toxic. Cyclopeptides containing a latent quinoniminium methide electrophile[32,33] induced the complete, rapid, and irreversible inhibition of the DPP IV activity of CD26. The failure of these inhibitors to affect cell-surface-expressed DPP IV-β, is an example of the kind of specificity sought. The synthesis and utility of potent boronic acid DPP IV inhibitors has been reviewed.[34]

Results

Chemistry

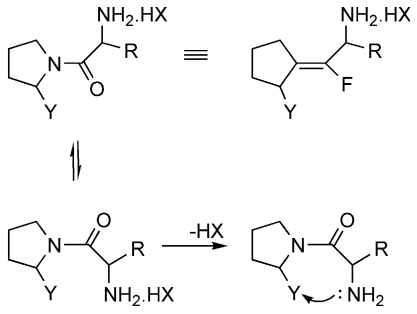
Many of the problems associated with the inefficiency and lack of stability of DPP IV inhibitors are a consequence of the requirement for a free N-terminal amino group. The cyclization reaction of the free N-terminal amino group with the reactive site of the inhibitor does, however, require the molecule to assume the *cis* amide conformation, the conformation previously proposed to be unreactive with DPP IV (Scheme 1).

In order to obviate this mode of inactivation and rigorously examine the *cis, trans* selectivity of DPP IV, a series of conformationally constrained fluoroolefin containing dipeptide isostere have been prepared. Recently, we described the synthesis of a series of fluoroolefin containing dipeptide isosteres, that is, a (*Z*)-fluoroolefin containing *N*-peptidyl-*O*-hydroxylamine, as inhibitors of DPP IV, with K_i in the submicromolar range.[35,36]

The most potent inhibitors that have been reported to date are the boroproline analogues. However, the relative instability of these materials at neutral pH[37] made it necessary to develop inhibitors that were more potent and stable such as the 2-cyanopyrrolidides **1** and 4-cyanothiazolidides[30,31] **2** which have K_i values generally in the range of 1 nM and 0.5 nM, respectively (Fig. 1).

Total synthesis of the target molecule

2-(2-Amino-1-fluoro-propylidene)-cyclopentanecarbonitriles ***u*-3** and ***l*-3** have been prepared to test the influence



Scheme 1. Conformational control by fluoroolefin peptide isostere.

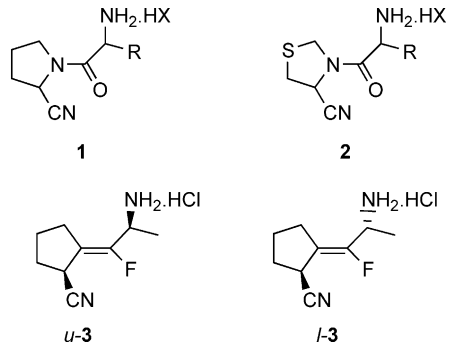
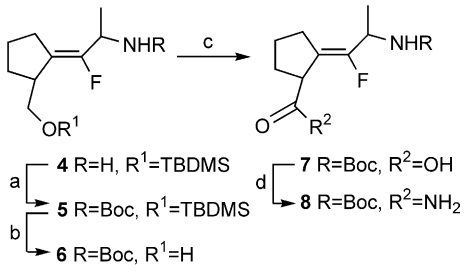


Figure 1.

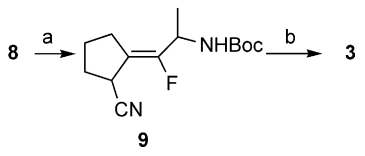
of the conformational constraint imposed by the ψ [CF=C] isostere on **1.** A key precursor for the entire synthetic sequence is the fluoroolefin bearing the appropriate latent functionalities. Compound **4** was prepared by the fluoroolefination strategy previously described.[35] Even though it was possible to separate the diastereomers of **5** by chromatography, diastereomer separation was more convenient after deprotection to the alcohol (Scheme 2).

The choice of the amine protecting group for the target compound, *tert*-butoxycarbonyl (Boc), was crucial. The Boc group was sufficiently labile to be removed without affecting the nitrile, yet was compatible with standard N-terminal peptide synthetic protocols. The mixture of diastereomers **7** was protected using 2-(Boc-oxyimino)-2-phenylacetonitrile (Boc-ON) according to the standard procedure in good yield. The selective cleavage of the silyl ether **5**, in the presence of the Boc-group, was accomplished by treatment with acetic acid-water-THF[38] liberating the primary alcohol **6** in 91% yield as a 1.2:1 ratio of diastereomers (Scheme 2). Following separation of the diastereomers by column chromatography, we initially employed Jones oxidation of the alcohols, *l*-**6** and *u*-**6**, to form the corresponding crystalline carboxylic acids, *l*-**7** and *u*-**7**.[35] (Scheme 2)

Conversion of the carboxylic acids, *l*-**7** and *u*-**7**, into amides, *l*-**8** and *u*-**8**, by sequential treatment with *N*-hydroxysuccinimide and concentrated aqueous ammonia in 95% yield, was followed by dehydration with phosphorus oxytrichloride and imidazole in pyridine (Scheme 3). The yield was moderate, 45% and 42% for *l*-**9** and *u*-**9**, respectively. The principal advantage of this approach is that the stereochemistry of the amides was not affected during the reaction, facilitating the evalua-



Scheme 2. (a) Boc-ON, dioxane, Et_3N; (b) AcOH, H_2O, THF; (c) Jones; (d) *N*-hydroxysuccinimide, NH_4OH, DCC.



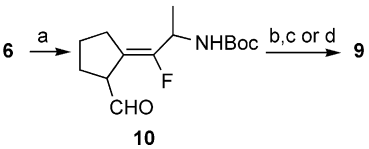
Scheme 3. (a) $POCl_3$, pyr; (b) 10% HCl–H_2O.

tion of alternative strategies for the introduction of the nitrile.

Of the approaches examined, the direct conversion of aldehyde **10** to the nitrile was the best. The primary alcohols, *l*-**6** and *u*-**6**, were converted into the corresponding aldehydes under the standard Swern condition with 92% yield. (Scheme 4)

Epimerization was observed during the Swern oxidation. Although it was possible to separate the diastereomers by chromatography at this step, it was more desirable to isolate the nitrile. Treatment of the diastereomeric mixture of aldehyde with hydroxylamine hydrochloride and triethylamine in anhydrous acetonitrile at 0 °C for 30 min, followed by addition of phthalic anhydride and heating under reflux for 7 h, according to Wang's procedure,[39] gave nitrile in low yield (42%) and numerous unidentifiable by-products (Scheme 3). Attempts to improve the yield by varying the reaction medium to dimethylformamide, dimethylsulfoxide or methylene chloride, were not successful. Treatment of the diastereomeric mixture of aldehydes **10** with 1.3 equivalents of hydroxylamine-*O*-sulfonic acid in water at room temperature per Fizet,[40] resulted in the formation of the nitrile in 51% yield. The yield of the nitrile was improved to 65%, when the reaction was effected in a saturated methylene chloride-water system instead of water. Taking into account the recovered starting material, the combined yield was nearly quantitative. Separation of the diastereomeric mixture by column chromatography resulted in a 1.3:1 ratio of diastereomers *l*-**9** and *u*-**9**. The relative stereochemistry of these diastereomeric pairs was determined by comparison of the NMR spectra of the products with the spectra of related previously prepared compounds.[35]

Since the enzyme DPP IV requires a free N-terminus for binding, the Boc-protecting group must be removed in order to carry out the enzyme binding assays. Earlier attempts to liberate the amino group by trifluoroacetic acid, trifluoroacetic acid in methylene chloride and 3M hydrochloric acid in ethyl acetate were unsuccessful. The *N*-Boc-protected nitrile remained intact by treatment with silica gel under high vacuum[41] for 24 h. Treatment with trimethylsilyl iodide,[42] trimethylsilyl chloride/3M phenol[43] or tin tetrachloride/ethyl acetate led to formation of mixtures.



Scheme 4. (a) Swern; (b) NH_2OH, Et_3N, CH_3CN; (c) phthalic anhydride; (d) NH_2OSO_2H, CH_2Cl_2–H_2O.

Removal of the Boc-protecting group was effectively accomplished by employing 1M hydrochloric acid solution in acetic acid to give the amino hydrochloride salt **3**. Treatment of compounds **9**, with 1M hydrochloric acid solution in acetic acid at room temperature for 1 h, afforded the final target compounds *u*-**3** and *l*-**3** in 75% and 65% yields. The crude products were purified by washing with cold diethyl ether.

Binding studies

Initial inhibition studies. The inhibitory activity of *l*-**3** and *u*-**3** towards the serine protease dipeptidyl peptidase IV (DPP IV) was measured and compared with the well-known *N*-peptidyl-*O*-acylhydroxylamine inhibitor, Ala-Pro-NHO-Bz(4-NO_2).[44,45] A substrate-initiated assay was employed to determine percentage inhibition (I%). The target enzyme was first incubated with an excess of the inhibitors for 2 min or 30 min; next the peptidase reaction was initiated by adding the incubated enzyme solution to a solution of the substrate, Gly-Pro-*p*-nitroanilide, in buffer. The residual enzyme activity ε was determined by monitoring the UV absorbance of the hydrolysis product, *p*-nitroaniline, at 385 nm. The percentage of inhibition (I%) was calculated using the following relationship,

$$I\% = 100\% - \varepsilon\% = (1 - v/v_0) \times 100\%$$

where v_0 is the rate of formation of the hydrolyzed product, *p*-nitroaniline, in a control experiment, in the absence of inhibitors. The results of initial inhibition studies of DPP IV by inhibitors *l*-**3** and *u*-**3** are shown in Table 1.

The initial results indicated that:

(a) Both pairs of diastereomers *l*-**3** and *u*-**3** exhibited inhibitory activity against DPP IV.

(b) Both pairs of diastereomers *l*-**3** and *u*-**3** had inhibitory activity superior to Ala-Pro-NHO-Bz(4-NO_2). The enhancement in inhibitory potency for the fluoroolefin-containing isosteres, pairs of diastereomers *l*-**3** and *u*-**3,** can be partly attributed to the efficient mimicking of the

Table 1. Inhibition of DPP IV by designed inhibitors *l*-**3** and *u*-**3**

		% Inhibition	
		Incubation time	
Inhibitor	[I] μM	2 min	30 min
u-**3** (*Z*)-Ala-Ψ[CF = C]-Pro-CN	1	16%	22%
	10	50%	49%
l-**3** (*Z*)-Ala-Ψ[CF = C]-Pro-CN	1	14%	12%
	10	47%	52%
Ala-Pro-NHO-Bz(4-NO_2)	1100	29%	60%

trans peptide bond between the P_1 and P_2 positions of the substrate by the (*Z*)-fluoroolefin double-bond conformation.

(c) Unexpectedly, the difference in inhibitory potency between *l*-**3** and the other diastereomer pair *u*-**3** was not substantial. At a concentration of 10 μM and an incubation time of 2 min, *l*-**3** inhibited 47% of the DPP IV enzymatic activity, while *u*-**3** inhibited 50% of the enzymatic activity. DPP IV requires the L configuration of the amino acid residue, both in the penultimate and *N*- terminal position. Apparently the lack of DPP IV selectivity between inhibitors *l*-**3** and *u*-**3** may be a consequence of the fact that these inhibitors are binding only loosely as transition state, reversible inhibitors. The relatively modest steric demand of the nitrile group in comparison with other functional groups may contribute to the lack of discrimination. An understanding of the intimate mechanism underlying the lack of selectivity for *l*-**3** and *u*-**3** remains under investigation.

(d) Inhibition was principally dependent on inhibitor concentration and was independent of incubation time. At an inhibitor concentration of 10 μm, for incubation times of 2 min and 30 min, the inhibition for the *l*-**3** inhibitors was 47% and 52%, respectively, while the inhibition for *u*-**3** was 50% and 49% respectively.

Inhibitor effect. Both enzyme-initiated assays (S + I) + E, and substrate-initiated assays (E + I) + S were employed to analyze the effect of inhibitors *l*-**3** and *u*-**3** on the hydrolysis of substrate (S) by DPP IV (E). In Figures 2 and 3, the curve labled 'control' represents hydrolysis in the absence of inhibitor, the curve '(S + I) + E' represents the enzyme-initiated assay and curve '(E + I) + S)' is for substrate-initiated assay. In all cases, the concentration of the product generated from hydrolysis of substrate by enzyme DPP IV increased linearly within 50 min.

Typically in such studies, the initial hydrolysis rate in an enzyme-initiated assay is larger than for a substrate-initiated assay; in this case the similar hydrolysis rate in both assays indicates that both the *l*-**3** and *u*-**3** inhibitors very rapidly inactivate DPP IV. The inactivation is so efficient that the competing enzyme substrate did not significantly retard the inactivation process.

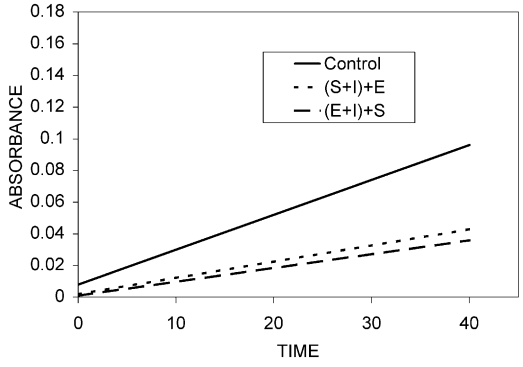


Figure 2. Effect of *u*-inhibitor on substrate hydrolysis by DPP IV.

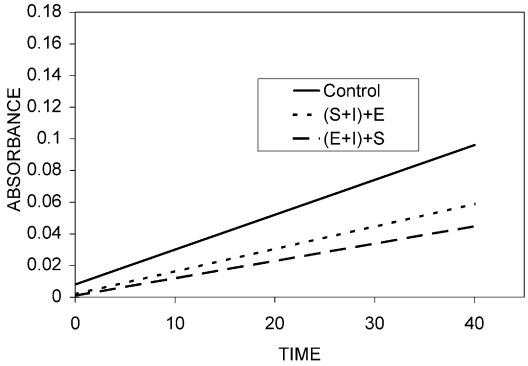


Figure 3. Effect of *l*-inhibitor on substrate hydrolysis by DPP IV.

Progress curves. The progress curves for *u*-**3** and *l*-**3** (see Figs 4 and 5) indicated that the inactivation of DPP IV did not follow pseudo-first order reaction kinetics. The concentration of hydrolyzed product increased linearly with time, at different inhibitor concentrations, for both diastereomeric pairs. Increasing inhibitor concentrations, resulted in decreasing rates of reaction. The ratio of the rate of reaction at an inhibitor concentration of 0.1 μM to that of the reaction at an inhibitor concentration of 10 μM is 0.59, suggesting that the inhibitor is more efficient at low inhibitor concentrations rather than high concentrations. This observation is reasonable when considering that the inhibitors are reversible inhibitors against DPP IV.

Determination of K_i. The inhibition constant K_i was determined by the method of Dixon. The inhibition

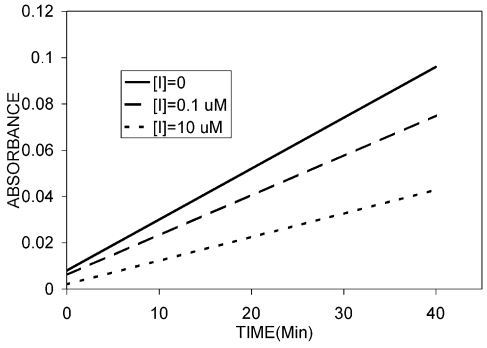


Figure 4. Progress curve of inhibitor *u*-**3**.

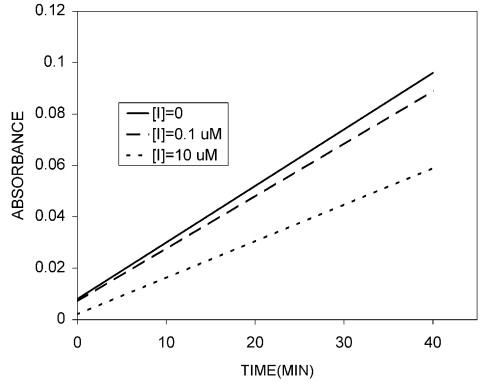


Figure 5. Progress curve of inhibitor *l*-**3**.

Table 2. Inhibition constants of DPP IV, *l*-**3** and *u*-**3**

Inhibitor	K_i (μM)
l-**3** (*Z*)-Ala-Ψ[CF = C]-Pro-CN	6.03
u-**3** (*Z*)-Ala-Ψ[CF = C]-Pro-CN	7.69
Ala-Pro-NHO-Bz(4-NO_2)	30

constant (K_i) was evaluated by determining, for each inhibitor, the rates of DPP IV-catalyzed hydrolysis of Gly-Pro-*p*-nitroanilide substrate at six different inhibitor concentrations (0.0 to 10 μM). According to Dixon, both *l*-**3** and *u*-**3** are competitive inhibitors. The values of K_i for both ***l*-3** and ***u*-3** were obtained from the plots 1/v versus [I] (Table 2)

The affinity of both *l*-**3** and *u*-**3** was much greater than that of Demuth's inhibitor, Ala-Pro-NHO-Bz(4-NO_2), for which $K_i = 30$ μM, however the affinity of both *l*-**3** and *u*-**3** was less than that of the pyrrolide analogues. The affinity of the fluoroolefin-containing isosteres, compounds *l*-**3** and *u*-**3,** for the enzyme DPP IV can be partly attributed to the efficient mimicking of the *trans* peptide bond between the P_1 and P_2 position of the substrate by the (*Z*) fluoroolefin double-bond conformation. The enzyme DPP IV again showed no substantial selectivity towards the *l*-**3** and *u*-**3** inhibitors, which was consistent with the results from initial inhibition studies.

Stability of inhibitors. Stability of the inhibitors towards decomposition in buffer (pH 7.6) was followed by UV–vis-spectroscopy over the range of 190 to 1100 nm at 30 and 50 °C. Adventitious decomposition in deuterated methanol was monitored by ^{1}H NMR spectroscopy at ambient temperature. No changes were detected by either UV–vis or ^{1}H NMR spectroscopy during 24 hrs, indicating that both the *l*-**3** and *u*-**3** compounds were very stable under the test conditions.

Conclusion

(*Z*)-Fluoroolefin-containing compounds, *l*-**3** and *u*-**3,** have been tested as inhibitors of the dipeptidyl peptidase DPP IV. In comparision with Demuth's inhibitor, Ala-Pro-NHO-Bz(4-NO_2), both isosteres *l*-**3** and *u*-**3** were better inhibitors of DPP IV by virtue of their superior inhibitory potency ($K_i = 7.69$ μM and 6.03 μM for *l*-**3** and *u*-**3** inhibitors respectively) and stability. Presumably, the (*Z*) double-bond conformation of the fluoroolefin isosteres effectively mimics the *trans* P_2 proline bonds in the original dipeptides. The inactivation rate of DPP IV by compounds *l*-**3** and *u*-**3** was very high. In contrast to previous reports on the activity of comparable fluoroolefin containing hydroxamic acid inhibitors[36] where there was a significant difference in the activity of the *l* and *u* diastereomeric pairs, DPP IV showed very little selectivity for *l*-**3** or *u*-**3** . The observed activity of both *l*-**3** and *u*-**3** was comparable to that reported for the corresponding enantiomerically pure alanine derived aminoacyl 2-pyrrolidide,[30] $K_i = 7.00$ μM. Resolution of *u*-**3** might be expected to result in a further enhancement of the activity of the fluoroolefin derived compound. Clearly, incorporation of the cyclohexylglycine or related moieties[30] with the fluoroolefin isostere may lead to additional improvements in both affinity and stability.

Experimental

General methods

Infrared spectra were recorded on a Perkin–Elmer 1600 Series FTIR spectrometer. ^{1}H NMR spectra were recorded at 300 MHz on a Gemini-300 NMR spectrometer with $CDCl_3$ as solvent and tetramethylsilane (TMS) or residual chloroform as the internal standard. ^{13}C NMR spectra were recorded at 75.43 MHz on a Gemini-300 NMR spectrometer with $CDCl_3$ as solvent and tetramethylsilane (TMS) or residual chloroform as the internal standard. ^{19}F NMR spectra were recorded at 282.20 MHz on a Gemini-300 NMR spectrometer with $CDCl_3$ as solvent and chlorotrifluoromethane ($CFCl_3$) as the internal standard. Thin layer chromatography was performed with silica gel F_{254} (Merck) as the adsorbent on 0.2 mm thick, plastic-backed plates. The chromatograms were visualized either under UV light (254 nm), by staining with a 5% solution of phosphomolybdic acid in isopropanol followed by drying in an oven at 90°C, or by spraying with a 95:5 mixture of 0.2% ninhydrin in *n*-butanol and 10% aqueous acetic acid followed by heating. Column chromatography was performed using 60 silica gel(70–230 mesh, Merck) and flash silica gel 60 (0.0040–0.063 μm, 230–400 mesh, EM Science).

1-[(1′-Fluoro-2′-amino)propylidene-2-(*tert*-butyldimethylsilyloxy)methyl] cyclopentanone.[35] (4) A hexane solution of *n*-butyllithium (1.5 mL, 3.8 mmol, 2.5 M solution in hexane) was slowly added to a solution of 1,1,1,3,3,3-hexamethyldisilazane (0.67 g, 4.2 mmol) in diethyl ether (32 mL) cooled in an ice-bath. The cooling bath was removed and the solution was stirred at room temperature for 30 min. The mixture was cooled to −30 °C and to this lithium bis(trimethylsilyl)amide solution was added a solution of 0.87 g (3.2 mmol) of [2-(*tert*-butyldimethylsilanyloxymethyl) - cyclopentylidene] - fluoro - acetaldehyde[35] in 8 mL of ether. The mixture was stirred at −30 °C for 1 h, then cooled to −78 °C. The resulting solution containing *N*-trimethylsilyl imine was treated with methyllithium (4.3 mL, 6.4 mmol, 1.5 M solution in ether) at −78 °C. The mixture was stirred at −78 °C for 1 h and then at room temperature for an additional 2 h. The solution was cooled to 0 °C again, quenched with 32 mL of saturated aqueous NH_4Cl, and extracted with CH_2Cl_2 (4×50 mL). The combined organic layers were dried with $MgSO_4$, filtered and evaporated. To remove some nonpolar impurities, the residue was subjected to a purification on a very short silica column using hexane/CH_2Cl_2 (1:1) as the eluant to give a 1.3:1 ratio of diastereomers, **4** (0.86 g, 93%, colorless liquid). The mixture of diastereomers was carried over to the next step without isolation. ^{1}H NMR ($CDCl_3$) δ 3.73–3.56 (m, 2H), 3.37,3.36 (dt, $J = 9.4$, 9.6 Hz, 1H), 2.95–

2.82 (m, 1H), 2.78–2.10 (m, 2H), 1.80–1.47 (m, 6H), 1.21, 1.20 (d, $J=6.7$, 6.7 Hz, 3H), 0.86, 0.85 (s, 9H), 0.0010 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 155.51 (d, $J=249.5$ Hz), 155.33 (d, $J=248.7$ Hz), 117.85 (d, $J=15.9$ Hz), 117.74 (d, $J=15.9$ Hz), 64.00 (d, $J=3.4$ Hz), 63.96 (d, $J=3.4$ Hz), 46.58 (d, $J=28.5$ Hz), 46.47 (d, $J=28.5$ Hz), 43.27, 29.27, 28.26 (dd, $J=5.5$,4.4 Hz), 28.19, 28.13, 25.90, 24.82, 24.58, 20.56, 20.12, 18.31, 18.28, −5.31, −5.36, −5.53; ^{19}F NMR ($CDCl_3$) δ −132.08 (d, $J=25.8$ Hz), −130.02 (d, $J=27.1$ Hz)

(*Z*)-*N*-*tert*-Butyloxycarbonyl-1-[(1′-fluoro-2′-amino)propylidene-2-(*tert*-butyldimethylsilyloxy) methyl] cyclopentanes (5). To a solution of amine **4** (0.86 g, 3.0 mmol) in dioxane (960 mL) was added triethylamine (0.62 mL, 4.5 mmol) and 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile (Boc-ON) (0.88 g, 3.6 mmol). The mixture was stirred for 20 h at room temperature and the solvent was evaporated. Elution of the crude product by a slow column chromatography with hexane/EtOAc (20:1) gave 0.88 g of **5** (77%, colorless liquid, from [2-(*tert*-butyl-dimethylsilanyloxymethyl)-cyclopentylidene]-fluoro-acetaldehyde). ^{1}H NMR ($CDCl_3$) δ 4.76 (br s, 1H), 4.47 (br, d, $J=24.7$ Hz), 3.68, 3.63 (dd, $J=8.5$, 4.5 Hz, 9.5, 4.5 Hz 1H), 3.38, 3.32 (t, $J=8.3$, 9.6 Hz), 2.89 (m, 1H), 2.38 (m, 1H), 2.16 (m, 1H), 1.85–1.53 (m, 4H), 1.40 (s, 9H), 1.23, 1.22 (d, $J=6.9$, 6.9 Hz, 3H), 0.86, 0.85 (s, 9H), 0.00 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 154.80, 154.77, 153.13 (d, $J=248.2$ Hz), 119.68 (br, s), 79.37, 63.90 (d, $J=3.6$ Hz), 63.78 (d, $J=3.3$ Hz), 43.39, 43.26, 29.31, 28.36, 25.91, 25.88, 24.79, 24.44, 18.75, 18.38, 18.29, 18.26, −5.37, −5.41, −5.45; ^{19}F NMR ($CDCl_3$) δ −130.09 (d, $J=24.4$ Hz), −130.78 (d, $J=24.4$ Hz).

(*Z*)-*tert*-Butyloxycarbonyl-1-[(1′-fluoro-2′-amino)propylidene-2-hydroxymethyl)] cyclopentanes (6). A solution of **5** (0.77 g, 2.0 mmol) in AcOH/H_2O/THF (100 mL, 13:7:3) was stirred for 16 h at room temperature. The solvent was then removed under vacuum. The yellow liquid residue was treated with solid $NaHCO_3$ until the mixture was slightly basic, and H_2O (8 mL) was added. The mixture was extracted with EtOAc (4×40 mL). The combined organic layers were dried over $MgSO_4$ and concentrated. The residue was purified by chromatography (hexane/EtOAc, 4:1) to provide 0.28 g of one diastereomer ***l*-6** (51%) and 0.23 g of the other isomer, ***u*-6** (43%) as colorless liquids.

(***l*-6**) . ^{1}H NMR ($CDCl_3$) δ 4.74 (br s, 1H), 4.38 (br d, $J=28.5$ Hz, 1H), 3.57 (d, $J=4.5$ Hz, 2H), 2.97–2.88 (m, 1H), 2.49–2.35 (m, 1H), 2.28–2.13 (m, 1H) 1.92–1.51 (m, 5H), 1.40 (s, 9H), 1.24 (d, $J=7.0$ Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 154.97, 151.99 (d, $J=249.3$), 119.17 (d, $J=14.7$ Hz), 79.64, 64.13 (d, $J=3.8$ Hz), 46.05 (d, $J=26.8$ Hz), 43.45, 29.64, 28.41, 28.31, 24.85, 17.90; ^{19}F NMR ($CDCl_3$) δ −130.50 (d, $J=28.5$ Hz).

(***u*-6**) . ^{1}H NMR ($CDCl_3$) δ 4.80 (br s, 1H), 4.45 (br, d, $J=27.0$ Hz, 1H), 3.62 (dd, $J=1015.51$ Hz, 1H), 3.44 (t, $J=9.2$ Hz, 1H), 2.93 (m, 1H), 2.43 (m, 1H), 2.19 (m, 1H), 1.87 (m, 1H), 1.77–1.52 (m, 4H), 1.39 (s, 9H), 1.23 (d, $J=7.0$ Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 154.82, 152.67 (d, $J=248.1$ Hz), 119.32 (d, $J=14.1$ Hz), 79.50, 64.18 (d, $J=3.7$ Hz), 46.62 (d, $J=29.5$ Hz), 43.46, 29.39, 28.32, 28.13 (d, $J=5.3$ Hz), 24.77, 18.63; ^{19}F NMR ($CDCl_3$) δ −129.28(d, $J=29.8$ Hz) Anal. Calcd. for $C_{14}H_{24}FNO_3$ C, 61.52; H, 8.85. Found; C, 61.37; H, 8.64.

(*Z*)-*N*-*tert*-Butyloxycarbonyl-1-[(1′-fluoro-2′-amino)propylidene]-2-cyclopentane carboxylic acid (7). Jones reagent (0.30 mL, 2.8 mmol) was added dropwise to a solution of alcohol ***l*-6** (0.15 g, 0.56 mmol) in dry acetone (9 mL) at 0 °C. The solution turned from orange to green. The reaction mixture was stirred for 1 h at 0 °C, then was quenched with H_2O, extracted, dried over $MgSO_4$, filtered and concentrated in vacuo. Column chromatography (hexane/EtOAc, 3:2) yielded the carboxylic acid ***l*-7** (0.12 g, 73%) as a white solid, Mp = 122–126 °C: ^{1}H NMR ($CDCl_3$) δ 4.83 (br s, 1H), 4.47 (br d, $J=28.9$ Hz, 1H), 3.48 (br s, 1H), 2.62–2.40 (m, 1H), 2.32–2.17 (m, 1H), 2.10–1.84 (m, 4H), 1.41 (s, 9H), 1.25 (d, $J=6.9$ Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 179.34, 154.99, 153.16 (d, $J=254.5$ Hz), 117.79 (d, $J=13.9$ Hz), 79.69, 45.59 (d, $J=30.0$ Hz), 45.45, 31.65, 28.28, 28.09 (d, $J=3.8$ Hz), 25.74, 18.00; ^{19}F NMR ($CDCl_3$) δ −123.26 (d, $J=25.8$ Hz).

The other diastereomer ***u*-7** was prepared in the same manner in 74% yield as a white solid, mp = 98–103 °C.

^{1}H NMR ($CDCl_3$) δ 4.76 (br s, 1H), 4.49 (br d, $J=27.1$ Hz, 1H), 3.52 (m, 1H), 2.60–2.47 (m, 1H), 2.41–2.26 (m, 1H), 2.08–1.81 (m, 4H), 1.41 (s, 9H), 1.27 (d, $J=7.0$ Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 179.68, 154.91, 153.20 (d, $J=251.4$ Hz), 117.53 (d, $J=15.3$ Hz), 79.64, 45.59 (d, $J=30.0$ Hz), 45.22, 31.61, 28.35, 28.00 (d, $J=3.9$ Hz); ^{19}F NMR ($CDCl_3$) δ −124.90 (d, $J=25.8$ Hz). Anal. Calcd. for ($C_{14}H_{22}FNO_4$) C, 58.52; H, 7.72. Found: C, 58.19; H, 7.77.

(*Z*)-*N*-*tert*-Butyloxycarbonyl-1-[(1′-fluoro-2′-amino)propylidene]-2-cyclopentane amide (8). To a stirred mixture of *N*-*tert*-butyloxycarbonyl-1-[(1′-fluoro-2′-amino)- propylidene]-2-cyclopentane carboxylic acid **l–7** (0.26 g, 0.90 mmol) and *N*-hydroxysuccinimide (0.13 g, 1.1 mmol) in dichloromethane (5 mL) at 0 °C was added *N*, *N*′-dicyclohexylcarbodiimide (0.23 g, 1.1 mmol). The reaction mixture was stirred for 1.5 h under the same condition, and concentrated ammonium hydroxide (0.5 mL) was then added to the stirred solution. The reaction was allowed to continue for 1 h. The dicyclohexylurea deposited during the reaction was separated by filtration. The filtrate was washed with water, dried over $MgSO_4$ and concentrated under reduced pressure to give 0.23 g (92%) of thick oil as crude product **l–8**. The product ***u*-8** was obtained in the same manner (95%). Without purification, the products were used for the next step directly.

(*Z*)-*N*-*tert*-Butyloxycarbonyl-1-[(1′-fluoro-2′-amino)propylidene]-2-cyclopentane nitrile (9). Method A. To a magnetically stirred solution of the (*Z*)-*N*-*tert*-butyloxycarbonyl-1-[(1′-fluoro-2′-amino) propylidene]-2-cyclopentane amide **l–8** (0.23 g, 0.80 mmol) in pyridine (10

mL) at room temperature was added phosphorus oxychloride (1.2 g, 8.4 mmol), followed with imidazole (1.1 g, 17 mmol). The mixture was heated to 70 °C and the reaction was allowed to continue for 7 h. The mixture was cooled to room temperature, saturated aqueous $NaHCO_3$ was added and the mixture was stirred overnight. The mixture was extracted with EtOAc (3×25 mL). The combined organic layers were dried with $MgSO_4$, filtered and evaporated. The residue was chromatographed on silica gel eluting with EtOAc-hexane (2:8) to give (*Z*)-*N*-*tert*-butyloxycarbonyl-1-[(1′-fluoro-2′-amino) propylidene]-2-cyclopentane nitrile **l–9** (0.1 g, 45%) as a colorless liquid; ^{1}H NMR ($CDCl_3$) δ 4.76 (br, s, 1H), 4.47 (br, d, J = 27.8 Hz, 1H), 3.52 (br, s, 1H), 2.69–2.51 (m, 1H), 2.35–2.20 (m, 1H), 2.08–1.74 (m, 4H), 1.41 (s, 9H), 1.25 (d, J = 7.2 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 156.26, 153.05 (d, J = 260.6 Hz), 119.86, 114.67, 79.89, 45.43 (d, J = 25.3 Hz), 32.12, 29.51, 28.41, 27.33 (d, J = 4.3 Hz), 25.68, 17.93; ^{19}F NMR ($CDCl_3$) δ −120.30 (d, J = 24.3 Hz). Anal. ($C_{14}H_{21}FN_2O_2$) C, 62.67; H, 7.87. Found: C, 62.30; H, 7.80.

***u*-9** was obtained in the same manner (42%) as a colorless liquid.

^{1}H NMR ($CDCl_3$) δ 4.72 (br, s, 1H), 4.43 (d, J = 27.7 Hz, 1H), 3.55 (br, s, 1H), 2.65–2.45 (m, 1H), 2.32–2.17 (m, 1H), 2.08–1.66 (m, 4H), 1.40 (s, 9H), 1.29 (d, J = 7.2H, 3H); ^{13}C NMR ($CDCl_3$) δ 156.40, 154.58, 152.93 (d, J = 266.3), 120.02, 114.72, 79.70, 45.35 (d, J = 25.7 Hz), 32.02, 29.35, 28.33, 27.13 (d, J = 3.4 Hz), 25.64, 17.78; ^{19}F NMR ($CDCl_3$) δ −121.42 (d, J = 24.9). Anal. ($C_{14}H_{21}FN_2O_2$) C, 62.67; H, 7.87. Found: C, 62.32; H, 7.62.

Method B. A suspension of (*Z*)-*N*-*tert*-butyloxycarbonyl-1-[(1′-fluoro-2′-amino) propylidene]-2-cyclopentane aldehyde **10** (0.12 g, 0.43 mmol) in saturated water-CH_2Cl_2 solution (5 mL) was treated with hydroxylamine-*O*-sulfonic acid (0.07 g, 0.56 mmol) at room temperature. The reaction was allowed to continue for 4 h at ambient temperature, then 3 mL 10% NaOH solution was added and reaction was continued for an additional 30 min. The reaction mixture was extracted with CH_2Cl_2 (4×15 mL). The combined organic layers were dried over $MgSO_4$ and concentrated. The residue was purified by chromatography (hexane/EtOAc, 4:1) to provide 0.03 g of one diastereomer ***l*-9** (28%) and 0.05 g of the other isomer, ***u*-9** (39%).

(*Z*)-*N*-*tert*-Butyloxycarbonyl-1-[(1′-fluoro-2′-amino)propylidene]-2-cyclopentane aldehyde (10). A stirred solution of CH_2Cl_2 (4 mL) and oxalyl chloride (0.17 g, 1.4 mmol) was dropwise added to Me_2SO (0.17 mL, 2.8 mmol) at −50 °C to −60 °C. The reaction mixture was stirred for 2 min and (*Z*)-*N*-*tert*-butyloxycarbonyl-1-[(1′-fluoro-2′-amino) propylidene-2-(hydroxy) methyl] cyclopentanes **6** (0.35 g, 1.3 mmol) were added within 5 min. Stirring was continued for an additional 15 min. Triethylamine (0.9 mL) was added and the reaction mixture was stirred for 5 min and then allowed to warm to room temperature. Water (6.5 mL) was then added and the aqueous layer was reextracted with additional CH_2Cl_2 (4×15 mL). The combined organic layers were washed with saturated NaCl solution (8 mL), and dried over $MgSO_4$. The filtrate was concentrated under vacuum. The residue was chromatographed on silica gel eluting with EtOAc-hexane (1:9) to give colorless oily (*Z*)-*N*-*tert*-butyloxycarbonyl-1-[(1′-fluoro-2′-amino) propylidene]-2-cyclopentane aldehyde **10** (0.31g, 92%). ^{1}H NMR ($CDCl_3$) δ 9.48 (s, 1H), 4.77 (s, br, 1H), 4.50 (d, J = 29.4 Hz), 3.55, 3.47 (s, br, 1H), 2.61–2.40 (m, 1H), 2.35–2.17 (m, 1H), 2.08–1.60 (m, 4H), 1.38 (s, 9H), 1.28, 1.26 (d, J = 7.0 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 198.93, 155.44, 154.53, 152.11, 115.49, 79.68, 53.21, 45.48, 28.29, 26.96, 26.70, 18.48, 17.99; ^{19}F NMR ($CDCl_3$) δ −125.36 (d, J = 24.7 Hz), −125.90 (d, J = 24.2 Hz); IR (neat) 3348, 2976, 2942, 1719, 1511, 1369, 1248, 1170, 1058 cm^{-1}

(*Z*)-1-[(1′-Fluoro-2′-amino)propylidene]-2-cyanocyclopentane hydrochloride (3). To a 2 mL 10% HCl-AcOH solution was added (*Z*)-*N*-*tert*-butyloxycarbonyl-1-[(1′-fluoro-2′-amino)propylidene]-2-cyclopentane nitrile ***l*-9** (0.05g, 0.18 mmol) at room temperature. The reaction mixture was stirred for 30 min, then concentrated under vacuum. The residue was treated with 1 mL cold diethyl ether and the mixture was kept at 0 °C for overnight. Decanting the diethyl ether and drying the precipitate gave 0.03g (75%) ***l*-3** as white solid. Mp = 219–222 °C, decomposition. ^{1}H NMR (CD_3OD) δ 4.29 (br, d, J = 25.5 Hz, 1H), 3.82 (s, br, 1H), 2.56–2.44 (m, 2H), 2.26–1.75 (m, 4H), 1.50 (d, J = 7.0, 3H); ^{13}C NMR (CD_3OD) δ 153, 149.85 (d, J = 261.8 Hz), 122.04 (d, J = 12.3 Hz), 120.97, 46.67 (d, J = 25.3 Hz), 33.15, 30.35, 28.69, 27.00, 16.04; ^{19}F NMR (CD_3OD) δ −122.64 (d, J = 24.7 Hz). Anal. ($C_9H_{14}ClFN_2$) C, 52.81; H, 6.89. Found: C, 52.98; H, 6.76.

The other diastereomer ***u*-3** was obtained in the same manner (62%) as white solid. Mp = 180–182 °C. ^{1}H NMR (CD_3OD) δ 4.24 (br, d, J = 27.1 Hz, 1H), 3.77 (s, br, 1H), 2.60–2.31 (m, 2H), 2.18–1.70 (m, 4H), 1.43 (d, J = 6.7 Hz, 3H); ^{13}C NMR (CD_3OD) δ 152.90, 149.70 (d, J = 259.6 Hz), 122.39 (d, J = 11.3 Hz), 120.90, 47.62 (d, J = 26.1 Hz), 33.17, 30.83, 28.48, 26.49, 16.10; ^{19}F NMR (CD_3OD) δ-122.52 (d, J = 24.9 Hz). Anal. ($C_9H_{14}ClFN_2$) C, 52.81; H, 6.89. Found: C, 52.78; H, 6.66.

Materials. The DPP IV substrate Gly-Pro-*p*-nitroanilide was obtained from Sigma Chemical Co. The phosphate buffer (KH_2PO_4-Na_2HPO_4, 90 mM, pH 7.6), and Tris–HCl buffer (20 mM, PH 7.8) were prepared in our laboratory. The UV–visible spectra were obtained by employing a Shimadzu UV–visible Recording Spectrophotometer (UV-160).

Human placenta dipeptidyl peptidase IV (EC 3.4.14.), purchased from Calbiochem-Novabiochem Corporation, La Jolla, CA, was stored at −20 °C. The specific activity is 8,333 mU (milliunit) per mg of protein. One mU, specified by Calbiochem-Novabiochem Corporation, is defined as the amount of enzyme that will hydrolyze 1.0 μM of Ala-Pro-7-amino-4-trifluoromethyl coumarin per min at 30 °C, pH 7.8. Before use, the enzyme solution was freshly diluted 10-fold by adding

the appropriate amount of 10 mM Tris-glycerol buffer solution. A preservative buffer solution (pH 7.8) for enzyme DPP IV was made of 20 mM Tris–HCl, 2mM EDTA, 0.04% NaN_3 and 50% glycerol. It was made by mixing 5 mL of a solution containing 40 mM Tris, 4 mM EDTA and 0.08% NaN_3 with 5 mL of glycerol.

Substrate Gly-Pro-*p*-nitroanilide hydrochloride (8.2 mg, 25 μmol) was dissolved in 5 mL of water to give a 5 mM substrate stock solution. The 5 mM inhibitor stock solutions were prepared by dissolving the appropriate inhibitor ***u*-1** or ***l*-1** into water. They then were further diluted to 1 mM, 50 μM and 10μM stock solutions. They were stored at 0 to −4 °C.

Inactivation assays. All inhibition experiments were monitored by UV-160 at 30 ± 0.1 °C.

Method A (inactivation in the absence of substrate). The inhibitory activity of the compounds ***u*-3** and ***l*-3** was estimated from the residual activity of DPP IV in the solution of the substrate Gly-Pro-*p*-nitroanilide. An aliquot of inhibitor (20 μL, from 50 μM stock solution in H_2O) was added to 80 μL of a buffered enzyme solution (0.2 mU, in Tris–HCl buffer pH 7.6) to initiate the inactivation reaction. The concentration of inhibitor in the incubation mixture (total volume 100 μL) was 10 μM. After the enzyme and inhibitor were incubated for either 2 or 30 min at 30°C, the incubation mixture was added to a 1 mL cuvette containing 900 μL of substrate Gly-Pro-*p*-nitroanilide (0.1 mM) in 45 mM phosphate buffer (pH 7.6, μ = 0.123). The measuring cell was thermally equilibrated in the spectrophotometer for 2 min before enzyme-inhibitor preincubation solution was added. The rate of change in UV absorbance at 385 nm, with respect to a cuvette containing only 0.1 mM substrate in 45 mM buffer, gave a straight line with a slope proportional to the enzyme activity. The residual enzyme activity is expressed relative to a DPP IV control which was prepared by adding only enzyme to the substrate solution. The percentage inhibition (I%) was calculated as $I = [(1\text{-}\nu/\nu_o)] \times 100\%$, where ν and ν_o are the rates of change in absorbance at 385 nm, with and without inhibitor, respectively. The percentage inhibition (I%) at other inhibition concentrations was measured by the same method.

Method B (inactivation in the presence of substrate). To a cuvette containing 5 to 20 μL of the appropriate concentration of inhibitor, 20 μL of 5 mM substrate Gly-Pro-*p*-nitroanilide, 500 μL of 90 mM phosphate buffer (pH 7.6), and enough H_2O to bring the final volume to 1 mL, was added 20 μL of enzyme solution (0.2 mU) in pH 7.6 Tris buffer. The rate of change in the absorbance at 385 nm, with respect to a cuvette containing the same amount of inhibitor and substrate in buffer, gave the inactivation progress curves.

The effect of inhibitors on DPP IV action. The control experiment (without inhibitor) for Curve A: to a cuvette containing 20 μL of 5 mM substrate Gly-Pro-*p*-nitroanilide, 500 μL of 90 mM phosphate buffer (pH 7.6), and enough H_2O to bring the final volume to 1 mL, was added 20 μL of enzyme solution (0.2 mU) in pH 7.6 Tris buffer. The rate of change in the absorbance at 385 nm, with respect to a reference cuvette containing the same amount of substrate in buffer, gave the uninhibited progress curve (Curve A). (2) The experiment for Curve B was conducted by an enzyme-initiated assay, designated as (S + I) + E. To a cuvette containing 20 μL of 50 μM of inhibitor, 20 μL of 5 mM substrate Gly-Pro-*p*-nitroanilide, 500 μL of 90 mM phosphate buffer (pH 7.6), and 440 μL of H_2O, was added 20 μL of enzyme solution (0.2 mU) in pH 7.6 Tris buffer. The rate of change in the absorbance at 385 nm, with respect to a reference cuvette containing the same amount of substrate and substrate in buffer, gave the inactivation progress curve B. (3) A substrate-initiated assay, designated as (E + I) + S, was employed in the third experiment. An aliquot of inhibitor (20 μL, from 50 μM stock solution in H_2O) was added to 80 μL of a buffered enzyme solution (0.2 mU, in Tris buffer pH 7.6) to initiate the inactivation reaction. After the enzyme and inhibitor were incubated for 30 min at 30 °C, the incubation mixture (100 μL) was added to a 1 mL cuvette containing 20 μL of 5 mM substrate Gly-Pro-*p*-nitroanilide, 500 μL of 90 mM phosphate buffer (pH 7.6), and 380 μL of H_2O. The measuring cell was thermally equilibrated in the spectrophotometer for 2 min before enzyme-inhibitor preincubation solution was added. The rate of change in UV absorbance at 385 nm, with respect to a cuvette containing only the same amount of substrate in buffer, was recorded to give the inactivation progress curve C. In all cases, the final concentration of buffer B, substrate S, enzyme E and inhibitor I were 45 mM, 0.1 mM, 0.2 mU and 1 μM, respectively.

Determination of K_i values. For each inhibitor, the data for two Dixon plots (1/V versus [I]) were obtained by repeating method B at three concentrations of substrate (0.1 mM, 0.2 mM and 0.4 mM), varying five to six different concentrations of inhibitor (0, 0.25, 0.5, 1.0, 5.0, 10.0 μM). The correlation coefficients of all straight lines were greater than 0.98. The regression equation was obtained and the error was estimated by the standard methods.[46] The K_i values were calculated by solving the equation sets according to the Dixon method.[47]

Inhibitor stability. (Method A) An aliquot (40 μL) of inhibitor from 5 mM stock solution in H_2O was added into a 1 mL cuvette containing 500 μL of 90 mM phosphate buffer solution (pH 7.6) and 460 μL of H_2O at 30 C such that the final inhibitor concentration was 0.2 mM. Adventitious decomposition in buffer was followed UV–vis-spectrometry in the range of 190 to 1100 nm at 30 °C for 24 h. The experiment was repeated in the same manner at 50 °C. (Method B) Inhibitor, 8.0 mg, was dissolved in 0.6 mL CD_3OD in a NMR tube at 25 °C; spontaneous decomposition in CD_3OD was monitored by ^1H NMR spectroscopy every other h for 24 h. No change was detected by both methods.

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