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Chemistry

# Inhibition of dipeptidyl peptidase IV by fluoroolefin-containing *N*-peptidyl-*O*-hydroxylamine peptidomimetics

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**ABSTRACT** **Dipeptidyl peptidase IV (EC 3.4.14.5; DPP IV), also known as the leukocyte differentiation antigen CD26 when found as an extracellular membrane-bound proline specific serine protease, cleaves a dipeptide from the N terminus of a polypeptide chain containing a proline residue in the penultimate position. Here we report that known *(Z)*-Ala-ψ[CF=C]-Pro dipeptide isosteres 1 and 2, which contain *O*-acylhydroxylamines, were isolated as diastereomeric pairs *u*-1, *l*-1, and *l*-2. The effect of each diastereomeric pair as an inhibitor of human placental dipeptidyl peptidase DPP IV has been examined. The inhibition of DPP IV by these compounds is rapid and efficient. The diastereomeric pair *u*-1 exhibits very potent inhibitory activity with a $K_i$ of 188 nM. Fluoroolefin containing *N*-peptidyl-*O*-hydroxylamine peptidomimetics, by virtue of their inhibitory potency and stability, are superior to *N*-peptidyl-*O*-hydroxylamine inhibitors derived from an Ala-Pro dipeptide.**

Dipeptidyl peptidase IV (EC 3.4.14.5; DPP IV; CD26), discovered in 1966 (1), is a transmembrane serine peptidase found in a variety of human tissues and organs (2–4). In particular, DPP IV, when expressed on the surface of $CD4^+$ T cells, is identical with the leukocyte differentiation antigen CD26 and is considered to be a lymphocyte activation marker (5, 6). Although the involvement of DPP IV in the immune response and regulation of lymphocyte activation has been implicated, the mechanism of the involvement is not clear (7, 8). Of the many functions that have been postulated (9–14), the most intriguing is the role of DPP IV in T-cell activation and in the regulation of T-cell proliferation (13, 15–18). Recognized as a cell surface activation marker of lymphocytes (19), the failure to observe CD26 implies a reduced immune response (20). The presence of DPP IV is associated with the capacity of cells to produce interleukin 2 and to proliferate strongly in response to mitogen stimulation (20, 21). Importantly, binding of mAbs to CD26 suppresses interleukin 2 production (21). CD26 modulation also can lead to enhanced cell proliferation preceded by an increase in $Ca^{2+}$ mobilization (22). CD26 is associated physically with CD45, which regulates T-cell activation pathways through protein tyrosine phosphatase action. CD26 apparently modulates the activity of CD45 by affecting the accessibility of critical substrates, with the result that the CD2/CD3 path amplifies the immune response (23). DPP IV, known to be localized on the surface of T cells with adenosine deaminase, seems to form a complex with adenosine deaminase that is involved in an important immunoregulatory mechanism involving T-cell proliferation (24, 25). DPP IV appears to be not only up-regulated among proliferating thymocytes but also by those undergoing programmed cell death (26). The involvement of CD26 in HIV infection has been the subject of investigation for some time, with the initial report (27) that CD26 was a cofactor facilitating HIV entry in $CD4^+$ cells refuted (28–30). Reports that DPP IV enzymatic activity may decrease the efficiency of HIV infection (31) may be related to the binding of HIV glycoproteins gp120 and gp41, which have been shown to be responsible for cell killing by apoptosis in $CD4^+$ cells (32). The Tat protein of HIV-1, known to be capable of suppressing CD3 activation of T cells, also has been shown to bind to DPP IV with effects on cytokine production and DNA synthesis, implying that the DPP IV plays a role in Tat immunosuppression (33, 34).

DPP IV will cleave the dipeptides Xaa-Pro from the N terminus of a polypeptide while recognizing several key structural features in substrate proteins or peptides. It has been postulated (35) that DPP IV substrates require the presence of a proline at the $P_1$ position as well as a protonated free N terminus (36, 37). It also has been proposed that DPP IV possesses a high conformational specificity for a trans amide bond between the $P_1$ and N-terminal $P_2$ residues (38). There is the additional requirement for the L configuration of the amino acid residue, both in the penultimate and the N-terminal position (39, 40).

Obviously, inhibition of CD26 may critically affect T-cell activation and function and may potentially have therapeutic utility in the modulation of the immune response. Relatively few of the compounds reported thus far are effective inhibitors of DPP IV, with most inhibitors suffering from either instability or low reactivity. *N*-Peptidyl-*O*-acylhydroxylamines irreversibly inhibit DPP IV, but most of the inhibitor is enzyme-hydrolyzed during the inactivation process (41, 42). The boronic acids Ala-*boro*Pro, Pro-*boro*Pro, and Val-*boro*Pro are potent and specific reversible inhibitors of DPP IV with $K_i$ values in the nanomolar range. However, these compounds lose their inhibitory activity in aqueous solution at neutral pH because of the formation of cyclic species in which the N-terminal amine nitrogen coordinates to the boron atom (37, 43–45). Peptidyl (α-aminoalkyl) phosphonate esters (46) and diphenyl phosphonate esters (47) are moderate and specific DPP IV inhibitors. These compounds are quite stable because phosphonate esters are relatively unreactive with nitrogen nucleophiles or N-terminal amines. Aminoacylpyrrolidine-2-nitriles (48) and 4-cyanothiazolidides (49, 50) recently were reported as very potent and rather stable inhibitors of DPP IV. They were found to have $K_i$ values in the nanomolar to low submicromolar range and half-lives between 27 and 72 h.

Many of the problems associated with inefficient inactivation of DPP IV are a consequence of the importance of the trans conformation of the $P_1$-$P_2$ amide bond and the requirement for a protonated free N terminus. The cyclization

Abbreviation: DPP IV, dipeptidyl peptidase IV.

Data deposition: The atomic coordinates and structure factors have been deposited in the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, CB2 1EZ, United Kingdom (reference 103375).

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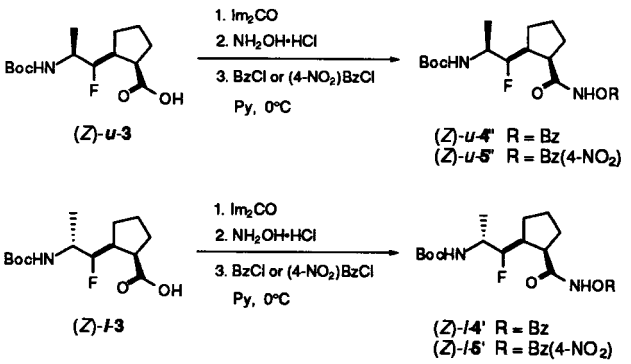
reaction of the free N-terminal amino group with the reactive site of the inhibitor does, however, require the molecule to assume the cis conformation, the conformation previously proposed to be unreactive with DPP IV (38). To obviate this mode of inactivation and to rigorously examine the cis–trans selectivity of DPP IV, we have prepared a series of conformationally constrained fluoroolefin dipeptide isosteres. The fluoroolefin dipeptide isostere was proposed as early as 1984 (51) as a superior isoelectronic and isosteric replacement for the amide bond. Theoretical studies strongly have supported the original hypothesis behind introduction of the fluoroolefin amide surrogate (52–54). The syntheses of fluoroolefin dipeptide surrogates, Ala-$\psi$[CF=C]-Pro containing *N*,*O*-diacylhydroxamic acid type protease inhibitors **1** and **2**, were reported recently by our laboratory (55).




SCHEME 1.

## MATERIALS AND METHODS

**Materials.** Human placenta dipeptidyl peptidase IV (EC 3.4.14.5) was purchased from Calbiochem–Novabiochem (La Jolla, CA). The specific activity is 8,333 milliunits per milligram of protein. One milliunit, specified by Calbiochem–Novabiochem, is defined as the amount of enzyme that will hydrolyze 1.0 $\mu$M of Ala-Pro-7-amino-4-trifluoromethyl coumarin per minute at 30°C, pH 7.8. The DPP IV substrate Gly-Pro-*p*-nitroanilide was obtained from Sigma. The phosphate buffer ($KH_2PO_4$-$NaHPO_4$, 90 mM, pH 7.6) and Tris·HCl buffer (20 mM, pH 7.8) were prepared in our laboratory.

$^1$H, $^{13}$C, $^{19}$NMR spectra were recorded on a Gemini-300 NMR spectrometer (Varian) with $CD_3OD$ as solvent and residual methanol or $CFCl_3$ as the internal standard. Thin layer chromatography was performed with $F_{254}$ (Merck) silica gel as the adsorbent on 0.2-mm thick, plastic-backed plates. The chromatograms were visualized under UV (254 nm) and by spraying with a 95:5 mixture of 0.2% ninhydrin in *n*-butanol and 10% aqueous acetic acid followed by heating. The UV-visible spectra were determined by using a Shimadzu UV-visible recording spectrophotometer (UV-160).

**Syntheses of Inhibitors 1–2.** The general procedure for amine deprotection is shown in Scheme 3. Compound *l*-**4** (14.7 mg, 0.025 mmol), prepared as described (55), was treated with 1 M HCl in acetic acid (1 ml), stirring at room temperature for 1–2 h. The solvent was removed under high vacuum. Diethyl ether (2 ml) was added to the residue. This mixture was stored at −4°C overnight. After the supernatant was decanted, the resultant white solid was washed with ether, and dried, to yield the sufficiently pure hydrochloride salts *(Z)*-(*R, R*), *(Z)*-(*S, S*)-1-[(1′-fluoro-2′-amino)propylidene]-2-cyclopentane-*O*-benzoyl hydroxamate hydrochloride (*l*-**1**) (10.7 mg, 86%). Data for *l*-**1**: $^{19}$NMR ($CD_3OD$) δ -124.37 (d, $J$ = 27.1 Hz); $^1$H NMR ($CD_3OD$) δ 8.07 (d, 2H, $J$ = 7.3 Hz), 7.69 (t, 1H, $J$ = 7.4 Hz), 1.84–1.72 (m, 1H), 1.47 (d, $^3$H, $J$ = 6.8 Hz); $^{13}$C NMR ($CH_3OD$) δ 171.81, 164.08, 148.52 (d, $J$ = 249.7 Hz), 133.67, 129.14, 128.29, 126.74, 123.34 (d, $J$ = 13.6 Hz), 44.71, 43.23 (d, $J$ = 27.6 Hz), 31.76, 28.03 (d, $J$ = 1.8 Hz), 25.26, 14.46.

*(Z)*-(*R, S*), *(Z)*-(*S, R*)-1-[(1′-fluoro-2′-amino)propylidene]-2-cyclopentane-*O*-benzoyl hydroxamate hydrochloride (*u*-**1**) was prepared in the same manner from *u*-**4** in 51% yield. Data for *u*-**1**: $^{19}$NMR ($CD_3OD$) δ -124.42 (d, $J$ = 24.4 Hz); $^1$H NMR ($CD_3OD$) δ 8.07 (d, 2H, $J$ = 8.5 Hz), 7.69 (t, 1H, $J$ = 7.5 Hz), 7.53 (t, 2H, $J$ = 7.3 Hz), 4.29 (dq, 1H, $J$ = 26.8, 6.9 Hz), 3.68–3.59 (m, 1H), 2.59–2.39 (m, 2H), 2.26–1.93 (m, 4H), 1.84–1.68 (m, 1H), 1.49 (d, $^3$H, $J$ = 6.9 Hz); $^{13}$C NMR ($CD_3OD$) δ 172.84, 165.57, 149.98 (d, $J$ = 249.7 Hz),135.13, 130.67, 129.78, 128.30, 124.69 (d, $J$ = 13.7 Hz), 47.35 (d, $J$ = 27.5 Hz), 45.30, 33.20, 29.67 (d, $J$ = 3.2 Hz), 26.99, 16.32.

*(Z)*-(*R, R*), *(Z)*-(*S, S*)-1-[(1′-fluoro-2′-amino)propylidene]-2-cyclopentane-(4-nitro)-*O*-benzoyl hydroxamate hydrochloride (*l*-**2**) was prepared in the same manner from *l*-**5** in 63% yield. Data for *l*-**2**: $^{19}$NMR ($CD_3OD$) δ -124.33 (d, $J$ = 27.1 Hz); $^1$H NMR ($CD_3OD$) δ 8.39 (d, 2H, $J$ = 9.1 Hz), 8.29 (d, 2H, $J$ = 9.0 Hz), 4.29 (dq, 1H, $J$ = 27.1, 6.9 Hz), 3.67–3.59 (m, 1H), 2.62–2.40 (m, 2H), 2.26–2.11 (m, 1H), 2.10–1.93 (m, 2H), 1.87–1.70 (m, 1H), 1.48 (d, $^3$H, $J$ = 6.9 Hz); $^{13}$C NMR ($CD_3OD$) δ 173.22, 164.03, 152.51, 150.00 (d, $J$ = 249.7 Hz), 133.66, 132.03, 124.84 (d, $J$ = 13.7 Hz), 124.83, 47.47 (d, $J$ = 27.1 Hz), 45.07, 33.43, 29.70 (d, $J$ = 2.8 Hz), 26.93, 16.16.

**Crystal Structure Determination of *l*-3.** Diastereomeric pair *l*-**3** prepared as described (55) was recrystallized from a mixture of hexanes and ethyl acetate (1:1). Crystal data: $C_{14}H_{21}FNO_3$, M = 287.3, monoclinic, space group $P2_1/n$, $a$ = 9.607 (4) Å, $b$ = 9.300 (3) Å, $c$ = 17.204 (6) Å, $\beta$ = 95.66 (3) °, $V$ = 1529.7 (9) Å$^3$, $Z$ = 4, $D_c$ = 1.248 g cm$^{-1}$, $\mu$ = 0.98 cm$^{-1}$, $\lambda$(MoK$\alpha$) = 0.71073 Å, $F$(000) = 616, T = 298 K. Nicolet R3 m/V diffractometer was used to collect 2,038 reflections ($3° < 2\theta < 45°$) on a colorless crystal 0.15 × 0.15 × 0.40 mm$^3$. Of these, 1,961 were unique and 1,193 were observed ( $F_o > 6\sigma F_o$ ). Lorentz and polarization corrections were applied to the data. The non-hydrogen atoms were located by direct methods. $R$ = 0.070, $R_w$ = 0.068, GOF = 2.14.

**Inactivation Assays.** Method A (inactivation in the absence of substrate): The inhibitory activity of the compounds, *u*-**1**, *l*-**1**, and *l*-**2**, was estimated from the residual activity of DPP IV in a solution of the substrate Gly-Pro-*p*-nitroanilide. An aliquot of inhibitor (20 $\mu$l, from 50 $\mu$M stock solution in water) was added to 80 $\mu$l of a buffered enzyme solution [0.2 milliunit in Tris·HCl buffer (pH 7.6)] to initiate the inactivation reaction. The concentration of inhibitor in the incubation mixture (total volume 100 $\mu$l) was 10 $\mu$M. 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 $\mu$l of substrate Gly-Pro-*p*-nitroanilide (0.1 mM) in 45 mM phosphate buffer (pH 7.6, $\mu$ = 0.123). The measuring cell had been equilibrated thermally 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 the 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 % $I = [(1 - v_i/v_o)] \times 100\%$, where $v_i$ and $v_o$ are the rate 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 $\mu$l of appropriate concentration of inhibitor, 20 $\mu$l of 5 mM substrate Gly-Pro-*p*-nitroanilide, 500

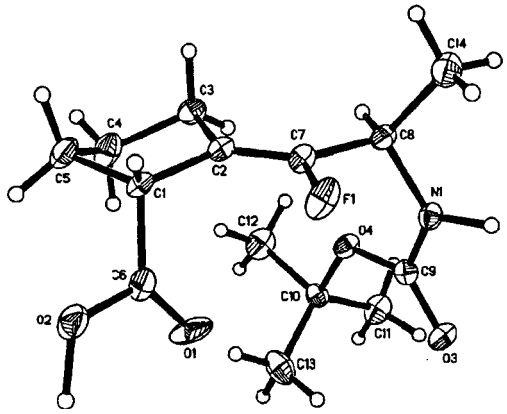



FIG. 1. ORTEP drawing of the x-ray structure of (*Z*)-*l*-Boc-Ala-ψ[CF=C]-Pro (*l*-**3**).

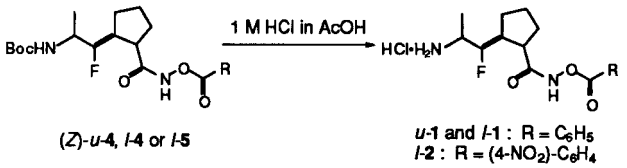
solution (0.2 milliunit) in pH 7.6 Tris buffer. The rate of change in the absorbance at 385 nm, with respect to a cuvette containing the same amount of inhibitor and substrate in buffer, gave the inactivation progress curves. All inhibition experiments were monitored by using a Shimadzu UV-160 at 385 nm and 30 ± 0.1°C.

**Determination of $K_i$ Values.** For inhibitors *u*-**1**, the data for two Dixon plots (1/V vs. [I]) were obtained by repeating method B at two concentrations of substrate (0.2 mM and 0.4 mM) and six to seven different inhibitor concentrations (0, 0.25, 0.5, 0.75, 1.0, 5.0, and 10.0 μM). The correlation coefficients of both lines were >0.994. The $K_i$ value of *u*-**1** was calculated according to the method of Dixon (56). The $K_i$ value for compound *l*-**1** was determined by the method described above.

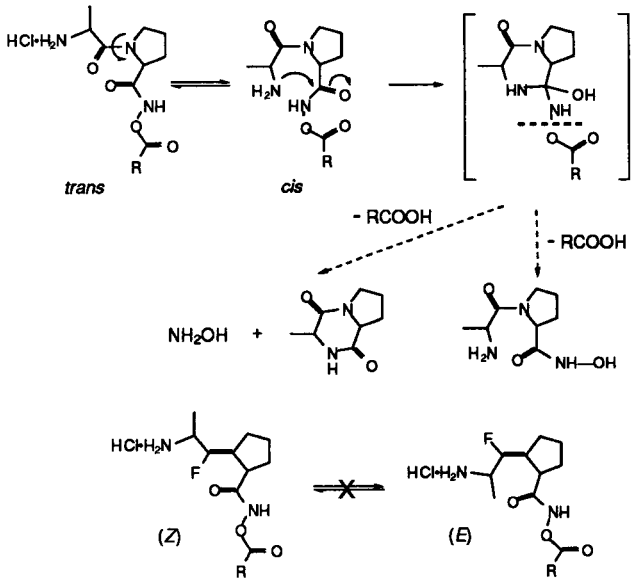
**Determination of the Half-Life of Inhibitor *u*-1.** An aliquot (40 μl) of inhibitor *u*-**1** from 5 mM stock solution in $H_2O$ was added to a 1-ml cuvette containing 500 μl of 90 mM phosphate buffer solution (pH 7.6) and 460 μl of water at 30°C, such that the final inhibitor concentration was 0.2 mM. Spontaneous decomposition was monitored by following the decrease in absorbance at 229 nm at various time intervals. The absorbance data points at 229 nm were recorded and plotted as function of time, which gave a spontaneous degradation curve. The half-life was obtained from first-order plot of $\ln(A/A_0)$ vs. time, where $A$ is the absorbance of the mixture at time $t$, and $A_0$ is the absorbance at initial time ($t = 0$).

## RESULTS AND DISCUSSION

**Chemistry.** (*Z*)-*N*-*tert*-Butyloxycarbonyl-1-[(1′-fluoro-2′-amino)propylidene]-2-cyclopentane carboxylic acid **3** was synthesized and isolated as two diastereomeric pairs **3′** and **3′′**, as described in a previous report (55). The relative stereochemistry of these diastereomeric pairs was determined by single crystal x-ray diffraction studies. The structure of compound **3′**, crystallized from a mixture of hexanes and ethyl acetate (1:1), is shown in Fig. 1. The absolute configurations at C1 and C8 of **3′** were confirmed as *R* and *R* or *S* and *S*, respectively. Therefore, **3′** can be designated as the "like" diastereomeric pair (*l*-**3**). Obviously, compound **3′′**, as "unlike" diastereomeric pair (*u*-**3**), contains the *S*, *R* isomer corresponding to the natural amino acid L-(*S*)-Ala-L-(*S*)-Pro and would be predicted to have higher biological activity.




The two diastereomeric pairs were converted independently to *l*-**4** and *u*-**4** after three step transformation (55). The analog *l*-**5** was prepared in the same manner by using 4-nitrobenzoyl chloride instead of benzoyl chloride, as shown in Scheme 2. Removal of the Boc-groups was accomplished by using 1 M HCl in AcOH to give compounds **1** and **2** (Scheme 3).




SCHEME 3.

**Inhibition of DPP IV.** The results of initial inhibition studies of DPP IV by diastereomeric pairs *u*-**1**, *l*-**1**, and *l*-**2** are shown in Table 1. At the same inhibitor concentration with 2-min incubation time or 30-min incubation time, the percentages of inhibition of DPP IV by *u*-**1**, *l*-**1**, and *l*-**2** unexpectedly remained the same or changed only slightly with an increase in incubation time.

The results presented in Table 1 revealed the following phenomena: (*i*) Inactivation of DPP IV by *u*-**1** did not follow pseudo-first order reaction kinetics. The inactivation process was principally dependent on inhibitor concentration and independent of incubation time. At a concentration of 0.01 mM, inhibitor *u*-**1** showed nearly the same percentages of inhibition, 42% and 39% inhibition, at both 2 min and 30 min incubation time, respectively. At 0.25 mM, after both 2 min and 30 min incubation time, 100% inhibition of the activity of DPP IV was observed. For inhibitors *l*-**1** and *l*-**2**, the percentages of inhibition (% *I*) increased or remained the same within experimental error with increasing incubation time at the same inhibitor concentrations (shown in Table 1). (*ii*) Inhibitory potency of *u*-**1** was much greater than that of the other diastereomeric pair *l*-**1**. At a concentration of 0.01 mM and a 2-min incubation time, compound *u*-**1** inhibited 42% of the enzymatic activity of DPP IV; however, the isomer *l*-**1** was nearly ineffective (4% inhibition) under the same conditions. As mentioned above, DPP IV has an absolute requirement for the L configuration of the amino acid residue, both in penultimate and N-terminal positions. Because the pair *u*-**1** contains the compounds with the desired configuration (L, L), it was more reactive with DPP IV. (*iii*) Replacement of the benzoyl group (*l*-**1**) by a *para*-nitrobenzoyl group (*l*-**2**) enhanced the inhibitory activity slightly. This may be because the electron

Table 1. Inhibition of DPP IV by fluoroolefin-containing *N*-peptidyl-*O*-hydroxylamines

| Inhibitors | [I], mM | % Inhibition* 2 min | % Inhibition* 30 min |
|---|---|---|---|
| *I*-**1** (*Z*)-Ala-ψ[CF=C]-Pro-NHO-Bz | 0.01 | 4 | 1 |
| | 0.50 | 17 | 25 |
| *I*-**2** (*Z*)-Ala-ψ[CF=C]-Pro-NHO-Bz(4-$NO_2$) | 0.50 | 22 | 34 |
| *u*-**1** (*Z*)-Ala-ψ[CF=C]-Pro-NHO-Bz | 0.01 | 42 | 39 |
| | 0.25 | 100 | 100 |
| Ala-Pro-NHO-Bz(4-$NO_2$) | 1.10 | 29† | 60 |

*Percentage inhibition was measured after 2- or 30-min incubation in 45 mM phosphate buffer, pH 7.6, at 30°C. Gly-Pro-*p*-nitroanilide was used as substrate.
†Incubation time was 10 min (41).

inhibitory activity superior to the previously prepared Ala-Pro-NHO-Bz(4-$NO_2$) compounds (41, 42) It has been proposed that the trans $P_2$-Pro peptide bonds of the substrates are essential to the reactivity of enzyme DPP IV. The enhancement in inhibitory potency of our fluoroolefin containing dipeptide isosteres can be attributed to the efficient mimicking of the trans $P_1$-$P_2$ amide bonds of the original dipeptides by the (*Z*) fluoroolefin double bond conformation.

The effect of inhibitor *u*-**1** on the hydrolysis of substrate (S) by DPP IV (E) was demonstrated in two different ways: enzyme-initiated assay, (S + I) + E, and substrate initiated assay, (E + I) + S (Fig. 2). The results were unexpected. In both cases (curves B and C), the rates of hydrolysis of substrate by DPP IV increased linearly over 50 min and were nearly identical (curves B and C overlapped). The initial rate (u) of hydrolysis of substrate in an enzyme initiated assay is often larger than that found in a substrate initiated assay (57). The curves B and C shown in Fig. 1 indicate that inhibitor *u*-**1** very rapidly inactivates DPP IV in a process much faster than the rate of hydrolysis of the substrate by DPP IV; thus, the presence of competing substrate did not slow down the inactivation process.

To determine the values of the inhibition constant $K_i$ for both compound *u*-**1** and *l*-**1**, the rates of DPP IV-catalyzed hydrolysis of Gly-Pro-*p*-nitroanilide substrate were estimated at six to seven different concentrations for each inhibitor (0.25 to 10 μM) in a competitive hydrolysis fashion. The $K_i$ values reported in Table 3 for compounds *u*-**1** and *l*-**1** were obtained

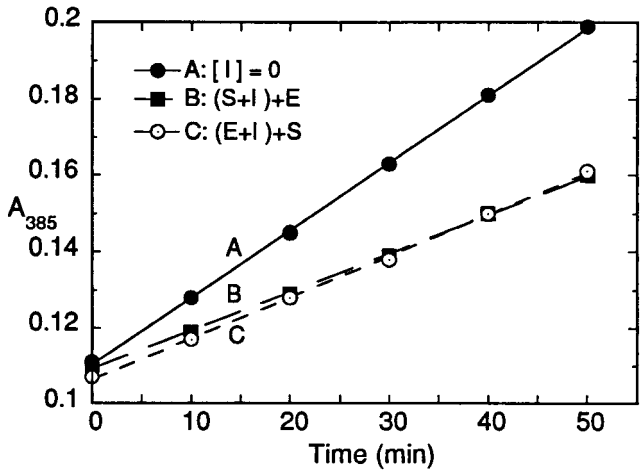



FIG. 2. Hydrolysis of substrate Gly-Pro-4-nitroanilide was monitored by the change in absorbance at 385 nm with time. In all cases, the final

Table 2. Inhibition constants of inhibitors of DPP IV, *I*-**1**, and *u*-**1**

| Inhibitors | $K_i$, nM |
|---|---|
| *I*-**1** (*Z*)-Ala-ψ[CF=C]-Pro-NHO-Bz | 14,400 |
| *u*-**1** (*Z*)-Ala-ψ[CF=C]-Pro-NHO-Bz | 188 |
| Ala-Pro-NHO-Bz(4-$NO_2$) | 30,000 (58) |

from the plots 1/v versus [I] according to the method of Dixon (56). The Dixon plot revealed that compounds *u*-**1** and *l*-**1** act as competitive inhibitors.

As shown in Table 2, the diastereomeric pair *u*-**1** (containing the L, L isomer) exhibited very potent inhibitory activity with a small $K_i$ value in the nanomolar range (0.19 μM). The affinity of this isomer for DPP IV is two orders of magnitude greater than the other diastereomeric pair *l*-**1** ($K_i$ = 14.4 μM). The Demuth's inhibitor, Ala-Pro-NHO-Bz(4-$NO_2$), also has a larger $K_i$ value (30 μM) (58) and is a poorer inhibitor.

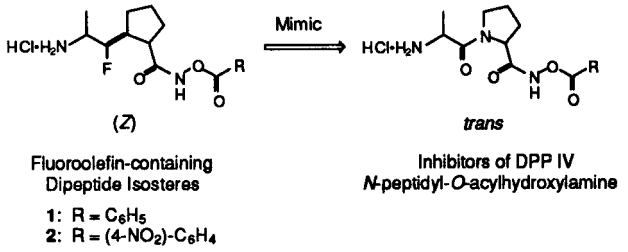
**Stability of Inhibitors.** As shown in Table 3, inhibitor *u*-**1** was very stable in buffer (pH 7.6) with a decomposition rate constant $k_d$ of $1.1 \times 10^{-4}$ $min^{-1}$ and a half-life of 103 h. In contrast, the stability of inhibitor Ala-Pro-NHO-Bz(4-$NO_2$) under assay conditions was limited because of a 10-fold higher decomposition rate constant $k_d$ ($1.3 \times 10^{-3}$ $min^{-1}$), resulting in a shorter half-life of only 8.8 h (42).

We believe that intramolecular cyclization is probably responsible for facile, spontaneous degradation of natural peptide-based hydroxamic acid inhibitors. In a manner similar to enzyme-induced *N-O* fission, the free amino group at the N terminus nucleophilically attacks the amide carbonyl carbon, thus forming a tetrahedral intermediate and thereby promoting *N-O* scission and subsequent generation of the reactive acylnitrene intermediate. The six-membered cyclic intermediate is hydrolyzed further either to hydroxamic acid or diketopiperazine products (Scheme 4). Clearly, the findings of our study support this postulate. Considerable improvement in stability of our compounds in buffer at neutral pH can be ascribed to the constrained double bond conformation of (*Z*)-fluoroolefin, excluding the possibility of intramolecular cyclization caused by the amide bond rotation in the dipeptides.

(*Z*)-Fluoroolefin-containing dipeptides *u*-**1**, *l*-**1**, and *l*-**2**, designed as the mimics of *N*-peptidyl-*O*-acylhydroxylamines, have been synthesized and tested as inhibitors of dipeptidyl peptidase DPP IV. One diastereomeric pair *u*-**1** exhibits very potent inhibitory activity with a $K_i$ of 188 nM. The inhibitory potency of this isomer is ≈70-fold higher than the other diastereomer *l*-**1** ($K_i$ = 14, 400 nM). In comparison with the Ala-Pro-NHO-Bz(4-$NO_2$) analog, the dipeptide isosteres **1** and **2** are better inhibitors of DPP IV by virtue of their superior inhibitory potency and stability; presumably, the (*Z*) double bond conformation of the fluoroolefin dipeptide isosteres efficiently mimics the trans $P_2$-Pro amide bonds of the original dipeptides. In addition, the rates of inactivation of DPP IV by compounds **1** and **2** appeared to be very fast. More detailed biological studies, kinetic analysis for inactivation rate constant $k_{inact}$, and investigations of inhibition mechanism are in progress at present. The results of this study reveal that a series of known inhibitors of DPP IV such as dipeptide boronic acids (43–45), dipeptide phosphonates (46, 47), peptidyl nitriles (49–51), and others can be modified by replacing the amide bonds by fluoroolefin moieties. Because of the anticipated high affinity and stability, the fluoroolefin containing dipeptide peptiomimetics should prove to be very promising inhibitors of

Table 3. Spontaneous degradation rate constants $k_d$ and half-life $t_{1/2}$

| Inhibitors | $k_d \cdot 10^4$, $min^{-1}$ | $t_{1/2}$, h |
|---|---|---|




SCHEME 4.

DPP IV and therefore helpful in elucidating the biological functions of DPP IV in T-cell activation. These agents may also be potential therapeutic agents useful in modifying and controlling the immune response.

Dedicated to Professor Dieter Seebach on the occassion of his 60th birthday. Financial support of this work by the National Science Foundation Grant CHE 9413004 and National Institutes of Health Grant A133690 is gratefully acknowledged.

1. Hopsu-Havu, V. K. & Sarimo, S. R. (1967) *Hoppe-Seylers Z. Physiol. Chem.* **348,** 1540–1550.
2. Mentlein, R., Heymann, E., Scholz. W., Feller, A. C. & Flad, H.-D. (1984) *Cell. Immunol.* **89,** 11–19.
3. Feller, A. C., Heijnen, C. J., Ballieux, R. E. & Parwaresc, M. R. (1982) *Br. J. Haematol.* **51,** 227–234.
4. Schön, E., Demuth, H. U., Barth, A. & Ansorge, S. (1984) *Biochem. J.* **223,** 255–258.
5. Mattern, T., Flad, H. D., Feller, A. C., Heymann, E. & Ulmer, A. J. (1989) in *Leukocyte Typing IV*, ed. Knapp, E. (Oxford Univ. Press, New York), pp. 417–418.
6. Barton, R. W., Prendergast, J. & Kennedy, C. A. (1990) *J. Leukocyte Biol.* **48,** 291–296.
7. Sedo, A. & Kraml, J. (1994) *Sb. Lek.* **95,** 285–288.
8. Ansorge., S., Buhling., F., Kahne, T., Lendeckel, U., Reinhold, D., Tager, M. & Wrenger, S. (1997) *Adv. Exp. Med. Biol.* **421,** 127–140.
9. Hanski, C., Huhle, T. & Reutler, W. (1985) *Biol. Chem. Hoppe-Seyler* **366,** 1169–1176.
10. Hapsu-Havu, V. K., Rintola, P. & Glenner, G. G. (1968) *Acta Chem. Scand.* **22,** 299–308.
11. Frohman, L. A., Downs, T. R., Williams, T. C., Heimer, E. P., Pan, Y.-C. & Felix, A. M. (1986) *J. Clin. Invest.* **78,** 906–913.
12. Frohman, L. A., Downs, T. R., Heimer, E. P. & Felix, A. M. (1989) *J. Clin. Invest.* **83,** 1533–1540.
13. Schon, E., Mansfield, H. W., Demuth, H. U., Barth, A. & Ansorge, S. (1985) *Biomed. Biochim. Acta* **44,** K9–K15.
14. Gruber, M., Scholz, W. & Flad, H. D. (1988) *Cell Immunol.* **113,** 423–434.
15. Kahne, T., Kroning, H., Thiel, U., Ulmer, A. J., Flad, H. D. & Ansorge, S. (1996) *Cell Immunol.* **170,** 63–70.
16. Hegen, M., Kameoka, J., Dong, R. P., Morimoto, C. & Schlossman, S. F. (1997) *Adv. Exp. Med. Biol.* **421,** 109–116.
17. Ruiz, P., Hao, L., Zucker, K., Zacharievich, N., Viciana, A. L., Shenkin, M. & Miller, J. (1997) *Transpl. Immunol.* **5,** 152–161.
18. Willheim, M., Ebner, C., Baier, K., Kern, W., Schrattbauer, K., Thien, R., Kraft, D., Breiteneder, H., Reinisch, W. & Scheiner, O. (1997) *J. Allergy Clin. Immunol.* **100,** 348–355.
19. Ulmer, A. J., Mattern, T., Feller, A. C., Heymann, E. & Flad, H. D. (1990) *Scand. J. Immunol.* **31,** 429–435.
20. Mentlein, R., Staves, R., Rix-Matzen, H. & Tinneberg, H. R. (1991) *Eur. J. Clin. Chem. Clin. Biochem.* **29,** 477–480.
21. Mattern, T., Scholz, W., Feller, A. C., Flad, H. D. & Ulmer, A. J. (1991) *Scand. J. Immunol.* **33,** 737–748.
22. Dang, N. H., Torimoto, Y., Sugita, K., Daley, J. F., Schow, P., Prado, C., Schlossman, S. F. & Morimoto, C. (1990) *J. Immunol.* **145,** 3963–3971.
23. Torimoto, Y., Dang, N. H., Vivier, E., Tanaka, T., Schlossman, S. F. & Morimoto, C. (1991) *J. Immunol.* **147,** 2514–2517.
24. Dong, R. P, Kameoka, J., Hegen, M., Tanaka, T., Xu, Y.,
25. Proost, P., De Meester, I., Schols, D., Struyf, S., Lambeir, A. M., Wuyts, A., Opdenakker, G., DeClercq, E., Scharpe, S. & Van-Damme, J. (1998) *J. Biol. Chem.* **273,** 7222–7227.
26. Ruiz, P., Nassiri, M., Steele, B. & Viciana, A. L. (1996) *Cytometry* **23,** 322–329.
27. Callebaut, C., Krust, B., Jacotot, E. & Hovanessian, A. G. (1993) *Science* **262,** 2045–2050.
28. Broder, C. C., Nussbaum O., Gutheil, W. G., Bachovchin, W. W., Berger, E. A., Patience, C., McKnight, A., Claphan, P. R., Boyd, M. T., Weiss, R. A., *et al.* (1994) *Science* **264,** 1156–1165.
29. Dalgleish, A. (1995) *Nat. Med.* **1,** 881–882.
30. Oranecz, T., Koderiquez, G., Koffi, J., Wang, J., Ditto, M., Bou-Habib, D. C., Lusso, P. & Norcross, M. A. (1995) *Nat. Med.* **1,** 1995.
31. Morimoto, C., Lord, C. I., Duke-Cohan, J. S., Letvin, N. L. & Schlossman, S. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9960–9964.
32. Jacotot, E., Callebaut, C., Blanco, J., Riviere, Y., Krust, B. & Hovanessian, A. G. (1996) *Virology* **223,** 318–330.
33. Reinhold, D., Wrenger, S., Bank, U., Buhling, F., Hoffmann, T., Neubert, K., Kraft, M., Frank, R. & Ansorge, S. (1996) *Immunobiology* **195,** 119–128.
34. Jiang, J. D., Wilk, S., Li, J., Zhang, H. & Bekesi, J. G., (1997) *Res. Virol.* **148,** 255–266.
35. Yaron, A. & Naider, F. (1993) *Crit. Rev. Biochem. Mol. Biol.* **28,** 31–81.
36. Barth, A., Heins, J., Fischer, G., Neuber, K., Neubert, K. & Scheneeweiss, B. (1984) *Wiss. Beitr. Martin-Luther-Univ. Halle-Wittenberg* **46**, 297–339.
37. Flentke, G. R., Munoz, E., Huber, B. T., Plaut, A. G., Kettner, C. A. & Bachovchin, W. W. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 1556–1559.
38. Fischer, G., Heins, J. & Barth, A. (1983) *Biochim. Biophys. Acta* **742,** 452–462.
39. Heins, J., Welker, P., Schonlein, C., Born, J., Hartrodt, B., Neubert, K., Tsuru, D. & Barth, A. (1988) *Biochim. Biophys. Acta* **954,** 161–169.
40. Gutheil, W. G. & Bachovchin, W. W. (1993) *Biochemistry* **32,** 8723–8731.
41. Fischer, G., Demuth, H. U. & Barth, A. (1983) *Pharmazie* **38,** 249–251.
42. Demuth, H. U., Baumgrass, R., Schaper, C. & Frischer, G. (1988) *J. Enzyme Inhib.* **2,** 129–142.
43. Snow, R. J., Bachovchin, W. W., Barton, R. W., Campbell, S. J., Coutts, S. J., Freeman, D. M., Gutheil, W. G., Kelly, T. A., Kennedy, C. A., Krolikowski, D. A., *et al.* (1994) *J. Am. Chem. Soc.* **116,** 10860–10869.
44. Kelly, T. A., Adams, J., Bachovchin, W. W., Barton, R. W., Campbell, S. J., Coutts, S. J., Kennedy, C. A. & Snow, R. J. (1993) *J. Am. Chem. Soc.* **115,** 12637–12638.
45. Bachovchin, W. W., Plaut, A. G., Flentke, G. R., Lynch, M. & Kettner, C. A. (1990) *J. Biol. Chem.* **265,** 3738–3743.
46. Bodusek, B., Oleksyszyn, J., Kam, C.-H., Selzler, J., Smith, R. E. & Powers, J. C. (1994) *J. Med. Chem.* **37,** 3969–3976.
47. Lambeir, A. M., Borloo, M., De Meester, I., Belyaev, A., Augustyns, K., Hendriks, D., Scharpe, S., Haemers, A. (1996) *Biochim. Biophys. Acta* **1290,** 76–82.
48. Li, J., Wilk, E. & Wilk, S. (1995) *Arch. Biochem. Biophys.* **323,** 148–154.
49. (1993) Patent Cooperation Treaty (PCT) WO 95/15309.
50. Ashworth, D. M., Atrash, B., Baker, G. R., Baxter, A. J., Jenkins, P. D., Jones, D. M. & Szelke, M. (1996) *Bioorg. Med. Chem. Lett.* **6,** 1163–1166.
51. Ellison, S. L. R. (1984) Ph. D. dissertation (Univ. of Liverpool, Liverpool, U.K.).
52. Abraham, R. J., Ellison, S. L. R., Schonholzer, P. & Thomas, W. A. (1986) *Tetrahedron* **42,** 2101–2110.
53. Allmendinger, T., Furet, P. & Hungerbühler, E. (1990) *Tetrahedron Lett.* **31,** 7297–7300.
54. Cieplak, P. & Kollman, P. A. (1993) *J. Comput. Aided Mol. Des.* **7**, 291–304.
55. Welch, J. T. & Lin, J. (1996) *Tetrahedron* **52,** 291–304.
56. Dixon, M. (1953) *Biochem. J.* **55,** 170–171.
57. Kettner, C. A. & Shenvi, A. B. (1984) *J. Biol. Chem.* **259,** 15105–15114.