Specific and Irreversible Cyclopeptide Inhibitors of Dipeptidyl Peptidase IV Activity of the T-Cell Activation Antigen CD26

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Received September 26, 1997

The dipeptidyl peptidase IV (DPP IV) activity of CD26 is characterized by its post-proline-cleaving capacity that plays an important but not yet understood role in biological processes. Here we describe a new family of specific and irreversible inhibitors of this enzyme. Taking into account the substrate specificity of DPP IV for P_2-P_1>-<P_1' cleavage, we have designed and synthesized cyclopeptides c[($^{\alpha}H_2N^+$)-Lys-Pro-Aba-(6-CH_2-S^+R_2)-Gly_n] 2TFA^- (Aba = 3-aminobenzoic acid, R = alkyl) possessing a proline at the P_1 position and a lysine in the P_2 position, which allows the closing of the cycle on its side chain. These molecules show a free N-terminus, necessary for binding to the CD26 catalytic site, and a latent quinoniminium methide electrophile, responsible for inactivation. Treatment of c[$^{\alpha}$Z-Lys-Pro-Aba-(6-CH_2-OC_6H_5)-Gly_n], obtained by peptide synthesis in solution, with R_2S/TFA simutaneously cleaved the Z protecting group and the phenyl ether function and led to a series of cyclopeptide sulfonium salts. These cyclopeptides inhibited rapidly and irreversibly the DPP IV activity of CD26, with IC_{50} values in the nanomolar range. Further studies were carried out to investigate the effect of the modification of the ring size (n = 2 or 4) and the nature of the sulfur substituents (R = Me, Bu, Oct). Cycle enlargement improved the inhibitory activity of the methylsulfonio cyclopeptide, whereas the increase of the alkyl chain length on the sulfur atom had no apparent effect. Other aminopeptidases were not inhibited, and a much weaker activity was observed on a novel isoform of DPP IV referred to as DPP IV-β. Thus, this new family of irreversible inhibitors of DPP IV is highly specific to the peptidase activity of CD26.

Introduction

Dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5), a membrane-bound exopeptidase, has been classically associated to the T-cell activation antigen CD26, a multifunctional sialoglycoprotein expressed on a variety of different epithelia and also by different hematopoietic cell types (for some recent reviews, see Yaron and Naider.[1] and Fleischer et al.[2]). Dipeptidyl peptidase IV (CD26) is a serine protease which has the unique specificity to cleave dipeptides from the N-terminus of polypeptides provided that proline is the penultimate residue.[1] In HIV infection, CD26 has been implicated in the viral entry process and its cytopathic effect.[3] Furthermore, DPP IV activity inhibition by HIV-1 Tat protein has been proposed as the mechanism of the lack of response to recall antigens observed in early stages of HIV infection;[4] however, the relevance of this inhibition in physiological conditions is unclear.[5] Whatever is the case, the addition of soluble CD26 can restore response to recall antigens of HIV-infected individuals in vitro.[6]

Irrespective of its peptidase activity, CD26 is associated with other molecules on the cell surface. It has been shown to be the main receptor of adenosine deaminase,[7] and on T-lymphocytes, CD26 is associated with CD45,[8] a cell-surface-expressed phosphotyrosine phosphatase involved in signal transduction. Both features of CD26, its signaling capacity and its peptidase activity, contribute to the costimulatory function of CD26 in T-cell activation events. However, the role of DPP IV activity of CD26 in these events is unclear. Some authors have described that small synthetic inhibitors of DPP IV impair mitogen and antigen stimulation of PBMC and other lymphocytic cell types.[9] In contrast, others have found no effect of DPP IV inhibitors on stimulated T-cells.[10] Similarly, contradictory results on the function of the DPP IV activity of CD26 have been reported by using cell lines expressing a mutated, catalytically inactive form of CD26.[11,12] The failure to understand the role of DPP IV activity is, in part, due to the fact that no physiological substrates have been identified. However, a broad spectrum of bioactive peptides, including some interleukines, chemokines, neuropeptides, and growth factors, can be potentially cleaved by DPP IV.

The availability of stable specific irreversible inhibitors or highly potent reversible inhibitors of DPP IV should be useful in studies for the determination of the physiological and pathological role(s) of this enzyme. Several competitive, tight-binding, or irreversible inhibitors of the enzyme are already known: oligopeptides with the N-terminal X-Pro sequence (X = various amino acid residues) such as the diprotins A and B (Ile-Pro-Ile or Val-Pro-Leu),[13,14] X-pyrrolidides and X-thiazolidides,[14−16] X-cyanoPro and X-cyanoThia,[17−19] X-phosphonoPro or Pip aryl esters,[20,21] X-boroPro,[22−25] X-ProCH_2N^+-

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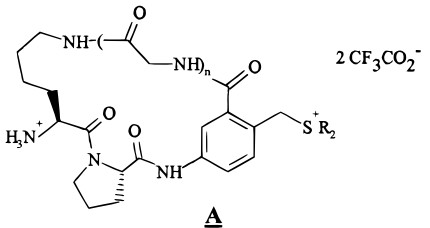
S0022-2623(97)00640-7 CCC: $15.00
Published on Web 05/13/1998

Chart 1. General Structure of the Cyclopeptides **A** Designed for Irreversible Inhibition of the DPP IV Activity of CD26



A

Me$_3$,[26] X-azaPro derivatives,[27] X-Pro-*N*-(arylcarbonyloxy)-amides,[28,29] and one of its ψ(CF=C) fluoro olefin isosteres.[30,31] Owing to the presence of a free amino N-terminus and the flexibility of the imino peptide bond, several of these inhibitors easily cyclize and are not very stable in solution.[32] The diacylated hydroxylamines are mechanism-based irreversible inhibitors of the protease: a demasked acylnitrene can react directly with an active site nucleophile or can lead, through a Lossen rearrangement, to an electrophilic isocyanate.[33]

We have previously designed and studied functionalized cyclopeptides containing a latent quinoniminium methide electrophile as suicide substrates for serine proteases.[34] Taking into account the substrate specificity of the DPP IV enzyme for P$_2$-Pro><-P′$_1$ cleavages (P$_1$ = Pro; Schechter and Berger notation[35]), we have now designed, synthesized, and studied cyclopeptides **A** (Chart 1): c[($^{\alpha}$H$_2$N$^+$)-Lys-Pro-Aba-(6-CH$_2$-S$^+$R$_2$)-Gly$_n$] 2TFA$^-$ (Aba = 3-aminobenzoic acid, R = alkyl), possessing the same latent electrophile, as selective suicide substrates for this exoprotease. These cyclopeptides were able to induce the complete, rapid, and irreversible inhibition of the DPP IV activity of CD26 with IC$_{50}$ in the nanomolar range. Their specificity was demonstrated by the lack of its effect on the activity of other peptidases, including the cell-surface-expressed DPP IV-β, the recently described protein with typical DPP IV activity.[36]

Results

From the relative weak importance of the nature of the P$_2$ side chain on the rate of hydrolysis of the enzyme substrates (vide infra), we hypothesized that the cyclization to the ϵ-amino function of a P$_2$ lysine residue would result in new molecules able to bind the catalytic site of the DPP IV activity in CD26. Moreover, this cyclization would leave the N-terminal α-amino group free and protonated, a necessary condition for the recognition by the enzyme (Chart 1). In macrocycles **A**, the substituted P′$_1$ aminobenzoic acid residue Aba-(6-CH$_2$-S$^+$R$_2$) is a precursor of the latent electrophilic quinoniminium methide cation. The presence of different numbers of glycine residues (*n*) in the cyclopeptides **A** allows the variation of the ring size, whereas the nature of the sulfur substituents (R) will modify the bulk and the lipophilicity in this part of the molecule. The influence of the leaving group ability on the inhibition efficiency and selectivity has been also examined by replacing the benzylic sulfonium substituent by an acetate group.

Chemistry. Cyclopeptides **A** were prepared according to the reaction sequence shown in Scheme 1. Peptide synthesis in solution, using DCC/HOBt for the formation of the amide bonds, was applied for the synthesis of the linear precursors of these macrocycles. The strategy involved the use of a nitro substituent as a latent amino group.[37] Coupling of the 2-(phenoxymethyl)-5-nitrobenzoic acid (Nba-[CH$_2$OPh], **5**[38]) with either ethyl diglycinate or ethyl tetraglycinate gave the substituted ethyl nitrobenzoyl polyglycinates **6a**,**b**. Selective catalytic hydrogenation of the nitro substituent, without hydrogenolysis of the benzyl ether function, occurred in the presence of platinium oxide in MeOH/DMF. The unstable aminobenzoyl polyglycine derivatives were rapidly acylated with *N*-Boc-L-proline to generate compounds Boc-Pro-Aba-[CH$_2$OPh]-Gly$_n$-OEt **7a**,**b**. Cleavage of the N-protecting group occurred in trifluoroacetic acid. In the following coupling step, the orthogonally diprotected Z-L-Lys(Boc)-OH derivative was used. The linear peptides **8a**,**b** were obtained in good yields. Cyclization of these precursors was achieved by the azide method: hydrazinolysis of the ester function leading to protected hydrazides **9a**,**b**, selective cleavage of the N$^{\epsilon}$-Boc group of the lysine residue in the presence of the N$^{\alpha}$-Z protecting group, treatment of the resulting hydrazides with an alkyl nitrite, and dilution in DMF in the presence of a tertiary amine. The cyclization yields were 45% and 44% for **10a**,**b**, respectively.

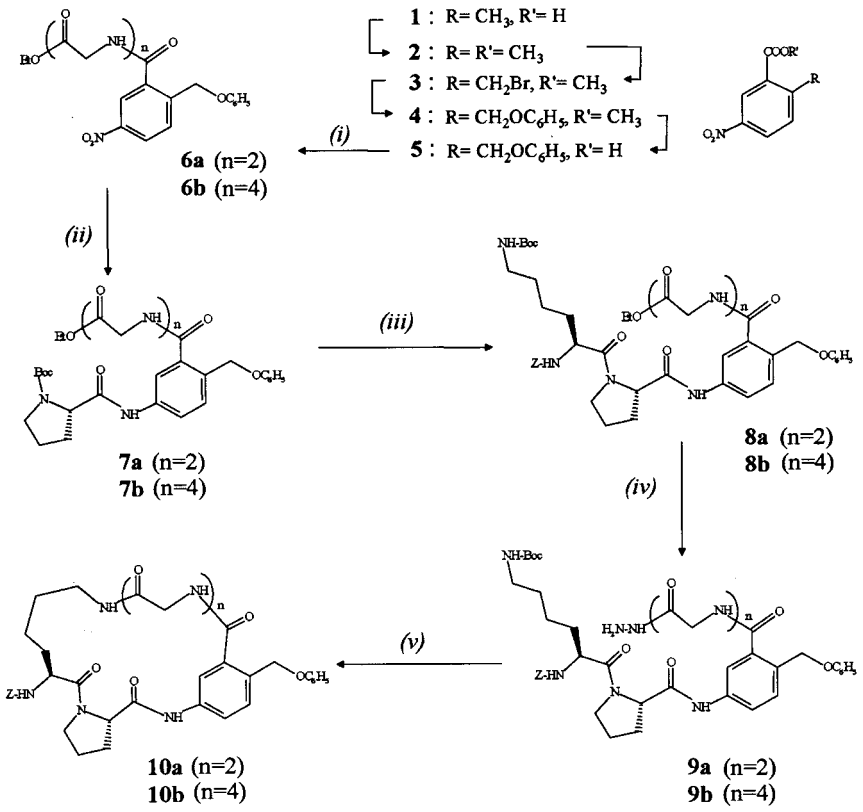
Organic sulfides, such as thioanisole, in trifluoroacetic acid are known to deprotect *O*-benzyltyrosine without the formation of O-to-C rearrangement products[39] and also to cleave the *N*-benzyloxycarbonyl protecting group.[40] The reactions occur by a "push−pull mechanism": nucleophilic attack of the sulfide lone pair on the benzylic carbon of a protonated ether or carbamate function. The byproducts in these deprotection reactions are sulfonium salts. We reasoned that treatment of the c[$^{\alpha}$Z-Lys-Pro-Aba-(6-CH$_2$-OC$_6$H$_5$)-Gly$_n$] cyclopeptides with various dialkyl sulfides in trifluoroacetic acid should cleave both the Z protecting group and the phenyl ether function (Scheme 2), thus leading to sulfonium salts having a protonated N-terminus. Effectively, such a treatment gave the bis trifluoroacetate salts **A**: **11a** (*n* = 2, R = Me, 62% yield), **11b** (*n* = 4, R = Me, 60% yield), **12a** (*n* = 2, R = Bu, 74.5% yield), **13a** (*n* = 2, R = Oct, 71% yield).

For the preparation of the cyclopeptide having a benzylic acetoxy substituent, the corresponding dimethylsulfonium salt **11a** was treated with potassium acetate in dry DMF. The resulting acetate was not very stable and decomposed during purification. The crude product was therefore reacted with di-*tert*-butyl dicarbonate to give the stable N-protected derivative which was easily purified by chromatography. Treatment with trifluoroacetic acid cleaved the Boc protecting group and led to the expected acetate salt **15a** in 76% yield.

Finally, selective hydrogenolysis of the Z protecting group of the cyclopeptide in the presence of the benzylic phenyl ether function was achieved by using a palladium on carbon catalyst in aqueous methanol and gave the cyclopeptide **16a** possessing a phenoxy substituent (Scheme 2).

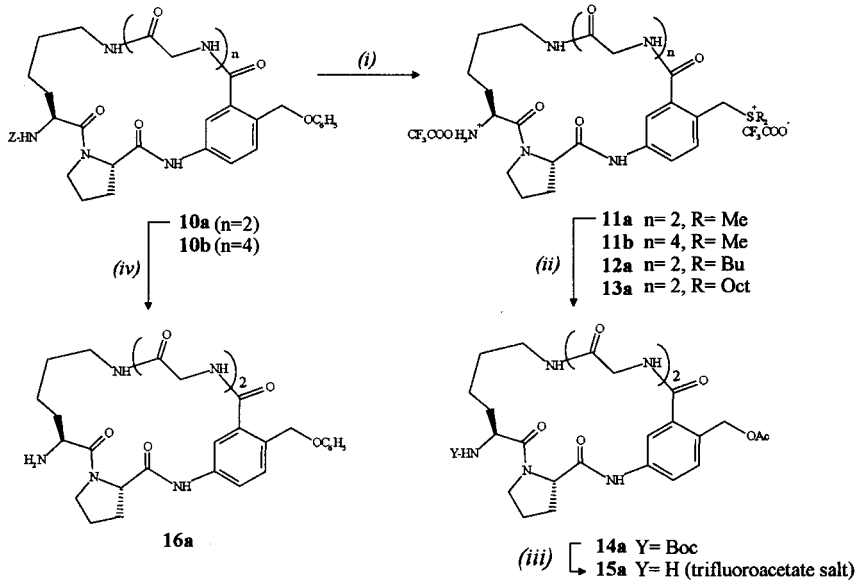
For comparison, simplified linear analogues of the cyclopeptides **A** were studied. The trifluoroacetate salts H$_2^+$-Ala-Pro-Aba(6-CH$_2$-S$^+$Me$_2$)-OMe and H$_2^+$-Lys(H$^+$)-

Scheme 1. Synthesis of the Cyclopeptides **10a**,**b** Having a Benzylic Phenoxy Substituent and a Terminal N-Z Protecting Group[a]



[a] (i) Gly_nOEt, DCC/HOBt; (ii) 1. H_2/PtO_2, MeOH−DMF, 2. Boc-Pro-OH, DCC/HOBt; (iii) 1. CF_3CO_2H or HCl/CH_2Cl_2, 2. Z-Lys(Boc)-OH, DCC/HOBt; (iv) NH_2NH_2/MeOH; (v) 1. CF_3CO_2H/CH_2Cl_2, 2. HCl/DMF−THF, 3. *i*-$PrNEt_2$/DMF.

Scheme 2. Last Steps of the Synthesis of Cyclopeptides **11a**−**16a**[a]



[a] (i) R_2S/CF_3CO_2H; (ii) 1. KOAc/DMF, 2. Boc_2O; (iii) CF_3CO_2H; (*iv*) H_2/Pd/C, MeOH.

Pro-Aba(6-CH_2-S^+Me_2)-OMe (**18a**,**b**) were prepared as above from the corresponding benzylic ethers H_2^+-X-Pro-Aba(6-CH_2-OC_6H_5)-OMe (**17a**,**b**).

Biological Studies. In all the studies described here, the dipeptidyl peptidase activity of CD26 and DPP IV-β was investigated using the enzymes in their natural habitat, i.e., by using either crude cell extracts or intact cells[36] as described in the Experimental Section. Indeed, CD26 and DPP IV-β are expressed on the cell surface, and thus their enzymatic activity could be assayed by using intact cells.[36] As a source of CD26 and DPP IV-β, we used either human CEM cells overexpressing CD26 by transfection[49] or human C8166 cells which express only DPP IV-β.[36] In some experiments partially purified enzyme preparations were also used.[51] In the case of CD26, we also used immunoaffinity-purified preparations of CD26 as we have described previously.[36] For the measurement of the

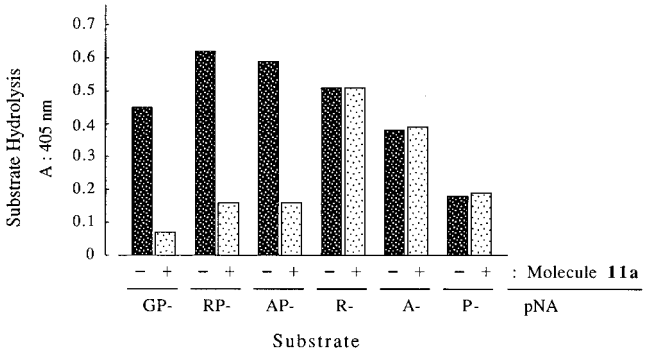


Figure 1. Specificity of molecule **11a**. Effect of inhibitor **11a** on different types of peptidase activities. Crude MOLT4 cell extracts[36] were assayed for the effect of 10 μM compound **11a** on different aminopeptidase activities. The peptidases tested were as follows: DPP IV by the cleavage of GP-pNA, RP-pNA, and AP-pNA; Arg-peptidase by the cleavage of R-pNA; Ala-peptidase by the cleavage of A-pNA; and Pro-peptidase by the cleavage of P-pNA. At 10 μM **11a**, the DPP IV activity against different substrates was inhibited by more than 75%, while no apparent effect on the other peptidases was observed. Abbreviations of the amino acids: G = glycine, P = proline, A = alanine, and R = arginine.

activity of different peptidases, extracts from human MOLT4 cells were used.[36]

Preliminary results pointed out that molecule **11a** was a potent inhibitor of DPP IV activity of CD26. Indeed, the IC_{50} value for the inhibition of the DPP IV activity of a purified preparation of CD26[36] was found to be 0.01 μM. For this reason, we first studied the specificity of this inhibitor by determining its effect on different aminopeptidases found in crude cell extracts. The peptidases tested were as follows: arginine-peptidase (EC 3.4.11.6) by the cleavage of Arg-pNA, alanine-peptidase (EC 3.4.11.2) by the cleavage of Ala-pNA, proline-peptidase (EC 3.4.11.5) by the cleavage of Pro-pNA, and DPP IV by the cleavage of different substrates (Gly-Pro-, Arg-Pro-, and Ala-Pro-pNA).[36] At 10 μM molecule **11a**, which is 3 orders of magnitude higher than its IC_{50}, the DPP IV activity monitored with the different substrates was inhibited by more than 80% (Figure 1). The remaining residual 20% activity was probably due to the background (i.e., a nonspecific and CD26-independent cleavage of the substrates) since it was observed even at 100 μM molecule **11a** (not shown). In contrast, molecule **11a** exerted no inhibitory effect on the activity of arginine-, alanine-, and proline-aminopeptidase (Figure 1). These results therefore demonstrated the specific nature of the DPP IV inhibition by molecule **11a**.

To investigate the irreversible nature of the inhibitory molecule **11a**, its effect on the DPP IV activity of CD26 expressed on the cell surface was studied. For this purpose, we established by transfection of CD26 cDNA, human CEM cells which express high levels of CD26, i.e., cells which express high levels of DPP IV activity on the cell surface. Consequently, intact cells (clone H01) could be assayed for DPP IV activity by incubation with an appropriate substrate, such as Gly-Pro-pNA.[36] Under these experimental conditions, molecule **11a** was found to be a potent inhibitor of cell-surface DPP IV activity, with maximum inhibition occurring at 2 μM. First, we investigated the kinetics of inhibition of cell-surface DPP IV activity in these CEM cells preincubated

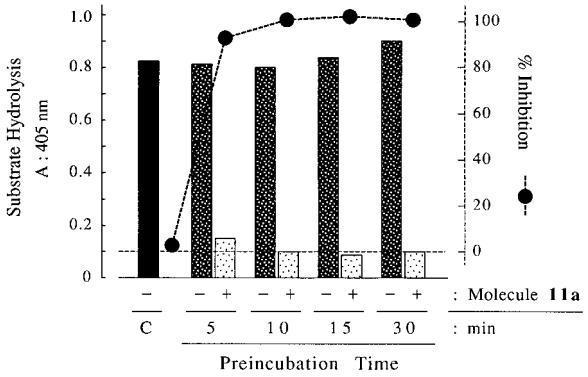


Figure 2. Kinetics of molecule **11a**-mediated inhibition of the cell-surface-expressed DPP IV activity of CD26. Intact CEM cells expressing high levels of CD26 (clone H01)[49] were incubated for 5, 10, 15, and 30 min in PBS with or without 5 μM compound **11a** at 37 °C and then washed twice with PBS. DPP IV activity of CD26 was then monitored by the cleavage of GP-pNA as described in the Experimental Section. The binding of the inhibitor **11a** to DPP IV active site is very rapid since 5 min is sufficient to obtain more than 75% inhibition.

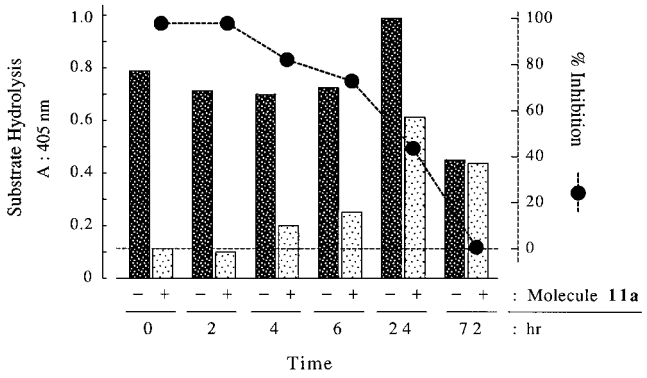


Figure 3. Irreversibility of DPP IV inhibition by molecule **11a** demonstrated by the cell-surface-expressed CD26. Intact CEM cells overexpressing CD26 (clone H01) were preincubated for 15 min in PBS in the absence or presence of 5 μM **11a** at 37 °C, then washed twice in PBS, and cultured in RPMI supplemented medium. Aliquots of cells were taken at the indicated times, washed twice with PBS, and then assayed for DPP IV activity by incubating cells with the substrate GP-pNA for 1 h at 37 °C. The samples at time 0 represent the DPP IV activity just after the 15 min of preincubation.

with 5 μM molecule **11a**. At times of 5, 10, 15, and 30 min, cells were washed extensively to remove unbound molecule **11a** before assay of the DPP IV activity. As shown in Figure 2, inhibition was almost maximal after 5 min of incubation, since the degree of inhibition was only slightly increased at 30 min. These results demonstrated that molecule **11a** has the capacity to bind CD26 rapidly and thus inhibit irreversibly its DPP IV activity. Second, to confirm the irreversible nature of molecule **11a**, cells were incubated with 5 μM inhibitor for 15 min before extensive washing and further incubation in the culture medium for up to 3 days. At different times during this period, cells were monitored for cell-surface-expressed DPP IV activity (Figure 3). The maximum inhibition observed following the 15-min incubation of cells with molecule **11a** (time 0 h) was found to last for several hours. At 6 h, there was a very slight difference on the maximum inhibition, whereas at 24 h, there was about 50% inhibition. Interestingly, this 50% reduction of the inhibition at 24 h coincided with the doubling time of cells, which results in the

Table 1. Inhibition of DPP IV Activity by the Cyclopeptide Compounds[a]

cyclopeptides **A**			inhibition of Gly-Pro-pNa hydrolysis (IC_{50}, μM)	
ref	glycine (*n*)	leaving group	CD26	DPP IV-β
11a	2	S^+Me_2	0.012	1
11b	4	S^+Me_2	0.003	0.61
12a	2	S^+Bu_2	0.02	0.57
13a	2	S^+Oct_2	0.03	0.63
15a	2	OAc	1.5	3.5
16a	2	OPh	10	25

[a] The DPP IV activity was monitored by the cleavage of Gly-Pro-pNA in purified preparations of CD26 and DPP IV-β (Experimental Section). CD26 was purified using extracts of CEM H01 cells expressing very high levels of CD26.[49] DPP IV-β was purified using extracts of CD26-negative C8166 cells.[36] Both purification procedures were as described elsewhere.[51] Such purified preparations of CD26 and DPP IV-β were free of any significant contamination by other peptidases. CD26 and DPP IV-β preparations were preincubated for 15 min with different concentrations of each inhibitor ranging from 1 nM to 10 μM before adding the substrate Gly-Pro-pNA as indicated.

production of cells that express newly synthesized CD26 molecules. At 3 days, no inhibition of cell-surface DPP IV activity was observed. Taken together, these data indicate that the inhibitory effect of molecule **11a** is irreversible, since the DPP IV activity could be resumed only by the newly synthesized CD26 (Figure 3). CD26 being a cell-surface-expressed protein, its expression on the cell surface could be modified with respect to the different phases of the cell cycle. Consequently at 24 and 72 h, the DPP IV activity was variable in control cells (without preincubation with the inhibitor) (Figure 3). It should also be noted that the cell-surface expression of CD26 was not affected by coupling with molecule **11a**. Indeed, cells in the absence or presence of preincubation with molecule **11a** manifested similar levels of cell-surface-expressed CD26, as monitored by FACS analysis using anti-CD26-specific monoclonal antibodies (data not shown).

The other cyclopeptides, possessing different leaving groups (**12a**, **13a**, **15a**, **16a**) or a larger ring (**11b**) were also assayed for their capacity to inhibit the DPP IV activity of CD26 using crude extracts from CEM cells (clone H01). The IC_{50} values for the inhibition of Gly-Pro-pNA hydrolysis are given in Table 1. All of these cyclopeptides showed different IC_{50} values in the nanomolar or micromolar range. However, no correlation was observed between these IC_{50} values and the length or lipophilicity of the leaving group. Molecules **15a** and **16a** with leaving groups as acetate and phenoxy, respectively, manifested significantly reduced IC_{50} values. In contrast, increasing the ring size in a given molecule generated a compound with an increased inhibitory activity (Table 1, compare molecules **11a**,**b**).

By use of the CD26-negative T-lymphoblastoid cell line C8166, we have recently described a CD26-like cell-surface protein with typical DPP IV activity.[36] This novel form of DPP IV, referred to as DPP IV-β, was found to be distinct from CD26. However the pH optimum and the profiles for substrate molecules were found to be indistinguishable for both CD26 and DPP IV-β. Similarly, several previously described inhibitors of DPP IV exerted a very similar mode of action on both CD26 and DPP IV-β.[36] Consequently, it remained

Table 2. Characterization of the Inhibition Kinetics of CD26 and DPP IV-β by the Cyclopeptide **11a**

	inhibition constants[b]	
enzyme[a]	K_I (μM)	k_{inact} (s^{-1})
CD26	0.085	$11.\ 10^{-4}$
DPP IV-β	0.470	$22.\ 10^{-5}$

[a] The DPP IV activities of CD26 and DPP IV-β were assayed by the use of extracts from CEM H01 (cells expressing very high levels of recombinant CD26) and C8166 cells (cells expressing only DPP IV-β), respectively. The cleavage of the substrate Gly-Pro-pNA (0.5 mM) was monitored in the presence of increasing concentrations of inhibitor **11a** by measuring absorbance at 405 nm. Preparation of extracts and assay conditions were as described before[36] and as in the Experimental Section. [b] Calculations were done as indicated in the Experimental Section. K_I is the equilibrium constant of the inhibitor binding to the enzyme, whereas k_{inact} is the constant of the irreversible reaction that leads to the inactivation of the enzyme.

essential to assess the action of the cyclopeptide inhibitors on DPP IV-β. The results given in Table 1 show that the inhibitory effect of the different irreversible cyclopeptide inhibitors is much more pronounced for CD26 compared to DPP IV-β. For example, it is interesting to note that the inhibitory effect of molecules **11a**,**b** is 83- and 203-fold higher on the DPP IV activity of CD26 compared to that of DPP IV-β, respectively (Table 1). In contrast to such a significant selectivity, molecules **15a** and **16a** exerted only about 2-fold difference between the two enzymes. These data might suggest that the higher specificity of the inhibitors on the DPP IV activity of CD26 could be related to the reactivity of the cyclopeptide sulfonium salts.

To further investigate the significant differences in the effect of the mostly studied inhibitorory molecule**11a** on DPP IV activity of CD26 and DPP IV-β (Table 1), we studied the kinetics of inhibition of both enzymes, by using the approach previously described for irreversible inhibitors of trypsin-like proteases.[34] This approach considers two steps in the inhibition process: first, the reversible binding of the inhibitor to the enzyme and, second, the cleavage and subsequent irreversible covalent modification of the enzyme leading to the loss of catalytic activity. The inhibition kinetics of the DPP IV activities of CD26 and DPP IV-β fit well to this model, and the equilibrium constant of the first step (K_I) as well as the kinetic constant of the second step, was calculated. The results are summarized in Table 2 and show that the higher potency of inhibitor **11a** on the DPP IV activity of CD26 is the consequence of a higher affinity for this enzyme as demonstrated by the K_I values, along with a faster inactivation rate as pointed out by the k_{inact} values found for CD26.

The difference in the inhibitory effect of irreversible inhibitors was further emphasized by testing the cell-surface-expressed enzymes. For this purpose, we used CEM cells expressing high levels of CD26 (clone H01) as a source of CD26 and C8166 cells as a source of DPP IV-β. Figure 4 shows the effect of different concentrations of molecule **11a** and a previously described reversible inhibitor, Lys-[Z(NO_2)]-pyrrolidide. As we had reported previously,[36] Lys-[Z(NO_2)]-pyrrolidide inhibited to a similar extent the DPP IV activity of both CD26 and DPP IV-β, with comparable IC_{50} values. On the other hand, the molecule **11a** completely inhibited the DPP IV activity of cell-surface-expressed CD26 without

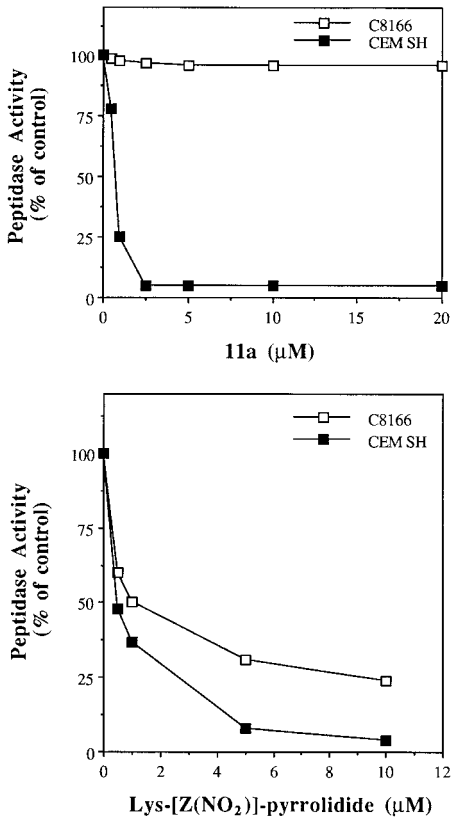


Figure 4. Molecule **11a** inhibits the DPP IV activity of the cell-surface-expressed CD26 but not that of DPP IV-β. Intact CEM cells overexpressing CD26 (clone H01) and C8166 cells expressing only DPP IV-β were preincubated for 15 min in PBS containing different concentrations of molecule **11a** (upper panel) or Lys-[Z(NO_2)]-pyrrolidide (lower panel), before adding GP-pNA to monitor residual DPP IV activity. Control activity (100%) was determined in the absence of inhibitor.

any apparent effect on cell-surface-expressed DPP IV-β, even when used at high concentrations such as 10−20 μM (Figure 4). It should be noted that molecule **11a** was active against the soluble form of DPP IV-β with an IC_{50} value of 1 μM. Thus, the inability of molecule **11a** to affect cell-surface-expressed DPP IV-β appears to be correlated with the structure of this protein when expressed on the cell surface. Whatever is the case, these results emphasize specific differences in the overall structure of DPP IV-β compared to that of CD26.

Discussion

The DPP IV enzyme has an absolute requirement for the L-configuration of the amino acid residues, both in the penultimate and in the N-terminal positions. The amide bond between the N-terminal P_2 residue and the P_1 proline residue must be in a trans conformation. A protonated amino end is necessary for enzymatic hydrolysis. Amino acids with aliphatic side chains at P_2 are slightly favored over aromatic ones, but the effect is not very large. On the basis of these literature data and the results of directed mutagenesis studies, Brandt et al. have recently proposed a model of the enzyme active site[41] and a new catalytic mechanism for this glycoprotein,[42] which has not yet been crystallized. Compared to usual serine proteases, the main difference is the stabilization of the first tetrahedral intermediate

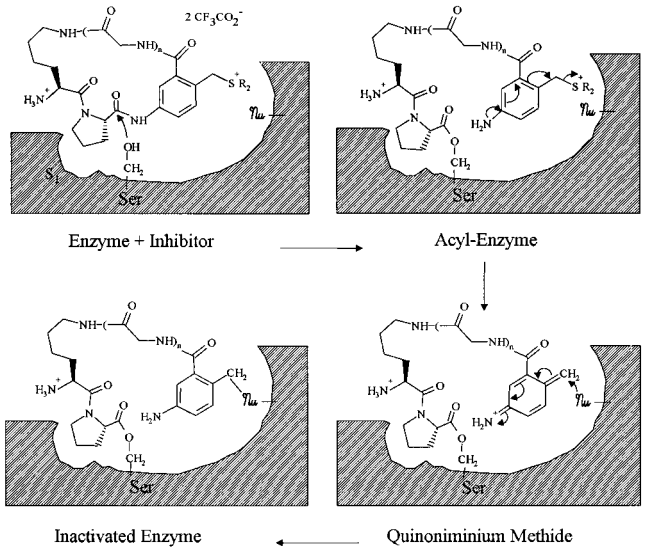


Figure 5. Postulated mechanism of enzyme inactivation. Two covalent bonds are formed between the inhibitor and the enzyme (double-hit mechanism). First, an acyl-enzyme is formed which should involve the catalytic serine 630 of CD26 and proline residue P_1 of the inhibitory molecule (step 1). Second, by means of the unmasked quinoniminium methide (step 2), a second covalent bond is formed implicating a nucleophile residue Nu in the vicinity of the catalytic serine (step 3). Consequently, this second bond is responsible for the irreversible blockade of the catalytic site of the enzyme.

IT_1 by formation of an oxazolidine ring (IT_2). Proton transfer from the terminal ammonium function leads to a third tetrahedral intermediate (IT_3) and then preferentially to a cis acyl-enzyme which isomerizes to the trans isomer.

For the herein described cyclopeptides **A**, the postulated mechanism of the observed enzyme inactivation is presented in Figure 5. Provided that the protease can accommodate the large molecule **A**, formation of an acyl-enzyme, by selective nucleophilic attack of the hydroxyl function of serine 630 on the P_1 proline carbonyl of the cyclopeptide (step 1), would unmask a P'_1-substituted aniline having a good benzylic leaving group. Owing to the strong electron-releasing property of the amino substituent ($\sigma^+_{p-NH_2} = -1.31$, compared to $\sigma^+_{p-NHCOR} = -0.69$[43] in the starting cyclopeptide), a fast elimination of a neutral dialkyl sulfide (or an anionic acetate) should give a substitued quinoniminium methide ion[34] (step 2) tethered in the active site during the lifetime of the acyl-enzyme by means of the peptide chain. A Michael-type addition of a second nucleophile Nu, in the vicinity of the active serine, on this demasked electrophile (step 3) would establish a second covalent enzyme−inhibitor bond (elimination−addition process).[44] Such a bridge between the active site serine and another nucleophilic residue active site leads to an irreversible loss of the enzymatic activity. Moreover, the inactivation is expected to be very selective as the inhibitory activity of suicide substrates (mechanism-based inhibitors) requires discrimination in the binding steps, catalytic activation by the target enzyme, and irreversible modification of the active center.[45]

It should be emphasized that the macrocyclic sulfonium salts **A** are obtained in just one step by reacting an alkyl sulfide in trifluoroacetic acid with the cyclopeptides having a benzylic phenoxy substituent and an

N^{α}-Z protecting group, themselves readily obtained by peptide synthesis in solution and cyclized by the azide method. These cyclopeptide sulfonium salts, as all compounds **11**−**16**, belonging to the **A** series of molecules, are stable and water-soluble, and their activity does not decrease even after several months in solution. Their cyclic structure prevents their decomposition by cyclization due to the attack of the free amine on the carbonyl of the proline. Moreover, the toxicity of these compounds on CEM cells was observed only at concentrations higher than 250 μM.

Similar inhibitory activities were observed among the compounds **11a**, **12a**, and **13a**, suggesting that the length of the alkyl chains on the sulfonium group has little or no influence on the inhibition of DPP IV activity of CD26. Interestingly, the size of the macrocycle appeared to play an important role, since a strong inhibition was observed with compound **11b** which contains four glycine residues in its cycle. The inhibitory activity of this compound is 4 times more effective than that of its homologue molecule **11a**, which possesses only two glycine residues. The weak inhibition observed with compounds **15a** and **16a** is not surprising, since the acetate and the phenoxy groups are not good leaving groups compared to the alkyl sulfide ones. For the linear analogues of the cyclopeptides, no inhibition was observed at 100 μM. In this case, a fast diffusion of the quinoniminium methide cation out of the active center may occur due to the lack of the peptide chain which tethers the electrophile in the active site. Taken together, our results show that large substituents on the sulfur atom did not significantly modify the inhibition reaction. In contrast the ring size enlargement improved it, probably by allowing more favorable interactions with the enzyme active center and/or facilitating the approach to the nucleophilic group Nu in the active site.

Comparative studies on the inhibition of the DPP IV activity of CD26 and DPP IV-β revealed that the two enzymes should be distinct from each other. In soluble enzyme preparations, the irreversible cyclopeptide inhibitors were less active on DPP IV-β compared to CD26. This latter is most probably due to both a different affinity and a different inactivation rate, as we have demonstrated to be the case with the cyclopeptide **11a** (Table 2). Consequently, it is plausible to suggest the presence of a less reactive active site nucleophile Nu−DPP IV-β compared to the corresponding Nu residue in CD26 and/or to a different localization of this group with respect to the active catalytic serine residue. These point out that there should be important structural differences between DPP IV-β and CD26. Such structural differences probably become accentuated with the cell-surface-expressed proteins. Indeed, cyclopeptide inhibitors active at nanomolar concentrations on the cell-surface-expressed CD26 manifest no apparent activity on the cell-surface-expressed DPP IV-β. Accordingly, the catalytic domain necessary for the DPP IV activity could be differentiated between CD26 and DPP IV-β. For the cell-surface-expressed DPP IV-β, the particular structure of its catalytic domain presumably cannot accommodate the inhibitor. On the other hand, the solubilized DPP IV-β can bind the functionalized cyclopeptide and therefore be inhibited.

We have previously reported that the irreversible inhibitor **11a** affects HIV-1 Lai infection of CEM cells in a dose-dependent manner, with an IC_{50} value of 10 μM.[46] In contrast, another potent but reversible inhibitor of the DPP IV activity, Lys-[$Z(NO_2)$]-pyrrolidide, has no apparent effect on HIV infection of CEM cells, even at 100 μM.[46] Interestingly, molecule **11a**, which has no inhibitory activity on the cell-surface-expressed DPP IV-β, manifests no effect on the HIV-1 infection of CD26-negative but DPP IV-β expressing C8166 cells.[46] These observations indicate that the irreversible inhibitors behave as specific compounds affecting the DPP IV activity of CD26, and consistent with previously published reports,[3,36,47−49] they demonstrate once again the implication of CD26 in the mechanism of HIV infection. Recently, Bekesi and collaborators have synthesized reversible inhibitors of the type X-Pyrr-2-CN and have shown that they inhibit HIV-1 infection with IC_{50} values of 2.4−5.3 μM.[17,50]

Taken together, our results describe for the first time potent irreversible inhibitors of DPP IV which could be used specifically on CD26 and could be used in different types of studies for better understanding the role of CD26 in biological processes.

Experimental Section

Synthetic Procedures. Melting points were recorded on a Tottoli (Büchi) or a Mettler FP61 and are uncorrected. The ^{1}H NMR measurements were performed on a Brüker AM 300 instrument. The chemical shifts are reported in ppm, the deuterated solvents being used as internal standards: CD_3OD (3.31 ppm) and CD_3COOD (2.10 ppm). The coupling constants are given in hertz. The optical rotations were obtained on a Perkin-Elmer 241 polarimeter. Analyses were performed by the Services of CNRS at ICSN (Gif sur Yvette). The mass spectrometry measurements were performed by Mr. Deroussent (Inst. G. Roussy, Villejuif) or Ms. Kargar (Ecole Polytechnique) and for the FAB HRMS data, Mr. Fabre-Bonvin (CNRS, Vernaison). Analytical thin-layer chromatography (TLC) and preparative column chromatography were performed on Kieselgel F 254 and Kieselgel 60, 0.063−0.5 and 0.040−00.063 mm (Merck), respectively, with the following solvent systems (by vol): I (*x*/*y*), MeOH *x*%−CH_2Cl_2 *y*%; II, AcOEt−*n*-BuOH−AcOH−H_2O (1/1/1/1). UV light (254 nm) allowed visualization of the spots after TLC runs for all compounds, even at low concentration.

Nba-[CH_2OPh]-Gly$_2$-OEt (6a). 2-(Phenoxymethyl)-5-nitrobenzoic acid (**5**) (2.5 g, 9.15 mmol), prepared as previously described[38] was dissolved in a 50-mL solution of CH_2Cl_2/THF (1/1). DCC (2.45 g, 11.89 mmol) and HOBt (1.36 g, 10.05 mmol) were added, and the solution was stirred at 0 °C for 15 min. Diglycine ethyl ester hydrochloride (1.98, 10.05 mmol) and a solution of triethylamine (3.9 g, 27.45 mmol) in CH_2Cl_2/THF (1/1) (40 mL) were then added. The reaction mixture was stirred for 1 h at 0 °C and for 24 h at room temperature. Solvents were evaporated under reduced pressure at 50 °C, the residue was dissolved in acetone, and the DCU was filtered. The filtrate was then evaporated, dissolved in AcOEt, and washed successively with H_2O (100 mL), 0.5 N aqueous HCl (100 mL), H_2O (100 mL), 5% aqueous $NaHCO_3$ (100 mL), and H_2O (100 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated. Chromatography on silica gel with eluent I (2/98) led to 3.23 g (85.5%) of pure peptide **6a** as a white powder. Mp: 132.4 °C. R_f (I: 2/98) = 0.5. ^{1}H NMR (CD_3OD): 8.56, d (2.3), 1H (ArH); 8.39, dd (2.3, 8.5), 1H (ArH); 7.97, d (8.5), 1H (H3); 7.32, m, 2H and 7.01, m, 3H (ArH); 5.45, s, 2H (CH_2OPh); 4.21, q (7.1), 2H (OEt); 1.30, t (7.1), 3H (OEt). Anal. Calcd for $C_{20}H_{21}N_3O_7 \cdot 0.5H_2O$: C, 56.59; H, 5.18; N, 9.90. Found: C, 56.61; H, 5.11; N, 10.21.

Boc-Pro-Aba-[CH_2OPh]-Gly$_2$-OEt (7a). The peptide **6a** (2.5 g, 6.02 mmol) was dissolved in hot DMF/MeOH (25/75;

60 mL), and platinium oxide (0.2 g) was added to the warm solution, which was hydrogenated in a Parr apparatus at room temperature for 2 h. The solution was filtered in order to remove the catalyst. Analytical TLC of the filtrate with eluent I (15/85) showed a new unique spot, highly fluorescent on UV light at 254 and 366 nm, corresponding to the intermediate aromatic amine. This compound was not isolated and was used directly in the next step, because of its instability in solution. Thus methanol and DMF were rapidly evaporated in vacuo at 40 °C. The residue was dissolved in CH_2Cl_2/THF (1/1; 30 mL). The clear solution was cooled at 0 °C, and a solution of Boc-L-proline (1.295 g, 6.02 mmol) and HOBt (8.94 g, 6.62 mmol) in CH_2Cl_2/THF (1/1; 70 mL) and then DCC (1.613 g, 7.82 mmol) were added. The reaction mixture was stirred from 0 °C during 2 h to room temperature overnight. The solvents were evaporated, ethyl acetate (300 mL) was added, and the dicyclohexylurea precipitate was removed by filtration. The filtrate was extracted successively with 0.5 M aqueous HCl (2 × 100 mL), H_2O (100 mL), 5% aqueous $NaHCO_3$ (2 × 100 mL), and H_2O (2 × 100 mL), dried over Na_2SO_4, filtered, and evapored. The residue was chromatographed on a column of silica gel. Elution with eluent system I (2/98) led to 2.05 g (58%) of the pure peptide **7a**. R_f (I: 10/90) = 0.5. ^{1}H NMR (CD_3OD): 7.84, d (1.8), 1H (ArH); 7.68, dd (1.8, 8.4), 1H (ArH); 7.54, d (8.4), 1H (ArH); 7.24, m, 2H and 6.94, m, 3H (ArH); 5.23, s, 2H (CH_2OPh); 4.31, md, 1H (HαPro); 4.14, q (7.1), 2H (OEt,); 3.89 and 3.68, 2s, 4H (HαGly); 3.50, m, 2H (Hδ Pro); 2.29, m, 1H (Hβ Pro); 1.97, m, 3H (Hβ Pro, Hγ Pro); 1.47 and 1.36, 9H (Boc); 1.24, t (7.1), 3H (OEt). Anal. Calcd for $C_{30}H_{38}N_4O_8 \cdot 0.5H_2O$: C, 60.89; H, 6.64; H, 9.47. Found: C, 60.86; H, 6.57; N, 9.45.

Boc-Pro-Aba-[CH_2OPh]-Gly$_4$-OEt (7b): obtained as above from **6b**[32] (3.5 g, 6.6 mmol). Yield: 1.4 g (30%), white powder. R_f (I: 10/90) = 0.5. ^{1}H NMR (CD_3OD): 7.85, s, 1H (ArH); 7.65, m, 1H (ArH); 7.52, m, 1H (ArH); 7.25, m, 2H and 6.95, m, 3H (ArH); 5.16, s, 2H (CH_2OPh); 4.26, md, 1H, (HαPro); 4.10, q, 2H (OEt); 4.05, s, 2H (HαGly); 3.90, 3.89 and 3.88, m, 6H (HαGly); 3.53, m, 2H (Hδ Pro); 2.29, m, 1H (Hβ Pro); 2.03, m, 2H (Hγ Pro); 1.89, m, 1H (Hβ Pro); 1.47 and 1.36, 9H (Boc); 1.24, t, 3H (OEt). Anal. Calcd for $C_{34}H_{44}N_6O_{10} \cdot H_2O$: C, 57.12; H, 6.48; H, 11.75. Found: C, 57.11; H, 6.38; N, 11.44.

$^{\alpha}$Z-Lys($^{\epsilon}$Boc)-Pro-Aba-[CH_2OPh]-Gly$_2$-OEt (8a). The peptide **7a** (1.5 g, 2.57 mmol) was dissolved in CH_2Cl_2 (15 mL) at 0 °C, and TFA (15 mL) was added. The reaction mixture was stirred at 0 °C for 1.5 h. Excess of TFA was evaporated in vacuo. The residue was dissolved in CH_2Cl_2, and the solution was washed with 5% aqueous $NaHCO_3$, dried over Na_2SO_4, filtered, and evaporated. The residue was dissolved in CH_2Cl_2/THF (1/1; 15 mL) and added to a mixture of Z-Lys(Boc)-OH (0.98 g, 2.57 mmol), DCC (0.689 g, 3.34 mmol), and HOBt (0.382 g, 2.82 mmol) in CH_2Cl_2/THF (1/1; 30 mL) at 0 °C. The reaction mixture was stirred from 0 °C during 2 h to room temperature overnight. The solvents were evaporated, ethyl acetate (300 mL) was added, and the dicyclohexylurea preciptate was removed by filtration. The filtrate was extracted successively with 0.5 M aqueous HCl (2 × 100 mL), H_2O (100 mL), 5% aqueous $NaHCO_3$ (2 × 100 mL), and H_2O (2 × 100 mL), dried over Na_2SO_4, filtered, and evapored. The residue was chromatographed on a column of silica gel. Elution with eluent system I (6/94) led to 1.4 g (65%) of the pure peptide **8a**. Mp: 78.7 °C. R_f (I: 5/95) = 0.5. ^{1}H NMR (CD_3OD): 7.74, d (2.0), 1H (ArH); 7.60, dd (2.0, 8.4), 1H (ArH); 7.45, d (8.4), 1H (ArH); 7.26, m, 7H (ArH); 6.92, m, (ArH); 5.19, s, 2H (CH_2OPh); 5.06, s, 2H (CH_2Z); 4.57, m, 1H (HαPro); 4.41, m, 1H (HαLys); 4.13, q (7.1), 2H (OEt); 4.03, s and 3.89, s, 4H (HαGly); 3.86 and 3.69, m, 2H (Hδ Pro); 3.03, m, 2H (Hε Lys); 2.19−1.48, m, 10H (Hβ Pro, Hγ Pro, Hδ Lys, Hβ Lys, Hγ Lys); 1.41, s, 9H (Boc); 1.23, t (7.1), 3H (OEt). Anal. Calcd for $C_{44}H_{56}N_6O_{11}$: C, 62.54; H, 6.68; N, 9.94. Found: C, 62.35; H, 6.63; N, 9.53.

$^{\alpha}$Z-Lys($^{\epsilon}$Boc)-Pro-Aba-[CH_2OPh]-Gly$_4$-OEt (8b). The peptide **7b** (0.25 g, 0.35 mmol) was dissolved in ethanol (5 mL), and a solution of HCl (3 N) in CH_2Cl_2 (20 mL) was added. The reaction mixture was stirred at 0 °C for 1.5 h and evaporated in vacuo. The residue was dissolved in diethyl ether, triturated, and evaporated. After this operation was repeated three times, the residue was dissolved in THF (20 mL) and *N*-methylmorpholine (40 μL, 0.35 mmol) was added. This solution **a** was stirred at 0 °C for 30 min. To a solution of Z-Lys(Boc)-OH (136 mg, 0.35 mmol) and *N*-methylmorpholine (40 μL, 0.35 mmol) in THF (20 mL) cooled at − 10 °C was added ethyl chloroformate (34 μL, 0.35 mmol). This mixture **b** containing the mixed anhydride was stirred at −10 °C for 15 min and the former solution **a** was added. The reaction mixture was stirred for 1 h at −10 °C and at room temperature overnight. After evaporation of the solvents, the residue was chromatographed on a column of silica gel. Elution with eluent system I (10/90) led to the pure peptide **8b** (0.14 g, 36%). Mp: 149.5 °C. R_f (I: 10/90) = 0.4. ^{1}H NMR (CD_3OD): 7.75, s, 1H (ArH); 7.56, m, 1H (ArH); 7.40, m, 1H (ArH); 7.28, m, 7H (ArH); 6.88, m, 3H (ArH); 5.15, s, 2H (CH_2OPh); 5.01, s, 2H (CH_2Z); 4.36, m, 1H (HαPro); 4.05, m, 5H (HαLys, OEt, HαGly); 3.85, ∼s, 6H (HαGly); 3.54, m, 2H (Hδ Pro); 2.99, m, 2H (Hε Lys); 2.21−1.58, m, 10H (Hβ Pro, Hγ Pro, Hβ Lys, Hγ Lys, Hδ Lys); 1.43, s, 9H (Boc); 1.16, t, 3H (OEt). Anal. Calcd for $C_{48}H_{62}N_8O_{13}$: C, 60.11; H, 6.52; N, 11.68. Found: C, 59.42; H, 6.57; N, 10.51.

$^{\alpha}$Z-Lys($^{\epsilon}$Boc)-Pro-Aba-[CH_2OPh]-Gly$_2$-NH-NH$_2$ (9a). The peptide **8a** (1 g, 1.18 mmol) was dissolved in methanol (50 mL). A large excess of hydrazine monohydrate (6 mL, 118 mmol) was added, and the solution was stirred at room temperature for 24 h. Methanol and the excess of hydrazine were evaporated in vacuo. The residue was evacuated at 40 °C/0.1 mmHg for 1 h, solubilized in methanol (20 mL), and precipitated by addition of ether (200 mL). The precipitate was filtered, washed with ether, and dried, leading to 0.69 g (70%) of hydrazide **9a** as a white powder. R_f (I: 10/90) = 0.5. ^{1}H NMR (CD_3OD): 7.83, s, 1H (ArH); 7.63, d (8.4), 1H (ArH); 7.51, d (8.4), 1H (ArH); 7.28, m, 7H (ArH); 6.76, m, 3H (ArH); 5.22, s, 2H (CH_2OPh); 5.07, s, 2H (CH_2Z); 4.57, m, 1H (HαPro); 4.41, m, 1H (HαLys); 4.23, 4.07, 4.04, and 3.94, 2sd, 4H (HαGly); 3.89 and 3.71, md, 2H (Hδ Pro); 3.04, m, 2H (Hε Lys); 1.99−1.46, m, 10H (Hβ Pro, Hγ Pro, Hγ Lys, Hγ Lys, Hδ Lys); 1.41, s, 9H (Boc). Anal. Calcd for $C_{42}H_{54}N_8O_{10} \cdot 0.5H_2O$: C, 60.05; H, 6.60; N, 13.34. Found: C, 60.32; H, 6.75; N, 12.76.

$^{\alpha}$Z-Lys($^{\epsilon}$Boc)-Pro-Aba-[CH_2OPh]-Gly$_4$-NH-NH$_2$ (9b): obtained as above from the peptide **8b** (0.058 g, 0.6 mmol), white powder (0.041 g, 72%). R_f (I: 8/92) = 0.1. ^{1}H NMR (CD_3OD): 7.77, s, 1H (ArH); 7.61, d (8.4), 1H (ArH); 7.45, m, 1H (ArH); 7.32, m, 7H (ArH); 6.92, m, 3H (ArH); 5.18, dd∼s, 2H (CH_2OPh); 5.05, dd∼s, 2H (CH_2Z); 4.55, m, 1H (HαPro); 4.40, m, 1H (HαLys); 4.04, s, 2H (HαGly); 3.87, s, 3.86, s and 3.80, s, 6H (HαGly); 3.68, m, 2H (Hδ Pro); 3.03, m, 2H (Hε Lys); 2.21−1.62, m, 10H (Hβ Pro, Hγ Pro, Hβ Lys, Hγ Lys, Hδ Lys); 1.40, s, 9H (Boc). Anal. Calcd for $C_{46}H_{60}N_{10}O_{12} \cdot 2H_2O$: C, 56.31; H, 6.57; N, 14.27. Found: C, 56.15; H, 6.55; N, 14.87.

Cyclo(-Lys($^{\alpha}$Z)-Pro-Aba-[CH_2OPh]-Gly$_2$-) (10a). The peptide **9a** (0.640 g, 0.77 mmol) was dissolved in CH_2Cl_2 (10 mL) at 0 °C, and TFA (15 mL) was added. The solution was stirred for 2 h. Then CH_2Cl_2 and excess of TFA were evaporated in vacuo. The residue was evacuated at 40 °C/0.1 mmHg for 1 h and triturated several times in diethyl ether to give a white powder. This compound was dissolved in DMF (17 mL). After cooling at −40 °C, a solution of 5.5 N HCl in THF (1.5 mL, 8.47 mmol) and isoamyl nitrite (0.145 mL, 1.08 mmol) were successively added. The reaction mixture was stirred at −40 °C for 0.5 h. The resulting peptide azide was diluted with cold DMF (200 mL). The solution was then brought to pH 9 by addition of diisopropylethylamine (2 mL, 11.55 mmol) and kept in a refrigerator for 24 h. DMF was evaporated, and distilled water was added to the residue. The resulting solid was filtered, dissolved in MeOH, and chromatographed on a column of silica gel. Elution with eluent system I (10/90) led to 0.242 g (45%) of the pure cyclopeptide **10a**. Mp: 144.6 °C. R_f (I: 10/90) = 0.6. ^{1}H NMR (DMSO-d_6): 9.86, s, 1H (NHAr); 8.85, ∼t (5.5), 1H (NHGly1); 8.33, ∼t (5.5), 1H (NHGly2); 8.09, s, 1H (ArH); 7.62, ∼t (5.4), 1H (NHε Lys); 7.50, m, 3H (2 ArH and NHαLys); 7.32, m, 7H (ArH); 6.91, m, 3H (ArH); 5.23 and

5.16, 2d (12.3), 2H (CH_2OPh); 5.01 and 5.00, 2d (12.7), 2H (CH_2Z); 4.35, m, 2H (HαPro, HαLys); 3.72, m, 5H (HαGly1, HαGly2, Hδ Pro); 3.56, ~dd (15.9, 5), 1H (HαGly2); 3.09, m, 2H (HεLys); 2.19, m, 1H (Hβ Pro); 3.79, m, 3H (Hβ Pro, Hγ Pro); 1.39, m, 5H (H_2βLys, Hδ Lys, Hγ Lys); 0.85, m, 1H (Hβ Lys). ^{1}H NMR (CD_3OD): 8.44, d, 1H (ArH); 7.62, d (8.3), 1H (ArH); 7.34, m, 5H (ArH); 7.32, m, 3H (ArH); 6.93, m, 3H (ArH); 5.28 and 5.23, 2d (12.7), 2H (CH_2OPh); 5.07, s, 2H (CH_2Z); 4.51, m, 2H (HαPro, HαLys); 4.11 and 3.97, 2d (16.6), 2H (HαGly1); 3.93 and 3.86, 2d (16.8), 2H (HαGly2); 3.72 and 3.54, 2m, 2H (Hδ Pro); 3.25, m, 2H (HεLys); 2.34 and 2.10, 2m, 2H (Hβ Pro); 2.04, m, 2H (Hγ Pro); 1.81, m, 2H (Hβ Lys); 1.54, m, 2H (Hδ Lys); 1.35, m, 2H (Hγ Lys). Anal. Calcd for $C_{37}H_{42}N_6O_8 \cdot H_2O$: C, 61.90; H, 6.18; N, 11.70. Found: C, 61.53; H, 6.07; N, 11.31.

Cyclo(-Lys(αZ)-Pro-Aba-[CH_2OPh]-Gly_4-) (10b): obtained as above from the peptide **9b** (0.05 g, 0.053 mmol). Yield: 0.019 g (44%), white powder. R_f (I: 10/90) = 0.4. ^{1}H (CD_3OD): 7.66, s, 1H (ArH); 7.54, d, 1H (ArH); 7.34, m, 8H (ArH); 6.92, m, 3H (ArH); 5.21, s, 2H (CH_2OPh); 5.06, s, 2H (CH_2Z); 4.56, m, 1H (HαPro); 4.43, m, 1H (HαLys); 4.10−3.80, m, 10H (HαGly1,HαGly2, HαGly3, HαGly4, Hδ Pro); 3.13, m, 2H (HεLys); 2.20−1.50, m, 10H (Hβ Pro, Hγ Pro, Hβ Lys, Hγ Lys, Hδ Lys). MS (FAB$^+$): 813 (MH^+), 835 (MNa^+), 851(MK^+).

Cyclo(-Lys(αH_2^+)-Pro-Aba-[$CH_2SMe_2^+$]-Gly_2-), 2CF_3COO^- (11a). To a mixture of cyclopeptide **10a** (0.05 g, 71 μmol) and dimethyl sulfide (0.52 mL, 71 mmol) was added TFA (2.7 mL). The solution was stirred at room temperature for 48 h. Addition of a large excess of ether led to precipitation of a white solid. The precipitate was collected by centrifugation, repeatedly washed with ether, and then centrifuged, dried, and chromatographed on a column of silica gel. Elution with eluent system II, followed by complete evaporation of the solvents in vacuo at 25−30 °C, dissolution of the residue in ca. 2 mL of methanol, precipitation with ether, repeated washings of the precipitate with ether, centrifugation, and drying in vacuo at 25 °C, led to 0.025 g of the title product **11a** (62%). Mp: 124.8 °C. R_f (II) = 0.05. ^{1}H (CD_3COOD): 8.95, s, 1H (ArH); 7.64, d (8.2), 1H (ArH); 7.45, m~s (8.2), 1H (ArH); 4.83, s, 2H ($ArCH_2S^+$); 4.76, t, 1H (HαPro); 4.61, t, 1H (HαLys); 4.33, 2d (16.9), 2H (HαGly1); 4.09, s, 2H (HαGly2); 3.91 and 3.71, 2m, 2H (Hδ Pro); 3.34, m, 2H (HεLys); 2.97, s, 6H (S^+Me_2); 2.44, m, 2H (Hβ Pro); 2.08, m, 4H (Hβ Lys,Hγ Pro); 1.52, m, 4H (Hδ Lys, Hγ Lys). ^{1}H NMR (CD_3OD): 8.33, 1H (ArH); 7.57, d (8.2), 1H (ArH); 7.31, m~s (8.2), 1H (ArH); 4.72, s, 2H ($ArCH_2S^+$); 4.59, m, 1H (HαPro); 4.50, m, 1H (HαLys); 4.26, 2d (16.9), 2H (HαGly1); 3.72, s, 2H (HαGly2); 3.74, m, 2H (Hδ Pro); 3.25, m, 2H (HεLys); 2.91, s, 6H (S^+Me_2); 2.41, m, 2H (Hβ Pro); 1.98, m, 4H (Hβ Lys,Hγ Pro); 1.55, m, 4H (Hδ Lys, Hδγ Lys). HRMS (FAB$^+$): calcd for $C_{25}H_{37}N_6O_5S^+$ (M^+), 533.2546; found, 533.2567; calcd for $C_{23}H_{31}N_6O_5$ ($M^+ - Me_2S$), 471.2356; found, 471.2386.

Cyclo(-Lys(αH_2^+)-Pro-Aba-[$CH_2S^+Me_2$]-Gly_4-), 2CF_3COO^- (11b): obtained as above from the peptide **10b** (0.01 g, 0.012 mmol). Yield: 6.5 mg (60%), white powder. R_f (II) = 0.5. ^{1}H NMR (CD_3OD): 8.57, s, 1H (ArH); 7.57, m, 1H (ArH); 7.34, m, 1H (ArH); 4.72, s, 2H ($ArCH_2S^+$); 4.21−3.92, m, 12H (HαPro, HαLys, HαGly, Hδ Pro); 3.19, m, 2H (HεLys); 2.91, s, 6H (S^+Me_2); 2.36 to 1.28, m, 10H (Hβ Pro, Hγ Pro, Hβ Lys, Hγ Lys, Hδ Lys). MS (FAB$^+$): 647 (M^+), 585 ($M^+ - Me_2S$). HRMS (FAB$^+$): calcd for $C_{29}H_{43}N_8O_7S^+$ (M^+), 647.2975; found, 647.2997.

Cyclo(-Lys(αH_2^+)-Pro-Aba-[$CH_2S^+Bu_2$]-Gly_2-), 2CF_3COO^- (12a): obtained as above from the peptide **10a** (0.05 g, 71 μmol) and dibutyl sulfide (0.52 mL, 71 mmol) in TFA (2.7 mL). Yield: 0.045 g (74,5%), white powder. Mp: 176 °C dec. R_f (II) = 0.1. ^{1}H NMR (CD_3OD): 8.28, sd (2.2), 1H (ArH); 7.22, d (8.4), 1H (ArH); 7.08, dd (2.2; 8.2), 1H (ArH); 4.57, m, 1H (HαPro); 4.35, t~m, 1H (HαLys); 4.09, s, 2H ($ArCH_2S^+$); 4.1−3.8, m, 4H (HαGly); 3.79 and 3.67, 2m, 2H (Hδ Pro); 3.54, t (6.4), 4H ($2CH_2S^+$); 3.20, m, 2H (HεLys); 2.36, m, 2H (Hβ Pro); 2.05, m, 3H (Hβ Lys, Hγ Pro); 1.87, m, 1H (Hγ Pro); 1.60−1.30, m, 12H (Hδ Lys, Hδγ Lys, 4CH_2Bu); 0.93, t, 6H (2CH_3Bu). MS (FAB$^+$): 617 (M^+), 471 ($M^+ - Bu_2S$). HRMS (FAB$^+$): calcd for $C_{31}H_{49}N_6O_5S^+$ (M^+), 617.3485; found, 617.3498.

Cyclo(-Lys(αH_2^+)-Pro-Aba-[$CH_2S^+Oct_2$]-Gly_2-), 2CF_3COO^- (13a): obtained as above from the peptide **10a** (0.035 g, 50 μmol) and dioctyl sulfide (0.5 mL, 50 mmol) in TFA (2 mL). Yield: 0.034 g (71%), white powder. Mp: 174 °C dec. R_f (II) = 0.6. ^{1}H NMR (CD_3OD): 8.29, s, 1H (ArH); 7.60, d (8.8), 1H (ArH); 7.28, m, 1H (ArH); 4.58, m, 1H (HαPro); 4.22, m, 1H (HαLys); 4.09, m, 2H (CH_2S^+); 3.98−3.80, m, 4H (HαGly); 3.67, m, 2H (Hδ Pro); 3.54, t (6.2), 4H ($2CH_2S^+$); 3.32 and 3.20, 2m, 2H (HεLys); 2.38, m, 2H (Hβ Pro); 2.04, m, 8H (Hβ Lys, Hγ Pro, 2CH_2Oct); 1.80, m, 4H (Hδ Lys, Hδγ Lys); 1.50, m, 8H (CH_2-Oct); 1.29, s, 12H (6CH_2Oct); 0.90, t, 6H (2CH_3Oct). MS (FAB$^+$): 729 (M^+), 471 ($M^+ - Oct_2S$). HRMS (FAB$^+$): calcd for $C_{39}H_{65}N_6O_5S^+$ (M^+), 729.4737; found, 729.4758.

Cyclo(-Lys(αBoc)-Pro-Aba-[CH_2OAc]-Gly_2-) (14a). A mixture of peptide **11a** (0.02 g, 0.031 mmol) and dry potassium acetate (0.05 g, 0.63 mmol) in 5 mL of DMF was stirred at room temperature for 1.5 h. The solvent was evaporated in vacuo. The residue was dissolved in dioxane (1 mL) and aqueous NaOH (1.5 mg, 0.046 mmol) (1 mL). Boc_2O (8.5 mg, 0.046 mmol) was added, and the reaction mixture was stirred from 0 °C during 2 h to room temperature overnight. Solvents were evaporated. Column chromatography of the residue (silica gel, eluent I: 8/92), followed by evaporation of the product containing pooled fractions, dissolution of the residue in methanol, filtration, concentration to ca. 3 mL, precipitation with ether, and treatment of the precipitate as before (centrifugation, repeated washings with ether, and drying), yielded 0.011 g (70%) of acetate **14a** as a white powder. R_f (I: 8/92) = 0.4. ^{1}H NMR (CD_3OD): 8.41, d (2.5), 1H (ArH); 7.57, d (8.0), 1H (ArH); 7.31, dd (2.5, 8.0), 1H (ArH); 5.28, dd~q, 2H (CH_2-OAc); 4.53, m, 1H (HαPro); 4.42, m, 1H (HαLys); 4.08, m, 2H (HαGly1); 3.89, s, 2H (HαGly2); 3.71, m, 2H (Hδ Pro); 3.22, m, 2H (HεLys); 2.34 and 2.10, 2m, 2H (Hβ Pro); 2.06, s, 3H (CH_3-Ac); 1.94, m, 2H (Hβ Lys); 1.62, m, 6H (Hγ Pro, Hδ Lys, Hγ Lys); 1.43, s, 9H (Boc). MS (FAB$^+$): 631 (MH^+), 653 (MNa^+), 669 (MK^+). HRMS (FAB$^+$): calcd for $C_{30}H_{43}N_6O_9$ (M^+), 631.3091; found, 631.3109.

Cyclo(-Lys(αH_2^+)-Pro-Aba-[CH_2OAc]-Gly_2-), CF_3COO^- (15a). The peptide **14a** (0.010 g, 0.016 mmol) was dissolved in CH_2Cl_2 (10 mL) at 0 °C, and TFA (15 mL) was added. The reaction mixture was stirred for 2 h. CH_2Cl_2 and excess of TFA were evaporated in vacuo. The residue was evacuated at 40 °C/0.1 mmHg for 1 h and triturated several times in diethyl ether to give a white powder (7.7 mg, 76%). R_f (II) = 0.7. ^{1}H NMR (CD_3OD): 8.40, s, 1H (ArH); 7.60, d (8.0), 1H (ArH); 7.30, d (8.0), 1H, (ArH); 5.30, dd~q, 2H (CH_2OAc); 4.55, m, 1H (HαPro); 4.30, m, 1H (HαLys); 4.10, m, 2H (HαGly1); 3.90, s, 2H (HαGly2); 3.70, m, 2H (Hδ Pro); 3.25, m, 2H (HεLys); 2.40 and 2.10, 2m, 2H (Hβ Pro); 2.10, s, 3H (OAc); 1.95, m, 2H (Hβ Lys); 1.65, m, 6H (Hγ Pro, Hδ Lys, Hγ Lys). MS (FAB$^+$): 531 (MH^+), 471 ($M^+ - OAc$). HRMS (FAB$^+$): calcd for $C_{25}H_{35}N_6O_7$ (MH^+), 531.2567; found, 531.2591.

Cyclo(H-Lys-Pro-Aba-[CH_2OPh]-Gly_2-) (16a). The peptide **10a** (0.055 g, 0.078 mmol) was dissolved in a mixture of MeOH (5 mL) and H_2O (1 mL). Palladium on charcoal (0.2 g) was added, and the solution was hydrogenated in a Parr apparatus at room temperature for 1 h. The solution was filtered in order to remove the catalyst, and the solvent was evaporated. The residue was chromatographed on a column of silica gel. Elution with solvent system I (20/80), followed by evaporation of the product containing pooled fractions, dissolution of the residue in methanol, filtration, concentration to ca. 3 mL, precipitation with ether, and treatment of the precipitate as before (centrifugation, repeated washings with ether, and drying), yielded 38.2 mg (80%) of pure peptide **16a**. Mp: 140.1 °C. R_f (I: 20/80) = 0.1. ^{1}H NMR (CD_3OD): 8.43, d, 1H (ArH); 7.53, d (8.1), 1H (ArH); 7.22, m, 3H (ArH); 6.89, m, 3H (ArH); 5.22, dd~q (12.5), 2H (CH_2OPh); 4.57, m, 2H (HαPro, HαLys); 4.10−3.71, m, 6H (2HαGly, Hδ Pro); 3.25, m, 2H (HεLys); 2.36−1.99, m, 4H (Hβ Pro, Hγ Pro); 1.54−1.23, m,

6H (HβLys, HδLys, HγLys). MS (FAB$^+$): 565 (MH^+), 471 ($MH^+ - OPh$). HRMS (FAB$^+$): calcd for $C_{29}H_{37}N_6O_6$ (MH^+), 565.2775.

Enzymatic Studies. Cells and Preparation of Extracts: MOLT4 cells are lymphoblastoid CD4$^+$ T-cells routinely used in our laboratory for the assay of different peptidases.[36] Cells (from Dr. G. Farrar) were generously provided by the Medical Research Council AIDS Directed Programm Reagent Project, U.K. CEM clones expressing enhanced levels of human CD26 were obtained in the laboratory by transfection of CEM cells as described previously;[49] clones were selected by their very high level of CD26 expression determined by FACS analysis and by DPP IV activity measurement. All cells were cultured in suspension medium RPMI-1640 (BioWhittaker, Verviers, Belgium) containing heat-inactivated (56 °C, 30 min) 10% (by vol) fetal calf serum. For preparation of cell extracts, cells were first washed extensively with phosphate-bufferd saline (PBS) before lysis in buffer E (20 mM Tris/HCl, pH 7.6, 150 mM NaCl, 5 mM $MgCl_2$, 0.2 mM phenylmethanesulfonyl fluoride ($PhMeSO_2F$), 5 mM 2-mercaptoethanol, aprotinin (100 U/mL), and 0.5% Triton X-100) (75 μL/10^7 cells), and the nuclei were pelleted by centrifugation (1000*g* for 8 min). The supernatant was diluted with 1 volume of buffer I (20 mM Tris/HCl, pH 7.6, 400 mM NaCl, 50 mM KCl, 1 mM EDTA, 0.2 mM $PhMeSO_2F$, aprotinin (100 U/mL), 5 mM 2-mercaptoethanol, 1% Triton X-100, and 20% glycerol) and centrifugated at 12000*g* for 10 min. The supernatants (cell extracts) were stored at −80 °C. Under these extraction conditions, extracts from CEM cells (clone H01) showed more than 95% of DPP IV activity associated to CD26; thus no further purification was necessary. Extracts from C8166 cells showed important levels of DPP II contamination; for this reason DPP IV-β was purified by gel filtration and ion-exchange chromatography using a FPLC system (Pharmacia, Uppsala, Sweden) as described elsewhere.[51]

The DPP IV peptidase activity was also measured on the surface of intact cells (2×10^6) in a total reaction volume of 0.5 mL of peptidase buffer PB (100 mM Hepes, pH 7.6, 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 8 mM glucose, and 1% BSA). Cells were incubated with 0.55 mM dipeptide-pNa at 37 °C for 90 min. The reactions were stopped by addition of 1 mM sodium acetate, pH 4.5 (1 mL). After centrifugation at 12000*g*, the production of *p*-nitroaniline in the supernatant was assessed by measurement of the absorbance at 405 nm. In this case, DPP II contamination of C8166 cells was not detectable, due to the intracellular localization of this enzyme. For determination of DPP IV peptidase activity in extracts from CEM cells or partially purified preparations of DPP IV-β, 50 μL of appropriately diluted samples was incubated in a total volume of 200 μL of buffer PB with 1 mM Gly-Pro-pNA at 37 °C for 120 min in flat-bottom 96-well microplates; activity was determined by measuring absorption at 405 nm.

Inhibitory effect upon DPP IV in soluble preparations was assayed in 96-well microplates, by preincubating CD26 and DPP IV-β preparations with different concentrations of each inhibitor ranging from 1 nM to 10 μM, for 15 min, before adding the substrate as indicated. The inhibitory effect on cell-surface-expressed cells was performed similarly; however, cells were extensively washed after preincubation with the inhibitor. The DPP IV activity-reversible inhibitor Lys-[Z(NO_2)]-pyrrolidide was kindly provided by Dr. A. Barth (Halle, Germany). When its inhibitory effect was studied, no washes were performed, due to the reversibility of this inhibitor. IC_{50} values were calculated by nonlinear regression as described.[5] The values of K_I and k_{inact} were calculated using the simplified approach described previously,[34] which ignores the inactivation of the inhibitor by the enzyme. Inhibition kinetics of the DPP IV activities of both enzymes tested (CD26 and DPP IV-β) fit well to this model. K_m values were calculated as decribed.[51] Briefly, in 96-well microtiter plates, aliquots of each enzyme preparation were added to wells containing 0.5 nM Gly-Pro-pNA and increasing concentrations of inhibitor (ranging from 0.3- to 3-fold IC_{50} values) in a total volume of 200 μL. Plates were incubated at 37 °C, and absorbance at 405 nM was determined every 15 min. Data were plotted semilogarithmically as ln(absorbance increase/time increase) against time, to obtain a straight line with slope $-\pi$. Plotting this slope against $1/[I(S/K_m + S)]$, where I = absorbance increase/time increase and S = substrate concentration, yields another straight line with an x-intercept of $-K_I$ and a y-intercept of k_{inact}.[34]

Acknowledgment. This work was supported by grants from the Institut Pasteur. J.B. is a recipient of a postdoctoral fellowship from the Spanish Ministerio de Educacion y Ciencia. We thank Mrs. Nadine Robert and Josette Svab for technical assistance.

References

(1) Yaron, A.; Naider, F. Proline-Dependent Structural and Biological Properties of Peptides and Proteins. *Crit. Rev. Biochem. Mol. Biol.* **1993**, *28*, 31−81.
(2) Fleischer, B. *Dipeptidyl peptidase IV* (*CD26*) *in Metabolism and in the Immune Response*; Springer-Verlag: New York, 1995.
(3) Callebaut, C.; Krust, B.; Jacotot, E.; Hovanessian, A. G. T Cell Activation Antigen, CD26, as a Cofactor for Entry of HIV in CD4$^+$ Cells. *Science* **1993**, *262*, 2045.
(4) Gutheil, W. G.; Subramanyam, M.; Flentke, G. R.; Sanford, D. G.; Munooz, E.; Huber, B. T.; Bachovchin, W. W. Human Immunodificiency Virus 1 Tat Binds to Dipeptidyl Aminopeptidase IV (CD26): A Possible Mecanism for Tat's Immunosuppressive activity. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 6594−6598.
(5) Blanco, J.; Marié, I.; Callebaut, C.; Jacotot, E.; Krust, B.; Hovanessian, A. G. Specific Binding of Adenosine Deaminase but not HIV-1 Transactivator Protein Tat to Human CD26. *Exp. Cell. Res.* **1996**, *225*, 102−111.
(6) Schmitz, T.; Underwood, R.; Khiroya, R.; Bachovchin, W. W.; Huber, B. T. Potentiation of the Immune response in HIV-1$^+$ Individuals. *J. Clin. Invest.* **1996**, *97*, 1545−1549.
(7) Kameoka, J.; Tanaka, T.; Nojima, Y.; Schlossman, S. F.; Morimoto, C. Direct Association of Adenosine Deaminase with a T Cell Activation Antigen, CD26. *Science* **1993**, *261*, 166−169.
(8) Torimoto, Y.; Dang, N. H.; Vivier, E.; Tanaka, T.; Schlossman, S. F.; Morimoto, C. Coassociation of CD26 (Dipeptidyl Peptidase IV) with CD45 on the Surface of Human T Lymphocytes. *J. Immunol.* **1991**, *147*, 2514−2517.
(9) Ansorge, S.; Bühling, F.; Hoffmann, T.; Käne, T.; Neubert, K.; Reinhold, D. DPP IV/CD26 on Human Lymphocytes: functional Roles in Cell Growth and Cytokine Regulation. *Dipeptidyl peptidase IV* (*CD26*) *in Metabolism and in the Immune Response*; Springer-Verlag: New York, 1995; pp 163−184.
(10) Fleisher, B. CD26: a Surface Protease Involved in T-Cell Activation. *Immunol. Today* **1994**, *15*, 180−184.
(11) Tanaka, T.; Kameoka, J.; Yaron, A.; Schlossman, S. F.; Morimoto, C. The Costimulatory Activity of the CD26 Antigen Requires Dpeptidyl Peptidase IV Enzymatic Activity. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 4586−4590.
(12) Steeg, C.; Hartwig, U.; Fleisher, B. Unchanged Signaling Capacity of Mutant CD26/Dipeptidylpeptidase IV Molecules Devoid of Enzymatic Activity. *Cell Immunol.* **1995**, *164*, 311−315.
(13) Rahfeld, J.; Schierhorn, M.; Hartrodt, B.; Neubert, K.; Heins, J. Are Diprotin A (Ile-Pro-Ile) and Diprotin B (Val-Pro-Leu) Inhibitors or Substrates of Dipeptidyl Peptidase IV? *Biochim. Biophys. Acta* **1991**, *1076*, 314−316.
(14) Schön, E.; Born, I.; Demuth, H.-U.; Faust, J.; Neubert, K.; Steinmetzer, T.; Barth, A.; Ansorge, S. Dipeptidyl Peptidase IV in the Immune System. *J. Biol. Chem. Hoppe-Seyler* **1991**, *372*, 305−311.
(15) Stöckel, A.; Demuth, H.-U.; Neubert, K. Competitive Inhibition of Proline Specific Enzymes by Amino Acid Thioxopyrrolidides and Thiazolidides. *Peptides*: *Chem., Struct. Biol.* **1996**, 709−710.
(16) Augustyns, K. J. L.; Lambeir, A.-M.; Borloo, M.; De Meester, I.; Vedernikova, I.; Vanhoof, G.; Hendriks, D.; Scharpé, S.; Haemers, A. Pyrrolidides: Synthesis and Structure−Activity Relationship as Inhibitors of Dipeptidyl Peptidase IV. *Eur. J. Med. Chem.* **1997**, *32*, 301−309.
(17) Li, J.; Wilk, E.; Wilk, S. Aminoacylpyrrolidine-2-Nitriles: Potent and Stable Inhibitors of Dipeptidyl-Peptidase IV (CD26). *Arch. Biochem. Biophys.* **1995**, *3*, 148−154.
(18) Ashworth, D. M.; Atrash, B.; Baker, G. R.; Baxter, A. J.; Jenkins, P. D.; Jones, D. M.; Szelke, M. 2-Cyanopyrrolidides as Potent, Stable Inhibitors of Dipeptidyl Peptidase IV. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1163−1166.
(19) Ashworth, D. M.; Atrash, B.; Baker, G. R.; Baxter, A. J.; Jenkins, P. D.; Jones, D. M.; Szelke, M. 4-Cyanothiazolidides as Very Potent, Stable Inhibitors of Dipeptidyl Peptidase IV. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2745−2748.

(20) Boduszek, B.; Oleyksyszyn, J.; Kam, C.-M.; Selzler, J.; Smith, R. E.; Powers, J. C. Dipeptide Phosphonates as Inhibitors of Dipeptidyl Peptidase IV. *J. Med. Chem.* **1994**, *37*, 3969−3976.
(21) Lambeir, A.-M.; Borloo, M.; De Meester, I.; Belyaev, A.; Augustins, K.; Hendricks, D.; Sharpe, S.; Hemers, A. Dipeptide-Derived Diphenyl Phosphonate Esters: Mechanism-Based Inhibitors of Dipeptidyl Peptidase IV. *Biochim. Biophys. Acta* **1996**, *1290*, 76−82.
(22) Kelly, T. A.; Adams, J.; Bachovchin, W. W.; Barton, R. W.; Campbell, S. J.; Coutts, S. J.; Kennedy, C. A.; Snow, R. J. Immunosuppressive Boronic Acid Dipeptides: Correlation Between Conformation and Activity. *J. Am. Chem. Soc.* **1993**, *115*, 12637−12638.
(23) Gutheil, G. W.; Bachovchin, W. W. Separation of L-Pro-D-Pro-BoroPro into its Component Diastereomers and Kinetic Analysis of Their Inhibition of Dipeptidyl Peptidase IV. A New Method for the Analysis of Slow, Tight-Binding Inhibition. *Biochemistry* **1993**, *32*, 8723−8731.
(24) Coutts, S. J.; Kelly, T. A.; Snow, R. J.; Kennedy, C. A.; Barton, R. W.; Adams, J.; Krolikowski, D. A.; Freeman, D. M.; Campbell, S. J.; Ksiazek, J. F.; Bachovchin, W. W. Structure−Activity Relationships of Boronic Acid Inhibitors of Dipeptidyl Peptidase IV. 1. Variation of the P_2 Position of A_{aa}-BoroPro Dipeptides. *J. Med. Chem.* **1996**, *39*, 2087−2094.
(25) Pargellis, C. A.; Campbell, S. J.; Pav, S.; Graham, E. T.; Pittner, T. P. Inhibition of Dipeptidyl Peptidase IV (CD26) by Peptide Boronic Acid Dipeptides. *J. Enzyme Inhib.* **1997**, *11*, 151−169.
(26) Steinmetzer, T.; Silberring, J.; Mrestani-Klaus, C.; Fittkau, S.; Barth, A.; Demuth, H.-U. Peptidyl Ammonium Methyl Ketones as Substrate Analogue Inhibitors of Proline-Specific Peptidase. *J. Enzyme Inhib.* **1993**, *7*, 77−85.
(27) Borloo, M.; Augustyns, K.; Belyaev, A.; Lambeir, A.-M.; Goosens, F.; Bollaert, W.; Rajan, P.; Scharpé, S.; Haemers, A. Synthesis and Evaluation of Azaproline Peptides as Potential Inhibitors of Dipeptidyl Peptidase IV and Prolyl Oligopeptidase. *Lett. Pept. Sci.* **1995**, *2*, 198−202.
(28) Demuth, H.-U.; Baumgrass, R.; Schaper, C.; Fischer, G.; Barth, A. Dipeptidyl Peptidase IV−Inactivation with *N*-Peptidyl-*O*-aroyl Hydroxylamines. *J. Enzyme Inhib.* **1988**, *2*, 129−142.
(29) Demuth, H.-U.; Schlenzig, D.; Schierhorn, A.; Grosche, G.; Chapot-Chartier, M.-P.; Gripon, J. C. Design of [ω-N-(O-Acyl) Hydroxy Amide]Aminodicarboxylic Acid Pyrrolidides as Potent Inhibitors of Proline-Specific Peptidases. *FEBS Lett.* **1993**, *320*, 23−27.
(30) Welch, J. T.; Lin, J. Fluoroolefin Containing Dipeptide Isosteres as Inhibitors of Dipeptidyl Peptidase IV (CD26). *Tetrahedron* **1996**, *52*, 291−304.
(31) Welch, J. T.; Lin, J.; Boros, L. G.; DeCorte, B.; Bergrmann, K.; Gimi, R. Fluoroolefin isosteres as peptidomimetics. *ACS Symp. Ser.* **1996**, *639*, 129−142.
(32) Demuth, H.-U.; Heins, J. Catalytic Mechanism of Dipeptidyl Peptidase IV in Fleisher, B. *Dipeptidyl peptidase IV* (*CD26*) *in Metabolism and in the Immune Response*; Springer-Verlag: New York, 1995; pp 1−35.
(33) Ding, X.; Rasmussen, B. F.; Demuth, H.-U.; Ringe, D.; Steinmetz, A. C. U. Nature of the Inactivation of Elastase by *N*-Peptidyl-*O*-aroyl Hydroxylamine as a Function of pH. *Biochemistry* **1995**, *34*, 7749−7756.
(34) Wakselman, M.; Xie, J.; Mazaleyrat, J.-P.; Bogetto, N.; Vilain, A.-C.; Montagne, J.-J.; Reboud-Ravaux, M. New Mechanism-Based Inactivators of Trypsin-like Proteinases. Selective Inactivation of Urokinase by Functionalized Cyclopeptides Incorporating a Sulfoniomethyl-Substitued *m*-Aminobenzoic Acid Residue. *J. Med. Chem.* **1993**, *36*, 1539−1547.
(35) Schechter, I.; Berger, A. On the Size of the Active Site in Proteases. I. Papain. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157−162.
(36) Jacotot, E.; Callebaut, C.; Blanco, J.; Krust, B.; Neubert, K.; Barth, A.; Hovanessian, A. G. Dipeptidyl-peptidase IV-β, a Novel Form of Cell-Surface-Expressed Protein with Dipeptidyl-Peptidase IV Activity. *Eur. J. Biochem.* **1996**, *239*, 248−258.
(37) Hamuro, Y.; Geib, S. J.; Hamilton, A. D. Oligoanthranilamides. Non-Peptide Subunits that Show Formation of Specific Secondary Structure. *J. Am. Chem. Soc.* **1996**, *118*, 7529−7541.
(38) Wakselman, M.; Mazaleyrat, J. P.; Xie, J.; Montagne, J. J.; Vilain, A. C.; Reboud-Ravaux, M. Cyclopeptididic Inactivators for Chymotrypsin-Like Proteinases. *Eur. J. Med. Chem.* **1991**, *26*, 699−707.
(39) Kiso, Y.; Ukawa, K.; Ito, K.; Akita, T. Efficient Removal Protecting group by a "Push-Pull" Mecanism. II. Deprotection of *O*-Benzyltyrosine with Thioanisole-Trifluoroacetic Acid System Without *O* to *C* Rearrangement. *Chem. Pharm. Bull.* **1980**, *28*, 673−676.
(40) Kiso, Y.; Ukawa, K.; Akita, T. Efficient Removal of *N*-benzyloxycarbonyl Group by a "Push-Pull" Mechanism Using Thioanisole-Trifluroroacetic Acid, Exemplified by a Synthesis of Met-Enkephalin. *J. Chem. Soc., Chem. Commun.* **1980**, 101−102.
(41) Brandt, W.; Lehmann, T.; Thondorf, I.; Born, I.; Schutkowski, M.; Rahfeld, J.-U.; Neubert, K.; Barth, A. A Model of the Active Site of Dipeptidyl Peptidase IV Predicted by Comparative Molecular Field Analysis and Molecular Modelling Simulations. *Int. J. Pept. Protein Res.* **1995**, *46*, 494−507.
(42) Brandt, W.; Ludwig, O.; Thondorf, I.; Barth, A. A New Mechanism in Serine Proteases Catalysis Exhibited by Dipeptidyl Peptidase IV (DP IV). *Eur. J. Biochem.* **1996**, *236*, 109−114.
(43) Hansch, C.; Leo, A.; Taft, R. W. A Survey of Hammett Substituent Constant and Resonnance and Field Parameters. *Chem. Rev.* **1991**, *91*, 165−195.
(44) Wakselman, M. 1,4- and 1,6-Eliminations From Hydroxy- and Amino-Substitued Benzyl Systems: Chemical and Biochemical Applications. *Nouv. J. Chim.* **1983**, *7*, 439−447.
(45) Silverman, R. B. *Mechanism-Based Enzyme Inactivation*: *Chemistry and Enzymology*; CRC Press: Boca Raton, FL, 1988; Vol. 1, pp 3−22.
(46) Wakselman, M.; Nguyen, C.; Mazaleyrat, J. P.; Callebaut, C.; Blanco, J.; Jacotot, E.; Krust, B.; Hovanessian, A. G. Inhibition of HIV-1 Infection of $CD26^+$, but not $CD26^-$ cells, by a cyclopeptidic Inhibitor of the DPP IV Activity of CD26. In *Peptides 1996*; Ramage, R., Epton, R., Eds.; Mayflower Worlwide Ltd., 1998; pp 897−898.
(47) Oravecz, Z.; Rodriquez, G.; Koffi, J.; Wang, J.; Ditto, M.; Bou-Habid, D. C.; Lusso, P.; Norcross, M. A. CD26 Expression Correlates with Entry, Replication and Cytopathicity of Monocytotrophic HIV-1 Strains in a T-Cell Line. *Nature Med.* **1995**, *1*, 919.
(48) Jacotot, E.; Callebaut, C.; Blanco, J.; Rivière, Y.; Krust, B.; Hovanessian, A. G. HIV Envelope Glycoproteins-Induced Cell Killing by Apoptosis is Enhanced with Increased Expression of CD26 in $CD4^+$ T Cells. *Virology* **1996**, *223*, 1−13.
(49) Callebaut, C.; Jacotot, E.; Blanco, J.; Krust, B.; Hovanessian, A. G. The Level of CD26 determines the Rate of HIV Entry in a $CD4^+$ T-Cell Line. In *Cellular Peptidases in Immune System and Diseases*; Adv. Exp. Med. Biol., Vol. 421; Ansorge, S., Langner, J., Eds.; Plenum Press: New York, 1997, Vol. 421, pp 179−184.
(50) Jiang, J. D.; Wilk, S.; Li, J.; Zhang, H.; Bekesi, J. G. Inhibition of Human Immunodeficiency Virus Type 1 Infection in T-Cell Line (CEM) by New Dipeptidyl-Peptidase IV (CD26) Inhibitors. *Res. Virol.* **1997**, *148*, 255−266.
(51) Blanco, J.; Nguyen, C.; Callebaut, C.; Jacotot, E.; Krust, B.; Mazaleyrat, J. P.; Wakselman, M.; Hovanessian, A. G. DPP IV β: further Characterization and Comparison to DPP IV activity of CD26. Submitted.

JM970640L