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Aminoacylpyrrolidine-2-nitriles: Potent and Stable Inhibitors of Dipeptidyl-Peptidase IV (CD 26)

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Dipeptidyl-peptidase IV (EC 3.4.14.5) also known as CD26 is a membrane-bound serine peptidase which cleaves N-terminal dipeptides from a peptide chain containing a proline residue in the penultimate position. The enzyme is believed to play an important role in neuropeptide metabolism and T-cell activation. A series of aminoacylpyrrolidine-2-nitriles, in which the carboxyl group of proline is replaced by a nitrile group, was synthesized as inhibitors of dipeptidyl-peptidase IV. All compounds were found to competitively inhibit a homogeneous preparation of the rat kidney enzyme with K_i values in the low to submicromolar range. The nitriles presumably react with the active-site serine to form an imidate adduct. The compounds were stable following incubation either for 20 h at 37°C or 72 h at room temperature. They proved to be poor inhibitors of dipeptidyl-peptidase II and prolyl oligopeptidase. These studies demonstrate that the generally held concept that nitriles are poor inhibitors of serine proteinases needs to be reconsidered. Aminoacylpyrrolidine-2-nitriles by virtue of their ease of synthesis, stability, specificity, and inhibitory potency appear to be superior to other described dipeptidyl-peptidase IV inhibitors. © 1995 Academic Press, Inc.

Dipeptidyl-peptidase IV (DPP IV[2]; EC 3.4.14.5) was first detected in a commercial preparation of acylase I and in rat liver as an enzyme which released 2-naphthylamine from the substrate glycyl-prolyl-2-naphthylamide (1). It was localized to the microsomal fraction and its molecular weight was estimated by gel filtration to be about 250,000 (2). The specificity of the purified enzyme was subsequently shown to be directed toward aminoacylprolyl-peptide bonds, although aminoacylalanyl-peptide bonds were also cleaved at a much lower efficiency (3). DPP IV is classified as a serine peptidase and is readily inactivated by diisopropylfluorophosphate (DFP). Active site titration of the pig kidney enzyme with radiolabeled DFP yielded a monomer molecular weight of 130,000 and established the pig kidney enzyme as a symmetrical dimer (4). More recently the DFP-modified residue of the rat liver enzyme has been identified as Ser^{631}. Site directed mutagenesis of this residue abolished enzymatic activity (5). The sequential order of the catalytic triad in DPP IV (Ser . . . Asp . . . His) differs from that of classical serine proteinases (His . . . Asp . . . Ser) (6). A comparison of amino acid sequences places DPP IV in a newly defined subfamily of serine proteinases which include prolyl oligopeptidase (EC 3.4.21.26), and acylaminoacyl-peptidase (EC 3.4.19.1) (7).

There has been considerable interest in the role of DPP IV both in the metabolism of neuropeptides and in the immune response. DPP IV degrades substance P with sequential removal of the Arg-Pro and Lys-Pro residues (8). It also removes the terminal Tyr-Pro from neuropeptide Y and peptide YY (9). The latter reaction is of significance since binding to Y1 receptors but not to Y2 receptors requires the intact amino terminus (10). Histochemical detection of DPP IV in lymphatic tissue (11) was followed by its biochemical characterization on membranes of T-lymphocytes (12). On the basis of localization with monoclonal antibodies, DPP IV has been assigned to the CD26 cluster (13). Considerable evidence exists in support of a role for DPP IV as a T-lymphocyte activation antigen (see 14 for a review).

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[2] Abbreviations used: DPP IV, dipeptidyl-peptidase IV; DPP II, dipeptidyl-peptidase II; 2-NA, 2-naphthylamide; Boc, *tert*-butyloxycarbonyl; Fmoc, 9-fluorenylmethyloxycarbonyl; pyrr-2-CN, pyrrolidine-2-nitrile; DMSO, dimethyl sulfoxide; DMF, dimethylformamide; THF, tetrahydrofuran; TFAA, trifluoroacetic anhydride; HOBT, 1-hydroxybenzotriazole; DCC, dicyclohexylcarbodiimide; PMC, 2,2,5,7,8-pentamethyl chroman-6-sulfonyl; Ot-Bu, *tert*-butyl ester; DFP, diisopropylfluorophosphate; BSA, bovine serum albumin.

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CD 26 (DPP IV) was recently proposed as a coreceptor which facilitates the entry of HIV into T-cells. Inhibitors of DPP-IV were reported to prevent the entry of virus into $CD4^+$ T lymphocytes (15). By contrast it has been reported that although DPP IV may play a role in HIV infectivity, DPP enzymatic activity may decrease the efficiency of HIV infection (16).

Few inhibitors of DPP IV are described in the literature and all of these have limitations in terms of potency, stability, or toxicity. The simple tripeptides Ile-Pro-Ile (diprotin A) and Val-Pro-Leu (diprotin B) are modest competitive inhibitors (IC_{50}'s = 8 and 920 μM, respectively) (17). Moreover these peptides should be degraded by the soluble form of X-Pro aminopeptidase (aminopeptidase P) (EC 3.4.11.9). *N*-Peptidyl-*O*-aroyl hydroxylamines are mechanism-based inhibitors of DPP IV of only modest potency (18). The reversible inhibitor Lys[Z-4-NO_2]-pyrrolidide is more effective ($IC_{50} = 2$ μM) but also highly toxic (17). The most potent DPP IV inhibitors thus far synthesized are dipeptides containing boroPro, the boronic acid analog of proline, at the C-terminus. The K_i of Ala-boroPro is 2 nM and the K_i of Pro-boroPro is 3 nM. These compounds however are quite unstable. Thus the $t_{1/2}$ of Ala-boroPro was reported to be between 2 and 30 min and the $t_{1/2}$ of Pro-boroPro was reported to be 1.5 h in aqueous solution (19). More recently, a series of dipeptide phosphonates were described as irreversible inhibitors of DPP IV (20). Although these compounds are specific, they are not very potent.

Peptidyl nitriles are regarded as good inhibitors of cysteine proteinases but rather poor inhibitors of serine proteinases (21, 22). However, several peptidyl nitriles were recently reported as potent inhibitors of prolyl oligopeptidase (23). Since prolyl oligopeptidase and DPP IV are members of the same subfamily of serine proteinase and since both enzymes cleave peptide bonds at the carboxyl end of a prolyl residue, we investigated whether nitriles would also inhibit DPP IV. We report here the synthesis and properties of aminoacyl-pyrrolidine-2-nitriles. These compounds are readily synthesized, stable, specific, and potent (submicromolar K_i's) competitive inhibitors of DPP IV.

EXPERIMENTAL PROCEDURES

Materials

Amino acid intermediates were obtained from Bachem, Inc. (Philadelphia, PA). 2-Naphthylamine and Lys-Ala-2-NA were obtained from the Sigma Chemical Co. (St. Louis, MO). Silica gel, Merck, grade 9385, 230–400 mesh, and all other chemicals were obtained from the Aldrich Chemical Co. (Milwaukee, WI). Bovine pituitaries were obtained from Pel-Freez Inc. (Rogers, AK). Rat kidneys for the purification of DPP IV were obtained from rat cadavers supplied by other investigators in this department, and stored frozen at −80°C. Sephadex G-200 and Phenyl Sepharose were products of Pharmacia (Piscataway, NJ). DE-52 was obtained from Whatman, Inc. (Clifton, NJ). Rat kidney prolyl oligopeptidase (EC 3.4.21.26) was purified through the DE-52 chromatography step as previously described for the rabbit brain enzyme (24). Membrane alanyl aminopeptidase (EC 3.4.11.2) was purified to apparent homogeneity from rabbit kidneys as previously described (25).

TLC Analysis

TLC was performed on polygram Sil G/UV 254 precoated plastic sheets (Brinkmann Instruments, Westbury, NY). Compounds were visualized by one or more of the following: tolidine reagent (26), ninhydrin, U.V.

HPLC Analysis

HPLC was performed on a Waters 600E liquid chromatograph equipped with a Beckman Ultrasphere 4.6-mm × 15-cm 5-μm column. The column was equilibrated with 15% acetonitrile, 0.05% TFA at a flow rate of 1 ml/min, and elution was carried out by linearly increasing the acetonitrile concentration to 70% over a period of 34 min. The products were monitored by measurement of absorbance at 210 nm.

NMR

NMR spectra were recorded in DMSO-d6 on a Varian XL-400 instrument. Chemical shifts are given as ppm with TMS as internal standard.

Mass Spectroscopy

Mass spectroscopic analysis was conducted on a Hewlett–Packard HP5988 instrument.

SDS–PAGE

SDS–PAGE was conducted on 10% slab gels in a 25 mM Tris, 0.192 M glycine buffer, pH 8.3, as described (27). Proteins were stained with Coomassie blue.

Measurement of Enzymatic Activities

DPP IV activity was determined by measuring the release of 2-naphthylamine (NA) from the substrate Ala-Pro-2-NA. The incubation mixture contained 10 μl 10 mM substrate in DMSO, enzyme, and 0.05 M Tris–HCl, pH 7.5, at a final volume of 250 μl. The released 2-NA was measured by a colorimetric procedure (28). The chromogen was measured spectrophotometrically at 580 nm. DPP II was determined by measuring the release of 2-NA from the substrate Lys-Ala-2-NA at pH 5.5. The incubation mixture contained 10 μl 10 mM substrate in DMSO, enzyme, and 0.05 M sodium acetate buffer, pH 5.5, at a final volume of 250 μl. Prolyl oligopeptidase (24) and membrane alanyl aminopeptidase (25) were determined as described.

Measurement of Enzyme Inhibition

K_i values for all compounds were obtained by the method of Dixon (29). Each experiment was performed at five concentrations of inhibitor and three concentrations of substrate. Reactions were initiated by addition of enzyme to solutions containing substrate and inhibitor.

Preparation of DPP IV

DPP IV was purified to electrophoretic homogeneity from rat kidney. Briefly, 120 g rat kidney were homogenized in 4 vol H_2O and the homogenate centrifuged for 15 min at 1000*g*. The supernatant was adjusted to pH 3.9 and the enzyme was solubilized by autolysis for 18 h at 37°C. The pH of the supernatant collected after centrifuga-

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tion was adjusted to 7.4 and the enzyme was precipitated with $(NH_4)_2SO_4$ at 90% saturation. The solubilized precipitate was chromatographed on Sephadex G-200 (1 m × 5 cm) equilibrated with 10 mM Tris–HCl, pH 7.5, 0.1 M NaCl, and developed from the bottom. Fractions containing enzymatic activity were pooled, chromatographed on DE-52 (16 × 2.5 cm) equilibrated with 10 mM Tris–HCl, pH 7.5, and eluted with a 250-ml linear 0–0.4 M NaCl gradient. DPP IV was then resolved from other brush border peptidases by chromatography on a Phenyl Sepharose column (12 × 2 cm) equilibrated with 25% $(NH_4)_2SO_4$ in 0.05 M Tris–HCl, pH 7.5. The enzyme was eluted in a homogeneous form with a 200-ml linear gradient of 25–0% $(NH_4)_2SO_4$.

Preparation of DPP II

DPP II was partially purified from 25 g bovine pituitaries. A homogenate in 25 mM Tris–HCl, pH 7.5, was centrifuged for 1 h at 70,000*g*. The resulting supernatant was fractionated with $(NH_4)_2SO_4$ (40–70%). The precipitate was dissolved in 10 mM sodium phosphate buffer, pH 7.0, and dialyzed against the same buffer. The dialysate was applied to a 2.5 × 10-cm DE-52 column equilibrated with the same buffer. The column was washed with the same buffer until the absorbance of the eluate at 280 nm was close to zero. The enzyme was eluted with a 400-ml linear 0–0.5 M NaCl gradient in the equilibrating buffer. Fractions containing DPP II activity were used for specificity studies. The resulting preparation was devoid of DPP IV and aminopeptidase activities.

Synthesis of Ala-Pro-2-NA

(a) Boc-Pro-2-NA

To a solution of 1 g 2-naphthylamine (7 mmol) in 10 ml dry THF was added 1.5 g Boc-Pro (7 mmol). The solution was cooled to −20°C. Seven millimoles of *N*-methylmorpholine and 7.7 mmol of isobutylchloroformate were then added and the reaction allowed to proceed for 20 min. The reaction mixture was allowed to come to room temperature and then filtered. The solvent was evaporated, the residue was dissolved in chloroform, and the chloroform layer was washed sequentially with sat. $NaHCO_3$, H_2O, 10% citrate, and H_2O. After drying over Na_2SO_4, the chloroform was evaporated. Ethyl ether was added to the residue and Boc-Pro-2-NA crystallized out in 67% yield.

(b) Pro-2-NA · HCl

Ten milliliters 4 N HCl in dioxane was added to (a) and the solution was stirred at room temperature for 30 min. Pro-NA · HCl crystallized from solution in a quantitative yield.

(c) Boc-Ala-Pro-2-NA

To a suspension of (b) in 40 ml dimethylformamide, equimolar amounts of triethylamine and Boc-Ala were added. The solution was cooled to 4°C in an ice–salt bath. Equimolar amounts of 1-hydroxybenzotriazole (HOBt) and dicyclohexylcarbodiimide (DCC) were added. The reaction was then allowed to proceed at 4°C for 20 h. After filtration the solvent was removed by evaporation. The residue was dissolved in chloroform and washed sequentially as described above. Boc-Ala-Pro-2-NA was obtained in quantitative yield after evaporation of solvent.

(d) Ala-Pro-2-NA · HCl

Ten milliliters 4 N HCl in dioxane was added to (c) and the reaction was stirred for 30 min at room temperature. The solvent was evaporated. When added to the residue, ether precipitated the product in quantitative yield. TLC: Ethyl acetate/methanol/NH_4OH (7:3:0.5), single spot, R_f = 0.18.

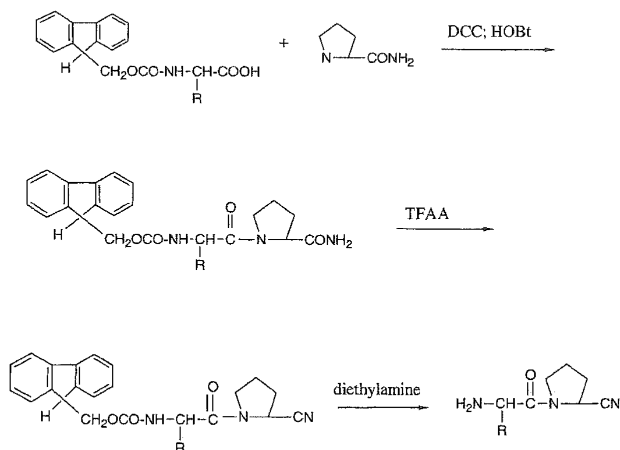

FIG. 1. Synthesis of aminoacylpyrrolidine-2-nitriles.

Synthesis of Aminoacylpyrrolidine-2-nitriles

All compounds were prepared by the synthetic route outlined in Fig. 1.

1. Phe-Pyrr-2-CN

(a) Fmoc-Phe-Pro-NH$_2$. Pro-NH_2 (3 mmol) was dissolved in 10 ml THF + 5 ml DMF. Equimolar amounts of Fmoc-Phe, DCC, and HOBT were added and the mixture was stirred at room temperature for 72 h. After filtration, the filtrate was evaporated to dryness, the residue was dissolved in chloroform, and then sequentially washed with 10% citrate, H_2O, brine, and H_2O. After drying over Na_2SO_4, the chloroform was removed by evaporation. Ether was then added to the residue and the suspension was reevaporated to dryness. The white crystalline material obtained (1.17 g) was used without further purification.

(b) fMoc-Phe-Pyrr-2-CN. fMoc-Phe-Pro-NH_2 (1.17 g) was dissolved in 30 ml THF and cooled to 5°C. Two equivalents of trifluoroacetic anhydride were added to the stirred solution in four equal portions over a period of 5 min. Stirring was continued for 3 h. The solvent was removed by evaporation and the residue was dissolved in chloroform. The nitrile was purified on a 2.5 × 30-cm silica gel column developed with 97.5% chloroform/2.5% ethanol. Evaporation yielded 850 mg white crystalline material. HPLC: single peak.

(c) Phe-Pyrr-2-CN· HCl. To 850 mg Fmoc-Phe-Pyrr-2-CN 10 ml 20% diethylamine in DMF was added, and the solution was stirred for 2 h at room temperature. The solvent was removed by evaporation and the residue was dissolved in ether. The amine was extracted into 0.1 N HCl and following lyophilization, 285 mg white crystalline material was obtained.

MS (EI) *m/z* 243 (M^+).

NMR (δ) 1.60 (m,1H), 1.81 (m,1H), 1.98 (m,1H), 2.10 (m,1H), 2.97 (dd,1H, J = 6.7, 16.0 Hz), 3.15 (m,1H), 3.40 (dd, 1H, J = 8.1, 16.0 Hz), 3.55 (m, 1H), 4.27 (br s 1H), 4.75 (dd, 1H, J = 4.2, 7.7 Hz), 7.20 (br d, 2H, J = 6.9 Hz), 7.30 (m,5H).

2. Arg(PMC)-Pyrr-2-CN

(a) FmocArg(PMC)-Pro-NH$_2$. Pro-NH_2 was coupled to Fmoc-Arg(PMC) on a 3 mmol scale as described above for 1a. The product

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was dissolved in chloroform and sequentially washed with 10% citrate, H_2O, brine, and H_2O. After drying over Na_2SO_4, the solvent was removed and ether added. The product (1.62 g) was collected as a white crystalline material.

TLC chloroform:ethanol 8:2 single uv positive, tolidine positive spot, $R_f = 0.53$.

(b) Fmoc-Arg(PMC)-Pyrr-2-CN. Fmoc-Arg(PMC)-Pro-NH_2 (758 mg; 1 mmol) was dissolved in 10 ml THF and cooled to 5°C. Two equivalents trifluoroacetic anhydride was added in 4 equal portions over a period of 5 min to the stirred solution, and stirring continued for 3 h. The solvent was evaporated, the residue dissolved in chloroform and passed over a 2.5 × 30 cm silica gel column equilibrated with chloroform. The product was eluted with 97.5% chloroform/2.5% ethanol. After evaporation, addition of ether facilitated the crystallization of 590 mg product.

TLC chloroform:ethanol 9.5:0.5 single spot, $R_f = 0.69$.

(c) Arg(PMC)-Pyrr-2-CN. Four hundred ninety milligrams Fmoc-Arg(PMC)-Pyrr-2-CN was dissolved in 5 ml THF. Diethylamine (1.5 ml) was added and the mixture was stirred at room temperature for 2.5 h. When all of the starting material disappeared, as judged by TLC, the solvent was evaporated, ether was added and the product (330 mg) was crystallized following cooling in a dry-ice methyl cellosolve bath.

HPLC: single peak.

MS (EI) m/z 518 (M^+).

NMR (δ) 1.30 (s, 6H), 1.45 (m, 2H), 1.51 (m, 2H), 1.80 (t, 2H, J = 6.6 Hz), 2.01 (m, 2H), 2.06 (s, 3H), 2.15 (m, 2H), 2.54 (s, 6H), 2.61 (br t, 2H, J = 6.6 Hz), 3.10 (br d, 2H, J = 6.4 Hz), 3.40 (m, 1H), 3.52 (br s, 2H), 4.73 (m, 1H), 6.41 (br s, 1H).

3. *Arg-Pyrr-2-CN·2CF$_3$COOH*

One hundred fifty milligrams Arg(PMC)-Pyrr-2-CN was dissolved in 10 ml of 50% TFA in chloroform and stirred for 2 h at room temperature. The reaction mixture was evaporated, and the product was crystallized by addition of ether. Removal of the blocking group was verified by TLC and by HPLC. Yield, 50%.

HPLC: single peak.

MS (EI) m/z 252 (M^+).

NMR (δ) 1.550 (m, 2H), 1.729 (m, 2H), 2.077 (dd, 2H, J = 6.68, 13.19 Hz), 2.245 ~ 2.141 (m, 2H), 3.109 (m, 2H), 3.652 ~ 3.540 (m, 2H), 4.148 (br t, 1H, J = 5.33 Hz), 4.824 (br d, 1H, J = 5.33, 7.97 Hz), 7.583 (br s, 2H).

4. *Ala-Pyrr-2-CN*

(a) Fmoc-Ala-Pro-NH$_2$. This compound was prepared in an identical manner to that described for 1a starting with 2 mmol of reactants. The product (650 mg; 1.6 mmol) obtained was used without further purification.

(b) Fmoc-Ala-Pyrr-2-CN. A solution of 1.2 mmol (490 mg) (4a) in 15 ml THF + 10 ml DMF was cooled to 5°C. To the cooled solution 2 eq trifluoroacetic anhydride in four equal portions was added over a period of 5 min. The solution was allowed to stir for 2 h at room temperature. The solvent was evaporated and the product purified by silica gel chromatography as described for 1b to yield 456 mg (1.1 mmol) product.

(c) Ala-Pyrr-2-CN. This compound was prepared in 90% yield in a manner identical to that described for 2c.

MS (EI) m/z 167 (M^+).

NMR (δ) 1.10 (d, 3H, J = 7.0), 2.03 (m, 2H), 2.14 (m, 2H), 3.59 (m, 2H), 3.61 (m, 1H), 4.72 (dd, 1H, J = 4.0, 7.8 Hz), 6.98 (br d, 2H, J = 7.0 Hz).

RESULTS

The procedure described for the purification of DPP IV employs phenyl sepharose chromatography as the final step. DPP IV which binds only weakly to this column, is totally resolved from other more strongly binding brush border peptidases such as glutamyl aminopeptidase (EC 3.4.11.7), membrane alanyl aminopeptidase (EC 3.4.11.2) and neprilysin (EC 3.4.24.11). The resulting preparation is homogeneous as judged by SDS–PAGE (Fig. 2). The molecular mass of 110 kDa is in excellent agreement with published values for this enzyme (5).

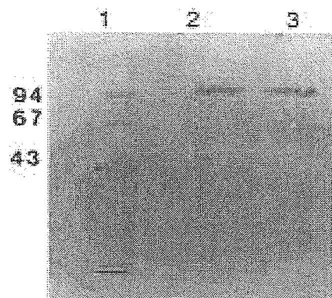

FIG. 2. SDS–PAGE of rat kidney DPP IV after the final step of purification. Lane 1, molecular mass marker proteins (kDa); lanes 2 and 3, DPP IV. Gels stained with Coomassie blue.

Aminoacylpyrrolidine-2-nitriles were efficiently synthesized by a simple three-step method (Fig. 1). An Fmoc-amino acid was first coupled to prolinamide by the dicyclohexylcarbodiimide method. Fmoc-aminoacylpyrrolidine-2-nitriles were then formed by dehydration of the amide with trifluoroacetic anhydride. The Fmoc-aminoacylpyrrolidine-2-nitrile intermediates were synthesized in high yield and purified by silica gel chromatography. Aminoacylpyrrolidine-2-nitriles were obtained by removal of the Fmoc group generally with a 20% solution of diethylamine in DMF. With the exception of Phe-Pyrr-2-CN, the free amine could be crystallized from a cooled ether solution. Phe-Pyrr-2-CN was extracted into 0.1 N HCl and obtained after lyophilization as the hydrochloride salt. Compound purity was confirmed by HPLC (Fig. 3) or by TLC for the hydrochlorides. The structures were confirmed by mass spectrometry and NMR.

Four aminoacylpyrrolidine-2-nitriles were synthesized for study. These compounds were selected to vary the nature of the N-terminal amino acid, i.e., hydrophobic (Phe-Pyrr-2-CN), basic (Arg-Pyrr-2-CN), small neutral amino acid (Ala-Pyrr-2-CN), and the side chain protected analog Arg(PMC)-Pyrr-2-CN. Inhibition by all compounds was evaluated by the method of Dixon (29). The Dixon plot revealed that all acted as competitive (or mixed) inhibitors (Fig. 4). Analysis of the data by the Cornish–Bowden plot of S/v vs i (30) yielded parallel lines, demonstrating competitive inhibition (not shown). All compounds were fairly potent inhibitors of DPP IV with K_i values in the low to submicromolar range (Table I). Although the least potent inhibitor Phe-Pyrr-2-CN

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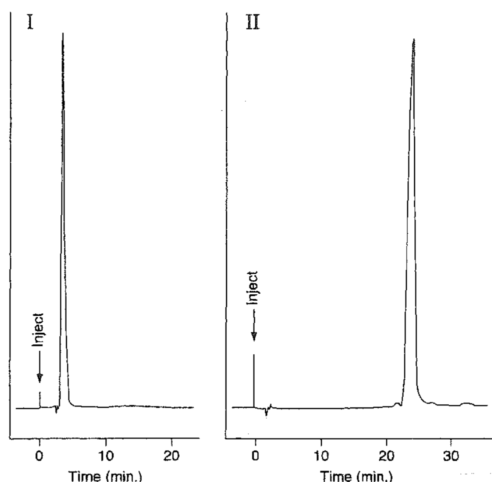

FIG. 3. HPLC analysis of 50 μg each Ala-Pyrr-2-CN (I), and Arg(PMC)-Pyrr-2-CN (II). Peaks monitored at 210 nm at a sensitivity of 1.0 absorbance unit for full-scale deflection. HPLC conditions as described under Experimental Procedures.

TABLE I

Comparison of Potencies of Aminoacylpyrrolidine-2-nitriles as Inhibitors of DPP IV

Compound	K_i (μM)
Arg(PMC)-Pyrr-2-CN	0.19
Arg-Pyrr-2-CN	0.37
Ala-Pyrr-2-CN	0.2
Phe-Pyrr-2-CN	1.2

Note. K_i's determined by the method of Dixon (1953) at three substrate concentrations and five concentrations of inhibitor.

was obtained as the hydrochloride salt, a comparison of Phe-Pyrr-2-CN as the free base or the hydrochloride revealed no difference in inhibitory potency. The presence of a free α-amino group is required for inhibition. Thus Fmoc-Phe-Pyrr-2-CN tested at a concentration of 400 μM negligibly inhibited DPP IV (6%).

To evaluate the specificity of aminoacylpyrrolidine-2-nitriles, the effect of Ala-Pyrr-2-CN and Arg(PMC)-Pyrr-2-CN on the activities of DPP II, prolyl oligopeptidase, and membrane alanyl aminopeptidase was evaluated. DPP II (EC 3.4.14.2), a lysosomal serine peptidase, has a substrate specificity resembling DPP IV. It differs from DPP IV in its ability to more readily cleave an aminoacyl–alanyl bond than an aminoacyl–prolyl bond and in its acidic pH optimum (31). Prolyl oligopeptidase also cleaves peptide bonds after a proline residue, and is a member of the same subfamily of serine proteinases as DPP IV (7). Moreover prolyl oligopeptidase is potently inhibited by N-blocked peptidylpyrrolidine-2-nitriles (23; and manuscript submitted). Membrane alanyl aminopeptidase, a zinc metalloproteinase is a fairly nonselective aminopeptidase (32). Ala-Pyrr-2-CN weakly inhibited DPP II (IC_{50} = 110 μM). Arg(PMC)-Pyrr-2-CN was an even weaker inhibitor (39% inhibition at 400 μM). For prolyl oligopeptidase Ala-Pyrr-2-CN was the weaker inhibitor (22% inhibition at 400 μM). Arg(PMC)-Pyrr-2-CN was more effective (IC_{50} = 75 μM), but still 100-fold less potent than its effect on DPP IV. Membrane alanyl aminopeptidase was unaffected by these compounds.

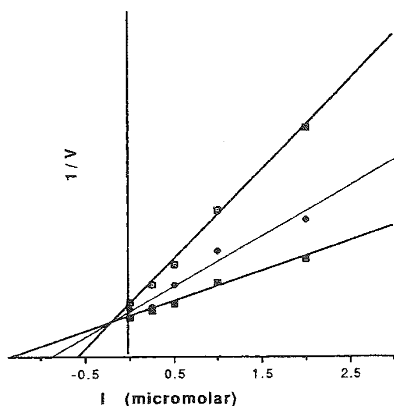

FIG. 4. Dixon plot for the inhibition of DPP IV by Ala-Pyrr-2-CN. Substrate concentrations of 3.5, 7, and 10 mM were used.

The instability of Pro-Boro-Pro limits its usefulness as a DPP IV inhibitor (19). To determine the stability of aminoacylpyrrolidine-2-nitriles, their effectiveness as inhibitors was determined after incubation of solutions at room temperature for 72 h and at 37°C for 20 h compared to solutions of freshly prepared inhibitors. There was negligible loss of inhibitory activity except at the lowest concentration tested (0.4 μM), where some loss was observed (Fig. 5).

DISCUSSION

The studies presented here establish aminoacylpyrrolidine-2-nitriles as potent, stable, specific, and easily synthesized inhibitors of DPP IV. These inhibitors contain a nitrile group in place of the carboxyl group of proline. Earlier experimentation with peptidyl nitriles led to the conclusion that such compounds although effective inhibitors of the class of cysteine proteinases were poor inhibitors of serine proteinases. This conclusion was based on limited information, i.e., the rather poor inhibition of α-chymotrypsin by peptidyl nitriles. Thus, although nitriles are strong inhibitors of papain, 2-DL-(*N*-acetyl-L-leucylamino)-3-phenylpropionitrile is a very weak inhibitor of α-chymotrypsin (K_i >50 mM) (22). Similarly acetylphenylalanyl nitrile is also a poor inhibitor of α-chymotrypsin (21).

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