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2-CYANOPYRROLIDIDES AS POTENT, STABLE INHIBITORS OF DIPEPTIDYL PEPTIDASE IV

Doreen M. Ashworth, Butrus Atrash, Graham R. Baker, Andrew J. Baxter, Paul D. Jenkins*, D. Michael Jones and Michael Szelke

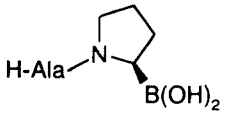
Ferring Research Institute, Chilworth Research Centre, Chilworth, Southampton, U.K., S016 7NP. Fax +44 (1703)766253; e-mail pdj@ferring.demon.co.uk

Abstract: A novel series of stable, potent inhibitors of dipeptidyl peptidase IV has been developed. A number of dipeptide analogues, incorporating a 2-cyanopyrrolidide, were found to have K_i values of less than 5 nM versus human DP-IV and half-lives of >48h in aqueous solution (pH 7.4).

Dipeptidyl peptidase IV (DP-IV, EC 3.4.14.5) is a serine protease which catalyses the cleavage of dipeptides from the N-terminus of proteins with the sequence H-X-Pro-Y- or H-X-Ala-Y- (where X, Y= any amino acid, Y≠ Pro).[1] DP-IV is widely distributed in mammalian tissues and is found in great abundance in the kidney, liver, intestinal epithelium and placenta.[2] In the human immune system, DP-IV is identical to the T cell activation marker, CD26. Recent evidence has also shown CD26 to be an activation marker of natural killer cells[3] and of a main population of B cells.[4]

Our interest in DP-IV was stimulated by the publication of data which showed that either simple inhibitors or antibodies of the enzyme were effective as inhibitors of T cell proliferation and were thus potential immunomodulators.[5-7]

Substrates and inhibitors of DP-IV require a free N-terminus, which means that potential dipeptide serine protease inhibitors (e.g. C-terminal aldehydes, boronic acids, α-ketoacids, trifluoromethylketones, or chloromethylketones) are inherently unstable at neutral pH due to intramolecular cyclisation.[8]



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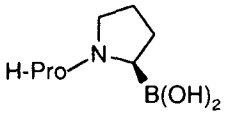

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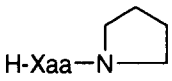
The most potent DP-IV inhibitors reported to date are the boroproline analogues **1**, (K_i=2nM) and **2**, (K_i=3nM). However, these boronic acids are unstable at neutral pH ($t_{½}$=30min and 90min for **1** and **2**

respectively).[7] Other, more stable classes of DP-IV inhibitors have been reported. These include tripeptides;[6] aminoacyl pyrrolidides and thiazolidides;[6] dipeptide phosphonates;[9,10] azaprolines;[11] and the irreversible *N*-peptidyl-*O*-aroylhydroxylamines.[12] Although specific for DP-IV, these compounds exhibit, at best, only modest levels of inhibition.

We felt that it was necessary to develop more potent, stable inhibitors of DP-IV. These would help elucidate the physiological role of the enzyme and may have therapeutic potential in a number of disease states such as inflammation, graft versus host disease (GVHD), cancer, or AIDS.[5]

Table I. *Inhibition of human DP-IV by aminoacyl pyrrolidides.*[13]



Compound N°	Xaa	K_i (μM)[13]
5	Cyclohexylglycine [Chg]	0.064 ± 0.01
6	(R,S)-Cyclopentylglycine [Cpg]	0.21 ± 0.04
7	Ile	0.41 ± 0.01
8	*allo*-Ile	0.44 ± 0.04
9	Val	0.47 ± 0.02
10	Lys(Cbz)	0.52 ± 0.07
11	tert-Butylglycine [Tbg]	0.88 ± 0.20
12	Thr(Me)	0.90 ± 0.15
13	Orn(Cbz)	0.91 ± 0.20
14	2-Aminohexanoic acid [Aha]	1.20 ± 0.20
15	Glu	2.00 ± 0.40
16	Pro	2.10 ± 0.20
17	Cyclohexylalanine [Cha]	2.15 ± 0.50
18	Glu(OBn)	2.70 ± 0.30
19	Thr	4.90 ± 0.90
20	Phenylglycine [Phg]	5.30 ± 0.10
21	Ser(Bn)	6.00 ± 1.50
22	Ala	7.00 ± 1.00
23	Asp	14.50 ± 1.90

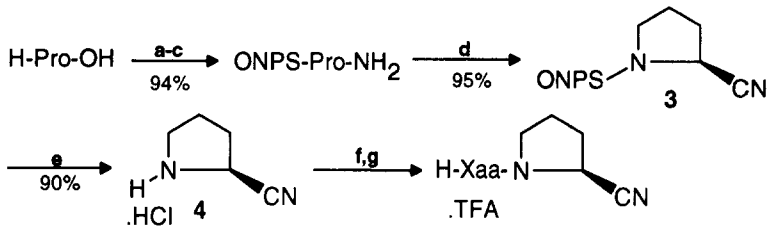
Our attention was drawn by a patent claiming 2-cyanopyrrolidides as inhibitors of prolyl endopeptidase[14] (PEP, EC 3.4.21.26), an enzyme belonging to the same subfamily of serine proteases as DP-IV. PEP differs from DP-IV by being an endopeptidase but the two enzymes share the common specificity for cleaving peptides at the carboxyl side of proline peptidyl bonds.

We now wish to report on the synthesis and biological activity of a series of dipeptide nitriles[15] with potencies versus human DP-IV comparable to the boroprolines **1** and **2** but with superior stability in aqueous solution. One other group has recently described similar compounds as inhibitors of DP-IV[16] but whereas they confirm that such derivatives possess good stability, their series exhibit only modest potency (four compounds with K_i values versus rat DP-IV of 0.19-1.2 μM).

To establish an optimal *N*-terminal residue, we prepared a series of amino acid pyrrolidides.[6] These compounds were prepared by reaction of the *O*-succinimide, (ONSu), ester of the required Boc protected amino acid with a slight excess of pyrrolidine in dichloromethane. Subsequent acid catalysed deprotection (4N HCl/dioxane) afforded the inhibitor as its hydrochloride salt. As expected, from the substrate specificity of DP-IV, only (S)-amino acid derivatives showed any activity and, as can be seen in **Table I**, lipophilic amino acids gave more potent compounds. In particular, β-branched α-amino acid derivatives were the most potent compounds with the non-proteinogenic amino acid, (S)-cyclohexylglycine providing the most active pyrrolidide (compound **5** possessing a K_i value of 64 nM).

We then applied these findings to a series of 2-cyanopyrrolidides. The preparation of these compounds required a large scale synthesis of 2-cyanopyrrolidine **4** (**Scheme I**). *N*-Boc-2-cyanopyrrolidine was readily prepared from Boc-Pro-NH_2 using a dehydrating mixture of phosphorous oxychloride, pyridine and imidazole but the usual acidic conditions required to remove the Boc protecting group led to decomposition of the 2-cyanopyrrolidide. Employment of the *o*-nitrophenylsulfenyl (ONPS) protecting group[17] however, enabled a very mild deprotection to be used in the final step. Adding three equivalents of 4N HCl/dioxane to **3** in a large volume of diethyl ether afforded the hydrochloride salt **4** as an off-white precipitate in excellent yield.

Scheme I. *Preparation of dipeptide nitriles.*

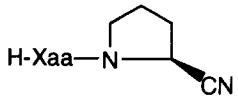


Reagents: **a**.ONPS-Cl, 2N NaOH. **b**. HONSu, Water soluble carbodiimide. **c** conc. NH_4OH, dioxane.
d. imidazole (2 equiv.), $POCl_3$ (4 equiv.), pyridine. **e**. 4N HCl/dioxane (3 equiv.), diethyl ether.
f. Boc-Xaa-OH, pyBop, NEt_3, CH_2Cl_2. **g**. Trifluoroacetic acid.

The series of dipeptide nitriles described in **Table II** were prepared via a pyBop[18] mediated coupling of **4** with the required Boc protected amino acid, followed by deprotection with TFA (**Scheme I**).

We were gratified to find that these compounds were potent inhibitors of DP-IV. The S.A.R. for the N-terminal residue developed in the pyrrolidide series correlated well for the dipeptide nitrile series and the most potent compounds **24**, **25**, **26** and **27** possessed activity comparable to the boroprolines, **1** and **2**. Stability studies[19] revealed excellent half-lives ($t_{½}$) in aqueous solution (pH 7.4) at room temperature (**Table II**) with several examples having $t_{½}$ greater than 48h. Further work on optimisation of the pyrrolidine ring will be reported shortly.

Table II. *Dipeptide nitriles: Potency versus human DP-IV and stability in aqueous solution (pH 7.4).*



Compound N°	Xaa	K_i (nM)[13]	$t_{½}$ (h)[19]
24	Cpg	1.1 ± 0.2	48
25	Chg	1.4 ± 0.5	>48
26	Ile	2.2 ± 0.5	48
27	Tbg	3.8 ± 0.8	>48
28	Lys(Z)	5.2 ± 1.0	24
29	Pro	22.0 ± 4.0	7.5

These compounds were found to be non-toxic in T cell assays up to 72h and inhibitor **26** had no acute toxicity when injected into mice (up to 10mg/Kg). We are currently exploring the effects of these compounds on lymphocytes (*e.g.* proliferation and cytokine release) and further details will be reported in due course.

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13) All compounds were tested in vitro against pure human DP-IV (purchased from M&E, Copenhagen, Denmark). Inhibition was determined using the fluorogenic substrate, H-Ala-Pro-AFC at three concentrations per inhibitor. A typical assay (total volume 0.4 mL) comprised sodium HEPES 83.3 mM, EDTA 1.67 mM, BSA 1.5 mg mL^{-1}, pH 7.8, DP-IV 25 μU mL^{-1}, inhibitor (in 10 mM acetate pH 4.0). The reaction was started by the addition of substrate and readings taken every 30 sec for 7.5 min, excitation at 395 nm, emission 450nm. K_i values were determined using Dixon plots.
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