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4-Substituted boro-proline dipeptides: Synthesis, characterization, and dipeptidyl peptidase IV, 8, and 9 activities

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ABSTRACT

The boroProline-based dipeptidyl boronic acids were among the first DPP-IV inhibitors identified, and remain the most potent known. We introduced various substitutions at the 4-position of the boroProline ring regioselectively and stereoselectively, and incorporated these aminoboronic acids into a series of 4-substituted boroPro-based dipeptides. Among these dipeptidyl boronic acids, Arg-(4*S*)-boroHyp (**4q**) was the most potent inhibitor of DPP-IV, DPP8 and DPP9, while (4S)-Hyp-(4*R*)-boroHyp (**4o**) exhibited the most selectivity for DPP-IV over DPP8 and DPP9.

Dipeptidyl peptidase IV (DPP-IV, E.C. 3.4.14.5), a serine protease that degrades the incretin hormones GLP-1 and GIP, is now a validated target for the treatment of type 2 diabetes. The Xaa-boroPro class of dipeptidyl boronic acid inhibitors was among the first, and remain among the most potent known inhibitors of DPP-IV. Typical K_i values for boroPro-based dipeptides are in the picomolar range, making them 100- to 1000-fold more potent than DPP-IV inhibitors in clinical use or currently known to be in development.[1] Extensive studies have been done using large compound libraries with variations in the P2 position of Xaa-boroPro dipeptides to examine the substrate specificity of DPP-IV.[2] However, as of yet, there are very limited reports of P1 proline modification and their effects on specificity.[3] Although the known Xaa-boroPro dipeptides exhibit excellent inhibition against DPP-IV, they provide very poor selectivity for DPP-IV over DPP8 and DPP9, a potentially crucial property conferring safety to these inhibitors.[4] Thus, continued exploration of modifications of these lead compounds is still necessary. Compounds with substitutions in the 4-position of proline residues, like 4-hydroxyproline (Hyp), play very important roles in the chemistry, structure, physiological properties, and development of amino acid and peptide-based drugs containing such modified amino acid residues. For example, recently a 4-fluoro-2-cyanopyrrolidine dipeptide derivative was reported to exhibit better DPP-IV inhibitory activity than the corresponding compound lacking substitution at the 4-position.[5] In light of the importance of such a substitution, we attempted to introduce substitutions at the 4-position of the boroPro pyrrolidine ring to obtain more potent and selective inhibition with respect to DPP-IV.

Regioselectively and stereoslectively introducing a substituent to the 4-position of boroPro is challenging. Sunose et al.,[6] reported that *N*-Boc-3-hydroxypyrrolidine can undergo a directed C-lithiation at C-5. The resulting dilithiated intermediate can then be trapped by an eletrophile to regioselectively give the 2-substituted-4-hydroxypyrrolidine. This strategy was utilized in the lithiation of *N*-Boc-(*S*)-(+)-3-pyrrolidinol, which was then trapped with an alkyl borate to regioselectively introduce the boronic acid group at the 2-position of the pyrrolidine ring. The (+)-pinanediol protecting group was subsequently introduced as a chiral auxiliary to enable the C-2 diastereomers to be resolved by diastereomeric crystallization, following which **1a** (*cis*-*N*-Boc-L-boroHyp-pn) was obtained as a single, pure diastereomer. This key intermediate allowed for the facile introduction of other substituents at the 4-postion of the boroPro pyrrolidine ring.

As outlined in Scheme 1 the commercially available *N*-Boc-(*S*)-(+)-3-pyrrolidinol was lithiated using *sec*-BuLi (2.2 equiv) in THF–TMEDA at −78 °C and the solution was then allowed to warm to −46 °C for 2 h to complete the lithiation. The reaction mixture was re-cooled to −78 °C and triisopropyl borate was added. The reaction was allowed to warm to room temperature and stirred overnight. After sequential aqueous work-ups with NaOH and then

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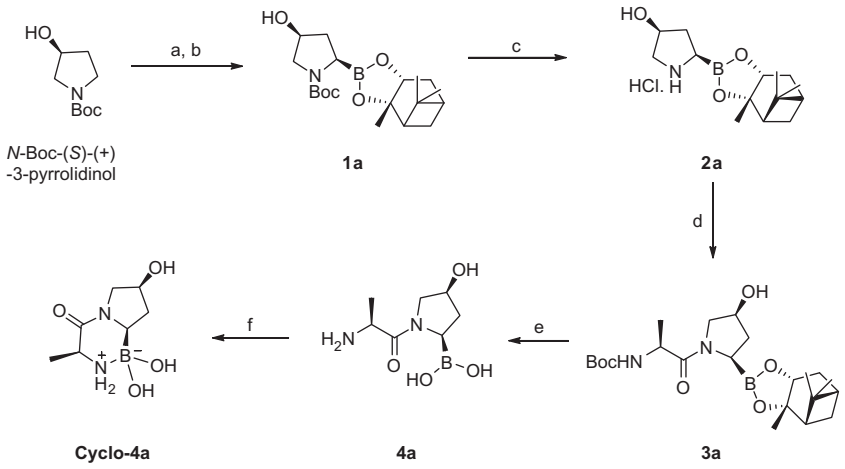


Scheme 1. Reagents and conditions: (a) *s*-BuLi, TMEDA, (*i*-PrO)$_3$B, THF, −78 °C, then NaOH, HCl work-ups; (b) (+)-Pinanediol, Et$_2$O, 35% over two steps; (c) 4 N HCl in dioxane, 98%; (d) L-Boc-Ala-OH, HATU, DIEA, DMF, 94%; (e) BCl_3, CH_2Cl_2, −78 °C, 75%; (f) D_2O, pH 8.8.

HCl[2] the crude boronic acid converted to the (+)-pinanediol ester by addition of 1.0 equiv of (+)-pinanediol in ethyl ether. A mixture of *cis*-2, 4-disubstituted (**1a**) and *trans*-2,4-disubstituted adducts was yielded in a ratio of 7:3, where the desired *cis*-2,4-disubstituted was the majority. Pure, **1a** was separated successfully from the L, D-mixture by crystallization from ethyl acetate in 35% overall yield. Removal of the *tert*-butoxycarbonyl (Boc) group with 4 N HCl in dioxane afforded **2a** as a hydrochloride salt in 98% yield. Compound **2a** was then coupled with *N*-Boc-L-Ala-OH in the presence of HATU and DIPEA to give **3a**, after which the Boc and pinanediol groups were simultaneously removed by treatment with BCl_3 to afford dipeptide boronate **4a** in 70% yield over two steps.

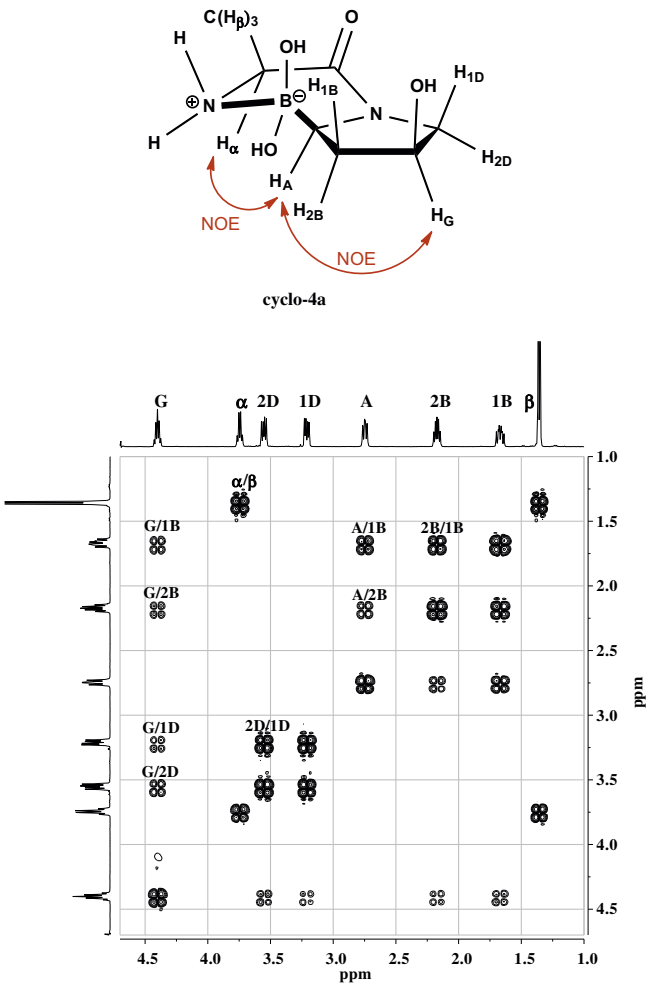


Figure 1. Presaturated ^1H–^1H COSY spectrum of 0.03 M **Cyclo-4a** in D_2O at pH 8.8 and 25 °C.

Compound **4a**, in addition to being the desired target compound, served to facilitate the determination of both the regiochemistry and stereochemistry of the introduced boronate substituent in **1a** and its derivatives. Conversion of **1a** to **4a** and subsequently to the corresponding cyclic species (**Cyclo-4a**) provides a means by which the stereochemistry and regiochemsitry can be unambiguously assigned. Compound **1a** itself is unsuitable for stereochemistry and regiochemistry determination, as the (+)-pinanediol group obfuscates the 2D COSY and NOESY NMR spectra. Furthermore, removal of the Boc and (+)-pinanediol groups results in a compound that provides less clear spectral data for this purpose than **Cyclo-4a**. Compound **4a** has similar difficulties as the deprotected compound, as the same protons would be used for stereochemistry and regiochemistry determination. However, there is an added complication in that compound **4a** has *cis* and *trans* isomers that complicate the spectra. The rigid bicyclic structure of **Cyclo-4a** eliminates the complication of *cis*/*trans* isomerization, and provides added NOEs for stereochemical assignment.

Previous studies have demonstrated that dipeptidyl boronic acids undergo a pH-dependent cyclization through the formation of a dative B→N bond. This cyclic species is favored over the linear species above physiological pH.[7] Compound **4a** was converted to **Cyclo-4a** via incubation in D_2O at pH 8.8. **Cyclo-4a** has a very similar 1D ^1H NMR spectrum with regard to the boroProline ring as cyclo(Xaa-boroPro), and thus aided in our assignments.[8] Stereospecific assignment of all the protons of **Cyclo-4a** is shown in Figure 1 (^1H–^1H COSY spectrum) and Figure 2 (^1H–^1H NOESY spectrum). Cross-peaks between the G proton and each of the 1B, 2B, 1D, and 2D protons were observed in the COSY spectrum; however, there was no cross-peak observed between G and A, clearly indicating that the boronic acid and hydroxyl groups are 2, 4-disubstituted, not 2, 3-disubstituted on the pyrrolidine ring. The stereochemistry of the carbon alpha to the boronic acid group was established as *R* (or *L*) by the presence of cross-peaks between G and A protons as well as between the A and α protons in the ^1H–^1H 2D-NOESY spectrum.

Having synthesized and confirmed the structure of key intermediate compound **1a**, we were then able to introduce other substituents at the 4-position of the boroPro pyrrolidine ring and

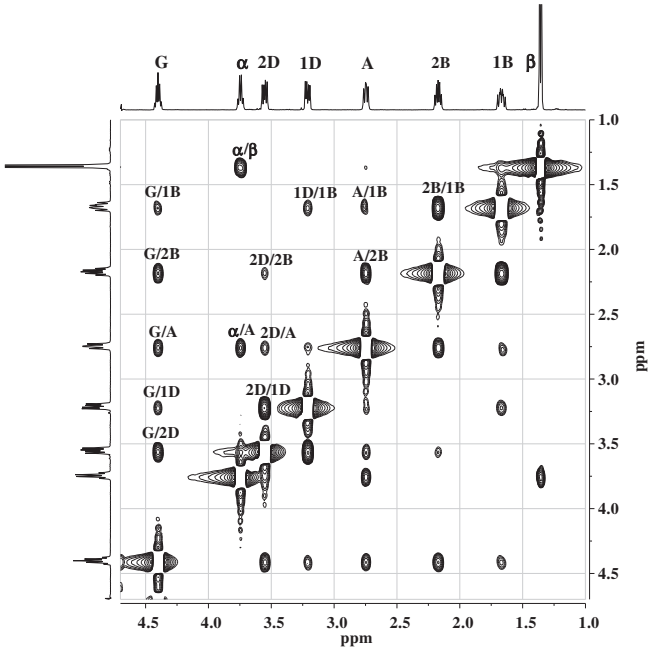
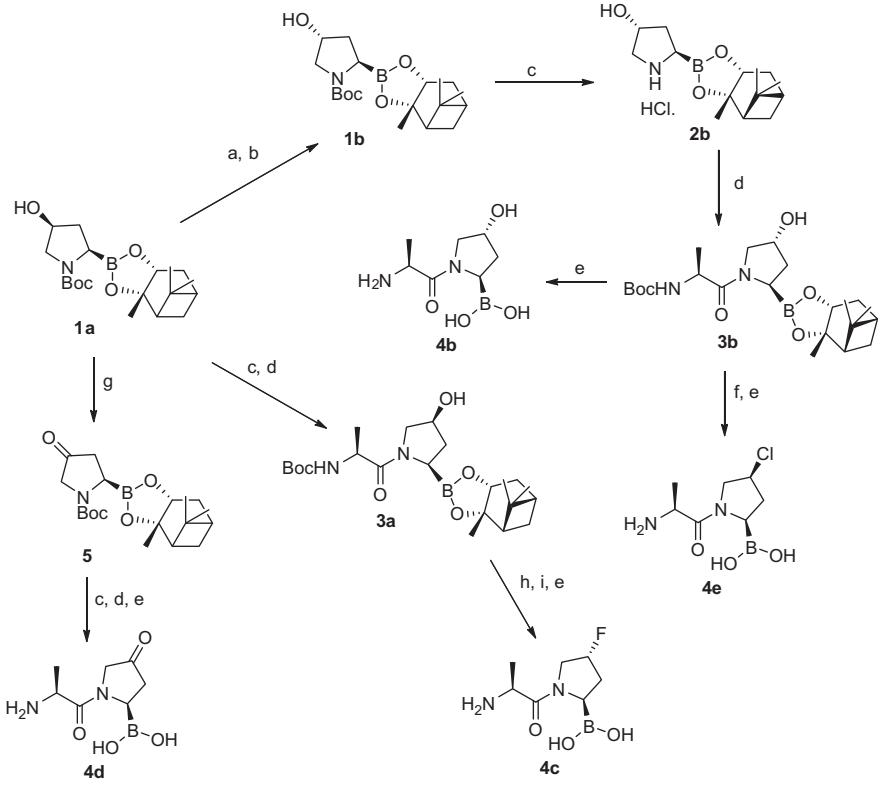


Figure 2. Presaturated ^{1}H–^{1}H NOESY spectrum of 0.03 M **Cyclo-4a** in D_2O at pH 8.8 and 25 °C.

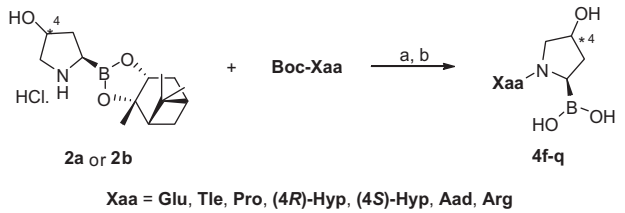
incorporate these novel aminoboronic acids into dipeptide derivatives. The (4*R*)-boroHyp derivative, **1b**, was obtained in 62% yield by inverting the configuration at the C-4 atom of **1a** via the Mitsunobu reaction[9] (Scheme 2). Removal of the *tert*-butoxycarbonyl (Boc) group in **1b** with 4 N HCl in dioxane gave another important intermediate: the *trans*-boroHyp pinanediol ester, **2b**. After coupling with Boc-L-Alanine, followed by the removal of the protecting groups, **4b** was obtained in similar yield as **4a**. The (4*S*)-hydroxyl intermediate (**3a**) was treated with tosyl chloride in pyridine and then refluxed with TBAF in THF to give the (4*R*)-fluoro derivative. This (4*R*)-fluoro compound was then deprotected to afford **4c**[10] in 20% total yield. It should be noted that several other fluorination reagents including KF and DAST were tried, but proved unsuccessful. Swern oxidation worked well in converting (4*S*)-hydroxyl derivative **1a** to **5**, but it failed to convert **3a** or **3b** to their corresponding 4-oxo derivatives enroute to the synthesis of **4d**. The obtained 4-oxo derivative **5** was converted to **4d** by a series of reactions similar to those used in the preparation of **4a** from **1a**. The intermediate (4*R*)-hydroxyl derivative **3b** was converted to (4*S*)-chloride using Ph_3P/CCl_4, which was then deprotected to give **4e**.

Finally, we also varied the P2 residues and coupled these to either (4*S*)-boroHyp or (4*R*)-boroHyp, giving **4f**–**q** (Xaa = Glu, Tle, Pro, (4*R*)-Hyp, (4*S*)-Hyp, Aad and Arg), as outlined by Scheme 3.

IC_{50} values of a series of 4-substituted boro-proline dipeptides against DPP-IV, DPP8 and DPP9[11] were determined to facilitate a further understanding of the structure-activity relationships and selectivity preferences of each of these enzymes, in particular with respect to compounds containing various 4-substituted pyrrolidine P1 aminoboronic acids (Table 1). In contrast to the unsubstituted analogue, Ala-boroPro (**6**)[4], introducing a substituent at position 4 of the boroProline ring generally resulted in a reduction in DPP-IV, DPP8 and DPP9 inhibitory activity, while (4*S*)-hydroxyl (**4a**) and (4*R*)-fluoro derivatives (**4c**) still retained most of their DPP-IV inhibitory activity. The inhibitory activity **4a** (IC_{50}: 2.3 nM) was only fivefold less potent than **6** (IC_{50}: 0.46 nM), while a similar change on the 2-cyanopyrrolidine dipeptide derivative was reported to result in a 250-fold decrease.[5] However, introduction of a keto to the 4-possition (**4d**) indeed greatly decreased the DPP-IV inhibitory activity (IC_{50}: 200 nM; 430-fold less potent than **6**). The stereochemical configuration of the 4-hydroxyl substituent



Scheme 2. Reagents and conditions: (a) DEAD, Ph_3P, *p*-NO_2-$PhCO_2H$, THF, 62%; (b) LiOH, THF–H_2O, 93%; (c) 4 N HCl in dioxane, 95–98%; (d) L-Boc-Ala-OH, HATU, DIEA, DMF, 92–95%; (e) BCl_3, CH_2Cl_2, −78 °C, 70–85%; (f) CCl_4–PPh_3, 72%; (g) $(COCl)_2$, DMSO, NEt_3, CH_2Cl_2, −78 °C to rt, 76%; (h) TsCl, Py, 85%; (i) TBAF, THF, reflux, 35%.

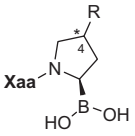


Scheme 3. Reagents and conditions: (a) HATU, DIEA, DMF, 90–95%; (b) BCl_3, CH_2Cl_2, −78 °C, 70–85%.

on the boroHyp residue generally does not significantly affect the DPP-IV inhibitory activity, except in the case of **4a** (4*S*-boroHyp derivative), which demonstrated 10-fold greater potency than **4b** (4*R*-boroHyp derivative). However, this configuration does play an important role in the inhibitory activity of these compounds against DPP8 and DPP9. In general, (4*R*)-boroHyp derivatives were much less potent than the corresponding (4*S*)-boroHyp derivatives against DPP8 and DPP9, thus exhibiting good selectivity for DPP-IV over DPP8 and DPP9. For example, with the Ala-boroHyp compound pair, **4b** ((4*R*)-boroHyp, IC_{50}: 480 nM against DPP8, 125 nM against DPP9) was about 40-fold less potent than **4a** ((4*S*)-boroHyp, IC_{50}: 9.2 nM against DPP8, 3.5 nM against DPP9) for both DPP8 and DPP9. In the case of the Pro-boroHyp and Hyp-boroHyp compound pairs, the 4*R*-boroHyp compounds **4k** (IC_{50}: 110 nM against DPP8, 98 nM against DPP9) and **4m** (IC_{50}: 500 nM against DPP8, 200 nM against DPP9) were about 20-fold less potent than the corresponding 4*S*-boroHyp compounds **4j** (IC_{50}: 5.9 nM against DPP8, 2.1 nM against DPP9) and **4l** (IC_{50}: 27 nM against DPP8, 9.3 nM against DPP9) with respect to DPP8 and DPP9 inhibition.

Of all the compounds evaluated herein, **4o** (IC_{50}: 16 nM against DPP-IV, 530 nM against DPP8, 240 nM against DPP9) exhibited the greatest selectivity for DPP-IV over DPP8 and DPP9 (33-fold and 15-fold, respectively). As expected, variation of the P2 amino acid residue resulted in a wide range of inhibitory activities. The greatest inhibition against each of DPP-IV, DPP8 and DPP9 was observed for **4q** (IC_{50}: 0.3 nM against DPP-IV, 1.2 nM against DPP8, 0.31 nM against DPP9), which had the basic amino acid, arginine, in the P2 position. When the P2 amino acid was Glu (**4f**) or Aad (**4p**)—both containing acidic P2 side-chains, only moderate inhibitory activity was observed (20–120 nM against DPP-IV, DPP8, and DPP9). Increasing the size of the alkyl group of the P2 residue from a

Table 1
Inhibition of dipeptidases by 4-substituted boro-proline dipeptides



Compound	Xaa	R	DPP-IV inhibition IC_{50} (nM)	DPP8 inhibition IC_{50} (nM)	DPP9 inhibition IC_{50} (nM)
4a	Ala	–OH (4*S*)	2.3	9.2	3.5
4b	Ala	–OH (4*R*)	23	480	125
4c	Ala	–F (4*R*)	2.1	23	11
4d	Ala	=O	200	510	180
4e	Ala	–Cl (4*S*)	18μM[a]	27μM[a]	6.9μM[a]
4f	Glu	–OH (4*S*)	29	54	35
4g	Glu	–OH (4*R*)	17	320	86
4h	Tle	–OH (4*S*)	53	17	6.4
4i	Tle	–OH (4*R*)	59	43	14
4j	Pro	–OH (4*S*)	6.2	5.9	2.1
4k	Pro	–OH (4*R*)	6.5	110	98
4l	Hyp(4R)	–OH (4*S*)	20	27	9.3
4m	Hyp(4R)	–OH (4*R*)	48	500	200
4n	Hyp(4S)	–OH (4*S*)	15	19	9.5
4o	Hyp(4S)	–OH (4*R*)	16	530	240
4p	Aad	–OH (4*R*)	17	114	40
4q	Arg	–OH (4*S*)	0.30	1.2	0.31
6	Ala	H	0.46	13	2.8
MK-0431[12]	—	—	6.0[b]	11μM[b]	20μM[b]

[a] The inhibitor stock solutions (1 mg/ml) for enzymatic assays were all prepared at pH 2.0 and equilibrated overnight except **4e** which behaved strangely in the enzyme assays at this pH. The listed data for **4e** was thus obtained at pH 7.8.
[b] This is in-house data (the assay has been conducted under the same conditions as for **4a** etc.).

methyl group (**4a** or **4b**) to the bulkier *tert*-butyl group (**4h** or **4i**) reduced the inhibitory potency against DPP-IV, but this tendency was not consistent with regard to DPP8 and DPP9, as evidenced by the fact that **4i** (IC_{50}: 43 nM against DPP8, 14 nM against DPP9) was more potent against DPP8 and DPP9 than **4b** (IC_{50}: 480 nM against DPP8, 125 nM against DPP9). Replacement of the P1 Pro residue (**4j**, **4k**) with the more polar Hyp residue (**4l**, **4m**, **4n**, **4o**) also slightly decreased the inhibition with respect to DPP-IV, DPP8, and DPP9. However, the configuration of the 4-hydroxy group of Hyp does not appear to drastically affect either the activity or the selectivity of these compounds for DPP-IV, DPP8, and DPP9.

In summary, we have introduced a hydroxyl group regioselectively and stereoselectively into the 4-position of boroProline ring, and obtained c*is*-*N*-Boc-L-BoroHyp-pn (**1a**) as a single, pure diastereomer following crystallization. By converting **4a** to a cyclic species (**Cyclo-4a**) the regiochemistry and stereochemistry of **1a** were then further characterized with 2D NMR. This key intermediate enabled the successful design and synthesis of a series of 4-substituted boroPro, including boroHyp-containing dipeptides. The inhibitory activity of these dipeptides against DPP-IV, DPP8 and DPP9 was determined, and the structure-activity relationships ascertained. Among the compounds examined, Arg-(4*S*)-boroHyp (**4q**) demonstrated the most potent inhibitory activity against DPP-IV, DPP8 and DPP9; while (4*S*)-Hyp-(4*R*)-boroHyp (**4o**) exhibited the greatest selectivity for DPP-IV over DPP8 and DPP9.

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Supplementary data

Supplementary data (experimental procedures and compounds characterization data) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.07.033.

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10. *Characterization data for* ***4c***: ^{1}H NMR (D_2O, 300 MHz) δ 1.46 (d, J = 6.9 Hz, 3H, CH_3CHNH_2), 1.80–2.05 (m, 1H, CH_2CHB), 2.30–2.45 (m, 1H, CH_2CHB), 3.29 (dd, J = 6.6 Hz, 6.6 Hz; 1H, CH_2CHB), 3.60–4.00 (m, 2H, NCH_2CHF), 4.29 (q, J = 6.9 Hz, 1H, H_2NCHCO), 5.47 (d, $^2J_{H-F}$ = 52 Hz, 1H, NCH_2CHF). LC–MS (ESI$^+$) m/z (rel intensity): 373.2, ([2 × (M−H_2O)+H]$^+$, 100); 227.3, ([M+Na]$^+$, 18). HRMS: calcd for $C_7H_{13}BFN_2O_2$, [M−H_2O+H]$^+$, 187.1054, found, 187.1054.
11. Enzymatic assays for human DPP-IV, DPP8 and DPP9 were determined at ambient temperature with the chromogenic substrate Ala-Pro-*p*-nitroanilide hydrochloride salt (Bachem) in 50 mM Phosphate Buffer, pH 7.8. Inhibitor stock solutions (1 mg/ml) were prepared in 0.01 N HCl, pH 2.0. Approximately 1 nM of human DPP-IV (R&D Systems), DPP8, or DPP9 (both supernatant from transfected HEK 293T cells) enzyme was incubated with concentrations of inhibitor ranging from 10^{-4} to 10^{-11} M for 10 min prior to the addition of the substrate (substrate concentration was 25 μm in DPP-IV assay and 60 μM in DPP8/9 assays). The mixture was then incubated for 30 min and measured the absorbance at 410 nM. IC_{50} values were determined by a nonlinear regression fit of the data to a variable-slope sigmoidal dose–response curve using Prism 5.0.4 (Graphad, Inc.).
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