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Probing for improved selectivity with dipeptide-derived inhibitors of dipeptidyl peptidases 8 and 9: the impact of P1-variation.

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Abstract

Selected pyrrolidines, 2-cyanopyrrolidines and heteroaromatic isoindoline analogues were evaluated as P1-residues in dipeptide-derived inhibitors of DPP8/9. Potency testing indicates that DPP8 or DPP9 specificity cannot be obtained with the selected set of P1-and P2-fragments. Nonetheless, the nanomolar DPP8/9 potencies and remarkable selectivities with respect to DPP IV and DPPII, makes inhibitors 4c and 4h suitable "leads" for future inhibitor optimization effort.

Dipeptidyl peptidases 8 and 9 (DPP8, DPP9) are two highly homologous, proline selective serine peptidases. They are closely related to dipeptidyl peptidase IV (DPP IV) and fibroblast activation protein (FAP, FAP-alpha). The two latter enzymes have been extensively investigated as drug targets over the last two decades. As a result, several DPP IV inhibitors have been marketed as diabetes

drugs during recent years.[1] In addition, non-selective inhibitors of FAP have been evaluated clinically in oncology but results of these studies have not been equivocally positive. The recent advent of very selective, orally bioavailable inhibitors might nonetheless spark renewed interest in the evaluation of FAP as a drug target.[2] Next to oncology, several other disease domains with FAP-implication have meanwhile been identified, including fibrosis, arthritis, wound healing and inflammation.[3]

The status of DPP8 and DPP9 as potential drug targets is far less clear. So far, basic research has convincingly linked the enzymes to a number of biological processes, including cancer biology, cell behavior and differentiation, and the immune response. Contributions from De Meester et al., Gorrell et al. and Geiss-Friedlander et al. have recently shed more light on the cell biology of, mainly, DPP9 and have demonstrated its implication in macrophage differentiation, cell adhesion and epidermal growth factor signaling.[4] Detailed understanding of the pathophysiological role of the enzymes and the functional differentiation between DPP8 and DPP9, is nonetheless not within close reach. Contributing to this knowledge void and to the scarcity of reliable translational research results, is the absence of inhibitors that are able to discriminate between DPP 8 and 9. Indeed, both enzymes share a remarkably high sequence homology, amounting to an overall 77% amino acid similarity (aas). The aas further increases to 100% within the enzymes' active sites, rendering discovery of selective DPP8 or DPP9 inhibitors a very challenging task. In addition, both enzymes have intracellular localization and are present simultaneously in most cell types that so far have been investigated.[5] The latter further complicates the interpretation of functional characterization experiments. Finally, it deserves mentioning that inhibition of DPP8/9 has been proposed earlier as the mechanism underlying toxicity of the non-selective DPP8/9 inhibitor *allo*-Ile-isoindoline (compound **1, Figure 1**). Follow-up research however has delivered convincing evidence that this claim is incorrect.[6]

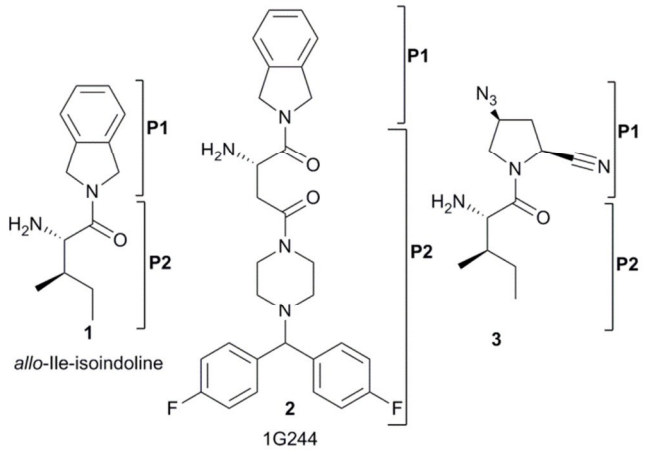


Figure 1: Reference DPP8/9 inhibitors used in this study.

This manuscript furthers on our previous work in DPP8/9 inhibitor discovery and on the effort to discover compounds that are able to discriminate between both enzymes.[5,7-8] So far, Structure-Activity Relationship (SAR) data reported for inhibitors of both enzymes have been generally highly analogous, as illustrated by the assay data for reference compounds **1-3** used in this study. (**Figure 1**, **Table 1**) With respect to this issue, we have earlier reported the possibility to selectively increase DPP8 affinity of analogues of **2** by structural modification of the P2-region.[5] Here, we will report on the possibilities offered by P1-modification of the reference molecules to optimize compound selectivity for one of both enzymes.

Table 1. IC_{50}-data of reference compounds.

IC_{50} (µM)[a]							Ref.
Compound	DPP8	DPP9	DPPIV	DPPII	FAP	SI (DPP8/DPP9)[b]	
1	0.12±0.01	0.29±0.02	90±4	29±1	>100	2.4	[5]
2	0.012 ± 0.001	0.084 ± 0.002	>50	>50	>100	7	[7a]
3	0.011 ± 0.0004	0.0063 ± 0.0003	0.29 ± 0.01	24 ± 2	>100	0.57	[7b]

[a] Values determined using our own assay conditions

[b]SI= Selectivity Index, calculated as IC_{50}(DPP9)/IC_{50}(DPP8)

The choice of P1-residues was governed by SAR-information that we generated earlier: within the evaluated parts of chemical space, we had already confined the range of potentially interesting structures to isoindolines, pyrrolidines and 2-cyanopyrrolidines, eventually decorated with small substituents.[7] Only one promising P1 residue identified during this earlier work ((*2S*,*4S*)-4-azido-2-cyanopyrrolidine, **5**), was retained for this study. (**Figure 2**, "**P1-Building Blocks"**) Next to leading to potent DPP8/9 inhibitors, this fragment had already displayed limited but consistent potential for increased DPP9- over DPP8-selectivity, a feature we wanted to evaluate further. In addition, potentially interesting P1 residues were selected that hitherto had not been evaluated: a series of pyrrolidines with small aliphatic substituents and a set of pyrrolidines annellated with small (hetero-)cycles. Known stereochemical restrictions in the SAR of the 3- or 4-position of the pyrrolidine ring in DPP8/9 inhibitors, led to the selection of stereochemically defined diastereomers **5**, **6** and **15**. Racemates were used for pyrrolidines **8**, **10** and **11**, because the desired enantiomers of these molecules were not readily available (*vide infra*). As the P2 complements of these fragments, the P2-part of 1G244 ("$\mathbf{R^{1}}$"), and/or *allo*-isoleucine ("$\mathbf{R^{2}}$") were chosen. (**Figure 2, "P2-building blocks"**). Selection of these P2-residues was again based on earlier work which had identified them as having the highest potential for general DPP8/9 inhibition. Moreover, given the presence of R^1 and R^2 in references **1-3,** they allow to directly assess the influence on DPP8 or DPP9 selectivity exerted by the different P1-residues.

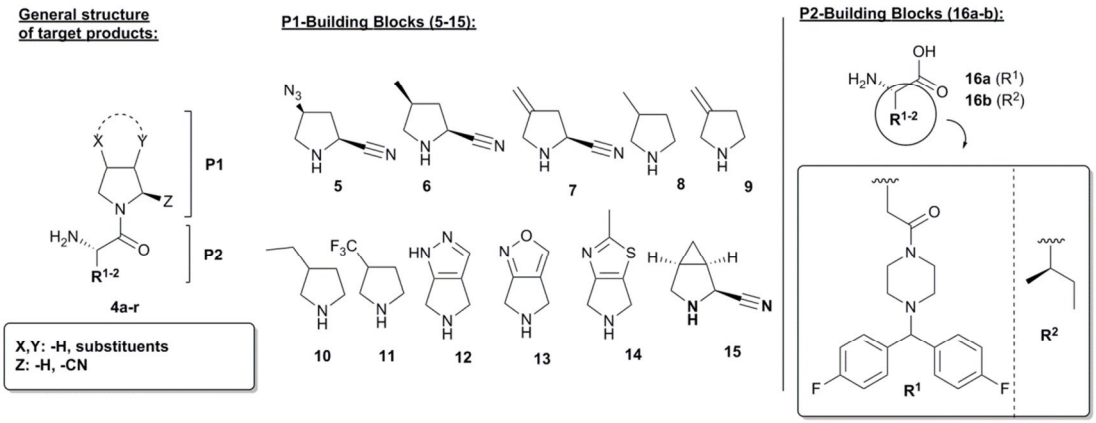
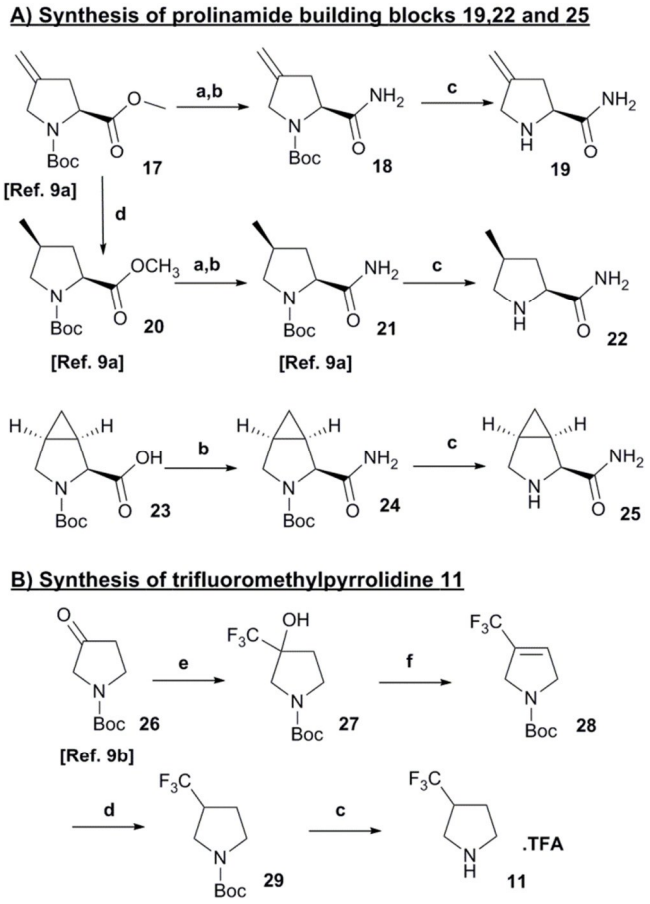


Figure 2: Structure of the target compounds of this study

For the synthesis of most target compounds, P1 building blocks were first prepared separately. Published procedures were used for residues **5, 8-10** and **12**.[7b,9] An identical synthesis to the one we devised for **13**, with comparable yields, was published by Christoffers et al. during the course of this work.[10] For fragments **6** and **7**, the unreported prolinamide precursors **19** and **22** were prepared as critical synthetic intermediates from the corresponding *N*-Boc protected proline derivatives. (**Scheme 1**) For 4-methylene-substituted prolinamide **19**, the required proline derivative **17** was obtained as an intermediate *en route* to **22.** Ester hydrolysis in **17** was followed by amidation and acidolytic cleavage of the Boc-group in **18**. Proline derivative **20**, obtained by hydrogenation of **17**, was similarly transformed into prolinamide **22**. **(Scheme 1, entry A)** The 3,4-methanopyrrolidine-2-carboxamide **25** was obtained using an identical amidation-dehydration sequence from the corresponding, commercially available proline derivative **23**. (**Scheme 1, entry A**) Unreported trifluoromethylpyrrolidine **11** was obtained by applying a synthetic strategy that had been described in the literature for 4-trifluoromethylproline.[11] (**Scheme 1, entry B**) Boc-4-oxopyrrolidine **26** was subjected to nucleophilic trifluoromethylation, rendering intermediate **27**. The latter was then subjected to dehydration and hydrogenation, respectively, to yield **28**. Boc-deprotection of the latter delivered the desired pyrrolidine **11**. Finally, effort to prepare the non-methylated analogue of thiazolopyrrolidine **14** using a Hantzsch protocol identical to the literature procedure used, was unsuccessful.[9d] Synthetic and analytical details for the unreported P1 fragments in **Scheme 1** can be found in the supporting information part of this manuscript.

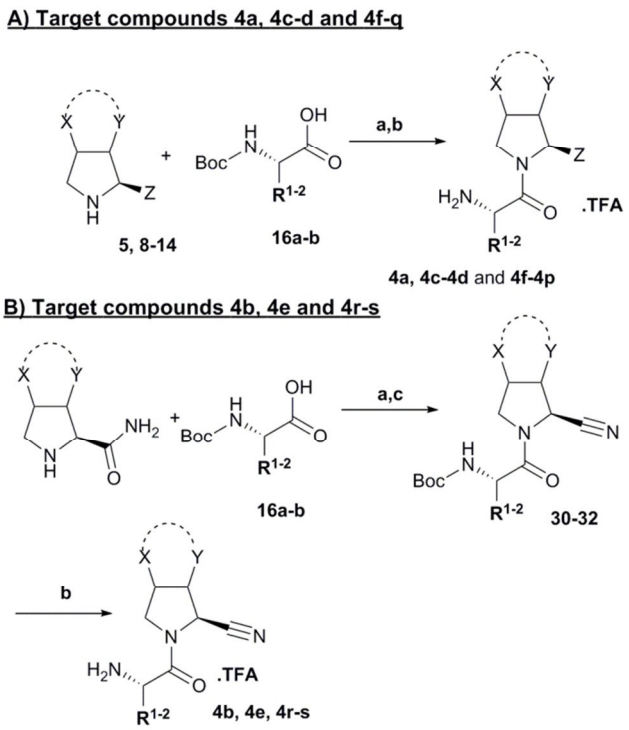
Scheme 1. Synthesis of intermediates 19, 22, 25 and 11.



Reagents and conditions: (a) KOH, CH_3OH/H_2O (b) DCC, *N*-Hydroxysuccinimide, NH_3/CH_3OH, DCM (c) trifluoroacetic acid/DCM (50:50), rt (d) H_2, Pd/C, methanol (e) tetrabutyl ammonium fluoride trihydrate, trimethyl(trifluoromethyl)silane, THF (f) thionyl chloride, pyridine

From here on, final products were synthesized by coupling of P1-building blocks **5, 8-14** and prolinamides **19, 22** and **25** to P2-fragments **16a-b,** using peptide coupling reagents HATU or TBTU. (**Scheme 2**). To obtain target compounds **4a**, **4c-d** and **4f-q,** the corresponding Boc-protected intermediates were treated with TFA. For target compounds **4b**, **4e and 4r**, the coupling products of, respectively, **19**, **22 and 25** were dehydrated to install the carbonitrile functions of intermediates **29-32**. The latter were subsequently Boc-deptrotected using TFA to yield **4b** and **4e**. All final products were then evaluated biochemically.

Scheme 2: General Inhibitor assembly strategy.

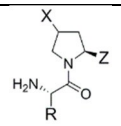
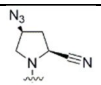
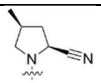
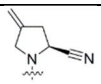


Reagents and conditions: (a) HATU, DIPEA, DCM, rt, 8 h or TBTU, DIPEA, DCM, rt, 8 h (b) trifluoroacetic acid/DCM (50:50) rt (c) $POCl_3$, pyridine, DCM

All compounds were investigated for their potential to inhibit DPP8, DPP9, DPP IV and DPPII. Based on SAR data for dipeptide-based FAP-inhibitors and the presence of a basic P2-amine function, the target compounds can be anticipated to lack appreciable FAP-affinity.[2] This is also illustrated by the assay data for references **1-3,** and explains why this enzyme is not part of the evaluation panel. Potencies of the mono-substituted pyrrolidine- and 2-cyanopyrrolidine-based compounds (**4a-j**) are shown in **Table 2**. As indicated by their DPP8/9-Selectivity Indices (SIs), all representatives of this series still have grossly comparable affinities for both enzymes, and only **4h** has the same limited DPP8 preference as reference **2** (1G244). Nonetheless, it deserves mentioning that the nanomolar dual DPP8/9-inhibory potency of 1G244 analogues **4a-c**, **4e** and **4h** implies potential to select these

compounds as "leads" for further optimization effort, e.g. via modification of their P2 residues. With respect to the 2-cyanopyridines (**4a**-**4b** and **4e**) in this set, it is remarkable that the presence of a carbonitrile warhead does not lead to a significant potency increase (typically 1-2 orders of magnitude for the enzymes in the panel) compared to their closest analogues that do not contain this functional group. This is apparent when comparing **4b** with its pyrrolidine analogue **4c** and carbonitrile **4e** with the corresponding pyrrolidine **4f**. A similar trend is also present for **4a** and reference **3.** The direct azidopyrrolidine analogue of **4a** was not included in this series: this P1-residue has been investigated by us in the past and was identified as less promising.[7b] A similar lack of contribution to affinity by the carbonitrile warhead in the (2-cyano)isoindoline analogue of 1G244 has also been reported by us.[5] Although several factors might contribute to this recurrent finding, it is very likely that the sterically demanding P2 residue of 1G244 either directly or by induced-fit interactions, precludes a correct orientation for covalent bond formation with DPP8/DPP9's catalytic serine-OH group. Noteworthy, DPP IV- and DPPII-potencies do not seem to be affected to the same extent: in the case of the analogue pairs **4b/4c** and **4e/4f**, the warhead does seem to have a significant affinity-increasing impact for these enzymes. This consideration could be of importance for "lead" prioritization of these and other DPP8/9 inhibitors, since the choice for a warhead-labeled P1 residue can clearly impact on the selectivity toward DPPIV and DPPII. The data in **Table 2** also indicate that for P1-residues lacking a warhead functionality, coupling to 1G244's P2- residue generally leads to higher DPP8/9-potencies and selectivities (with respect to DPPIV and DPPII) than an an *allo*-isoleucyl P2-residue. This trend is visible for inhibitor pairs **4c/4d**, **4f/4g** and **4h/4i**. The same observation had already been made earlier with isoindoline references **1** and **2**. Nonetheless, older SAR data generated by us and inhibitor pair **3**/**4a** also suggest that an *allo*-Ile, or similarly less bulky P2 residue, might be better compatible with a carbonitrile or diaryl phopshonate warhead strategy. Taking all this information together, within this series the methyl- and ethyl-substituted 1G244 analogues **4c** and **4h** seem to have the highest potential for future inhibitor discovery effort. Both compounds were also found to lack appreciable FAP affinity (IC_{50} > 100 μM).

Table 2. Biochemical evaluation results for inhibitors **4a**-**4j** with a substituted pyrrolidine P1-residue.

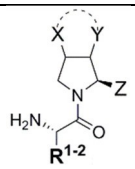
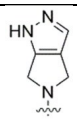
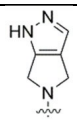
							
			IC50 (μM)				
Compound	P1	R	DPP8	DPP9	DPPIV	DPPII	SI (DPP8/DPP9)[a]
4a		R^1	0.08±0.01	0.23±0.01	>100	>25	2.9
4b		R^1	0.017±0.005	0.012±0.004	2.6±0.1	1.1±0.06	0.7
4c		R^1	0.037±0.001	0.17±0.01	19.4±1.1	23±2	4.6
4d		R^2	1.7±0.1	4.7±0.1	16.2±2.0	12.9±0.5	2.8
4e		R^1	0.119±0.007	0.083±0.003	0.68±0.05	0.025±0.001	0.7
4f		R^1	0.113±0.005	0.41±0.03	34±2	>100	3.6
4g		R^2	7.09±0.57	16±0.5	>50	>100	2.3
4h		R^1	0.050±0.001	0.36±0.02	>100	40.0±3.5	7.2
4i		R^2	1.98±0.07	5.7±5.0	>100	14.8±1.4	2.9
4j		R^1	0.55±0.01	3.5±0.3	>100	10.9±0.5	6.4

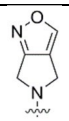
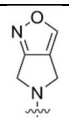
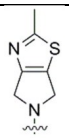
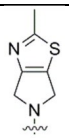
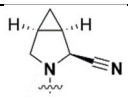
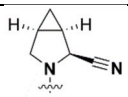
[a]SI= Selectivity Index, calculated as IC_{50}(DPP9)/IC_{50}(DPP8)

Table 3 groups inhibitors with a bicyclic P1 residue. Compounds **4k**-**4p** can be envisaged as heteroaromatic analogues of isoindoline based references **1** and **2**, while the methanopyrrolidine-based **4q**-**4r** can be regarded as a conformationally constrained counterpart of **4b**. For the first

subset, it is clear that any of the investigated P1-residues leads to a drastic loss of affinity for DPP8/9 compared to **1** and **2**. Given the comparable spatial dimensions of these P1-fragments and isoindoline, sterical factors are less likely to be held accountable for this effect. Contributing factors are more likely to be identified in the electron density distribution of the heteroaromatic systems compared to benzene. Nonetheless, mainly the isoxazole-based compounds **4m** and **4n** still lead to appreciable DPP8/9 inhibition, albeit with a slightly less pronounced selectivity toward DPPIV and DPPII than reference 1G244 (**2**). In addition, the low micromolar DPP8-potency of thiazole-based **4o** might suggest that it could be worthwhile to evaluate the corresponding desmethyl-analog as a P1 residue. As mentioned however, the significant effort that has already been put in the synthetic preparation of this residue, so far has not been successful. Finally, the methanopyrrolidine-based P1-fragment of **4q-r** does seem to have potential to deliver compounds with significant DPP8/9 affinities. The grossly comparable propensity of the methanopyrrolidine part to bind to DPP IV's active center, makes this a slightly less interesting candidate for future lead-optimization programs.

Table 3. Biochemical evaluation data for inhibitors **4k-4r** with a bicyclic P1 residue.

							
			IC_{50} (μM)				
Compound	P1	R	DPP8	DPP9	DPPIV	DPPII	SI (DPP8/DPP9)[a]
4k		R^1	>100	>25	>100	>100	n.a.[b]
4l		R^2	>100	>100	>100	>100	n.a.

4m		R^1	0.37±0.02	2.4±0.3	>100	31±3	6.5
4n		R^2	10.0±0.4	42±2	56±1	9.4±0.4	4.2
4o		R^1	3.7±0.2	30±4	>100	>100	8.1
4p		R^2	>25	>100	>100	>100	n.a.
4q		R^1	0.036±0.001	0.0218±0.0006	0.79±0.04	22±3	0.6
4r		R^2	4.7±0.4	2.84±0.07	4.0±0.2	>100	0.6

[a]SI= Selectivity Index, calculated as IC_{50}(DPP9)/IC_{50}(DPP8)

[b]n.a.= not applicable.

Conclusions

Based on earlier SAR-results, a number of substituted pyrrolidines, 2-cyanopyrrolidines and heteroaromatic isoindoline analogues were selected and synthesized as P1-building blocks of DPP8/9 inhibitors. These were linked to known DPP8/9-affinity conferring P2 residues: either the P2-part of 1G244 or an *allo*-isoleucyl fragment. The biochemical data of the resulting dipeptide-derived inhibitors indicate that DPP8 or DPP9 specificity cannot be obtained using the selected set of P1-and P2-fragments. Nonetheless, **4c** and **4h** were found to combine nanomolar DPP8/9 potencies and remarkable selectivities with respect to DPP IV and DPPII. In additional assays that were carried out, both compounds were also found to lack appreciable FAP-affinity (IC_{50}> 100 µM). All these features make **4c** and **4h** suitable for future inhibitor optimization effort. Such effort could consist of pursuing

DPP8 or DPP9 specificity via modification of their P2 part, similar to a strategy that we explored earlier.[5] Ideally, further research would greatly benefit from the availability of experimental high-resolution structures for DPP8 and DPP9. To date, only homology model structures have been published for both enzymes.[12] While, as presented in the Supporting Information part of the manuscript, **4c** and **4h** can indeed be docked in a satisfactory manner into these homology models, the models' predictive value for selective inhibitor discovery remains to be demonstrated. Finally, the obtained results warn against inconsiderate use of warhead functionalities in DPP8/9 inhibitors, since they can have the potential to reduce selectivity against DPP IV and DPPII.

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Electronic Supporting Information

Electronic Supplementary Information (ESI) available: experimental details for unreported P1-residues, analytical data of final products and enzymatic assay protocols.

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