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Structure−Activity Relationship Studies on Isoindoline Inhibitors of Dipeptidyl Peptidases 8 and 9 (DPP8, DPP9): Is DPP8-Selectivity an Attainable Goal?

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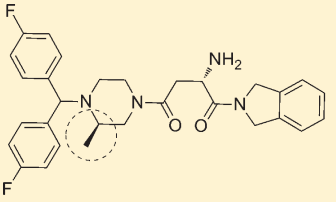
Supporting Information

ABSTRACT: This work represents the first directed study to identify modification points in the topology of a representative DPP8/9-inhibitor, capable of rendering selectivity for DPP8 over DPP9. The availability of a DPP8-selective compound would be highly instrumental for studying and untwining the biological roles of DPP8 and DPP9 and for the disambiguation of biological effects of nonselective DPP-inhibitors that have mainly been ascribed to blocking of DPPIV's action. The cell-permeable DPP8/9-inhibitor **7** was selected as a lead and dissected into several substructures that were modified separately for evaluating their potential to contribute to selectivity. The obtained results, together with earlier work from our group, clearly narrow down the most probable DPP8-selectivity imparting modification points in DPP8/9 inhibitors to parts of space that are topologically equivalent to the piperazine ring system in **7**. This information can be considered of high value for future design of compounds with maximal DPP8 selectivity.



IC_{50}(DPP8)= 0.05 ± 0.005 μM
IC_{50}(DPP9)= 0.54 ± 0.04 μM
IC_{50}(DPP IV, DPP II) > 25 μM

INTRODUCTION

In recent years, several serine proteases belonging to clan SC have gained pharmaceutical interest in the areas of immunology, metabolic disorders, and cancer.[1,2] The most widely investigated protein in this class of enzymes is dipeptidyl peptidase IV (DPPIV/CD26). Other members of the so-called DPPIV activity and/or structure-homologues (DASH) family are DPPII, DPP8, DPP9, fibroblast activating protein (FAP), and prolyl oligopeptidase (POP/PREP).[3−6] These prolyl specific peptidases are unique among the proteases because they are able to cleave a peptide bond flanked by a proline residue. Because of the specific recognition of the pyrrolidine ring, these serine proteases are responsible for the cleavage of what is accepted to be a relatively resistant peptide bond, giving unique biochemical properties. The biological importance of the prolyl peptidases is underscored by the fact that many bioactive peptides, i.e. hormones, chemokines, and neuropeptides, contain one or more proline residues and the processing and degradation of such biologically important regulatory peptides requires prolyl specific peptidases.[7] Despite their enzymatic and structural similarity, the DASH proteins are most likely to have distinct physiological functions, as indicated by their difference in expression and localization.

Since the discovery of DPPIV in the late 1960s, more than 30 pharmaceutical and biotechnology companies have started up development projects for DPPIV inhibitors as therapeutics for the treatment of type 2 diabetes.[8] By prolonging the lifetime of the incretin hormones glucagon-like-peptide 1 (GLP-1) and glucose dependent insulinotropic peptide (GIP), DPPIV inhibitors are able to stimulate postprandial insulin production and blood glucose clearance.[9] Up until now sitagliptin **1**, vildagliptin **2**, and saxagliptin **3** have all gained regulatory approval, while others have successfully advanced into late stage clinical trials and several other "gliptins" are still under investigation.[10−14] (Figure 1, Table 1) Besides these incretin enhancers, other potential therapies comprise GLP-1 receptor agonists or incretin mimetics, i.e. long acting GLP-1 analogues.[15−17] Besides the important function as incretin regulating protein, the ubiquitously expressed DPPIV is under investigation for its role in cell biological processes and immune function. This is extensively reviewed in recent literature reports.[2,3,18−20]

Fibroblast activation protein (FAP), a 95 kD membrane-bound glycoprotein, has both endopeptidase and exopeptidase

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Table 1. Potency and Selectivity of Reference Compounds

		IC_{50}[a] (μM)				
compd	name	DPP8	DPP9	DPPIV	DPPII	reference
1	sitagliptin	>50	>100	0.04 ± 0.001	>100	*b*
2	vildagliptin	9.0 ± 0.1	0.68 ± 0.07	0.12 ± 0.01	>1000	*b*
3	saxagliptin			0.60 ± 0.06 nM	*c*	12
4	UAMC-00039	142 ± 27	78.6 ± 9.1	165 ± 9	0.48 ± 0.04 nM	25
5	UAMC-00141	0.53 ± 0.11	1.57 ± 0.07	>500	>1000	45
6	UAMC-00491	0.160 ± 0.016	0.07 ± 0.04	>100	21.5 ± 3.2	43
7		0.014	0.053	>100	>100	46

[a] Values expressed in μM unless stated otherwise. [b] *In house* data. [c] **3** did not show any significant inhibition of DPPII at concentrations up to 30 μM.

activity and is a member of the S9B family most closely related to DPPIV. In general, FAP is absent in the adult tissues. It is expressed in pathologies comprising tissue remodelling, such as tumors and liver disease.[20] Research on small molecule inhibitors of FAP is currently ongoing in our group as well as in other laboratories.[21]

Dipeptidyl peptidase II (DPPII, QPP) is assigned to family S28 of class SC. Despite the numerous reports with suggestions on possible functions of the enzyme, the physiological role of this serine protease still has to be elucidated.[22] To unravel the physiological role of the latter, our group developed the most potent and specific hitherto described, orally bioavailable DPPII inhibitor **4**.[23−25]

During the past decade, DPP8 and DPP9 have been discovered as two members of the prolyl oligopeptidase S9b subfamily, which also contains DPPIV and FAP. Compared to the two latter extensively studied enzymes, relatively little is known about DPP8 and DPP9.[26] Neither the tertiary structures nor in vivo substrates or the physiological role of these two enzymes have been firmly established. The open reading frame (ORF) of DPP8 codes for two splice variants of 882 or 898 amino acids. DPP8 shares 27% amino acid (aa) identity and 51% aa similarity with the protein sequences of DPPIV, and this increases to 35% aa identity and 57% aa similarity in the hydrolase domain.[27] Unlike DPPIV and FAP, DPP8 is a soluble protein localized in the cytoplasm.[28] DPP9 was identified in silico by Abbott et al. and was first cloned and expressed recombinantly by Olsen and Wagtmann.[26,28] Recently, natural DPP9 was purified and identified from bovine testes.[29,30] Similar to DPP8, DPP9 is also ubiquitously expressed as two variants comprising the short 863 aa version and the longer 892 aa version. Whether both forms are biologically active remains controversial.[30−32] DPP8 shares 57% aa identity and 77% aa similarity with DPP9, and this increases to more than 90% and 100% respectively within the active site, following an in-house homology modeling study. This study is based on the X-ray structures of DPPIV (pdb code 3KWJ), FAP (pdb code 1Z68), and PREP (pdb code 1H2W), and on the homology models of DPP8 and DPP9, constructed by Rummey et al.[33] The depicted sequence alignment clearly shows that in DPP8 and DPP9 almost identical amino acid residues make up the active sites: only at positions 174^{DPP8} and 433^{DPP8} a difference is seen. However, this alteration is moderate with possibly little overall effect, as the basic aa Lys at position 174 in DPP8 is replaced by the analogous amino acid residue Arg in DPP9. Similarly, the hydrophobic residue Ile at position 433 in DPP8 is replaced by Val^{DPP9}, also a hydrophobic amino acid.

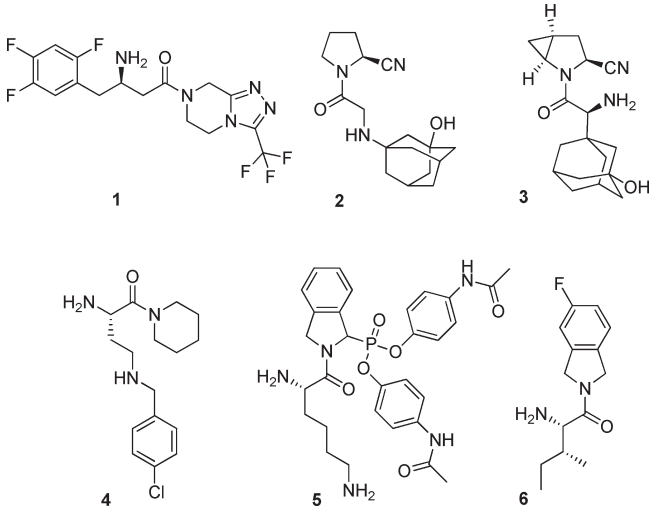

Figure 1. DPPIV inhibitors **1**, **2**, and **3**, DPPII inhibitor **4**, irreversible DPP8/9 inhibitor **5**, and reversible DPP8/9 inhibitor **6**.

The distribution of the cytosolic enzymes, DPP8 and DPP9, and their expression in different types of endothelial cells is summarized by Yu et al.[34] Because of the recent insight that DPP8 and DPP9 have a similar enzymatic activity profile as DPPIV, it is very likely that some physiological functions attributed to the latter could in fact be credited to DPP8 and/or DPP9.[19,35] Furthermore, because studies on the involvement of DPPIV in various pathologies such as inflammatory bowel disease, rheumatoid arthritis, and multiple sclerosis were performed with nonselective DPPIV inhibitors, it is suspected that DASH proteins other than DPPIV could be responsible for this effect.[19] Recent literature reports show DPP8/9 activity in human leukocytes as well as in B and T cell lines and in endothelial cells.[35,36] Moreover, several cytokines, e.g. SDF1, ITAC, and IP-10, are demonstrated to be in vitro substrates for DPP8/9.[32] In addition, DPP9 appears to be a rate-limiting enzyme for degradation of $RU1_{34-42}$, a proline containing antigenic peptide.[37] These findings suggest a potential role for DPP8/9 in the immune system. Furthermore, these enzymes may be involved in other physiological processes, such as spermatogenesis, because bovine testes appear to be a source of DPP9 and DPP8 could be detected in spermatogonia and spermatids.[28−30,38] Previously, attention was drawn to DPP8 and DPP9, following research results reported by Lankas et al., suggesting that the use of the DPP8/9 inhibitor *allo*-Ile-isoindoline is associated with severe toxicity in animal

Enzyme	Residue									
DPPIV	Arg125	Trp201	Glu204	Glu205	Glu206	Val207	Phe208	Ser209	Asp302	Arg356
FAP	Arg123	Trp199	Glu202	Glu203	Glu204	Met205	Leu206	Ala207	Trp295	Gly349
DPP8	***Lys174***	Phe255	Gln258	Glu259	Glu260	Phe261	Asp262		Arg364	***Ile433***
DPP9	***Arg163***	Phe244	Gln247	Glu248	Glu249	Phe250	Asp251		Arg354	***Val423***
PREP	Arg643			Phe 173		Met235				

DPPIV	Phe357	Arg358	Pro359	Tyr547	Gly549	Pro550	Cys551	Ser552	Leu598	Ser630
FAP	Phe350	Phe351	Val352	Tyr541	Gly543	Pro544	Cys545	Ser546	Leu592	Ser624
DPP8	His434	Asp435	Ile436	Tyr653	Gly655	Pro656	Gln657	Val658	Met706	Ser739
DPP9	His424	Asp425	Ile426	Tyr644	Gly646	Pro647	Gln648	Val649	Met697	Ser730
PREP		Arg252		Tyr473						Ser554

DPPIV	Tyr631	Val656	Trp659	Tyr662	Asp663	Tyr666	Arg669	Asp708	Asn710	Val711	His740
FAP	Tyr625	Val650	Trp653	Tyr656	Ala657	Tyr660	Arg663	Asp702	Asn704	Val705	His734
DPP8	Tyr740	Val765	Trp768	Tyr771	Asp772	Tyr775	Arg778	Asp817	Asn819	Val820	His849
DPP9	Tyr731	Val756	Trp758	Tyr762	Asp763	Tyr766	Arg769	Asp808	Asn810	Val811	His740
PREP	Asn555	Val580	Tyr599	Trp595	Ile591	Ala594	Cys255	Asp641	Arg643	Val644	His680

Figure 2. Protein sequence comparison of the active sites of DPPIV, FAP, PREP, DPP8, and DPP9. After alignment and superposition of the proteins amino acid sequences, residues were selected within 4.5 Å from a docked inhibitor. Differences in the secondary structure between DPP8 and DPP9 are highlighted in italic and bold.

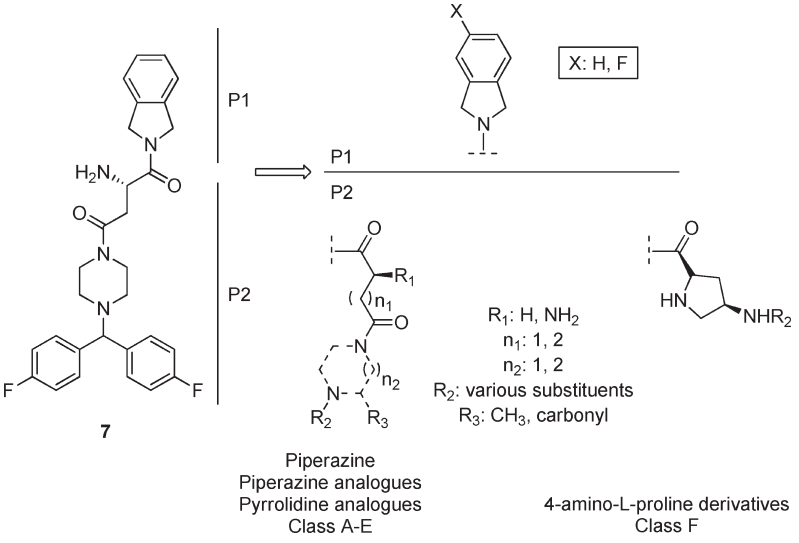

Figure 3. Lead compound **7** and structural modifications investigated in this study.

models.[39] Whether the observed toxicity was DPP8/DPP9 related, or solely due to compound mediated off-target effects, remains to be unambiguously established.[40] Nevertheless, several groups provide sufficient evidence to question the alleged toxicity caused by DPP8/9 inhibition.[41,42]

As depicted in Figure 2, the high degree of sequence homology between DPP8 and DPP9 on one side and DPPIV and FAP on the other makes even dual DPP8/9 inhibitor design a challenging task that hitherto has been the subject of only a limited number of publications, several of which have come from our groups.[29,43−47] Strong inhibition of DPP8 was reported by us using several irreversible, in-house synthesized, isoindolin-1-yl phosphonate analogues of **5**.[45] However, after our reported successful investigations to force access to highly purified bovine DPP9, the compounds turned out to show concomitant affinity for this enzyme.[29] Subsequently, we investigated an extended group of pyrrolidine analogues and a focused series of substituted isoindolines and azaheteroaromatic analogues as P1 fragments in DPP8/9 inhibitors. From this series, we selected isoindoline and 5-fluoroisoindoline as fragments that offer an optimal balance of potency and selectivity for DPP8/9 with respect to DPP IV and DPP II (e.g., **6**).[43] Additionally, this study indicated the limited potential of modifying the P1 position for obtaining compounds able to discriminate between DPP8 and DPP9. Furthermore, our previously described (2*S*,4*S*)-4-azido-2-cyanopyrrolidines yielded nanomolar DPP8/9 inhibitors with modest overall selectivity toward DPPIV and DPPII.[44] Thus far, neither our strategies, nor the attempts of other groups, resulted in compounds with either only DPP8 or only DPP9 affinity. To establish the biological functions and identify the in vivo substrates of the enzymes, the availability of at least a highly selective inhibitor of DPP8 (or a highly selective inhibitor of DPP9) is very desirable.

The principal objective of this work was to establish topological insight in a representative DPP8/9 inhibitor structure for identifying positions in its molecular framework that, via chemical modification, allow improvement of DPP8 selectivity. As the model inhibitor in this study, compound **7** (1G244) was selected

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and synthesized by Jiaang et al.[46] (Figure 3) This lead compound, already displaying some selectivity for DPP8, possesses the overall dipeptide-derived structure of all known DPP8/9 inhibitors, is the most active DPP8/9 inhibitor described to date and readily penetrates plasma membranes.[42] In addition, compared to the dimensions of several other reported DPP8/9 inhibitors, **7** is a large molecule that can be expected to probe a proportionally larger part of DPP8's and DPP9's active site, hence offering higher probability for detecting differences between both. As the starting point of our study, compound **7** was dissected in four substructures that conceptually can each be envisaged as modifiable modules: (1) the P1-isoindoline, (2) the P2 Asp-residue, (3) the piperazine ring system, and (4) the bis(4-fluorophenyl)-methyl substituent. In all molecules prepared under this study, however, isoindoline or 5-fluoroisoindoline were incorporated at the P1 position. As explained earlier, these two residues were selected from a large number of P1 candidates based on earlier work by our group that among other things also indicated that the P1 position is not an obvious modification point for imparting DPP8 (or DPP9) selectivity. Structural modifications of the P2 position investigated included homologation and deletion of the free amino function (Figure 3). As for the piperazine module, this was altered into (i) acyclic aminoalkyl chains, (ii) substituted piperazines, and (iii) piperazine analogues like homopiperazine and 3-aminopyrrolidine. Finally, a series of both alkyl- and acyl-replacements of comparable bulk size or smaller were selected for the bis(4-fluorophenyl)methyl group in **7**. In the following parts, we will describe the synthesis, the obtained biochemical evaluating data, and the derived SAR for the prepared analogues of **7**. For clarity's sake, the different compounds prepared are grouped into six classes (A–F) as summarized in Figure 4.

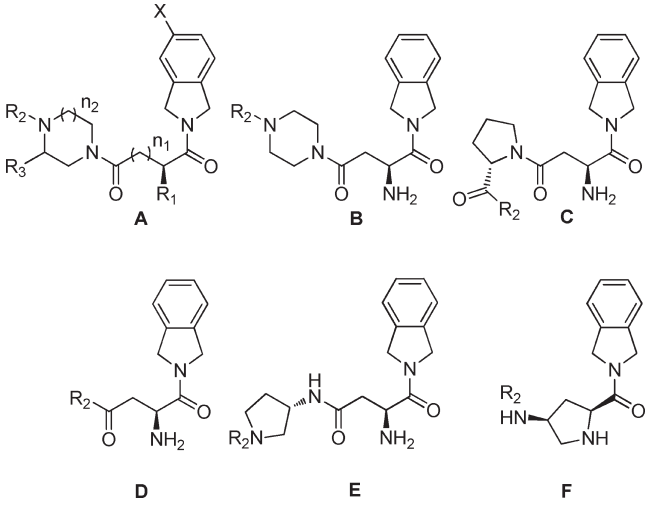


Figure 4. Overview of compounds divided in six classes. (A) Alkylated piperazine analogues **7** and **12**(**a**–**o**); (B) acylated piperazine analogues **12**(**q**–**s**); (C) L-proline derivatives **12**(**t**–**u**); (D) derivatized aspartic acid compounds **12**(**v**–**y**); (E) 3-aminopyrrolidine derivative **12z**; (F) 4-amino-L-proline derivatives **16**(**a**–**b**).

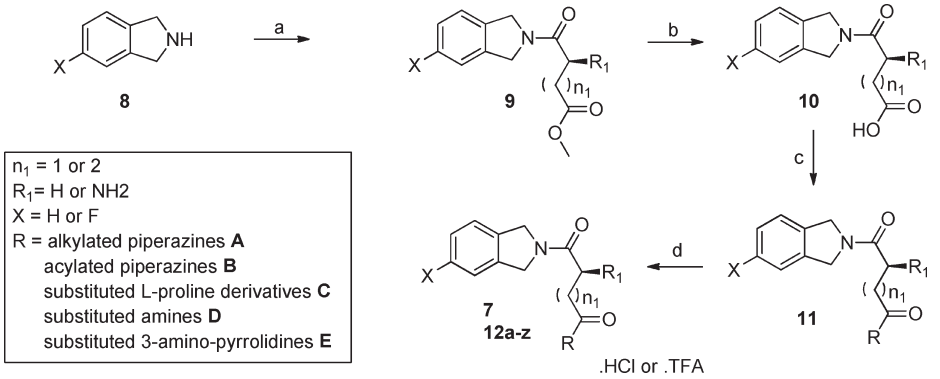
CHEMISTRY

Target compounds from class A–F were prepared using the following synthetic strategy (Scheme 1) and are listed in Table 2. Starting from isoindoline (X = H) or 5-fluoroisoindoline (X = F), which was synthesized as reported previously,[43] these favorable P1 L-proline surrogates were coupled with succinyl acid 4-methyl ester, *N*-Boc-aspartic acid 4-methyl ester, or *N*-Boc-glutamic acid 4-methyl ester, yielding succinyl (R_1 = H and n_1 = 1), *N*-Boc protected glutamylisoindoline (R_1 = NH-Boc and n_1 = 2), or *N*-Boc protected aspartylisoindoline (R_1 = NH-Boc and n_1 = 1) derivatives, respectively. Standard peptide coupling procedures were employed. Subsequent hydrolysis with potassium hydroxide in methanol proceeded smoothly to give the aminoacylisoindoline derivatives ready for peptide coupling with a diverse set of primary and secondary amines. Final deprotection with trifluoroacetic acid (TFA) or hydrochloric acid (HCl) yielded the final compounds.

For the preparation of the piperazine analogues, mainly five synthetic strategies were followed. First, the piperazine moieties from target compounds **7** and **12**(**a**–**o**) were bought as such or were synthesized starting from commercially available piperazine analogues, which were first *N*-Boc protected using Boc-anhydride and Et_3N. These intermediates were employed as nucleophiles in an S_N2 reaction on different alkyl halides using Et_3N as base in dimethylformamide at 70 °C. Subsequently, this alkylation was followed by acidolytic cleavage of the Boc functionality with TFA in dichloromethane (DCM), furnishing the corresponding alkylated piperazines.

In a second strategy, two different approaches to form the acylated piperazine analogues **12**(**p**–**s**) were selected. The first approach started with mono-Boc-piperazine, which was coupled to different commercially available carboxylic acids, for example,

Scheme 1. General Synthesis of DPP8/9 Inhibitors[a]



[a] Reagents: (a) TBTU, Et_3N, aa derivative, CH_2Cl_2; (b) KOH, MeOH; (c) TBTU, Et_3N, various amines, CH_2Cl_2 ; (d) TFA, CH_2Cl_2 or HCl in dioxane.

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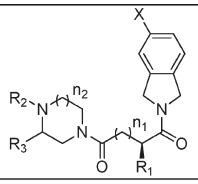
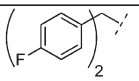
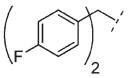
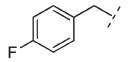
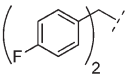
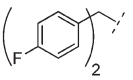
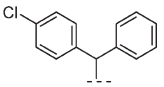
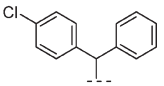
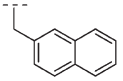
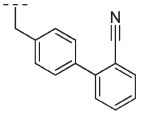
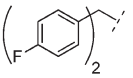
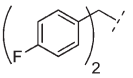
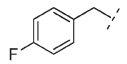
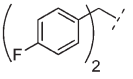
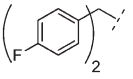
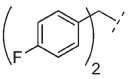
Table 2. Structural Characteristics of Isoindoline Compounds

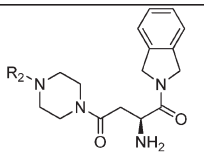
Class A

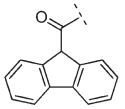
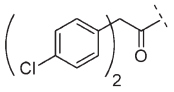
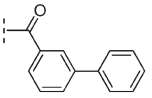
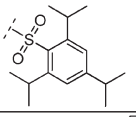


Compound	X	R_1	R_2	R_3	n_1	n_2
7	H	NH_2		H	1	1
12a	H	NH_2		H	2	1
12b	H	NH_2		H	2	1
12c	H	H		H	1	1
12d	5-F	NH_2		H	1	1
12e	5-F	NH_2		H	1	1
12f	H	NH_2		H	1	1
12g	H	NH_2		H	1	1
12h	H	NH_2		H	1	1
12i	H	NH_2		H	1	1
12j	H	NH_2		H	1	2
12k†	H	NH_2		H	1	1
12l	H	NH_2		H	1	1
12m	H	NH_2		(*S*)CH_3	1	1
12n	H	NH_2		(*R*)CH_3	1	1
12o	H	NH_2		carbonyl	1	1

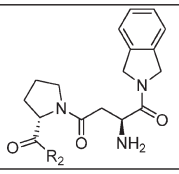
† bridged piperazine ((1*R*,4*R*)-2,5-diazabicyclo[2.2.1]heptane).

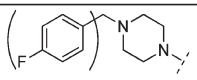
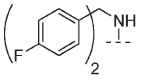
Class B



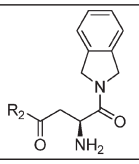
Compound	X	R_1	R_2	R_3	n_1	n_2
12p	-	-		-	-	-
12q	-	-		-	-	-
12r	-	-		-	-	-
12s	-	-		-	-	-

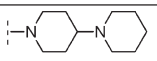
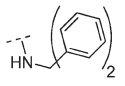
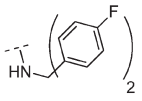
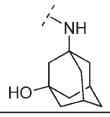
Class C



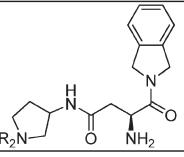
Compound	X	R_1	R_2	R_3	n_1	n_2
12t	-	-		-	-	-
12u	-	-		-	-	-

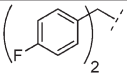
Class D



Compound	X	R_1	R_2	R_3	n_1	n_2
12v	-	-		-	-	-
12w	-	-		-	-	-
12x	-	-		-	-	-
12y	-	-		-	-	-

Class E



Compound	X	R_1	R_2	R_3	n_1	n_2
12z	-	-		-	-	-

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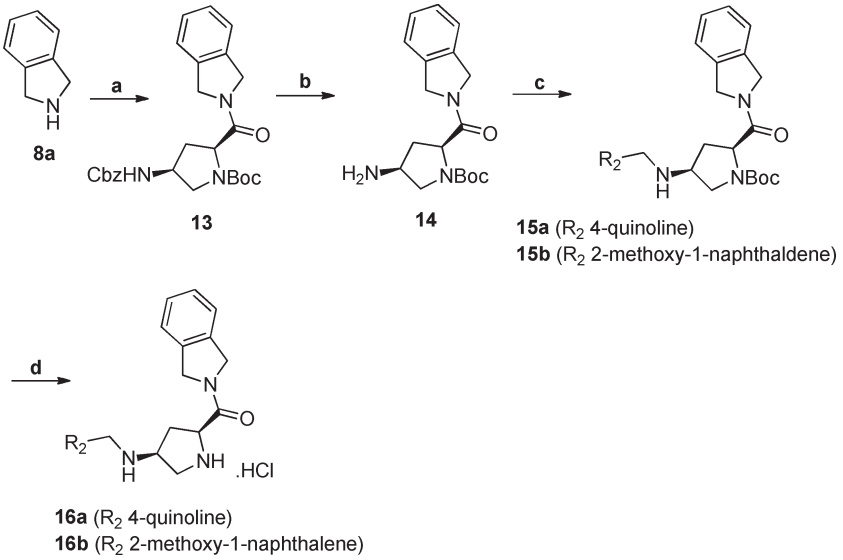
Scheme 2. General Synthesis of 4-Amino-L-proline Derivatives 16a and 16b[a]



[a] Reagents: (a) TBTU, Et_3N, (2*S*,4*S*)-4-(((benzyloxy)carbonyl)amino)-1-(*tert*-butoxycarbonyl)pyrrolidine-2-carboxylic acid, CH_2Cl_2; (b) Pd/C, H_2, methanol; (c) **15a**: quinoline-4-carboxaldehyde, acetic acid, $NaCNBH_3$, DCM; **15b**: 2-methoxy-1-carboxaldehyde, acetic acid, $NaCNBH_3$, CH_2Cl_2; (d) TFA, CH_2Cl_2 followed by HCl in isopropyl alcohol, CH_2Cl_2.

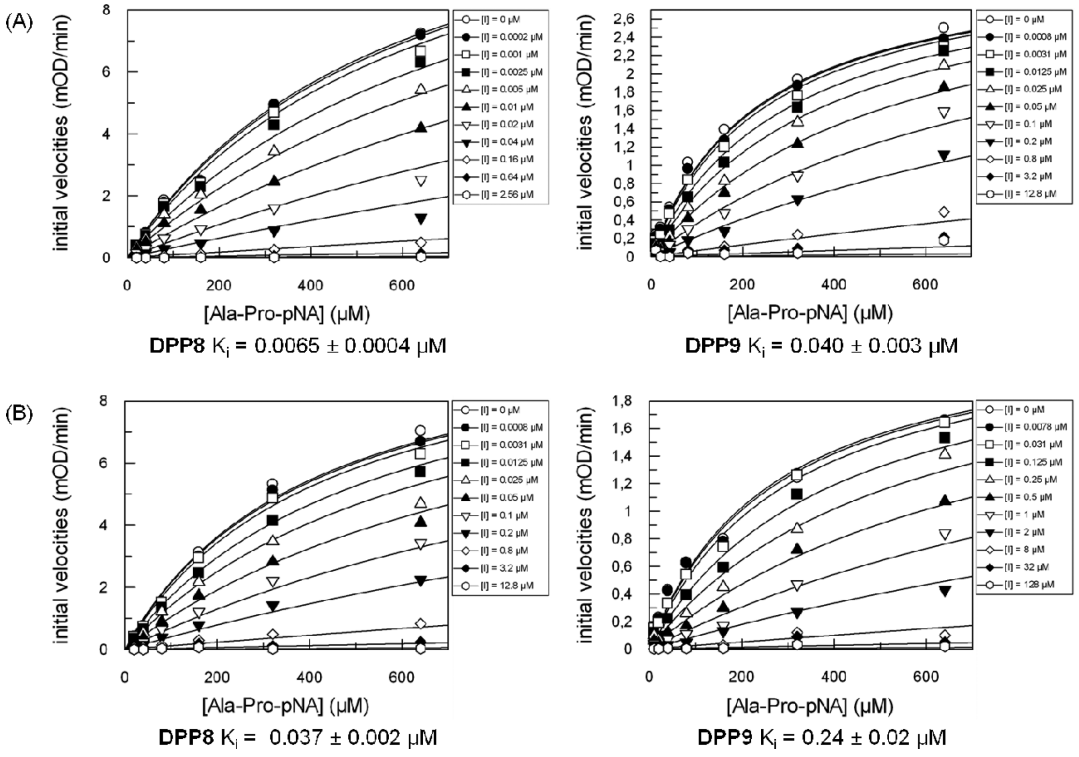


Figure 5. Determination of the K_i values for compound **12f** (A) and **12n** (B) against both DPP8 and DPP9, assuming competitive inhibition.

9*H*-fluorene-9-carboxylic acid, under normal peptide coupling conditions as described above. In a second approach, mono-Boc-piperazine was reacted with different acid chlorides and Et_3N in DCM. Finally, the treatment of Boc-protected acylated intermediates with TFA gave four acylated piperazine analogues, using conditions as described above.

Third, preparation of the L-proline derivatives began with the coupling of the free carboxylic acid from *N*-Boc-proline to commercially available amines: bis(4-fluorophenyl)methanamine and 1-(bis(4-fluorophenyl)methyl)piperazine. A Boc-deprotecting step yielded a piperazine-nitrogen ready for coupling with the P1 moiety with formation of compounds **12**(**t**−**u**).

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Table 3. Inhibitory Properties of Isoindoline Compounds

		IC_{50} (μM)[a]			
compd	class	DPP8	DPP9	DPPIV	DPPII
7	A	0.012 ± 0.001	0.084 ± 0.002	>50	>50
12a	A	0.96 ± 0.04	4.0 ± 0.17	>100	19.3 ± 0.7
12b	A	4.0 ± 0.1	5.0 ± 0.4	>100	14.0 ± 0.6
12c	A	>10	>10	>100	>100
12d	A	0.0094 ± 0.0004	0.0254 ± 0.0025	>50	>50
12e	A	0.0088 ± 0.0007	0.0269 ± 0.0026	>100	>25
12f	A	0.0099 ± 0.0004	0.050 ± 0.003	>50	>50
12g	A	0.063 ± 0.002	0.224 ± 0.023	>100	>50
12h	A	0.141 ± 0.003	0.53 ± 0.06	>100	>25
12i	A	34.8 ± 1.4	54 ± 2	>100	>100
12j	A	0.080 ± 0.004	0.26 ± 0.02	>100	3.3 ± 0.17
12k	A[c]	0.23 ± 0.03	1.13 ± 0.12	>100	>25
12l	A	0.08 ± 0.01	0.24 ± 0.01	>100	>25
12m	A	0.032 ± 0.002	0.26 ± 0.02	>100	>50
12n	A	0.05 ± 0.005	0.54 ± 0.04	>50	>25
12o	A	0.52 ± 0.03	2.6 ± 0.2	>100	>50
12p	B	0.166 ± 0.006	0.63 ± 0.08	>100	1.9 ± 0.1
12q	B	0.278 ± 0.006	0.68 ± 0.03	>100	17.3 ± 1.1
12r	B	0.029 ± 0.001	0.065 ± 0.006	>100	>25
12s	B[b]	0.31 ± 0.01	0.54 ± 0.05	>100	>25
12t	C	6.8 ± 0.4	22.6 ± 2.5	>100	23 ± 3
12u	C	0.91 ± 0.07	1.7 ± 0.1	>100	2.10 ± 0.07
12v	D	0.73 ± 0.02	1.4 ± 0.1	>100	>100
12w	D	9.4 ± 0.3	29.1 ± 2.2	>100	0.17 ± 0.01
12x	D	4.5 ± 0.2	35.0 ± 1.8	>100	0.65 ± 0.03
12y	D	>10	>10	>100	15.2 ± 0.8
12z	E	3.91 ± 0.18	16.4 ± 1.9	>100	0.033 ± 0.001
16a	F	1.1 ± 0.1	2.1 ± 0.1	17.7 ± 0.7	3.6 ± 0.1
16b	F	15.9 ± 0.18	>50	>100	0.29 ± 0.01

[a] IC_{50} values expressed in μM, except stated otherwise. [b] Sulfonylated R_2. [c] Bridged piperazine ((1*R*,4*R*)-2,5-diazabicyclo[2.2.1]heptane).

Alternatively, converting commercially available amines, e.g. *tert*-butyl (2-aminoethyl)carbamate, 2,2-diphenylethanamine, and bis(4-fluorophenyl)methanamine, into primary and secondary amides with *N*-Boc aspartylisoindoline yielded target compounds **12**(**v**−**y**). The same procedure was used for 4-hydroxyamantadine, which was synthesized in excellent yield following a literature procedure.[48] Finally, the amino-pyrrolidine moiety of **12z** was synthesized as follows. The starting product 4*S*-hydroxy-L-proline was decarboxylated to the corresponding 3-hydroxypyrrolidine, which was *N*-Boc protected. Successive mesylation of the alcohol was accomplished using methanesulfonyl chloride and Et_3N in DCM. Nucleophilic substitution of the mesylated alcohol with sodium azide gave compound X in quantitative yield. The Boc protecting group was converted to the base-labile trifluoroacetic acid (TFA) protecting group. Catalytic hydrogenation of the azide functionality yielded the primary amine, which was immediately Boc protected. Removal of the TFA protecting group, subsequent alkylation of 4,4′-(chloromethylene)bis(fluorobenzene), Boc deprotection, and final coupling afforded compound **12z**.

Compounds **16a** and **16b** were synthesized, starting from isoindoline, which was coupled to the 4-amino-L-proline building block (2*S*,4*S*)-4-(((benzyloxy)carbonyl)amino)-1-(*tert*-butoxycarbonyl)pyrrolidine-2-carboxylic acid, the preparation of which is described in the Supporting Information section of this paper. Prior to reductive alkylation of the 4-amino functionality with the commercially available aldehydes quinoline-4-carbaldehyde and 2-methoxy-1-naphthaldehyde, the benzyloxycarbonyl (Cbz) protecting group was removed with catalytic hydrogenation. Final deprotection yielded compounds **16a** and **16b** (Scheme 2).

RESULTS AND DISCUSSION

For all compounds synthesized, inhibitory potency toward DPP8, DPP9, DPPIV, and DPPII was assessed using IC_{50} measurements. Experimentally determined K_i values were found to be in line with the IC_{50} results as depicted in Figure 5.[29] Table 3 summarizes the inhibitory profiles of these inhibitors: **7**, **12a**−**z**, and **16a**−**b**. In general, the compounds tested showed no affinity for DPPIV, comparable to what was observed for lead **7**. Because of the presumed larger S2-pockets of DPP8 and DPP9, selectivity over DPP IV is most likely achieved by the combined presence of a P1 isoindoline and the sterically demanding substituents of the P2 aspartyl residue.[33] In general, all synthesized compounds consistently display at least some selectivity for DPP8 over DPP9, a feature already present in the lead. During our initial attempts to increase this DPP8 selectivity, we homologated the P2 aspartyl fragment to a glutamyl residue, yielding compounds **12a** and **12b**, which proved to be less favorable inhibitors of both DPP8 and DPP9 and displayed limited but measurable affinity for DPP II. Therefore, no further analogues containing a P2-glutamyl residue were prepared. In addition, removing the amine functionality from the P2 fragment in **12c** resulted in a dramatic decrease in inhibitory potency that was not compensated for by affinity contribution from the P2 substituting (bis(4-fluorophenyl)methyl)piperazine fragment. This finding indicates the pivotal importance of the free amine group, reflecting the substrate selectivity of the dipeptidyl peptidases, and was convincing enough not to further explore this type of derivatization. Subsequently, modification of the piperazine ring was investigated. First, piperazine analogues or substituted acyclic secondary amines were found to induce a decrease in potency toward both DPP8 and DPP9 or, again, to diminish the selectivity with regards to DPPII. Inhibitors **12j**, **12**(**t**−**y**), and **12z** might serve as illustrative examples thereof. Surprisingly, compound **12z** roughly lost all DPP8/9 potency and turned out to be a potent DPPII inhibitor, albeit less pronounced than reference DPP II inhibitor **4**. Compound **12z** is characterized by the presence of a less conformationally constrained 3-aminopyrrolidine ring. As a rationale for its DPPII activity, one might hypothesize this molecule, if bound to the enzyme, to be able to align its basic amine functions to the same positions as the corresponding amines of the dibasic 1,4-diaminobutyryl pharmacophore in **4**. From the foregoing data, a 1,4-diazacyclohexane ring was deduced to be preeminently tolerated by both DPP8 and DPP9. Next, *C*-substitution of the piperazine ring with a methyl group, was investigated for its potential to uncouple DPP9 activity in the diastereomeric pair **12m** and **12n**. Both were found to possess a roughly 10-fold selectivity for DPP8 over DPP9 and approximately thousand-fold selectivity over DPPIV and DPPII, with the *S*-methyl congener displaying slightly higher affinity. However, converting the piperazine ring into a piperazin-2-one in compound **12o** was shown not to be beneficial, as it reduced potency toward both DPP8 and DPP9. Additionally, the

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bridged piperazine ((1*R*,4*R*)-2,5-diazabicyclo[2.2.1]heptane) of compound **12k** did not result in increased potency nor selectivity. Finally, modification of the bis(4-fluorophenyl)methyl group with piperazine *N*-substituents of comparable or slighty decreased bulk size, was investigated. This effort involved the preparation of compounds **12f**−**i** and **12l**, grouping inhibitors with *N*-alkyl type substituents and **12p**−**s** in which susbtituents were grafted by acylation or sulfonylation. Of all substituents introduced by alkylation, only the 1,1-phenyl-(4-chloro)phenylmethyl substituent of **12f** led to a comparable inhibitory profile compared to the lead compound. Mono *N*-substitution of the piperazine ring with a 4-fluorobenzyl moiety in compound **12l** did not result in increased DPP8/9 potency. For the acylated piperazine compounds **12p**−**s**, no general trend could be observed. While compounds **12p**−**q** and **12s** are just reasonable DPP8/9 inhibitors, compound **12r** is a potent inhibitor of DPP8 and DPP9 with a favorable selectivity profile toward the other assessed DPPs. In summary, these results indicate that both acylated and alkylated piperazine *N*-substitution types allow for preparation of potent DPP8/9 inhibitors, but neither is significantly better at inducing selectivity for DPP8. Finally, combining the P1-(5-fluoroisoindoline), optimized by us earlier, in conjunction with optimized P2 fragments, gave compounds **12d** and **12e**, with potencies equivalent to that of the reference compound **7**.

CONCLUSIONS

This work represents the first directed study to identify modification points in the topology of a representative DPP8/9-inhibitor, capable of rendering selectivity for DPP8 over DPP9. The highly challenging character of this task was discussed in view of the remarkably similar amino acid sequences within these enzymes' active sites. As the starting point of our investigations, the cell-permeable DPP8/9-inhibitor **7** was selected as a lead and dissected into several substructures that were modified separately for evaluating their potential to contribute to selectivity. The analogues of **7** generated in this way were assessed for DPP8 and DPP9 activity and, in addition, for activity toward DPPIV and DPPII. SAR data obtained from this study reflect the anticipated difficulties in designing DPP8-selective compounds, with the most promising structures possessing a selectivity index of approximately 1 order of magnitude in favor of DPP8. In summary, the series of inhibitors presented here all contain an isoindoline and 5-fluoroisoindoline as the optimal P1 residues, a feature investigated earlier in extenso by our group. The SAR generated in this publication demonstrates that an aspartyl residue at the P2 position is preferable over succinyl and glutamyl moieties for inhibitory potency. Further, a piperazin-1-yl moiety, with a bulky alkyl or acyl substituent at its 4-position, seems to be the most evident choice to supplement the optimal P2 aspartyl residue. Practically all synthesized compounds, compared to lead structure **7**, turned out to conserve excellent selectivity with respect to DPPIV. Compounds, however, in which the P2-supplementing piperazine was either deleted or replaced by a 3-aminopyrrolidine analogue, turned out to regain binding potential toward DPPII. Maximal selectivity for DPP8 over DPP9, roughly in the range of 1 order of magnitude, was observed with compounds **12m** and **12n**, methylpiperazine analogues of **7**. While this degree of selectivity toward DPP8 is still modest, *C*-alkylation of the piperazine ring was found to be the only modification type to further improve uncoupling of DPP9 activity, compared to the lead molecule. Further structural modification is currently being investigated as a means to obtain compounds with maximal affinity and selectivity for DPP8. Finally, by narrowing down the most probable DPP8-selectivity imparting modification points in DPP8/9 inhibitors to parts of space that are topologically equivalent to the piperazine ring system, this study can be considered valuable to future DPP8-selective inhibitor discovery programs, eventually involving novel compound chemotypes.

EXPERIMENTAL SECTION

Amino acids and TBTU were purchased from Novabiochem. Other reagents were obtained from Sigma-Aldrich or Acros and used as such, unless otherwise specified. Characterization of intermediates and final compounds was done using NMR spectroscopy and mass spectrometry, and final purity was controlled using HPLC analysis. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker Advance III ultrashield 400 spectrometer. $CDCl_3$, CD_3OD, or DMSO-d_6 were used as the solvents. Chemical shifts in the spectra are given in ppm and coupling constants (J) in Hz. ES mass spectra were obtained with an Esquire 3000plus ion trap mass spectrometer from Bruker Daltonics, using the direct infusion mode.

Purity of final products was controlled using HPLC analysis. (1) LC/MS chromatograms were recorded on an AGILENT 1100 series HPLC system equipped with an Alltech Prevail C18 column (2.1 mm × 50 mm × 3 μm) connected to an Esquire 3000plus ion trap mass spectrometer from Bruker Daltonics. A 5−100% acetonitrile/water, 20 min gradient was used with a flow rate of 0.2 mL/min. Formic acid (0.1%) was added to both solvents. (2) In addition, reversed phase HPLC chromatograms were recorded on a Gilson instrument equipped with an Ultra sphere ODS column (4.6 mm × 250 mm × 5 μm) and a UV detector. A 10−100% acetonitrile, 35 min gradient was used with a flow rate of 1 mL/min. Then 0.1% trifluoroacetic acid was added to both solvents. An indicated purity of 100% indicates that no other peaks in the chromatogram occur. All compounds reported in this publication were determined to have purities of ≥95%, except for compounds **12i** (93%) and **12p** (92%), which could not be purified further.

General Experimental and Analytical Data for Key Products 12e, 12f, 12m, and 12n. Detailed experimental and analytical data for all intermediates and final products in the manuscript can be found in the Supporting Information part of this publication.

(2*S*)-2-Amino-4-(4-((4-chlorophenyl)(phenyl)methyl)piperazin-1-yl)-1-(5-fluoroisoindo-lin-2-yl)butane-1,4-dione bis-(2,2,2-trifluoroacetate) (12e). Compound **11e** (0.1 mmol) was dissolved in dichloromethane (2 mL). Next, trifluoroacetic acid (2 mL) was added and the reaction was stirred for 30 min. The volatiles were evaporated under reduced pressure. The crude product was purified by precipitation with cold, dry diethyl ether and obtained as a white, amorphous powder; yield 94%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59−7.54 (m, 4H), 7.41−7.28 (br m, 5H), 7.19−7.13 (m, 1H), 7.01−6.89 (m, 2H), 4.98−4.83 (m, 2H), 4.79−4.73 (m, 1H), 4.68−4.57 (m, 3H), 4.12−4.07 (m, 1H), 3.83−3.71 (m, 3H), 3.14−3.07 (m, 4H), 2.97−2.93 (m, 2H). ESI$^+$-MS: m/z = 521 (M + H). Purity determination: (1) LC/MS t_R = 14.8 min, 99%; ESI+-MS m/z = 521.3 (M + H), 1063.2 (2M + Na); (2) HPLC t_R = 17.3 min, 100%

(2*S*)-2-Amino-4-(4-((4-chlorophenyl)(phenyl)methyl)piperazin-1-yl)-1-(isoindolin-2-yl)butane-1,4-dione bis(2,2,2-trifluoroacetate) (12f). This product was obtained as a white, amorphous solid by acidolytic removal of the Boc-protecting group in **11f**, using an identical procedure as described for **12e**; yield 87%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.57−7.56 (m, 4H), 7.44−7.38 (m, 5H), 7.28−7.26 (m,2H), 7.21−7.19 (m, 2H), 4.99−4.93 (m, 2H), 4.84−4.81 (m, 1H),

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4.74−4.70 (m, 2H), 4.63−4.60 (m, 1H), 3.88−3.77 (m, 2H), 3.27−3.10 (m, 5H), 2.88 (s, 3H). ESI$^+$-MS: m/z = 503 (M + H). Purity determination: (1) LC/MS t_R = 14.5 min, purity: 99% ESI+-MS m/z = 503 (M + H), 1027 (2M + Na); (2) HPLC t_R = 16.5 min, 98%.

(*S*)-2-Amino-4-((*S*)-4-(bis(4-fluorophenyl)methyl)-3-methylpiperazin-1-yl)-1-(isoindolin-2-yl)butane-1,4-dione Bis(2,2,2-trifluoroacetate) (12m). This product was obtained as a white, amorphous solid by acidolytic removal of the Boc-protecting group in **11m**, using an identical procedure as described for **12e**; yield 91%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71−7.59 (m, 4H), 7.21−6.08 (m, 8H), 4.98 (s, 1H), 4.82−4.68 (m, 5H), 4.43 (m, 1H), 3.69 (m, 2H), 3.31 (m, 1H), 3.16−2.76 (br m, 5H), 1.30−1.15 (m, 3H). ESI$^+$-MS: m/z = 519 (M + H). Purity determination: (1) LC/MS t_R = 14.1 min, purity 97%; (2) HPLC t_R = 15.9 min, 98%.

(2*S*)-2-Amino-4-((3*R*)-4-((3-fluorophenyl)(4-fluorophenyl)-methyl)-3-methylpiperazin-1-yl)-1-(isoindolin-2-yl)butane-1,4-dione Bis(2,2,2-trifluoroacetate) (12n). This product was obtained as a white, amorphous solid by acidolytic removal of the Boc-protecting group in **11n**, using an identical procedure as described for **12e**; yield 84%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.50−7.44 (m, 4H), 7.26−6.99 (m, 8H), 5.00 (s, 1H), 4.82−4.68 (m, 5H), 4.20 (m, 1H), 3.65 (m, 2H), 3.30 (m, 1H), 3.14 (m, 2H), 2.91−2.73 (m, 2H), 2.64 (m, 1H), 1.11−1.05 (m, 3H). ESI$^+$-MS: m/z = 519 (M + H). Purity determination: (1) LC/MS t_R = 14.2 min, purity 96%; (2) HPLC t_R = 15.9 min, 97%.

■ ASSOCIATED CONTENT

Supporting Information. General synthetic procedures, compound characterization data, and enzymatic assay conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

DPP, dipeptidyl peptidase; DASH, DPPIV activity and/or structure homologue; FAP, fibroblast activation protein; GLP-1, glucagon-like peptide-1; GIP, glucose dependent insulinotropic peptide; IP-10, interferon γ-induced protein-10; POP, prolyl oligopeptidase; PREP, prolyl endopeptidase; QPP, quiescent cell proline dipeptidase, DPPII

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