Irreversible Inhibition of Dipeptidyl Peptidase 8 by Dipeptide-Derived Diaryl Phosphonates

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Abstract: Dipeptide-derived compounds, bearing various P2 residues and a diaryl pyrrolidin-2-yl phosphonate at the P1 position, were evaluated as dipeptidyl peptidase 8 (DPP8) inhibitors. With these products, irreversible inhibition of DPP8 was observed. To obtain inhibitors with an improved activity and selectivity profile, a set of selected analogues containing a diaryl isoindolin-1-ylphosphonate at P1 was synthesized and evaluated. Within this latter series, compound **2e** was shown to be a potent, irreversible inhibitor of DPP8, demonstrating very low affinity for DPP IV and DPP II.

Proline-selective dipeptidyl peptidases have become the subject of intense research since it has become clear that several metabolically important peptides, for example, the insulin-releasing incretin hormone glucagon-like peptide 1, are substrates of dipeptidyl peptidase IV (DPP IV[a], EC 3.4.14.5). Small-molecule inhibitors of DPP IV have been selected for development as antidiabetic drugs, with one compound currently approved by FDA and EMEA (sitagliptin/Januvia) and several others having reached different stages of clinical investigation. Next to the intensively studied DPP IV, a number of DPP IV-related enzymes have been described, fibroblast activation protein α (FAPα), dipeptidyl peptidase II (DPP II, EC 3.4.14.2), DPP8, and DPP9. They all belong to clan SC of the peptidases with a serine-type mechanism and share a remarkable selectivity for substrates with an N-terminal penultimate proline residue. For these related enzymes, however, neither in vivo substrates nor a physiological role has been firmly established.[1−3]

Recently, inhibition of DPP8 has been associated with severe toxicity following in vivo studies with *allo*-Ile-isoindoline (**1**), a potent DPP8-targeting inhibitor.[4] To verify whether this observed toxicity was caused by inhibition of DPP8 or by off-target compound-related events and, more general, for the characterization of the enzyme's physiological role, other structurally or mechanistically distinct inhibitors can be expected to be valuable research tools. Irreversible inhibitors have some advantages for this purpose; (1) it is often favorable to obtain "long-term" inhibition in biological systems, reducing the need for frequent dosing of the compound, and (2) the extent of in vivo inhibition is easier to estimate. The aim of the present study was to develop dipeptide-based diaryl phosphonates as irreversible inhibitors for DPP8. The diaryl phosphonate moiety is known to be capable of interacting irreversibly with the catalytic serine alcohol function of serine proteases and to do so with high selectivity with regard to other groups of proteolytic enzymes, including cysteine proteases. The process of enzyme inactivation comprises a nucleophilic substitution reaction during which a covalent bond is formed between the serine alcohol and the phosphonate part of the inhibitor, while one of the two *P*-aryloxysubstituents is expelled.[5]

Hitherto, the number of publications reporting potent DPP8 inhibitors is limited to only two examples, both describing structures with a dipeptide basic structure and isoindoline at the P1 position as the most promising compounds.[6] By retaining this basic structure and introducing a diaryl phosphonate moiety at the P1 position, we wanted to synthesize potent, irreversible DPP8 inhibitors (Figure 1).

Expecting a comparable structure−activity relationship for DPP8 inhibition between our target structures and pyrrolidin-2-yl phosphonates reported earlier by our groups, we first evaluated a small library of these compounds. As such, we aimed at identifying useful P2 building blocks for the isoindolin-1-yl phosphonate inhibitors (Figure 2 and Tables 1 and 2, compounds **3**−**7**).[7] Some of these compounds have been used in in vivo studies for prolonged inhibition of dipeptidyl peptidase IV.[8]

Table 1 lists three inhibitors (compounds **3**−**5**) with a common prolylproline-like skeleton and different aryl substituents. In addition, it contains, as a reference, the evaluation data under our assay conditions of *allo*-Ile-isoindoline (**1**). Evaluation of the activity of compounds **3**, **4**, and **5** revealed their considerable potential to inhibit DPP8. Although less pronounced than reported earlier for DPP IV, higher potency toward DPP8 was observed for the compounds with a 4-acetamidophenyl or 4-(ethyl hippuryl) phosphonate function, (**4** and **5**). For reasons of chemical stability, however, only the synthesis of diphenyl and bis(4-acetamidophenyl) isoindolin-1-yl phosphonate containing inhibitors was considered as feasible.[7]

Table 2 lists the biochemical evaluation data of a series of diaryl pyrrolidin-2-yl phosphonates with varying P2 residues. In this series, the P2 lysyl containing inhibitors **6g** and **7a** combine low micromolar IC_{50} values in the DPP8 assay, with at least 10-fold selectivity toward both DPP II and DPP IV. Therefore, a lysine residue was expected to be a useful P2 fragment in the construction of isoindolin-1-yl phosphonate inhibitors targeting DPP8. Again, on the basis of the, albeit less convincing, activity or selectivity profiles of compounds **6k**, **6h**, and **7b**, an isoleucyl or an ϵ-*N*-benzyloxycarbonyllysyl (Lys(*Z*)) residue was also selected for this purpose. Noteworthy is that compound **7b**, a bis(4-acetylamidophenyl) phosphonate, has a decreased potency toward DPP8 when compared to its diphenyl analogue **6h**.

To assess whether DPP8 inactivation mediated by these phosphonates indeed has an irreversible character, a dilution experiment was performed. The enzyme was incubated with the inhibitor (15 min, 37 °C), and then, the enzyme−inhibitor complex was diluted 100 times in the assay mixture. In the presence of the inhibitor, the DPP8 activity was not recovered to the extent expected for the final concentration of inhibitor, indicating the irreversible inactivation of DPP8 by compound **7a**. Progress curves (Figure 3A) for the DPP8-catalyzed generation of *p*-nitroaniline from the chromogenic substrate Ala-Pro-*p*-nitroanilide in the presence of different concentrations

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[a] Abbreviations: DPP, dipeptidyl peptidase; FAPα, fibroblast activation protein α.

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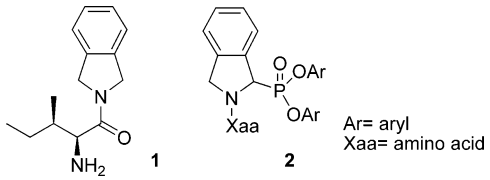


Figure 1. Example of a reported DPP8 inhibitor, *allo*-Ile-isoindoline (**1**), and the template structure of target irreversible inhibitors synthesized in the framework of this project (**2**).

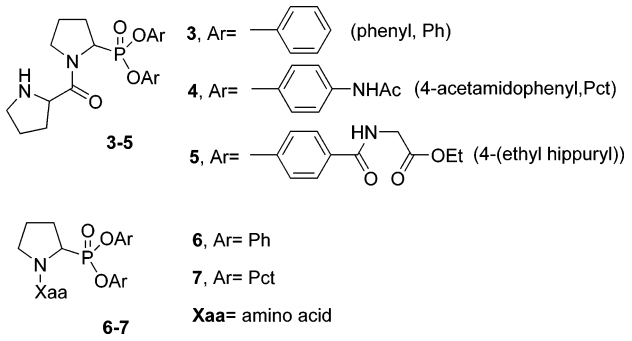


Figure 2. Overview of diaryl pyrrolidin-2-yl phosphonates evaluated as DPP8 inhibitors.

Table 1. IC_{50} Values for Compound **1** and Pyrrolidin-2-yl Phosphonates **3**−**5**

	IC_{50} (μM)		
compound	DPP8	DPP IV	DPP II
1	0.122 ± 0.011	90 ± 4	28.7 ± 1.2
3	71 ± 5	55 ± 13	>1000
4	56 ± 18	5.6 ± 2.7	>1000
5	0.53 ± 0.03	0.014 ± 0.001	>100

Table 2. IC_{50} Values for Pyrrolidin-2-yl Phosphonates **6**−**7**[a]

			IC_{50} (μM)		
compound	Xaa	Ar	DPP8	DPP IV	DPP II
6a	Gly	Ph−	25 ± 1.4	16 ± 1.6	79 ± 48
6b	Val	Ph−	126 ± 16	89 ± 6	>1000
6c	Phe	Ph	>1000	142 ± 16	>1000
6d	Asp	Ph	>1000	>1000	>1000
6e	Asn	Ph	>1000		>125[a]
6f	Ser	Ph	>500	503 ± 55	>1000
6g	Lys	Ph	8.1 ± 0.8	117 ± 21	>1000
6h	Lys(*Z*)	Ph	8.6 ± 0.4	4.8 ± 0.2	>500
6i	His	Ph	>1000	326 ± 40	60 ± 7.5
6j	Dab	Ph	86 ± 3	261 ± 55	20 ± 1
6k	Ile	Ph	71 ± 4	>125	>500
7a	Lys	Pct	0.91 ± 0.35	8 ± 0.5	85 ± 4
7b	Lys(*Z*)	Pct	43 ± 5	4.2 ± 0.3	77 ± 4

[a] Ph = phenyl; Pct = 4-acetamidophenyl.

of **7a** are also indicative for an active-site-directed inactivation of the enzyme. The observed pseudo-first-order rate constants show a linear dependency upon inhibitor concentration, with an intercept very close to zero. (Figure 3B).

Summarizing, results obtained from evaluating these pyrrolidin-2-yl phosphonates inspired us to synthesize both diphenyl and bis(4-acetamidophenyl) isoindolin-1-yl phosphonates having either a Lys, Lys(*Z*), or an Ile residue at the P2 position. In addition, inhibitors with a P2 *allo*-Ile residue (present in compound **1**) were deemed equally interesting target structures. The general synthetic route followed for these compounds is outlined in Scheme 1 and contains, similar to the procedure applied for the preparation of pyrrolidin-2-yl phosphonate containing inhibitors, a Birum−Oleksyszyn phosphonylation reaction on a cyclic hemiaminal as a key transformation.[6] Commercially available 2-cyanobenzaldehyde was first reduced

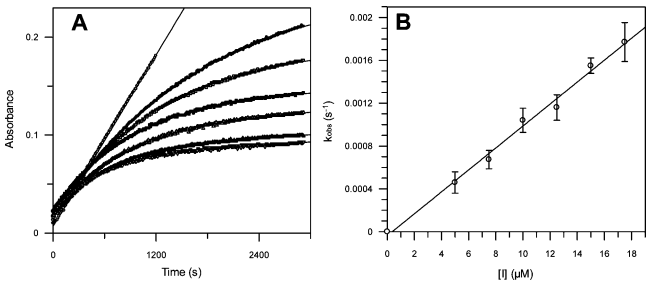
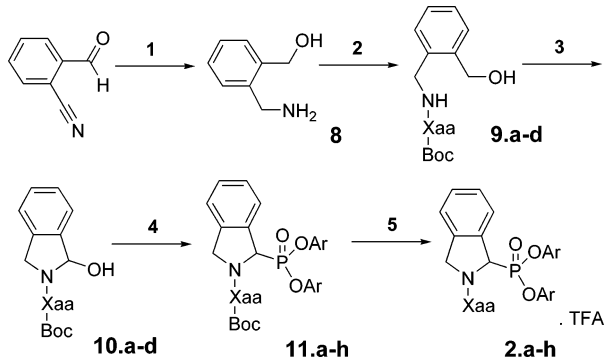


Figure 3. Kinetic analysis of compound **7a** binding to DPP8. (A) Progress curves of *p*-nitroaniline release from the chromogenic substrate Ala-Pro-*p*-nitroanilide in the absence or presence of different concentrations (5, 7.5, 10, 12.5, 15, and 17.5 μM) of **7a**. (B) The observed pseudo-first-order rate constants show a linear dependency upon inhibitor ([I], **7a**) concentration. The k_{app} was found to be 100 ± 4 M^{-1} s^{-1}.

Scheme 1. Synthesis of Diaryl Isoindol-1-yl Phosphonate Inhibitors[a,b,c]



[a] Reagents and conditions: (1) (i) $LiAlH_4$, THF, 0 °C, 1 h, 88%, (ii) aq. NaOH; (2) (i) *N*-Boc-Xaa-OH, DCC, HONSu, CH_2Cl_2, 1 h, (ii) **8**, CH_2Cl_2, 74−96%; (3) PCC, CH_2Cl_2, 2 h, 47−81%; (4) $P(OAr)_3$, $Cu(OTf)_2$, CH_2Cl_2, 2 h, 43−89% TFA; (5) TFA, CH_2Cl_2, 0.5 h, 88−99%. [b]Xaa = Lys, Lys(Z), Ile, *allo*-Ile. In the case of Xaa = Lys, the ϵ-NH_2 is protected with a Boc group, that is, simultaneously cleaved with the α-*N*-Boc functionality in step 5. [c]Ar = phenyl (Ph) or 4-acetamidophenyl (Pct).

using $LiAlH_4$, and 2-aminomethylbenzyl alcohol (**8**) obtained from this reaction was coupled to the *N*-hydroxysuccinimidates of selected *N*-α-Boc-protected P2 amino acids. After oxidation of the alcohol group present in intermediates **9a**−**d** using pyridinium chlorochromate (PCC), resulting hemiaminals **10a**−**d** were subjected to a modified Birum−Oleksyszyn protocol using either triphenyl phosphite or tris(4-acetamidophenyl) phosphate and a Lewis acid catalyst.[9] Final products were obtained as trifluoroacetic acid salts after acidolytic deprotection of intermediates **11a**−**h**.

Upon biochemical evaluation, these isoindoline-derived inhibitors were found to be (1) slightly more potent, irreversible DPP8 inhibitors than their pyrrolidine-based counterparts and, most importantly, (2) to generally exhibit pronounced selectivity for the target enzyme (Table 3). In this series again, the combined presence of a dibasic P2 lysine residue and a bis(4-acetamidophenyl) phosphonate group (**2e**) gives rise to the most favorable activity/selectivity profile. Second, DPP8's preference for compounds containing a P2 *allo*-isoleucine residue found with other dipeptide-based inhibitors was not observed for the diaryl phosphonates.

A comparative kinetic analysis of DPP8 inactivation by selected pyrrolidine and isoindoline phosphonates is summarized in Table 4. Progress curve analysis revealed a hyperbolic relationship between the observed first-order rate constant and the inhibitor concentration indicating saturation kinetics. This allows calculation of the equilibrium constant of the initial

Table 3. IC_{50} Values of Diaryl Isoindolin-2-yl Phosphonates (**2a**−**h**)[a]

			IC_{50} (μM)		
compound	Xaa	Ar	DPP8	DPP IV	DPP II
2a	Lys	Ph−	2.11 ± 0.23[c]	>500	n.a.[d]
2b	Lys(*Z*)	Ph−	7.8 ± 1.5[b]	>50	2.2 ± 0.12
2c	Ile	Ph	36 ± 2	>250	>250
2d	*allo*-Ile	Ph	16.0 ± 1.0	>1000	>250
2e	Lys	Pct	0.53 ± 0.11[c]	>500	>1000
2f	Lys(*Z*)	Pct	12.5 ± 2.5[b]	>250	>250
2g	Ile	Pct	48 ± 3	>1000	>1000
2h	*allo*-Ile	Pct	17.5 ± 0.2	>250	>100

[a] Ph = phenyl; Pct = 4-acetamidophenyl. [b] Mean of two independent measurements. [c] Mean of three to four independent measurements. [d] n.a.= not analyzed.

Table 4. Kinetic Analysis of DPP8 Inhibition by Selected Compounds

compound	K_d(μM)	k_{inact} (s^{-1})	k_{app} (M^{-1} s^{-1})
7a	7.3 ± 1.1[a]	0.005 ± 0.0006[a]	680[b]
2e	0.77 ± 0.26	0.0029 ± 0.0004	3800[b]
2d	39 ± 6	0.0017 ± 0.0001	40[b]
2h	9.1 ± 1.3	0.0013 ± 0.000046	140[b]

[a] Mean of two measurements. [b] Calculated value (k_{inact}/ K_d).

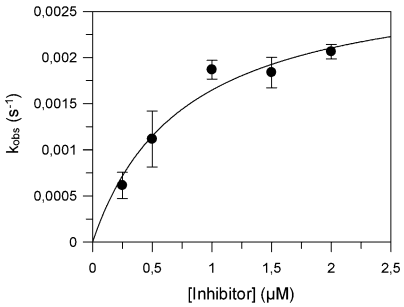


Figure 4. Kinetic analysis of compound **2e** inhibition of DPP8. The observed pseudo-first-order rate constants (k_{obs} (s^{-1})) exhibit a hyperbolic relationship with the inhibitor concentration. The dissociation constant K_d was determined to be 0.77 μM, and the k_{inact} was 0.0029 ± 0.0004 s^{-1}.

enzyme−inhibitor complex as well as the first-order rate constant for the irreversible inactivation of the enzyme.

Figure 4 shows a typical hyperbolic relation between the observed first-order rate constant and the inhibitor concentration (as exemplified with data for compound **2e**).

In conclusion, we developed a series of irreversible DPP8 inhibitors. Potent inhibition of DPP8 could be obtained with bis(4-acetamidophenyl) pyrrolidin-2-yl and isoindolin-1-yl phosphonate derivatives **7a** and **2e**, both containing a dibasic P2 lysine residue. Compound **2e** proved to combine a good affinity (K_d) and pronounced selectivity for DPP8 with regard to DPP V and DPP II. This molecule promises to be a useful tool in the study of the non-DPP II/non-DPP IV members of the proline-selective dipeptidyl peptidases (DPP8 and DPP9). The compounds' inhibitory potential with respect to the latter enzyme will be determined.[10]

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Supporting Information Available: General synthetic procedures, spectral and analytical data for new compounds, and detailed descriptions of all biochemical experiments used for their characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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