Phosphinic Peptide Matrix Metalloproteinase-9 Inhibitors by Solid-Phase Synthesis Using a Building Block Approach

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Abstract: The solid-phase synthesis of an array of different pseudopeptides containing a phosphinic glycine–leucine moiety (-GΨ{P(O)OH-CH_2}L-)[1] is described. The resulting pseudopeptides were shown to act as matrix metalloproteinase-9 (MMP-9) inhibitors. Starting from available materials, the protected amino acid isosters benzyloxycarbonyl aminomethylphosphinic acid (glycine analogue) and ethyl α-isobutylacrylate (leucine analogue) were synthesized and coupled with the bis(trimethylsilyl)phosphonite. Protective group interchange yielded a protected phosphinic dipeptide building block **1** ready for use in solid-phase peptide synthesis. Solid-phase peptide synthesis was performed with 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry on a polyethylene glycol polyamide (PEGA) support and the coupling of **1** (1.5 equiv) was carried out with standard activation. The P_4-P_2 and $P_2'-P_4'$ positions of the pseudopeptides were designed by analogy of the cleavage sequences of different natural extracellular matrix protein substrates with a synthetic peptide substrate of MMP-9. The crude peptides were obtained in high yield and purity as determined by RP-HPLC, and were characterized by electrospray mass spectrometry and amino acid analysis after purification. Enzyme kinetic investigations with MMP-9 of the purified peptide inhibitors showed K_i values in the range from mM to nM.

Keywords: enzyme inhibitors · matrix metalloproteinases · peptide isosters · solid-phase synthesis

Introduction

Matrix metalloproteinases (MMPs),[2, 3] also known as matrixins, are a family of zinc-containing endoproteases involved in a wide variety of biological processes. These include embryo development, reproduction, tissue resorption, blood vessel formation, and wound healing. MMPs are also believed to play a major role in pathological conditions such as arthritis, tumor invasion, and metastasis. In particular, the resorption of organic bone constituents is controlled by MMPs produced by both bone-forming osteoblasts,[4] and bone-degrading osteoclasts.[5, 6] Cysteine proteases, such as cathepsin K, and MMPs,[6, 8–13] in particular MMP-9, MMP-12, and MMP-14,[14] are produced by osteoclasts.[5, 7, 8] In addition to bone resorption, the migration of osteoclasts[15, 16] and their anchoring to the extracellular bone matrix is controlled by MMPs, although their specific roles in these processes as well as the resorption process still remain to be elucidated.

The evaluation of substrate specificity of MMP-9 towards small peptide substrates[17] showed that P, L, G/A, and L/I are preferred in the P_3, P_2, P_1, and P_1' subsites, respectively. This has been utilized in a quenched fluorogenic substrate Mca-PLG–L-Dpa-AR-NH_2,[18–20] which has high k_{cat}/K_M values for MMP-1, MMP-2, and MMP-3, in addition to the high activity to MMP-9. Truncation of the substrate yielded poor k_{cat}/K_M values;[17] thus a good substrate must cover the P_3-P_2' subsites. Recently, specific peptide substrates for MMP-9 containing P, Hyp, and M or L in the P_3, P_1, and P_1' subsites, respectively, have also been found by solid-phase combinatorial methods.[21] In the case of natural protein substrates, the specificity of MMP-12 has been proven to be similar.[6] Thus, MMP-12 cleaves the bone matrix proteins osteopontin (OPN) and bone sialo protein (BSP) from different species at conserved sites with G or A, and L or I in the P_1 and P_1' subsites, respectively.

The observed preference of MMP-9 to cleave between the amino acids G/A and L/I suggests that good inhibitors of MMP-9 could be obtained by replacing, for example, the G–L cleavage site in a good substrate with a phosphinic -GΨ-

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{P(O)OH-CH_2}L- segment. Phosphinic peptides have previously been used to prepare protease inhibitors;[22–24] in some cases the actual G–L phosphinic segment -GΨ{P(O)OH-CH_2}L- has been used.[25–27] In these reports the phosphinic part was placed at the N-terminus of a relatively short sequence of about two to four amino acids including the phosphinic part. However, as only relatively long peptides seem to act as good substrates of MMP-9, longer sequences might be necessary in order to prepare potent inhibitors. This phenomenon was reported for MMP-3,[28] where the elongation of the P_3 subsite of a phosphinic peptide increased the affinity.

The inhibitory effect of phosphorus acids on esterases has been known for a long time.[29] This type of compounds has been used extensively for the construction of peptide isosters as competitive protease inhibitors, especially by Bartlett and co-workers,[27, 30–34] with traditionally three different types of isosters being used, namely the phosphonamidic, phosphonic, and phosphinic group. Phosphinic compounds have proven to be as potent as the other compounds,[25, 27, 35] and in addition are particularly stable towards hydrolysis.[36] By coordinating to zinc, the oxy-anion of phosphinic acids mimics the unstable tetrahedral transition state of the peptide bond cleavage site, a feature which is crucial to their function as inhibitors.[27, 36] The phosphinic moiety has been utilized for the construction of many inhibitors of a variety of enzymes such as MMP-3,[28, 35] collagenases,[25, 37, 38] other zinc metalloproteases,[22, 26, 27, 39–41] HIV-protease,[42, 43] other aspartic proteases,[32, 44, 45] ligases,[23, 24, 46, 47] and yet other enzymes.[48–50] Phosphinic peptides have been prepared both in solution[27, 28, 37, 44] and more recently on the solid phase.[22, 39, 40, 51–53] The solid-phase synthesis technique[54] has a number of advantages in the synthesis of sequential oligomeric molecules. Recently, Yiotakis et al.[52] have developed a strategy for preparing suitably protected phosphinic dipeptides that meet the existing peptide synthesis protocol requirements with the use of Fmoc-amino acids.

The specific roles of MMPs during bone resorption, osteoclast migration, and anchoring still remain to be solved. Therefore, specific and strong competitive inhibitors of MMPs are desired as a tool to investigate these processes, not only under physiological conditions, but also under pathological conditions such as osteoporosis and bone metastasis. Adapting the strategy of Yiotakis et al.,[52] we set out to prepare the protected G–L pseudodipeptide **1** (see Scheme 1), and subsequently incorporate the phosphinic segment into peptides on the solid phase. The sequences of these pseudopeptides were based on the cleavage sites of MMP-12 in the bone matrix proteins OPN and BSP,[6] and the frequently encountered MMP cleavage site in the synthetic peptide substrate H-Abz-GPLG–LY(NO_2)AR-NH_2.[55] Thus, replacement of G and L at the cleavage sites in these substrates by a -GΨ{P(O)OH-CH_2}L- phosphinic segment would produce putative inhibitors (Figure 1).

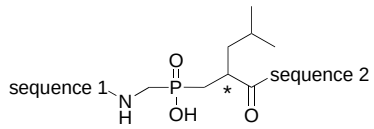

Figure 1. Putative phosphinic peptide MMP inhibitor containing the phosphinic -GΨ{P(O)OH-CH_2}L- segment.

Abstract in Danish: *Fastfase syntese af et antal forskellige pseudopeptider indeholdende en glycin-leucin phosphinsyredel (-GΨ{P(O)OH-CH_2}L-[1]) er beskrevet. Pseudopeptiderne viste sig at være effektive inhibitorer af matrix metalloproteinase 9. De beskyttede aminosyreanaloger, benzyloxycarbonyl aminomethyl phosphinsyre (glycin-analog) og ethyl α-isobutylakrylat (leucin-analog), blev syntetiseret udfra kommercielt tilgængelige stoffer og koblet via den til phosphinsyren svarende bis(trimethylsilyl)phosphonit. Efter udskiftning af beskyttelsesgrupper opnåedes en phosphinsyre dipeptid byggeblok **1** designet til brug ved fastfase peptidsyntese. Fastfase peptidsyntesen blev udført vha. Fmoc-kemi på en PEGA-resin og koblingen af byggeblokken **1** (1.5 equiv) blev foretaget med en gængs aktiveringsmetode. Aminosyrerne i P_4–P_2 og P_2'–P_4' positionerne i pseudopeptiderne var udvalgt således, at de opnåede pseudopeptider lignede MMP-9 substrater, heriblandt både matrixprotein substrater og et syntetisk peptidsubstrat. Råprodukterne fra peptidsyntesen havde ifølge RP-HPLC høj renhed og blev isoleret i højt udbytte. Efter oprensning vha. preparativ HPLC blev peptiderne karakteriseret ved elektrospray massespektrometri og aminosyre analyse. Enzymkinetiske undersøgelser af de oprensede peptid-inhibitorer med MMP-9 gav K_i værdier i området fra m*M* til n*M*.*

Results and Discussion

Building block synthesis: The key step in the strategy for the preparation of **1** is the formation of the pseudopeptide bond by coupling of the amino acid isosters, benzyloxycarbonyl aminomethyl phosphinic acid (**2b**) (protected glycine analogue) and ethyl α-isobutylacrylate (protected leucine analogue). This is followed by exchange of protective groups (Scheme 1).

Ethyl α-isobutylacrylate was prepared analogous to the previously described procedure for ethyl acrylates;[56] however, preparation of the intermediate compound **2a** was not immediately achieved. Neither the method by Baylis et al.[57] nor the most recently described preparation[58] were successful in our hands. Therefore, a third procedure[59, 60] was attempted and proved to be successful. Thus, reaction of ethyl (diethoxymethyl)phosphinate[61, 62] with 1,3,5-(diphenylmethyl)-hexahydro-*s*-triazine[57] followed by complete deprotection with concentrated aqueous HBr gave the desired compound **2a**. Since large amounts of the final building block were needed for combinatorial investigations, **2a** was prepared on a multigram scale, while the synthesis was still efficient (73% yield). Benzyloxycarbonyl (Cbz) protection of **2a** was achieved by a standard procedure[63] and the product **2b** was obtained in pure form by crystallization, although at this point the product was difficult to analyze by NMR spectroscopy. Both ^1H and ^{13}C NMR spectra recorded in organic solvents were impossible to assign; the proton spectrum contained only broad signals with few visible couplings. However, when acquired in ≈0.3% NaOD in D_2O, a useful spectrum with

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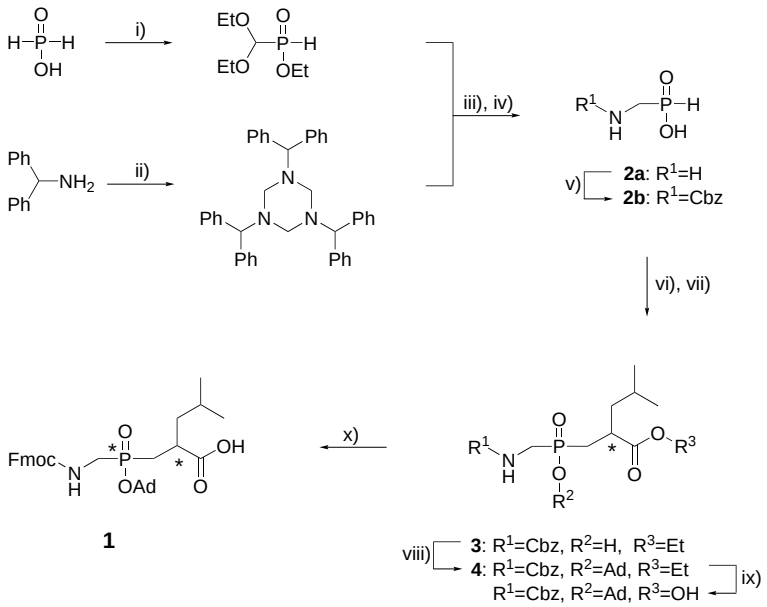
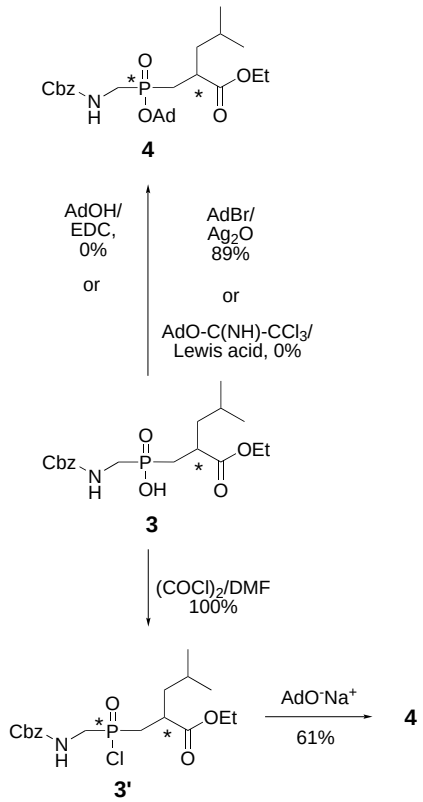


Scheme 1. Preparation of building block **1**, suitable for the solid-phase synthesis of phosphinic peptides containing the phosphinic -GΨ{P(O)OH-CH_2}L- moiety: i) $CH(OEt)_3$, RT, 55% (distilled); ii) aqueous CH_2=O, cat. KOH, 90°C, 87%; iii) toluene, 100°C; iv) 48% aqueous HBr, Δ, 73%; v) Cbz-Cl, K_2CO_3, H_2O, RT, 72%; vi) HMDS, 110°C; vii) CH_2=CH(*i*Bu)-CO_2Et, 90°C, 96%; viii) AdBr, Ag_2O, $CHCl_3$, Δ, 89%; ix) NaOH (EtOH), RT; x) H_2/Pd(C), Fmoc-OSu, $NaHCO_3$, MeOH/EtOAc/H_2O, RT, 65%.

sharp peaks was obtained and the significant double triplet from the phosphorus-bonded proton was clearly visible. This phenomenon has not previously been reported for other similar phosphinic acids. The broad peaks persisted until the phosphinic acid had been protected, suggesting that aggregation or micelle formation occurred as a result of hydrogen bonding in the amphiphilic compound (phosphinic acids are known to form hydrogen-bonded dimers in nonpolar solvents).[64]

Conversion of **2b** into **3** was performed by Michael addition of the phosphinate to ethyl α-isobutylacrylate.[52, 65] Reaction of **2b** with five equivalents of hexamethyldisilazane (HMDS) produced the corresponding bis(trimethylsilyl)phosphonite in situ, and subsequent addition to the acrylate yielded the pseudodipeptide **3**. The NMR spectral analysis of **3** was rendered difficult for the same reason as for **2b**. The problems were overcome by recording the spectra in aqueous base. The basic conditions gave rise to a partial hydrolysis of the carboxylic ester as seen by slow disappearance of a broad signal at $\delta = 4.12$ (ethyl ester) and formation of a sharp quartet at $\delta = 3.64$ (EtOD). Similarly, in the ^{31}P NMR spectrum, a peak at $\delta = 36.5$ slowly replaced the peak at $\delta = 34.5$, when the sample was left at room temperature. The crude product was collected in 96% yield and was essentially pure according to analysis by thin-layer chromatography (TLC) and NMR spectroscopy. However, the analysis of the product by electrospray mass spectrometry (ES-MS) was difficult because of the low intensity of the molecular ion.

A previously suggested procedure for the subsequent Ag^I-mediated protection of the phosphinic acid with 1-adamantyl bromide (AdBr)[52] was not reproducible in our hands. However, rearranging the order of reagent addition gave a satisfactory reaction (yield of **4** 89%) on a medium scale. Performing this reaction on a larger scale (74.5 mmol) was more problematic. Work-up of the complex product mixture was difficult and the yield decreased to 35%. Thus, this reaction did not seem suitable for large-scale synthesis particularly considering the amount of expensive silver reagent required. Alternative procedures for esterification were therefore considered (Scheme 2). Esterification of the phosphinic acid with 1-adamantanol (AdOH) mediated by ethyl-(dimethylamino)-propylcarbodiimide (EDC)[66] was not successful. However, reaction of the corresponding phosphinic acid chloride (**3′**) (generated cleanly in situ with $(COCl)_2$/DMF[67] as confirmed by ^{31}P NMR spectroscopy) with sodium 1-adamantanolate in THF at 60°C gave **4** in 61% yield. The moderate yield is most probably a result of the fact that both the phosphinic acid and the nucleophile (AdO^-) are very sterically hindered. Thus, an S_N1-type reaction must be considered as the most effective reaction type for the esterification of phosphinic acids with a tertiary alcohol. Therefore, the reaction of



Scheme 2. Reaction conditions investigated to improve the phosphinic acid esterification in dipeptide analogue **3**.

1-adamantyl trichloroacetimidate with **3** catalyzed by different acids and Lewis acids was investigated. However, only trace amounts of **4** were formed in this reaction. 1-Adamantyl trichloroacetimidate was prepared in 96% yield by a standard procedure.[68] These observations indicate that the Ag^I-mediated S_N1-type reaction with 1-adamantyl bromide is a superior method for esterification of the phosphinic dipeptide **3**.

Both mass spectrometric and NMR spectral analysis of the compound were possible after the esterification of the phosphinic acid. However, the esterification produced a chiral center at the phosphinic acid[27, 52] resulting in a mixture of two diastereomeric sets of enantiomers. This complicated the analysis of ^1H and ^{13}C NMR spectra, and gave rise to two sets of resonances in the ^{13}C and ^{31}P NMR spectra.

Carboxylic ester hydrolysis of **4** was followed by the exchange of Cbz at the α-amino group for Fmoc (9-fluorenylmethyloxycarbonyl), which has previously been performed on other phosphinic dipeptides by a two-step procedure.[52] However, the desired product **1** was not observed when following the reported method. Although it has previously been indicated that the phosphinic adamantyl ester is unstable to standard hydrogenolytic conditions,[52] a one-pot procedure could be developed in which 9-fluorenylmethyl succinimidyl carbonate (Fmoc-OSu) was added directly to the hydrogenation mixture containing $NaHCO_3$ as a basic buffer. The Fmoc group is known to be rather stable towards hydrogenolytic conditions.[69] However, prolonged exposure of the mixture to hydrogen at atmospheric pressure resulted in decomposition of the formed Fmoc-protected dipeptide analogue **1**. Therefore, simultaneous hydrogenolysis and Fmoc protection were performed for 80 min, leading to almost complete removal of the Cbz group and a reasonable yield (65%) of **1** on a millimolar scale. A tenfold scale-up of this procedure resulted in lower yield (41%), plus the formation of other by-products.

In summary, a straight-forward procedure for preparation of a protected glycine–leucine phosphinic dipeptide building block **1** was developed. For a difficult step in this synthesis, the protection of the phosphinic acid as its 1-adamantyl ester, it was found that the procedure[52] with 1-adamantyl bromide and Ag_2O was superior to other methods.

Phosphinic peptide synthesis: Phosphinic peptides **5a/b–11a/b** (Table 1) were prepared in a multiple-column peptide synthesizer[70] by using a standard peptide synthesis[71] protocol on the PEGA[72] (polyethylene glycol polyamide)-resin. The couplings of building block **1** performed well in the Fmoc-peptide synthesis approach. The acid lability of the phosphinic adamantyl ester is comparable to that of normal *t*Bu-protected amino acids, and the protected derivatives can therefore be converted into the phosphinic acid by the trifluoroacetic acid (TFA) treatment, which simultaneously cleaves the peptide side chain protective groups. Coupling of **1** was performed with 1.5 equiv after activation with *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU)[73] and *N*-ethylmorpholine.

The peptides contained the racemic building block **1**, and each peptide was therefore a mixture of two diastereomers (indicated by **a** and **b**). This was characteristically observed in analytical HPLC as two closely eluting components of equal peak intensity. The impurities, which were minor, also frequently appeared as two close peaks. The representative analytical HPLC chromatogram of the crude peptides **6a** and **b** in Figure 2 shows the high purity of the crude pseudopeptides. The individual diastereomers were separated on a semipreparative column. Not all diasteromers could be completely separated to the baseline. However, from a synthetic point of view a troublesome resolution of the valuable building block prior to peptide assembly would not be sensible, since the diastereomers of the final peptide analogues could all be separated and isolated in good yield.

The properties and inhibitory activities of the phosphinic peptides **5a/b–11a/b** are presented in Table 2. The sequences of the inhibitors **5a/b–10a/b** were selected from cleavage sites in proteins known to be natural substrates of MMP-12 and MMP-14.[6] These proteins, bone sialo protein (BSP) and osteopontin (OPN), are noncollagenous proteins present in the extracellular matrix of the bone. Both MMP-12 and MMP-14 cleave bovine OPN selectively at

Table 1. Structures of the phosphinic peptide inhibitors **5a/b–11a/b** containing the -GΨ{P(O)OH-CH_2}L- phosphinic moiety.

	Sequence[a]									Amino acid analysis[b]									
	P_5	P_4	P_3	P_2	P_1–P_1'	P_2'	P_3'	P_4'	P_5'	A	G	K	L	P	Q[c]	R	S	V	Y
5a		V	A	Y	GΨ{PO_2H-CH_2}L	K	S	R	G	1.01	1.02	1.02	–	–	–	1.04	0.94	0.99	0.99
5b		V	A	Y	GΨ{PO_2H-CH_2}L	K	S	R	G	1.01	1.02	1.00	–	–	–	1.04	0.94	1.00	0.97
6a			A	Y	GΨ{PO_2H-CH_2}L	K	S	G		1.00	1.03	1.02	–	–	–	–	0.94	–	1.01
6b			A	Y	GΨ{PO_2H-CH_2}L	K	S	G		1.01	1.02	1.02	–	–	–	–	0.94	–	1.01
7a			V	Y	GΨ{PO_2H-CH_2}L	R	S	G		–	1.02	–	–	–	–	1.06	0.94	0.99	0.98
7b			V	Y	GΨ{PO_2H-CH_2}L	R	S	G		–	1.03	–	–	–	–	1.05	0.96	0.97	0.98
8a		G	L	A	GΨ{PO_2H-CH_2}L	W	L	P	G	0.98	1.99	–	2.04	0.98	–	–	–	–	-
8b		G	L	A	GΨ{PO_2H-CH_2}L	W	L	P	G	0.98	2.00	–	2.06	0.95	–	–	–	–	-
9a			L	A	GΨ{PO_2H-CH_2}L	W	L	G		0.97	1.03	–	2.00	–	–	–	–	–	–
9b			L	A	GΨ{PO_2H-CH_2}L	W	L	G		0.99	1.02	–	1.99	–	–	–	–	–	–
10a			L	A	GΨ{PO_2H-CH_2}L	Q	L	G		0.99	1.01	–	1.99	–	1.01	–	–	–	–
10b			L	A	GΨ{PO_2H-CH_2}L	Q	L	G		0.99	1.01	–	1.99	–	1.02	–	–	–	–
11a	A	G	P	L	GΨ{PO_2H-CH_2}L	Y	A	R	G	1.97	2.00	–	1.06	0.95	–	1.05	–	–	0.97
11b	A	G	P	L	GΨ{PO_2H-CH_2}L	Y	A	R	G	1.99	2.00	–	1.08	0.94	–	1.03	–	–	0.97

[a] All peptides are of the type H-phosphinic peptide-OH (as depicted in Figure 1); the absolute configuration of the -GΨ{PO_2H-CH_2}L- moiety was not determined. [b] W-residues were not detected because the samples were analyzed with aqueous 6 M HCl which results in partial decomposition of the indole ring. [c] Q-residues were detected as E-residues.

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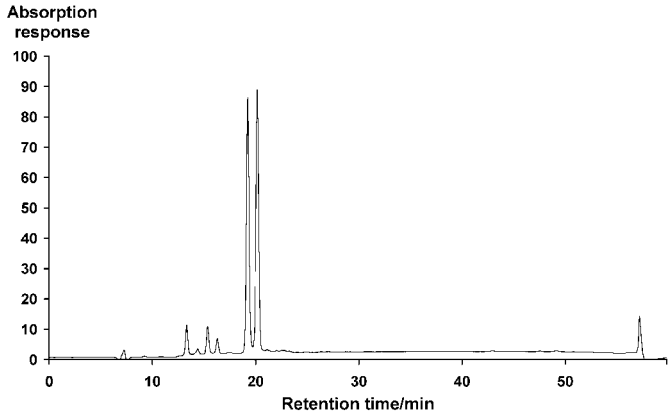

Figure 2. Analytical HPLC chromatogram of the crude phosphinic peptide inhibitor **6**.

Table 2. Characteristics of the phosphinic peptide inhibitors **5a/b**–**11a/b** containing the -GΨ{P(O)OH-CH_2}L- phosphinic moiety.

Compound	MW	Found ES-MS $[M+H]^+$	R_f value [min][a]	Yield in SPPS[b] [mg] → [%]	K_i [μM]
5a	985.1	985.6	20.0	8 → 24	0.29
5b	985.1	985.5	21.5	8 → 24	7.8
6a	729.8	730.6	19.0	6 → 24	3.5
6b	729.8	730.4	20.0	7 → 28	70
7a	785.8	786.1	20.0	5 → 19	0.68
7b	785.8	786.3	21.0	7 → 26	16
8a	918.1	918.5	23.0	9 → 29	17
8b	918.1	918.5	25.0	7 → 22	390
9a	763.9	764.4	29.0	6 → 23	11
9b	763.9	764.5	31.0	5 → 19	150
10a	705.8	706.4	23.0	6 → 25	29
10b	705.8	706.4	25.5	5 → 21	8.8
11a	1009.1	1009.5	25.0	5 → 15	0.0006
11b	1009.1	1009.4	25.5	7 → 20	0.0034

[a] Each HPLC analysis was carried out by going from 0 to 100 % B-buffer in 50 min, i. e., a gradient of 2 % B min^{-1} (see Experimental Section). [b] Yields are based on the loading of the resin measured after the first coupling.

VAYG[159]–L[160]KSR which is analogous to the human OPN sequence, VVYG[159]–L[160]RSK. Similarly, both proteases cleave bovine BSP selectively at GLAA[134]–I[135]WLP and human BSP at GLAA[129]–I[130]QLP.[6] Since MMPs generally share similar substrate specificities, it was assumed that these substrate sequences for MMP-12 and MMP-14 could be used to construct potential inhibitors of MMP-9. Thus, the G–L and A–I cleavage sites of OPN and BSP, respectively, were replaced with the -GΨ{P(O)OH-CH_2}L- phosphinic part to obtain inhibitors. As seen in Table 2, the K_i values are generally in the μM range, and thus considerably higher than the values for the inhibitors derived from the small peptide substrates **11a/b**. These inhibitors were based on a sequence derived from a quenched fluorogenic MMP substrate,[18] H-Abz-GPLG–LY(NO_2)AR-NH_2, which has previously been used in our investigations ($k_{cat}/K_M = 1.5 \times 10^5\,M^{-1}s^{-1}$ for MMP-9). Replacement of the G- and L-residues at the cleavage site by a -GΨ{P(O)OH-CH_2}L- phosphinic part gave the very effective inhibitors **11a/b**. In fact, **11a** ($K_i = 0.6$ nM for MMP-9) is almost as effective as the well known highly potent hydroxamate/sulfide-based general MMP inhibitor BB-94[74] ($K_i = 0.05$ nM for MMP-9).

The individual diastereomers displayed up to two orders of magnitude difference in inhibitory activities towards MMP-9. This is expected as a result of the strict stereochemical requirements in the active site of the enzyme. Noteworthy, for all peptides a tendency was observed for the later eluting compound to give the highest K_i values; this indicates the same order of elution of the seven diastereomeric pairs. However, this conclusion would require independent proof by the structures. It does, however, suggest that there is a correlation between the overall polarity of the compound and its potency as an inhibitor. When comparing similar sequences (such as **5a/b** and **6a/b**, or **8a/b** and **9a/b**), there is no clear tendency for the longer sequence to constitute the most effective inhibitor: **5a/b** is one order of magnitude more potent than **6a/b**, while there is no significant difference in K_i value between the pairs **8a/b** and **9a/b**.

The substrate specificity of MMPs is determined not only by the amino acid sequence in the vicinity of the cleavage site. There is evidence that MMP-2, a smaller gelatinase, contains two substrate recognition sites, one at the active site, controlling the specificity of small peptide substrates, and one further away from the active site, which is responsible for the specificity of large protein substrates such as gelatin and collagen. This is also true for MMP-9, known to bind to extracellular bone matrix proteins with the hemopexin domain or the fibronectin-like insert, and this may account for the lower activity of the inhibitors based on the MMP cleavage site of protein substrates **5a/b**–**10a/b** compared with the peptide substrates **11a/b**.

Conclusion

The synthesis of phosphinic pseudopeptides containing the -GΨ{P(O)OH-CH_2}L- phosphinic part was described. These were prepared by standard solid-phase peptide synthesis using a protected phosphinic dipeptide building block **1** to introduce the phosphinic moiety. The building block was prepared by a useful, but somewhat lengthy procedure starting from available materials. Although analysis of the intermediate phosphinic acids was difficult, the procedure gave access to the final building block **1** well designed for the solid-phase peptide synthesis by the Fmoc-based strategy. Thus, coupling of a small excess of the building block in a standard Fmoc-based peptide synthesis with conventional coupling procedures in a multiple-column synthesizer provided 14 inhibitors as seven diastereomeric pairs, which could easily be separated. The crude pseudopeptides obtained from the solid-phase synthesis were of high purity according to HPLC analysis. They proved to be moderate to excellent inhibitors of MMP-9. In fact, one of the inhibitors (**11a**) has a K_i value which is in the range of the most potent existing inhibitors for MMP-9. Finally, this work is a further example, in which the replacement of the amino acids in the P_1 and P_1' subsites of a good protease substrate with a phosphinic dipeptide may result in an efficient inhibitor of the same protease.

Current work in our laboratory is aiming at the extension of this method to the synthesis of pseudopeptide split-and-combine solid-phase inhibitor libraries with various phos-

phinic acid isosteric dipeptides as building blocks in combination with substrate indicators.[76]

Experimental Section

Abbreviations: AMPA: 4-aminophenylmercuric acetate, DCM: dichloromethane, DMF: *N*,*N*-dimethyl formamide, DIPEA: diisopropylethylamine, HMBA: hydroxymethylbenzoic acid, MSNT: 2,4,6-mesitylenesulfonyl-3-nitro-1,2,4-triazole, Dhbt-OH: (3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine), VLC: vacuum liquid chromatography. The IUPAC recommendations for amino acid notation with one letter codes has been employed, see http://www.chem.qmw.ac.uk/iupac.

General procedures: Anhydrous solvents were obtained by storing analytical quality solvents over 3 or 4 Å activated molecular sieves; the water content was then verified to be below 30 ppm by Karl Fischer titration, except for DMF which was fractionally distilled and stored over 4 Å molecular sieves. PEGA-resin was purchased from Polymer Laboratories, England. All other synthetic starting materials were purchased from Fluka, Aldrich, NovaBiochem, or Bachem and used without further purification. NMR data were acquired on a Bruker Avance DRX 250 or Varian 500 MHz Unity Inova spectrometer and were referenced to $CHCl_3$ ($\delta = 7.24$, ^{1}H and $\delta = 77.0$, ^{13}C), HDO ($\delta = 4.72$, ^{1}H), MeOD ($\delta = 3.30$, ^{1}H), or 85% aqueous H_3PO_4 ($\delta = 0$, ^{31}P). Electrospray mass spectra were obtained on a Fisons VG Quattro 5098 mass spectrometer (mobile phase 50% aq. MeCN, 8 μL min^{-1}, sample: 10 μL ≈ 20 pmol μL^{-1}). Melting points were measured on a Büchi B-540 and were uncorrected. VLC[77] was performed with a tightly packed column of Merck silica gel 60 H; TLC plates used were Merck silica gel 60 F_{254} on aluminum. TLC plates were visualized with a UV lamp, and developed with the AMC reagent (21 g $(NH_4)_6Mo_7O_{24}$, 1 g $Ce(SO_4)_2$, 31 mL H_2SO_4, 500 mL water) or ninhydrin spray (5 g ninhydrin in 100 mL EtOH). Analytical HPLC was performed on a Waters system (490E detector, two 510 pumps with gradient controller and ∅ 8 mm RCM C_{18} column); the semipreparative purification of peptide analogues **5a/b**–**11a/b** was carried out on a Merck-Hitachi system (L-4250 UV/Vis detector and L-6250 pump) connected to a Waters ∅ 25 mm RCM C_{18}-column. All RP-HPLC procedures were carried out with a linear gradient system consisting of two buffers: A (0.1% TFA in water) and B (0.1% TFA in MeCN/H_2O 9:1). A Kontron SFM-25 spectrofluorometer used for inhibitor assays.

Aminomethylphosphinic acid (2a):[78] Phosphinic acid **2a** was efficiently prepared analogous to a literature procedure[59] on a 300 mmol scale, although the pure amino acid could not be successfully precipitated with propylene oxide as previously reported.[57–60] Therefore, the aqueous solution (≈300 mL) of the crude hydrobromide was concentrated to dryness with silica gel (≈200 g), split in two portions, each portion placed on a short VLC column (∅ 10 cm, L 10 cm, 250 mL fractions) and purified with acetone (2 to 4 fractions) and acetic acid/water/acetone/isopropyl alcohol 2:2:8:5 (15 to 20 fractions). The product was obtained in 73% yield with this procedure, which was pure according to the ^{1}H and ^{31}P NMR spectra,[57] but still contained 16% hydrobromide as determined by elemental analysis.

Benzyloxycarbonyl aminomethylphosphinic acid (2b): Phosphinic acid **2b** was prepared from **2a** analogous to a standard procedure.[63] Compound **2a** (5.00 g, 52.6 mmol) was dissolved in water with K_2CO_3 (17.0 g, 123 mmol, 2.34 equiv), and Cbz-Cl (95%, 10.3 mL, 68.4 mmol, 1.3 equiv) was added dropwise at 0 °C. After the solution was stirred at RT for 19 h, further Cbz-Cl (2 × 0.5 equiv) was added and the solution was stirred for 1 h until complete consumption of **2a** was observed. The aqueous phase was diluted with water (50 mL) and washed with ethyl acetate (3 × 50 mL) and toluene (30 mL). It was then mixed with ice (150 g) and acidified with concentrated aqueous HCl (21.8 mL, 5 equiv). The product was dissolved in ethyl acetate (300 mL) and the aqueous phase was extracted with ethyl acetate (2 × 150 mL). The organic phase was dried with $MgSO_4$ and concentrated to yield a white solid (8.68 g, 72%). The crude product was crystallized from ethyl acetate/light petroleum to afford white crystals in 63% yield. M.p. 95–96 °C; ^{1}H NMR (250 MHz, 0.3% NaOD in D_2O, 25 °C): $\delta = 3.18$ (dd, ^2J(H,P) = 10.5 Hz, ^3J(H,H) = 1.6 Hz, 2H), 5.07 (s, 2H), 6.86 (dt, ^1J(H,P) = 523.3 Hz, ^3J(H,H) = 2.0 Hz, 1H), 7.36 (s, 5H); ^{31}P NMR (250 MHz, 1% NaOD in D_2O, 25 °C): $\delta = 21.6$.

***P*-(Benzyloxycarbonylaminomethyl)-*P*-(ethyl 2-isobutylpropionate-3-yl)-phosphinic acid (3)**: A mixture of **2b** (5.00 g, 21.8 mmol) and HMDS (23 mL, 109 mmol, 5 equiv) was stirred under Ar in a dried 250 mL round-bottom flask. The flask was heated to 115 °C, where **2b** melted and then reprecipitated forming a suspension in HMDS. The reaction was accompanied by the liberation of NH_3. The temperature was maintained at 115 °C for 2 h after which time the temperature was lowered to 95 °C within 35 min. Ethyl α-isobutylacrylate (4.76 mL, 28.3 mmol, 1.3 equiv) was added dropwise to the opaque mixture within 35 min. After stirring for 3.5 h, the mixture became clear and colorless. The temperature was lowered to 70 °C and EtOH (65 mL) was carefully added. Cooling to room temperature and subsequent concentration in vacuo yielded a white solid, which was redissolved in ethyl acetate (570 mL) and washed with HCl (50 mL, 1M). The organic phase was separated and the aqueous phase was extracted with ethyl acetate (3 × 25 mL). The combined organic extracts were washed with water (2 × 50 mL), saturated brine (50 mL), dried with Na_2SO_4, and concentrated to give a white solid, which was dried at high vacuum for 4 h. Yield 8.03 g (96%). The product could also be precipitated in chloroform by addition of light petroleum. However, no significant increase in purity was observed. The crude product, which was pure according to TLC (R_f = 0.21, chloroform/methanol 5:1 + 5% acetic acid) and NMR spectroscopy, was characterized by 1D and 2D NMR (^{1}H and ^{1}H COSY NMR). ^{1}H NMR (500 MHz, 0.3% NaOD in MeOD/C_5D_5N 1:1, 25 °C): $\delta = 0.81$ (m, 3H), 0.86 (m, 3H), 1.15 (t, ^3J(H,H) = 7.5 Hz, 3H), 1.42 (m, 1H), 1.49 (m, 1H), 1.56 (m, 1H), 1.79 (m, 1H), 2.10 (m, 1H), 3.05 (m, 1H), 3.40 (m, 1H), 3.50 (m, 1H), 4.12 (m, 1H), 5.80 (m, 1H); NH was not visible due to H/D exchange. The aromatic signals were superimposed with residual C_5H_5N in the deuterated pyridine, but were visible in another solvent mixture (250 MHz, 0.3% NaOD in D_2O/MeOD 1:1, 25 °C) at $\delta = 7.50$ (s, 5H); ^{31}P NMR (250 MHz, 1% NaOD in D_2O/MeOD 1:1, 25 °C): $\delta = 34.5$; ^{13}C NMR spectral data could not be obtained due to low solubility in aqueous NaOD; ES-MS *m/z* (%): 386.1 $[M+H]^+$; calcd for $C_{18}H_{28}NO_6P$: 385.2.

***O*-Adamantyl *P*-(benzyloxycarbonyl-aminomethyl)-*P*-(ethyl 2-isobutyl-propionate-3-yl)phosphinate (4)**:

Procedure A [from *P*-(benzyloxycarbonylaminomethyl)-*P*-(ethyl 2-isobutylpropionate-3-yl)phosphinic acid (**3**)]: A mixture of phosphinic acid **3** (820 mg, 2.12 mmol) and Ag_2O (986 mg, 4.25 mmol, 2 equiv) was refluxed under Ar in dry chloroform (3 mL) for 15 min, after which a solution of AdBr (503 mg, 2.34 mmol, 1.1 equiv) was added dropwise to the refluxing suspension over a period of 30 min. Reflux was continued for 1 h and the mixture was stirred overnight. The crude mixture was filtered through celite, concentrated, and purified by VLC with toluene/ethyl acetate 3:1 as eluent to afford **4** as a highly viscous syrup (977 mg, 89%). When pure, the compound crystallized as a white solid. TLC: $R_f = 0.40$ (toluene/ethyl acetate 1:3).[79] The ^{1}H NMR spectrum was assigned with ^{1}H COSY and ^{13}C NMR with ^{1}H–^{13}C COSY experiments. ^{1}H NMR (250 MHz, $CDCl_3$, 25 °C): $\delta = 0.86$ (d, ^3J(H,H) = 7.0 Hz, 3H), 0.92 (d, ^3J(H,H) = 7.0 Hz, 3H), 1.22 (t, ^3J(H,H) = 7.0 Hz, 3H), 1.22 (t, ^3J(H,H) = 7.0 Hz, 3H), 1.33 (m, 1H), 1.49 (m, 1H), 1.56 (m, 1H), 1.58 (br s, 6H), 1.72 (m, 1H), 2.01 (br s, 6H), 2.23 (br s, 3H), 2.18 (m, 1H), 2.80 (m, 1H), 3.50 (br m, 2H), 4.10 (m, 2H), 5.10 (s, 2H), 5.30 (m, 1H), 7.31 (s, 5H); ^{13}C NMR (250 MHz, $CDCl_3$, 25 °C): $\delta = 14.1$, 22.3 (2d), 25.8, 31.1, 31.4 (2d), 35.6, 37.4 (d), 41.4 (2d), 43.4 (2d), 44.7 (d), 60.6 (d), 67.6, 83.3, 128.1–130.0; ^{31}P NMR (250 MHz, $CDCl_3$, 25 °C): $\delta = 44.1$ and 44.9; ES-MS *m/z* (%): 520.5 (90) $[M+H]^+$, 542.4 (100) $[M+Na]^+$, 557.8 (5) $[M+K]^+$; $C_{28}H_{42}NO_6P$ (519.6): calcd C 64.72, H 8.15, N 2.70; found C 64.40, H 7.92, N 2.74.

Procedure B [from *P*-(benzyloxycarbonyl-aminomethyl)-*P*-(ethyl 2-isobutylpropionate-3-yl)phosphinic acid chloride (**3′**)]: A suspension of phosphinic acid **3** (60 mg, 0.16 mmol) and DMF (24 μL, 0.32 mmol, 2 equiv) in anhydrous THF (1.5 mL) was cooled to 0 °C under Ar and $(COCl)_2$ (15 μL, 0.17 mmol, 1.1 equiv) was added dropwise. The reaction was accompanied by evolution of gas. After 20 min, the formation of the acid chloride **3′** was complete as verified by ^{31}P NMR spectroscopy (250 MHz, $CDCl_3$, 25 °C): $\delta = 62.8$ and 63.9. NaH (60% in oil, 6 mg, 0.16 mmol, 1 equiv) was added to a solution of 1-adamantanol (26 mg, 0.17 mmol, 1.1 equiv) in anhydrous THF (1 mL) and heated to 65 °C. The mixture was stirred for 20 min after which the solution of **3′** in THF was added dropwise over a period of 5 min. Precipitation of NaCl was observed shortly after addition of **3′**. After stirring for 1 h, the mixture was concentrated and purified by preparative TLC (2 mm silica-coated plates on glass, toluene/ethyl acetate 1:1) to yield 49 mg (61%).

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***O*-Adamantyl *P*-(9-fluorenylmethyloxycarbonyl-aminomethyl)-*P*-(2-isobutylpropionic acid-3-yl)phosphinate (1)**: Aqueous NaOH (11.8 mL, 4 M, 11.8 mmol, 4 equiv) was added dropwise to a solution of phosphinate **4** (5.55 g, 11.8 mmol) in EtOH (100 mL). The resulting opaque solution was stirred for 24 h, after which **4** was consumed. The solution was concentrated, partitioned between ethyl acetate (100 mL) and water (50 mL), cooled to 0 °C, and aqueous HCl (20 mL 2.4 M, then 8 mL 1 M) was slowly added to adjust the pH to 2. The aqueous phase was extracted twice with ethyl acetate (50 mL), the combined organic phases were dried with $MgSO_4$ and concentrated to dryness to afford a white solid carboxylic acid (6.30 g; 100 % yield corresponds to 5.82 g) that consisted of one major product according to TLC ($R_f = 0.40$, toluene/ethyl acetate 1:3 + 5 % acetic acid). The crude carboxylic acid (200 mg, 407 μmol) was dissolved in ethyl acetate/MeOH/water (11 + 6 + 0.5 mL) and hydrogenated at atmospheric pressure in the prescence of Pd (5 % on activated carbon, 86 mg, 0.1 equiv), $NaHCO_3$ (171 mg, 2.0 mmol, 5 equiv) and Fmoc-OSu (206 mg, 610 mmol, 1.5 equiv) for 80 min. Vacuum (10 mm Hg) was then applied for 30 min and the mixture was stirred overnight. Filtration through celite and concentration gave the crude product as a sticky solid (411 mg), which was purified by VLC with chloroform/methanol 30:1 as eluent to afford pure **1** as a solid foam. Yield: 154 mg (65 %). TLC: $R_f = 0.49$ (toluene/ethyl acetate 1:3 + 5 % acetic acid). The ^{1}H NMR-spectrum was assigned with ^{1}H – ^{1}H COSY and ^{1}H – ^{13}C COSY spectra. ^{1}H NMR (250 MHz, $CDCl_3$, 25 °C): $\delta = 0.89$ (m, 6H), 1.40 (m, 1H), 1.47 (s, 6H), 1.65 (m, 1H), 1.66 (m, 1H), 1.70 (m, 1H), 1.95 (s, 6H), 1.99 (s, 6H), 2.39 (m, 1H), 2.84 (m, 1H), 3.53 (m, 1H), 3.78 (m, 1H), 4.22 (m, 1H), 4.22 (m, 1H), 4.38 (m, 1H), 6.89 (d, 1H), 7.14 – 7.74 (m); ^{13}C NMR (250 MHz, $CDCl_3$, 25 °C): 22.3 (2d), 25.5 (d), 30.7 (2d), 31.1, 35.5, 37.8 (t), 42.0 (2d), 42.5 (t), 44.2 (d), 47.0 (s), 67.5 (s), 84.0 (2d), 119.7 (s), 125.3 (d), 125.6, 127.0, 127.5, 141.1, 143.7 (d), 144.1, 156.9 (t), 177.8; ^{31}P NMR (250 MHz, $CDCl_3$, 25 %): $\delta = 46.1$ and 48.1; ES-MS *m/z* (%): 580.3 (100) $[M+H]^+$; $C_{33}H_{42}NO_6P$ (579.7): calcd C 68.38 H 7.30, N 2.42; found C 67.68, H 7.50, N 2.30.

Phosphinic peptides 5a/b – 11a/b: Synthesis protocol: Amino acid couplings were allowed to run for at least 3 h and complete coupling was monitored by a negative Kaiser test.[71] After coupling of one amino acid or building block **1**, the resin was washed with DMF, followed by deprotection of α-amino group with piperidine (20 % in DMF) for 2 and 10 min. After the resin was washed with DMF, the cycle was repeated with another amino acid or building block **1**. Volumes of washing solvent were 1 to 2 times the volume necessary to swell the resin and washings were 6 × 1 min unless stated otherwise. The peptides were synthesized by multiple-column peptide synthesis[70] (MCPS) in a 20 well teflon block with sintered teflon filters. For each diastereomeric pair of peptides a PEGA-resin (0.45 μmol mg^{-1}, 75 mg, 34 μmol) derivatized with Fmoc-Gly-HMBA was used. This was prepared as follows:

A preactivated (10 min) mixture of HMBA (657 mg, 4.32 mmol, 3 equiv), TBTU (1.33 g, 4.15 mmol, 2.88 equiv) and *N*-ethylmorpholine (727 μL, 662 mg, 5.76 mmol, 4 equiv) in anhydrous DMF (25 mL) was added to the resin (3.01 g swelled in DMF). After 2 h, the resin was washed with DMF, DCM, and then lyophilized overnight. The resin was swollen in dry DCM (40 mL) and Fmoc-G-OH was coupled with MSNT[80] (two couplings, 65 min and 75 min): Fmoc-G-OH (1.28 g, 4.32 mmol, 3 equiv) was dissolved in dry DCM (20 mL) together with *N*-methyl imidazole (258 μL, 266 mg, 3.24 mmol, 2.25 equiv), and when dissolved, MSNT (1.28 g, 4.32 mmol, 3 equiv) was added. The mixture was immediately added to the resin. After the second reaction, the resin was washed with DCM, lyophilized, and a fraction (525 mg, 238 μmol) of the resin was weighed out for the synthesis. It was then swelled in DMF and equally distributed between seven wells, the remaining 13 wells being sealed off. Couplings of normal amino acids were performed with N^{α}-Fmoc-protected amino acid pentafluorophenyl esters (3 equiv) in dry DMF and Dhbt-OH (1 equiv) as catalyst, by the protocol described above. Coupling of the building block **1** (1.5 equiv) was accomplished with TBTU activation (1.44 equiv) as above, and was completed in 4.5 h. After the last amino acid was attached to the resin, Fmoc was removed and the resins were washed with DMF and 12 × DCM, dried by lyophilization for 1.5 h and then treated with a cocktail composed of TFA/DCM/H_2O/MeSPh/$(CH_2SH)_2$/triisopropylsilane 66.5:20:5:5:2.5:1 for 2.5 h to remove peptide side chain protective groups. The resins were washed with 4 × AcOH (95 % in H_2O, 5 min), 2 × DMF, 2 × DIPEA (5 % in DMF), 2 × DMF, 15 × DCM, and lyophilized (1 h). The deprotected peptides were cleaved off the resin by treatment with NaOH (0.1 M, 2 × 1.5 mL each, 1 h) and each portion of the resin was washed with H_2O (4 × 0.5 mL) and MeOH (2 × 0.5 mL). The resulting solutions of **5a/b – 11a/b** (≈ 5 mM) were neutralized with HCl (0.1 M, 2.5 mL) and characterized by analytical HPLC, electrospray mass spectrometry, and amino acid analysis as presented in Tables 1 and 2.

The individual diastereomers of the peptides **5a/b – 11a/b** were isolated by preparative HPLC: The crude peptide solutions were lyophilized and redissolved in a suitable mixture of H_2O, MeOH and TFA. After filtration, the solutions were loaded to a semipreparative reversed-phase HPLC column and purified using the standard buffers A and B (2 % B min^{-1}, 10 mL min^{-1}) and UV detection at 215 nm. Fractions containing the correct compounds according to matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF) were collected and lyophilized.

Enzyme kinetic assays: The K_i values of inhibitors **5a/b – 11a/b** against MMP-9 were determined with increasing concentrations of inhibitors, which were preincubated with a constant amount (0.2 nM final concentration) of MMP-9 for one hour at 37 °C. APMA-activated mouse recombinant MMP-9 was obtained and purified from the cell culture medium of a baby hamster kidney (BHK) cell line overexpressing MMP-9.[81] The remaining free enzyme was then analyzed by fivefold dilution of the preincubation mixture into an assay buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 50 μM $ZnSO_4$, 0.05 % (*v*/*v*) Brij-35) containing 6.5 μM of the quenched fluorogenic MMP-substrate Mca-PLG – L-Dpa-AR-NH_2[18] and incubated at 37 °C. The time course of the reaction was followed by recording the increase in fluorescence ($\lambda_{ex} = 320$ nm, $\lambda_{em} = 387$ nm). The initial velocities were calculated and plotted against the concentration of inhibitor. The K_i value was determined by nonlinear regression fit.

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[78] The synthesis of **2a** involves the use of the pentavalent hypophosphorus acid derivative, ethyl (diethoxymethyl)phosphinate. Anhydrous hypophosphorus acid is reported to be required for the preparation of this compound, but we observed that *explosive* substances were formed in this reaction when hypophosphorus acid containing less than 5% water was used (rigorously dried by coevaporation several times with 1,4-dioxane below RT). Instead, 95% aqueous hypophosphorus acid obtained by simple rotary evaporation of the commercial 50% acid at high vacuum was found to be sufficient for this purpose.
[79] The acid-protected phosphinic dipeptide building blocks are present as two diastereomeric sets of enantiomers. These are indicated by two closely running spots on TLC, but also by the multiple signals observed for some resonances in both ^{1}H, ^{13}C, and ^{31}P NMR. R_f values given here are mean values for the two spots.
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