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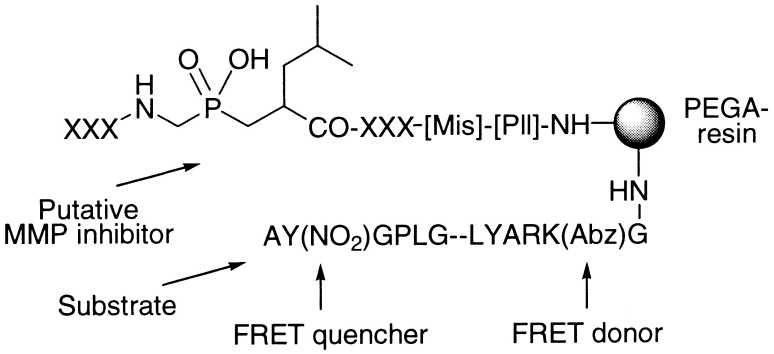

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Solid Phase Combinatorial Library of Phosphinic Peptides for Discovery of Matrix Metalloproteinase Inhibitors†

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A solid phase combinatorial library of 165 000 phosphinic peptide inhibitors was prepared and screened for activity against MMP-12. The inhibitors of the library had the structure XXX-GΨ{PO_2H-CH_2}L-XXX, in which X is an arbitrary amino acid and GΨ{PO_2H-CH_2}L is a Gly-Leu phosphinic dipeptide analogue. The library was constructed as a one-bead-two-compounds library so that every bead contained a common quenched fluorogenic substrate and a different putative inhibitor. In addition, the inhibitor part was prepared by ladder synthesis. After incubation with MMP-12, beads containing active inhibitors were selected, and the inhibitor sequences were recorded using MALDI-TOF MS. Statistical analysis of the sequences obtained from 86 beads gave rise to a consensus sequence which was resynthesized along with 20 related sequences. Three truncated sequences and 16 sequences originally present on beads were also resynthesized. The inhibitors were investigated in an enzyme kinetic assay with MMP-12 showing that the compounds derived from the consensus sequence were strong inhibitors with K_i values down to 6 nM, whereas the sequences originally present on beads varied in potency with K_i values from micromolar to nanomolar. Truncated sequences derived from the consensus sequence were poor inhibitors of MMP-12.

Introduction

Most physiological events involve proteolytic cleavage as a key transformation triggering cascades of biochemical reactions leading to cellular maturation, migration, resorption, or host invasion. These events may be modulated by application of inhibitors that prevent the proteolytic activity. The development of protease inhibitors has traditionally focused on rational design from knowledge about either structure of substrates or the active site of the protease from an X-ray structure of the enzyme.[1] Compounds designed by this concept are synthesized individually and tested in an enzyme kinetic solution assay to determine K_i values. This methodology has provided a large number of potent inhibitors of a large variety of proteases, including MMPs.[2] These enzymes have been in focus as targets for drug development because of their involvement in a number of physiological and pathological conditions.[3] MMPs present in the extracellular matrix of bone are of importance in relation to bone physiology and pathology due to their ability to degrade proteins, among these collagen. Thus, it is believed that MMPs, together with cysteine proteases, are responsible for the increased resorption of the organic bone constituents occurring under pathological conditions such as bone metastasis and osteoporosis. Inhibition of MMPs is therefore important in the treatment of these conditions. Although the field of synthetic MMP inhibitors has been dominated by compounds containing a hydroxamic acid function,[2] several examples of potent inhibitors based on phosphorus peptide isosteres have been reported.[4−10]

As the speed of biochemical testing of potent inhibitors has increased, a bottleneck in the development of inhibitors has appeared at the stage of chemical synthesis. The design, preparation, and screening of chemical libraries,[11−15] both in solution and on solid support, has therefore become an important part of fast development of protease inhibitors. An obvious advantage of combinatorial libraries compared to structure based design is that a huge number of compounds are screened simultaneously, and from these the most potent inhibitors are selected. Another important advantage is that

† Abbreviations. Abz: 2-aminobenzoyl; AcODhbt: 3-acetoxy-3*H*-benzo-[*d*][1,2,3]triazin-4-one; Ad: 1-adamantyl; Alloc: allyloxycarbonyl; Boc: *tert*-butoxycarbonyl; Bu^t: *tert*-butyl; Bu^i: isobutyl; DCM: dichloromethane; DIPEA: diisopropylethylamine; DMF: dimethylformamide; Dpa: *N*-3-(2,4-dinitrophenyl)-l-2,3-diaminopropionyl; Fmoc: 9-fluorenylmethoxycarbonyl; FRET: fluorescence resonance energy transfer; GΨ(PO_2H-CH_2)L or {GL}: Gly-Leu phosphinic dipeptide obtained by replacing the conventional peptide bond (-CO-NH-) with -PO_2H-CH_2-; KQ: designation for Lys and Gln; LIJ: designation for Leu, Ile, and Hyp; MALDI-TOF MS: matrix assisted laser desorption/ionization time-of-flight mass spectrometry; Mca: (7-methoxycoumarin-4-yl)acetyl; MCPS: multiple column peptide synthesis; MMP: matrix metalloproteinase; [Mis]: mass/ionization spacer; NEM: *N*-ethylmorpholine; PEGA: poly(ethylene glycol)-polydimethyl acrylamide resin; Pfp: pentafluorophenyl; [Pll]: photolabile linker; Pmc: 2,2,5,7,8-pentamethylchroman-6-sulfonyl; RP-HPLC: reversed phase high-pressure liquid chromatography; TBTU: *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TFA: trifluoroacetic acid; Trt: triphenylmethyl; VLC: vacuum liquid chromatography; Y(NO_2): 3-nitrotyrosine. Three- and one-letter abbreviations for amino acids are used according to the recommendations from IUPAC, see http://www.chem.qmw.ac.uk/iupac. Hyl or U was used for δ-(*R*/*S*)-δ-hydroxy-DL-lysine, and Hyp or J was used for *trans*-4-hydroxy-l-proline.

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the combinatorial library approach does not require the crystal structure of the enzyme. Although the field of combinatorial chemistry has emerged relatively recently and no procedures within the field are trivial, there are already several examples of successful drug lead identification by this method,[12−14] and inhibitors of MMPs have also been identified.[16]

To create a true combinatorial library in which the number of compounds are greater than the number of reaction vessels,[17] mixtures of compounds have to be encountered. The well-known split and combine method[18] has been employed for preparation of one-bead-one-compound solid phase combinatorial libraries,[19] a type of library which has the unique property of using single resin beads as microreactors. Although consisting of a compound mixture, these libraries display a high degree of order because each bead contains only a single compound. The generation of such libraries has been described; however, they have been used less frequently than spatially addressable libraries due the fact that direct screening and analysis is difficult without a laborious deconvolution procedure. The structure of the resin is determining for the outcome of an on-bead screening.[20] By using the hydrophilic, high-swelling resin PEGA,[21] enzymes have access to all reactive sites of the resin, allowing direct screening with large enzymes.[20,22−24] Several methods have been used for tagging individual beads during synthesis in order to enable identification of active compounds after a screening.[14] Identification of inhibitors of peptide-like nature may be accomplished by employing capping during so-called ladder synthesis of the inhibitor followed by mass spectral analysis.[25]

In the present work, potent inhibitors of MMP-12 have been identified from a solid phase combinatorial library of phosphinic peptides XXX-GΨ{PO_2H-CH_2}L-XXX, in which X is an arbitrary amino acid and GΨ{PO_2H-CH_2}L is a Gly-Leu phosphinic dipeptide analogue. It was prepared and screened using a variety of different techniques and concepts, some of which have been used previously to identify inhibitors of Subtilisin Carlsberg[15] and Cruzipain.[26] The library was designed as a *one-bead-two-compounds* library which, by combining the use of a PEGA resin, split and combine synthesis, ladder synthesis, and a quenched fluorogenic substrate, resulted in discovery of novel, potent phosphinic peptide inhibitors of MMP-12.

Results

Structure and Principle. The overall structure of the library (**1**) is depicted in Figure 1. It consists of a PEGA resin in which each bead contains two different amino groups bearing different substituents. The system of two different amino groups is designated a functional biantenna. On one part of the biantenna the putative inhibitor, XXX-GΨ{PO_2H-CH_2}L-XXX, is attached through a mass/ionization spacer [Mis] of sequence TISRTI and a photolabile linker [Pll].[27] On the other part, the quenched fluorogenic substrate[28] AY-(NO_2)GPLG- -LYARK(Abz)G is attached (k_{cat}/K_M 1.9×10^5 M^{-1} s^{-1} for MMP-12; - - indicates cleavage site). The sequence of the mass spacer was chosen because of its low susceptibility to MMP cleavage, and the Arg in the sequence

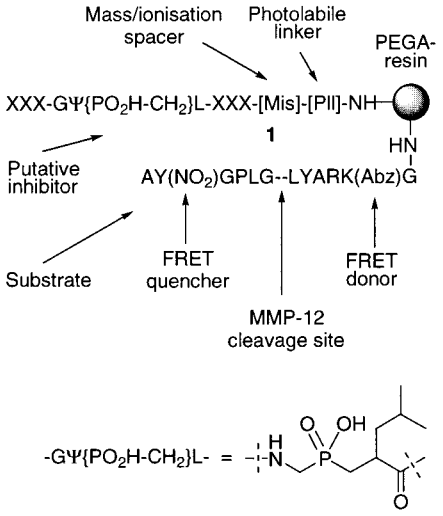


Figure 1. Structure of the one-bead-two-compounds solid phase combinatorial library **1** used to identify potent phosphinic peptide inhibitors of MMP-12. X's correspond to arbitrary α-amino acids.

facilitated desorption of the whole inhibitor sequence during MALDI-TOF mass spectrometric analysis. Preparation of the construct was accomplished using an orthogonal set of amine protective groups: the Fmoc group susceptible to base, the Alloc group which is cleaved using $Pd(PPh_3)_4$, and the Boc group susceptible to acid, along with the acid labile protective groups on amino acid side chains (Bu^t, Trt, and Pmc).

When an MMP molecule enters a bead with this construct, a competition occurs between substrate and inhibitor: the stronger the inhibitor present on a specific bead, the less substrate is cleaved. To use the library to identify inhibitors, two tasks have to be addressed: identification of beads containing potent inhibitors and determination of the structural identity of the inhibitors present on these beads.

The first problem was addressed by the incorporation of a quenched fluorogenic substrate (Figure 2). As every bead contained a different inhibitor sequence, some beads would contain potent inhibitors and others would contain poor inhibitors. A bead containing a potent inhibitor would remain unchanged with respect to fluorescence. In contrast, a bead containing a poor inhibitor would allow the enzyme to cleave the quenched fluorogenic substrate and the bead would consequently light up when viewed under a fluorescence microscope. In reality, the illumination was differentiated as varying amounts of substrate were cleaved on each bead depending on potency of the inhibitor on the specific bead. Only the very darkest beads were selected as containing potent inhibitors.

Conventional Edman sequence analysis[29,30] was not useful to determine the structural identity of the inhibitors present on the beads selected by the fluorescence assay, due to the incorporation of the phosphinic dipeptide inhibitor element. The technique of ladder synthesis[25] with capping was therefore applied to the synthesis of the inhibitor part of the library. However, instead of using β-alanine for capping as originally suggested,[25] a mixture of Fmoc- and Boc-amino acids in the ratio 9:1 was used during the couplings in every step beginning with the last Thr of the mass/ionization spacer.[31] Thus, treatment with 20% piperidine in DMF after each coupling released only 90% of the free amines for

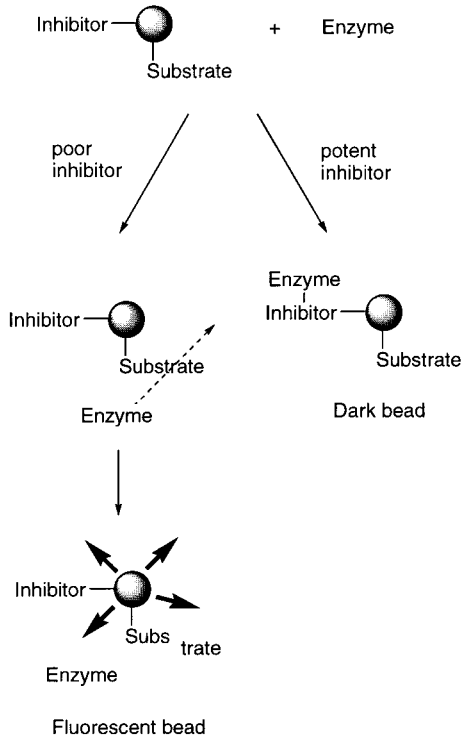


Figure 2. Principle in the use of the fluorescence quenched substrate as a marker in the inhibitor library. If the bead contains a potent inhibitor, the substrate is not cleaved and the bead remains dark. If the bead contains a poor inhibitor, the substrate is cleaved and the fluorescence from the Abz group is emitted. Thus, dark beads containing potent inhibitors are picked after incubation.

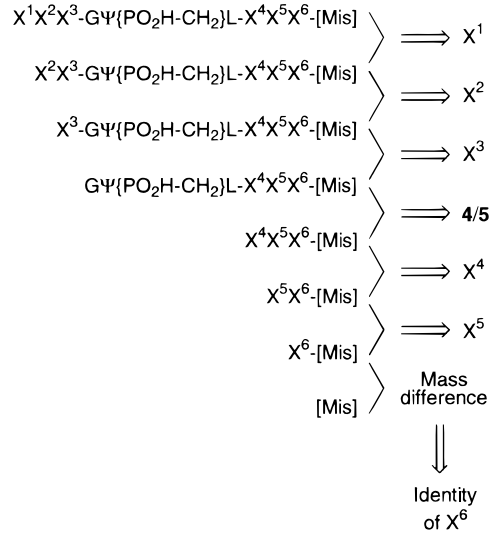
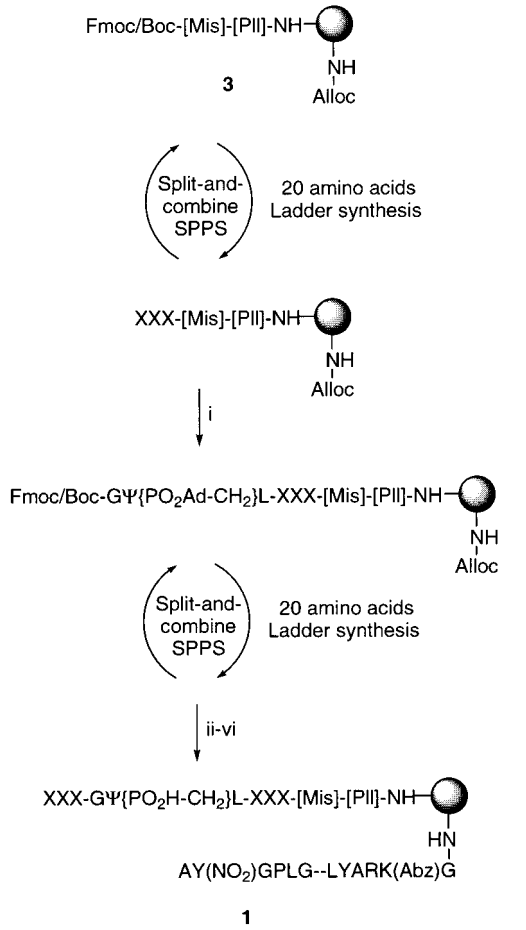


Figure 3. As a result of the ladder synthesis, a mixture of all truncated peptide fragments was obtained upon photocleavage from the resin. Thus, the sequence of the inhibitor was determined by the differences in mass between the fragments. The mass spacer [Mis], which is common for all the resin beads, has the amino acid sequence TISRTI and functioned as the base peak.

further reaction, giving rise to a mixture of truncated peptides on the inhibitor part of the functional biantenna (Figure 3). By UV irradiation with a Hg lamp, the mixture of truncated inhibitors was released from the photolabile linker, and a single MALDI-TOF MS then afforded the sequence of the active compound.

Preparation. Prior to preparation of the complete library **1** (Scheme 1) designed for screening, initial experiments were

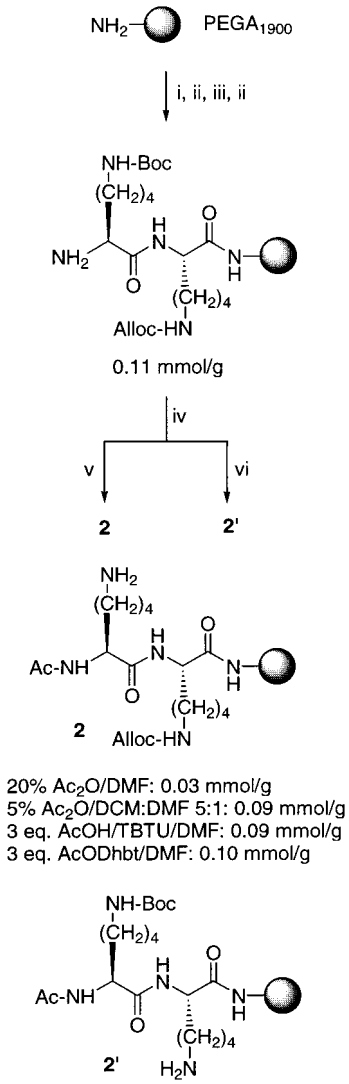
Scheme 1. Generation of the One-Bead-Two-Compounds Library of Phosphinic Peptides **1** Starting from Resin **3** (Scheme 3)[a]



[a] Including split and combine solid phase peptide synthesis using Fmoc- and Boc-amino acids in a 9:1 ratio, coupling of the Gly-Leu phosphinic dipeptide building block (**4**:**5** 9:1), and fragment condensation of a preformed protected MMP-12 substrate. Reagents and conditions: (i) **4** and **5**, TBTU, NEM; (ii) $Pd(PPh_3)_4$, AcOH, NEM; (iii) **8**, TBTU, NEM; (iv) 20% piperidine (DMF); (v) TFA/PhSMe/$(CH_2SH)_2$/$(Pr^i)_3SiH$; (vi) 0.1 M NaOH.

performed in order to secure the reliability of the fluorescence based assay for selecting active beads and of the ladder synthesis and sequencing of the inhibitors. For this purpose the functional biantenna (**2**) (Scheme 2) was prepared on which the sequence AGPL-GΨ{PO_2H-CH_2}L-YAR was assembled using ladder synthesis (Scheme 3). This sequence has previously been shown to be a strong inhibitor of MMP-9[10] and is also an inhibitor of MMP-12, though considerably less potent.[32] Preparation of the biantenna **2** resulted in minor problems. Assembly of Lys(Boc)-Lys(Alloc) on the PEGA resin proceeded by standard activation of the parent Fmoc amino acids using TBTU[33] and NEM, or using Fmoc amino acid Pfp esters. However, after the subsequent acetylation of the *N*-terminal amine with 20% Ac_2O in DMF and cleavage of the Boc group by TFA treatment, the amount of free amine present was reduced from 0.11 mmol/g of the original resin to about 0.03 mmol/g which was unacceptably low. Other acetylation conditions were investigated, and the best was found to be AcODhbt[34] (1.5−3 equiv) which resulted in a loading of 0.10 mmol/g. The allyl carbamate seemed to be less affected by the acetylation conditions as

Scheme 2. Preparation of the Functional Biantenna **2** on a $PEGA_{1900}$-Resin[a]

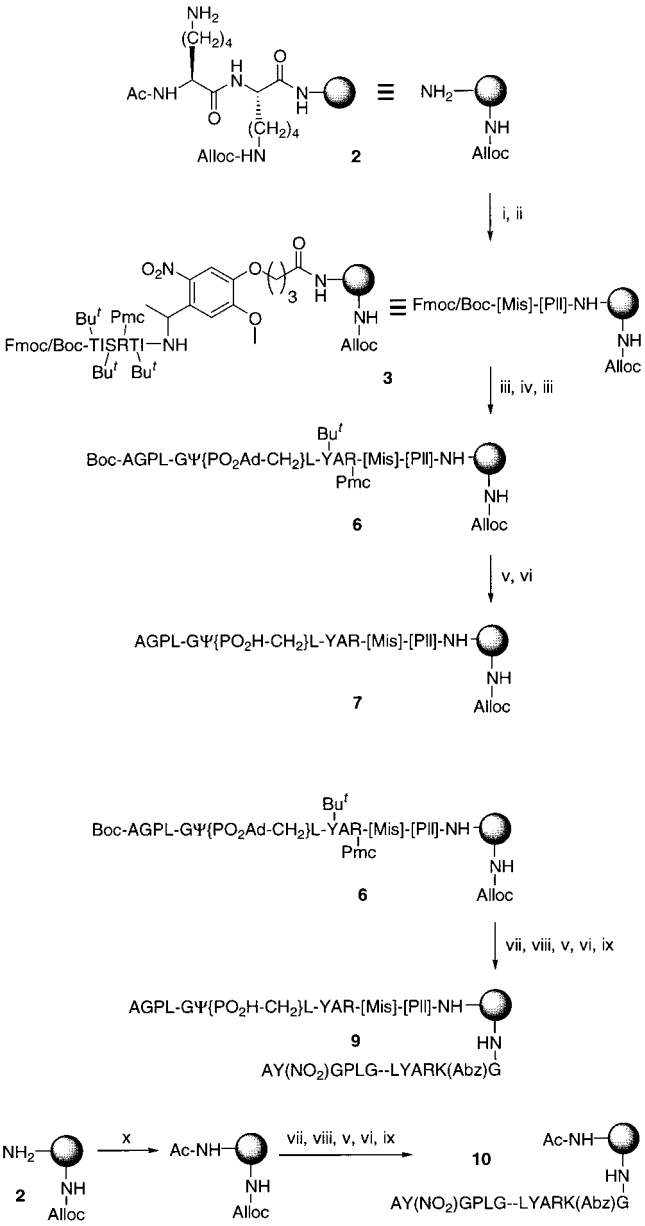


[a] Acetylation with 3 equiv of AcODhbt was found to be superior. Reagents and conditions: (i) Fmoc-Lys(Alloc)-OH, TBTU, NEM; (ii) 20% piperidine (DMF); (iii) Fmoc-Lys(Boc)-OH, TBTU, NEM; (iv) acetylation; (v) TFA:H_2O:DCM 19:1:20; (vi) $Pd(PPh_3)_4$, AcOH, NEM.

a reasonable loading of 0.09 mmol/g was found on the substrate side of the biantenna after using 20% Ac_2O in DMF (Scheme 2).

Coupling of the photolabile linker[27] on **2** was carried out using TBTU activation, and the amino acids constituting the mass/ionization spacer were coupled using Fmoc amino acid Pfp esters to obtain resin **3** (Scheme 3). Subsequent assembly of the inhibitor sequence AGPL-GΨ{PO_2H-CH_2}L-YAR with ladder capping proceeded without problems using Fmoc- and Boc-amino acids or phosphinic dipeptide building blocks **4** and **5** in the ratio 9:1. The amino acids as well as the building block mixture was coupled using TBTU activation. The phosphinic dipeptide building block, Fmoc-GΨ{PO_2Ad-CH_2}L-OH (**4**), was prepared as previously described[10] while Boc-GΨ{PO_2Ad-CH_2}L-OH (**5**) was prepared in 64% yield from **4** (Scheme 4). After the last coupling, resin **6** was treated with piperidine, TFA-scavenger cocktail, and finally 0.1 M NaOH to obtain resin **7**. A single

Scheme 3. Ladder Synthesis of the Known Inhibitor Sequence AGPL-GΨ{PO_2H-CH_2}L-YARG[a]

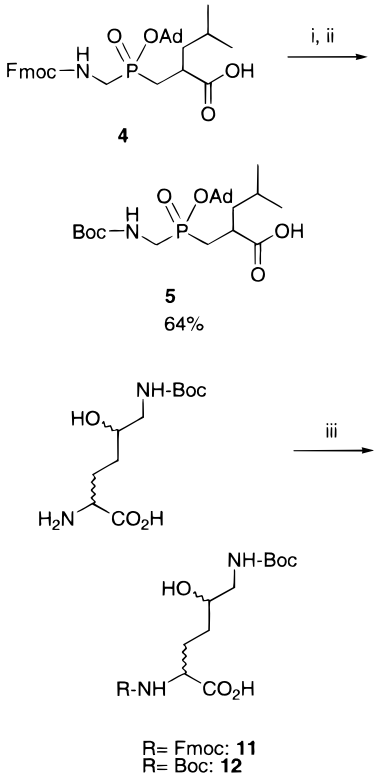


[a] The sequence of the inhibitor was recorded by MALDI-TOF MS both prior to (**7**) and after (**9**) incubation with MMP-12. Beads with (**9**) and without (**10**) inhibitor were observed under a fluorescence microscope and found to be significantly different with respect to fluorescence emission. Reagents and conditions: (i) coupling of photolabile linker [Pll], TBTU, NEM; (ii) assembly of mass/ionization spacer [Mis]; (iii) Fmoc-/Boc-amino acids 9:1, TBTU, NEM; (iv) **4** and **5**, TBTU, NEM; (v) TFA/PhSMe/$(CH_2SH)_2$/$(Pr^i)_3SiH$; (vi) 0.1 M NaOH; (vii) $Pd(PPh_3)_4$, AcOH, NEM; (viii) coupling of **8**, TBTU, NEM; (ix) MMP-12, 100 nM, pH 7.5; (x) 1.5 equiv AcODhbt.

bead was placed on a MALDI target, and after exposure to UV light the bead was swelled in a matrix solution. The mass spectrum was acquired, it clearly showed all fragments resulting from the ladder synthesis, and the sequence was easy to read. When the sodium hydroxide treatment was omitted, a considerable amount of amine trifluoroacetylation was observed in the mass spectrum.

To test the reliability of the fluorescence based assay in the selection of beads containing potent inhibitors, the resin **6** was treated with $Pd(PPh_3)_4$ and *N*-ethylmorpholinium acetate to remove the Alloc-group[35] on the substrate part of

Scheme 4. Preparation of the Boc-Protected Phosphinic Dipeptide Building Block (**5**) and δ-Hydroxylysine Building Blocks (**11** and **12**)[a]



[a] Reagents and conditions: (i) 20% piperidine (DMF); (ii) Boc_2O, Et_3N; (iii) Fmoc-OSu, Na_2CO_3 or Boc_2O, Et_3N.

the functional biantenna. A protected preformed quenched fluorogenic substrate, Boc-AY(NO_2)GPLG- -LY(Bu^t)AR-(Pmc)K(Abz(Boc))G-OH (**8**) was then coupled to the resulting free amine using TBTU activation. The substrate was prepared by standard SPPS procedures[30] using the base labile hydroxymethylbenzoic acid linker on a PEGA-resin, and it was purified by RP-HPLC using neutral eluents. After full deprotection of the peptides, resin **9** was obtained. Another resin, **10**, was prepared which also contained the quenched fluorogenic substrate. Resin **10** did, however, not contain any inhibitor, and the two resins, **9** and **10**, therefore acted as models for the fluorescence based assay. After incubation of these resins with MMP-12, **9** was expected to remain dark while the fluorescence of **10** should increase. Incubation of the resins was performed with 100 nM MMP-12 in a buffered solution containing Zn^{2+}. Inspection of the two resins under the fluorescence microscope showed that resin **9** was significantly darker than **10**. Furthermore, **10** was much darker before than after incubation.

With the synthetic and analytical methodology being established, library **1** was prepared as outlined in Scheme 1. Resin **3** was prepared with 10% capping in the last coupling of the mass/ionization spacer (Thr). Subsequent couplings were carried out with mixtures of Fmoc- and Boc-amino acids (Table 1) and TBTU activation. Twenty naturally occurring amino acids were selected. With the aim of mimicking natural substrate sequences of MMP-12, cysteine and tryptophan, which are not encountered in collagen and elastin, were substituted by 4-*trans*-L-hydroxyproline and δ-(*R*/*S*)-hydroxy-DL-lysine, both of which are amino acids

Table 1. Amino Acid Derivatives Used in the Preparation of Library **1**

amino acid	MW, Fmoc[a]	MW, Boc[a]	Fmoc-derivative, each 12 × 2.7 equiv (mg)	Boc-derivative, each 12 × 0.3 equiv (mg)
Ala	311.3	189.3	66.6	4.5
Arg(Pmc)[b]	736.4	540.8	157.5	12.8
Asn(Trt)[c]	596.7	232.2	127.6	5.5
Asp(Bu^t)	411.5	289.5	88.0	6.9
Gln(Trt)[c]	610.7	246.3	130.6	5.9
Glu(Bu^t)	425.5	303.5	91.0	7.2
Gly	297.3	175.3	63.6	4.2
His(Trt)[d]	645.5	428.2	138.0	10.2
Ile	353.4	231.4	75.6	5.5
Leu[e]	353.4	249.4	75.6	5.9
Lys(Boc)	468.5	346.5	100.2	8.2
Met	371.5	249.5	79.4	5.9
Phe	387.4	265.4	82.8	6.3
Pro	337.4	215.4	72.1	5.1
Ser(Bu^t)	383.4	261.4	82.0	6.2
Thr(Bu^t)	397.5	275.5	85.0	6.5
Tyr(Bu^t)	459.6	337.6	98.3	8.0
Val	339.4	217.4	72.6	5.2
Hyl(Boc)[f]	484.5	385.6	103.6	9.2
Hyp(Bu^t)[g]	409.5	231.4	87.6	5.5

[a] Molecular masses were corrected according to notes *b*−*g*. [b] Fmoc: 90%. [c] Boc: without Trt on side chain. [d] Fmoc: 96%. Boc: include crystal benzene and had a Boc instead of Trt. [e] Boc: included crystal H_2O. [f] Boc: 94%, contained 6% toluene. [g] Boc: without Bu^t on side chain.

present in collagen. Useful racemic building blocks of δ-(*R*/*S*)-hydroxy-DL-lysine, Fmoc-Hyl(Boc)-OH (**11**),[36] and Boc-Hyl(Boc)-OH (**12**), were prepared (Scheme 4) from the commercial racemic H-Hyl(Boc)-OH and used in the library synthesis. The one-bead-one-compound ladder of the inhibitor part of the library was prepared by MCPS[37] to facilitate split and combine library synthesis. Resin **3** was equally distributed in the 20 wells, and couplings were carried out with one activated amino acid mixture in each well. After each coupling, the resin was washed, mixed, and redistributed. Fmoc was then removed with piperidine, and the next coupling was performed. After three couplings with conventional amino acids, a 9:1 mixture of **4** and **5** was coupled, and this was followed by another three couplings of conventional amino acids by the split and combine protocol. This completed the synthesis of the inhibitor part of the library (Scheme 1). The Alloc group was removed, and the quenched fluorogenic substrate **8** was attached as described above. A few beads were removed for test-sequencing by MALDI-TOF MS, and the sequences were easily recorded in all cases (Figure 4 A).

Incubating, Selecting, and Sequencing Beads. Incubation of the library (approximately 165 000 beads) was performed for a period of 22 h. After deactivation of the enzyme with acid and thorough washing, the resin was placed under a fluorescence microscope, and dark beads were collected manually. Figure 5 shows a view of the resin as it was seen under the fluorescence microscope. A large variation of fluorescence intensity from individual beads depending on the amount of cleaved substrate was observed. Approximately 1000 beads were selected, and from these initially 20 and afterwards 92 were selected for sequencing, leading to 86 unambiguously determined sequences.

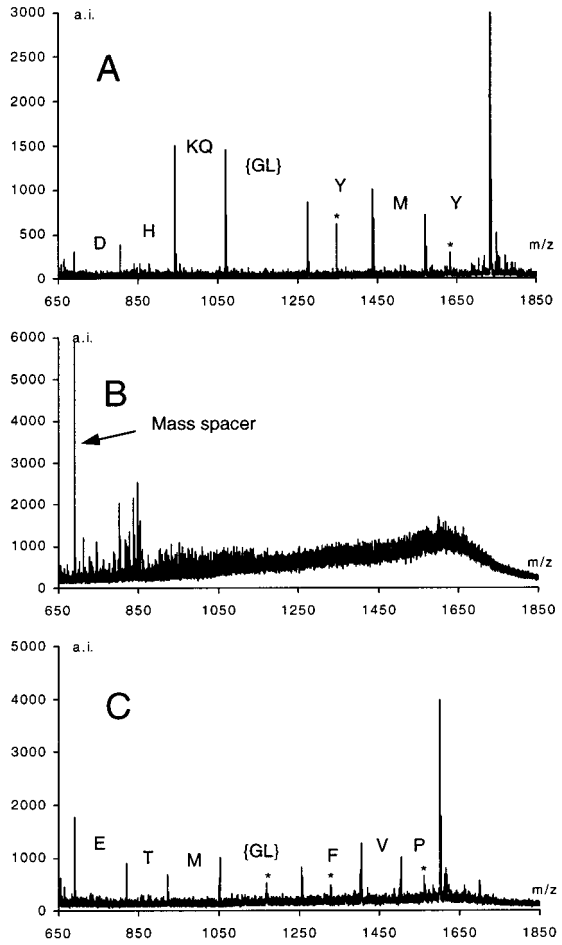


Figure 4. Examples of MALDI-TOF mass spectrometrical sequencing of material from beads. Peaks marked with an asterisk did not give rise to any meaningful sequence. {GL} denotes $G\Psi\{PO_2H\text{-}CH_2\}L$. A: Arbitrary bead before incubation. B and C: an active bead from the second batch of 92 beads. B: Before wash, the mass spacer which was common for all beads in the library is the only distinct peak; the broad bump has a maximum at about *m*/*z* 1600. C: After wash, all peaks necessary to make a full sequence determination could be identified.

Mass spectra were acquired on a high-resolution MALDI-TOF mass spectrometer. Peaks corresponding to the different ladder fragments present on a bead were assigned a mass, and a sequence assignment program was used to calculate all possible differences between the assigned masses and compare them with a list of the molecular masses of the amino acids and the Gly-Leu phosphinic dipeptide isoster. The program could therefore automatically assign the amino acid sequence.

Most of the amino acids used in the library had a unique mass, except for Leu, Ile, and Hyp (short LIJ) which are isobaric with mass 113.1, and Lys and Gln (short KQ) which both have mass 128.1. As a consequence, it was not possible to distinguish these amino acids in the sequences obtained. This was a problem of major concern since many of the sequences on the active beads turned out to contain LIJ (Figure 6). Five active beads containing LIJ were subjected to three cycles of Edman degradation (to the phosphinic dipeptide) while the peptides were still attached to the beads,[30] and it was observed that only Ile and Leu were present in the P_4 and P_3 of these sequences.

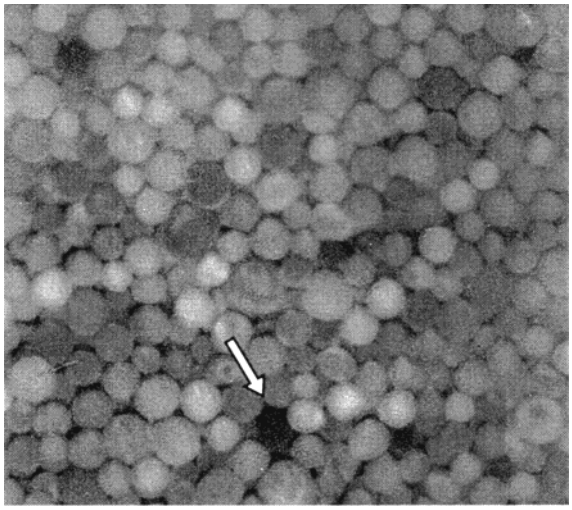


Figure 5. View of library **1** under a fluorescence microscope after incubation with MMP-12. Only the most persistently dark beads (as indicated with an arrow) were selected for identification of potent inhibitors.

Resynthesis and Enzyme Kinetic Investigations. To show that the library was in fact yielding leads to inhibitors, some of the sequences present on dark beads were resynthesized and tested in an enzyme kinetic assay with MMP-12. However, due to the multiple structures arising form the lack of discrimination between LIJ and KQ, it was decided not to prepare individual sequences. Instead a consensus sequence, LMY-$G\Psi\{PO_2H\text{-}CH_2\}$L-YAPG (**13**), based on the most frequently observed amino acids in the individual subsites, was constructed. As seen from Figure 6, LIJ were the most frequently observed amino acids in subsites P_4, P_3, P_2', and P_4'. However, it was only selected (arbitrarily as Leu) in the P_4 subsite of the consensus sequence. The reason for this was that the frequency of each of the amino acids (Leu, Ile, and Hyp) might have been one-third of the total frequency observed for LIJ. Hyp was ruled out in P_4 and P_3 by means of conventional Edman sequencing, and this led to the decision that Leu should be selected for P_4 in which the frequency LIJ was more than twice the frequency of Met (Figure 6). In contrast, in P_3, the frequency of Met was more than half the frequency of LIJ. Therefore, Met was selected for P_3. The same was true for P_2' and P_4' for which Ala and Pro were selected, respectively. By single substitutions in **13** with Leu, Ile, and Hyp or Lys and Gln in the subsites where LIJ and KQ were frequently observed, analogues of the consensus sequence (**14**−**32**) (Table 2) were used to identify which amino acids gave the superior inhibitors.

Determination of K_i values were performed using the substrate Mca-PLGL-Dpa-AR-NH_2[38] (k_{cat}/K_M 1.1×10^5 M^{-1} s^{-1} for MMP-12). The results from the enzyme kinetic assays with the inhibitors **13**−**32** showed that the consensus sequence (**13**) was a good choice being a strong inhibitor of MMP-12 (K_i 16 nM). In the sequences **14**−**16**, **17**−**18**, **22**−**24**, **25**−**27**, and **30**−**32**, the influence of changing the amino acid (Leu, Ile, and Hyp or Lys and Gln) was investigated in the subsites P_4, P_3, P_2', P_3', and P_4', respectively. As seen from Table 2, only marginal changes in inhibitor potency were observed upon changing the amino acids in these subsites. This was also the case when some of the more frequently observed amino acids in P_3 (Pro **19**), P_2 (Arg **20**

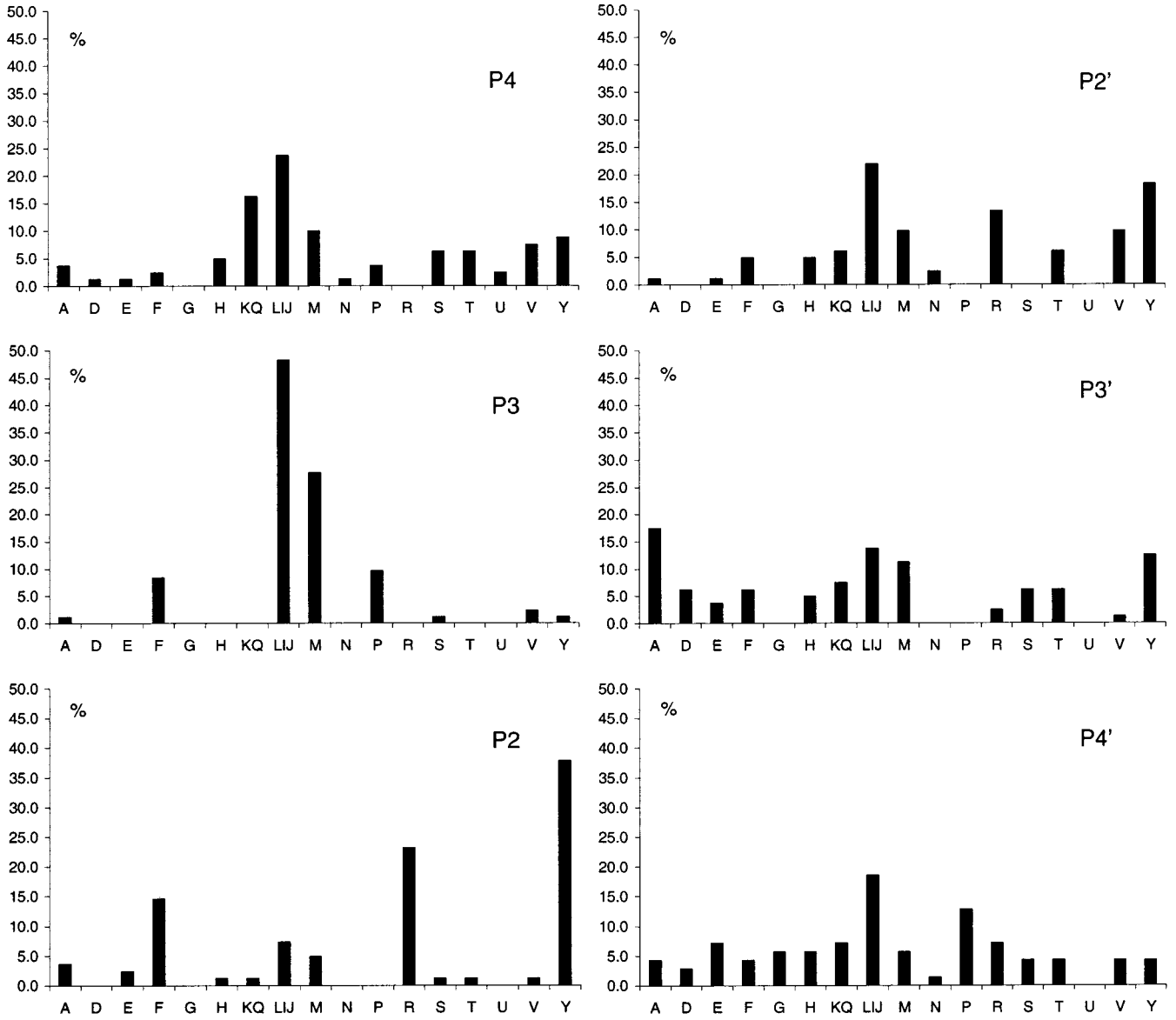


Figure 6. Frequencies of amino acids in the various subsites of identified sequences from 86 beads.

and Phe **21**), and in P_3' (Met **28** and Tyr **29**) were inserted. Most inhibitors of the series had K_i values between 6 and 30 nM. The only exception was when Tyr in P_2' was exchanged with Hyp (**24**). This led to a 1000-fold decrease in potency, most probably a consequence of introducing conformational constrains in the inhibitor.

Sixteen out of the first 20 sequences actually determined on beads (**33**−**48**) were also prepared, and the amino acids Leu, Ile, or Hyp were selected according to the results obtained from the substitutions on the consensus sequence. The amino acid providing inhibitors with the lowest K_i values was selected in each subsite. Thus, Ile was chosen for subsites P_4 and P_4' and Leu for P_3, P_2', and P_3', except for the few cases in which Leu and Ile had been identified unambiguously in P_4−P_2 by Edman degradation. In subsite P_4, which was the only subsite where KQ was encountered to a significant degree (Figure 6), Lys was selected. When KQ was encountered in subsites P_3−P_4', Lys was arbitrarily chosen, since these residues were not significant in the substitution of the consensus sequence. The K_i values for these compounds are given in Table 2, and they varied more in the potencies than those originating from the consensus sequence.

A "second generation" consensus sequence was made by constructing **49**. This sequence contained those amino acids which for each subsite gave the most potent inhibitor in the previous modifications of the consensus sequence (**14**−**32**). Although there were only insignificant differences in potencies among the sequences **14**−**32**, sequence **49** could have been more potent than any of these as a result of small additive effects from the different amino acids. However, **49** had a K_i of 29 nM which is in the range of most of the sequences **13**−**32**.

Simultaneously with synthesis of sequences **33**−**49**, three other sequences (**50**−**52**) were prepared in order to investigate the effect of truncating the potent sequence **28** at both the *N*- and *C*-terminal. In contrast to **28**, sequences **50**−**52** were all poor inhibitors of MMP-12 (Table 2).

Compounds **13**−**52** were synthesized by MCPS using a procedure previously reported and purified by RP-HPLC.[10]

Table 2. Sequences, K_i's, Masses, and Purities of the Resynthesized Compounds[a]

compd	P_4	P_3	P_2	P_2'	P_3'	P_4'	P_5'	K_i MMP-12, nM[b]	MW	found MALDI-TOF[c]	purity,[d] %
13	L	M	Y	Y	A	P	G[e]	16 ± 4	1019.2	1019.8	90
14	I	M	Y	Y	A	P	G	13 ± 2	1019.2	1019.7	90
15	K	M	Y	Y	A	P	G	17 ± 5	1034.2	1034.7	100
16[f]	Q	M	Y	Y	A	P	G	23 ± 5	1034.2	1034.6	100
17	L	L	Y	Y	A	P	G	13 ± 5	1001.2	1001.7	100
18	L	I	Y	Y	A	P	G	22 ± 4	1001.2	1001.7	100
19	L	P	Y	Y	A	P	G	28 ± 7	985.1	985.6	100
20	L	M	R	Y	A	P	G	20 ± 6	1012.2	1012.6	100
21	L	M	F	Y	A	P	G	19 ± 3	1003.2	1003.6	100
22	L	M	Y	L	A	P	G	9 ± 2	969.2	969.6	100
23	L	M	Y	I	A	P	G	12 ± 2	969.2	969.6	100
24	L	M	Y	J	A	P	G	41000 ± 18000	969.2	969.5	70
25	L	M	Y	Y	L	P	G	8 ± 2	1061.3	1061.6	100
26	L	M	Y	Y	I	P	G	16 ± 3	1061.3	1061.7	100
27	L	M	Y	Y	J	P	G	85 ± 14	1061.3	1061.4	100
28	L	M	Y	Y	M	P	G	6 ± 1	1079.3	1079.5	100
29	L	M	Y	Y	Y	P	G	6 ± 1	1111.3	1111.8	100
30	L	M	Y	Y	A	L	G	30 ± 8	1035.3	1035.6	100
31	L	M	Y	Y	A	I	G	20 ± 4	1035.3	1035.6	100
32	L	M	Y	Y	A	J	G	15 ± 5	1035.3	1035.6	100
33	I	L	L	N	L	I	G	23000 ± 1000	959.7	961.2	100
34[g,h]	T	L	Y	L	D	G		66 ± 2	885.5	887.1	100
35	V	L	Y	T	L	S	G	985 ± 315	956.6	958.1	70
36	I	M	Y	V	K	F	G	74 ± 9	1061.7	1063.2	100
37[g,h]	L	I	E	R	K	G		≥389000 ± 38000	919.6	921.1	100
38[h]	T	L	Y	R	A	I	G	875 ± 465	997.7	999.1	80
39	E	F	Y	K	Y	N	G	3700 ± 500	1124.6	1126.1	80
40	T	L	R	L	F	F	G	22 ± 5	1057.7	1059.2	100
41	I	L	R	M	A	P	G	25 ± 4	961.6	963.1	100
42	S	L	F	R	D	I	G	762 ± 8	1011.6	1013.1	100
43[h]	L	M	F	Y	L	S	G	129 ± 5	1034.6	1036.0	90
44	I	M	Y	Y	M	T	G	8 ± 4	1082.6	1084.0	95
45	K	F	Y	L	Y	A	G	150 ± 18	1065.6	1067.2	100
46[h]	Y	I	Y	T	M	P	G	19 ± 15	1048.6	1050.1	60
47[g]	S	M	A	Y	H	G		303 ± 9	869.5	870.9	100
48	I	M	R	L	S	E	G	23 ± 8	1009.6	1010.9	95
49	I	L	F	L	M	I	G	28 ± 2	1010.7	1012.3	95
50			Y	Y	M			14600 ± 2100	680.4	681.7	95
51		M	Y	Y				2500 ± 500	680.4	681.8	95
52			Y	Y				350000 ± 90000	549.3	550.6	100

[a] P_1-P_1' consisted of the phosphinic Gly-Leu dipeptide analogue $G\Psi\{PO_2H\text{-}CH_2\}L$; amino acid analyses were consistent for all peptides. [b] Values are for the most potent diastereomer, in all cases the first eluting from the HPLC.[10] [c] $[M + H]^+$. In all cases both protonated, sodiated and potassiated ions were observed. [d] RP-HPLC, 215 nm, only contaminants were the corresponding diastereomers which were generally 50-fold less potent. [e] *C*-terminal Gly was incorporated because of synthetic convenience.[10] [f] A considerable amount of pyro-Glu was observed for compound **16**. [g] For compounds **34**, **37**, and **47**, only two amino acids were observed on the P′-side by MALDI-TOF sequencing. [h] In underlined sequences, Leu and Ile were unambiguously determined in the P-subsites by Edman degradation.

Discussion

Preparation. Apart from minor synthetic problems, the library **1** could easily be synthesized. Most steps in the preparation involved peptide couplings, and the selected three-dimensional protective strategy was critical to the formation of the one-bead-two-compounds construct. One problem was the low loading initially obtained for resin **2**. This was believed to originate from partial acetylation of the Boc-NH as the loading was increased by using lower amounts of Ac_2O. However, using AcODhbt only a minor reduction in loading was observed.

Initial experiments with resins **7**, **9**, and **10** showed that sequencing by MALDI-TOF MS was indeed possible. In addition, the experiments showed that the 22 kDa MMP-12 (catalytic domain) was able to penetrate into resin beads and cleave the substrate and that the presence of an inhibitor on the bead would prevent the enzyme from cleaving the substrate (Figure 2).

Selecting and Sequencing Beads. A few problems were observed while selecting and sequencing beads. First of all it was noted that since the inhibitor was attached to the resin by a photolabile linker, a considerable amount of inhibitor was cleaved during the selection process because the excitation light of the fluorescence microscope was in the UV region. This was a problem that became apparent when sequencing the second batch of 92 beads. As the selection of beads was carried out with swelled resin, material cleaved from all beads (including all ladder fragments from these) was present in the supernatant. Thus, a dark bead removed for sequencing inevitably contained a huge amount of different compounds absorbed in it. For the second batch of 92 beads, sequencing was rendered extremely difficult due

to a very large noise level originating from the large number and high concentration of "alien" compounds present in the beads. The problem was solved by washing the 92 beads after selection (Figure 4B,C); however, even after this wash the sequencing process was considerably more difficult than in the case of the 20 beads initially selected, due to less signal-to-noise and to the time required. The 20 beads initially selected were easily sequenced without additional washing. Thus, the more time beads were exposed to UV under the microscope, the more difficult sequencing of compounds present on beads became, and from the experiments presented, a critical time of approximately 45 min was determined after which the sequencing by MALDI-TOF was virtually impossible. This figure is of course dependent on the flux of the exiting light and, therefore, on the actual microscope used. Other linkers may be used in order to avoid these problems completely. However, the photolytic cleavage has the great advantage that it is solvent free and leaves no salts interfering with the ionization of the sample during MALDI-TOF acquisition. This is important when performing single bead analysis. Therefore a linker labile to UV below excitation wavelength of the fluorescence microscope would be preferred. However, the excitation wavelength of the fluorescence microscope was determined by the properties of the selected FRET donor/acceptor pair Abz and Y(NO_2).

Sequencing of active inhibitors was fast and reliable as long as the bead selection process had not been too lengthy. In most cases, more peaks were present in a given spectrum than the number corresponding to an amino acid sequence. However, these peaks were also included in the calculation routine, and most frequently they did not give rise to any meaningful assignment. For example, in the spectrum shown in Figure 4A there is a peak at *m*/*z* 1347.3. The differences between this peak and any other peak in the spectrum did not correspond to the mass of any amino acid, and it could therefore be assigned to an impurity. On the other hand, the difference between a peak at *m*/*z* 1631.5 and the full length peak at *m*/*z* 1732.5 corresponded to the molecular mass of Thr. However, assuming that Thr was the *N*-terminal amino acid instead of Tyr, the sequence would be incomplete since no other peaks appeared with a suitable distance to *m*/*z* 1631.5, and for this reason Thr was ruled out. This reasoning was often employed in the interpretation of the MALDI-TOF spectra. No systematic reasons were found for the extra peaks, although they may originate from unprecedented reactions during photocleavage. Oxidation of Met leading to M+16 ion was also frequently encountered, and for compounds containing two or more methionines, the mass spectra were complicated. However, it was always possible to interpret these spectra and determine the sequence. Capping of the phosphinic dipeptide building block, i.e., the use of **5**, was not a necessity in order to determine sequences; however, it was desired as its unique mass placed a marker in mass spectra which was useful in the automated sequencing process.

The lack of discrimination between the amino acids Leu, Ile, and Hyp, and between Lys and Gln, was a difficulty of the method, and as a result the resynthesized sequences **33**−**48** may differ from what was actually present on beads. Additional procedures involving different capping for Leu, Ile, and Hyp and Lys and Gln (e.g., the series acetyl, propionyl, butyroyl, pentanoyl, and hexanoyl), leading to an unambiguous identification of these isobaric residues, might have been employed. However, capping reagents other than the Boc-amino acids result in a less reliable ladder synthesis due to difference in coupling rates of Fmoc-amino acids and the capping reagents, which in addition may be sequence dependent. Furthermore, sequences capped with completely different groups could have a strong effect on inhibitor potency leading to false positives.

Surprisingly, approximately 8% of the sequences determined contained only two amino acids on the *C*- or *N*-terminal side of the phosphinic dipeptide, which is reflected in the sequences **34**, **37**, and **47**. This was easily observed due to the unique mass of the phosphinic dipeptide fragment (*m*/*z* 205.2) and could be the result of some beads not being subjected to any activated amino acid in one particular step of the assembly. However, a more plausible reason is that all the deletions originated from Hyl (**11** and **12**) which was seldom observed in the analyzed beads and was shown to form the δ-lactone during activation by TBTU due to the unprotected δ-hydroxyl.

Analysis of Sequence and K_i Data. The conclusions drawn from Figure 6 are interesting. In the discussion it is assumed that the inhibitors bind to MMP-12 with the phosphinic dipeptide in the S_1-S_1' subsites. Generally it is seen that apolar amino acids dominate, the main exception being Arg. Although no crystal structure exist for MMP-12, it is believed that the catalytic site is similar to that of other MMPs except MMP-1 and -7. Subsite S_4, which has not previously been described,[1] showed some selectivity for amino acids KQ and LIJ. In S_3 there was a pronounced preference for amino acids LIJ and Met. This is surprising since most studies[1] state that S_3 is a small cleft resulting in the frequently observed preference for Pro in this subsite.[39] As seen from Figure 6, Pro is also frequently observed in S_3, however, not to the extent of LIJ and Met. In subsite S_2, only Phe, Arg, and Tyr seemed to be allowed. The space including subsites S_2-S_1 is considered a shallow region without any specific grooves or clefts. Thus, in contrast to the common view that subsites S_3-S_1 in MMPs in general are of minor importance to binding of inhibitors,[2] Figure 6 indicates that S_2 and S_3 in MMP-12 are pockets/regions that only allow certain amino acids in order to maintain inhibitory activity. In general, it must be concluded from these data that the S-subsites seem to give much more well-defined interactions than the S′-subsites, which is also supported by the data for the sequences **50**−**52**. Thus, sequence **51**, in which P_3 is occupied by Met, is 140 times more potent than the shorter sequence **52** with empty P_3 and P_3' positions, and approximately 6 times more potent than the sequence where the P_3' is occupied by Met (**50**). These findings are interesting since the large majority of inhibitors developed for MMPs to date are so-called right-hand succinylhydroxamates,[2] compounds that only interact with the S′-subsites. Subsite S_2' is known to be a small, solvent exposed cleft. It showed a distinct preference for certain amino acids, Arg and Tyr. The S_3'- and S_4'-subsites of most MMPs are considered to

confer little or no contribution to the binding specificity,[1,2] and indeed the library did not show much preference among the various amino acids in these two subsites.

The fact that each bead contained a mixture of ladder fragments opens for the possibility that one of these shorter ladder fragments might be the most active compound present on the bead. However, as is evident from Table 2, the truncated sequences **50**−**52** derived from sequence **28** were several orders of magnitude less potent than **28**. Although short phosphinic peptides have been shown to be potent inhibitors of MMPs,[9] it is generally believed that, in contrast to, e.g., hydroxamic acid inhibitors, these compounds require several backbone and/or subsite interactions to compensate for the relatively poor affinity of the phosphinic acid for the catalytic Zn.[1,10] Another important issue is the aim for selectivity of inhibitors among different MMPs, a property which seems more difficult to achieve for small compounds than for larger.

The special amino acids Hyp and Hyl often found in collagens were used instead of Cys and Trp in the library. However, they were not found to be important. Hyp was part of the isobaric set with Leu and Ile and was therefore not directly determined. The five sequences in which the P-subsites were determined by Edman sequencing did not show any Hyp, and moreover it seemed that Hyp gave rise to decreased potency when incorporated in, e.g., P_2' or P_3'.

Synergistic and/or antagonistic effects between amino acid residues often play an important role in protease specificity of peptide substrates,[40,41] and these effects are also anticipated in specificity of peptide-like inhibitors. Therefore, it is somewhat surprising that it was possible to obtain potent inhibitors based on the consensus sequence (**13**−**32**). On the other hand, the consensus sequence was based on data from 86 sequences, which is a considerable amount of data. The larger the amount of data, the more clearly the amino acid preference is determined, and this yields more reliable trends. On the other hand, it is necessary to design the library such as to force the enzyme to select only the most potent inhibitors, i.e., to shift the detection limit to the lowest possible K_i value. This can be achieved either by performing multiple, sequential incubations, by increasing the enzyme concentration, by using a substrate with high affinity, or by lowering the amount (concentration) of inhibitor, i.e., lowering the loading on the inhibitor part of the functional biantenna. In the present study, these effects were well balanced, and the number of active beads was approximately 0.6% of the original number, leading to inhibitors such as **28** and **29** (K_i 6 nM) which are among the most potent inhibitors of MMP-12.

The results from the resynthesis also indicate that there is, in general, a good correlation between inhibitor activity observed on the solid support and in solution. Thus, 14 out of 17 of the sequences **33**−**48** originating from actual beads had K_i values well below 1 μM. This indicates that the detection limit of the fluorescence based assay is in the range of K_i 0.1−1 μM, i.e., beads containing inhibitors of K_i 0.1−1 μM and below cannot be distinguished. There were three exceptions, namely **33**, **37**, and **39**, that all had K_i above 1 mM.

It is worth noting that only five of the inhibitors found on active beads (**40**, **41**, **44**, **46**, and **48**) had K_i values which were in the same range as the consensus sequence (**13**) and most of its analogues (**14**−**32**). This is probably due to the detection limit of discrimination in the fluorescence based assay. Sequence **44** resembles **28** in many subsites which may account for its high potency; sequences **40**, **41**, **46**, and **48** have more different amino acids incorporated. It is difficult to draw conclusions from the K_i data of sequences **33**−**48** since many residues are different, and substantial synergistic and/or antagonistic effects may be present. However, most of the residues are hydrophobic and/or aromatic as was also the case for the consensus sequence (**13**), and it is seen that especially P_2' seems sensitive to the polarity of the side chain: sequences **35**, **37**−**39**, and **42** all have charged or polar residues in this subsite, and these have significantly higher K_i value. The activity of **37** is particularly low, indicating that a negative charge in P_2 (Glu) is not accepted.

Conclusion

The present study clearly demonstrates that potent MMP inhibitors can indeed be identified from a one-bead-two-compounds library of phosphinic peptides, XXX-GΨ{PO_2H-CH_2}L-XXX. Apart from obtaining potent inhibitors, the study also provided information about the subsite preference for MMP-12 with peptide-like inhibitors, information which is valuable for further development of drug candidates based on inhibition of this important enzyme. MMPs are believed to undergo substantial conformational changes during substrate/inhibitor binding.[2] This phenomenon has limited the success of structure based design of MMP inhibitors. In the combinatorial library approach these problems are completely alleviated due to the fact that the enzyme, in a process allowing induced fit of the active site, will select the most potent inhibitors. Thus, it is possible to take into account interdependency between amino acids.

The library was prepared in a straightforward manner using mainly Fmoc solid phase peptide chemistry. It was designed such that screening with MMP-12 and structure determination of active inhibitors was highly efficient, utilizing a quenched fluorogenic substrate and MALDI-TOF mass spectrometry, respectively. Most of the inhibitors present on active beads were shown to be potent inhibitors of MMP-12. Furthermore, despite possible antagonistic effects between amino acids in the inhibitors, it was shown that with sufficient data available a consensus sequence with high potency against MMP-12 was obtained.

Data concerning inhibition of a series of MMPs with sequences **13**−**52** will be reported elsewhere.

Experimental Section

Anhydrous solvents were obtained by storing analytical quality solvents over 3 or 4 Å activated molecular sieves after which the H_2O content was verified to be below 30 ppm by Karl Fischer titration, except for DMF which was fractionally distilled at reduced pressure and stored over 4 Å molecular sieves. PEGA library resin with a PEG_{1900} cross-

linker ($PEGA_{1900}$, reported loading 0.20 mmol/g) was purchased from Polymer Laboratories, England. All commercial starting materials were used without further purification. NMR data was acquired on a Bruker 250 MHz Avance DRX 250 spectrometer and were referenced to $CHCl_3$ ($\delta = 7.24$, ^1H), HDO ($\delta = 4.75$, ^1H), or MeOD ($\delta = 3.34$, ^1H). Analytical RP HPLC was performed on a Waters system (490E detector, two 510 pumps with gradient controller and Ø 8 mm RCM C_{18} column), and preparative RP HPLC purification of phosphinic peptides **13**−**52** was carried out on a Waters system (991 photodiode array detector and 600 E system controller) connected to a Waters Ø 25 mm RCM C_{18}-column. All RP HPLC procedures were carried out with a linear gradient. Buffers: A (0.1% TFA in H_2O) and B (0.1% TFA in MeCN:H_2O 9:1). VLC[42] was performed using a tightly packed column of Merck silica gel 60 H. Electrospray mass spectra were obtained on a Fisons VG Quattro 5098 mass spectrometer (mobile phase 50% aqueous MeCN, 8 μL/min, sample: 10 μL ~20 pmol/μL). MALDI-TOF mass spectra were acquired on a Bruker Reflex III high-resolution MALDI-TOF mass spectrometer, and sequencing was facilitated using the Aura macro LabelDelta which was supplied with the spectrometer software. Conventional Edman sequence analysis was carried out on an automated Applied Biosystems 477A protein sequencer/120A analyzer. Library beads were examined for inhibitor activity under an Optical Star fluorescence microscope with a 320 nm pass filter for excitation (20 nm wide) and a 410 nm low pass filter for detection.

Deprotection of N^{α} Fmoc was performed with piperidine (20% in DMF) for 2 and 10 min. Volumes of washing solvent were 1−2 times the volume necessary to swell the resin, and washings were 6 × 1 min unless otherwise stated. Minimum volume was used for couplings. Amounts of amino acid derivatives or other reagents which were too small to weigh out were determined as an aliquot of a more concentrated solution for which 10 or 100 times the amount were weighed out. Amino group loadings were determined by spectrophotometric determination of released piperidine-dibenzofulvene adduct after coupling with Fmoc-Gly-OPfp/DhbtOH and treatment of a known sample with 20% piperidine in DMF.

Ac-Lys-Lys(Alloc)-PEGA (2). A solution of Fmoc-Lys-(Alloc)-OH (0.264 mmol, 119.5 mg), NEM (0.352 mmol, 44.5 μL), and TBTU (0.253 mmol, 81.4 mg) in anhydrous DMF was preactivated for 10 min, added to $PEGA_{1900}$-resin (0.11 mmol/g, 1.20 g, 0.132 mmol), and allowed to react for a period of 3.5 h. The resin was washed with DMF, Fmoc was removed, and it was washed with DMF. A solution of Fmoc-Lys(Boc)-OPfp (0.396 mmol, 251.5 mg), and DhbtOH (0.132 mmol, 22 mg) in anhydrous DMF was added to the resin, and after a period of 1 h reaction was complete. The resin was washed with DMF, Fmoc was removed, and it was washed with DMF. It was treated with a solution of AcODhbt (0.198 mmol, 40.6 mg) in anhydrous DMF for a period of 18 h. The resin was washed with DMF, DCM, and treated with trifluoroacetic acid:H_2O 19:1 for a period of 1 h after which it was washed with AcOH × 3, DCM × 3, DMF × 3, (5% DIPEA in DMF) × 1, and DMF.

Ac-Lys{Fmoc/Boc-Thr(Bu^t)-Ile-Ser(Bu^t)-Arg(Pmc)-Thr-(Bu^t)-Ile-[Pll]}-Lys(Alloc)-PEGA (3). The photolabile linker[27] (0.396 mmol, 206 mg) was mixed with NEM (0.528 mmol, 67 μL) and TBTU (0.380 mmol, 122 mg) in anhydrous DMF, preactivated for 10 min and added to resin **2** (1.20 g, 0.132 mmol). Coupling was complete after a period of 17 h. All resin manipulations beyond this point were carried out with the reaction vessels wrapped in aluminum foil avoiding direct light. Fmoc was removed, and after the resin was washed with DMF, the amino acids Ile, Thr, Arg, Ser, and Ile were attached to resin using Fmoc amino acid pentafluorophenyl esters (3 equiv, 0.396 mmol, amounts: Ile: 206 mg; Thr-(Bu^t): 223 mg; Arg(Pmc): 328 mg; Ser(Bu^t): 218 mg) and DhbtOH (1 equiv, 0.132 mmol, 22 mg). Amino acids and DhbtOH were dissolved in anhydrous DMF and added to the resin. Coupling times varied; complete acylation was ensured by a negative Kaiser test.[43] Then Thr (a mixture of Fmoc-Thr(Bu^t)-OH (2.7 equiv, 0.356 mmol, 142 mg) and Boc-Thr(Bu^t)-OH (0.3 equiv, 0.040 mmol, 11 mg)) was coupled using NEM (0.528 mmol, 67 μL) and TBTU (0.380 mmol, 122 mg). The resin was washed with DMF and DCM and lyophilized.

Ladder Synthesis of Known Inhibitor AGPL-GΨ-{PO_2H-CH_2}L-YAR with and without Substrate 8 Attached (Resins 6, 7, and 9). Fmoc was removed from resin **3** (23 mg, 2.3 μmol), and it was washed with DMF. The protected Fmoc- and Boc-amino acids/building block Arg, Ala, Tyr, GΨ{PO_2Ad-CH_2}L, Leu, Pro, Gly, and Ala were coupled sequentially: the amino acid (Fmoc- and Boc-derivative mixed for each amino acid, for last Ala only Boc) was dissolved in DMF; NEM (9.3 μL, 73 μmol/8 couplings) and TBTU (17 mg, 53 μmol/8 couplings) was added, and the mixture was preactivated for 10 min before it was added to the resin. Amounts for each coupling were as follows: Arg(Pmc): Fmoc/Boc 4.1/0.37 mg; Ala: Fmoc/Boc 2.0/0.26 mg; Tyr(Bu^t): Fmoc/Boc 2.9/0.23 mg; Leu: Fmoc/Boc 2.2/0.16 mg; Pro: Fmoc/Boc 2.1/0.15 mg; Gly: Fmoc/Boc 1.8/0.12 mg; Ala: Boc 2.6 mg. After each coupling the resin was washed with DMF, Fmoc was removed, and the resin was washed with DMF before it was ready for another coupling with preactivated amino acid. After the final coupling, the resin was washed with DMF, MeOH × 3, and DCM × 3 and lyophilized to obtain resin **6**.

Half of this resin (11 mg, 1.1 mmol) was treated with a mixture composed of TFA:DCM:H_2O:MeSPh:$(CH_2SH)_2$:TIPS 66.5:20:5:5:2.5:1 for 3 h, after which it was washed with AcOH × 3, DCM × 3, MeOH, and H_2O × 3. It was then treated for 1 h with 0.1 M NaOH and washed with H_2O to give resin **7**. The sequence of the inhibitor was verified by MALDI-TOF MS by the method described below for library **1**.

The other half of resin **6** (1.1 mg, 1.1 μmol) was treated with $Pd(PPh_3)_4$ (3 equiv, 3.3 μmol, 3.5 mg) to remove Alloc, and substrate **8** (6.6 mg, 3.3 μmol) was coupled for a period of 12 h using TBTU (1.0 mg, 3.2 μmol) and NEM (0.56 μL, 4.4 μmol) as described below under library **1**. Fmoc was removed, and the resin was washed with DMF and DCM followed by treatment with TFA, NaOH treatment, and wash as described for the conversion of resin **6** into **7** above.

Finally the resin was washed with a pH 7.5 buffer (50 mM tris,150 mM NaCl, 10 mM $CaCl_2$, 50 μM $ZnSO_4$, 0.05% (v/v) Brij-35) to obtain resin **9**.

Substrate Resin without Inhibitor (10). A solution of AcODhbt (0.7 mg, 3.3 mg) in anhydrous DMF was added to resin **2** (11 mg, 1.1 μmol), and it was allowed to react for 30 min. The Alloc group was removed, and substrate **8** was attached as described below for library **1** to obtain resin **10**.

Incubations of Resins 9 and 10 with MMP-12. Resins **9** and **10** (approximately 1 mg of each) were placed on a glass plate, and MMP-12 (20 μL, 100 nM) was added to each. After a period of 1 h, a significant difference in brightness was observed between the two resins: **9** was remaining as dark as before and **10** was illuminating. After incubation for 30 h, resin **9** was washed with $H_2O \times 3$ and the sequence of the inhibitor was verified by MALDI-TOF MS by the method described below for library **1**.

***O*-Adamantyl *P*-(Boc-aminomethyl)-*P*-(2-isobutylpropionic acid-3-yl)phosphinate (5).** *O*-Adamantyl *P*-(Fmoc-aminomethyl)-*P*-(2-isobutylpropionic acid-3-yl)phosphinate (**4**)[10] (100 mg, 172 μmol) was dissolved in DMF (2 mL), and piperidine (0.5 mL) was added, reacted for a period of 5 min, and concentrated to dryness. The solid residue was dissolved in DMF (2 mL) and added to a solution of di-*tert*-butyl dicarbonate (50 mg, 259 μmol) in DMF (2 mL) followed by Et_3N (72 μL, 345 μmol). The reaction mixture was stirred for 30 min after which it was concentrated to dryness. Acidic workup of the crude product did not result in any purification, and the product was therefore purified directly by VLC (Ø 25 mm, gradient: neat $CHCl_3$ to $CHCl_3$:MeOH:AcOH 40:1:1). Yield: 50 mg white solid (64%). ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.87 + 0.89 (two m, -CH_2-CH(CH_3)$_2$, 6 H), 1.32 + 1.67 (two m, -CH_2-CH(CH$_3$)$_2$ 2 H), 1.43 (s, -O-C(CH_3)$_3$, 9 H), 1.60 (br s, -O-C-CH_2CHCH_2, 6 H), 1.63 (m, CH_2-CH(CH$_3$)$_2$, 1 H), 1.73 + 2.18 (two m, -PO_2Ad-CH_2-CHBu^i-, 2 H), 2.05 (br s, -O-C-CH_2CHCH_2, 6 H), 2.15 (br s, -O-C-CH_2CHCH_2, 3 H), 2.78 (m, -PO_2Ad-CH_2-CHBu^i-, 1 H), 3.41 + 3.64 (two m, -NH-CH_2-PO_2Ad, 2 H), 5.40 + 5.75 (two m, -NH-CH_2-PO_2Ad). Anal. Calcd for $C_{23}H_{40}NO_6P$: C, 60.38; H, 8.81; N, 3.06. Found: C, 60.11; H, 8.55; N, 2.83; MS (ESI) calcd for $C_{23}H_{40}NO_6P$ 457.3. Found: 458.4 $[M + H]^+$.

***N*$^\alpha$-Fmoc-*N*$^\epsilon$-Boc-δ-hydroxy-DL-lysine (11).** 9-Fluorenylmethyl *N*-succinimidyl carbonate (252 mg, 0.75 mmol) was dissolved in dioxane (3 mL) and added dropwise to an aqueous solution (2 mL) containing δ-(R/S)-*N*$^\epsilon$-(Boc)-δ-hydroxy-DL-lysine (200 mg, 0.76 mmol) and Na_2CO_3 (81 mg, 0.76 mmol) at room temperature while stirring briskly. A pH of 9−10 was maintained by occasional addition of a few drops of Na_2CO_3 (1 M). The reaction mixture was stirred for a period of 24 h after which it was concentrated to dryness. The residue was dissolved in DCM (10 mL) and H_2O (10 mL), and pH was adjusted to 2 by careful addition of HCl (1 M, approximately 3 mL). The aqueous phase was extracted 3 times with DCM (10 mL), and the combined extracts were dried with $MgSO_4$ and concentrated to dryness to obtain a white solid (309 mg). The crude product was purified by VLC (Ø 25 mm) using a gradient of eluents from neat $CHCl_3$ + 5% AcOH to $CHCl_3$:MeOH 20:1 + 5% AcOH. Fractions were freed from AcOH by coevaporation with toluene and dried at high vacuum. Yield: 276 mg (76%), white powder. R_f ($CHCl_3$:MeOH:AcOH 9:1:1) 0.31.^{1}H NMR (250 MHz, MeOD), δ 1.19 (s, 9 H, (CH_3)$_3$-C-O-), 1.21 (m, 2 H, Hγ), 1.60 (m, 2 H, Hβ), 2.71 (dd, ^3J = 7.2 Hz, ^2J = 13.8 Hz, 1 H, Hϵ'), 2.96 (dd, ^3J = 4.1, Hz, ^2J = 13.8 Hz, 1 H, Hϵ), 3.38 (m, 1 H, Hδ), 3.96 (m, 1 H, Hα), 3.99 (br t, 1 H, Ar-CH-CH_2-C-O-), 4.15 (m, 1 H, Ar-CH-CH_2-C-O-), 6.42 (d, J = 7.2 Hz, 1 H, NHα), 7.04 (dt, ^3J = 7.5 Hz, ^4J = 1.3 Hz, 2 H, -CH=CH-CH=CH-), 7.11 (dt, ^3J = 7.2 Hz, ^2J = 1.0 Hz, 2 H, -CH=CH-CH=CH-), 7.37 (br d, J = 7.2 Hz, 2 H, -CH=CH-CH=CH-), 7.50 (d, 2 H, J = 7.5 Hz, -CH=CH-CH=CH-). Hϵ' and Hϵ are two protons from the same diastereomer. The shifts of the sets of Hϵ' and Hϵ from the two diastereomers were separated ca. 1 Hz. MS (ESI) calcd for $C_{26}H_{32}N_2O_7$ 484.2. Found: 485.4 $[M + H]^+$.

***N*$^\alpha$-Boc-*N*$^\epsilon$-Boc-δ-hydroxy-DL-lysine (12).** A solution of *N*$^\epsilon$-(Boc)-5-hydroxy-DL-lysine (100 mg, 0.38 mmol) and Et_3N (127 μL, 0.92 mmol) in H_2O (2 mL) was added dropwise to a solution of di-*tert*-butyl dicarbonate (100 mg, 0.46 mmol) in DMF (2 mL) at room temperature and stirred for 24 h. It was concentrated to dryness, and the residue dissolved in $CHCl_3$ (10 mL) and H_2O (10 mL) and HCl (0.5 mL, 1 M) was added to adjust pH to 2. The aqueous phase was extracted with $CHCl_3$ (3 × 10 mL), and the combined extracts were dried with $MgSO_4$ and concentrated to dryness to obtain a clear syrup, 198 mg. It was purified by VLC (Ø 25 mm) using a gradient from neat $CHCl_3$ + 5% AcOH to $CHCl_3$:MeOH 9:1 + 5% AcOH. Fractions were coevaporated with toluene to remove AcOH and lyophilized from dioxane to remove toluene. Yield: 126 mg (87%), white solid. R_f ($CHCl_3$:MeOH:AcOH 9:1:1) 0.22. ^{1}H NMR (250 MHz, 0.3% NaOD in MeOD), δ 1.21 (m, 2 H, Hγ), 1.23 (s, 18 H, (CH_3)$_3$-C-O-), 1.58 (m, 2 H, Hβ), 2.83 (dd, ^3J = 6.6 Hz, ^2J = 13.8 Hz, 1 H, Hϵ'), 2.94 (dd, ^3J = 3.8, Hz, ^2J = 13.8 Hz, 1 H, Hϵ), 3.43 (m, 1 H, Hδ), 3.78 (m, 1 H, Hα). MS (ESI) calcd for $C_{16}H_{30}N_2O_7$ 362.2. Found: 363.3 $[M + H]^+$.

Library 1. Resin **3** (1.2 g, 0.132 mmol) was distributed equally between the wells in a 20 well multiple column peptide synthesizer.[37] This was done most conveniently by swelling the resin in DMF and placing it in the synthesizer together with excess DMF (40 mL). A lid was placed on top of the synthesizer, it was turned up-side-down and shaken thoroughly for 1 min, after which it was quickly turned up-side-up. The suspension of resin then distributed equally between the wells, and after draining the synthesizer for DMF, Fmoc was removed and the resin was washed with DMF. The split and combine synthesis was carried out by coupling 9:1 mixtures of 20 different Fmoc- and Boc-amino acids as given in Table 1 by the cyclic protocol: (i) Coupling of amino acids. Each amino acid was placed in a specific well. Stock solutions of Fmoc- + Boc-amino acids were prepared by dissolving each set of amino acids in anhydrous DMF (2400 μL). As seen from Table 1, the amounts weighed out corresponded to 12 couplings with each set of amino acids containing 2.7 equiv of Fmoc- and 0.3 equiv of Boc-derivative, thus 200 μL solution/coupling. Stock solutions of TBTU (20 × 2.88 equiv, 0.380 mmol, 122 mg) and NEM (20 × 4 equiv, 0.528 mmol, 67 μL) in anhydrous DMF (2000

μL and 133 μL, respectively) were prepared before each coupling. Thus, for each of the 20 amino acids, 200 μL of amino acid solution (Fmoc- + Boc-derivative), 10 μL of NEM solution, and 100 μL of TBTU solution was mixed in an Eppendorf tube and allowed to activate for 10−20 min. DMF (250 μL) was added to each well in the synthesizer to avoid leaking from the wells during addition. Finally, the activated mixtures of Fmoc- and Boc-amino acids were added to their individual wells, resulting in a total reaction volume of 560 μL in each well (60 mg resin). All couplings were allowed to proceed overnight. (ii) Wash with DMF. (iii) Mixing the resin. Excess DMF (40 mL) was added to the synthesizer, the lid was closed, and it was shaken thoroughly up-side-down for 1 h. (iv) Removal of Fmoc. (v) Wash with DMF. (vi) Back to step i.

After three couplings with amino acids, a 9:1 mixture of the building blocks **4** (2.7 equiv, 0.356 mmol, 207 mg) and **5** (0.3 equiv, 0.040 mmol, 18 mg) dissolved in DMF (11 mL) was activated using NEM (0.528 mmol, 67 μL) and TBTU (0.380 mmol, 122 mg) and added to the wells (550 μL each). Reaction was allowed to proceed for 42 h. Three more couplings of amino acids were performed with intermediate combining/mixing by the above protocol. After the last coupling, the resin was transferred to a flat-bottomed syringe with Teflon fritte and washed with DMF, split into three equal portions which were washed with DCM and lyophilized.

One-third of the resin, which corresponded to 400 mg of underivatized resin or approximately 165 000 beads, was used in the screening. The number of beads was established by weighing out 1.85 mg of resin on an analytical balance, swelling the resin in H_2O, and counting the number of beads under the microscope. A number of 409/mg was determined corresponding to approximately 165 000 beads in 400 mg. The resin was treated with $Pd(PPh_3)_4$ (0.132 mmol, 139 mg) in a degassed mixture of $CHCl_3$:AcOH:NEM 92.5:5:2.5 for a period of 4.5 h and washed with $CHCl_3$ × 3, DMF × 3, 0.5% Et_2NCS_2Na in DMF × 1 and DMF. A solution of the protected peptide MMP-substrate, Boc-Ala-Tyr(NO2)-Gly-Pro-Leu-Gly-Leu-Tyr(Bu^t)-Ala-Arg(Pmc)-Lys(Abz(Boc))-Gly-OH (**8**, 0.132 mmol, 264 mg), NEM (0.176 mmol, 22.5 μL), and TBTU (0.127 mmol, 40.6 mg) in anhydrous DMF was preactivated for 10 min, added to the resin, and allowed to react for a period of 14 h. The resin was washed with DMF, Fmoc was removed, and the resin was washed with DMF and DCM. The resin was dried by lyophilization for 1.5 h and treated with a cocktail composed of TFA:DCM:H_2O:MeSPh:$(CH_2SH)_2$:TIPS 66.5:20:5:5:2.5:1 for 3 h to remove peptide side chain protective groups. The resin was washed with AcOH × 3, DCM × 3, methanol × 3, H_2O × 3, 0.1 M NaOH (aqueous), treated with 0.1 M NaOH (aqueous) for a period of 40 min, and washed with H_2O × 3, methanol × 5, DCM, and lyophilized to obtain resin **1**.

Incubation of Library 1 with MMP-12, Selection of Beads, and Sequencing of Active Inhibitors. A solution of MMP-12 (8 mL, 100 nM in buffer 50 mM Tris pH = 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 50 μM $ZnSO_4$, 0.05% (v/v) Brij-35) was added to the dried library resin **1** (400 mg, 0.132 mmol) in a flat-bottomed syringe with Teflon fritte. Incubation was carried out for a period of 22.5 h at room temperature after which the resin was washed with H_2O, 10 mM TFA × 3, H_2O, saturated $NaHCO_3$ × 3, H_2O.

An amount of H_2O just enough to swell it was added to the resin, and approximately 1/50 of resin was placed on a glass plate (8 × 8 cm). The other side of the glass plate had been furnished with a line of a yellow, fluorescent highlighter pen, and the line was covered with a piece of transparent tape. Portions of the resin were placed in a line beside the yellow line, and while looking in the fluorescence microscope the beads were sorted in such a way that only those appearing to be approximately as dark as the black support underneath were pushed (with a bent steel needle) to the side to lie on top of the yellow, fluorescent line. After a batch of resin was sorted, all dark beads were transferred to another glass plate, and sorting was continued with a new batch. Approximately 1000 dark beads were collected manually by inspection under a fluorescent microscope, and from these 20 of the most persistently dark beads were selected and sequenced (see below). Further, 92 dark beads were selected; however, as described sequencing was more difficult for these due to prolonged exposure to UV light. They were transferred to a well in a 96 well ELISA plate and washed with MeCN:H_2O 7:3 × 3 by adding 100 μL solvent, waiting 2 min, and removing the solvent with a bent needle on a syringe. The aperture of the needle was reduced to avoid unwanted removal of beads. All manipulations of single beads were carried out in such a manner that the beads could be viewed under a microscope at all times. Otherwise beads were too easily lost. Therefore this was a rather specialized washing procedure.

Each bead was moved from the glass plate to a MALDI-TOF target by means of a needle and irradiated for 1 h with a Hg lamp. H_2O (0.2 μL) and matrix solution (0.2 μL, 10 mg of α-cyano-4-hydroxycinnamic acid in 1 mL of MeCN:H_2O 7:3) were added to each bead, and they were allowed to dry out before the mass spectrum was acquired.

Resynthesis of Inhibitor Sequences 13−52. MCPS of sequences **13−52** was carried out on a $PEGA_{800}$-resin using Fmoc amino acid Pfp esters, building block **4**, and the base labile 4-hydroxymethylbenzoic acid as previously reported.[10] For each peptide, a PEGA-resin (0.45 μmol/mg, 60 mg, 27 μmol) derivatized with Fmoc-Gly-HMBA was used. Purification was carried by preparative HPLC, and the products were characterized by MALDI-TOF MS and amino acid analysis.

Enzyme Kinetic Assays for Determination of K_i Values. Equilibrium constants K_i were determined from 16 point titration experiments with 60 min preincubation of the enzyme−inhibitor solution at 37 °C. Activity was measured (320 nm excitation, 387 nm emission at 37 °C) on a SPECTRAmax Gemini plate reader mixing the enzyme−inhibitor solution 1:1 with 10 μM of a quenched fluorescent substrate Mca-PLGL-Dpa-AR-NH_2.[38] As buffer was used 10 mM Tris, 0.1 M NaCl, 10 mM $CaCl_2$, 50 μM $ZnCl_2$, 0.05% w/v Brij, pH 7.50. The final concentration of the substrate and enzyme were 5 μM and 3 nM, respectively, and the total volume of the assays was 200 μL. The K_i was calculated

from plots of inhibition versus inhibitor concentration using $I = I_{max}\,[I_0]/(K_i + [I_0])$, assuming that $E_0 < K_i$, where I is inhibition, K_i is the dissociation constant, $[I_0]$ is the concentration of inhibitor added.

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