Rational Design and Combinatorial Evaluation of Enzyme Inhibitor Scaffolds: Identification of Novel Inhibitors of Matrix Metalloproteinases

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The discovery of a novel series of heterocyclic matrix metalloproteinase (MMPs) inhibitors is described. Published crystal structures of peptidyl hydroxamates bound to MMPs were the basis for the rational design of diketopiperazine (DKP) inhibitors. Combinatorial libraries were prepared and evaluated for their ability to inhibit collagenase-1, stromelysin-1, and gelatinase-B substrate hydrolysis. Deconvolution of active pools resulted in the identification of potent inhibitors (IC_{50}'s < 100 nM) of collagenase-1 and gelatinase-B, with the most potent inhibitor exhibiting an IC_{50} of 30 nM against collagenase-1. A description of the combinatorial evaluation process, as well as initial SAR interpretation for this novel series, is provided.

Introduction

Combinatorial chemistry has now gained acceptance throughout the pharmaceutical industry as a tool to assist in the identification and development of therapeutic agents.[1] The two most commonly utilized strategies are (1) high-throughput screening of naive libraries against an array of proteins to identify a lead structure and (2) screening of directed libraries based upon an already existing lead compound in order to optimize the physicochemical and biochemical properties of the inhibitor. The lead compound that forms the basis of the directed libraries may be generated from either traditional sources or naive library screens. A third approach that has not been reported at this time is the identification of a novel inhibitor from combinatorial libraries that have been prepared based upon the rational design of a new inhibitor series. The inherent challenges of this strategy are substantial but if successful would represent a powerful new application of the technology. The ability to construct libraries around a core scaffold uniquely positions combinatorial strategies to thoroughly investigate a proposed inhibitor scaffold. In this article we describe our successful efforts in identifying potent collagenase-1 and gelatinase-B inhibitors using combinatorial strategies that were based upon the rational design of a new inhibitor class.

The matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes involved in the degradation and remodeling of the extracellular matrix.[2] They are important therapeutic targets with indications in cancer,[3] arthritis,[4] autoimmunity,[5] and cardiovascular disease.[6] Structural features common to most MMP inhibitors include a ligand that interacts with the catalytic site zinc metal and a peptidic fragment that binds to a subset of the enzyme's specificity pockets. Although it is relatively straightforward to identify potent in vitro MMP inhibitors using this approach,[7] in general they exhibit low oral availability and poor plasma stability due to the peptidic fragments they contain. It has proven very difficult to improve the in vivo profiles of these inhibitors; as a result, only a limited number have progressed into clinical trials. Our goal was to design a heterocyclic scaffold that would replace the peptidyl-succinate portion of these inhibitors while retaining the appropriate spatial relationship between a zinc ligand and side chains.[8]

On the basis of published crystal structures of succinyl hydroxamate inhibitors bound with various MMPs,[9] a pharmacophore model that incorporated the P_1' and P_2' side chains as well as the zinc ligand was constructed. This was compared with a number of internally generated heterocyclic scaffolds. Acceptable overlap was obtained with a 2,5-diketopiperazine (DKP) scaffold. Although the DKP scaffold was able to array its three side chains in the required orientation, it was also apparent from these models that there was negligible overlap with the peptide backbone of the succinyl hydroxamates. Consequently, hydrogen bonds that form between the enzyme active site and peptidic inhibitors might be unavailable to DKP-based inhibitors. These hydrogen bonds have been identified as an important component of protease−inhibitor binding interactions and their loss could potentially result in a significant reduction in binding energy for DKP-based inhibitors. However, one attractive feature of the DKP scaffold compared to many of the other heterocyclic scaffolds that were considered is that hydrogen-bonding sites are present within the scaffold itself, thereby maintaining the potential to form new interactions within the catalytic site.

Chemistry

The synthesis of DKPs on a solid support has been described in detail previously (Scheme 1).[10] The sequence begins with esterification of the solid support with an amino acid to yield **1**, followed by reductive alkylation of the amino acid and acylation of the resulting secondary amine to yield **3**. Deprotection of the N-alkylated dimer followed by cyclative cleavage of

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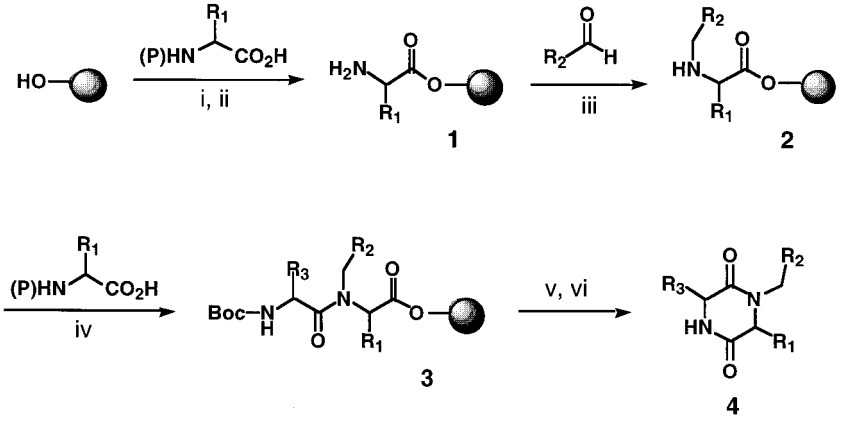
Scheme 1. Solid-Phase Diketopiperazine Synthesis Cycle[a]



[a] (i) DIC, DMAP, DCM or 1,3-dimethyl-2-fluoropyridinium 4-toluenesulfonate (DMFP), DIEA, DCM; (ii) deprotect; (iii) $NaCNBH_3$, MeOH or HOAC, trimethyl orthoformate; (iv) HATU, DIEA, DCM; (v) TFA; (vi) toluene.

the linear precursor from the resin yields a soluble DKP (**4**). As a consequence of the mild conditions required for cyclization, it is effectively a purification step. Any failed sequences (including unalkylated dimer, resulting from incomplete reductive alkylation followed by amino acid acylation, which requires heat (>80 °C) in order to cyclize) remain immobilized to the resin and do not contaminate samples submitted for bioassay. We typically observe only products in greater than 90% purity by HPLC, while failed reaction sequences do not yield any material. This provides assurances that the DKPs are actually assayed and reduces the likelihood of false-positives during bioassay.

Library construction was automated and was performed on instruments developed in house. The machines are able to perform split and pool manipulations with the resin in an automated fashion greatly facilitating the ability to construct combinatorial libraries. One advantage of this automated approach is that in the same time it takes to synthesize a single DKP (3 days) a library of DKPs can be constructed.

Results and Discussion

A fluorogenic assay that was compatible with the efficient screening of the DKP libraries against the four targeted enzymes, collagenase-1, gelatinase-B, stromelysin-1, and matrilysin, was developed.[11] Library pools were rank-ordered based upon inhibition of substrate proteolysis at a single concentration. This was followed by traditional 8-point IC_{50} determinations for pools of interest. Pools exhibiting the best inhibition were then deconvoluted and the crude products assayed to identify the active component. Compounds with desired potencies were resynthesized, purified, fully characterized, and assayed to obtain accurate IC_{50} values.

DKP libraries were constructed utilizing cysteine as the zinc ligand, and the remaining two diversity sites were expected to interact with the enzyme's specificity pockets. Initial libraries incorporated both L- and D-cysteine. Two different substitution patterns, DKP-I (R_1 derived from cysteine) and DKP-II (R_3 derived from cysteine), were investigated (Tables 1 and 2, respectively).[12] In addition to varying the spatial arrangement of the side chains relative to one another, it is important to note that the positions of the side chains relative to the DKP's hydrogen-bonding sites also differed in both series. As a result of the pure material obtained following cyclative cleavage, the concentration of pools could be determined using an Ellman's assay to quantify the amount of free thiol present, enabling the accurate assessment of each pool's activity.

The first library constructed was in the DKP-I format. A 684-membered library (Table 1) was prepared that incorporated L- and D-cysteine (R_1), 19 aldehydes (R_2), and 18 amino acids (R_3). The library consisted of 36 pools, each defined by cysteine stereoconfiguration and amino acid R_3, with 19 DKPs per pool (aldehyde R_2 pooled). The most active pool (D-cysteine at R_1 and pyridylalanine at R_3) was retested and exhibited an IC_{50} of 15 μM against collagenase-1 (Figure 1). Deconvolution of this pool identified two DKPs with low-micromolar activity, R_2 = *n*-heptyl and *n*-pentyl. Another library was constructed with a portion of the building blocks selected based upon the structure−activity relationship (SAR) from these initial results with the remainder chosen in a nonbiased fashion. No further increase in binding affinity was observed with this library. On the basis of the results obtained with the DKP-II format libraries described below, no further efforts were expended attempting to optimize these structures.

A 684-member DKP-II library was prepared (Table 2) that incorporated 19 amino acids (R_1), 18 aldehydes (R_2), and both isomers of cysteine (R_3). The library consisted of 36 pools, each defined by cysteine stereoconfiguration and aldehyde R_2, with 19 DKPs per pool (amino acid R_1 pooled). MMP inhibition assays identified a number of pools with sub-micromolar activities against collagenase-1 and gelatinase-B (Figure 2). Interestingly none of the pools in this library exhibited significant activity against matrilysin or stromelysin-1. It was apparent from these results that a wide range of substitutions at the R_2 position was tolerated while retaining inhibitor potency. Sterically demanding substituents with limited flexibility (Figure 2, jj, mm, rr) exhibited the least activity. The IC_{50} values for a cross section of the pools incorporating L-cysteine were determined for collagenase-1 and gelatinase-B, and all had values between 0.5 and 1 μM.

Pools containing L-cysteine on average exhibited only 10% more inhibitory activity than their D-cysteine counterparts. This observation led us to suspect that

Table 1. Diversity Set for DKP-I

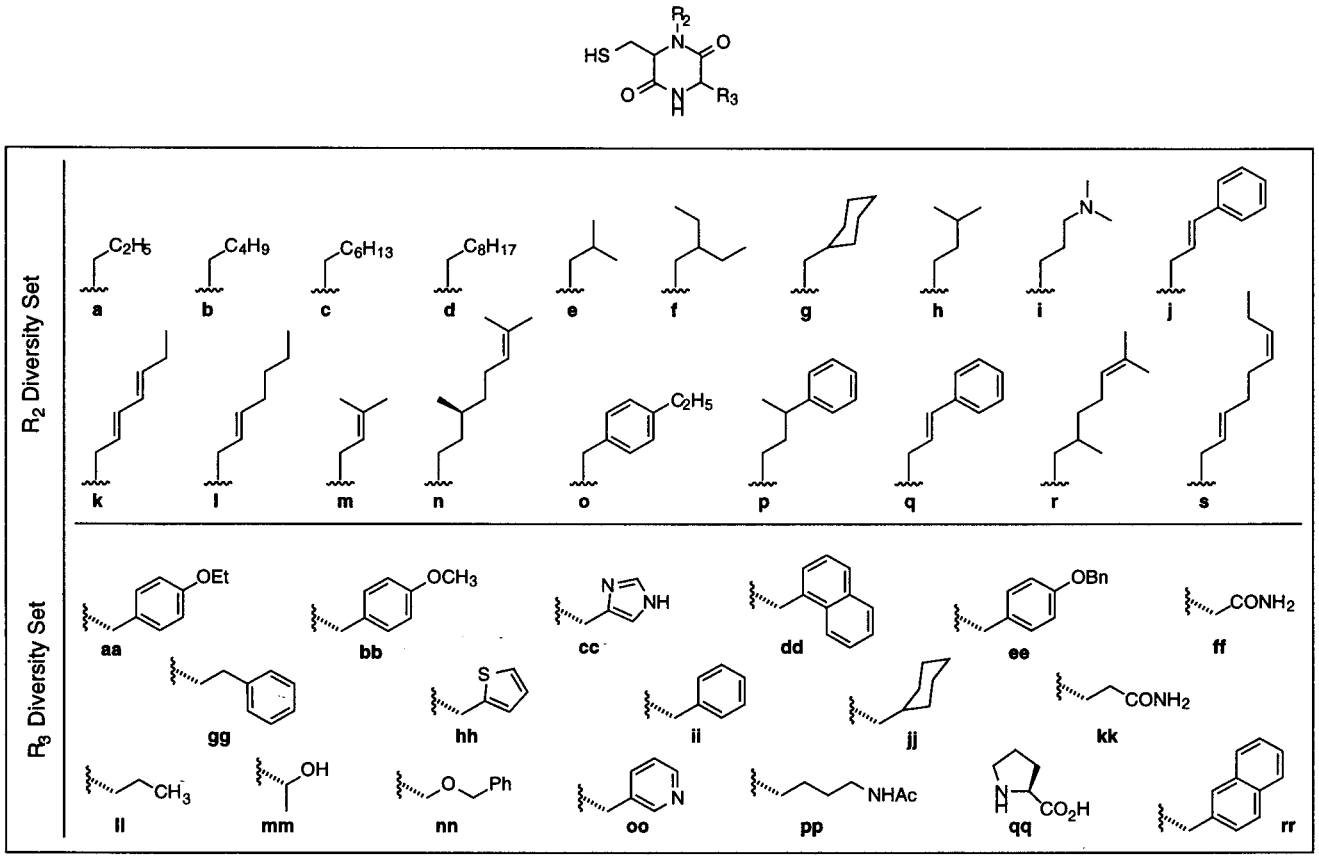
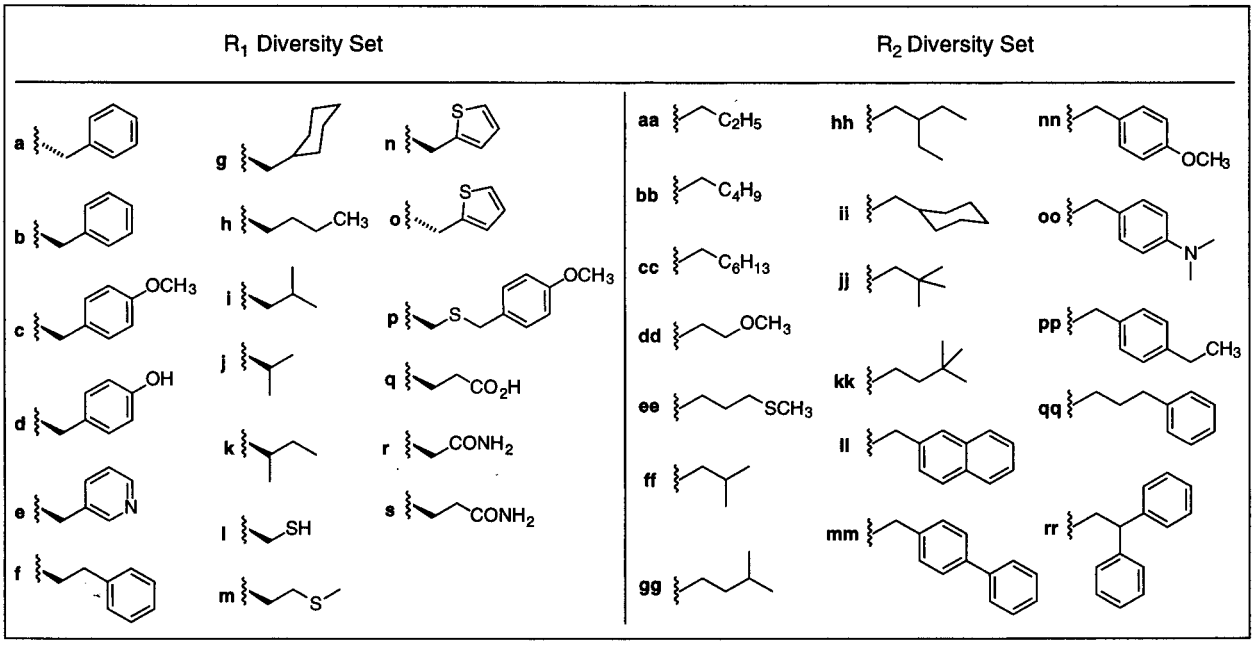


Table 2. Diversity Set for DKP-II



epimerization of cysteine was occurring during DIC/DMAP coupling of cysteine to the secondary amine. This was confirmed upon further investigation of the coupling reaction. A number of different coupling reagents were tested, and the most useful, with respect to both coupling yields and extent of epimerization, was 1,3-

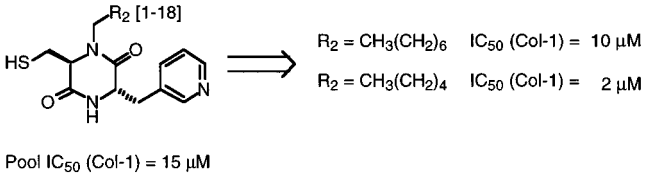


Figure 1. DKP-I deconvolution results.

dimethyl-2-fluoropyridinium 4-toluenesulfonate (DMFP). For the remainder of the experiments, DMFP was utilized as the coupling reagent.

The anisaldehyde pool (Figure 2, pool nn) was selected for deconvolution. As can be seen from Figure 3, variations in the amino acid (R_1) position had a significant effect on inhibition potency with variations of ≥10-fold observed, in contrast to the tolerance for substitutions at the aldehyde (R_2) position. Some of the more obvious SAR observations are (i) the preference for L-configuration at R_1 (a vs b), (ii) the hydrophobic nature of the R_1 binding pocket (c vs d; e, q, r, s), (iii) gelatinase-B's preference for extended R_1 groups compared with collagenase-1 (f and p), and (iv) the loss of activity with α-methyl branching (j and k).

The compound exhibiting the most potent inhibition of both collagenase-1 and gelatinase-B incorporated cyclohexylalanine at R_1 (Figure 3, sample g). Parallel synthesis of DKPs holding cyclohexylalanine constant and varying all 18 aldehydes at R_2 confirmed the wide latitude allowed at this position: inhibition of proteolysis for the majority of the DKPs was within 2-fold of one another (Figure 4).

Some of the more active compounds were resynthesized on a larger scale to allow full characterization and determination of accurate IC_{50} values (Table 3). The IC_{50} values were in general agreement with the library data supporting the SAR established during library screening. The low-nanomolar potencies exhibited by these compounds against collagenase-1 are comparable to those of the succinyl hydroxamate inhibitors that have been described in the literature. As such they represent a truly novel class of MMP inhibitors. To gain additional insight into the SAR for this class of MMP inhibitors, the four stereoisomers of **5** were synthesized and assayed (Figure 5). Changes in the configuration of any of the stereocenters resulted in a substantial loss in activity.

Conclusion

The design and development of metalloproteinase inhibitors has been an area of active investigation since the 1960s and represents a thoroughly investigated field. The ability to design and identify a novel inhibitor class in such a mature field highlights and begins to realize the immense potential of combinatorial strategies, especially when integrated with structural information. Rational design provides guidance in selecting a scaffold out of the nearly infinite number of possibilities that could be attempted, while combinatorial strategies enable the synthesis and testing of many compounds in order to more fully investigate a proposed structural class compared to traditional approaches involving serial synthesis. In addition, a combinatorial library incorporating a diverse set of building blocks may still interact with a protein that has undergone an unexpected conformational shift when interacting with the new scaffold.[13]

As a result of this investigation, we now have a general understanding of the SAR for this class of MMP inhibitors (Figure 6). A DKP incorporating L-amino acids is required for activity. The R_1 position has a significant effect on inhibitor potency, while the R_2 position can undergo substantial variations and retain inhibitor potency. Interpretation of this SAR is consistent with R_1 interacting at the S_1' subsite and R_2 with the S_2' subsite. On the basis of the crystal structures of these proteins with succinyl hydroxamate inhibitors, the S_1' subsite is a pocket that acts as the primary specificity determinant, while S_2' is usually described as a narrow cleft pointing out toward the solvent. The ability to vary the R_2 position should become important as this class of MMP inhibitors undergo further development, as it may be possible to vary the in vivo profile of these inhibitors without affecting inhibitor potency. It is important to note that this investigation was accomplished primarily through library analysis and required minimal compound scaleup (the rate-limiting step in the process), used primarily to authenticate the library analysis. Further investigations of this class of MMP inhibitors are in progress and will be disclosed in due course.

Experimental Section

General Methods. All reagents were purchased from Aldrich, Bachem Bioscience, or Novabiochem and used without further purification. Resins were purchased from Rapp Polymere (TentaGel S OH) or Argonaut Technologies, Inc. (Argo-Gel-OH). NMR spectra were obtained on a Varian Gemini 400 instrument. Mass spectra (flow-injection ESI) were obtained on a Finnigan TSQ 7000 instrument. High-resolution mass spectra were obtained on a VG ZAB 2SE instrument (U.C. Berkeley). Microanalysis was done in the microanalytical lab at U.C. Berkeley. All compounds were purified by flash column chromatography using 5% methanol/DCM. Mercapto DKPs were handled in degassed solvents and stored at −20 °C or lower temperatures.

General Procedure for the Synthesis of Discrete Cys-DKPs. Loading of amino acids on hydroxy resin and reductive alkylations were performed as previously described.[10] BocCys-(Trt)-OH was coupled to the secondary amine via the symmetric anhydride using 2 equiv of BocCys(Trt)-OH and 1 equiv of dicylohexylcarbodiimide in anhydrous dichloromethane at a 0.5 M concentration overnight. The resin was washed several times with dimethylformamide, methanol, and dichloromethane. For Boc and trityl deprotection a mixture of 45% dichloromethane, 50% trifluoroacetic acid, and 5% triethylsilane was added and the resin agitated for 30 min. The solvent was drained and the solid support washed several times with dichloromethane until it was pH neutral. For the cyclative cleavage, 1% acetic acid in degassed methanol was added and the resin shaken for 5−10 h. After that time, the supernatant was filtered and the resin washed several times with degassed methanol. The combined filtrate and washings were concentrated, and the crude product was purified by flash chromatography. After removal of the chromatography solvents under vacuum, the samples were lyophilized from *tert*-butyl alcohol.

(6*S*,3*R*)-1,6-Bis(cyclohexylmethyl)-3-(sulfanylmethyl)-hexahydro-2,5-pyrazinedione (5): ^{1}H NMR ($CDCl_3$) δ 7.10 (s, 1H), 4.08 (m, 1H), 3.91 (m, 2H), 3.16 (m, 1H), 2.95 (m, 1H), 2.48 (m, 1H), 1.94−1.55 (m, 15H), 1.33−1.10 (m, 5H), 1.05−0.83 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 168.43, 164.55, 58.60, 58.09, 50.80, 42.68, 35.91, 34.39, 34.31, 32.88, 31.39, 31.44, 30.83, 30.62, 26.62, 26.56, 26.38, 26.21, 26.09, 25.93; MS *m*/*z* 353 (M + H); $[\alpha]^{22}_D$ = 18.9 (ethanol). Anal. ($C_{19}H_{32}N_2O_2S$) C, H, N.

(6*S*,3*R*)-6-(Cyclohexylmethyl)-1-(2-quinolylmethyl)-3-(sulfanylmethyl)hexahydro-2,5-pyrazinedione (6): ^{1}H NMR

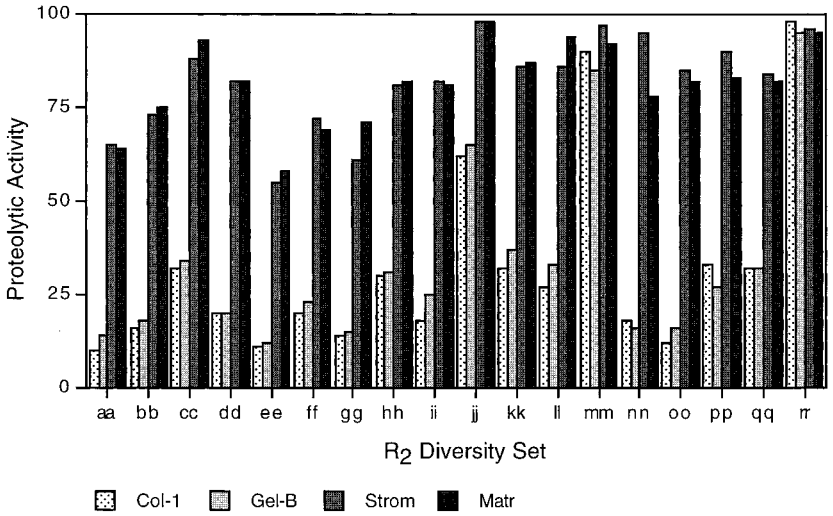


Figure 2. Proteolytic activity (%) in the presence of 6 μM L-cysteine-DKP-II pools.

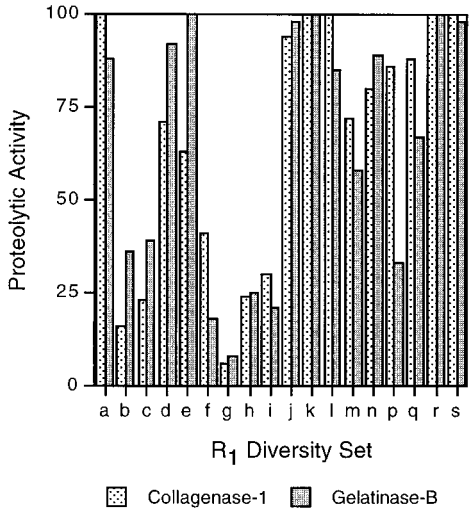


Figure 3. Proteolytic activity (%) of L-cysteine-DKP-II deconvolution (diversity set o was not prepared/assayed).

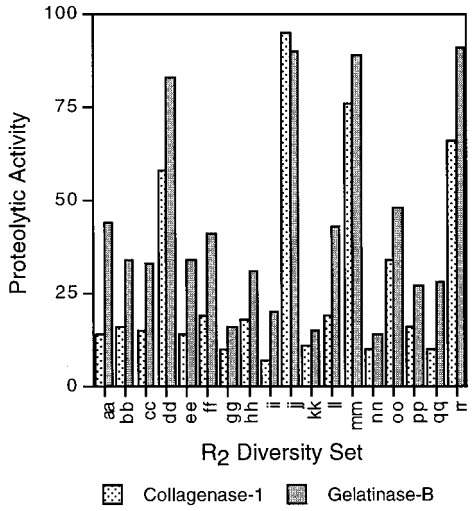
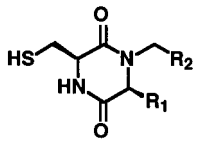


Figure 4. Proteolytic activity (%) varying aldehyde position and holding R_1 constant as cyclohexylalanine.

$(CDCl_3)$ δ 8.14 (m, 1H), 8.02 (m, 1H), 7.81 (m, 1H), 7.72 (m, 1H), 7.54 (m, 1H), 7.41 (m, 1H), 6.76 (s, 1H), 5.43 (d, J = 15.4 Hz, 1H), 4.40 (d, J = 15.4 Hz, 1H), 4.18 (m, 2H), 3.18 (m, 1H), 2.88 (m, 1H), 1.90−1.50 (m, 8H), 1.28−0.86 (m, 5H); ^{13}C NMR $(CDCl_3)$ δ 168.20, 164.72, 155.73, 147.67, 137.31, 129.91, 129.28, 127.65, 127.50, 126.80, 120.20, 58.40, 58.11, 50.38, 41.72, 34.50, 34.25, 33.03, 30.73, 26.59, 26.41, 26.21; MS m/z 398 (M + H). Anal. ($C_{22}H_{27}N_3O_2S\cdot0.4H_2O$) C, H, N.

Table 3. IC_{50} Values for Purified DKPs



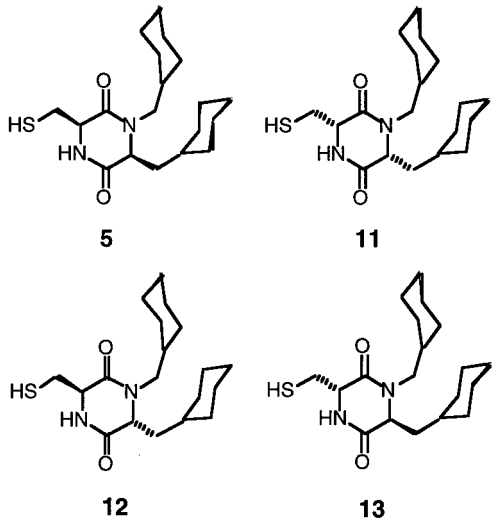
			IC_{50} (nM)		
no.	R_1	R_2	MMP-1	MMP-3	MMP-9
5	$C_6H_{11}CH_2$	C_6H_{11}	47	2910	121
6	$C_6H_{11}CH_2$	2-quinolyl	35	2400	87
7	$C_6H_{11}CH_2$	*p*-$CH_3OC_6H_4$	30	3800	79
8	$PhCH_2$	*p*-$CH_3OC_6H_4$	64	4045	2365
9	$PhCH_2$	*n*-propyl	319	19000	2365
10	*n*-butyl	C_6H_{11}	220	3400	370

(6*S*,3*R*)-6-(Cyclohexylmethyl)-1-(4-methoxybenzyl)-3-(sulfanylmethyl)hexahydro-2,5-pyrazinedione (7): ^{1}H NMR $(CDCl_3)$ δ 7.16 (d, J = 8.6 Hz, 2H), 7.10 (s, 1H), 6.86 (d, J = 8.6 Hz, 2H), 5.33 (d, J = 15.2 Hz, 1H), 4.14 (m, 1H), 3.83 (m, 1H), 3.80 (s, 3H), 3.77 (d, J = 15.2 Hz, 1H), 3.15 (m, 1H), 2.81 (m, 1H), 1.87−1.52 (m, 9H), 1.30−1.13 (m, 2H), 1.00−0.80 (m, 2H); ^{13}C NMR $(CDCl_3)$ δ 168.38, 164.46, 159.45, 129.78, 129.59, 127.33, 114.42, 58.50, 56.17, 55.58, 46.86, 41.70, 34.40, 34.37, 33.05, 32.99, 31.34, 30.65, 26.59, 26.42, 26.22; MS m/z 377 (M + H), 399 (M + Na). Anal. ($C_{20}H_{28}N_2O_3S\cdot0.7$tBuOH) C, H, N.

(6*S*,3*R*)-6-Benzyl-1-(4-methoxybenzyl)-3-(sulfanylmethyl)hexahydro-2,5-pyrazinedione (8): ^{1}H NMR $(CDCl_3)$ δ 7.35−7.09 (m, 7H), 6.89 (m, 2H), 6.35 (s, 1H), 5.55 (m, 1H), 4.18 (m, 1H), 3.95 (m, 1H), 3.91 (s, 3H), 3.66 (m, 1H), 3.25 (m, 2H), 2.63 (m, 1H), 1.05 (m, 1H); ^{13}C NMR $(CDCl_3)$ δ 166.18, 164.29, 159.63, 134.68, 130.42, 130.14, 129.02, 128.01, 126.87, 114.55, 59.19, 57.69, 55.63, 46.49, 36.31, 31.56, 29.75; MS m/z 371 (M + H); HRMS (M + H) $C_{20}H_{23}N_2O_3S$ calcd 371.1429, found 371.1428.

(6*S*,3*R*)-6-Benzyl-1-propyl-3-(sulfanylmethyl)hexahydro-2,5-pyrazinedione (9): ^{1}H NMR $(CDCl_3)$ δ 7.31 (m, 3H), 7.10 (m, 2H), 6.44 (s, 1H), 4.30 (m, 1H), 4.12 (m, 1H), 3.61 (m, 1H), 3.34 (m, 1H), 3.14 (m, 1H), 2.89 (m, 1H), 2.58 (m, 1H), 1.65 (m, 3H), 0.98 (m, 3H); ^{13}C NMR $(CDCl_3)$ δ 166.15, 164.06, 134.53, 130.51, 128.97, 127.98, 60.50, 57.58, 46.09, 36.96, 29.58, 20.33, 11.67; HRMS (M + H) $C_{15}H_{21}N_2O_2S$ calcd 293.1324, found 293.1321. Anal. ($C_{15}H_{20}N_2O_2S$) C, H, N.

(6*S*,3*R*)-6-Butyl-1-(cyclohexylmethyl)-3-(sulfanylmethyl)hexahydro-2,5-pyrazinedione (10): ^{1}H NMR $(CDCl_3)$ δ 7.01 (s, 1H), 4.09 (m, 1H), 3.93 (m, 2H), 3.20 (m, 1H), 2.72 (m, 1H), 2.55 (m, 1H), 2.00 (m, 1H), 1.82 (m, 1H), 1.78−1.58 (m,



	Collagenase-1	Gelatinase-B	Stromelysin	Matrilysin
5	47	113	2.300	590
11	14,000	>40,000	>40,000	>40,000
12	1,600	12,000	>40,000	4,800
13	2,600	7,000	>40,000	>40,000

Figure 5. Dependence of IC_{50} values (nM) upon stereoconfiguration of DKP centers.

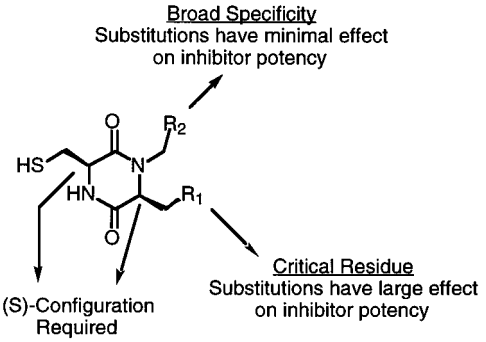


Figure 6. General SAR for DKP inhibitors.

7H), 1.40−1.05 (m, 8H), 1.08−0.88 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 168.08, 164.43, 60.03, 58.17, 50.33, 35.57, 32.91, 31.37, 30.75, 30.60, 27.65, 26.56, 26.07, 25.91, 22.74, 14.20; MS *m/z* 313 (M + H); HRMS (M + H) $C_{16}H_{29}N_2O_2S$ calcd 313.1950, found 313.1958.

(6*R*,3*S*)-1,6-Bis(cyclohexylmethyl)-3-(sulfanylmethyl)-hexahydro-2,5-pyrazinedione (11): ^{1}H NMR ($CDCl_3$) δ 7.11 (s, 1H), 4.09 (m, 1H), 3.89 (m, 2H), 3.13 (m, 1H), 2.75 (m, 1H), 2.46 (m, 1H), 1.94−1.55 (m, 15H), 1.33−1.10 (m, 5H), 1.05−0.83 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 168.57, 164.59, 58.58, 58.08, 50.79, 42.69, 35.91, 34.42, 34.31, 32.89, 31.40, 30.84, 30.57, 26.63, 26.57, 26.39, 26.22, 26.10, 25.93; $[\alpha]^{22}_D$ = 18.0 (ethanol); HRMS (M + H) $C_{19}H_{33}N_2O_2S$ calcd 353.2263, found 353.2273. Anal. ($C_{19}H_{32}N_2O_2S$) C, H, N.

(6*R*,3*R*)-1,6-Bis(cyclohexylmethyl)-3-(sulfanylmethyl)-hexahydro-2,5-pyrazinedione (12): ^{1}H NMR ($CDCl_3$) δ 6.79 (s, 1H), 4.13 (m, 1H), 3.91 (m, 2H), 3.12 (m, 1H), 2.98 (m, 1H), 2.47 (m, 1H), 1.90 (m, 1H), 1.86−1.55 (m, 12H), 1.31−1.05 (m, 6H), 1.01−0.82 (m, 4H), 1.48 (m, 1H); ^{13} C NMR ($CDCl_3$) δ 169.16, 165.35, 59.42, 55.31, 51.37, 40.15, 36.26, 34.12, 33.93, 33.10, 31.33, 30.83, 27.86, 26.63, 26.59, 26.37, 26.24, 26.11, 25.95; $[\alpha]^{22}_D$ = 40.0 (ethanol); MS *m/z* 353 (M + H). Anal. ($C_{19}H_{32}N_2O_2S$) C, H, N.

(6*S*,3*S*)-1,6-Bis(cyclohexylmethyl)-3-(sulfanylmethyl)-hexahydro-2,5-pyrazinedione (13): ^{1}H NMR ($CDCl_3$) δ 6.90 (s, 1H), 4.11 (m, 1H), 3.90 (m, 2H), 3.11 (m, 1H), 2.99 (m, 1H), 2.44 (m, 1H), 1.90 (m, 1H), 1.86−1.55 (m, 12H), 1.31−1.05 (m, 6H), 1.01−0.82 (m, 4H), 1.48 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 169.66, 165.62, 59.47, 55.39, 51.33, 40.10, 36.18, 34.05, 33.90, 33.06, 31.26, 31.21, 30.77, 27.67, 26.53, 26.26, 26.14, 26.01, 25.85; MS *m/z* 353 (M + H); $[\alpha]^{22}_D$ = 57.8 (ethanol). Anal. ($C_{19}H_{32}N_2O_2S\cdot0.1H_2O$) C, H, N.

Library Synthesis and Deconvolutions. Libraries and discrete DKPs were synthesized on a 36-channel Affymax ESL synthesizer. Amino acids were manually loaded to the resin as previously described.[10] In each reaction vessel was placed 200−300 mg of resin, and it was reductively alkylated with the aldehyde. After washing, the resins were automatically mixed and redistributed to individual vessels. BocCys(Trt)-OH was coupled to all pools as described for the synthesis of discrete compounds. After Boc cleavage and DKP cyclizations, the supernatants were collected for each pool and concentrated in a Savant SpeedVac system.

Protease Inhibition Assays. Recombinant C-terminally truncated forms of human stromelysin-1 and matrilysin were prepared as described previously.[14] The truncated form of human collagenase-1 was prepared using the same methodology. Recombinant C-terminally truncated gelatinase-B was prepared from *Pichia pastoris*. IC_{50} values were determined using the quenched-fluorescence substrate (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-(3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl)-Ala-Arg-NH_2 (Bachem, Torrance, CA), developed by Knight et al.[11] Assays were conducted at room temperature in a total volume of 0.2 mL in assay buffer (20 mM Hepes, 5 mM $CaCl_2$, 0.02% Brij35, 0.5 mM cysteine) in black 96-well assay plates.[15] Compounds were dissolved in methanol that was degassed by bubbling argon on ice for >30 min and then diluted 1:1 with assay buffer before use. In each assay, enzyme was diluted to its final concentration (collagenase, stromelysin-1, matrilysin, and gelatinase-B at 10, 10, 0.5, and 2 nM, respectively). After addition of inhibitor (final [methanol] = 2%), the assay was initiated 3 min later by the addition of substrate to a final concentration of 10 μM. The progress of the reaction was monitored by observing fluorescence (excitation 330 nm (30-nm bandwith)/emission 430 nm (60-nm bandwith)) in a Dynatek Fluorlite 10000 fluorescence plate reader. The fluorescence of each well was recorded once a minute for 30 min. The IC_{50} values were determined by fitting the data to the equation $v_i = v_0/(1+ [\text{inhibitor}]/IC_{50})$, where v_i is the rate of fluorescence change at a given inhibitor concentration and v_0 the rate of fluorescence change in the absence of inhibitor.

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