Identification of Highly Selective Inhibitors of Collagenase-1 from Combinatorial Libraries of Diketopiperazines

Anna Katrin Szardenings, Valery Antonenko, David A. Campbell,† Nuria DeFrancisco, Satoru Ida, Lihong Shi, Nikolai Sharkov, David Tien, Yongwen Wang, and Marc Navre*

Affymax Research Institute, 3410 Central Expressway, Santa Clara, California 95051

Received August 13, 1998

Thiol-containing diketopiperazines have been recently identified as novel heterocyclic inhibitors of matrix metalloproteinase (MMPs). The compounds described had similar activities against the MMPs collagenase-1 and gelatinase-B. An inhibitor that showed greater than 10-fold selectivity for collagenase-1 over gelatinase-B was desired. Previously published work with peptidyl hydroxamates and thiols indicated that while preparing gelatinase selective inhibitors was straightforward, there was not an obvious route to selective inhibitors of collagenase-1. Combinatorial libraries were prepared and evaluated for their ability to inhibit collagenase-1 and gelatinase-B substrate hydrolysis. A method for estimating the IC_{50} values of compounds generated by high-throughput parallel synthesis aided in the identification of compounds with the desired properties. We have found that thiol diketopiperazines derived from nitrophenylalanine are both potent and selective inhibitors of collagenase-1. In addition, we have demonstrated that combinatorial chemistry can be utilized to identify molecules with a desired selectivity profile without access to the traditional tools of rational drug design.

Introduction

There are now many examples of the application of combinatorial chemistry in the identification of enzyme inhibitors.[1] We recently described the use of combinatorial chemistry to design and evaluate the activity of a new scaffold, the thiol-containing diketopiperazines (DKPs), as inhibitors of the matrix metalloproteases (MMPs).[2] Starting with inhibitors that showed IC_{50} values in the range of 10−20 μM, compounds as potent as 30 nM against collagenase-1 were identified in libraries of DKPs.

The MMPs are a family of zinc-dependent enzymes involved in the degradation and remodeling of extracellular matrix.[3−5] They are important therapeutic targets, especially in cancer and arthritis.[6−12] In the past decade, numerous new classes of MMP inhibitors, both peptidic and nonpeptidic, have been developed as potential pharmaceutical agents.[6,13,14] Although many of these inhibitors were potent, the first generation of these compounds lacked selectivity. Second generation compounds were developed to exhibit selective inhibition on different subsets of MMPs. In the case of peptide-based succinyl hydroxamate inhibitors, selectivity for gelatinase and stromleysin was easily obtained by incorporating large substituents into the portion of the molecule mimicking the P_1' side chain of the peptide substrate.[15−17]

Prior to the characterization of the enzyme collagenase-3 (MMP-13),[18−20] collagenase-1 (MMP-1) was considered an important target in the development of MMP inhibitors for the treatment of rheumatoid arthritis.[21−23] In addition, anecdotal information available at that time suggested that the use of nonselective MMP inhibitors in treatment regimens could result in undesirable side effects, possibly due to the inhibition of gelatinase or stromelysin. Thus, there was a requirement for collagenase-1 inhibitors selective against gelatinase-B and stromelysin. Known structure−activity relationships (SAR) provided very limited information as to how to design a selective collagenase-1 inhibitor.[24] SAR and structural studies indicated that collagenase-1 would only tolerate small substituents (such as the isobutyl moiety of leucine) in its P_1' binding pocket. Gelatinase and stromelysin were inhibited just as potently by these inhibitors.[13,25]

The challenge we were presented with was to rapidly identify selective DKP inhibitors of collagenase-1 without access to X-ray coordinates or insight into collagenase-1 selectivity from peptide hydroxamate SAR. We had previously shown that through iterative screening of libraries of thiol diketopiperazines, we could improve the *potency* of this class of compounds from 15 μM to 30 nM. In this report, we demonstrate the use of various methodologies of combinatorial chemistry to identify and improve highly *selective* inhibitors of collagenase-1.

Chemistry

DKPs were synthesized on solid support as described previously[26] (see Schemes 1 and 2). For an automated library and parallel synthesis, instruments developed in house were utilized. The multicomponent condensation was especially suited for a high-throughput parallel synthesis and all Ugi DKPs were synthesized in a parallel fashion in a 96-well plate format. The crude compounds were analyzed by LC/MS to confirm structures and purities.

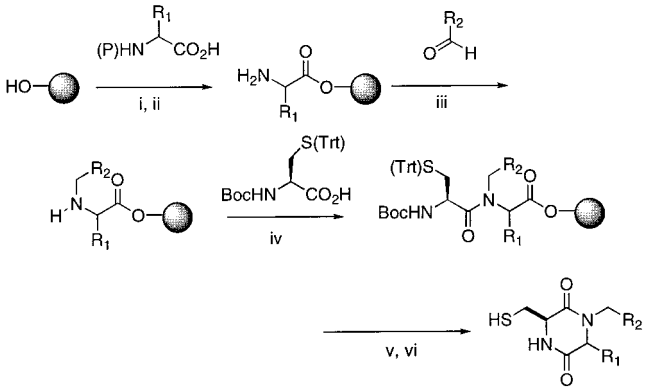
Results and Discussion

To identify compounds from combinatorial libraries that were selective as well as potent inhibitors of

* To whom correspondence should be addressed. Phone: (408) 522-5718. Fax: (408) 481-0522. E-mail: Marc_Navre@affymax.com.
† Current address: Bayer Corp., Institute of Chemistry, West Haven, CT 06516.

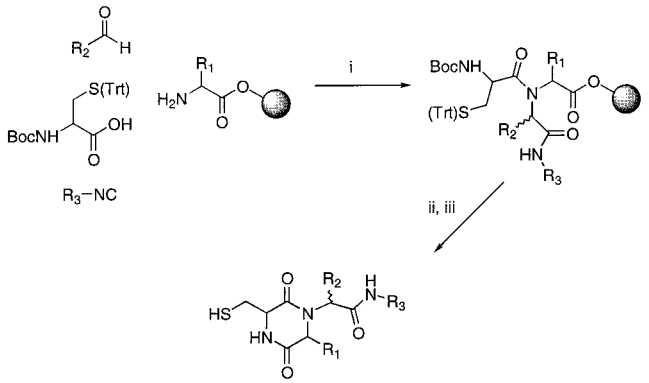
10.1021/jm980475p CCC: $18.00
Published on Web 03/31/1999

Scheme 1. Solid Phase DKP Synthesis I[a]



[a] Reagents and conditions: (i) DIC, DMAP, DCM -or- 1,3-dimethyl-2-fluoropyridinium 4-toluenesulfonate (DMFP), DIEA, DCM; (ii) deprotect; (iii) $NaCNBH_3$, MeOH or HOAC, trimethylorthoformate; (iv) DIC, DCM; (v) TFA, TES (vi) toluene or methanol.

Scheme 2. Solid Phase DKP Synthesis II (Ugi Route)[a]



[a] Reagents and conditions: (i) DCM, MeOH; (ii) TFA/TES/DCM; (iii) methanol.

collagenase-1, the following process was implemented: libraries of thiol diketopiperazines were prepared as described above with instrumentation to facilitate the pooling and splitting process. Because of the cyclative cleavage step, the crude compounds were typically > 90% pure.[26] We took advantage of this property of the DKP pools to make more reliable measurements of inhibitor concentration by determining the concentration of free thiol in the sample using Ellman's reagent (see Experimental Section). A fluorogenic assay for the four targeted enzymes, collagenase-1, gelatinase-B, stromelysin, and matrilysin was utilized.[2] Library pools were rank-ordered based upon inhibition of substrate proteolysis. Pools exhibiting the best inhibition were then deconvoluted and the crude products assayed to identify the active components. Compounds with desired potencies were resynthesized, purified, fully characterized, and assayed by traditional eight-point determinations to obtain accurate IC_{50} values.

We initiated our search with ACL0938, a 1225-member library (Tables 1 and 2). This library was prepared with L-cysteine, 35 amino acids (R_1), and 35 aldehydes (R_2). Each of the 35 pools in this library was fixed at the aldehyde position and degenerate with regard to the amino acids. Each pool of 35 compounds was assayed at two dilutions against all four MMPs. The results are shown in Figure 1 of the Supporting Information. The observation of a relatively flat SAR with respect to the aldehyde in R_2 was consistent with results from our previous study.[2] Pool 32, carrying a quinoline group, however, showed better activity for collagenase-1 than the others and was selected for further study.

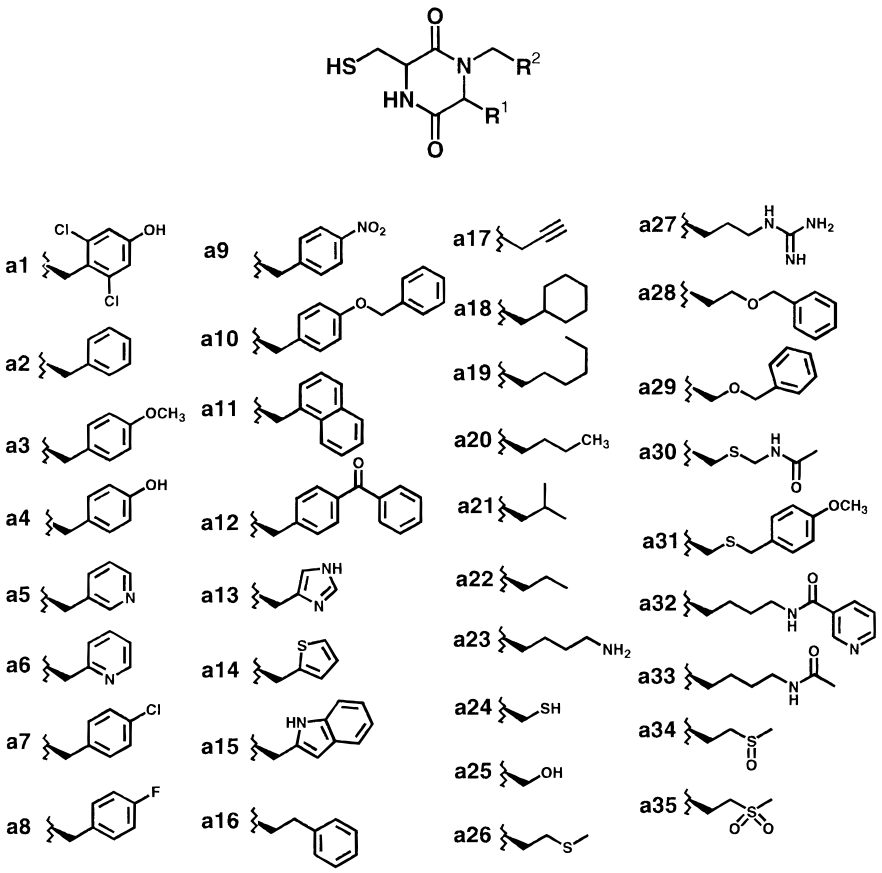
Pool 32 was deconvoluted by preparing 35 discrete compounds, each fixed with the 4-quinoline group at R_2 and with one of the 35 amino acid building blocks at R_1. Each pool was assayed at two dilutions, as above, against collagenase-1 and gelatinase-B. The results are shown in the Supporting Information (top panel of Figure 2). In contrast to SAR observed when the aldehyde position was varied, the results of the deconvolution showed that the potency of the DKPs was significantly affected by the group at R_1, which was again consistent with our earlier studies. We had previously observed that DKPs derived from cyclohexylalanine at R_1 showed good potency against collagenase-1 and gelatinase-B. This is consistent with SAR observed from peptide hydroxamate MMP inhibitors. In contrast, we were surprised to see potency with phenylalanine derivatives such as chloro-, fluoro-, and nitrophenylalanine. While substitution of these amino acids into the P_1 position of peptide hydroxamate MMP inhibitors would result in active stromelysin and gelatinase inhibitors, they would not be expected to show good activity against collagenase-1. Moreover, it was completely unexpected that these substituents could lead to selectivity over gelatinase, as the results suggest.

To further explore this relationship, a second deconvolution library was prepared. This time the amino acid at position R_1 was kept fixed with nitrophenylalanine, and the aldehyde at R_2 was varied. Thirty-five compounds were prepared and tested against the enzymes, the results of which are shown in Figure 3 of the Supporting Information. Most compounds showed good potency against collagenase-1 and exhibited selectivity against gelatinase-B.

To verify the results of these experiments, several DKPs were resynthesized and purified. The IC_{50} values of these compounds against both enzymes were determined, and the results are shown in Table 3. DKPs incorporating nitrophenylalanine (**3**, **4**, and **5**) showed good potency against collagenase-1 and, more importantly, very good selectivity (greater than 20-fold) against gelatinase-B. Replacement of the nitro functionality in **5** with aminoacetate in **6** results in a complete loss of activity. Removal of the nitro group from **3,** resulting in **2,** causes only a 2-fold loss of activity for collagenase-1, but this is accompanied by a 7-fold loss in selectivity against gelatinase-B. Similarly, the replacement of nitrophenylalanine with cyclohexylalanine in **1** also causes a 10-fold loss in selectivity. Note that replacing the R_1 position with a larger substituent results in better activity for gelatinase-B than collagenase (**7**).

Improvements in Estimation of Pool Potency and Selectivity. An important issue in the screening of focused combinatorial libraries is the quantitative determination of compound concentration. The nature of parallel library synthesis makes the weighing of individual products impractical. We were able to circumvent this problem by virtue of the fact that the crude DKPs had excellent purities. As a consequence of this,

Table 1. Diversity Set Used for ACL0938 (Amino Acids)



we could ascertain inhibitor concentration in the pools (or discretes) by determining the concentration of free thiol groups in the sample using Ellman's reagent (see Experimental Section).[2]

This additional knowledge created a new dilemma. Typically, combinatorial library samples are bioassayed at one dilution in duplicate or once at each of two dilutions. As an example of this, if compounds A and B are members of a library, and compound A is present at twice the concentration of compound B, but compound A shows 2 times the inhibition of compound B, which is the more potent compound? The answer is not obvious as the relationship between inhibitor concentration and IC_{50} is not linear. A more concrete example of this problem is illustrated in Figure 1. The top panel shows the percentage activity of the 18 discrete compounds (from the deconvolution of ACL0938 pool 32) when assayed at one dilution (the results of assay at lower dilution are not shown). The center panel shows the results of Ellman's determination of the concentration of the compound stocks. Again, relating compound concentration and percentage inhibition to compound potency for two enzymes simultaneously is of little value.

The solution to this problem relates to the relationship between enzyme activity and inhibitor concentration. Under proper assay conditions this relationship is described by eq 1

$$\nu = \frac{V_0}{1 + ([I]/IC_{50})} \qquad (1)$$

where V_0 is the rate of the enzyme reaction in the absence of inhibitor, [I] is inhibitor concentration, and ν is the observed reaction rate. Normally IC_{50} values are solved through nonlinear curve fitting of eq 1 with eight pairs of experimentally measured values of [I] and ν.

When screening combinatorial libraries, it is not common practice, nor is it reasonable, to expect eight-point assays for all samples. In general, only one (or possibly two) dilutions are tested, and reliable concentration data are not available. In our studies, we already had two such pairs of data: ν was determined at two dilutions of the pools, and [I] was known from the Ellman's assay. V_0, of course, was known from control reactions. We thus fit eq 1 with the two data points. Since the result of this process was not precise enough to be considered as a reliable IC_{50} value, we referred to such a result as an EIF ("estimated IC_{50}") to ensure there would be no confusion between EIF values and true IC_{50} determinations. Numerous trials indicated that EIF results were good predictors of IC_{50} values for MMPs (data not shown) unless the values were very high (in which case precision was not required) or very low. In the latter case, potent inhibition is suggested and warranted retesting.

The panel at the bottom of Figure 1 demonstrates the application of this approach. The EIF values for collagenase-1 and gelatinase-B for the pool 32 deconvolution are shown. In contrast to the results in the top panel, the SAR among the building blocks becomes quite clear. In this graph, the superior potency and *selectivity* of compound 9 (nitrophenylalanine) stands out without

Table 2. Diversity Set Used for ACL0938 (Aldehydes)

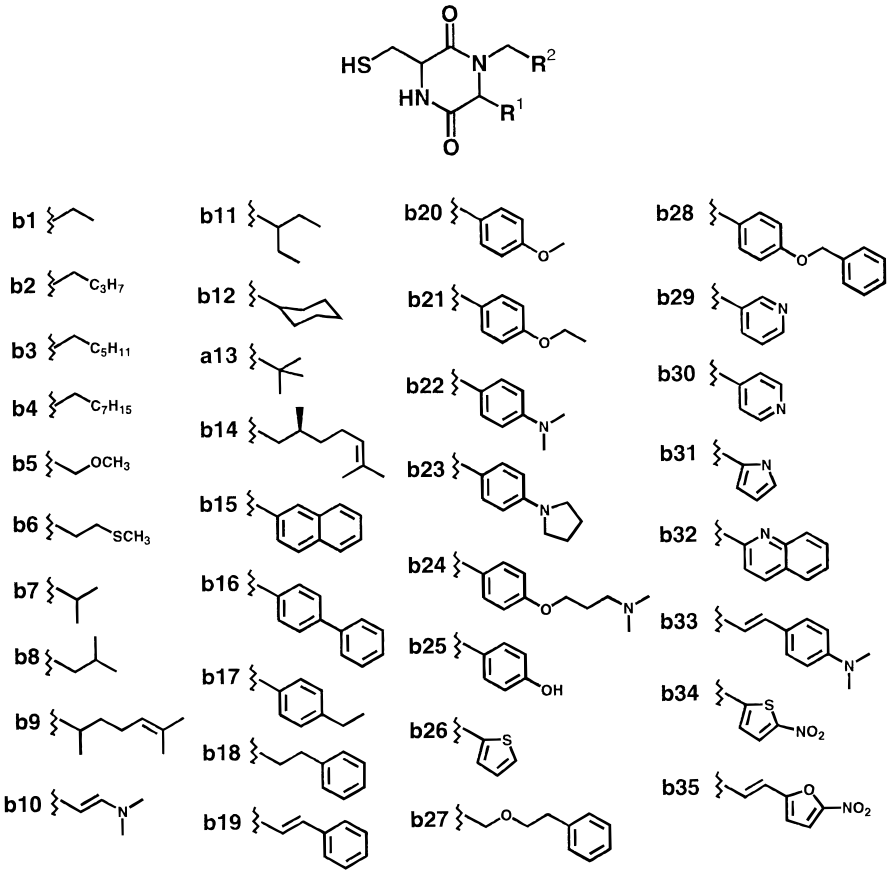
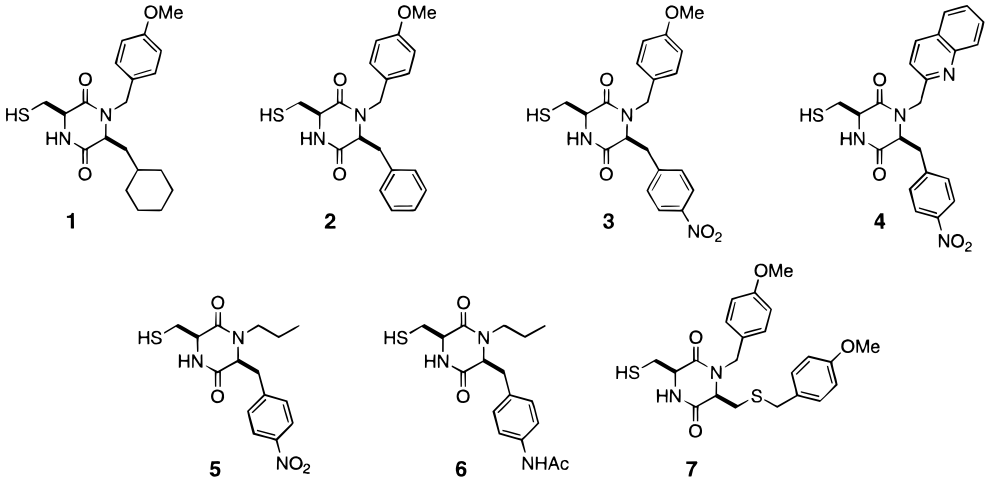


Table 3. IC_{50} Values for Purified DKPs



	IC_{50} values (nM)			
no.	collagenase 1	gelatinase-B	stromelysin	coll-1:gelB ratio
1	30	79	3800	1:2.6
2	110	400	4000	1:3.6
3	47	1200	>40000	1:26
4	74	1700	>20000	1:23
5	65	2900	>20000	1:45
6	>40000	>40000	>40000	
7	310	52	1100	6:1

effort. The use of EIF calculations improves SAR analysis and gives greater confidence in the selection of building blocks for further studies.

Screening Parallel Synthesis Libraries. To further improve the properties of this inhibitor class, we chose to add additional functionality to the DKP mol-

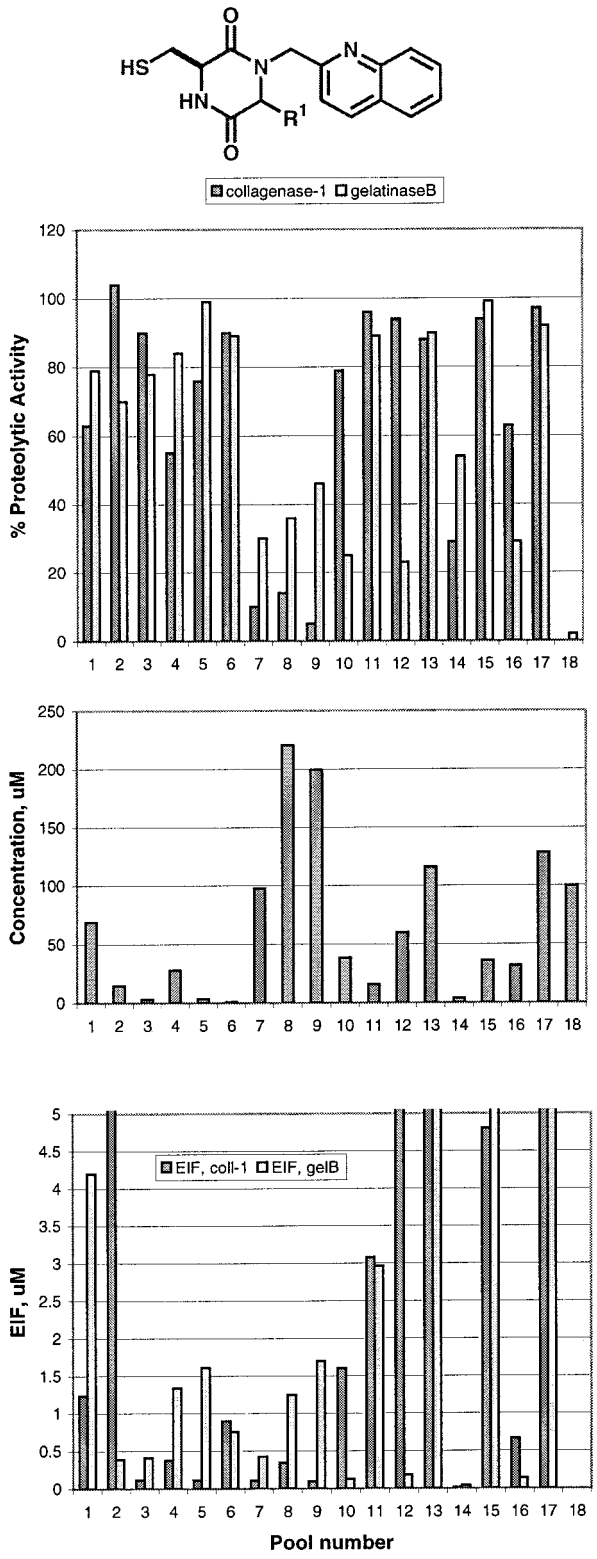


Figure 1. Proteolytic actitivity (percent of control) of 18 compounds from the deconvolution of ACL0938 pool 32. The pool number corresponds to the amino acid building block in Table 1 (e.g., pool 2 incorporates amino acid b2 at R1). Top panel: Pools assayed at 100-fold dilution. Middle panel: Concentration of the pool stock solutions as determined by Ellman's assay. Bottom panel: EIF values of the 18 pools calculated as described in the text.

ecule. As described in the Chemistry section, this was accomplished through the use of the Ugi multicomponent condensation reaction. This synthetic strategy presented several advantages: (1) it added a third center of diversity on the DKP molecule (Scheme 2), (2) the yields of the multicomponent reaction were better than the previous synthesis route, and (3) the Ugi reaction required fewer manipulations and thus lent itself effectively to the methods of parallel synthesis. We thus chose to prepare libraries of DKP compounds in which the substituents in the R_1, R_2, and R_3 positions were varied simultaneously. The Ugi DKP syntheses were performed in deep 96-well plates using HiTOPS manifolds.

Nine plates of Ugi DKPs were prepared, generally with 80−96 compounds per plate. Ellman's assays were performed for each sample, and each plate was assayed at two dilutions against the four MMPs. Over 3000 curve fittings were carried out (920 compounds × 4 assays). Curve fitting was automated through the use of the RoboSage program (Mike Lutz, Glaxo Wellcome USA). The results for two of these plates are shown in Figures 2 and 3.

In ACL1602 (Figure 2), four different phenylalanine derivatives were incorporated into the DKPs. Nitrophenylalanine gave the best inhibition overall, followed by chlorophenylalanine. The nitrophenylalanine also yielded the most selective compounds, ranging from 10- to 30-fold selective over gelatinase-B. It is interesting to observe that in these compounds selectivity appears to derive strongly from the nitrophenylalanine. Variation of the aldehyde or isocyanide group used in each compound only had small effects on potency or selectivity over gelatinase. In contrast, for matrilysin, the aldehyde group did have some effect on selectivity, which ranged from about 20- to 100-fold.

Nitrophenylalanine and chlorophenylalanine were also the most active amino acids in ACL1608 (Figure 3) whereas methyltyrosine and norvaline DKPs showed very little potency. The range of activities for the active amino acid containing compounds was similar to that seen in ACL1602.

Seven other plates of compounds were prepared. The generic structure of the compounds in these plates is shown in Figure 4. For each plate, two amino acids were used (amino acids used shown in Figure 4), in combination with one isocyanide only (the *tert*-butyl) and 44 different aldheyde groups. Some compounds with cyanophenylalanine and tryptophan showed weak (EIF 100−200 nM) activity. Otherwise, most compounds did not inhibit collagenase-1. With only rare exceptions, all the compounds examined in this series showed EIFs of 10 μM or greater against stromelysin.

To test the SAR generated from the parallel synthesis screening, a selection of compounds was resynthesized on a larger scale to allow full characterization and standard eight-point IC_{50} determinations (Table 4). All compounds had IC_{50} values of approximately 100 nM or better and showed selectivity versus gelatinase-B from 30- to 60-fold.

It is intriguing to speculate as to the source of the selectivity of the nitrophenyl DKPs. One model that could account for this selectivity of these compounds would involve the presence of an arginine at the bottom of the S1′ pocket of collagenase-1. Most of the MMPs studied by X-ray crystallography have deep S1′ pockets, which allow the entry of large aliphatic or aromatic

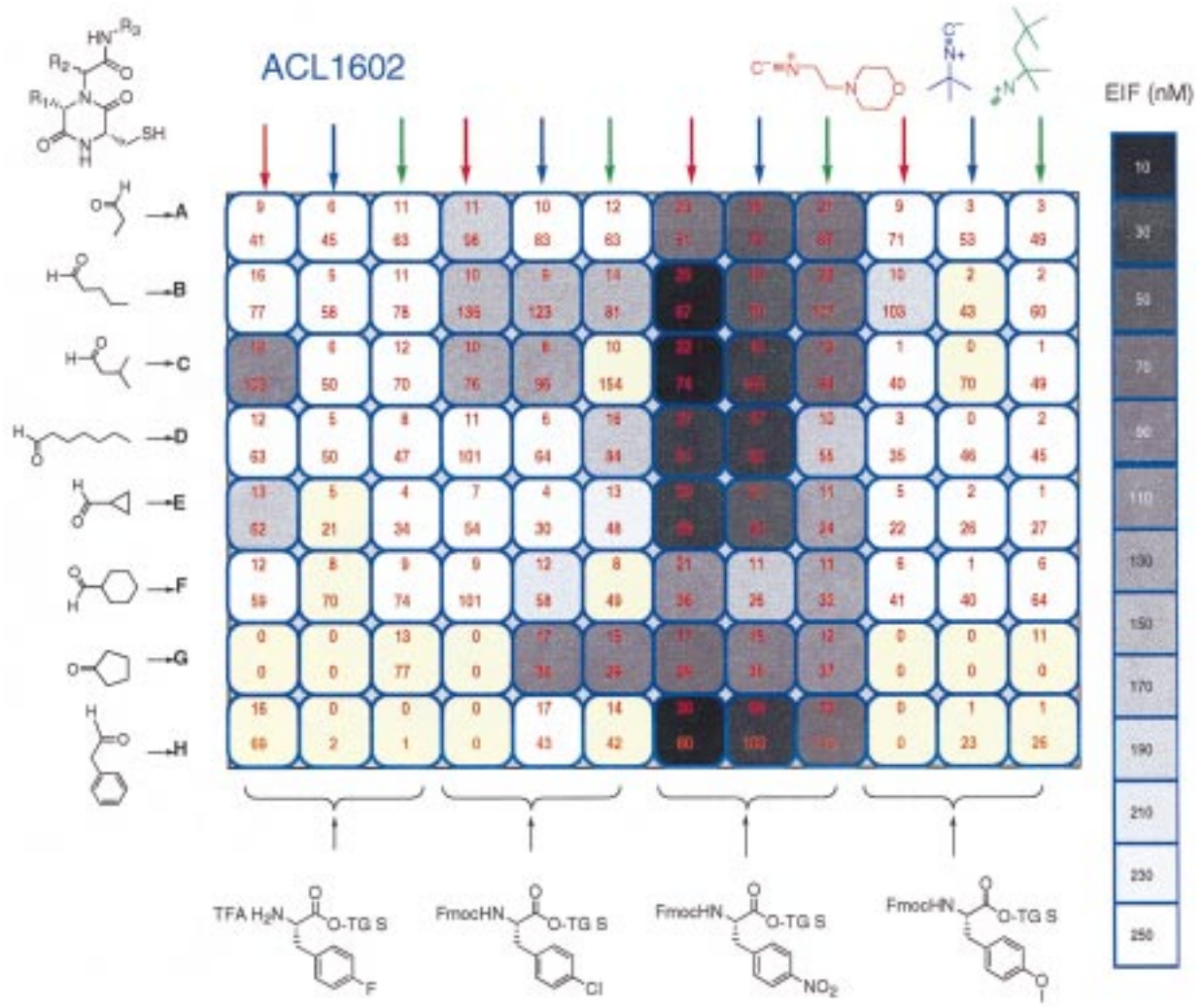


Figure 2. EIF analysis of the 96 discrete Ugi DKPs prepared by parallel synthesis of library ACL1602. Each sample from the 96-well plate is represented as a box. The gray scale in the box corresponds to the EIF against collagenase-1 (see the "thermometer" for gray scale). The selectivity of each compound is represented as a ratio. The top number in each box is the ratio of the EIF for gelatinase-B to the EIF for collagenase-1. The lower number is the corresponding ratio for matrilysin. A value of zero indicates that either the ratio was less than 1 or the activity of the sample for collagenase-1 was too high to determine reliably. Boxes shaded yellow indicate that the EIF value was deemed to be unreliable.

substituents. In collagenase-1, this pocket is blocked primarily by the side chain of an arginine residue, which is held rigidly in place by a network of hydrogen bonds.[27−29] It is possible that the selectivity of the nitrophenyl DKPs is due to the interaction of the aromatic nitro group with the arginine side chain. Whether this contact is based on ionic interactions or π-stacking is unclear. The SAR from HiTOPs plates, which showed that fluoro- and chloro-phenyl DKPs also show potency and selectivity, supports the latter model, but is insufficient at this point to be definitive.

Conclusion

We have previously demonstrated that thiol DKPs can serve as potent inhibitors of MMPs.[2] Further preparation and screening of thiol DKP libraries has revealed that when the substituent that presumably binds to the active site S1′ pocket contains a nitrophenyl group, the DKP shows unexpected selectivity for collagenase-1 over gelatinase-B. The basis of this selectivity is unclear, but comparisons of the activities of **2** and **3** indicate the selectivity is a result of both increased potency for collagenase-1 and decreased potency for gelatinase-B.

In the last five years, combinatorial chemistry has been shown to be a powerful tool for the development of potent inhibitors of therapeutically interesting targets. As more compounds are progressed into the progression pathway of drug development, the chemists and biologists that practice combinatorial technologies will be called upon to improve the properties of molecules other than potency. In this work, we have demonstrated that combinatorial approaches can be utilized to rapidly achieve selectivity in a compound class without input from the usual tools of rational drug design (e.g., crystal structure, etc.).

Due to the ability of combinatorial chemistry to generate large numbers of compounds, the investigator can quickly become overwhelmed with data. Trying to get sense of SAR from the results of 3000 assays can be a daunting challenge. The use of the EIF measurements, which relies on the accurate determination of inhibitor concentration, coupled with innovative approaches to data visualization, provides a means for a more meaningful analysis of the screening process. The extraction of quantitative data from an essentially qualitative

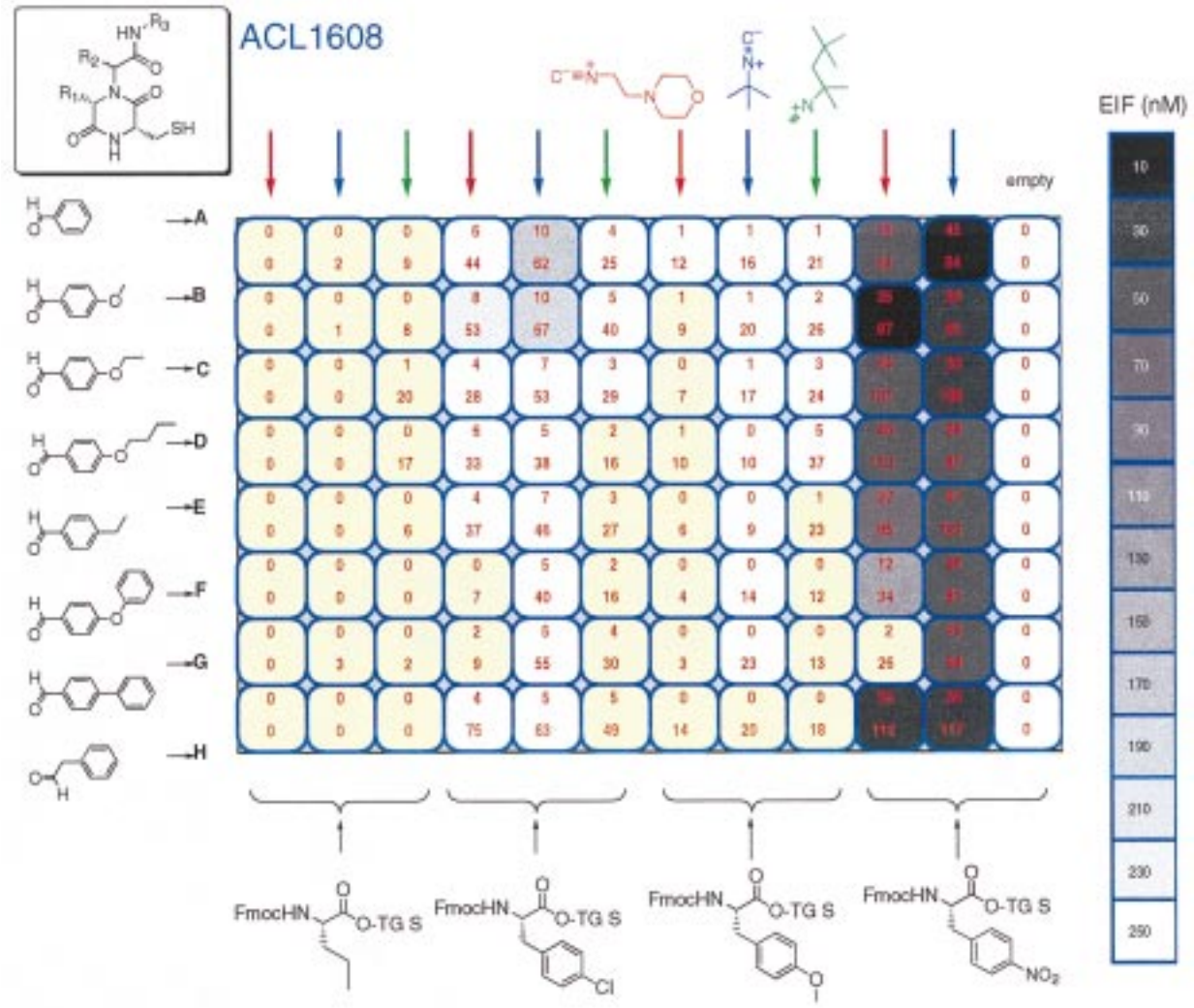


Figure 3. EIF analysis of the 88 discrete Ugi DKPs prepared by parallel synthesis of library ACL1608.

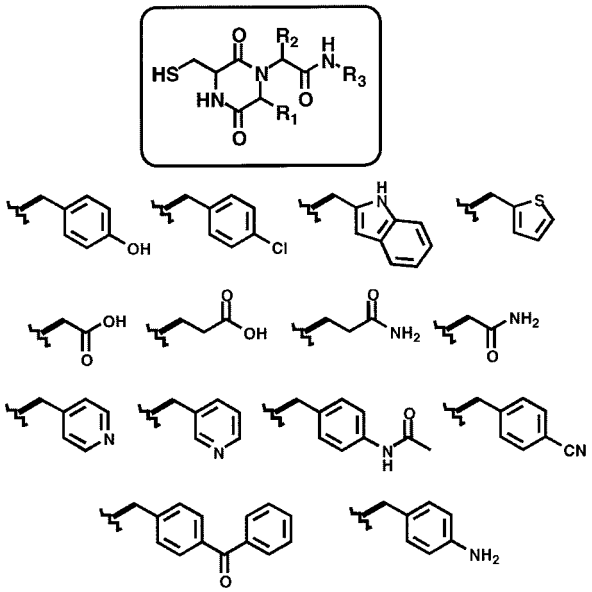


Figure 4. Amino acid building blocks used in the remaining parallel synthesis libraries. The generic structure of the library is highlighted. Amino acids were substituted at R1, aldehydes at R2.

process gives the investigator greater confidence in the selection of compounds for follow-up studies.

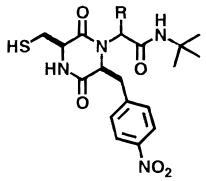
In the aggregate, we have been able to use combinatorial chemistry to design and evaluate new concepts in enzyme inhibition, resulting in the development of thiol DKPs as potent MMP inhibitors. In this work, we have demonstrated that the combinatorial approach can enable one to "dial in" a desired selectivity profile without any preconceived notions or insight as to how that selectivity should be achieved.

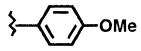
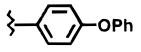
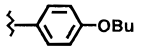
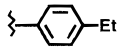
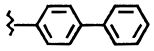
Experimental Section

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. High-resolution mass spectra were obtained on a VG ZAB 2SE (U. C. Berkeley). Microanalysis was done in the Microanalytical lab at U. C. Berkeley. All compounds were purified either by flash column chromatography using 5% methanol/DCM or preparative TLC using 10% 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.[26] 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

Table 4. IC_{50} Values (in nM) for Purified Ugi Derived DKPs



		IC50 Value [nM]		
no.	R	collagenase 1	gelatinase-B	selectivity
8		37	1180	1 : 32
9		24	570	1 : 24
10		37	1200	1 : 32
11		36	1500	1 : 42
12		21	1300	1 : 62
13		108	4400	1 : 41
14		73	3200	1 : 44
15		41	2600	1 : 63
16		102	4300	1 : 42
17		25	1300	1 : 52

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 purified. After removal of the chromatography solvents under vacuum, the samples were lyophilized from *tert*-butyl alcohol.

Library and Parallel Synthesis. DKP libraries were synthesized on a 36-channel Affymax ESL synthesizer. Discrete DKPs were synthesized on a HiTOPs manifold.[30] Amino acids were manually loaded to the resin as previously described. In each HiTOPs well was placed 20−50 mg resin, and the Ugi reaction was performed as described previously.[26] After Boc cleavage and DKP cyclization, the supernatants were collected into a 96-well plate and concentrated in a Savant SpeedVac system and analyzed by LC/MS.

Thiol Determinations. The concentration of free thiol in the parallel synthesis libraries was determined using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), more commonly known as Ellman's reagent.[31] Aliquots of compounds (stored in methanol in 96-well microtiter plates) were diluted into an aqueous solution containing 200 μM DTNB in 0.1 M Tris, pH 8.0. The optical density at 405 nM of the resulting solution was obtained. A dilution series of cysteine was used to prepare a standard curve.

Enzyme Assays. Recombinant matrilysin, as well as C-terminally truncated forms of collagenase-1, gelatinase-B, and stromelysin were prepared as described previously.[2] IC50 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-NH2 (Bachem, Torrance, CA), developed by Knight et al.[32] as described previously.[2] The results shown are the average of two or more independent determinations and have a standard deviations of ±20% or better. Assays were peformed in the presence of 0.5 mM cysteine to prevent any potential dimerization of the mercaptan compounds.

(6*S*,3*R*)-1-(4-Methoxybenzyl)-6-(4-nitrobenzyl)-3-sulfanylmethylhexahydro-2,5-pyrazinedione (3): ^{1}H NMR ($CDCl_3$) δ 8.19 (d, J = 8.4 Hz, 2H), 7.28 (d, J = 8.4 Hz, 2H), 7.19 (d, J = 8.4 Hz, 2H), 6.89 (d, J = 8.4 Hz, 2H), 6.77 (s, 1H), 5.44 (d, J = 14.4 Hz, 1H), 4.23 (m, 1H), 3.97 (d, J = 14.4 Hz, 1H), 3.82 (s, 3H), 3.35 (m, 2H), 2.79 (m, 1H), 1.26 (m 1H), 1.09 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 165.55, 164.06, 159.80, 147.44, 142.71, 131.16, 130.11, 128.41, 126.60, 123.96, 114.66, 59.20, 57.57, 55.64, 47.16, 37.01, 30.30; HRMS (M + H) $C_{20}H_{21}N_2O_3S$ calcd 416.1280, found 416.1276. Anal. ($C_{20}H_{21}N_2O_3S$) C, H, N.

(6*S*,3*R*)-6-(4-Nitrobenzyl)-1-(2-quinolylmethyl)-3-sulfanylmethylhexahydro-2,5-pyrazine-dione (4): ^{1}H NMR ($CDCl_3$) δ 8.37 (d, J = 8.4 Hz, 1H), 8.13 (d, J = 8.8 Hz, 2H), 7.98 (m, 2H), 7.81 (m, 1H), 7.64 (m, 1H), 7.54 (d, J = 8.4 Hz, 1H), 7.43 (d, J = 8.8 Hz, 2H), 5.32 (d, J = 15.6 Hz, 1H), 4.71 (d, J = 15.6 Hz, 1H), 4.62 (m, 1H), 4.17 (m, 1H), 3.57 (m 2H), 2.62 (m, 1H), 1.94 (m, 1H); HRMS (M + H) $C_{22}H_{20}N_4O_4S$ calcd 437.1284, found 437.1282.

(6*S*,3*R*)-6-(4-Nitrobenzyl)-1-(4-propyl)-3-sulfanylmethylhexahydro-2,5-pyrazinedione (5): ^{1}H NMR ($CDCl_3$) δ 8.22 (d, J = 8.4 Hz, 2H), 7.38 (d, J = 8.4 Hz, 2H), 6.72 (s, 1H), 4.36 (m, 1H), 4.09 (m, 1H), 3.81 (m, 1H), 3.45 (m, 1H), 3.30 (m, 1H), 2.81 (m, 2H), 1.66 (m, 2H), 1.23 (m, 1H), 0.93 (m, 3H);

MS *m*/*z* (M + Na); HRMS (M + H) for $C_{15}H_{19}N_3O_4S$ calcd 338.1170, found 338.1170.

***N*1-{4-[(2*S*,5*R*)-3,6-Dioxo-1-propyl-5-sulfanylmethylhexahydro-2-pyrazinylmethyl]-phenyl}acetamide (6):** ^{1}H NMR ($CDCl_3$) δ 7.72 (s, 1H), 7.50 (d, J = 8.4 Hz, 2H), 7.12 (s, 1H), 7.06 (d, J = 8.4 Hz, 2H), 4.27 (m, 1H), 4.11 (m, 1H), 3.70 (m, 1H), 3.31 (m, 1H), 3.11 (m, 1H), 2.89 (m, 1H), 2.63 (m, 1H), 1.68 (m, 1H), 1.22 (m, 1H), 0.98 (m, 3H), 0.61 (m, 1H); MS *m*/*z* 350 (M + H); HRMS (M + H) for $C_{17}H_{23}N_3O_3S$ calcd 350.1538, found 350.1548.

(6*R*,3*R*)-1-(4-Methoxybenzyl)-6-(4-methoxybenzylsulfanylmethyl)-3-sulfanylmethylhexa-hydro-2,5-pyrazinedione (7): ^{1}H NMR (CD_3OD) δ 7.33 (m, 4H), 6.99 (m, 4H), 5.15 (d, J = 15.2 Hz, 1H), 4.31 (m, 1H), 4.21 (m, 1H), 4.17 (d, J = 15.2 Hz, 1H), 3.79 (s, 3H), 3.43 (s, 3H), 3.26 (m, 2H), 3.15 (m, 2H), 1.42 (m, 1H), 1.08 (m, 1H); HRMS (M + H) $C_{22}H_{26}N_2O_4S_2$ calcd 447.1412, found 447.1411. Anal. ($C_{22}H_{26}N_2O_4S_2$) C, H, N.

***N*1-(*tert*-Butyl)-2-cyclopropyl-2-[(2*S*,5*R*)-2-(4-nitrobenzyl)-3,6-dioxo-5-sulfanylmethylhexa-hydro-1-pyrazinyl]-acetamide (8):** ^{1}H NMR ($CDCl_3$) δ 8.17 (d, J = 8.8 Hz, 2H), 7.54 (d, J = 8.8 Hz, 2H), 6.79 (m 2H), 4.30 (m, 1H), 4.12 (m, 1H), 3.91 (m, 1H), 3.49 (m, 2H), 2.87 (d, J = 10.8 Hz, 1H), 2.72 (m, 1H), 1.72 (m, 1H), 1.53 (m, 1H), 1.41 (s, 9H), 0.82 (m, 1H), 0.73 (m, 1H), 0.44 (m, 2H); HRMS (M + H) $C_{21}H_{28}N_4O_5S$ calcd 485.1859, found 485.1853. Anal. ($C_{21}H_{28}N_4O_5S$) C, H, N.

***N*1-(*tert*-Butyl)-4-methyl-2-[(2*S*,5*R*)-2-(4-nitrobenzyl)-3,6-dioxo-5-sulfanylmethylhexa-hydro-1-pyrazinyl]pentanamide (9):** ^{1}H NMR ($CDCl_3$) δ 8.16 (d, J = 8.8 Hz, 2H), 7.45 (d, J = 8.8 Hz, 2H), 6.90 (s, 1H), 6.83 (s, 1H), 4.83 (m, 1H), 4.36 (m, 1H), 3.99 (m, 1H), 3.44 (m, 2H), 2.82 (m, 1H), 1.99 (m, 1H), 1.53 (m, 3H), 1.43 (s, 9H), 1.24 (m, 6H); ^{13}C NMR ($CDCl_3$) δ 168.72, 166.07, 165.82, 147.25, 143.46, 131.37, 123.70, 57.86, 56.75, 51.91, 39.11, 36.19, 30.15, 29.07, 25.25, 23.11, 22.80; HRMS (M + H) $C_{22}H_{32}N_4O_5S$ calcd 465.2171, found 465.2177. Anal.($C_{22}H_{32}N_4O_5S$) C, H, N.

***N*1-(*tert*-Butyl)-2-[(2*S*,5*R*)-2-(4-nitrobenzyl)-3,6-dioxo-5-sulfanylmethylhexahydro-1-pyrazinyl]-3-phenylpropanamide (10):** ^{1}H NMR (CDCl3) δ 8.16 (d, J = 8.8 Hz, 2H), 7.45 (d, J = 8.8 Hz, 2H), 7.32−7.20 (m, 5H), 6.51 (s, 1H), 6.25 (s, 1H), 5.04 (dd, $J1$ = 8.0 Hz, $J2$ = 8.4 Hz, 1H), 4.51 (m, 1H), 3.79 (m, 2H), 3.43 (dd, J = 14.0 Hz, J = 8.4 Hz, 1H), 3.06 (dd, J = 14.0 Hz, J = 8.0 Hz, 1H), 2.79 (m, 2H), 1.34 (s, 9H); ^{13}C NMR (CDCl3) δ 167.89, 166.07, 165.54, 147.28, 143.39, 135.46, 131.41, 129.09, 128.91, 127.36, 123.74, 59.78, 58.30, 57.71, 52.04, 39.27, 34.09, 31.55, 30.05, 28.95; HRMS (M + H) $C_{25}H_{30}N_4O_5S$ calcd 499.2015, found 499.2003. Anal. ($C_{25}H_{30}N_4O_5S$) C, H, N.

***N*1-(*tert*-Butyl)-2-[(2*S*,5*R*)-2-(4-nitrobenzyl)-3,6-dioxo-5-sulfanylmethylhexahydro-1-pyrazinyl]nonanamide (11):** ^{1}H NMR ($CDCl_3$) δ 8.16 (m, 2H), 7.45 (m, 2H), 6.82 (s, 1H), 6.55 (s, 1H), 4.67 (m, 1H), 4.41 (m, 1H), 3.94 (m, 1H), 3.47 (m, 2H), 2.82 (m, 1H), 2.08 (m, 2H), 1.63 (m, 2H), 1.42 (s, 9H), 1.45−1.23 (m, 9H), 0.89 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 168.66, 166.08, 165.97, 147.25, 143.44, 131.42, 123.70, 58.96, 57.85, 57.82, 51.95, 39.04, 31.85, 30.13, 29.32, 29.09, 27.62, 26.33, 22.85, 14.40; HRMS (M + H) $C_{24}H_{37}N_4O_5S$ calcd 493.2485, found 493.2489. Anal. ($C_{24}H_{37}N_4O_5S$) C, H, N.

***N*1-(*tert*-Butyl)-2-(4-methoxyphenyl)-2-[(2*S*,5*R*)-2-(4-nitrobenzyl)-3,6-dioxo-5-sulfanyl-methylhexahydro-1-pyrazinyl]acetamide (12):** ^{1}H NMR ($CDCl_3$) δ 8.05 (m, 2H), 7.40 (d, J = 8.8 Hz, 2H), 7.14 (d, J = 8.4 Hz, 2H), 6.97 (m, 2H), 6.37 (s, 1H), 5.60 (s, 1H), 5.30 (s, 1H), 4.06 (m, 1H), 3.96 (m, 1H), 3.87 (s, 3H), 3.47 (m, 1H), 3.34 (m, 1H), 2.87 (m, 1H), 1.62 (m, 1H), 1.36 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 167.61, 166.18, 165.12, 160.56, 146.97, 143.71, 131.60, 131.11, 124.64, 123.48, 115.07, 94.43, 66.17, 60.43, 58.05, 55.74, 52.25, 39.49, 31.29, 30.43, 28.89; HRMS (M + H) $C_{25}H_{31}N_4O_6S$ calcd 515.1964, found 515.1978. Anal. ($C_{25}H_{31}N_4O_6S$) C, H, N.

***N*1-(*tert*-Butyl)-2-[(2*S*,5*R*)-2-(4-nitrobenzyl)-3,6-dioxo-5-sulfanylmethylhexahydro-1-pyrazinyl]-2-(4-phenoxyphenyl)acetamide (13):** ^{1}H NMR ($CDCl_3$) δ 8.07 (d, J = 8.8 Hz, 2H), 7.42 (m, 4H), 7.23 (m, 3H), 7.10−7.03 (m, 4H), 6.40 (s, 1H), 5.66 (s, 1H), 5.36 (s, 1H), 4.07 (m, 1H), 3.96 (m, 1H), 3.48 (m, 1H), 3.37 (m, 1H), 2.87 (m, 1H), 1.67 (m, 1H), 1.37 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 167.45, 166.13, 165.22, 159.14, 155.70, 147.02, 143.74, 131.79, 131.09, 130.22, 126.64, 124.68, 123.51, 120.09, 118.79, 65.77, 60.22, 58.05, 52.34, 39.55, 31.29, 30.42, 28.89; HRMS (M + H) $C_{30}H_{33}N_4O_6S$ calcd 577.2121, found 577.2132. Anal. ($C_{30}H_{33}N_4O_6S$) C, H, N.

***N*1-(*tert*-Butyl)-2-(4-butoxyphenyl)-2-[(2*S*,5*R*)-2-(4-nitrobenzyl)-3,6-dioxo-5-sulfanyl-methylhexahydro-1-pyrazinyl]acetamide (14):** ^{1}H NMR ($CDCl_3$) δ 8.05 (m, 2H), 7.38 (d, J = 8.4 Hz, 2H), 7.14 (d, J = 8.8 Hz, 2H), 6.96 (m, 2H), 6.46 (s, 1H), 5.59 (s, 1H), 5.28 (s, 1H), 4.07 (m, 1H), 4.10 (t, J = 6.4 Hz, 2H), 3.96 (m, 1H), 3.47 (m, 1H), 3.34 (m, 1H), 2.83 (m, 1H), 1.79 (m, 2H), 1.64 (m, 1H), 1.53 (m, 2H), 1.34 (s, 9H), 1.02 (t, J = 7.2 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 167.65, 166.25, 165.09, 146.96, 143.74, 131.57, 131.10, 124.35, 123.48, 115.54, 68.18, 66.27, 60.47, 58.05, 52.34, 39.51, 31.51, 30.44, 28.89, 19.59, 14.22; HRMS (M + H) $C_{28}H_{36}N_4O_6S$ calcd 557.2434, found 557.2445. Anal. ($C_{28}H_{36}N_4O_6S$) C, H, N.

***N*1-(*tert*-Butyl)-2-(4-ethylphenyl)-2-[(2*S*,5*R*)-2-(4-nitrobenzyl)-3,6-dioxo-5-sulfanyl-methylhexahydro-1-pyrazinyl]acetamide (15):** ^{1}H NMR ($CDCl_3$) δ 8.02 (m, 2H), 7.37 (d, J = 8 Hz, 2H), 7.29 (d, J = 8 Hz, 2H), 7.05 (d, J = 8.8 Hz, 2H), 6.59 (s, 1H), 5.60 (s, 1H), 5.31 (s, 1H), 4.07 (m, 1H), 4.00 (m, 1H), 3.49 (m, 1H), 3.33 (m, 1H), 2.87 (m, 1H), 2.72 (q, J = 7.6 Hz, 2H), 1.81 (m, 1H), 1.36 (s, 9H), 1.30 (t, J = 7.6 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 167.54, 166.32, 165.14, 146.90, 146.34, 143.87, 130.92, 130.24, 130.06, 129.23, 123.43, 66.43, 60.47, 58.03, 57.93, 52.28, 39.70, 30.48, 28.92, 28.86, 15.89; HRMS (M + H) $C_{26}H_{32}N_4O_5S$ calcd 513.2172, found 513.2186. Anal. ($C_{26}H_{32}N_4O_5S$) C, H, N.

***N*1-(*tert*-Butyl)-2-[(2*S*,5*R*)-2-(4-nitrobenzyl)-3,6-dioxo-5-sulfanylmethylhexahydro-1-pyrazinyl]-2-(4-phenylphenyl)acetamide (16):** ^{1}H NMR ($CDCl_3$) δ 8.03 (m, 2H), 7.70−7.41 (m, 9H), 7.12 (d, J = 8.4 Hz, 2H), 6.72 (s, 1H), 5.66 (s, 1H), 5.34 (s, 1H), 4.18 (m, 1H), 4.03 (m, 1H), 3.50 (m, 1H), 3.39 (m, 1H), 2.87 (m, 1H), 1.82 (m, 1H), 1.37 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 167.34, 166.29, 165.30, 146.96, 143.76, 142.77, 139.63, 131.82, 130.96, 130.58, 129.17, 128.27, 128.23, 127.18, 123.49, 66.50, 60.84, 58.02, 57.93, 52.38, 39.74, 30.43, 28.88; HRMS (M + H) $C_{30}H_{32}N_4O_5S$ calcd 561.2172, found 561.2180. Anal. ($C_{30}H_{32}N_4O_5S$) C, H, N.

***N*1-(*tert*-Butyl)-2-[(2*S*,5*R*)-2-(4-nitrobenzyl)-3,6-dioxo-5-sulfanylmethylhexahydro-1-pyrazinyl]-2-phenylacetamide (17):** ^{1}H NMR ($CDCl_3$) δ 8.02 (d, J = 8.8 Hz, 2H), 7.48 (m, 5H), 7.06 (d, J = 8.8 Hz, 2H), 6.47 (s, 1H), 5.58 (s, 1H), 5.31 (s, 1H), 4.08 (m, 1H), 4.00 (m, 1H), 3.48 (m, 1H), 3.33 (m, 1H), 2.85 (m, 1H), 1.76 (m, 1H), 1.36 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 167.29, 166.15, 165.23, 146.97, 143.65, 133.15, 130.97, 130.21, 129.91, 129.78, 123.48, 66.82, 60.83, 58.02, 52.35, 39.62, 31.55, 30.47, 28.86; HRMS (M + H) $C_{24}H_{29}N_4O_5S$ calcd 485.1859, found 485.1853. Anal. ($C_{24}H_{29}N_4O_5S$) C, H, N.

Supporting Information Available: Figures presenting proteolytic activity of ACL0938. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Dolle, R. E. Discovery of enzyme inhibitors through combinatorial chemistry. *Mol. Diversity* **1997**, *2*, 223−236.

(2) Szardenings, A. K.; Harris, D.; Lam, S.; Shi, L.; Tien, D.; Wang, Y.; Patel, D. V.; Navre, M.; Campbell, D. A. Rational Design and Combinatorial Evaluation of Enzyme Inhibitor Scaffolds: Identification of Novel Inhibitors of Matrix Metalloproteinases. *J. Med. Chem.* **1998**, *41*, 2194−2200.

(3) Woessner, J. F., Jr. The family of matrix metalloproteinases. *Ann. N.Y. Acad. Sci.* **1994**, *732*, 11−21.

(4) Nagase, H. In *Zinc Metalloproteases in Health and Disease*; Hooper, N. M., Ed.; Taylor & Francis: London, 1996; p 153−204.

(5) Cawston, T. Matrix metalloproteinases and TIMPs: properties and implications for the rheumatic diseases. *Mol. Med. Today* **1998**, *4*, 130−137.

(6) Bottomley, K. M.; Johnson, W. H.; Walter, D. S. Matrix metalloproteinase inhibitors in arthritis. *J. Enzyme Inhib.* **1998**, *13*, 79−101.

(7) Yu, A. E.; Hewitt, R. E.; Connor, E. W.; Stetler-Stevenson, W. G. Matrix metalloproteinases: novel targets for directed cancer therapy. *Drugs Aging* **1997**, *11*, 229−244.

(8) Wojtowicz-Praga, S.; Torri, J.; Johnson, M.; Steen, V.; Marshall, J.; Ness, E.; Dickson, R.; Sale, M.; Rasmussen, H. S.; Chiodo, T. A.; Hawkins, M. J. Phase I trial of marimastat, a novel matrix metalloproteinase inhibitor, administered orally to patients with advanced lung cancer. *J. Clin. Oncol.* **1998**, *16*, 2150−2156.
(9) Davidson, A.; Drummond, A. H.; Galloway, W. A.; Whittaker, M. The inhibition of matrix metalloproteinase enzymes. *Chem. Ind. (London)* **1997**, 258−261.
(10) Denis, L. J.; Verweij, J. Matrix metalloproteinase inhibitors: present achievements and future prospects. *Invest. New Drugs* **1997**, *15*, 175−185.
(11) Hagmann, W. K.; Larkk, M. W.; Becker, J. W. Inhibition of matrix metalloproteinases. *Annu. Rep. Med. Chem.* **1996**, *31*, 231−240.
(12) Watson, S. A.; Tierney, G. Matrix metalloproteinase inhibitors: a review. *BioDrugs* **1998**, *9*, 325−335.
(13) Levy, D.; Ezrin, A. Matrix metalloproteinase inhibitor drugs. *Emerging Drugs* **1997**, *2*, 205−230.
(14) Zask, A.; Levin, J. I.; Killar, L. M.; Skotnicki, J. S. Inhibition of matrix metalloproteinases: structure based design. *Curr. Pharm. Des.* **1996**, *2*, 624−661.
(15) Porter, J. R.; Beeley, N. R. A.; Boyce, B.; Mason, B. A.; Millican, A. W.; Millar, K. H.; Leonard, J.; Morphy, J. R.; O'Connell, J. P. Potent and selective inhibitors of gelatinase-A. 1. Hydroxamic acid derivatives. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 2741−2746.
(16) Sahoo, S. P.; Caldwell, C. G.; Chapman, K. T.; Durette, P. L.; Esser, C. K.; Kopka, I. r. E.; Polo, S. A.; Sperow, K. M.; Niedzwiecki, L. M.; Iizquierdo-Martin, M.; Chang, C. C.; Harrison, R. K.; Stein, R. L.; MacCoss, M.; Hagmann, W. K. Inhibition of matrix metalloproteinases by N-carboxyalkyl dipeptides: enhanced potency and selectivity with substituted P1′ homophenylalanines. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 2441−2446.
(17) Miller, A.; Askew, M.; Beckett, R. P.; Bellamy, C. L.; Bone, E. A.; Coates, R. E.; Davidson, A. H.; Drummond, A. H.; Huxley, P.; Martin, F. M.; Sariglou, L.; Thompson, A. J.; Van Dijk, S. E.; Whittaker, M. Inhibition of matrix metalloproteinases: an examination of the S1′ pocket. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 193−198.
(18) Freije, J. M. P.; Diez-Itza, I.; Balbin, M.; Sanchez, L. M.; Blasco, R.; Tolivia, J.; Lopez-Otin, C. Molecular cloning and expression of collagenase-3, a novel human matrix metalloproteinase produced by breast carcinomas. *J. Biol. Chem.* **1994**, *269*, 16766−73.
(19) Mitchell, P. G.; Magna, H. A.; Reeves, L. M.; Lopresti-Morrow, L. L.; Yocum, S. A.; Rosner, P. J.; Geoghegan, K. F.; Hambor, J. E. Cloning, expression, and type II collagenolytic activity of matrix metalloproteinase-13 from human osteoarthritic cartilage. *J. Clin. Invest.* **1996**, *97*, 761−8.
(20) Billinghurst, R. C.; Dahlberg, L.; Ionescu, M.; Reiner, A.; Bourne, R.; Rorabeck, C.; Mitchell, P.; Hambor, J.; Diekmann, O. Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. *J. Clin. Invest.* **1997**, *99*, 1534−1545.
(21) Cawston, T. E. Metalloproteinase inhibitors and the prevention of connective tissue breakdown. *Pharmacol. Ther.* **1996**, *70*, 163−182.
(22) Reboul, P.; Pelletier, J.-P.; Tardif, G.; Cloutier, J.-M.; Martel-Pelletier, J. The new collagenase, collagenase-3, is expressed and synthesized by human chondrocytes but not by synoviocytes. A role in osteoarthritis. *J. Clin. Invest.* **1996**, *97*, 2011−2019.
(23) Shingleton, W. D.; Hodges, D. J.; Brick, P.; Cawston, T. E. Collagenase: a key enzyme in collagen turnover. *Biochem. Cell Biol.* **1996**, *74*, 759−775.
(24) After the completion of this work, scientists from Roche published on a collagenase inhibitor, Ro 32-3555, that was selective over the gelatinases, but their publications gave no detailed insight into how this selectivity was achieved. Broadhurst, M. J.; Brown, P. A.; Lawton, G.; Ballantyne, N.; Borkakoti, N.; Bottomley, K. M. K.; Cooper, M. I.; Eatherton, A. J.; Kilford, I. R.; Malsher, P. J.; Nixon, J. S.; Lewis, E. J.; Sutton, B. M.; Johnson, W. H. Design and synthesis of the cartilage protective agent (CPA, Ro 32-3555). *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2299−2302.
(25) Morphy, J. R.; Millican, T. A.; Porter, J. R. Matrix metalloproteinase inhibitors: current status. *Curr. Med. Chem.* **1995**, *2*, 743−62.
(26) Szardenings, A. K.; Burkoth, T. S.; Lu, H. H.; Tien, D. W.; Campbell, D. A. A simple procedure for the solid-phase synthesis of diketopiperazine and diketomorpholine derivatives. *Tetrahedron* **1997**, *53*, 6573−6593.
(27) Borkakoti, N.; Winkler, F. K.; Williams, D. H.; D'Arcy, A.; Broadhurst, M. J.; Brown, P. A.; Johnson, W. H.; Murray, E. J. Structure of the catalytic domain of human fibroblast collagenase complexed with an inhibitor. *Nat. Struct. Biol.* **1994**, *1*, 106−110.
(28) Stams, T.; Spurlino, J. C.; Smith, D. L.; Wahl, R. C.; Ho, T. F.; Qoronfleh, M. W.; Banks, T. M.; Rubin, B. Structure of human neutrophil collagenase reveals large S1′ specificity pocket. *Nat. Struct. Biol.* **1994**, *1*, 119−123.
(29) Grams, F.; Reinemer, P.; Powers, J. C.; Kleine, T.; Pieper, M.; Tschesche, H.; Huber, R.; Bode, W. X-ray structures of human neutrophil collagenase complexed with peptide hydroxamate and peptide thiol inhibitors. Implications for substrate binding and rational drug design. *Eur. J. Biochem.* **1995**, *228*, 830−841.
(30) Antonenko, V. A. Automation in Combinatorial Chemistry. In *Combinatorial Chemistry and Combinatorial Technologies: Methods and Applications*; Miertus, S., Fassina, G., Eds.; Marcel Dekker: New York, 1998 (in press).
(31) Habeeb, A. F. S. A. Reaction of protein sulfhydryl groups with Ellman's reagent. In *Methods Enzymology*; Hirs, C. H. W., Ed.; Academic: New York, 1972; Vol. 25, pp 457−464.
(32) Knight, C. G.; Willenbrock, F.; Murphy, G. A novel coumarin-labeled peptide for sensitive continuous assays of the matrix metalloproteinases. *FEBS Lett.* **1992**, *296*, 263−266.

JM980475P