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Squaric Acid-Based Peptidic Inhibitors of Matrix Metalloprotease-1

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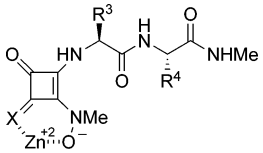

X = O or S

A series of squaric acid-peptide conjugates were synthesized and evaluated as inhibitors of MMP-1. The cyclobut-3-enedione core was substituted at the 3-position with several functional groups, such as −N(alkyl)OH, −NHOH, and −OH, that are designed to bind to the zinc atom in the active site of the metalloprotease. The 4-position of the cyclobut-3-enedione was derivatized with mono- or dipeptides that are designed to bind in the S1′ and S2′ subsites of the enzyme, and position the metal chelating group appropriately in the active site for binding to zinc. Positional scanning revealed that −N(Me)OH provided the highest level of inhibition among the chelating groups that were tested, and Leu-Tle-NHMe was the preferred amino acid sequence. A combination of these groups yielded an inhibitor with an IC_{50} value of 95 μM. For one inhibitor, conversion of one of the carbonyl groups on the cyclobut-3-enedione core to a thiocarbonyl group resulted in a 18-fold increase in potency, and yielded a compound with an IC_{50} value of 15 μM.

Introduction

Matrix Metalloproteases (MMPs). Matrix metalloproteases are a family of structurally related endopeptidases that degrade and remodel components of the extracellular matrix (ECM).[1] These enzymes regulate structure and sustain a balanced composition of the ECM, two processes that are important for maintaining normal physiology in a number of tissues. For example, MMPs play a crucial role in embryonic development, healing, and reproduction.

The activity of MMPs is normally regulated at three levels: (1) gene transcription, (2) activation of MMP propeptides, and (3) inhibition of MMPs by tissue inhibitors of metalloproteases (TIMPs).[2,3] Overexpression of MMPs and deregulation of their activity are associated with a variety of pathological conditions including tumor growth and metastasis,[4] angiogenesis,[5] destruction of joints that causes osteoarthritis[6] and rheumatoid arthritis,[7] periodontal disease,[8] and multiple sclerosis.[9] Therefore, there is significant interest in developing MMP inhibitors for therapeutic applications.

Biological Applications of Squaric Acids. Squaric acid is a molecule that has significant aromatic character. In one resonance form it has two π electrons and a negative charge on each of the carbonyl oxygen atoms (Figure 1). The conjugate base of squaric acid can serve as an electrostatic mimic of negatively charged groups that are common in biology including carboxylates and phosphate mono- and diesters. As a result, derivatives of squaric acid have been used as a replacement for these groups in a number of medicinal applications.

† These two authors contributed equally to this work.

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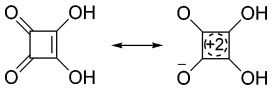

FIGURE 1. Resonance structures of squaric acid.

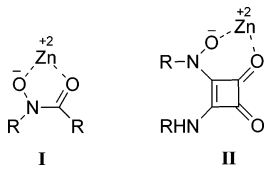

FIGURE 2. Known binding mode of hydroxamic acid **I** versus proposed binding mode by squaric acid derivative **II**.

Our research group has used squaric acid derivatives to mimic the phosphate group in phosphotyrosine residues. We prepared a number of 3-hydroxy-4-arylcyclobut-3-enediones as nonhydrolyzable isosteres of aryl phosphate esters and found that these compounds are effective inhibitors of protein tyrosine phosphatases.[10] Kim and co-workers have used phosphonocyclobutenedione as a mimic of pyrophosphate and found that it is a selective inhibitor of DNA polymerases from several viruses.[11] Sekine has used a diamide of squaric acid to replace a phosphate diester linkage in an oligodeoxynucleotide.[12]

Derivatives of squaric acid have also been used to mimic carboxylates by a number of investigators. Shinada replaced the γ-carboxylic acid of a glutamate residue within a polyamine toxin with a squaric acid derivative. The resulting compound was a selective agonist of ionotropic glutamate receptors.[13] Sun and coworkers used a derivative of squaric acid in their investigations of an NMDA antagonist that regulates the activation of glutamate receptors.[14] In this example, squaric acid mimics the natural glutamate agonist of the neuronal receptors. In its application as a guanidinium isotere, Butera used diaminocyclobutenedione as a replacement for the *N*-cyanoguanidine group in a bladder-selective potassium channel opener that is used as a treatment for urge urinary incontinence.[15]

Hydroxamic acids are potent inhibitors of zinc metalloproteases. X-ray crystal structures show that hydroxamic acids chelate to the active site zinc atom as shown in structure **I** (Figure 2).[16] In addition, Bruckner and coworkers have demonstrated that vinylogous hydroxamic acids that are derived from squaric acid are good metal chelators (see structure **II** in Figure 2).[17] These two observations prompted us to investigate the potential of vinylogous hydroxamic acids that are based upon squaric acid as inhibitors of MMPs. We have derivatized the vinylogous hydroxamic acids with peptides to target them to the active site of the proteases.

Results and Discussion

Synthesis of Compounds 4a−f. We began our studies by making a series of simple derivatives to determine what functional group is preferred at the R^1 position of the inhibitors (Scheme 1). Squaric acid **1** was converted to its dimethyl ester **2** by treating it with trimethyl orthoformate. Reaction of compound **2** with a series of hydroxylamines gave vinylogous hydroxamic acids **3a−e**. Substitution of the remaining methyl ester with several primary amines gave inhibitors **4a−f**.

These compounds were screened for activity against MMP-1. Assays were performed in a buffer of 200 mM NaCl, 50 mM Tris, 5 mM $CaCl_2$, 20 μM $ZnSO_4$, and 0.05% Brij 35 at pH 7.6 using the fluorogenic substrate Dnp-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(Nma)-NH_2. In this substrate, *N*-methylanthranilic acid (Nma) is a fluorophore and the dinitrophenyl (Dnp) group is a quencher.[18,19] The progress of the reactions was monitored by fluorescence spectroscopy with excitation at 340 nm and emission at 460 nm. For inhibitors **4a**,**b**, $R^1 = H$ while the R^2 position was varied between a branched and a straight-chain alkyl group. Between these two compounds, inhibitor **4b**, which incorporates a simple *n*-hexyl chain, had the better activity. We next screened Me, cyclohexyl, and benzyl groups at the R^1 position, and found that large groups are unfavorable at this site. Compounds **4e** and **4f**, where R^1 is cyclohexyl or benzyl, were poor inhibitors that showed no activity up to 10 mM concentration. Compound **4c**, which incorporates a smaller methyl group at the R^1 position, was approximately three times more active than compound **4b**, where $R^1 = H$. Both inhibitors **4c** and **4d** had IC_{50} values of 310 μM.

Monopeptide Inhibitors. We next turned our attention to inhibitors that incorporated a single amino acid to determine which amino acid side chain is preferred in the S1′ subsite within the context of these inhibitors. As shown in Scheme 2, inhibitors **6a−g** were synthesized by reaction of vinylogous hydroxamic acids **3b** or **3e** with a variety of amino acid methyl esters. We chose amino acids with hydrophobic side chains since data from the literature indicated that such structures are preferred in the S1′ subsite.[1]

Comparison of inhibitors **6a** and **6b** shows that both methyl and isopropyl groups at the R^1 position are small enough to be tolerated by the enzyme (Table 2). In addition, the enzyme appears to be fairly insensitive to the identity of the R^3 side chain, since all of the inhibitors in this series gave IC_{50} values that range from 190 to 380 μM. Among these compounds inhibitor **6a** had the best activity with an IC_{50} value of 190 μM.

Dipeptide Inhibitors. To improve the potency of the inhibitors, we extended their structure by incorporating

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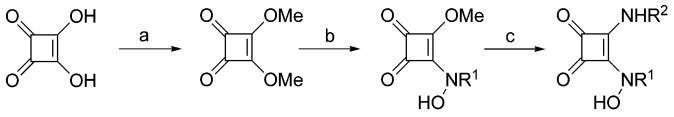
SCHEME 1[a]



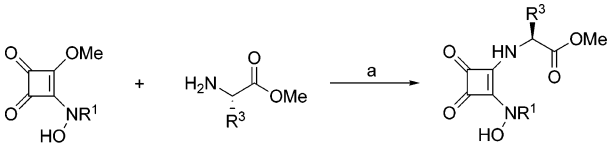
1 **2**

3a: R^1 = H
3b: R^1 = Me
3c: R^1 = cyclohexyl
3d: R^1 = benzyl
3e: R^1 = isopropyl

4a: R^1 = H, R^2 = $CH_2CH(CH_3)_2$
4b: R^1 = H, R^2 = $(CH_2)_5CH_3$
4c: R^1 = Me, R^2 = $(CH_2)_5CH_3$
4d: R^1 = Me, R^2 = $(CH_2)_4CH_3$
4e: R^1 = cyclohexyl, R^2 = $(CH_2)_5CH_3$
4f : R^1 = benzyl, R^2 = $(CH_2)_5CH_3$

[a] Reagents: (a) $CH(OCH_3)_3$, MeOH, Δ; (b) $HONHR^1{\cdot}HCl$, KOH, MeOH; (c) H_2NR^2, MeOH.

SCHEME 2[a]



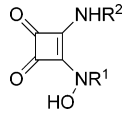
3b: R^1 = Me
3e: R^1 = $CH(CH_3)_2$

R^3 = side chain of amino acid
5a: R^3 = Ile
5b[b]: R^3 = Nle
5c: R^3 = Leu
5d: R^3 = Phe
5e: R^3 = Trp
5f : R^3 = Met

R^3 = side chain of amino acid
6a: R^1 = Me, R^3 = Ile
6b: R^1 = $CH(CH_3)_2$, R^3 = Ile
6c[b]: R^1 = Me, R^3 = Nle
6d: R^1 = Me, R^3 = Leu
6e: R^1 = Me, R^3 = Phe
6f : R^1 = Me, R^3 = Trp
6g: R^1 = Me, R^3 = Met

[a] Reagents: (a) MeOH, KOH. [b]Racemic Nle was used to prepare **5b** and **6c**.

TABLE 1. Inhibition of MMP-1 by Compounds 4a−f



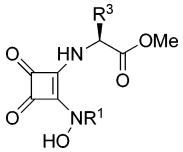
compd	R^1	R^2	IC_{50} (mM)[a]
4a	H	$-CH_2CH(CH_3)_2$	1.8 ± 0.3
4b	H	$-(CH_2)_5CH_3$	1.0 ± 0.2
4c	Me	$-(CH_2)_5CH_3$	0.31 ± 0.03
4d	Me	$-(CH_2)_4CH_3$	0.31 ± 0.03
4e	cyclohexyl	$-(CH_2)_5CH_3$	>10
4f	benzyl	$-(CH_2)_5CH_3$	>10

[a] All experiments were performed in duplicate.

a second amino acid that is designed to bind in the S2′ subsite. X-ray crystallographic studies have shown that peptide-based hydroxamic acid inhibitors bind in the active site of MMPs by occupying the primed subsites.[20] The synthesis of the inhibitors in this series is shown in Scheme 3. For these studies, we used the dibutyl ester of squaric acid **7** as the starting point. We found that this compound is more convenient to work with than the corresponding dimethyl ester, since the dibutyl ester has increased solubility in most common organic solvents.

Squaric acid was treated with tributyl orthoformate to give dibutyl ester **7**. Subsequent reaction of **7** with *N*-methylhydroxylamine yielded compound **8**. The dipeptide building blocks were prepared by coupling *N*-Boc amino acids **9a**−**g** with methylamine to give the corresponding *N*-methyl amides. These were treated with trifluoroacetic acid to give compounds **10a**−**g**. Coupling of **10a**−**g** with *N*-Boc-Ile or *N*-Boc-Leu, followed by Boc deprotection gave dipeptides **11a**−**h**. Finally, reaction of the dipeptides with compound **8** in methanol at room temperature provided the desired inhibitors **12a**−**h**.

TABLE 2. Inhibition of MMP-1 by Compounds 6a−g



compd	R^1	R^3 = side chain of amino acid	IC_{50} (μM)[a]
6a	Me	Ile	190 ± 30
6b	$-CH(CH_3)_2$	Ile	210 ± 10
6c	Me	Nle[b]	280 ± 20
6d	Me	Leu	320 ± 20
6e	Me	Phe	330 ± 20
6f	Me	Trp	380 ± 50
6g	Me	Met	300 ± 50

[a] All experiments were performed in duplicate. [b] Racemic Nle was used to prepare compound **6c**.

Most of the inhibitors in this series incorporate the side chain of Ile at the R^3 position because the data presented in Table 2 indicated that Ile is preferred in the S1′ subsite. The side chains at the R^4 position were chosen based upon literature precedent, which suggest that aromatic and hydrophobic amino acids are preferred at the S2′ subsite.[1] For inhibitors **12a**−**g**, which incorporate an Ile side chain at R^3, we did not observe any improvement in activity over the related monopeptide inhibitor **6a**. In addition, the activity did not depend strongly on the structure of R^4 since we observe a difference in IC_{50} values of only 2.5-fold among these seven compounds.

Compounds **12a** and **12h** provide an interesting comparison. Both incorporate a bulky *tert*-butyl group at R^4. Compound **12a** incorporates the side chain of Ile at R^3, and was the least active among **12a**−**g**. By contrast, **12h** has a Leu side chain at R^3 and it is 2−5-fold more active than any of the other mono- or dipeptide-based inhibitors. These data suggest that binding interactions in the S1′ and S2′ subsites are dependent on one another. For the monopeptide inhibitors that leave the S2′ subsite empty (Table 2, compare inhibitors **6a** and **6d**), Ile is preferred over Leu at S1′. This selectivity is reversed in the dipeptide inhibitors. With Tle in the S2′ subsite, Leu is preferred over Ile at S1′ (compare **12a** and **12h**).

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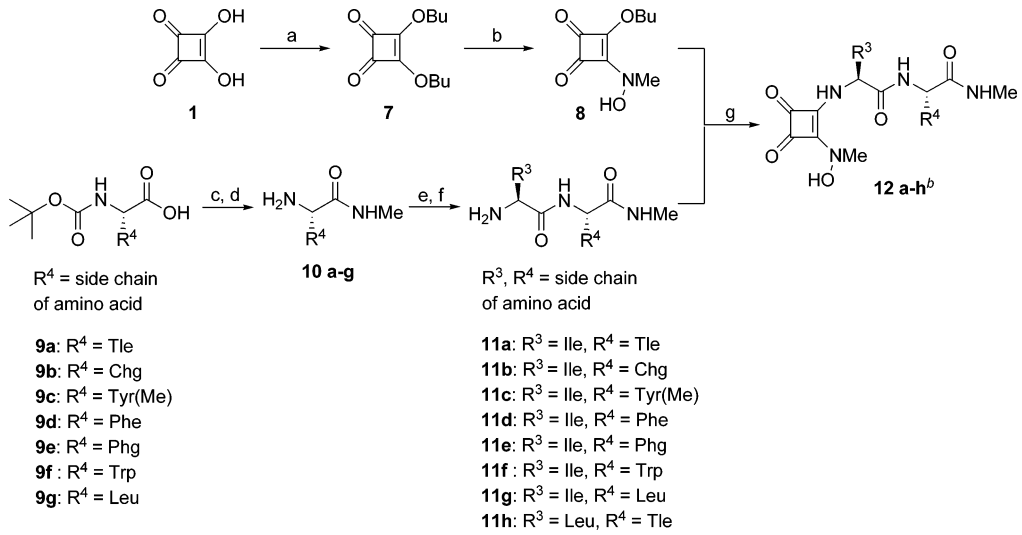
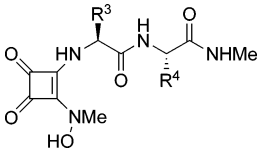
SCHEME 3[a]



[a] Reagents: (a) $(BuO)_3CH$, BuOH, reflux; (b) HONHMe·HCl, KOH, MeOH; (c) NH_2Me, *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), DMF; (d) 50% TFA, CH_2Cl_2; (e) NHS, *N*-Boc-Ile or *N*-Boc-Leu, EDC, DMF; (f) 50% TFA, CH_2Cl_2; (g) MeOH. Tle = *tert*-leucine, Chg = cyclohexylglycine, Phg = phenylglycine. [b]See Table 3 for specific structures.

TABLE 3. Inhibition of MMP-1 by Dipeptides 12a−h[a]



compd	R^3 = side chain of amino acid	R^4 = side chain of amino acid	IC_{50} (μM)[a]
12a	Ile	Tle	500 ± 100
12b	Ile	Chg	300 ± 100
12c	Ile	Tyr(Me)	280 ± 30
12d	Ile	Phe	270 ± 30
12e	Ile	Phg	260 ± 25
12f	Ile	Trp	210 ± 30
12g	Ile	Leu	200 ± 20
12h	Leu	Tle	95 ± 7

[a] All experiments performed in duplicate.

Squaric Acid Monoamides. We also made a cursory examination of a simple monoamide of squaric acid as a potential MMP-1 inhibitor. Scheme 4 shows the synthesis of compound **14**, which is the squaric acid analogue of the vinylogous hydroxamic acid inhibitor **12h**. Reaction of H-Leu-Tle-NHMe (**11h**) with dimethyl squarate **2** gave compound **13**, which was subsequently hydrolyzed under acidic conditions to give compound **14**.

When compound **14** was assayed against MMP-1, we observed no inhibition up to a concentration of 200 μM. In retrospect, this result is not surprising since the bite angle between the two oxygen atoms in squaric acid is known to be too large to form a strong five-membered chelate with Zn^{2+} (right side of Figure 3).[21] By comparison, the vinylogous hydroxamic acid inhibitors have the potential to form a six-membered chelate with zinc (left side of Figure 3). This structure has a reduced bite angle and a shorter distance between the two chelating oxygen atoms. Both of these factors favor binding to the metal center.[22]

Thiocarbonyl-Containing vs Carbonyl-Containing Inhibitors. Despite the fact that hydroxamic acids are potent inhibitors of metalloproteases, they have met with limited success in the clinic because of unfavorable oral bioavailability, stability in vivo, and side effects.[23] As a result, investigators have been working to discover alternate zinc-binding motifs as a substitute for hydroxamic acids. One such example has been published by Cohen and co-workers, who reported hydroxypyridinone and pyrone ligands as promising alternatives to hydroxamic acids.[24] Among the compounds assayed, the zinc chelators that incorporated a thiocarbonyl group had lower IC_{50} values when compared to their non-sulfur-containing analogues.

To apply this strategy to the squaric/hydroxamic acid hybrids, we first needed to develop a reliable method for incorporating sulfur into the inhibitors. Squaric acid monoester **3b** (Scheme 2) did not react with a variety of thionating agents including P_2S_5/hexamethyldisiloxane and Lawesson's reagent. By contrast, the squaric acid monoamide **6a** reacted with both P_2S_5 and Lawesson's reagent to give a product that incorporated a single sulfur atom in place of oxygen, as determined by mass spectrometry. Since Lawesson's reagent provided the cleaner of the two reactions, this method was used to convert **6a**, **12d**, **12g**, and **12h** into the corresponding thiocarbonyl compounds (Scheme 5).

Determining the Position of Sulfur Incorporation. The inhibitors in Scheme 5 contain three or four

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(22) An X-ray crystal structure of a hydroxamic acid/zinc complex shows that the bite angle in this complex is 81.1°. Ruf, M.; Weis, K.; Brasack, I.; Vahrenkamp, H. *Inorg. Chim. Acta* **1996,** *250,* 271−281. For comparison, we used HyperChem 3D to calculate the bite angle in a zinc complex of the squaric acid/hydroxamic acid hybrids, which was calculated to be 117.9°.

(23) Reich, R.; Katz, Y.; Hadar, R.; Breuer, E. *Clin. Cancer Res.* **2005**, *11*, 3925−3929.

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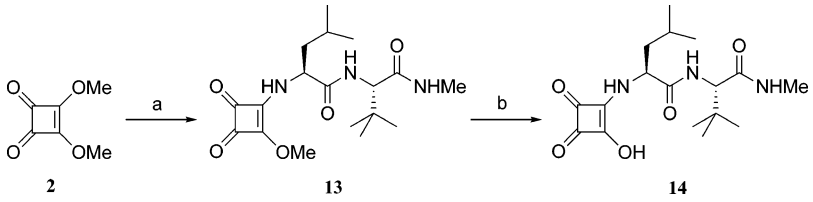
SCHEME 4[a]



[a] Reagents: (a) H-Leu-Tle-NHMe, MeOH, reflux; (b) 0.15 N HCl, MeOH, reflux.

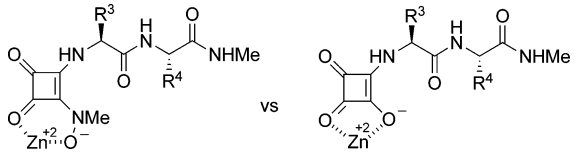
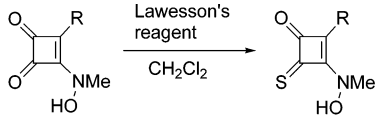


FIGURE 3. Six-membered vs five-membered zinc chelation models.

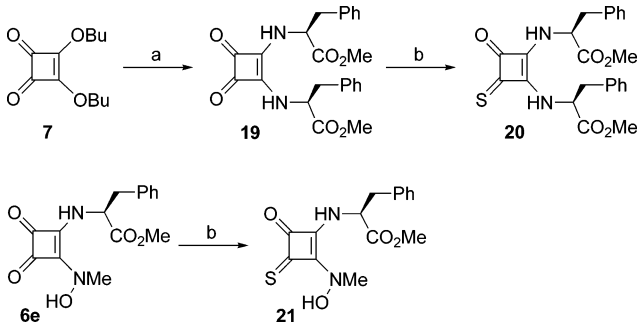
SCHEME 5. Thionation of Cyclobutenediones



6a: R = HN-Ile-OMe
12d: R = HN-Ile-Phe-NHMe
12g: R = HN-Ile-Leu-NHMe
12h: R = HN-Leu-Tle-NHMe

15: R = HN-Ile-OMe
16: R = HN-Ile-Phe-NHMe
17: R = HN-Ile-Leu-NHMe
18: R = HN-Leu-Tle-NHMe

SCHEME 6[a]



[a] Reagents: (a) L-Phe-OMe·HCl, KOH, MeOH; (b) Lawesson's reagent, CH_2Cl_2, 25 °C.

different carbonyl groups, any of which could be the site of reaction with Lawesson's reagent. To determine the specific site that sulfur was incorporated into the molecules, we prepared two model compounds to aid us with this analysis (Scheme 6). Dibutyl squarate **7** was treated with 2 equiv of NH_2-Phe-OMe to give compound **19**. When compound **19** was treated with <1 equiv of Lawesson's reagent, the reaction yielded compound **20** in which one carbonyl group on the cyclobutenedione core had been replaced by a thiocarbonyl as determined by mass spectrometry. Ester carbonyl groups do not react with Lawesson's reagent under the reaction conditions that we employed (25 °C). In a similar manner, compound **6e** was converted to **21**.

The ^{1}H NMR spectrum of compound **19** (Figure 4) has a resonance at 5.0 ppm that corresponds to the α-protons of the two Phe residues (H^a). When this compound is treated with Lawesson's reagent to give compound **20**, one of the α-protons remains at 5.0 ppm (H^c) while the other is shifted to 6.1 ppm (H^b shown in red). H^b shifts downfield since the neighboring nitrogen atom of this Phe residue is in conjugation with the thiocarbonyl group, which is a better electron acceptor than a standard carbonyl. In a similar manner, compound **6e** has a resonance at 5.0 ppm that corresponds the Phe α-proton (H^d). When this compound is converted to the monothiocarbonyl adduct **21**, this proton shifts downfield to 6.2 ppm (H^e shown in red). This observation, along with the fact that compound **3b** (Scheme 2) that also incorporates a −N(OH)Me group does not react with Lawesson's reagent, suggests that the carbonyl group opposite the Phe residue in **6e** was the site of reaction with Lawesson's reagent. Since we observe a similar shift for the α-proton of the amino acid that is attached directly to the cyclobutenedione ring in compounds **6a**, **12d**, **12g**, and **12h**, we infer that all of these compounds react with Lawesson's reagent on the opposite side of the ring from the peptide chain (see the Supporting Information for the spectra).

Additional evidence for the site of sulfur incorporation comes from the chemical shift of the −OH proton of the vinylogous hydroxamic acid in compounds **6e** and **21**. This proton appears at 10.7 ppm in compound **6e**, since it participates in a strong hydrogen bond with the neighboring carbonyl group on the cyclobutenedione ring. However, the analogous proton in compound **21** appears at 8.5−9.0 ppm. This change in chemical shift is partly due to the fact that the thiocarbonyl group is a weaker hydrogen bond acceptor than the carbonyl group.

Effect of the Thiocarbonyl Group on Inhibition. As shown in Table 4, two of the compounds show improved activity against MMP-1 on conversion to their thiocarbonyl analogues, while the activity of the other two remain unchanged. Compound **15** is 2−3 times more potent than **6a**, while the potency of **16** is increased by 18-fold compared to that of **12d**. By contrast, compounds **12g** and **12h** have similar IC_{50} values when compared to their thiocarbonyl analogues **17** and **18**. These results suggest that conversion of a carbonyl group on the cyclobutenedione core of the inhibitors to a thiocarbonyl can be an effective method for improving inhibition. However, this improvement is dependent on the specific structure of the inhibitor. One plausible explanation for the observation that the potencies of compounds **12g** and **12h** do not change upon thionation is that the improvement in chelation between the cyclobutene core and the active site zinc atom changes the position of the bound inhibitor in the active site. This geometry change could decrease binding interactions between the peptide portion of the inhibitors and the S1′ and S2′ subsites, and offset the improved binding to zinc. By contrast, conversion of compounds **6a** and **12d** to their thiocarbonyl analogues

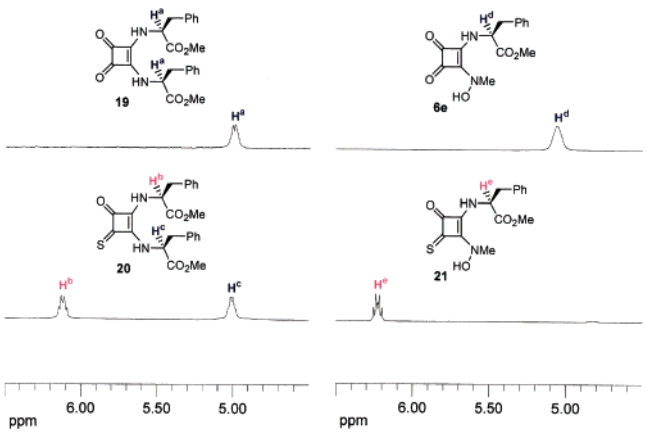
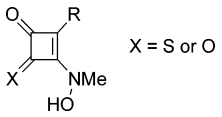


FIGURE 4. Changes that occur in the ^{1}H NMR spectra of compounds **19** and **6e** upon monothionation of the cyclobutenedione core.

TABLE 4. Inhibition of MMP-1 by Thiocarbonyl-Containing Inhibitors



R	compd no. for X = S	IC_{50} (μM)[a]	compd no. for X = O	IC_{50} (μM)
HN-Ile-OMe	**15**	70 ± 8	**6a**	190 ± 30
HN-Ile-Phe-NHMe	**16**	15 ± 1	**12d**	270 ± 30
HN-Ile-Leu-NHMe	**17**	170 ± 40	**12g**	200 ± 20
HN-Leu-Tle-NHMe	**18**	96 ± 30	**12h**	95 ± 7

[a] All experiments performed in duplicate.

could lead to improved binding with the zinc atom, and also may reposition the inhibitor in the active site so that it makes more favorable interactions with the distal enzyme subsites.

Conclusions

We have investigated the potential of a hybrid between squaric and hydroxamic acids to serve as a metal binding motif for the design of metalloprotease inhibitors. While hydroxamic acids are commonly used as MMP inhibitors, to the best of our knowledge this report represents the first use of a squaric acid derivative as a warhead for the design of inhibitors of metalloproteases. The squaric/hydroxamic acid hybrids are generally not as potent as hydroxamic acid-based inhibitors, many of which have inhibition constants in the nM range. However, since hydroxamic acids have not met with much success in the clinic, these hybrids could serve as an alternate starting point for the design of inhibitors with perhaps improved pharmacological properties. The structure of the peptidic portion of the inhibitors helps target them to the active site of zinc proteases, rather than to other classes of enzymes for which squaric acid derivatives can serve as inhibitors.

Among the alkyl groups that we have examined at the −N(OH)alkyl position of the inhibitors, small substituents such as Me and *i*-Pr are well accommodated in the active site of MMP-1, while sterically demanding groups such as Bn and cyclohexyl are too large and lead to poor activity. Among the inhibitors that do not contain a sulfur atom, we found that the dipeptide Leu-Tle-NHMe provided the highest level of activity when attached to the cyclobutenedione core.

We also developed a regioselective method for converting one of the carbonyl groups on the core into the corresponding thiocarbonyl compound. This reaction occurs specifically at the position on the opposite side of the cyclobutene ring from the peptide substituent. Thionation of the inhibitors leads to improved activity in some cases, but not in others. Compound **12d** gave the largest increase in potency upon thionation. The 18-fold improvement resulted in an inhibitor with an IC_{50} value of 15 μM against MMP-1.

Experimental Section

Full characterization for compounds**3b**, **3c**, and **3e** has been reported in the literature.[17]

3-(Hydroxyamino)-4-methoxy-3-cyclobutene-1,2-dione (3a). 3,4-Dimethoxy-3-cyclobutene-1,2-dione (**1**) (200 mg, 1.4 mmol) was added to 10 mL of MeOH and the solution was stirred until all of the solids had dissolved. To this mixture was added hydroxylamine hydrochloride (104 mg, 1.5 mmol), the reaction mixture was cooled to 0 °C, and KOH (94.3 mg, 1.7 mmol) dissolved in 5 mL of MeOH was added. At this point the reaction contained a white precipitate. The reaction was monitored by TLC and upon disappearance of the starting material the mixture was filtered and the solid that was collected was washed with cold MeOH. The filtrate and washes were combined and solvent was evaporated. The crude material was purified by column chromatography (1:9 MeOH/CH_2Cl_2) to give compound **3a** as a yellow solid (0.093 g, 0.650 mmol, 47%). ^{1}H NMR (300 MHz, CD_3OD) δ 4.37 (s, 3 H); ^{13}C NMR (100 MHz, CD_3OD) δ 184.9, 181.5, 174.4, 170.1, 60.1; HRMS-ESI (M + H^+) calcd for $C_5H_6NO_4$ 144.0297, found 144.0292.

3-[(Benzyl)hydroxyamino]-4-methoxy-3-cyclobutene-1,2-dione (3d). This compound was prepared with use of *N*-benzylhydroxylamine hydrochloride (0.367 g, 2.3 mmol) and **1** (0.30 g, 2.1 mmol) according to the procedure described above for the preparation of compound **3a**. Purification of **3d** was performed by column chromatography (5:95 MeOH/CH_2Cl_2) to obtain a white solid (0.318 g, 1.37 mmol, 65%). ^{1}H NMR (300 MHz, CD_3OD) δ 7.39 (m, 5 H), 4.88 (s, 3 H), 4.38 (s, 3 H); ^{13}C NMR (75 MHz, CD_3OD) δ 185.0, 181.3, 175.0, 169.0, 135.0, 129.0, 128.8, 128.5, 60.3, 57.3; HRMS-ESI (M + Na^+) calcd for $C_{12}H_{11}NO_4Na$ 256.0586, found 256.0590.

3-(Hydroxyamino)-4-[(2-methylpropyl)amino]-3-cyclobutene-1,2-dione (4a). To a solution of compound **3a** (0.055 g, 0.383 mmol) dissolved in MeOH (1 mL) was added isobutylamine (0.062 g, 0.844 mmol) and the mixture was stirred at room temperature for 25 h. The solvent was evaporated and the product was purified by column chromatography (1:9:90 30% aqueous NH_4OH/MeOH/CH_2Cl_2) giving **4a** (0.009 g, 0.049 mmol, 13%) as a white solid. ^{1}H NMR (400 MHz, CD_3OD) δ 3.39 (d, J = 6.8 Hz, 2 H), 1.85 (m, 1 H), 0.96 (d, J = 5.2 Hz, 6 H); ^{13}C NMR (100 MHz, CD_3OD) δ 181.1, 180.3, 168.9, 167.8, 52.8, 31.4, 20.0; HRMS-ESI (M + H^+) calcd for $C_8H_{12}N_2O_3$ 185.0926, found 185.0932.

3-(Hexylamino)-4-(hydroxyamino)-3-cyclobutene-1,2-dione (4b). Compound **3b** (100 mg, 0.70 mmol) was dissolved in MeOH (10 mL) and hexylamine (84.9 mg, 0.84 mmol) was added to the solution. The reaction was stirred for 12 h at room temperature, after which time TLC showed that all the starting material was consumed. The solvent was then removed by rotary evaporation. The crude material was purified by column chromatography (1:9:90 30% aqueous NH_4OH/MeOH/CH_2Cl_2) to yield **4b** as a yellow oil (17.8 mg, 0.084 mmol, 12%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.35 (m, 2 H),

1.50 (m, 2 H), 1.27 (m, 6 H), 0.86 (m, 3 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 184.2, 183.6, 170.2, 169.6, 44.1, 31.7, 31.6, 26.4, 23.0, 14.8; HRMS-ESI (M + H$^+$) calcd for $C_{10}H_{17}N_2O_3$ 213.1239, found 213.1250.

3-(Hexylamino)-4-(hydroxymethylamino)-3-cyclobutene-1,2-dione (4c). To a solution of **3b** (0.157 g, 1.0 mmol) in MeOH (1 mL) was added hexylamine (0.111 g, 1.1 mmol) and the mixture was stirred at room temperature for 4 h. The solvent was evaporated and the crude product was purified by column chromatography (1:9:90 30% aqueous NH_4OH/MeOH/CH_2Cl_2) giving **4c** (0.19 g, 0.84 mmol, 84%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.19 (s, 1 H), 7.50 (s, 1 H), 3.59 (m, 5 H), 1.64 (m, 2 H), 1.32 (m, 6 H), 0.88 (t, J = 6.5 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.9, 177.5, 166.8, 164.9, 45.0, 41.4, 31.4, 30.9, 26.1, 22.6, 14.0; HRMS-FAB (M + Na$^+$) calcd for $C_{11}H_{18}N_2O_3Na$ 249.1215, found 249.1220.

3-(Hydroxymethylamino)-4-(pentylamino)-3-cyclobutene-1,2-dione (4d). Compound **4d** (0.058 g, 0.27 mmol, 37%) was prepared from **3b** (0.118 g, 0.75 mmol) and pentylamine (0.65 g, 0.75 mmol) according to the procedure used for preparing **4c**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.16 (s, 1 H), 3.56 (m, 5 H), 1.64 (m, 2 H), 1.33 (m, 4 H), 0.89 (t, J = 6.8 Hz, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.9, 177.4, 166.7, 165.1, 45.0, 41.4, 30.7, 28.6, 22.3, 14.0; HRMS-FAB (M + Na$^+$) calcd for $C_{10}H_{16}N_2O_3Na$ 235.1059, found 235.1066.

3-[(Cyclohexyl)hydroxyamino]-4-(hexylamino)-3-cyclobutene-1,2-dione (4e). Compound **4e** (0.0047 g, 0.016 mmol, 13%) was prepared as a white solid from **3c** (0.027 g, 0.12 mmol) and hexylamine (0.015 g, 0.144 mmol) according to the procedure used for the preparation of **4b**. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.34 (br s, 2 H), 3.76 (br s, 1 H), 3.32 (s, 4 H), 3.52 (s, 2 H), 1.88 (m, 2 H), 1.70 (m, 2 H), 1.57 (s, 3 H), 1.27 (m, 10 H), 1.53 (m, 3 H), 0.86 (m, 3 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 181.7, 181.4, 167.3, 166.4, 51.5, 42.7, 33.2, 30.3, 30.2, 25.0, 24.3, 23.5, 21.5, 13.4; HRMS-ESI (M + H$^+$) calcd for $C_{16}H_{27}N_2O_3$ 295.2022, found 295.2018.

3-[(Benzyl)hydroxyamino]-4-(hexylamino)-3-cyclobutene-1,2-dione (4f). Compound **4f** (0.232 g, 0.77 mmol, 89%) was prepared as a white solid from **3d (**0.200 g, 0.86 mmol) and hexylamine (0.101 g, 1.00 mmol) according to the procedure used for the preparation of **4b**. ^{1}H NMR (300 MHz, CD_3OD) δ 7.36 (m, 5 H), 4.94 (s, 2 H), 3.58 (t, J = 7.0 Hz, 2 H), 1.60 (dd, J = 13.9, 6.9 Hz, 2 H), 1.34 (m, 6 H), 0.91 (t, J = 6.7 Hz, 3 H); ^{13}C NMR (75 MHz, CD_3OD) δ 180.4, 178.6, 168.2, 166.1, 135.6, 129.1 128.7, 128.3, 57.5, 44.4, 31.6, 26.1, 22.7, 13.4; HRMS-ESI (M + H$^+$) 303.1709, found 303.1711.

3-(Hydroxymethylamino)-4-(L-isoleucine methyl ester)-3-cyclobutene-1,2-dione (6a). To a solution of **3b** (0.219 g, 1.39 mmol) dissolved in MeOH (5 mL) was added the hydrochloride salt of L-isoleucine methyl ester (**5a**) (0.229 g, 1.26 mmol). To this stirred solution was added KOH (0.0713 g, 1.27 mmol), and immediately a white precipitate formed. The reaction was stirred at room temperature for 12 h and solvent was evaporated. Purification was performed by column chromatography (1:9:90 30% aqueous NH_4OH/MeOH/CH_2Cl_2) to yield a yellowish flaky solid (0.238 g, 0.88 mmol, 70%). ^{1}H NMR (300 MHz, CD_3OD) δ 5.04 (br s, 2 H), 4.81 (d, J = 7 Hz, 2 H), 3.78 (s, 3 H), 3.50 (s, 3 H), 2.03 (m, 1 H), 1.54 (m, 1 H), 1.30 (m, 1 H), 0.97 (m, 6 H); ^{13}C NMR (75 MHz, CD_3OD) δ 180.4, 180.0, 173.0, 167.6, 167.0, 62.4, 52.8, 41.4, 39.4, 25.8, 15.6, 11.6; HRMS-ESI (M + H$^+$) calcd for $C_{12}H_{19}N_2O_5$ 271.1294, found 271.1287.

3-[Hydroxy(1-methylethyl)amino]-4-(L-isoleucine methyl ester)-3-cyclobutene-1,2-dione (6b). Compound **6b** (0.034 g, 0.11 mmol, 26%) was prepared as a colorless solid starting from **3e** (0.080 g, 0.432 mmol) and **5a** (0.120 g, 0.56 mmol) according to the procedure used for the preparation of **4b**. ^{1}H NMR (300 MHz, CD_3OD) δ 7.28 (br s, 1 H), 4.83 (br s, 1 H), 4.52 (br s, 1 H), 3.73 (s, 3 H), 2.01 (s, 1 H), 1.49 (m, 1 H), 1.31 (m, 7 H), 0.93 (m, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 179.1, 179.0, 172.0, 166.7, 165.5, 61.8, 55.5, 52.7, 38.7, 25.1, 19.8, 15.5, 11.8; HRMS-ESI (M + H$^+$) calcd for $C_{14}H_{23}N_2O_5$ 299.1607, found 299.1602.

3-(Hydroxymethylamino)-4-(D,L-norleucine methyl ester)-3-cyclobutene-1,2-dione (6c). Compound **6c** (0.09 g, 0.33 mmol, 45%) was prepared from **3b** (0.118 g, 0.75 mmol), d,L-norleucine methyl ester HCl salt (0.136 g, 0.75 mmol), and KOH (0.042 g, 0.75 mmol) according to the procedure used for preparing **6a**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.15 (d, J = 8.4 Hz, 1 H), 4.79 (dt, J = 9.1, 4.8 Hz, 1 H), 3.73 (s, 3 H), 3.55 (s, 3 H), 1.95 (m, 1 H), 1.82 (m, 1 H), 1.36 (m, 4 H), 0.89 (t, J = 7.1 Hz, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.8, 178.2, 172.0, 165.9, 165.2, 57.0, 52.5, 41.3, 32.5, 27.6, 22.2, 13.8; HRMS-ESI (M + H$^+$) calcd for $C_{12}H_{19}N_2O_5$ 271.1294, found 271.1285.

3-(Hydroxymethylamino)-4-(L-leucine methyl ester)-3-cyclobutene-1,2-dione (6d). Compound **6d** was prepared as a white solid (0.023 g, 0.076 mmol, 38%) starting from **3b** (0.031 g, 0.20 mmol) and **5c** (0.047 g, 0.26 mmol) according to the procedure used to prepare **6a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.14 (br s, 1 H), 4.87 (br s, 1 H), 3.72 (s, 3 H), 3.55 (s, 3 H), 1.75 (m, 1 H), 0.96 (d, J = 4.8 Hz, 6 H); ^{13}C NMR (100 MHz, CD_3OD) δ 184.5, 183.5, 174.1, 170.3, 168.7, 54.8, 53.0, 31.3, 25.9, 23.4, 21.7; HRMS-ESI (M + Na$^+$) calcd for $C_{12}H_{18}N_2O_5$-Na 293.1113, found 293.1118.

3-(Hydroxymethylamino)-4-(L-phenylalanine methyl ester)-3-cyclobutene-1,2-dione (6e). Compound **6e** (0.135 g, 0.44 mmol, 59%) was prepared from **3b** (0.118 g, 0.75 mmol), l-phenylalanine methyl ester HCl salt (0.216 g, 1.0 mmol), and KOH (0.056 g, 1.0 mmol) according to the procedure used for preparing **6a**. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.75 (s, 1 H), 7.79 (s, 1 H), 7.25 (m, 5 H), 5.06 (s, 1 H), 3.70 (s, 3 H), 3.35 (s, 3 H), 3.28 (dd, J = 14.0, 3.9 Hz, 1 H), 3.18 (m, 1 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 180.0, 179.2, 171.9, 166.9, 166.0, 137.7, 129.6, 128.8, 127.0, 57.6, 52.8, 41.2, 37.7; HRMS-FAB (M + Na$^+$) calcd for $C_{15}H_{16}N_2O_5Na$ 327.0957, found 327.0948.

3-(Hydroxymethylamino)-4-(L-tryptophan methyl ester)-3-cyclobutene-1,2-dione (6f). Compound **6f** (0.16 g, 0.47 mmol, 62%) was prepared from **3b** (0.118 g, 0.75 mmol), L-tryptophan methyl ester HCl salt (0.255 g, 1.0 mmol), and KOH (0.056 g, 1.0 mmol) according to the procedure used for preparing **6a**. ^{1}H NMR (300 MHz, CD_3OD) δ 7.56 (d, J = 7.7 Hz, 1 H), 7.34 (d, J = 8.0 Hz, 1 H), 7.07 (m, 3 H), 5.13 (dd, J = 7.7, 5.1 Hz, 1 H), 3.76 (s, 3 H), 3.43 (m, 5 H); ^{13}C NMR (75 MHz, CD_3OD) δ 179.6, 179.0, 172.4, 166.9, 166.2, 137.0, 127.7, 123.7, 121.5, 119.0, 118.2, 111.3, 109.1, 57.7, 52.1, 40.3, 28.7; HRMS-ESI (M + Na$^+$) calcd for $C_{17}H_{17}N_3O_5Na$ 366.1066, found 366.1075.

3-(Hydroxymethylamino)-4-(L-methionine methyl ester)-3-cyclobutene-1,2-dione (6g). Compound **6g** (0.082 g, 0.28 mmol, 38%) was prepared from **3b** (0.118 g, 0.75 mmol), l-methionine methyl ester HCl salt (0.15 g, 0.75 mmol), and KOH (0.042 g, 0.75 mmol) according to the procedure used for preparing **6a**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 (d, J = 7.8 Hz, 1 H), 4.93 (dd, J = 12.7, 8.5 Hz, 1 H), 3.75 (s, 3 H), 3.55 (s, 3 H), 2.60 (m, 2 H), 2.27 (m, 1 H), 2.17 (td, J = 14.7, 7.2 Hz, 1 H), 2.10 (s, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.8, 178.3, 171.6, 165.9, 165.3, 56.0, 52.8, 41.4, 31.8, 30.1, 15.4; HRMS-FAB (M + Na$^+$) calcd for $C_{11}H_{16}N_2O_5SNa$ 311.0678, found 311.0685.

3-Butoxy-4-(hydroxymethylamino)-3-cyclobutene-1,2-dione (8). To a stirred solution of HONHMe HCl salt (2.631 g, 31.5 mmol) in MeOH (30 mL) were added KOH (1.767 g, 31.5 mmol) and compound **7** (4.766 g, 21.0 mmol). The mixture was stirred overnight at room temperature, the solvent was evaporated, and the crude product was washed with H_2O, and then dissolved in EtOAc. The aqueous layer was extracted with EtOAc. The organic extracts were combined and dried over $MgSO_4$, and the solvents were evaporated under reduced pressure. The crude product was purified by column chromatography (3% MeOH/CH_2Cl_2) to yield compound **8** as a pale yellow solid (3.416 g, 17.2 mmol, 82%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.70 (t, J = 6.6 Hz, 2 H), 3.52 (s, 3 H), 1.77 (m, 2 H), 1.44 (m, 2 H), 0.97 (t, J = 7.4 Hz, 3 H); ^{13}C NMR (75 MHz,

$CDCl_3$) δ 184.0, 180.7, 174.3, 169.1, 74.2, 41.6, 32.3, 18.9, 14.0; HRMS-FAB ($M + Na^+$) calcd for $C_9H_{13}NO_4Na$ 222.0742, found 222.0740.

L-*tert*-Leucine Methyl Amide (10a). Compound **10a** was synthesized starting from *N*-Boc-protected amino acid **9a**. To a stirred solution of *N*-hydroxysuccinimide (NHS, 0.925 g, 8.04 mmol) in DMF (20 mL) under N_2 was added NH_2Me (4.02 mL, 2.0 M in MeOH) via syringe and the mixture was stirred for 30 min at room temperature. Formation of a white precipitate was observed. Addition of *N*-Boc-protected amino acid **9a** (1.55 g, 6.7 mmol) and cooling the mixture to 0 °C was followed by addition of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 1.54 g, 8.04 mmol). The mixture was stirred for 1 h at 0 °C and 2 h at room temperature and then diluted with EtOAc. The solution was washed with H_2O, the organic layer was separated, and the aqueous layer was extracted with EtOAc. The combined organic extracts were washed with saturated $NaHCO_3$, H_2O, and brine and dried over $MgSO_4$, and the solvents were evaporated under reduced pressure. The crude product was purified by column chromatography (3% MeOH/CH_2Cl_2), washed with heptane, dried in vacuo, and dissolved in CH_2Cl_2 (15 mL). To this stirred solution was added TFA (15 mL) and the mixture was stirred for 40 min at room temperature. The solvents were removed in vacuo, and the residue was partitioned between CH_2Cl_2 (90 mL) and saturated aqueous $NaHCO_3$ (90 mL). The organic phase was separated and the aqueous phase was extracted with CH_2Cl_2. The organic extracts were combined and dried over Na_2SO_4, and the solvents were evaporated. Purification was performed by column chromatography (5:95 MeOH/CH_2Cl_2) yielding **10a** as a colorless oil (0.637 g, 4.42 mmol, 66%). ^{1}H NMR (300 MHz, CD_3OD) δ 2.99 (s, 1 H), 2.75 (s, 3 H), 0.98 (s, 9 H); ^{13}C NMR (75 MHz, CD_3OD) δ 175.2, 63.9, 34.0, 25.9, 24.9; HRMS-FAB ($M + Na^+$) calcd for $C_7H_{16}N_2ONa$ 167.1160, found 167.1163.

L-Cyclohexylglycine Methyl Amide (10b). Compound **10b** was synthesized starting from *N*-Boc-protected amino acid **9b**. To a stirred solution of *N*-Boc-protected amino acid **9b** (1.16 g, 4.52 mmol) in CH_2Cl_2 (20 mL) was added HOBt (760 mg, 4.97 mmol) and then EDC (911 mg, 4.75 mmol) and the mixture was stirred at 0 °C for 30 min. Formation of a white precipitate was observed. To this solution was added NH_2Me (2.49 mL, 2.0 M in MeOH) via syringe and the reaction mixture was stirred overnight. The solution was diluted with CH_2Cl_2 (50 mL) and washed once with 1 M citric acid (30 mL), once with saturated $NaHCO_3$ (30 mL), and then once with brine (30 mL). The organic layer was then dried over $NaSO_4$ and the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography (3% MeOH/CH_2Cl_2) and the product was dissolved in CH_2Cl_2 (15 mL). To this stirred solution was added TFA (15 mL) and the mixture was stirred at room temperature for 40 min. The solvents were removed in vacuo, and the residue was partitioned between 1:1 CH_2Cl_2/saturated aqueous $NaHCO_3$. The organic phase was separated and the aqueous phase was extracted with CH_2Cl_2. The organic extracts were combined and dried over Na_2SO_4, and solvents were evaporated. Purification was performed by flash chromatography (5% MeOH/CH_2Cl_2) yielding **10b** as a white solid (440 mg, 2.80 mmol, 62%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.34 (br s, 1 H), 3.22 (d, J = 3.8 Hz, 1 H), 2.80 (d, J = 5.0 Hz, 3 H), 1.27 (m, 14 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.5, 60.5, 41.5, 30.6, 27.0, 26.7, 26.6, 26.5, 26.0; HRMS-FAB ($M + Na^+$) calcd for $C_9H_{18}N_2NaO$ 193.1317, found 193.1320.

***O*-Methyl-L-tyrosine Methyl Amide (10c).** The synthesis and the ^{1}H NMR spectrum for this compound have been reported previously.[25] ^{13}C NMR (75 MHz, CD_3OD) δ 175.9, 158.6, 129.9, 129.3, 113.5, 56.5, 54.2, 40.3, 24.7; HRMS-ESI ($M + Na^+$) calcd for $C_{11}H_{16}N_2O_2Na$ 231.1109, found 231.1115.

L-Phenylalanine Methyl Amide (10d). Compound **10d** (105 mg, 0.590 mmol, 52%) was prepared as a white solid from **9d** (300 mg, 1.13 mmol), HOBt (190 mg, 1.24 mmol), EDC (228 mg, 1.19 mmol), and NH_2Me (624 μL of a 2.0 M solution in MeOH) according to the procedure used to prepare **10b**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.26 (m, 5 H), 3.60 (dd, J = 9.5, 4.0 Hz, 1 H), 3.28 (dd, J = 13.7, 4.0 Hz, 1 H), 2.82 (d, J = 5 Hz, 3 H), 2.67 (dd, J = 13.7, 9.5 Hz, 1 H), 1.39 (br s, 2 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.8, 138.0, 129.3, 128.7, 126.8, 56.5, 41.0, 25.8; HRMS-FAB ($M + Na^+$) calcd for $C_{10}H_{14}N_2NaO$ 201.1004, found 201.1007.

L-Phenylglycine Methyl Amide (10e). Compound **10e** (0.836 g, 5.1 mmol, 51%) was prepared from **6e** (2.513 g, 10 mmol), NH_2Me (6.0 mL of a 2.0 M solution in MeOH), NHS (1.381 g, 12 mmol), EDC (2.3 g, 12 mmol), and TFA (15 mL) according to the procedure used for preparing **10a**. The ^{1}H NMR and ^{13}C NMR spectra and the HRMS for this compound have been reported previously.[26]

L-Tryptophan Methyl Amide (10f). The synthesis and the ^{1}H NMR spectrum for this compound have been reported previously.[24] ^{13}C NMR (75 MHz, CD_3OD) δ 174.8, 135.3, 125.9, 121.8, 119.6, 116.9, 116.5, 109.4, 108.3, 54.0, 29.3, 23.3; HRMS-FAB ($M + Na^+$) calcd for $C_{12}H_{15}N_3ONa$ 240.1113, found 240.1117.

L-Leucine Methyl Amide (10g). Compound **10g** (0.548 g, 3.8 mmol, 42%) was prepared from **9g** (2.245 g, 9 mmol), NH_2-Me (5.4 mL of a 2.0 M solution in MeOH), NHS (1.243 g, 10.8 mmol), EDC (2.068 g, 10.8 mmol), and TFA (12 mL) according to the procedure used for preparing **10a**. ^{1}H NMR (300 MHz, CD_3OD) δ 3.33 (dd, J = 7.7, 6.5 Hz, 1 H), 2.76 (s, 3 H), 1.68 (qt, J = 12.9, 6.5 Hz, 1 H), 1.54 (ddd, J = 13.8, 7.4, 6.4 Hz, 1 H), 1.39 (m, 1 H), 0.95 (t, J = 6.6 Hz, 6 H); ^{13}C NMR (75 MHz, CD_3OD) δ 177.0, 53.2, 44.2, 24.8, 24.5, 22.0, 21.1; HRMS-FAB ($M + Na^+$) calcd for $C_7H_{16}N_2ONa$ 167.1160, found 167.1166.

L-Isoleucyl-L-*tert*-leucine Methyl Amide (11a). To a stirred solution of NHS (0.138 g, 1.2 mmol) in DMF (5 mL) under N_2 was added **10a** (0.144 g, 1.0 mmol) and the mixture was stirred at room temperature for 30 min. Addition of *N*-Boc-L-isoleucine (0.288 g, 1.2 mmol) and cooling the mixture to 0 °C was followed by addition of EDC (0.23 g, 1.2 mmol). The mixture was stirred for 1 h at 0 °C and 2 h at room temperature and then diluted with EtOAc. The solution was washed with H_2O, the organic layer was separated, and the aqueous layer was extracted with EtOAc. The combined organic extracts were washed with saturated $NaHCO_3$, H_2O, and brine and dried over $MgSO_4$, and the solvents were evaporated under reduced pressure. The crude product was purified by column chromatography (3:97 MeOH/CH_2Cl_2), washed with heptane, dried in vacuo, and dissolved in CH_2Cl_2 (2 mL). To this solution was added TFA (2 mL) and the mixture was stirred at room temperature for 40 min. The solvents and TFA were removed, and the residue was partitioned between CH_2Cl_2 (15 mL) and saturated aqueous $NaHCO_3$ (15 mL). The organic phase was separated and the aqueous phase was extracted with CH_2Cl_2. The organic extracts were combined and dried over Na_2SO_4, and the solvent was evaporated. Purification was performed by flash chromatography (5:95 MeOH/CH_2Cl_2) yielding **11a** as a colorless oil (0.162 g, 0.63 mmol, 63%). ^{1}H NMR (300 MHz, CD_3OD) δ 5.51 (s, 1 H), 4.19 (s, 1 H), 2.73 (s, 3 H), 1.79 (m, 1 H), 1.48 (m, 1 H), 1.16 (m, 1 H), 0.95 (m, 15 H); ^{13}C NMR (75 MHz, CD_3OD) δ 175.5, 171.8, 60.6, 59.4, 38.6, 33.8, 25.7, 24.6, 23.8, 14.8, 10.7; HRMS-FAB ($M + Na^+$) calcd for $C_{13}H_{27}N_3O_2Na$ 280.2001, found 280.2000.

L-Isoleucyl-L-cyclohexylglycine Methyl Amide (11b). *N*-Boc-L-Isoleucine (140 mg, 0.584 mmol) was dissolved in CH_2Cl_2 (20 mL) and the solution was cooled to 0 °C. To this solution were added HOBt (89.5 mg, 0.584 mmol) and EDC (201 mg, 0.558 mmol) and the mixture was stirred for 30 min. To this mixture was then added compound **10b** (83.5 mg, 0.531

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(26) Reichard, G. A.; Stengone, C.; Paliwal, S.; Mergelsberg, I.; Majmundar, S.; Wang, C.; Tiberi, R.; McPhail, A. T.; Piwinski, J. J.; Shih, N.-Y. *Org. Lett.* **2003**, *5*, 4249−4251.

mmol) and the reaction mixture was stirred overnight. The solution was diluted with CH_2Cl_2 (50 mL) and washed once with 1 M citric acid (30 mL), once with saturated $NaHCO_3$ (30 mL), and then once with brine (30 mL). The organic layer was dried over $NaSO_4$ and the solvents evaporated under reduced pressure. The crude product was purified by column chromatography (3% MeOH/CH_2Cl_2) and then dissolved in CH_2Cl_2 (15 mL). To this solution was added TFA (15 mL) and the mixture was stirred at room temperature for 40 min. The solvents and TFA were evaporated, and the residue was partitioned between a 1:1 mixture of CH_2Cl_2:saturated aqueous $NaHCO_3$. The organic phase was separated and the aqueous phase was extracted with CH_2Cl_2. The organic extracts were combined and dried over Na_2SO_4, and the solvent was evaporated. Purification was performed by flash chromatography (5% MeOH/CH_2Cl_2) yielding **11b** as a white solid (69 mg, 0.244 mmol, 46%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.94 (d, J = 9.0 Hz, 1 H), 6.98 (m, 1 H), 4.26 (m, 1 H), 3.28 (d, J = 4.0 Hz, 1 H), 2.78 (d, J = 4.8 Hz, 3 H), 1.93 (m, 1 H), 1.75 (m, 7 H), 1.39 (m, 2 H), 1.25 (m, 2 H), 1.13 (m, 4 H), 0.96 (d, J = 6.9 Hz, 3 H), 0.90 (t, J = 7.3 Hz, 3 H); ^{13}C NMR (100 MHz, CD_3OD) δ 174.6, 171.9, 59.8, 57.8, 39.7, 38.1, 29.8, 28.8, 26.1, 25.9, 25.8, 25.8, 24.0, 16.1, 11.9; HRMS-FAB (M + Na^+) calcd for $C_{15}H_{29}N_3NaO_2$ 306.2157, found 306.2150.

L-Isoleucyl-*O*-methyl-L-tyrosine Methyl Amide (11c). Compound **11c** (0.265 g, 0.82 mmol, 58%) was prepared as a white solid from **10c** (0.295 g, 1.42 mmol), NHS (0.196 g, 1.7 mmol), *N*-Boc-L-isoleucine (0.408 g, 1.7 mmol), EDC (0.326 g, 1.7 mmol), and TFA (3 mL) according to the procedure used for preparing **11a**. ^{1}H NMR (300 MHz, CD_3OD) δ 7.16 (d, J = 8.5 Hz, 2 H), 6.84 (d, J = 8.5 Hz, 2 H), 4.55 (dd, J = 8.6, 6.3 Hz, 1 H), 3.76 (s, 3 H), 3.17 (d, J = 5.0 Hz, 1 H), 3.07 (dd, J = 13.8, 6.2 Hz, 1 H), 2.86 (dd, J = 13.8, 8.8 Hz, 1 H), 2.69 (s, 3 H), 1.66 (m, 1 H), 1.18 (m, 1 H), 0.99 (m, 1 H), 0.83 (m, 6 H); ^{13}C NMR (75 MHz, CD_3OD) δ 175.6, 172.7, 158.7, 129.9, 128.8, 113.5, 59.6, 54.7, 54.2, 38.5, 37.0, 24.9, 23.5, 14.7, 10.6; HRMS-FAB (M + Na^+) calcd for $C_{17}H_{27}N_3O_3Na$ 344.1950, found 344.1942.

L-Isoleucyl-L-phenylalanine Methyl Amide (11d). Compound **11d** (373 mg, 1.28 mmol, 61%) was prepared as a white solid from **10d** (374 mg, 2.1 mmol), HOBt (322 mg, 2.1 mmol), EDC (383 mg, 2.0 mmol), and *N*-Boc-L-isoleucine (456 mg, 1.9 mmol) according to the procedure that was used to prepare **11b**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (d, J = 8.8 Hz, 1 H), 7.18 (m, 5 H), 4.78 (dt, J = 8.7, 6.3 Hz, 1 H), 2.98 (dd, J = 13.8, 8.8 Hz, 1 H), 2.68 (d, J = 4.8 Hz, 3 H), 1.77 (m, 1 H), 0.99 (m, 1 H), 0.88 (m, 1 H), 0.81 (d, J = 7.0 Hz, 3 H), 0.75 (t, J = 7.3 Hz, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.0, 171.9, 137.1, 129.2, 128.4, 126.7, 59.8, 54.0, 38.4, 26.0, 23.4, 15.9, 11.8; HRMS-FAB (M + Na^+) calcd for $C_{16}H_{25}N_3NaO_2$ 314.1844, found 314.1850.

L-Isoleucyl-L-phenylglycine Methyl Amide (11e). Compound **11e** (0.032 g, 0.115 mmol, 53%) was prepared from **10e** (0.036 g, 0.217 mmol), NHS (0.03 g, 0.26 mmol), *N*-Boc-L-isoleucine (0.062 g, 0.26 mmol), EDC (0.05 g, 0.26 mmol), and TFA (0.35 mL) according to the procedure used for preparing **11a**. ^{1}H NMR (400 MHz, CD_3OD) δ 7.44 (m, 2 H), 7.34 (m, 3 H), 5.42 (s, 1 H), 3.29 (d, J = 5.3 Hz, 1 H), 2.74 (s, 3 H), 1.77 (m, 1 H), 1.47 (m, 1 H), 1.15 (m, 1 H), 0.96 (d, J = 6.9 Hz, 3 H), 0.89 (t, J = 7.4 Hz, 3 H); ^{13}C NMR (100 MHz, CD_3OD) δ 175.1, 171.4, 137.7, 128.3, 127.9, 127.1, 59.2, 57.0, 38.8, 25.0, 23.9, 14.6, 10.6; HRMS-FAB (M + Na^+) calcd for $C_{15}H_{23}N_3O_2$-Na 300.1688, found 300.1680.

L-Isoleucyl-L-tryptophan Methyl Amide (11f). Compound **11f** (0.103 g, 0.313 mmol, 55%) was prepared from **10f** (0.124 g, 0.573 mmol), NHS (0.079 g, 0.688 mmol), *N*-Boc-L-isoleucine (0.165 g, 0.688 mmol), EDC (0.132 g, 0.688 mmol), and TFA (1 mL) according to the procedure used to prepare **11a**. ^{1}H NMR (300 MHz, CD_3OD) δ 7.57 (d, J = 7.8 Hz, 1 H), 7.29 (dd, J = 8.1, 0.9 Hz, 1 H), 7.02 (m, 3 H), 4.59 (t, J = 6.5 Hz, 1 H), 3.2 (d, J = 6.5 Hz, 1 H), 2.62 (d, J = 1.9 Hz, 3 H), 1.60 (m, 1 H), 1.14 (m, 1 H), 0.9 (m, 1 H), 0.75 (m, 6 H); ^{13}C NMR (75 MHz, CD_3OD) δ 174.3, 172.1, 135.6, 126.2, 122.1, 119.9, 117.2, 116.8, 109.7, 108.3, 58.3, 52.9, 37.2, 26.7, 23.8, 22.3, 13.4, 9.4; HRMS-ESI (M + H^+) calcd for $C_{18}H_{27}N_4O_2$ 331.2134, found 331.2138.

L-Isoleucyl-L-leucine Methyl Amide (11g). Compound **11g** (0.219 g, 0.851 mmol, 43%) was prepared from **10g** (0.285 g, 1.98 mmol), NHS (0.273 g, 2.375 mmol), *N*-Boc-L-isoleucine (0.57 g, 2.375 mmol), EDC (0.455 g, 2.375 mmol), and TFA (2.5 mL) according to the procedure used to prepare **11a**. ^{1}H NMR (300 MHz, CD_3OD) δ 4.4 (t, J = 7.5 Hz, 1 H), 3.23 (d, J = 5.5 Hz, 1 H), 2.73 (s, 3 H), 1.61 (m, 5 H), 1.18 (m, 1 H), 0.94 (m, 12 H); ^{13}C NMR (75 MHz, CD_3OD) δ 175.9, 174.2, 59.9, 51.9, 41.2, 39.3, 25.4, 24.9, 24.4, 22.5, 21.1, 15.1, 11.0; HRMS-FAB (M + Na^+) calcd for $C_{13}H_{27}N_3O_2Na$ 280.2001, found 280.2006.

L-Leucyl-L-*tert*-leucine Methyl Amide (11h). Compound **11h** (0.054 g, 0.208 mmol, 65%) was prepared from **10h** (0.046 g, 0.322 mmol), NHS (0.045 g, 0.386 mmol), *N*-Boc-L-leucine (0.096 g, 0.386 mmol), EDC (0.074 g, 0.386 mmol), and TFA (0.75 mL) according to the procedure that was used to prepare **11a**. ^{1}H NMR (400 MHz, CD_3OD) δ 4.22 (s, 1 H), 3.46 (t, J = 5.3 Hz, 1 H), 2.74 (s, 3 H), 1.76 (m, 1 H), 1.57 (m, 1 H), 1.41 (m, 1 H), 1.01 (m, 9 H), 0.97 (m, 6 H); ^{13}C NMR (100 MHz, CD_3OD) δ 176.8, 172.1, 60.8, 53.6, 44.5, 34.5, 26.2, 25.1, 24.9, 22.7, 21.2; HRMS-FAB (M + Na^+) calcd for $C_{13}H_{27}N_3O_2Na$ 280.2001, found 280.2010.

General Procedure for the Synthesis of Compounds 12a–h. To a stirred solution of **8** (1.0 equiv) dissolved in MeOH was added **11a–h** (1.0 equiv) and the mixture was stirred at room temperature overnight. The solvent was evaporated and the product was purified by column chromatography (3:97 MeOH/CH_2Cl_2).

3-(Hydroxymethylamino)-4-(L-isoleucyl-L-*tert*-leucine methyl amide)-3-cyclobutene-1,2-dione (12a). Compound **12a** (0.03 g, 0.077 mmol, 71%) was prepared from **8** (0.022 g, 0.109 mmol) and **11a** (0.028 g, 0.109 mmol) according to the general procedure. ^{1}H NMR (300 MHz, CD_3OD) δ 4.58 (d, J = 8.7 Hz, 1 H), 4.23 (s, 1 H), 3.49 (s, 3 H), 2.73 (s, 3 H), 1.93 (m, 1 H), 1.62 (m, 1 H), 1.23 (m, 1 H), 0.99 (s, 9 H), 0.94 (m, 6 H); ^{13}C NMR (75 MHz, CD_3OD) δ 179.5, 178.9, 172.0, 171.8, 166.5, 166.3, 62.4, 61.3, 40.2, 37.7, 34.2, 26.1, 24.9, 24.4, 14.5, 10.0; HRMS-ESI (M + Na^+) calcd for $C_{18}H_{30}N_4O_5Na$ 405.2114, found 405.2126.

3-(Hydroxymethylamino)-4-(L-isoleucyl-L-cyclohexylglycine methyl amide)-3-cyclobutene-1,2-dione (12b). Compound **12b** (0.0106 g, 0.026 mmol, 11%) was prepared from **8** (0.038 g, 0.24 mmol) and **11b** (0.068 g, 0.24 mmol) as a white solid according to the general procedure. ^{1}H NMR (300 MHz, CD_3OD) δ 4.56 (d, J = 9 Hz, 1 H), 4.14 (d, J = 6 Hz, 1 H), 3.49 (s, 3 H), 2.74 (s, 3 H), 1.92 (m, 1 H), 1.67 (m, 7 H), 1.22 (m, 5 H), 0.94 (m, 7 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 183.8, 182.7, 171.7, 171.1, 169.1, 168.1, 61.3, 58.4, 40.7, 39.2, 39.1, 31.1, 30.0, 29.2, 26.9, 26.8, 26.7, 24.4, 15.8, 12.0; HRMS-FAB (M + Na^+) calcd for $C_{20}H_{32}N_4NaO_5$ 431.2270, found 431.2261.

3-(Hydroxymethylamino)-4-(L-isoleucyl-*O*-methyl-L-tyrosine methyl amide)-3-cyclobutene-1,2-dione (12c). Compound **12c** (0.054 g, 0.12 mmol, 76%) was prepared from **8** (0.032 g, 0.16 mmol) and **11c** (0.051 g, 0.16 mmol) according to the general procedure. ^{1}H NMR (400 MHz, CD_3OD) δ 7.11 (d, J = 8.5 Hz, 2 H), 6.77 (d, J = 8.6 Hz, 2 H), 4.60 (dd, J = 9.6, 5.5 Hz, 1 H), 4.41 (d, J = 8.7 Hz, 1 H), 3.75 (s, 3 H), 3.49 (s, 3 H), 3.06 (dd, J = 13.8, 5.5 Hz, 1 H), 2.81 (dd, J = 13.9, 9.7 Hz, 1 H), 2.73 (s, 3 H), 1.87 (m, 1 H), 1.53 (m, 1 H), 1.18 (m, 1 H), 0.90 (m, 6 H); ^{13}C NMR (100 MHz, CD_3OD) δ 179.1, 178.5, 172.4, 171.3, 165.9, 165.7, 158.6, 129.8, 128.6, 113.4, 62.0, 54.5, 54.2, 40.0, 37.0, 36.7, 24.9, 24.2, 14.1, 9.5; HRMS-FAB (M + Na^+) calcd for $C_{22}H_{30}N_4O_6Na$ 469.2063, found 469.2078.

3-(Hydroxymethylamino)-4-(L-isoleucyl-L-phenylalanine methyl amide)-3-cyclobutene-1,2-dione (12d). Compound **12d** (0.224 g, 0.54 mmol, 75%) was prepared from **8** (0.113 g, 0.72 mmol) and **11d** (0.210 g, 0.72 mmol) as a white

solid according to the general procedure. ^{1}H NMR (300 MHz, CD_3OD) δ 7.21 (m, 5 H), 4.63 (dd, J = 9.6, 9.3 Hz, 1 H), 4.43 (d, J = 8.6 Hz, 1H) 3.49 (s, 3 H), 3.12 (dd, J = 6.3, 5.3 Hz, 1 H), 2.88 (dd, J = 9.7, 9.3 Hz, 1 H), 2.71 (s, 3 H), 1.86 (m, 1 H), 1.52 (m, 1 H), 1.16 (m, 1 H), 0.90 (m, 6 H); ^{13}C NMR (75 MHz, CD_3OD) δ 179.6, 178.9, 172.7, 171.7, 166.4, 166.2, 137.2, 129.2, 128.4, 126.7, 62.3, 54.9, 40.4, 38.1, 37.3, 25.4, 24.5, 14.5, 9.92; HRMS-FAB (M + Na^+) calcd for $C_{21}H_{28}N_4NaO_5$ 439.1957, found 439.1968.

3-(Hydroxymethylamino)-4-(L-isoleucyl-L-phenylglycine methyl amide)-3-cyclobutene-1,2-dione (12e). Compound **12e** (0.021 g, 0.053 mmol, 65%) was prepared from **8** (0.016 g, 0.08 mmol) and **11e** (0.023 g, 0.083 mmol) according to the general procedure. ^{1}H NMR (300 MHz, CD_3OD) δ 7.42 (m, 2 H), 7.35 (m, 3 H), 5.42 (s, 1 H), 4.6 (d, J = 6 Hz, 1 H), 3.47 (s, 3 H), 2.73 (s, 3 H), 1.96 (m, 1 H), 1.63 (m, 1 H), 1.26 (m, 1 H), 1.0 (m, 6 H); ^{13}C NMR (75 MHz, CD_3OD) δ 179.1, 178.5, 171.1, 171.0, 166.1, 165.9, 137.3, 128.4, 128.0, 127.2, 61.8, 57.3, 40.0, 37.7, 25.0, 24.2, 14.1, 9.8; HRMS-FAB (M + Na^+) calcd for $C_{20}H_{26}N_4O_5Na$ 425.1801, found 425.1810.

3-(Hydroxymethylamino)-4-(L-isoleucyl-L-tryptophan methyl amide)-3-cyclobutene-1,2-dione (12f). Compound **12f** (0.057 g, 0.125 mmol, 78%) was prepared from **8** (0.032 g, 0.16 mmol) and **11f** (0.053 g, 0.16 mmol) according to the general procedure. ^{1}H NMR (400 MHz, CD_3OD) δ 7.57 (d, J = 7.8 Hz, 1 H), 7.31 (d, J = 8.1 Hz, 1 H), 7.07 (m, 2 H), 6.99 (m, 1 H), 4.66 (dd, J = 8.1, 6.4 Hz, 1 H), 4.46 (d, J = 8.2 Hz, 1 H), 3.47 (s, 3 H), 3.25 (dd, J = 14.6, 6.3 Hz, 1 H), 3.11 (dd, J = 14.5, 8.1 Hz, 1 H), 2.67 (s, 3 H), 1.86 (m, 1 H), 1.50 (m, 1 H), 1.13 (m, 1 H), 0.88 (m, 6 H); ^{13}C NMR (100 MHz, CD_3OD) δ 179.1, 178.5, 172.8, 171.3, 166.0, 165.8, 136.6, 127.3, 123.3, 120.9, 118.4, 117.9, 110.9, 109.3, 62.0, 54.3, 40.0, 37.3, 27.8, 25.0, 24.1, 14.1, 9.7; HRMS-FAB (M + Na^+) calcd for $C_{23}H_{29}N_5O_5Na$ 478.2066, found 478.2060.

3-(Hydroxymethylamino)-4-(L-isoleucyl-L-leucine methyl amide)-3-cyclobutene-1,2-dione (12 g). Compound **12g** (0.051 g, 0.133 mmol, 82%) was prepared from **8** (0.032 g, 0.16 mmol) and **11g** (0.042 g, 0.163 mmol) according to the general procedure. ^{1}H NMR (400 MHz, CD_3OD) δ 4.57 (d, J = 8.1 Hz, 1 H), 4.41 (dd, J = 9.4, 5.4 Hz, 1 H), 3.50 (s, 3 H), 2.74 (s, 3 H), 1.96 (m, 1 H), 1.63 (m, 3 H), 1.53 (m, 1 H), 1.22 (m, 1 H), 0.94 (m, 12 H); ^{13}C NMR (100 MHz, CD_3OD) δ 179.1, 178.6, 173.5, 171.6, 166.3, 165.9, 61.9, 51.9, 40.6, 40.1, 37.6, 25.0, 24.5, 24.3, 22.0, 20.7, 14.3, 9.9; HRMS-FAB (M + H^+) calcd for $C_{18}H_{31}N_4O_5$ 383.2295, found 383.2304.

3-(Hydroxymethylamino)-4-(L-leucyl-L-*tert*-leucine methyl amide)-3-cyclobutene-1,2-dione (12h). Compound **12h** (0.028 g, 0.074 mmol, 74%) was prepared from **8** (0.02 g, 0.1 mmol) and **11h** (0.026 g, 0.1 mmol) according to the general procedure. ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.76 (br s, 1 H), 4.2 (d, J = 9.6 Hz, 1 H), 3.38 (s, 3 H), 2.57 (d, J = 4.4 Hz, 3 H), 1.76 (m, 1 H), 1.62 (m, 1 H), 1.51 (m, 1 H), 0.87 (s, 15 H); ^{13}C NMR (75 MHz, CD_3OD) δ 179.8, 178.9, 172.7, 171.9, 166.5, 166.3, 61.3, 56.6, 41.1, 40.4, 34.4, 26.1, 25.0, 24.9, 22.5, 20.8; HRMS-FAB (M + Na^+) calcd for $C_{18}H_{30}N_4O_5Na$ 405.2114, found 405.2105.

3-(L-Leucyl-L-*tert*-leucine methyl amide)-4-methoxy-3-cyclobutene-1,2-dione (13). To a stirred solution of **2** (0.021 g, 0.15 mmol) in MeOH was added **11h** (0.042 g, 0.165 mmol) and the mixture was heated at reflux overnight. The solvent was evaporated and the crude product was purified by column chromatography (3:97 MeOH/CH_2Cl_2) giving compound **13** (0.052 g, 0.136 mmol, 95%). ^{1}H NMR (300 MHz, CD_3OD) δ 4.40 (s, 4 H), 4.25 (d, J = 9 Hz, 1 H), 2.74 (s, 3 H), 1.68 (m, 3 H), 0.99 (d, J = 6 Hz, 15 H); ^{13}C NMR (75 MHz, CD_3OD) δ 188.7, 187.9, 184.2, 183.7, 178.0, 176.9, 173.1, 172.6, 172.0, 171.9, 171.4, 60.8, 60.7, 59.9, 56.8, 56.1, 25.6, 24.6, 24.5, 22.1, 20.0; HRMS-ESI (M + Na^+) calcd for $C_{18}H_{29}N_3O_5Na$ 390.2005, found 390.2010.

3-Hydroxy-4-(L-leucyl-L-*tert*-leucine methyl amide)-3-cyclobutene-1,2-dione (14). To a stirred solution of **13** (0.05 g, 0.136 mmol) in MeOH (3 mL) was added 0.15 N HCl (1 mL) and the mixture was heated at reflux overnight. The solvent was evaporated and the crude product was purified by column chromatography (3:97 MeOH/CH_2Cl_2) giving **14** (0.032 g, 0.091 mmol, 67%). ^{1}H NMR (300 MHz, CD_3OD) δ 4.22 (s, 1 H), 3.32 (s, 1 H), 2.74 (s, 3 H), 1.69 (m, 3 H), 0.96 (s, 15 H); ^{13}C NMR (75 MHz, CD_3OD) δ 196.3, 187.8, 180.7, 172.5, 171.9, 61.3, 55.8, 40.6, 34.5, 26.1, 25.1, 24.8, 22.5, 20.9; HRMS-ESI (M − H^+) calcd for $C_{17}H_{26}N_3O_5$ 352.1872, found 352.1880.

3-(Hydroxymethylamino)-2-(L-isoleucine methyl ester)-4-thioxo-2-cyclobuten-1-one (15). Compound **6a** (0.100 g, 0.370 mmol) was dissolved in 2 mL of CH_2Cl_2 and the solution was allowed to stir while Lawesson's reagent (0.150 g, 0.370 mmol) was added. The reaction was monitored by TLC until all the starting material had been consumed. The solvent was then evaporated and the crude material was purified by column chromatography (5:95 MeOH/CH_2Cl_2) providing **15** as a yellow solid (0.062 g, 0.23 mmol, 62%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.02 (br s, 1 H), 8.19 (br s, 1 H), 5.93 (br s, 1 H), 3.76 (s, 3 H), 3.67 (s, 3 H), 2.20 (m, 1 H), 2.07 (m, 1 H), 1.48 (m, 1 H), 1.26 (m, 1 H), 1.00 (d, J = 6.8 Hz, 3 H), 0.93 (t, J = 7.3 Hz, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 205.4, 201.6, 170.9, 170.8, 170.6, 61.2, 52.9, 39.4, 31.6, 25.3, 15.8, 12.2; ESI-MS (M + H^+) calcd for $C_{12}H_{19}N_2O_3S_2$ 271.1294, found 271.1287.

3-(Hydroxymethylamino)-2-(L-isoleucyl-L-phenylalanine methyl amide)-4-thioxo-2-cyclobuten-1-one (16). Compound **16** (0.093 g, 0.216 mmol, 71%) was prepared as a yellow solid according to the procedure used to prepare **15** starting from **12d** (0.127 g, 0.305 mmol) and Lawesson's reagent (0.123 g, 0.304 mmol). The crude material was purified by column chromatography (3:97 MeOH/CH_2Cl_2). ^{1}H NMR (300 MHz, CD_3OD) 7.15 (m, 5 H), 5.56 (d, J = 7.6 Hz, 1 H), 4.90 (s, 3 H), 4.68 (m, 1 H), 3.63 (s, 2 H), 3.10 (m, 1 H), 2.72 (d, J = 0.6 Hz, 3 H), 1.91 (m, 1 H), 1.49 (m, 1 H), 1.17 (m, 1 H), 0.87 (m, 6 H); ^{13}C NMR (75 MHz, CD_3OD) δ 206.7, 204.0, 172.7, 171.1, 170.7, 170.4, 137.1, 129.3, 128.4, 126.5, 60.8, 54.9, 38.3, 37.6, 30.4, 25.5, 24.4, 14.5, 10.3; ESI-MS (M + Na^+) calcd for $C_{21}H_{28}N_4O_4SNa$ 455.1729, found 455.1738.

3-(Hydroxymethylamino)-2-(L-isoleucyl-L-leucine methyl amide)-4-thioxo-2-cyclobuten-1-one (17). Compound **17** was prepared as a yellow solid (0.013 g, 0.032 mmol, 59%) from **12g** (0.021 g, 0.055 mmol) and Lawesson's reagent (0.022 g, 0.055 mmol) according to the procedure used to prepare compound **15**. The crude material was purified by column chromatography (1.3:98.7 MeOH/CH_2Cl_2). ^{1}H NMR (300 MHz, CD_3OD) δ 5.67 (d, J = 6.8 Hz, 1 H), 4.39 (dd, J = 9.5, 5.2 Hz, 1 H), 3.63 (s, 3 H), 2.73 (s, 3 H), 2.00 (m, 1 H), 1.56 (m, 4 H), 1.24 (m, 1 H), 0.96 (m, 12 H); ^{13}C NMR (75 MHz, CD_3OD) δ 206.6, 203.9, 173.5, 171.2, 170.9, 170.2, 60.5, 51.9, 40.6, 37.9, 29.9, 24.9, 24.5, 23.9, 22.0, 20.5, 14.2, 10.2; HRMS-FAB (M + Na^+) calcd for $C_{18}H_{30}N_4O_4SNa$ 421.1885, found 421.1880.

3-(Hydroxymethylamino)-2-(L-leucyl-L-*tert*-leucine methyl amide)-4-thioxo-2-cyclobuten-1-one (18). Compound **18** was prepared as a yellow solid (0.014 g, 0.035 mmol, 41%) from **12h** (0.033 g, 0.086 mmol) and Lawesson's reagent (0.035 g, 0.086 mmol) according to the procedure used to prepare compound **15**. The crude material was purified by column chromatography (1.3:98.7 MeOH/CH_2Cl_2). ^{1}H NMR (400 MHz, CD_3OD) δ 5.92 (s, 1 H), 4.21 (s, 1 H), 3.62 (s, 3 H), 2.74 (s, 3 H), 1.72 (m, 3 H), 0.98 (m, 15 H); ^{13}C NMR (100 MHz, CD_3OD) δ 206.1, 203.5, 171.7, 171.4, 170.7, 170.0, 61.2, 54.8, 40.7, 34.2, 30.2, 25.9, 24.8, 24.5, 22.2, 20.6; LRMS-FAB (M + Na^+) calcd for $C_{18}H_{30}N_4O_4SNa$ 421.0, found 421.1.

3,4-Di(L-phenylalanine methyl ester)-3-cyclobutene-1,2-dione (19). To a stirred solution of l-phenylalanine methyl ester HCl salt (0.646 g, 3.0 mmol) in MeOH (10 mL) were added KOH (0.168 g, 3.0 mmol) and compound **7** (0.256 g, 1.13 mmol). Formation of a white precipitate was observed. The mixture was stirred for 3 h at room temperature, the solvent was evaporated, EtOAc was added, and the solution was washed with H_2O. The organic layer was dried over $MgSO_4$, and the solvent was evaporated. The crude product was recrystallized from MeOH. The resulting white crystals were

washed with cold MeOH and dried in vacuo to give **19** as a white solid (0.363 g, 0.831 mmol, 74%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.91 (br s, 1 H), 7.27 (m, 6 H), 7.14 (d, J = 6.4 Hz, 4 H), 5.00 (d, J = 6.1 Hz, 2 H), 3.70 (s, 6 H), 3.17 (dd, J = 13.5, 4.9 Hz, 2 H), 3.06 (m, 2 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 183.3, 171.6, 167.5, 136.4, 129.8, 128.9, 127.3, 57.2, 55.3, 52.8; HRMS-ESI (M + H$^+$) calcd for $C_{24}H_{25}N_2O_6$ 437.1713, found 437.1722.

2,3-Bis(L-phenylalanine methyl ester)-4-thioxo-2-cyclobuten-1-one (20). To a stirred solution of **19** (0.112 g, 0.257 mmol) in dry CH_2Cl_2 (5 mL) was added Lawesson's reagent (0.021 g, 0.052 mmol). After TLC analysis indicated that the reaction was complete, the crude product was purified by column chromatography (gradient of 0.25−1.0% MeOH/CH_2Cl_2) to yield compound **20** as a yellow solid (0.038 g, 0.084 mmol, 40%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.76 (d, J = 7.9 Hz, 1 H), 8.31 (d, J = 9.1 Hz, 1 H), 7.21 (m, 10 H), 6.12 (dd, J = 15.0, 6.3 Hz, 1 H), 5.01 (dd, J = 12.7, 6.7 Hz, 1 H), 3.73 (s, 3 H), 3.70 (s, 3 H), 3.18 (m, 4 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 204.0, 180.4, 172.0, 171.4, 170.8, 168.9, 136.0, 129.8, 129.7, 129.0, 128.9, 127.5, 127.4, 58.1, 55.8, 53.1, 52.9; HRMS-ESI (M + H$^+$) calcd for $C_{24}H_{25}N_2O_5S$ 453.1484, found 453.1490.

3-(Hydroxymethylamino)-2-(L-phenylalanine methyl ester)-4-thioxo-2-cyclobuten-1-one (21). To a stirred solution of **6e** (0.171 g, 0.562 mmol) in dry CH_2Cl_2 (5 mL) was added Lawesson's reagent (0.227 g, 0.561 mmol). After TLC analysis indicated that the reaction was complete, the solvent was evaporated and the crude product was purified by column chromatography (CH_2Cl_2) to yield compound **21** as a yellow solid (0.104 g, 0.325 mmol, 58%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.87 (br s, 1 H), 8.56 (br s, 1 H), 7.24 (m, 5 H), 6.23 (t, J = 6.0 Hz, 1 H), 3.72 (s, 3 H), 3.50 (s, 3 H), 3.21 (m, 2 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 206.5, 203.2, 171.0, 170.7, 170.6, 135.7, 129.9, 129.0, 127.5, 56.3, 53.1, 30.8; HRMS-FAB (M + Na$^+$) calcd for $C_{15}H_{16}N_2O_4SNa$ 343.0729, found 343.0740.

Procedure for the Assay of MMP-1. MMP-1, prepared from a culture medium of human rheumatoid synovial fibroblasts, was obtained from Calbiochem. The assays were based on the enzymatic hydrolysis of the peptide substrate Dnp-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(Nma)-NH_2,[18] using the procedure described by Le Diguarher.[19] This is a fluorescence quenching assay in which *N*-methylanthranilic acid (Nma) is the fluorophore and dinitrophenyl (Dnp) is the quencher.

Pro-MMP-1 was dissolved in assay buffer (200 mM NaCl, 50 mM Tris, 5 mM $CaCl_2$, 20 μM $ZnSO_4$, 0.05% Brij 35, pH 7.6) at a concentration of 1.25 μg/mL. APMA solution (*p*-aminophenylmercuric acetate, 2 mM in 0.1 N NaOH) was prepared. Proenzyme activation was performed by mixing the proenzyme solution and the APMA solution in a 10:1 ratio. This reaction was incubated at 37 °C for 30 min and subsequently transferred to ice and then to a freezer. The substrate was dissolved in DMSO at a concentration of 2 mM, and then diluted to 0.2 mM with H_2O. Inhibitors were dissolved in a 10:90 DMSO/buffer solution. Fluorescence measurements were performed in 96-well plates by incubating assay buffer (76 μL), activated enzyme (4 μL), and the inhibitor solution (or buffer for the blank) (10 μL) at 37 °C for 30 min and then adding the substrate (10 μL) to the wells. The change in fluorescence was measured with use of excitation and emission wavelengths of 340 and 460 nm, respectively. IC_{50} values were calculated with the commercial graphing package Grafit (Erithacus Software Ltd.). Data were obtained for assays with at least five different concentrations, in duplicate, of each inhibitor.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra for all new compounds; Lineweaver−Burk plot for compound **16**. This material is available free of charge via the Internet at http://pubs.acs.org.

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