Structure-Based Design, Synthesis, and Biological Evaluation of Irreversible Human Rhinovirus 3C Protease Inhibitors. 4. Incorporation of P_1 Lactam Moieties as L-Glutamine Replacements

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The structure-based design, chemical synthesis, and biological evaluation of various human rhinovirus (HRV) 3C protease (3CP) inhibitors which incorporate P_1 lactam moieties in lieu of an L-glutamine residue are described. These compounds are comprised of a tripeptidyl or peptidomimetic binding determinant and an ethyl propenoate Michael acceptor moiety which forms an irreversible covalent adduct with the active site cysteine residue of the 3C enzyme. The P_1-lactam-containing inhibitors display significantly increased 3CP inhibition activity along with improved antirhinoviral properties relative to corresponding L-glutamine-derived molecules. In addition, several lactam-containing compounds exhibit excellent selectivity for HRV 3CP over several other serine and cysteine proteases and are not appreciably degraded by a variety of biological agents. One of the most potent inhibitors (AG7088, mean antirhinoviral $EC_{90} \approx 0.10\ \mu M$, $n = 46$ serotypes) is shown to warrant additional preclinical development to explore its potential for use as an antirhinoviral agent.

Introduction

The human rhinoviruses (HRVs) are members of the picornavirus family and are the single most significant cause of the common cold.[1,2] We recently described the design and development of substrate-derived[3,4] tripeptidyl HRV 3C protease (3CP) inhibitors which incorporate C-terminal Michael acceptor moieties (e.g., compound **1**, Figure 1, Table 1).[5−7] These compounds irreversibly inhibit 3CP by forming a covalent adduct with the active site cysteine residue of the enzyme and exhibit antirhinoviral activity in cell culture. In addition, the preceding paper describes the modification of such molecules by the introduction of a P_2-P_3 ketomethylene dipeptide isostere into the inhibitor design.[8] The resulting peptidomimetic compounds display substantially improved in vitro antiviral properties relative to the corresponding peptide-derived molecules. In an effort to further modify the structure of the initially studied inhibitor series, we sought to alter amide moieties other than that linking the P_2-P_3 amino acid residues of **1** without significantly interfering with 3CP recognition. The results of our efforts to derivatize the primary amide present in the P_1 Gln[9] side chain of **1** are described below.

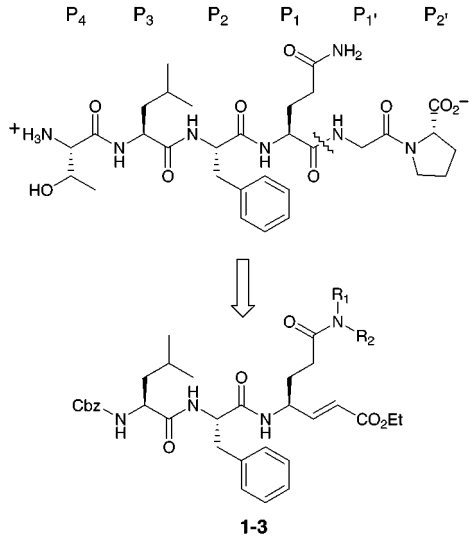

Figure 1. Design of HRV 3CP inhibitors.

Table 1

compd no.	R_1	R_2	formula[a]	serotype[b]	$k_{obs}/[I]$ ($M^{-1}\ s^{-1}$)[c]	EC_{50} (μM)[c]	CC_{50} (μM)[c]
1	H	H	$C_{32}H_{42}N_4O_7$		25 000	0.54	>320
				2	2 000	1.6	
2	CH_3	H	$C_{33}H_{44}N_4O_7$		750	5.6	>100
3	CH_3	CH_3	$C_{34}H_{46}N_4O_7$		60	4.0	>100

[a] Elemental analyses (C, H, N) of all compounds agreed to within ±0.4% of theoretical values. [b] Serotype-14 unless otherwise noted. [c] See ref 5 for assay method and error.

Inhibitor Design and Structure−Activity Studies

Analysis of the HRV-2 3CP-**1** X-ray crystal structure[5] indicated that only the trans P_1 Gln amide hydrogen atom interacted with the protease while the cis NH was solvent-exposed (Figure 2). Thus, selective alkylation of the latter was envisioned to allow for amide modification without adversely affecting 3CP inhibition properties. However, a molecule containing a monomethylated P_1 glutamine moiety (**2**)[6] displayed drastically reduced 3CP inhibition activity relative to the nonalkylated inhibitor **1** (Figure 1, Table 1).[10] A comparison of **1**, **2**, and the dimethylated analogue **3**[6] (Figure 1, Table 1) suggested

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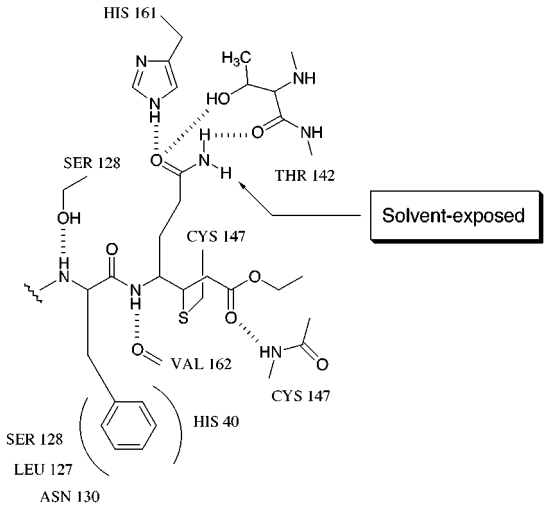


Figure 2. Schematic diagram of **1** bound in the HRV-2 3CP active site.[5] Hydrogen bonds are represented as dashed lines, and the residues which make up the enzyme binding subsites are depicted.

that the poor anti-3CP activity exhibited by **2** was primarily attributable to an unfavorable equilibrium in which the trans methyl amide isomer predominated over the desired cis isomer (Figure 3).[11] We therefore sought to enforce the cis amide geometry by the incorporation of a P_1 lactam moiety into the inhibitor design. Modeling studies (see below) utilizing the HRV-2 3CP-**1** crystal structure[5] suggested that such a lactam moiety could be accommodated in the 3CP S_1 binding pocket and predicted the (*S*)-stereochemistry at the lactam α-carbon to be the most favorable for effective 3CP recognition (Figure 3).

In the event, the (*S*)-*γ*-lactam-containing compound **4** displayed dramatically improved inhibitory activity against 3CPs from several rhinovirus serotypes relative to the corresponding glutamine-derived molecule **1** (Tables 1 and 2). Equally important, inhibitor **4** also exhibited improved antiviral properties relative to **1** and was not cytotoxic in cell culture to the limits of its solubility.[13] The corresponding (*S*)-*δ*-lactam-containing inhibitor **5** also displayed significantly improved anti-3CP and antiviral activity relative to **1**, while a molecule incorporating an (*R*)-*γ*-lactam (**6**) exhibited reduced 3CP inhibition properties in accordance with computational predictions (see below, Table 2). A similar reduction in anti-3CP activity was obtained by incorporation of a cyclic urea moiety into the inhibitor design (**7**). The poor inhibition properties of **7** relative to **4** paralleled reductions in anti-3CP activity observed previously with inhibitors containing acyclic P_1 urea functionalities.[6]

The promising antirhinoviral activity exhibited by the (*S*)-*γ*- and (*S*)-*δ*-lactam-containing Cbz-Leu-Phe-derived compounds prompted the incorporation of these lactam entities into other 3CP inhibitors. Thus, substitution of a P_3 Val amino acid residue in place of Leu (**8**, Table 2) significantly improved anti-3CP activity in direct analogy to structure−activity relationships observed for related glutamine-containing 3CP inhibitors.[6] This result indicated that the P_1 lactam-containing compounds bound to 3CP in a manner similar to that observed for the corresponding glutamine-derived molecules and suggested that other beneficial modifications of the latter series might improve the former as well.[14]

Accordingly, the P_1 lactam moiety was incorporated into tripeptidyl molecules containing a P_3 Val amino acid residue and an N-terminal amide derived from 5-methylisoxazole-3-carboxylic acid (**9** and **10**, Table 3).[15] The resulting compounds rapidly and irreversibly inhibited HRV-14 3CP and displayed very potent antiviral properties in cell culture. Inclusion of a Val-Phe ketomethylene dipeptide isostere[8] in the inhibitor design resulted in slightly diminished anti-3CP activity but further improved antirhinoviral properties in cell culture (compare **9** with **11** and **10** with **12**, Table 3). Similar activity differences between ketomethylene- and peptide-derived 3CP inhibitors were previously observed during the examination of non-P_1-lactam-containing molecules.[8] In addition to the compounds described above, a tripeptide-derived inhibitor which contained a P_3 tBuGly residue also exhibited impressive anti-3CP and antiviral properties (compound **13**, Table 3).

Two of the most active antiviral agents were also examined against multiple rhinovirus serotypes in cell culture (Table 4). The tripeptide-derived inhibitor **9** exhibited potent EC_{90} antiviral activity against all serotypes tested.[16] As anticipated from previous studies,[8] the ketomethylene-containing molecule **11** displayed more potent EC_{90} antiviral properties than the peptide **9**. This improvement, however, was not nearly as dramatic as that observed when examining P_1 glutamine-derived peptidyl and peptidomimetic 3CP inhibitors.[8] A comparison of the lactam-containing molecule **11** with its glutamine analogue **14**[8] indicated that incorporation of the P_1 lactam moiety into the inhibitor design improved EC_{90} antiviral activity by a factor of between 8 and 16 (depending on HRV serotype). Similar improvements in antiviral activity were noted when comparing other lactam- and glutamine-containing molecules (data not shown). Compounds **9** and **11** rank among the most potent known antirhinoviral agents, and they compare favorably with the capsid-binding molecules Pirodavir[17] and Pleconaril[18] (Table 4).

Due to the encouraging antiviral activity exhibited by the compounds depicted in Table 3, several of their other biological properties were examined in anticipation of possible preclinical development efforts. One such property was the ability of the molecules to selectively inhibit HRV 3CP without affecting other proteolytic enzymes. None of the Michael acceptor-containing compounds examined in this study appreciably inhibited a variety of serine proteases when incubated at relatively high concentrations for 10−15 min (Table 5). However, measurable inhibition of the cysteine protease cathepsin B by the tripeptidyl molecule **1** was observed under the above assay conditions. In contrast, the more potent antirhinoviral agents described in this study (**9** and **11**) did not affect any of the nonrhinoviral cysteine proteases examined, suggesting that optimization of their 3CP inhibition properties also led to improvements in enzyme selectivity.

In addition to the selectivity studies described above, the stability of many 3CP inhibitors toward several biologically relevant agents was also examined. As was the case with previously examined irreversible 3CP inhibitors,[5] the ethyl propenoate Michael acceptor was employed extensively in the current work due to its ease

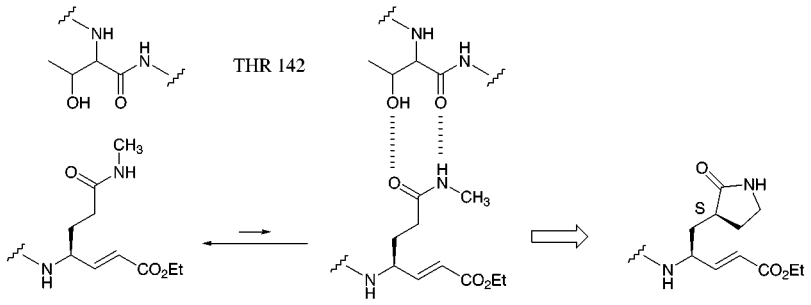
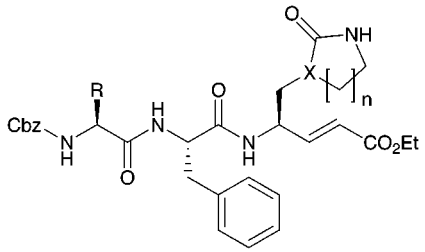


Figure 3. Design of P_1-lactam-containing HRV 3CP inhibitors.

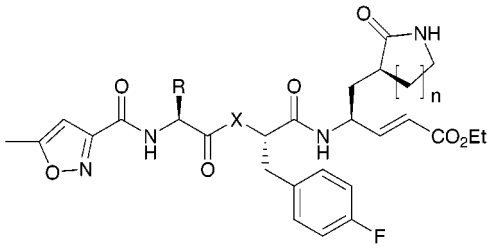
Table 2



compd no.	R	X	*n*	formula[a]	serotype[b]	k_{obs}/[I] (M^{-1} s^{-1})[c]	EC_{50} (μM)[c]	CC_{50} (μM)[c]
4	$CH_2CH(CH_3)_2$	(*S*)-CH	1	$C_{34}H_{44}N_4O_7 \cdot 0.50H_2O$		257 000	0.10	>100
					2	31 000	ND	
5	$CH_2CH(CH_3)_2$	(*S*)-CH	2	$C_{35}H_{46}N_4O_7 \cdot 0.50H_2O$		239 000	0.03	>100
6	$CH_2CH(CH_3)_2$	(*R*)-CH	1	$C_{34}H_{44}N_4O_7$		18 000	1.6	>100
7	$CH_2CH(CH_3)_2$	N	1	$C_{33}H_{43}N_5O_7$		10 900	0.60	>100
8	$CH(CH_3)_2$	(*S*)-CH	1	$C_{33}H_{42}N_4O_7$		500 000	0.03	>100

[a] Elemental analyses (C, H, N) of all compounds agreed to within ±0.4% of theoretical values. [b] Serotype-14 unless otherwise noted. [c] See ref 5 for assay method and error. ND = not determined.

Table 3



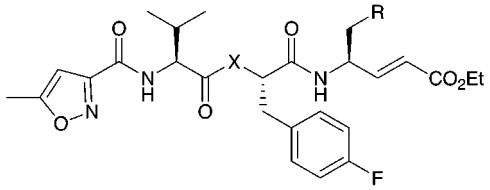
compd no.	R	X	*n*	formula[a]	k_{obs}/[I] (M^{-1} s^{-1})[b,c]	EC_{50} (μM)[b,c]	CC_{50} (μM)[c]
9	$CH(CH_3)_2$	NH	1	$C_{30}H_{38}FN_5O_7$	1 500 000	0.01	>100
10	$CH(CH_3)_2$	NH	2	$C_{31}H_{40}FN_5O_7$	900 000	0.02	>100
11	$CH(CH_3)_2$	CH_2	1	$C_{31}H_{39}FN_4O_7$	1 090 000	0.005	>100
12	$CH(CH_3)_2$	CH_2	2	$C_{32}H_{41}FN_4O_7$	500 000	0.001	>100
13	$C(CH_3)_3$	NH	1	$C_{31}H_{40}FN_5O_7$	980 000	0.01	>100

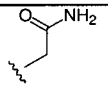
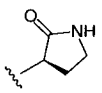
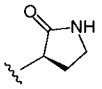
[a] Elemental analyses (C, H, N) of all compounds agreed to within ±0.4% of theoretical values. [b] Serotype-14. [c] See ref 5 for assay method and error.

of preparation. However, a concern existed that this moiety might undergo facile in vivo metabolism, thereby compromising the usefulness of molecules in which it was incorporated.[19] As expected based on previous studies,[5] inhibitor **11** was rapidly degraded in rat plasma, presumably due to hydrolysis of the α,β-unsaturated ethyl ester present in the molecule (Table 6).[20] However, the compound was quite stable toward human and dog plasmas and exhibited negligible degradation in the presence of hydrolytic enzymes such as chymotrypsin and peptidase. In addition, the molecule was unaffected by prolonged exposure to high concentrations of dithiothreitol (5 mM), suggesting that the inhibitor did not react readily with nonenzymatic thiols. Similar stability toward the various biological agents listed (and instability toward rat plasma) was noted for many other 3CP inhibitors related to the molecules described in this study (data not shown). Although the studies described above do not address every aspect of in vivo metabolism, they do examine some of the more obvious degradation events that might affect the compounds detailed in this work.

The extremely potent antirhinoviral activity displayed by compound **11**, along with its excellent protease selectivity, low cytotoxicity in cell culture, and stability toward various biological agents prompted its selection

Table 4. Antirhinoviral Activity of Selected 3CP Inhibitors against Several HRV Serotypes



Compd No.	R	X	EC_{90} vs Rhinovirus Serotype (μM)[a]			
			HRV-14	HRV-1A	HRV-2	HRV-10
14		CH_2	0.32	0.63	0.28	0.79
11		CH_2	0.020	0.041	0.030	0.10
9		NH	0.040	0.14	0.050	0.14
Pirodavir[b]	-	-	0.13	0.95	0.035	0.060
Pleconaril[c]	-	-	0.16	1.4	0.050	0.30

[a] See ref 5 for assay method and error. [b] Provided by Janssen Pharmaceuticals. [c] Prepared as described in ref 18.

Table 5. Inhibition of Serine and Cysteine Proteases by Selected Antirhinoviral Agents

compd no.[b]	% activity remaining after 10−15 min[a]					
	thrombin	chymotrypsin	trypsin	elastase	cathepsin B	calpain
1[c]	101	96	98	92	39	100
9	103	101	100	100	96	100
11	100	100	100	99	98	100
13	101	97	95	96	81	100
DMSO	100	100	100	100	100	100

[a] Each enzyme was present in the appropriate assay at ∼10 nM. [b] Tested at 10 μM unless otherwise noted. [c] Tested at 2 μM.

Table 6. Stability of Compound **11** toward Various Biological Agents

biological agent[a]	half-life (min)
rat plasma	<2
dog plasma	>60
human plasma	>60
chymotrypsin	>60
peptidase	>60
dithiothreitol (5 mM)	>30

[a] See the Experimental Section for assay conditions and details.

as a candidate for further development. This molecule (AG7088) is currently undergoing testing to evaluate its possible clinical use as an antirhinoviral agent, and additional data concerning these studies will be disclosed in due course.

Molecular Modeling Studies

Two related computational approaches were utilized to estimate the binding affinities of various lactam-containing molecules to HRV 3CP. The results of both methods are depicted in Table 7 and are compared with experimental values. Each analysis employed lactam model compounds constructed from the previously described HRV-2 3CP-**1** complex.[5] In the first approach, the expected covalent protein−inhibitor complex was examined, while in the second the noncovalent encounter complex between enzyme and ligand was studied.

Table 7. Computational Studies of P_1-Lactam-Containing HRV 3CP Inhibitors

compd no.	k_{obs}/[I] (M^{-1} s^{-1})	relative rate	measured affinity[a] (kcal/mol)	predicted affinity (no. 1)[a,b] (kcal/mol)	predicted affinity (no. 2)[a,c] (kcal/mol)
1	25 000	1	0.0	0.0	0.0
4	257 000	10	−1.4	−2.0	−1.4
5	239 000	9.6	−1.3	−1.7	−1.0
6	18 000	0.72	0.20	0.7	5.9

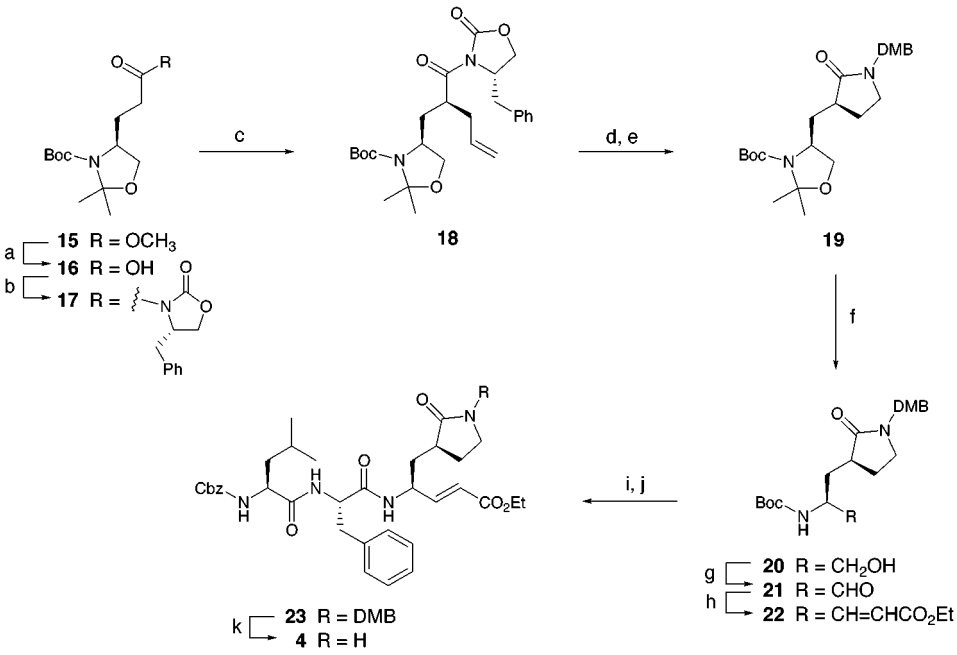
[a] Relative values; assumes changes in k_{obs}/[I] values are representative of changes in ligand-3CP affinity. [b] Calculated using the covalent protein−ligand complex. [c] Calculated using the noncovalent protein−ligand encounter complex. See the Experimental Section for additional details.

The 3CP affinity of compound **1** was calculated in both analyses to serve as a reference, and the latter calculations also included an entropic correction to account for the expected differences in flexibility between lactam- and glutamine-containing molecules.[21] As seen in Table 7, both methods qualitatively predicted the improved anti-3CP activity of (*S*)-lactam containing molecules **4** and **5** relative to glutamine-derived inhibitors such as **1**. Both methods also qualitatively anticipated the reduced 3CP affinity exhibited by the (*R*)-lactam containing compound **6**. Additional details of these molecular modeling studies are included in the Experimental Section.

Synthesis

The lactam-containing compounds utilized in this study were prepared by a variety of methods which incorporate several elements from previous syntheses of related 3CP inhibitors. The preparation of a tripeptidyl (*S*)-γ-lactam-containing inhibitor is illustrated in Scheme 1 by the synthesis of compound **4**. The known methyl ester **15**[22] (derived from L-glutamic acid in four steps) was hydrolyzed under basic conditions to provide the corresponding carboxylic acid (**16**) in quantitative yield. This material was not purified, but was instead coupled with (*S*)-(−)-4-benzyl-2-oxazolidinone to give

Scheme 1[a]



[a] DMB = 2,4-dimethoxybenzyl. Reagents and conditions: (a) 3.0 equiv of NaOH, CH_3OH, 23 °C, 3.5 h, 100%; (b) 3.0 equiv of Et_3N, 1.0 equiv of *t*-BuCOCl, 1.1 equiv of LiCl, 0.95 equiv of (*S*)-(−)-4-benzyl-2-oxazolidinone, THF, 0→23 °C, 19 h, 83%; (c) 1.0 equiv of $NaN(TMS)_2$, 3.0 equiv of allyl iodide, THF, −78→−45 °C, 2 h, 55%; (d) O_3, 2.0 equiv of CH_3OH, CH_2Cl_2, −78 °C, then 10 equiv of Me_2S, 0 °C, 1 h; (e) 4.0 equiv of $HCl \cdot H_2N-$DMB, 4.0 equiv of NaOAc, 2.0 equiv of $NaBH_3CN$, 2:1 THF:EtOH, 23 °C, 18 h; (f) 0.20 equiv of $TsOH \cdot H_2O$, CH_3OH, 50 °C, 2.5 h, 44% from **18**; (g) 3.0 equiv of DMSO, 1.5 equiv of oxalyl chloride, 6.0 equiv of Et_3N, CH_2Cl_2, −78 °C, 1.5 h, 100%; (h) 1.0 equiv of $NaN(TMS)_2$, 1.0 equiv of $(EtO)_2P(O)CH_2CO_2Et$, THF, −78→0 °C, 45 min, 61%; (i) HCl, 1,4-dioxane, 23 °C, 1.5 h; (j) 1.3 equiv of Cbz-L-Leu-L-Phe-OH, 4.0 equiv of NMM, 1.7 equiv of HOBt, 1.7 equiv of EDC, CH_2Cl_2, 23 °C, 20 h, 70%; (k) 4.9 equiv of DDQ, 10:1 $CHCl_3$:H_2O, 50 °C, 8 h, 55%.

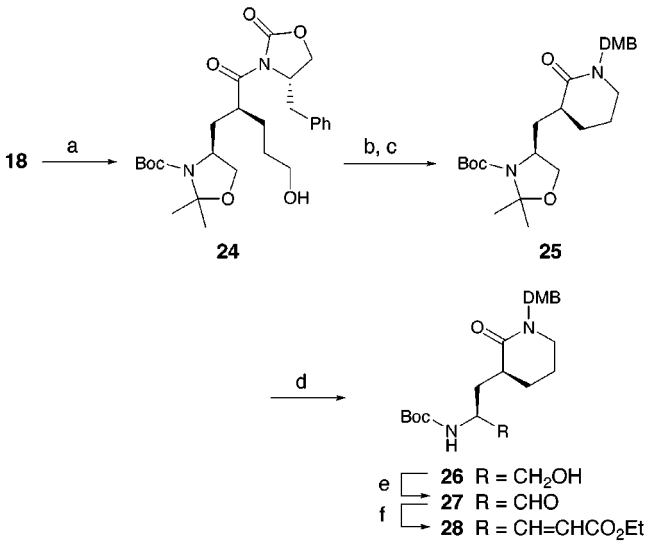
acyl oxazolidinone **17** in good yield.[23] Low-temperature alkylation of the sodium enolate derived from **17** with allyl iodide then provided the allyl derivative **18** after purification on silica gel.[24,25] This material was converted to lactam **19** by ozonolysis and subsequent reductive amination of the resulting aldehyde (not shown) with 2,4-dimethoxybenzylamine. Lactam **19** was difficult to separate completely from contaminating (*S*)-(−)-4-benzyl-2-oxazolidinone, and the mixture of the two was typically hydrolyzed under mildly acidic conditions to provide the more easily purified amino alcohol **20**.

The conversion of **20** into inhibitor **4** then paralleled several previous syntheses of tripeptide-derived Michael acceptors quite closely.[5,6] Thus, Swern oxidation[26] of **20** followed by olefination of the resulting aldehyde (**21**) provided the trans α,β-unsaturated ethyl ester **22** in moderate yield. As was observed previously,[5] only minor amounts (<5%) of the corresponding cis olefin isomer were formed during the conversion of **21** to **22**. Acidic removal (HCl) of the Boc protecting group from **22** and carbodiimide-mediated coupling of the resulting amine hydrochloride salt with commercially available Cbz-L-Leu-L-Phe-OH afforded the tripeptidyl molecule **23** in good overall yield. The 2,4-dimethoxybenzyl moiety was removed from **23** by treatment with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) at elevated temperature to give γ-lactam-containing inhibitor **4** in moderate yield.[27] Alternatively, this deprotection could be accomplished by exposure of **23** to ceric ammonium nitrate (CAN).[28] In both cases, flash column chromatography was necessary to remove impurities generated during the deprotection reaction from the desired inhibitor. The (*R*)-γ-lactam-containing compound **6** was prepared in a manner that was completely analogous to the synthesis of **4** described above, with the exception that (*R*)-(+)-4-benzyl-2-oxazolidinone was employed for coupling with carboxylic acid **16** (data not shown).[29]

The preparation of an intermediate required for the synthesis of inhibitors incorporating a P_1 (*S*)-δ-lactam moiety is illustrated in Scheme 2. Hydroboration of the allyl derivative **18** described in Scheme 1 afforded alcohol **24** in good yield after oxidative workup and chromatographic purification. This material was treated with SO_3·pyridine, and the resulting aldehyde (not shown) was condensed with 2,4-dimethoxybenzylamine utilizing the reductive amination conditions described above for the preparation of compound **19** to give intermediate **25**. As before, **25** was difficult to separate from the (*S*)-(−)-4-benzyl-2-oxazolidinone produced during lactam formation. Impure **25** was therefore hydrolyzed under acidic conditions to give alcohol **26** which could be satisfactorily purified by flash column chromatography. Swern oxidation[26] of **26** and subsequent olefination of the resulting aldehyde (**27**) then afforded α,β-unsaturated ester **28**. This intermediate was converted into inhibitors **5**, **10**, and **12** by methods that were completely analogous to other syntheses reported in this work (cf., Schemes 1, 4, and 5).

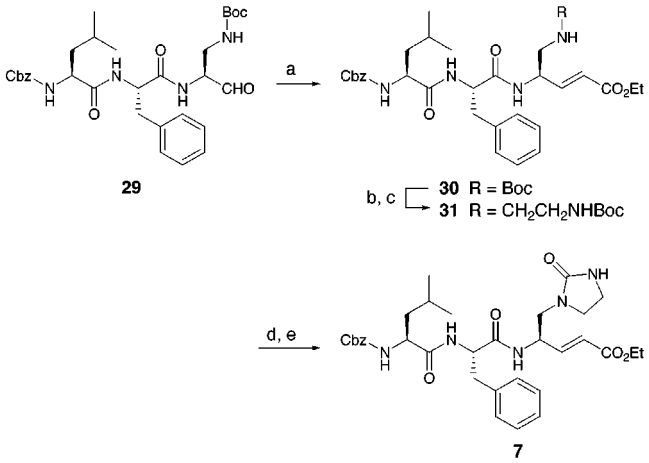
The preparation of the cyclic-urea-containing inhibitor **7** is depicted in Scheme 3. Attempts to synthesize the P_1 intermediate corresponding to **22** and **28** (Schemes 1 and 2) were unsuccessful in the case of the cyclic urea, and an alternate approach to the target compound was therefore utilized. Accordingly, olefination of the known tripeptide **29**[30] uneventfully provided the α,β-unsaturated ester **30**. Removal of the Boc protecting group from **30** and coupling of the resulting amine salt with N-Boc-

Scheme 2[a]



[a] DMB = 2,4-dimethoxybenzyl. Reagents and conditions: (a) 1.0 equiv of BH_3·THF, THF, 0 °C, 30 min, then 1.0 equiv of $NaBO_3$·$4H_2O$, 25 °C, 1 h, 72%; (b) 4.0 equiv of SO_3·pyridine, 3.6 equiv of Et_3N, DMSO, 0→23 °C, 3 h; (c) 4.0 equiv of HCl·H_2N−DMB, 4.0 equiv of NaOAc, 2.0 equiv of $NaBH_3CN$, 2:1 THF:EtOH, 23 °C, 20 h; (d) 0.20 equiv of TsOH·H_2O, CH_3OH, 50 °C, 2.5 h, 44% from **24**; (e) 4.0 equiv of DMSO, 3.0 equiv of oxalyl chloride, 5.0 equiv of Et_3N, CH_2Cl_2, −78 °C, 1.5 h; (f) 1.0 equiv of $NaN(TMS)_2$, 1.0 equiv of $(EtO)_2P(O)CH_2CO_2Et$, THF, −78→0 °C, 45 min, 48% from **26**.

Scheme 3[a]

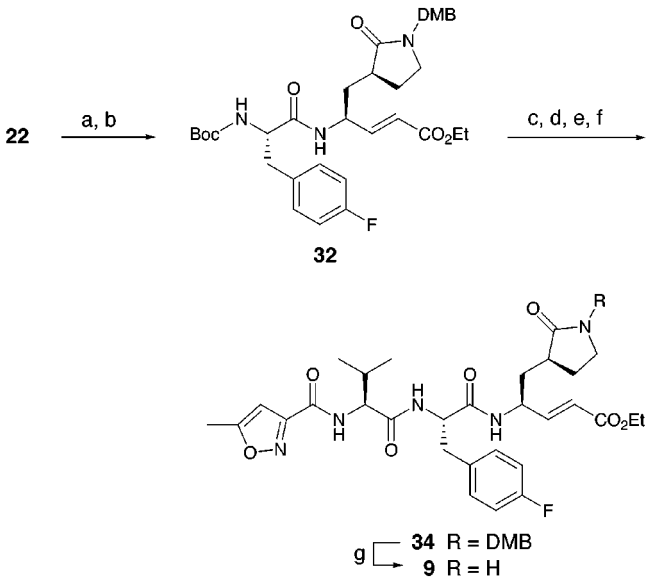


[a] Reagents and conditions: (a) 1.2 equiv of $Ph_3P{=}CHCO_2Et$, THF, 23 °C, 18 h; (b) HCl, 1,4-dioxane, 23 °C, 18 h; (c) 1.1 equiv of $BocNHCH_2CHO$, 1.0 equiv of $NaBH_3CN$, CH_3OH, 23 °C, 18 h, 44%; (d) TFA, CH_2Cl_2, 23 °C, 2 h, 92%; (e) 0.92 equiv of CDI, THF, 23 °C, 3.5 h, 54%.

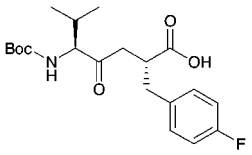
2-aminoethanal under reductive amination conditions provided the protected diamine compound **31** in moderate yield. Boc deprotection (TFA), neutralization of the amine salt thus obtained, and subsequent exposure of the free diamine to 1,1′-carbonyldiimidazole afforded the cyclic-urea-containing inhibitor **7** in good yield.

Inhibitors **9** and **11** were prepared by derivatization of intermediate **22** with amino acid residues or a ketomethylene dipeptide isostere, respectively. Thus, acidic deprotection (HCl) of **22** and carbodiimide-mediated coupling of the resulting amine salt with Boc-L-Phe(4-F)-OH afforded the dipeptide product **32** in moderate yield (Scheme 4). A second HCl deprotection and subsequent coupling with Boc-L-Val-OH then provided the corresponding tripeptide **33** (not shown). This material was also deprotected under acidic conditions (HCl), and the amine hydrochloride thus produced was derivatized with 5-methylisoxazole-3-carbonyl chloride to give intermediate **34** in moderate overall yield. As described for the preparation of **4** above, treatment of **34** with DDQ at elevated temperature provided the tripeptidyl inhibitor **9** after purification on silica gel. Similarly, coupling of the amine hydrochloride salt derived from HCl deprotection of **22** with the ketomethylene dipeptide isostere **35**[8] (used in crude form) gave intermediate **36** in moderate overall yield (Scheme 5). This material was transformed into inhibitor **11** in a manner analogous to that used for the conversion of intermediate **33** to inhibitor **9** described above.

Scheme 4[a]



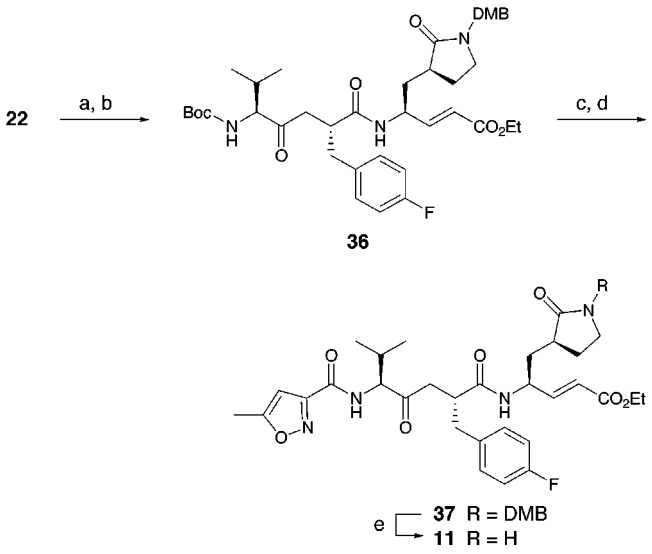
[a] DMB = 2,4-dimethoxybenzyl. Reagents and conditions: (a) HCl, 1,4-dioxane, 23 °C, 1.5 h; (b) 1.2 equiv of Boc-L-(4-F-Phe)-OH, 3.0 equiv of $(i\text{-}Pr)_2NEt$, 1.1 equiv of HATU, DMF, 0→23 °C, 1.5 h, 77%; (c) HCl, 1,4-dioxane, 23 °C, 1.5 h; (d) 1.2 equiv of Boc-L-Val-OH, 4.0 equiv of NMM, 1.7 equiv of HOBt, 1.7 equiv of EDC, CH_2Cl_2, 23 °C, 18 h, 88%; (e) HCl, 1,4-dioxane, 23 °C, 1.5 h; (f) 2.2 equiv of NMM, 1.1 equiv of 5-methylisoxazole-3-carbonyl chloride, CH_2Cl_2, 0→23 °C, 1 h, 69%; (g) 1.3 equiv of DDQ, 10:1 $CHCl_3$:H_2O, reflux, 9 h, 56%.



Conclusions

The studies presented above illustrate how the incorporation of P_1 lactam glutamine replacements into substrate-derived, irreversible human rhinovirus 3C protease (3CP) inhibitors substantially improves the anti-3CP and antiviral activity of such compounds. Both peptidyl and peptidomimetic molecules benefit from such incorporation, and the resulting optimized inhibitors rank among the most potent antirhinoviral agents described in the literature to date. In addition, most of the compounds presented in this work display excellent selectivity for HRV 3CP over other serine and cysteine

Scheme 5[a]



[a] DMB = 2,4-dimethoxybenzyl. Reagents and conditions: (a) HCl, 1,4-dioxane, 23 °C, 1.5 h; (b) 1.4 equiv of **35**,[8] 4.0 equiv of NMM, 1.7 equiv of HOBt, 1.7 equiv of EDC, CH_2Cl_2, 23 °C, 18 h, 63%; (c) HCl, 1,4-dioxane, 23 °C, 1.5 h; (d) 2.2 equiv of NMM, 1.1 equiv of 5-methylisoxazole-3-carbonyl chloride, CH_2Cl_2, 0→23 °C, 1 h, 85%; (e) 1.4 equiv of DDQ, 10:1 $CHCl_3$:H_2O, reflux, 20 h, 39%.

proteases and exhibit good stability toward a variety of biological agents. The above studies led to the selection of one inhibitor (compound **11**, AG7088) as a development candidate, and the molecule is currently undergoing testing to evaluate its potential clinical use as an antirhinoviral agent.

Experimental Section

General descriptions of experimental procedures, reagent purifications, and instrumentation along with conditions and uncertainties for enzyme and antiviral assays are provided elsewhere.[5] ^{1}H NMR chemical shifts are reported in ppm (δ) downfield relative to internal tetramethylsilane, and coupling constants are given in hertz. A simplified naming system employing amino acid abbreviations is used to identify some intermediates and final products. When utilizing this naming system, italicized amino acid abbreviations represent modifications at the C-terminus of that residue where acrylic acid esters are reported as "E" (trans) propenoates. In addition, the terminology "$AA_1\Psi[COCH_2]$-AA_2" indicates that, for any peptide sequence, two amino acids (AA_1 and AA_2) usually linked by an amide bond are replaced by a ketomethylene dipeptide isostere. The following abbreviations also apply: HATU [*O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate], HOBt [1-hydroxybenzotriazole hydrate], EDC [1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride], CDI [1,1′-carbonyldiimidazole], MTBE [*tert*-butyl methyl ether], DDQ [2,3-dichloro-5,6-dicyano-1,4-benzoquinone], (*S*)-Pyrrol-Ala [(2*S*,3′*S*)-2-amino-3-(2′-oxopyrrolidin-3′-yl)propionic acid], (*S*)-Piper-Ala [(2*S*,3′*S*)-2-amino-3-(2′-oxopiperidin-3′-yl)-propionic acid]. Pirodavir was kindly provided by Janssen Pharmaceuticals. Pleconaril was prepared as described in the literature.[18]

(4*S*)-4-(2′-Carboxyethyl)-2,2-dimethyloxazolidine-3-carboxylic Acid *tert*-Butyl Ester (16). Sodium hydroxide (27 mL of a 4.0 M solution in H_2O, 108 mmol, 3.0 equiv) was added to a solution of (4*S*)-4-(2-methoxycarbonylethyl)-2,2-dimethyloxazolidine-3-carboxylic acid *tert*-butyl ester **15**[22] (10.5 g, 36.5 mmol, 1 equiv) in CH_3OH (150 mL), and the resulting cloudy reaction mixture was stirred at 23 °C for 3.5 h. The mixture was concentrated under reduced pressure to ~30 mL volume and then was partitioned between 0.5 M HCl (150 mL) and EtOAc (2 × 150 mL). The combined organic layers were dried over $MgSO_4$ and were gravity-filtered. The filtrate was concentrated under reduced pressure and the residue dried under vacuum to afford **16** (10.0 g, 100% crude yield) as a colorless oil. This material was used without further purification: ^{1}H NMR ($CDCl_3$, mixture of isomers) δ 1.49 (s), 1.57 (s), 1.60 (s), 1.84−2.05 (m), 2.39−2.41 (m), 3.71−3.74 (m), 3.91−4.05 (m).

(4*S*,4″*S*)-4-[3′-(4″-Benzyl-2″-oxo-oxazolidin-3″-yl)-3′-oxopropyl]-2,2-dimethyloxazolidine-3-carboxylic Acid *tert*-Butyl Ester (17). Triethylamine (8.87 mL, 63.6 mmol, 3.0 equiv) and pivaloyl chloride (2.61 mL, 21.2 mmol, 1.0 equiv) were added sequentially to a solution of **16** (5.80 g, 21.2 mmol, 1 equiv) in THF (450 mL) at 0 °C. The cloudy reaction mixture was stirred at 0 °C for 3.5 h, and then lithium chloride (0.988 g, 23.3 mmol, 1.1 equiv) and (*S*)-(−)-4-benzyl-2-oxazolidinone (3.57 g, 20.1 mmol, 0.95 equiv) were added sequentially. After warming to 23 °C and being stirred for 19 h, the reaction mixture was partitioned between 0.5 M HCl (150 mL) and EtOAc (2 × 150 mL). The combined organic layers were washed with half-saturated Na_2CO_3 (150 mL), dried over $MgSO_4$, and gravity-filtered. The filtrate was concentrated under reduced pressure, and the residue was purified by flash column chromatography (30% EtOAc in hexanes) to give **17** (7.17 g, 83%) as a colorless oil which slowly crystallized over 7 days: mp = 60−64 °C; R_f = 0.69 (50% EtOAc in hexanes); IR (cm^{-1}) 2978, 1783, 1694; ^{1}H NMR ($CDCl_3$, mixture of isomers) δ 1.49 (s), 1.59 (s), 1.63 (s), 2.01−2.10 (m), 2.76 (dd, J = 13.5, 9.8), 2.82−3.13 (m), 3.30−3.41 (m), 3.76−3.82 (m), 3.90 (s, br), 3.97 (dd, J = 9.0, 5.6), 4.10−4.19 (m), 4.63−4.71 (m), 7.22−7.36 (m). Anal. ($C_{23}H_{32}N_2O_6$) C, H, N.

(2′*S*,4*S*,4″*S*)-4-[2′-(4″-Benzyl-2″-oxo-oxazolidine-3″-carbonyl)-pent-4′-enyl]-2,2-dimethyloxazolidine-3-carboxylic Acid *tert*-Butyl Ester (18). A solution of **17** (7.17 g, 16.6 mmol, 1 equiv) in THF (50 mL) was added to a solution of sodium bis(trimethylsilyl)amide (16.6 mL of a 1.0 M solution in THF, 16.6 mmol, 1.0 equiv) in the same solvent (150 mL) at −78 °C. The reaction mixture was stirred for 20 min at −78 °C, and then allyl iodide (4.55 mL, 49.8 mmol, 3.0 equiv) was added. After being stirred an additional 3 h at −78 °C, the reaction mixture was maintained at −45 °C for 2 h and then was partitioned between a 2:1 mixture of half-saturated NH_4Cl and 5% $Na_2S_2O_3$ (300 mL) and a 1:1 mixture of EtOAc and hexanes (2 × 200 mL). The combined organic layers were washed with H_2O (200 mL), dried over $MgSO_4$, and gravity-filtered. The filtrate was concentrated under reduced pressure, and the residue was purified by flash column chromatography (15% EtOAc in hexanes) to provide **18** (4.29 g, 55%) as a colorless oil: R_f = 0.44 (30% EtOAc in hexanes); IR (cm^{-1}) 2978, 1780, 1695; ^{1}H NMR ($CDCl_3$, mixture of isomers) δ 1.45 (s), 1.49 (s), 1.68−1.80 (m), 2.13−2.47 (m), 2.49−2.67 (m), 3.32 (dd, J = 13.4, 3.1), 3.69−3.97 (m), 4.11−4.21 (m), 4.66−4.74 (m), 5.06−5.13 (m), 5.74−5.88 (m), 7.20−7.36 (m). Anal. ($C_{26}H_{36}N_2O_6$) C, H, N.

(1*S*,3′*S*)-{2-[1′-(2″,4″-Dimethoxybenzyl)-2′-oxo-pyrrolidin-3′-yl]-1-hydroxymethylethyl}-carbamic Acid *tert*-Butyl Ester (20). Ozone was bubbled through a solution of **18** (4.29 g, 9.08 mmol, 1 equiv) in CH_2Cl_2 (200 mL) containing CH_3OH (0.735 mL, 18.1 mmol, 2.0 equiv) at −78 °C until a blue color persisted. The reaction mixture was then purged with argon until it became colorless. Methyl sulfide (6.67 mL, 90.8 mmol, 10 equiv) was added, and the mixture was stirred at −78 °C for 3.5 h and then was maintained at 0 °C for an additional 1 h. After partitioning the reaction mixture between H_2O (200 mL) and a 1:1 mixture of EtOAc and hexanes (2 × 200 mL), the combined organic layers were dried over $MgSO_4$ and were gravity-filtered. The filtrate was concentrated under reduced pressure, and the residue was immediately utilized without further purification.

The above residue was dissolved in a 2:1 mixture of THF and EtOH (240 mL) at 23 °C, and 2,4-dimethoxybenzylamine hydrochloride (7.40 g, 36.3 mmol, 4.0 equiv), sodium acetate (2.98 g, 36.2 mmol, 4.0 equiv), and sodium cyanoborohydride (1.14 g, 18.1 mmol, 2.0 equiv) were added sequentially. The resulting suspension was stirred for 18 h at 23 °C and then was partitioned between 0.5 M HCl (400 mL) and EtOAc (2 ×

200 mL). The combined organic layers were washed with half-saturated $NaHCO_3$ (300 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The residue was passed through a short silica gel column (eluting with 50% EtOAc in hexanes) to give **19** contaminated with (*S*)-(−)-4-benzyl-2-oxazolidinone.

This material was dissolved in CH_3OH (100 mL), and TsOH·H_2O (0.345 g, 1.81 mmol, 0.20 equiv) was added. The reaction mixture was heated to 50 °C and was maintained at that temperature for 2.5 h. After cooling to 23 °C, the reaction mixture was concentrated under reduced pressure to ∼20 mL volume and was partitioned between half-saturated $NaHCO_3$ (150 mL) and a 9:1 mixture of CH_2Cl_2 and CH_3OH (2 × 150 mL). The combined organic layers were dried over Na_2SO_4 and were concentrated under reduced pressure. Purification of the residue by flash column chromatography (3% CH_3OH in CH_2Cl_2) afforded **20** (1.62 g, 44% from **18**) as a foam: R_f = 0.03 (50% EtOAc in hexanes); IR (cm^{-1}) 3328, 1669; ^1H NMR ($CDCl_3$) δ 1.44 (s, 9H), 1.50−1.75 (m, 2H), 1.90−2.00 (m, 1H), 2.17−2.27 (m, 1H), 2.52−2.62 (m, 1H), 3.14−3.24 (m, 2H), 3.51−3.65 (m, 3H), 3.70−3.78 (m, 1H), 3.80 (s, 6H), 4.35 (d, 1H, J = 14.3), 4.48 (d, 1H, J = 14.3), 5.51−5.54 (m, 1H), 6.42−6.46 (m, 2H), 7.09−7.12 (m, 1H). Anal. ($C_{21}H_{32}N_2O_6$) C, H, N.

Ethyl-3-{Boc-L-[(*N*-2,4-dimethoxybenzyl)-*(S)*-Pyrrol-*Ala*]}-E-propenoate (22). DMSO (0.270 mL, 3.80 mmol, 3.0 equiv) was added dropwise to a −78 °C solution of oxalyl chloride (0.166 mL, 1.90 mmol, 1.5 equiv) in CH_2Cl_2 (14 mL). The reaction mixture was stirred 20 min at −78 °C and then a solution of **20** (0.518 g, 1.27 mmol, 1 equiv) in CH_2Cl_2 (13 mL) was added via cannula along the side of the reaction vessel. After an additional 20 min, triethylamine (1.06 mL, 7.60 mmol, 6.0 equiv) was added dropwise, and the reaction mixture was stirred for 1.5 h at −78 °C. Acetic acid (0.479 mL, 8.37 mmol, 6.6 equiv) was then added, and the reaction mixture was warmed to 0 °C for 5 min, then diluted with MTBE (200 mL), and washed with water (25 mL), saturated $NaHCO_3$ (25 mL), and brine (25 mL). The organic phase was dried over Na_2SO_4 and was concentrated to provide **21** as a white foam (0.516 g, 100%). This material was used without further purification.

Sodium bis(trimethylsilyl)amide (1.23 mL of a 1.0 M solution in THF, 1.23 mmol, 1.0 equiv) was added to a solution of triethyl phosphonoacetate (0.244 mL, 1.23 mmol, 1.0 equiv) in THF (15 mL) at −78 °C, and the resulting mixture was stirred for 20 min at that temperature. A solution of crude **21** (prepared above, 0.500 g, 1.23 mmol, 1 equiv) in THF (13 mL) was added via cannula along the side of the reaction vessel, and the resulting mixture was stirred for 45 min at −78 °C, warmed to 0 °C for 7 min, then was partitioned between 0.5 M HCl (20 mL) and MTBE (2 × 50 mL). The combined organic layers were dried over $MgSO_4$ and were concentrated. Purification of the residue by flash column chromatography (60% EtOAc in hexanes) provided **22** (0.356 g, 61%) as a white foam: R_f = 0.43 (60% EtOAc in hexanes); IR (cm^{-1}) 3307, 1708, 1678; ^1H NMR ($CDCl_3$) δ 1.28 (t, 3H, J = 7.2), 1.43 (s, 9H), 1.52−1.70 (m, 2H), 1.98−2.09 (m, 1H), 2.21−2.34 (m, 1H), 2.48−2.59 (m, 1H), 3.16−3.24 (m, 2H), 3.80 (s, 6H), 4.18 (q, 2H, J = 7.2), 4.27−4.40 (m, 1H), 4.41 (s, 2H), 5.40 (d, 1H, J = 8.1), 5.95 (dd, 1H, J = 15.6, 1.6), 6.41−6.48 (m, 2H), 6.86 (dd, 1H, J = 15.6, 5.3), 7.08−7.13 (m, 1H). Anal. ($C_{25}H_{36}N_2O_7$·0.25H_2O) C, H, N.

Ethyl-3-{Cbz-L-Leu-L-Phe-L-[(*N*-2,4-dimethoxybenzyl)-*(S)*-Pyrrol-*Ala*]}-E-propenoate (23). A solution of HCl in 1,4-dioxane (4.0 M, 4 mL) was added to a solution of **22** (0.139 g, 0.292 mmol, 1 equiv) in the same solvent (4 mL) at 23 °C. After the mixture was stirred for 1.5 h at that temperature, the volatiles were evaporated to give the crude amine hydrochloride salt. This material was dissolved in CH_2Cl_2 (7 mL), and Cbz-L-Leu-L-Phe-OH (0.156 g, 0.378 mmol, 1.3 equiv), 4-methylmorpholine (0.128 mL, 1.16 mmol, 4.0 equiv), HOBt (0.067 g, 0.50 mmol, 1.7 equiv) and EDC (0.095 g, 0.50 mmol, 1.7 equiv), were added sequentially. After being stirred for 20 h at 23 °C, the reaction mixture was partitioned between brine (15 mL) and a 1:9 mixture of CH_3OH and CH_2Cl_2 (3 × 25 mL). The combined organic layers were dried over Na_2SO_4 and were concentrated. Purification of the residue by flash column chromatography (60% EtOAc in hexanes) provided **23** (0.158 g, 70%) as a tan foam: R_f = 0.30 (5% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3289, 1713, 1655; ^1H NMR ($CDCl_3$) δ 0.87−0.92 (m, 6H), 1.28 (t, 3H, J = 7.2), 1.46−1.68 (m, 5H), 1.74−1.86 (m, 1H), 1.97−2.19 (m, 2H), 3.02 (dd, 1H, J = 13.7, 5.6), 3.11−3.24 (m, 3H), 3.78 (s, 3H), 3.79 (s, 3H), 4.17 (q, 2H, J = 7.2), 4.20−4.30 (m, 2H), 4.35−4.45 (m, 2H), 4.82−4.90 (m, 1H), 5.07 (d, 1H, J = 12.3), 5.13 (d, 1H, J = 12.3), 5.36 (d, 1H, J = 7.8), 5.82 (dd, 1H, J = 15.6, 1.2), 6.42−6.46 (m, 2H), 6.72 (dd, 1H, J = 15.6, 5.3), 6.88 (d, 1H, J = 8.7), 7.09 (d, 1H, J = 9.0), 7.13−7.20 (m, 5H), 7.29−7.37 (m, 5H), 8.09 (d, 1H, J = 6.5). Anal. ($C_{43}H_{54}N_4O_9$·0.5H_2O) C, H, N.

Ethyl-3-{Cbz-L-Leu-L-Phe-L-[*(S)-Pyrrol-Ala*]}-E-propenoate (4). DDQ (0.066 g, 0.29 mmol, 1 equiv) and **23** (0.225 g, 0.291 mmol, 1.0 equiv) were combined in a mixture of $CHCl_3$ (4 mL) and water (0.4 mL). After the mixture was stirred for 2 h in a 50 °C oil bath, additional DDQ (0.095 g, 0.42 mmol, 1.4 equiv) was added. After 2 h more at 50 °C, a third portion of DDQ (0.099 g, 0.44 mmol, 1.5 equiv) was added. The reaction mixture was stirred for 2 h at 50 °C, and then DDQ (0.065 g, 0.29 mmol, 1.0 equiv) was added for the fourth time. After stirring for 2 h more at 50 °C, the reaction mixture was allowed to cool overnight, then was diluted with EtOAc (250 mL), and washed with saturated $NaHCO_3$ (40 mL) and brine (25 mL). The organic phase was dried over Na_2SO_4 and was concentrated. Purification of the residue by flash column chromatography (2.4% CH_3OH in CH_2Cl_2) provided **4** (0.100 g, 55%) as a white foam: R_f = 0.26 (5% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3278, 1690, 1637; ^1H NMR ($CDCl_3$) δ 0.85−0.92 (m, 6H), 1.28 (t, 3H, J = 7.2), 1.39−1.65 (m, 4H), 1.68−1.93 (m, 2H), 2.08−2.20 (m, 1H), 2.27−2.38 (m, 1H), 3.02−3.13 (m, 2H), 3.24−3.32 (m, 2H), 4.11−4.20 (m, 1H), 4.18 (q, 2H, J = 7.2), 4.47−4.58 (m, 1H), 4.81−4.89 (m, 1H), 5.05 (d, 1H, J = 12.1), 5.12 (d, 1H, J = 12.1), 5.26 (d, 1H, J = 8.1), 5.78 (dd, 1H, J = 15.7, 1.2), 6.23 (s, 1H), 6.72 (dd, 1H, J = 15.7, 5.3), 7.13−7.25 (m, 6H), 7.30−7.37 (m, 5H), 7.54 (d, 1H, J = 7.2). Anal. ($C_{34}H_{44}N_4O_7$·0.5H_2O) C, H, N.

(2′*S*,4*S*,4″*S*)-4-[2′-(4″-Benzyl-2″-oxo-oxazolidine-3″-carbonyl)-5′-hydroxypentyl]-2,2-dimethyloxazolidine-3-carboxylic Acid *tert*-Butyl Ester (24). A solution of borane−tetrahydrofuran complex (0.96 mL of a 1.0 M solution in THF, 0.96 mmol, 1.0 equiv) was added to a 0 °C solution of **18** (0.455 g, 0.963 mmol, 1 equiv) in THF (3 mL). After the mixture was stirred for 30 min, water (3 mL) and sodium perborate tetrahydrate (0.148 g, 0.962 mmol, 1.0 equiv) were added and the ice bath was removed. After an additional hour, the reaction mixture was diluted with MTBE (125 mL) and was washed with water (15 mL) and brine (2 × 15 mL). The combined organic layers were dried over Na_2SO_4 and were concentrated. Purification of the residue by flash column chromatography (50% EtOAc in hexanes) provided **24** (0.339 g, 72%) as a colorless glass: R_f = 0.41 (50% EtOAc in hexanes); IR (cm^{-1}) 3486, 1780, 1693; ^1H NMR ($CDCl_3$, mixture of isomers) δ 1.42−1.85 (m), 2.13−2.24 (m), 2.70 (dd, J = 13.1, 10.0), 3.29−3.38 (m), 3.61−4.22 (m), 4.63−4.76 (m), 7.19−7.38 (m). Anal. ($C_{26}H_{38}N_2O_7$·0.50H_2O) C, H, N.

(1*S*,3′*S*)-{2-[1′-(2″,4″-Dimethoxybenzyl)-2′-oxo-piperidin-3′-yl]-1-hydroxymethylethyl}-carbamic Acid *tert*-Butyl Ester (26). A solution of sulfur trioxide−pyridine complex (5.04 g, 31.67 mmol, 4.0 equiv) in DMSO (150 mL) was added to an ice-cooled solution of **24** (3.88 g, 7.92 mmol, 1 equiv) in triethylamine (2.75 mL, 28.51 mmol, 3.6 equiv) at a rate which maintained the reaction temperature between 8 and 17 °C. The resulting mixture was then stirred at 23 °C for 3 h, cooled to 0 °C, and was quenched by the addition of H_2O (150 mL). The quenched reaction mixture was extracted with EtOAc (2 × 150 mL), and the combined organic layers were washed with 5% citric acid (100 mL) and brine (100 mL), then were dried over Na_2SO_4, and concentrated. The residue was dried further under vacuum to give a white foam (3.53 g).

To a solution of this material (3.53 g, 7.22 mmol, 1 equiv) in a 2:1 mixture of THF and EtOH (120 mL) were added 2,4-

dimethoxybenzylamine hydrochloride (5.88 g, 28.89 mmol, 4.0 equiv), NaOAc (2.37 g, 28.89 mmol, 4.0 equiv), and $NaBH_3CN$ (0.908 g, 14.45 mmol, 2.0 equiv) at 23 °C. The reaction mixture was stirred 20 h at 23 °C and then was diluted with MTBE (200 mL). The organic layer was washed with 10% $KHSO_4$ (100 mL), saturated $NaHCO_3$ (100 mL), and brine (100 mL), dried over Na_2SO_4, and concentrated to give **25** as a pale yellow foam. This material was utilized without further purification.

p-Toluenesulfonic acid monohydrate (0.275 g, 1.44 mmol, 0.20 equiv) was added to a solution of crude **25** (3.34 g, 7.22 mmol, 1 equiv) in CH_3OH (50 mL) at 23 °C. The reaction mixture was maintained at 50 °C for 2.5 h and then was partitioned between CH_2Cl_2 (100 mL) and saturated $NaHCO_3$ (100 mL). The organic layer was dried over Na_2SO_4 and was concentrated. Purification of the residue by flash column chromatography (3% CH_3OH in CH_2Cl_2) afforded **26** (1.33 g, 44% from **24**) as a white foam: ^{1}H NMR ($CDCl_3$) δ 1.44 (s, 9H), 1.71−1.85 (m, 2H), 1.92−1.98 (m, 2H), 2.40−2.48 (m, 1H), 2.71−2.78 (m, 1H), 3.19−3.32 (m, 2H), 3.45−3.69 (m, 4H), 4.11−4.20 (m, 2H), 4.68 (m, 1H), 5.47 (m, 1H), 6.44 (s, 1H), 7.20−7.33 (m, 2H).

Ethyl-3-{Boc-L-[(*N*-2,4-dimethoxybenzyl)-*(S)-Piper-Ala*]}-E-propenoate (28). DMSO (0.133 mL, 1.87 mmol, 4.0 equiv) was added dropwise to a −78 °C solution of oxalyl chloride (0.123 mL, 1.41 mmol, 3.0 equiv) in CH_2Cl_2 (7 mL). The reaction mixture was stirred 20 min at −78 °C, and then a solution of **26** (0.198 g, 0.468 mmol, 1 equiv) in CH_2Cl_2 (5 mL) was added via cannula along the side of the reaction vessel. After an additional 20 min, triethylamine (0.327 mL, 2.34 mmol, 5.0 equiv) was added dropwise, and the reaction mixture was maintained at −78 °C for 1.5 h. Acetic acid (0.148 mL, 2.59 mmol, 5.5 equiv) was added, and the reaction mixture was warmed to 0 °C for 5 min, then was diluted with MTBE (50 mL), and was washed with water (15 mL), saturated $NaHCO_3$ (15 mL), and brine (15 mL). The organic phase was dried over Na_2SO_4 and was concentrated to provide **27** as a foam which was used without further purification.

Sodium bis(trimethylsilyl)amide (0.468 mL of a 1.0 M solution in THF, 0.468 mmol, 1.0 equiv) was added to a solution of triethyl phosphonoacetate (0.093 mL, 0.47 mmol, 1.0 equiv) in THF (5 mL) at −78 °C, and the resulting solution was stirred for 20 min at that temperature. A solution of **27** (0.468 mmol, 1 equiv) in THF (4 mL) was added via cannula along the side of the reaction vessel, and the reaction mixture was stirred for 45 min at −78 °C, then warmed to 0 °C for 7 min, and was partitioned between 0.5 M HCl (20 mL) and MTBE (2 × 50 mL). The combined organic layers were dried over $MgSO_4$ and were concentrated. Purification of the residue by flash column chromatography (40% EtOAc in hexanes) provided **28** (0.110 g, 48%) as a white foam: R_f = 0.29 (40% EtOAc in hexanes); IR (cm^{-1}) 3296, 1716, 1616; ^{1}H NMR ($CDCl_3$) δ 1.28 (t, 3H, J = 7.2), 1.42−1.87 (m, 4H), 1.44 (s, 9H), 2.03−2.24 (m, 2H), 2.36−2.46 (m, 1H), 3.16−3.30 (m, 2H), 3.79 (s, 6H), 4.18 (q, 2H, J = 7.2), 4.26−4.38 (m, 1H), 4.53 (s, 2H), 5.50 (d, 1H, J = 7.8), 5.95 (dd, 1H, J = 15.6, 1.6), 6.42−6.48 (m, 2H), 6.86 (dd, 1H, J = 15.6, 5.1), 7.08−7.15 (m, 1H). Anal. ($C_{26}H_{38}N_2O_7$·0.25H_2O) C, H, N.

Ethyl-3-[Cbz-L-Leu-L-Phe-L-*N*-Boc-*aminoAla*]-E-propenoate (30). (Carbethoxymethylene)triphenylphosphorane (1.20 g, 3.28 mmol, 1.2 equiv) was added to a solution of **29**[30] (1.60 g, 2.73 mmol, 1 equiv) in THF (55 mL), and the reaction mixture was stirred at 23 °C overnight. The volatiles were then removed under reduced pressure, and the residue was purified by flash column chromatography (gradient elution, 0→1.5% CH_3OH in CH_2Cl_2) to give **30** (0.968 g, contaminated with triphenylphosphine oxide). This material was used without further purification.

Ethyl-3-[Cbz-L-Leu-L-Phe-L-*(2-Boc-2-aminoethyl)aminoAla*]-E-propenoate (31). A solution of HCl in 1,4-dioxane (4.0 M, 10 mL) was added to a solution of **30** (0.95 g, 1.46 mmol, 1 equiv) in the same solvent (20 mL) at 23 °C. The reaction mixture was stirred at that temperature for 1.5 h, and then additional HCl in 1,4-dioxane (4.0 M, 10 mL) was added. After the mixture was stirred overnight, the volatiles were removed under reduced pressure and the residue was triturated with Et_2O (20 mL). The resulting solids were collected by vacuum filtration and were washed with Et_2O (3 × 10 mL) to give the crude amine salt (0.63 g 73%, 1.05 mmol).

This material was dissolved in CH_3OH (10 mL) at 23 °C, and N-Boc-2-aminoethanal (Aldrich, 0.19 g, 1.16 mmol, 1.1 equiv) and $NaBH_3CN$ (0.069 g, 1.05 mmol, 1.0 equiv) were added sequentially. The reaction mixture was stirred at 23 °C overnight and then was concentrated. The residue was dissolved in EtOAc (25 mL), and the organic layer was washed with water (25 mL) and brine (25 mL), dried over $MgSO_4$, and concentrated. The residue was purified by flash column chromatography (gradient elution, 0→3% CH_3OH in CH_2Cl_2) to provide **31** (0.32 g, 44%) as a white amorphous solid: R_f = 0.20 (5% CH_3OH in $CHCl_3$); IR (cm^{-1}) 1712, 1649, 1537, 1252, 1175; ^{1}H NMR (DMSO-d_6) δ 0.79 (d, 3H, J = 6.6), 0.82 (d, 3H, J = 6.6), 1.21 (t, 3H, J = 7.0), 1.26−1.37 (m, 13H), 1.46−1.54 (m, 1H), 2.56−2.60 (m, 2H), 2.82−2.97 (m, 4H), 3.98−4.04 (m, 1H), 4.10 (q, 2H, J = 7.0), 4.42−4.49 (m, 2H), 4.98 (d, 1H, J = 12.5), 5.04 (d, 1H, J = 12.9), 5.59 (d, 1H, J = 15.8), 6.73−6.75 (m, 1H), 6.77 (dd, 1H, J = 15.8, 4.8), 7.20−7.34 (m, 10H), 7.41 (d, 1H, J = 8.1), 7.97 (d, 1H, J = 7.0), 8.07 (d, 1H, J = 7.0). Anal. ($C_{37}H_{53}N_5O_8$·0.50H_2O) C, H, N.

Ethyl-3-[Cbz-L-Leu-L-Phe-L-*1-(2-imidazolidone)Ala*]-E-propenoate (7). TFA (0.8 mL) was added to a solution of **31** (0.29 g, 0.42 mmol, 1 equiv) in CH_2Cl_2, (8 mL) and the reaction mixture was stirred at 23 °C for 2 h. The volatiles were then removed under reduced pressure, and the residue was dissolved in EtOAc (25 mL) and washed with saturated $NaHCO_3$ solution (25 mL), water (25 mL), and brine (25 mL). The organic layer was dried over $MgSO_4$ and was concentrated to give the crude diamine (0.23 g, 92%, 0.39 mmol) as a tan amorphous solid.

This material was dissolved in THF (4 mL) at 23 °C, and carbonyldiimidazole (0.06 g, 0.36 mmol, 0.92 equiv) was added. After being stirred for 3.5 h at 23 °C, the reaction mixture was concentrated under reduced pressure, and the residue was purified by flash column chromatography (gradient elution, 0→2% CH_3OH in CH_2Cl_2) to give **7** (0.12 g, 54%) as a white amorphous solid: mp = 161−164 °C; R_f = 0.21 (5% CH_3OH in $CHCl_3$); IR (cm^{-1}) 1701, 1647, 1535, 1277; ^{1}H NMR (DMSO-d_6) δ 0.79 (d, 3H, J = 6.6), 0.82 (d, 3H, J = 6.6), 1.21 (t, 3H, J = 7.0), 1.27−1.35 (m, 2H), 1.48−1.52 (m, 1H), 2.79−2.86 (m, 1H), 2.92−3.05 (m, 3H), 3.14−3.19 (m, 2H), 3.25−3.30 (m, 2H), 3.98−4.03 (m, 1H), 4.10 (q, 2H, J = 7.0), 4.47−4.49 (m, 1H), 4.59−4.63 (m, 1H), 4.97−5.02 (m, 2H), 5.72 (d, 1H, J = 15.8), 6.37 (s, 1H), 6.71 (dd, 1H, J = 15.8, 5.5), 7.15−7.39 (m, 10H), 7.42 (d, 1H, J = 8.1), 8.00 (d, 1H, J = 8.1), 8.18 (d, 1H, J = 8.1). Anal. ($C_{33}H_{43}N_5O_7$) C, H, N.

Ethyl-3-{Boc-L-Phe(4-F)-L-[(*N*-2,4-dimethoxybenzyl)-*(S)-Pyrrol-Ala*]}-E-propenoate (32). A solution of HCl in 1,4-dioxane (4.0 M, 12 mL) was added to a solution of **22** (0.432 g, 0.906 mmol, 1 equiv) in the same solvent (12 mL) at 23 °C. After the mixture was stirred for 1.5 h at 23 °C, the solvent was evaporated to give the crude amine salt which was dissolved in DMF (7 mL) and cooled to 0 °C. Boc-L-Phe(4-F)-OH (0.308 g, 1.09 mmol, 1.2 equiv), *N*,*N*-diisopropylethylamine (0.474 mL, 2.72 mmol, 3.0 equiv), and HATU (0.379 g, 0.997 mmol, 1.1 equiv) were added sequentially, and the reaction mixture was allowed to warm to 23 °C. After 1.5 h, the mixture was diluted with MTBE (200 mL), washed with 5% $KHSO_4$ (20 mL) and brine (20 mL), dried over $MgSO_4$, and concentrated. The residue was purified by flash column chromatography (60% EtOAc in hexanes) to provide **32** (0.447 g, 77%) as a white foam: R_f = 0.34 (60% EtOAc in hexanes); IR (cm^{-1}) 3258, 1705, 1666; ^{1}H NMR ($CDCl_3$) δ 1.28 (t, 3H, J = 7.2), 1.45 (s, 9H), 1.51−1.66 (m, 2H), 1.78−1.90 (m, 1H), 2.06−2.23 (m, 2H), 2.99 (dd, 1H, J = 13.7, 6.2), 3.11 (dd, 1H, J = 13.7, 5.3), 3.17−3.23 (m, 2H), 3.80 (s, 3H), 3.81 (s, 3H), 4.18 (q, 2H, J = 7.2), 4.35 (s, 2H), 4.38−4.51 (m, 2H), 5.29−5.37 (m, 1H), 5.76 (d, 1H, J = 15.8), 6.43−6.47 (m, 2H), 6.72 (dd, 1H, J = 15.8, 5.3), 6.83−6.91 (m, 2H), 7.09−7.17 (m, 3H), 7.92 (br, 1H). Anal. ($C_{34}H_{44}FN_3O_8$) C, H, N.

Ethyl-3-{Boc-L-Val-L-Phe(4-F)-L-[(*N*-2,4-dimethoxybenzyl)-*(S)-Pyrrol-Ala*]}-E-propenoate (33). A solution of HCl in 1,4-dioxane (4.0 M, 10 mL) was added to a solution of **32** (0.425 g, 0.662 mmol, 1 equiv) in the same solvent (10 mL) at 23 °C. After the mixture was stirred for 1.5 h at 23 °C, the solvent was evaporated to give the crude amine salt. This material was combined with Boc-L-Val-OH (0.173 g, 0.796 mmol, 1.2 equiv) and dissolved in CH_2Cl_2 (20 mL) at 23 °C. HOBt (0.152 g, 1.12 mmol, 1.7 equiv), 4-methylmorpholine (0.291 mL, 2.65 mmol, 4.0 equiv), and EDC (0.216 g, 1.13 mmol, 1.7 equiv) were then added sequentially. After being stirred overnight at 23 °C, the reaction mixture was diluted with MTBE (200 mL) and washed with 5% $KHSO_4$ (20 mL), saturated $NaHCO_3$ (20 mL), and brine (20 mL). The organic layer was dried over Na_2SO_4 and was concentrated. The residue was purified by flash column chromatography (gradient elution, 60→100% EtOAc in hexanes) to provide **33** (0.430 g, 88%) as a white foam: $R_f = 0.24$ (60% EtOAc in hexanes); IR (cm^{-1}) 3284, 1713, 1678 br, 1643; ^1H NMR ($CDCl_3$) δ 0.91 (d, 3H, $J = 6.8$), 0.97 (d, 3H, $J = 6.8$), 1.28 (t, 3H, $J = 7.2$), 1.45 (s, 9H), 1.50−1.62 (m, 2H), 1.66−1.82 (m, 1H), 1.90−2.02 (m, 1H), 2.08−2.21 (m, 2H), 2.94 (dd, 1H, $J = 13.5, 5.8$), 3.17−3.27 (m, 3H), 3.80 (s, 3H), 3.82 (s, 3H), 3.97−4.05 (m, 1H), 4.17 (q, 2H, $J = 7.2$), 4.27 (d, 1H, $J = 14.3$), 4.29−4.38 (m, 1H), 4.40 (d, 1H, $J = 14.3$), 4.86−4.93 (m, 1H), 5.10 (d, 1H, $J = 8.7$), 5.76 (dd, 1H, $J = 15.6, 1.2$), 6.45−6.52 (m, 2H), 6.70 (dd, 1H, $J = 15.6, 5.4$), 6.79−6.88 (m, 3H), 7.12−7.22 (m, 3H), 8.30 (d, 1H, $J = 5.9$). Anal. ($C_{39}H_{53}FN_4O_9$) C, H, N.

Ethyl-3-{(5′-methylisoxazole-3′-carbonyl)-L-Val-L-Phe(4-F)-L-[(*N*-2,4-dimethoxybenzyl)-*(S)-Pyrrol-Ala*]}-E-propenoate (34). A solution of HCl in 1,4-dioxane (4.0 M, 10 mL) was added to a solution of **33** (0.410 g, 0.553 mmol, 1 equiv) in the same solvent (10 mL) at 23 °C. After the mixture was stirred for 1.5 h at 23 °C, the solvent was evaporated to give the crude amine salt which was dissolved in CH_2Cl_2 (20 mL) and cooled to 0 °C. 4-Methylmorpholine (0.134 mL, 1.22 mmol, 2.2 equiv) and 5-methylisoxazole-3-carbonyl chloride (0.089 g, 0.61 mmol, 1.1 equiv) were added sequentially, and the ice bath was removed. After being stirred for 1 h at 23 °C, the reaction mixture was diluted with MTBE (200 mL), washed with 5% $KHSO_4$ (20 mL), saturated $NaHCO_3$ (20 mL), and brine (20 mL), dried over Na_2SO_4, and concentrated. The residue was purified by flash column chromatography (2% CH_3OH in CH_2Cl_2) to provide **34** (0.288 g, 69%) as a white foam: $R_f = 0.36$ (5% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3284, 1717, 1650; ^1H NMR ($CDCl_3$) δ 0.97 (d, 3H, $J = 6.8$), 1.01 (d, 3H, $J = 6.8$), 1.28 (t, 3H, $J = 7.2$), 1.51−1.64 (m, 2H), 1.72−1.84 (m, 1H), 1.95−2.05 (m, 1H), 2.11−2.33 (m, 2H), 2.48 (s, 3H), 2.98 (dd, 1H, $J = 13.7, 5.6$), 3.16−3.24 (m, 3H), 3.80 (s, 3H), 3.81 (s, 3H), 4.17 (q, 2H, $J = 7.2$), 4.23 (d, 1H, $J = 14.3$), 4.31−4.42 (m, 1H), 4.40 (d, 1H, $J = 14.3$), 4.44−4.50 (m, 1H), 4.88−4.96 (m, 1H), 5.79 (dd, 1H, $J = 15.6, 1.4$), 6.43−6.49 (m, 3H), 6.71 (dd, 1H, $J = 15.6, 5.3$), 6.80−6.88 (m, 2H), 6.94 (d, 1H, $J = 9.3$), 7.11−7.17 (m, 3H), 7.29 (d, 1H, $J = 8.7$), 8.33 (d, 1H, $J = 6.2$). Anal. ($C_{39}H_{48}FN_5O_9 \cdot 0.50H_2O$) C, H, N.

Ethyl-3-{(5′-methylisoxazole-3′-carbonyl)-L-Val-L-Phe(4-F)-L-[*(S)-Pyrrol-Ala*]}-E-propenoate (9). A suspension of **34** (0.263 g, 0.351 mmol, 1 equiv), water (2 drops), and DDQ (0.104 g, 0.458 mmol, 1.3 equiv) in $CHCl_3$ (10 mL) was refluxed for 9 h and then allowed to cool to room temperature over 8 h. The reaction mixture was diluted with CH_2Cl_2 (200 mL) and washed with a 2:1 mixture of saturated $NaHCO_3$ and 1 M NaOH (20 mL). The organic phase was dried over $MgSO_4$ and was concentrated. Purification of the residue by flash column chromatography (gradient elution, 2→3% CH_3OH in CH_2Cl_2) gave **9** (0.117 g, 56%) as a white solid: mp = 219−220 °C; $R_f = 0.23$ (5% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3401 br, 3295, 1655 br; ^1H NMR ($CDCl_3$) δ 0.94 (d, 3H, $J = 6.8$), 0.97 (d, 3H, $J = 6.5$), 1.29 (t, 3H, $J = 7.2$), 1.54−1.65 (m, 1H), 1.72−1.91 (m, 2H), 2.07−2.26 (m, 2H), 2.28−2.39 (m, 1H), 2.49 (d, 3H, $J = 0.9$), 3.01 (dd, 1H, $J = 13.8, 6.1$), 3.12 (dd, 1H, $J = 13.8, 6.4$), 3.26−3.38 (m, 2H), 4.18 (q, 2H, $J = 7.2$), 4.34 (dd, 1H, $J = 8.7, 7.2$), 4.43−4.54 (m, 1H), 4.90 (dt, 1H, $J = 9.0, 6.2$), 5.76 (dd, 1H, $J = 15.6, 1.6$), 6.00 (s, 1H), 6.42 (q, 1H, $J = 0.9$), 6.72 (dd, 1H, $J = 15.6, 5.4$), 6.86−6.94 (m, 2H), 7.01 (d, 1H, $J = 9.0$), 7.11−7.18 (m, 2H), 7.21 (d, 1H, $J = 8.7$), 7.76 (d, 1H, $J = 7.2$). Anal. ($C_{30}H_{38}FN_5O_7$) C, H, N.

Ethyl-3-{Boc-L-ValΨ[COCH$_2$]-L-Phe(4-F)-L-[(*N*-2,4-dimethoxybenzyl)-*(S)-Pyrrol-Ala*]}-E-propenoate (36). A solution of HCl in 1,4-dioxane (4.0 M, 10 mL) was added to a solution of **22** (0.342 g, 0.718 mmol, 1 equiv) in the same solvent (10 mL) at 23 °C. After the mixture was stirred for 1.5 h at that temperature, the volatiles were removed under reduced pressure to give the crude amine salt. This material was combined with crude **35**[8] (1.00 mmol, 1.4 equiv) and dissolved in CH_2Cl_2 (17 mL) at 23 °C. HOBt (0.165 g, 1.22 mmol, 1.7 equiv), 4-methylmorpholine (0.316 mL, 2.87 mmol, 4.0 equiv) and EDC (0.234 g, 1.22 mmol, 1.7 equiv) were added sequentially. After stirring overnight at 23 °C, the reaction mixture was partitioned between brine (30 mL) and a 1:9 mixture of CH_3OH and CH_2Cl_2 (3 × 70 mL). The organic layers were dried over Na_2SO_4 and were concentrated. The residue was purified by flash column chromatography (55% EtOAc in hexanes) to provide **36** (0.337 g, 63%) as a white foam: $R_f = 0.24$ (60% EtOAc in hexanes); IR (cm^{-1}) 3293, 1717, 1668; ^1H NMR ($CDCl_3$) δ 0.82 (d, 3H, $J = 6.8$), 1.01 (d, 3H, $J = 6.8$), 1.30 (t, 3H, $J = 7.2$), 1.51−1.65 (m, 2H), 1.84−1.96 (m, 1H), 2.16−2.37 (m, 2H), 2.47 (s, 3H), 2.49−2.55 (m, 3H), 2.85−3.01 (m, 2H), 3.12−3.25 (m, 3H), 3.77 (s, 3H), 3.78 (s, 3H), 4.18 (q, 2H, $J = 7.2$), 4.31−4.49 (m, 3H), 4.65−4.70 (m, 1H), 5.53 (dd, 1H, $J = 15.7, 1.4$), 6.39−6.44 (m, 3H), 6.63 (dd, 1H, $J = 15.7, 5.4$), 6.93−7.01 (m, 2H), 7.05−7.10 (m, 1H), 7.12−7.18 (m, 2H), 7.24 (d, 1H, $J = 8.7$), 7.47 (d, 1H, $J = 6.5$). Anal. ($C_{40}H_{54}FN_3O_9 \cdot 0.50H_2O$) C, H, N.

Ethyl-3-{(5′-methylisoxazole-3′-carbonyl)-L-ValΨ[COCH$_2$]-L-Phe(4-F)-L-[(*N*-2,4-dimethoxybenzyl)-*(S)-Pyrrol-Ala*]}-E-propenoate (37). A solution of HCl in 1,4-dioxane (4.0 M, 5 mL) was added to a solution of **36** (0.309 g, 0.418 mmol, 1 equiv) in the same solvent (5 mL) at 23 °C. After the mixture was stirred for 1.5 h at 23 °C, the volatiles were removed under reduced pressure to give the crude amine salt. This material was dissolved in CH_2Cl_2 (15 mL) and cooled to 0 °C. 4-Methylmorpholine (0.101 mL, 0.919 mmol, 2.2 equiv) and 5-methylisoxazole-3-carbonyl chloride (0.067 g, 0.46 mmol, 1.1 equiv) were added sequentially, and the ice bath was removed. After being stirred for 1 h at 23 °C, the reaction mixture was partitioned between brine (15 mL) and a 1:9 mixture of CH_3OH and CH_2Cl_2 (3 × 30 mL). The organic layers were dried over Na_2SO_4 and were concentrated. The residue was purified by flash column chromatography (70% EtOAc in hexanes) to provide **37** (0.266 g, 85%) as a white foam: $R_f = 0.24$ (60% EtOAc in hexanes); IR (cm^{-1}) 3293, 1717, 1668; ^1H NMR ($CDCl_3$) δ 0.82 (d, 3H, $J = 6.8$), 1.01 (d, 3H, $J = 6.8$), 1.30 (t, 3H, $J = 7.2$), 1.51−1.65 (m, 2H), 1.84−1.96 (m, 1H), 2.16−2.37 (m, 2H), 2.47 (s, 3H), 2.49−2.55 (m, 3H), 2.85−3.01 (m, 2H), 3.12−3.25 (m, 3H), 3.77 (s, 3H), 3.78 (s, 3H), 4.18 (q, 2H, $J = 7.2$), 4.31−4.49 (m, 3H), 4.65−4.70 (m, 1H), 5.53 (dd, 1H, $J = 15.7, 1.4$), 6.39−6.44 (m, 3H), 6.63 (dd, 1H, $J = 15.7, 5.4$), 6.93−7.01 (m, 2H), 7.05−7.10 (m, 1H), 7.12−7.18 (m, 2H), 7.24 (d, 1H, $J = 8.7$), 7.47 (d, 1H, $J = 6.5$). Anal. ($C_{40}H_{49}FN_4O_9 \cdot 0.5H_2O$) C, H, N.

Ethyl-3-{(5′-methylisoxazole-3′-carbonyl)-L-ValΨ[COCH$_2$]-L-Phe(4-F)-L-[*(S)-Pyrrol-Ala*]}-E-propenoate (11). A suspension of DDQ (0.102 g, 0.449 mmol, 1.4 equiv), **37** (0.240 g, 0.320 mmol, 1 equiv), and water (2 drops) in $CHCl_3$ (10 mL) was refluxed for 20 h and then allowed to cool. The reaction mixture was diluted with CH_2Cl_2 (200 mL) and washed with a 2:1 mixture of saturated $NaHCO_3$ and 1 M NaOH (20 mL). The organic phase was dried over Na_2SO_4 and was concentrated. Purification of the residue by flash column chromatography (2% CH_3OH in CH_2Cl_2) gave **11** (0.074 g, 39%) as a faintly yellow solid: mp = 178−181 °C; $R_f = 0.49$ (10% CH_3OH in $CHCl_3$); IR (cm^{-1}) 3295, 1678 br; ^1H NMR ($CDCl_3$) δ 0.85 (d, 3H, $J = 6.8$), 1.03 (d, 3H, $J = 6.5$), 1.30 (t, 3H, $J = 7.2$), 1.51−1.62 (m, 1H), 1.71−1.93 (m, 2H), 2.27−2.40 (m, 2H), 2.47 (s, 3H), 2.51−2.75 (m, 3H), 2.82−2.98 (m, 2H), 3.11−3.24 (m, 1H), 3.26−3.42 (m, 2H), 4.18 (q, 2H, $J = 7.2$), 4.41−4.53 (m, 1H), 4.63−4.72 (m, 1H), 5.50 (d, 1H, $J = 15.4$), 5.88 (s,

1H), 6.39 (s, 1H), 6.63 (dd, 1H, J = 15.4, 5.3), 6.92−7.03 (m, 2H), 7.08−7.31 (m, 4H). Anal. ($C_{31}H_{39}FN_4O_7$) C, H, N.

Protease Selectivity Assays. Assays were performed using commercially available proteases (at ~10 nM concentrations) essentially as described by the supplier. Human liver cathepsin B, porcine erythrocyte calpain I, and human neutrophil elastase were purchased from Calbiochem. Bovine chymotrypsin and human thrombin were purchased from Boehringer Mannheim. Bovine trypsin was purchased from Sigma.

Degradation Studies of Compound 11. Compound **11** (25 μM) and 100 mM potassium phosphate buffer (pH 7.4) were preincubated in test tubes for 5 min at 37 °C in a shaking water bath. Chymotrypsin (Sigma, 5 units/mL) or porcine intestinal peptidase (Sigma, 0.1 units/mL) was preincubated at 37 °C and then added to the appropriate test tube containing **11** to initiate the reaction. After 0 and 60 min, a 200 μL aliquot of each incubation mixture was transferred to a microcentrifuge tube containing 200 μL of CH_3CN. These tubes were capped, vortexed, and centrifuged in an Eppendorf (Hamburg) 5415C centrifuge at 14 000 rpm for 10 min. A 15 μL portion of each supernatant was then injected onto a Hewlett-Packard 1100 HPLC equipped with a Zorbax RX C8 (2.1 m × 150 mm) column (DuPont, Wilmington, DE). The aqueous component of the mobile phase was 0.1% Et_3N in water adjusted to pH 3.5 with approximately 0.05% trifluoroacetic acid. Compound **11** was eluted using a solvent gradient beginning with 55:25:20 buffer:CH_3CN:CH_3OH, increasing to 20:60:20 buffer:CH_3CN:CH_3OH over 10 min and held for 10 min before returning to the original conditions at a flow rate of 0.3 mL/min. Compound **11** was detected by UV at 220 nm. The hydrolysis of 1-naphthyl butyrate served as a positive control for the esterase activity of the peptidase under study.

Plasma Stability Studies of Compound 11. Human plasma anticoagulated with CPDA (citrate phosphate dextrose adenine) was obtained from the San Diego Blood Bank, San Diego, CA. Heparinized plasma from several animal species was purchased from Covance Research, Denver, PA. The plasma was distributed into 5 mL cryotubes and frozen at −70 °C until analysis. In preparation for the experiment, 1990 μL of plasma (n = 3) or 1990 μL of 100 mM potassium phosphate pH 7.4 buffer (n = 3) were placed into separate test tubes. Because of enhanced plasma esterase activity relative to that of other species, rat plasma was diluted 1:1 with 100 mM potassium phosphate pH 7.4 buffer (n = 3) prior to the experiments. All tubes were preincubated in a shaking water bath for a few minutes until the solutions had reached 37 °C. At the start of the incubation, 10 μL of a freshly made 5 mM solution of compound **11** in CH_3CN was pipetted into a test tube containing plasma or buffer to achieve a final concentration of 25 μM. After vortexing the tube, a 200 μL sample was collected, and the tube was returned to the water bath. The test tubes were then incubated at 37 °C and 200 μL samples were collected periodically for 1 h. These samples were immediately transferred to borosilicate tubes prefilled with 2.0 mL of CH_3CN, and the mixtures were vortexed to ensure protein precipitation and termination of metabolic transformations. After subsequent centrifugation for 10 min at 4000 rpm at 10 °C in a Sorvall RT 7 centrifuge, the clear supernatant was decanted into a new set of borosilicate tubes and the volatiles were removed under a gentle stream of nitrogen using a Dri-Block sample concentrator (Techne, Princeton, NJ) at 40 °C. The samples were reconstituted in 250 μL of mobile phase (60% 25 mM $NH_4H_2PO_4$ at pH = 5.1 and 40% CH_3CN) and subjected to HPLC analysis.

Chromatographic analyses were performed using a 1100 Hewlett-Packard HPLC with a Primesphere reversed phase column (5 μm, 4.6 m × 150 mm, Phenomenex, Torrance, CA) at flow rate 1 mL/min and a gradient elution of $NH_4H_2PO_4$ 25 mM buffer, pH = 5.1/CH_3CN/CH_3OH (95/3/2 to 70/28/2 over 5 min, changing to 50/48/2 over the next 5 min, and reverting to 95/3/2 for the final 5 min). A sample volume of 100 μL was injected onto the column, and compound **11** was detected by UV absorption at 212 nm utilizing a Hewlett-Packard multiwavelength detector. The standard curve for compound **11** ranged from 0.05 μg/mL to 40.0 μg/mL. Using the above HPLC conditions, the retention time for compound **11** was approximately 13 min. The hydrolysis of 1-naphthyl butyrate served as a positive control for carboxyl esterase activity in human plasma.

Molecular Modeling Studies. A simple computational approach was employed to estimate the binding free energies of complexes formed between the HRV 3C protein (P) and various ligands (L). This approach assumes that a single protein−ligand conformation predominates in the bound complex while another dominates in solution. The protein−ligand association was expressed as a two-step process involving hypothetical bound conformations of the ligand and protein in solution, where the binding energy is given by the sum of an interaction term and two strain energies (eq 1). Unlike free energy perturbation methods, this approach neglects entropic contributions to binding affinity and represents a zero-temperature enthalpic estimate for the binding free energy. A simple entropic correction was therefore applied to the calculated binding affinities of the noncovalent encounter complexes to account for this fact.[21]

$$\Delta G = E_{LP}^{bound} - E_{L}^{unbound} - E_{P}^{unbound}$$

$$= [E_{LP}^{bound} - E_{L}^{bound} - E_{P}^{bound}] + [E_{L}^{bound} - E_{L}^{unbound}] + [E_{P}^{bound} - E_{P}^{unbound}]$$

$$= \Delta E_{LP}^{interaction} + \Delta E_{L}^{strain} + \Delta E_{P}^{strain} \quad (1)$$

The binding energy of the complex was obtained by local minimization using the Amber* force field and GB/SA solvation correction contained in the Macromodel (v5.5) software package.[31] The ligand was assigned partial charges and minimized in an enzyme active site that was reduced to 631 atoms to allow truncated-Newton conjugate gradient (TNCG) minimization. Constraints to the protein were applied in concentric spheres with atoms far from the ligand fixed rigidly in their crystallographic positions. Atoms near the ligand were constrained with increasing flexibility, utilizing a harmonic force constant of 400 kJ/Å at the outer edge of the site, 200 kJ/Å between 10 and 15 Å from the ligand, 10 kJ/Å between 5 and 10 Å from the ligand, and no constraints on atoms less than 5 Å from the ligand. The strain energies were obtained in an analogous manner.

Method No. 1. A minimized model of the covalent adduct between the glutamine-derived compound **1** and HRV 3CP was prepared using the HRV-2 3CP-**1** cocrystal structure.[5] Covalent models of the lactam-containing molecules **4**, **5**, and **6** were similarly constructed. Nonbonded protein−ligand interactions were assumed to determine binding trends in the series. No additional constraints were applied to the covalent connection between the protein and the ligand since bond-making and bond-breaking terms were assumed to cancel for each ligand.

Method No. 2. Noncovalent encounter complexes between HRV 3CP and compounds **1**, **4**, **5**, and **6** were constructed from the HRV-2 3CP-**1** co-crystal structure[5] and were investigated as described above. For these complexes, an entropic correction[21] was applied to the calculated binding affinities.

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(11) The loss in anti-3CP activity of **2** relative to **1** was approximately equal to the expected energetic difference between the cis and trans amide isomers of **2** (2.2 kcal/mol extrapolated from measured k_{obs}/[I] values vs 2.6 kcal/mol reported for simple model systems).[12] This analysis assumes that changes in k_{obs}/[I] values approximate changes in ligand−3CP binding affinity and that the anti-3CP activities of *cis*- and *trans*-**2** are similar to those displayed by compounds **1** and **3**, respectively.

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