Structure-Based Design, Synthesis, and Biological Evaluation of Irreversible Human Rhinovirus 3C Protease Inhibitors. 1. Michael Acceptor Structure−Activity Studies

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The structure-based design, chemical synthesis, and biological evaluation of peptide-derived human rhinovirus (HRV) 3C protease (3CP) inhibitors are described. These compounds incorporate various Michael acceptor moieties and are shown to irreversibly bind to HRV serotype 14 3CP with inhibition activities (k_{obs}/[I]) ranging from 100 to 600 000 M^{-1} s^{-1}. These inhibitors are also shown to exhibit antiviral activity when tested against HRV-14-infected H1-HeLa cells with EC_{50}'s approaching 0.50 μM. Extensive structure−activity relationships developed by Michael acceptor alteration are reported along with the evaluation of several compounds against HRV serotypes other than 14. A 2.0 Å crystal structure of a peptide-derived inhibitor complexed with HRV-2 3CP is also detailed.

Introduction

The human rhinoviruses (HRVs) belong to the picornavirus family and are the single most significant cause of the common cold.[1,2] These viruses translate a positive-strand RNA genome into a large polyprotein which undergoes co- and posttranslational processing by virally encoded proteases to produce the structural and enzymatic proteins required for viral replication.[3] Specifically, cleavage between several glutamine−glycine residues is effected by the human rhinovirus 3C protease (3CP),[3] a cysteine protease with structural similarity to the trypsin protein family but possessing minimal homology to prevalent mammalian enzymes.[4,5] Importantly, the activity of 3CP is essential for viral replication, and its active site residues are believed to be highly conserved throughout the more than 100 known rhinovirus serotypes.[4−7]

Currently, no effective therapy exists to directly treat rhinoviral infections, and the numerous viral serotypes make the development of a vaccine unlikely.[1a,1e] Previous approaches toward the identification of antirhinoviral therapeutics include the use of interferon,[1a,c,e] disruption of virus−receptor (ICAM-1) interactions,[1b] the examination of capsid-binding antipicornaviral compounds,[8] and the use of other miscellaneous agents.[7,9] In addition, several examples of 3CP inhibitors have recently been described in the literature, and some of these compounds exhibit antiviral properties.[10] Due to the importance of the 3C protease in viral replication, we also sought to develop inhibitors of this enzyme which might function as broad-spectrum antirhinoviral agents.

Inhibitor Design and Structure−Activity Studies

Inhibition studies commenced by examination of the peptide sequence of 3CP substrates. A minimal substrate composition was previously determined to be H_2N-Thr-Leu-Phe-Gln-Gly-Pro-CO_2H with cleavage occurring between the Gln and Gly residues (Figure 1).[11,12] It was reasoned that a truncated 3CP peptide substrate in which the scissile amide carbonyl was replaced with a Michael acceptor might function as an irreversible 3CP inhibitor (Figure 1). Similar analyses have produced potent, irreversible inhibitors of several other cysteine proteases.[13] Accordingly, incorporation of a trans-α,β-unsaturated methyl ester moiety into a substrate-derived, Cbz-protected tripeptide afforded a compound which displayed relatively potent irreversible inhibition of HRV-14 3CP (**1**, Table 1).[14] Equally important, compound **1** exhibited moderate antiviral activity (EC_{50}) when tested against H1-HeLa cells infected with HRV-14 and was nontoxic (CC_{50}) to the limits of its solubility.[15,16] In addition, the inhibitory properties of the molecule were not affected by short exposure to high concentrations of dithiothreitol (see the Experimental Section), suggesting that Michael acceptors of this type would not react readily with ubiquitous biological thiols (e.g., glutathione). These encouraging results prompted an extensive SAR study of peptide-derived Michael acceptors to better define this class of 3CP inhibitors.

The corresponding cis-α,β-unsaturated methyl ester (**2**) exhibited 10-fold lower anti-3CP activity and reduced antiviral properties relative to **1** (Table 1). Further efforts were therefore focused on molecules containing Michael acceptors with trans geometry. Inclusion of an ethyl ester in the inhibitor design (**3**) produced both a better enzyme inhibitor and a more potent antiviral agent, while incorporation of an α-fluoro substituent drastically reduced 3CP inhibitory activity (**4**). Importantly, compound **3** irreversibly inhibited 3CPs obtained from several rhinovirus serotypes other than HRV-14

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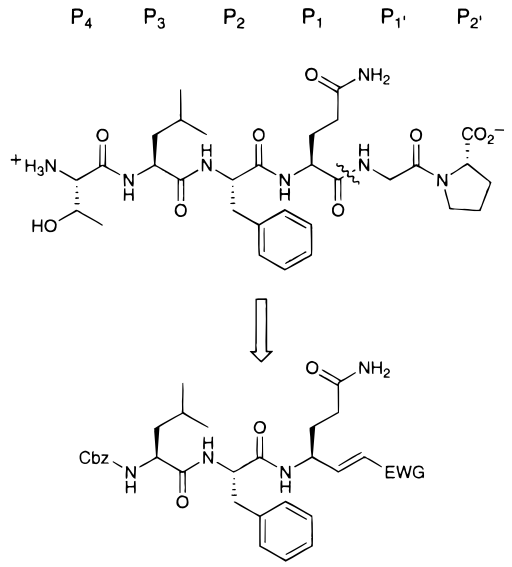


Figure 1. Design of irreversible HRV 3CP inhibitors. EWG = electron-withdrawing group.

and also exhibited antiviral properties when tested against these serotypes in cell culture. The related α,β-unsaturated carboxylic acid (**10**) was a poor 3CP inhibitor and did not exhibit antiviral activity at the highest concentration examined (100 μM). Compounds containing the simple esters described above were rapidly converted to the corresponding carboxylic acids upon incubation in rat plasma (Table 2), and efforts were therefore directed toward ester modification or replacement in order to increase the biological stability of the inhibitors under study.

Analysis of the X-ray crystal structure of compound **3** complexed with HRV-2 3CP (see below) suggested that additional substitution could be tolerated at the α-position of the Michael acceptor. Accordingly, inclusion of an α-methyl group in the inhibitor design (**5**) increased stability toward rat plasma (Table 2) but also reduced anti-3CP activity relative to compound **3**. The crystallographic analysis also indicated that additional functionality could be incorporated in the vicinity of the ethyl ester. Therefore, several other ester-derived Michael acceptors were evaluated in which the sterics opposite the α,β-unsaturated carbonyl moiety were altered (**6**−**9**). Most of these molecules displayed relatively good anti-3CP activity, particularly the benzyl analogue **8**, but some exhibited reduced stability toward rat plasma (Table 2, compound **7**). Importantly, the half-lives in rat plasma of all ester-containing compounds examined were sufficiently short to warrant the development of 3CP inhibitors which did not incorporate this functional group.

A series of amide-containing Michael acceptors was therefore also investigated (**11**−**26**, Table 1). These inhibitors typically displayed reduced anti-3CP activity, poorer antiviral activity, and/or increased toxicity compared to the esters described above. One exception was a molecule incorporating an indoline-derived Michael acceptor (**20**), and this discovery prompted an examination of inhibitors containing substituted indolines (**27**−**38**, Table 3). Although proper indoline modification increased anti-3CP activity to that observed for the corresponding ethyl ester (compare **3** and **29**), the antiviral activity displayed by all indoline-containing 3CP inhibitors did not substantially improve from that exhibited by the unsubstituted parent compound **20**. In addition to the indolines described above, amides derived from *N*,*O*-dialkylhydroxylamines were also potent 3CP inhibitors (**21**, **23**, and **24**). As expected, an indoline-containing compound displayed good stability toward rat plasma, but a related *N*,*O*-dimethylhydroxylamine amide was surprisingly short-lived (**20** and **21**, respectively, Table 2).

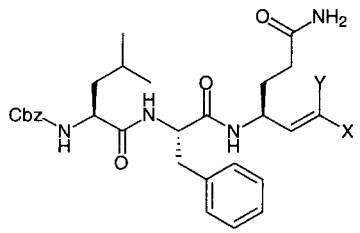
Several molecules incorporating aliphatic and aryl α,β-unsaturated ketones were also evaluated as 3CP inhibitors (**39**−**47**, Table 1). Most of these compounds displayed increased anti-3CP activity relative to the ester-containing inhibitors described above. However, the inhibitory activity of all ketone-derived Michael acceptors examined was substantially reduced or eliminated by short exposure to dithiothreitol, suggesting that such compounds might react rapidly with biological thiols (e.g., glutathione). In addition, the observed EC_{50}'s of the ketones were usually worse than those exhibited by related ester-containing compounds (see above) and were often not significantly distinct from toxic concentrations to be considered true antiviral activity ($CC_{50} > 10EC_{50}$). The ketone-containing molecules were also significantly more toxic than similar esters, possibly due to the greater reactivity of the ketones toward nonenzymatic thiols.

A variety of miscellaneous Michael acceptors were prepared in addition to the compounds described above (Table 1). Vinyl sulfones (**48** and **49**) displayed low levels of anti-3CP inhibition and were poor antiviral agents. Similar properties were exhibited by molecules which incorporated a vinyl nitrile (**50**), vinyl phosphonate (**53**), vinyl oxime (**52**), and several vinyl heterocycles (**54**−**56**). In contrast, a compound containing an α,β-unsaturated nitro moiety (**51**) demonstrated potent, irreversible 3CP inhibitory activity. However, this molecule was inactivated by exposure to dithiothreitol and did not display measurable antiviral properties. Likewise, compounds incorporating acyl lactam, acyl oxazolidinone, and acyl urea functionalities were also potent anti-3CP agents but were inactivated by exposure to nonenzymatic thiols (**57**, **58**, and **59**, respectively).

Since the preparation of compound **5** demonstrated that hydrocarbon substitution could be tolerated at the Michael acceptor α-position, the possibility of utilizing exocyclic α,β-unsaturated lactones as 3CP inhibitors was also examined (Table 4). The γ-lactone **60** exhibited irreversible 3CP inhibitory activity similar to that observed for ester **5** above and was stable toward rat plasma for much greater periods of time (Table 2). Surprisingly, the corresponding δ-lactone (**61**) displayed potent, *reversible* 3CP inhibition. The factors which determine whether a given lactone functions as a reversible or irreversible 3CP inhibitor are currently not understood. A molecule containing an *N*-acyl lactam-derived Michael acceptor (**62**) was a potent, irreversible 3CP inhibitor and exhibited good antiviral properties. However, the related carbamate and *N*-methoxy derivatives displayed reduced anti-3CP activity and were poor antiviral agents (compounds **63** and **64**, respectively).

Finally, compound **65** was prepared in an effort to assess the affinity of the peptidyl portion of the above

Table 1.



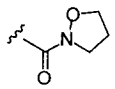
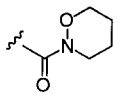
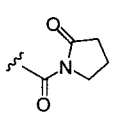
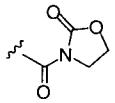
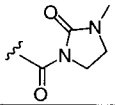
Compd No.	X	Y	Prep.[a]	Formula[b]	Serotype[c]	k_{obs}/[I] ($M^{-1}s^{-1}$)[d]	DTT inhib?[e]	EC_{50} (μM)[d]	CC_{50} (μM)[d]
1	CO_2CH_3	H	A	$C_{31}H_{40}N_4O_7$		20 000	no	1.3	>320
2	H	CO_2CH_3	A	$C_{31}H_{40}N_4O_7$		2 400	no	3.2	>320
3	$CO_2CH_2CH_3$	H	A	$C_{32}H_{42}N_4O_7$		25 000	no	0.54	>320
					16	6 500		2.3	
					2	2 000		1.6	
4	$CO_2CH_2CH_3$	F	A	$C_{32}H_{41}FN_4O_7$		60	ND	>320	>320
5	$CO_2CH_2CH_3$	CH_3	B	$C_{33}H_{44}N_4O_7 \cdot 0.75\ H_2O$		7 300	no	3	>320
6	CO_2Cyclopentyl	H	C	$C_{35}H_{46}N_4O_7$		14 600	no	0.56	>100
7	CO_2Cyclohexyl	H	C	$C_{36}H_{48}N_4O_7 \cdot 0.30\ H_2O$		3 700	ND	3.6	>320
8	CO_2CH_2Ph	H	C	$C_{37}H_{44}N_4O_7 \cdot 0.25\ H_2O$		39 400	ND	3.2	>100
9	$CO_2CH_2C(CH_3)_3$	H	C	$C_{35}H_{48}N_4O_7 \cdot 0.50\ H_2O$		9 800	ND	0.50	>100
10	CO_2H	H	B	$C_{30}H_{38}N_4O_7 \cdot 0.50\ H_2O$		30	ND	>100	>100
11	$C(O)NHCH_2CH_3$	H	C	$C_{32}H_{43}N_5O_6$		350	no	>320	>320
12	C(O)NHCyclopentyl	H	C	$C_{35}H_{47}N_5O_6 \cdot 1.3\ CH_2Cl_2$		250	ND	>100	>100
13	C(O)NHPh	H	C	$C_{36}H_{43}N_5O_6 \cdot 0.25\ H_2O$		200	ND	>320	>320
14	$C(O)N(CH_3)_2$	H	C	$C_{32}H_{43}N_5O_6 \cdot 0.60\ H_2O$		1 300	ND	56	>100
15	C(O)Pyrrolidine	H	C	$C_{34}H_{45}N_5O_6 \cdot 0.60\ H_2O$		2 100	ND	22	>100
16	C(O)3*R*-Pyrrolidinol	H	C	$C_{34}H_{45}N_5O_7 \cdot 0.75\ H_2O$		1 200	no	>320	>320
17	C(O)3-Pyrroline	H	C	$C_{34}H_{43}N_5O_6$		8 500	no	>44	44
18	$C(O)N(CH_3)Ph$	H	C	$C_{37}H_{45}N_5O_6$		580	ND	158	>320
19	C(O)Tetrahydroquinoline	H	C	$C_{39}H_{47}N_5O_7 \cdot 0.25\ H_2O$		1 000	ND	126	>320
20	C(O)Indoline	H	C	$C_{38}H_{45}N_5O_6 \cdot 1.0\ H_2O$		6 500	no	16	>320
21	$C(O)N(CH_3)OCH_3$	H	C	$C_{32}H_{43}N_5O_7 \cdot 0.60\ H_2O$		5 900	ND	4	>100
22	$C(O)N(CH_3)OH$	H	B	$C_{31}H_{41}N_5O_7$[f]		1 800	ND	42	42
23		H	C	$C_{33}H_{43}N_5O_7$		14 000	ND	45	>320
24		H	C	$C_{34}H_{45}N_5O_7 \cdot 0.50\ H_2O$		19 000	ND	16	100
25	C(O)Pyrrole	H	D	$C_{34}H_{41}N_5O_6$		400 000	ND	1.4	6.3
26	C(O)Indole	H	C	$C_{38}H_{43}N_5O_6 \cdot 0.20\ H_2O$		53 000	yes	1.8	29
39	$C(O)CH_3$	H	A	$C_{31}H_{40}N_4O_6$		54 000	yes	2.0	60
					16	8 900		ND	
					2	2 700		ND	
40	$C(O)C(CH_3)_3$	H	B	$C_{34}H_{46}N_4O_6 \cdot 1.25\ H_2O$		15 000	yes	1.7	5.6
41	C(O)Ph	H	B	$C_{36}H_{42}N_4O_6$		500 000	yes	4	16
42	C(O)Ph(4-OCH_3)	H	B	$C_{37}H_{44}N_4O_7$		150 000	yes	22	100
43	C(O)Ph[4-$N(CH_2CH_3)_2$]	H	B	$C_{40}H_{51}N_5O_6$		4 500	yes	>25	25
44	C(O)Ph(4-NO_2)	H	B	$C_{36}H_{41}N_5O_8 \cdot 0.50\ H_2O$		310 000	yes	32	32
45	C(O)Ph(4-CN)	H	B	$C_{37}H_{41}N_5O_6$		200 000	ND	50	>320
46	C(O)2-(1,3-Benzodioxole)	H	B	$C_{37}H_{42}N_4O_8$		120 000	ND	3.2	45
47	C(O)(2-Furan)	H	B	$C_{34}H_{40}N_4O_7 \cdot 0.50\ H_2O$		460 000	yes	2.4	19
48	SO_2CH_3	H	A	$C_{30}H_{40}N_4O_7S$		160	ND	>320	>320
49	SO_2Ph	H	A	$C_{35}H_{42}N_4O_7S$		270	ND	200	>320
50	CN[g]	H	A	$C_{30}H_{37}N_5O_5$		0	ND	18	>320
51	NO_2	H	D	$C_{29}H_{37}N_5O_7 \cdot 0.85\ CH_2Cl_2$		500 000	yes	>100	100
52	$C{=}N(OCH_3)$	H	D	$C_{31}H_{41}N_5O_6$		0	ND	100	>320
53	$P(O)(OCH_2CH_3)_2$	H	B	$C_{33}H_{47}N_4O_8P \cdot 2.5\ H_2O$		0	ND	>320	>320
54	2-Pyridine	H	B	$C_{34}H_{41}N_5O_5 \cdot 1.0\ H_2O$		120	ND	>100	>100
55	4-Oxazole	H	B	$C_{32}H_{39}N_5O_6 \cdot 0.50\ CHCl_3$		0	ND	>100	>100
56	5-Isoxazole	H	B	$C_{32}H_{39}N_5O_6 \cdot 0.30\ CHCl_3$		600	ND	>320	>320
57		H	C	$C_{34}H_{43}N_5O_7 \cdot 1.5\ H_2O$		126 000	yes	0.89	56

Table 1. (Continued)

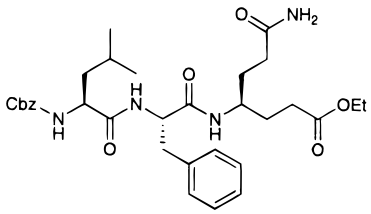
Compd No.	X	Y	Prep.[a]	Formula[b]	Serotype[c]	k_{obs}/[I] ($M^{-1}s^{-1}$)[d]	DTT inhib?[e]	EC_{50} (μM)[d]	CC_{50} (μM)[d]
58		H	C	$C_{33}H_{41}N_5O_8$		600 000	yes	1.6	>100
59		H	C	$C_{34}H_{44}N_6O_7 \cdot 1.0\ H_2O$		312 000	yes	5.0	>320

[a] Method of preparation: A = Scheme 1, B = Scheme 2, C = Scheme 3, D = see the Experimental Section. [b] Elemental analyses (C, H, N) of all compounds agreed to within ±0.4% of theoretical values unless otherwise noted. [c] Serotype 14 unless otherwise noted. [d] See the Experimental Section for assay method and error. [e] Indicates partial or complete loss of 3CP inhibitory activity after exposure of compound to 5 mM dithiothreitol for 2−3 min at 23 °C. [f] C, calcd 62.50, found 63.31; N, calcd 11.76, found 11.20. [g] 1:1 mixture of diastereomers. ND = not determined.

Table 2. Stability of Several 3CP Inhibitors in Rat Plasma

compd	$t_{1/2}$ rat plasma (min)	compd	$t_{1/2}$ rat plasma (min)
3	10	**20**	>80
5	20	**21**	12
7	4	**60**	>240

inhibitors for the target protease. This compound was a relatively weak, reversible 3CP inhibitor ($K_i = 20\ \mu M$) and did not display antiviral properties when tested to its solubility limit (100 μM). These results demonstrate



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the importance of the Michael acceptor moiety in the design of potent 3CP inhibitors and suggest that covalent 3CP modification is required for obtaining such inhibitors which function as highly active antirhinoviral agents.

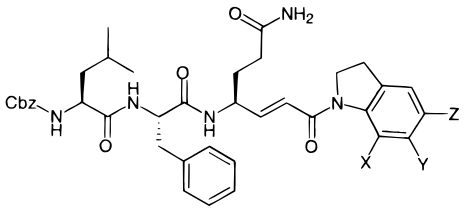
X-ray Structure Analysis

The iterative analysis of protein−ligand interactions by X-ray crystallography is an essential element of a structure-based inhibitor design program.[17] Accordingly, several crystal structures of 3CP−inhibitor− complexes were obtained during the development of the Michael acceptor-containing compounds described above. These structures confirmed the binding geometries that the inhibitors adopted when complexed with 3CP and suggested locations for Michael acceptor modification. However, the X-ray data were utilized somewhat cautiously for inhibitor design since the structures depicted the covalent protein−inhibitor product and not the presumably more-relevant transition state for adduct formation. The specific details of one protein−inhibitor complex are discussed below.

The 2.0 Å X-ray crystal structure of the covalent adduct formed between compound **3** and HRV-2 3CP[18] is shown in Figure 2, and key protein−inhibitor interactions are illustrated in Figure 3. In general, the inhibitor bound to the enzyme in a manner similar to that predicted by substrate modeling[4a,10c] and filled a series of shallow grooves on the protein surface N-terminal to the scissile amide bond of the substrate (S_1−S_4).[12] As expected, a covalent bond was observed between the 3CP active site cysteine residue (Cys-147) and the β-carbon of the Michael acceptor of **3**. A hydrogen bond between the carbonyl moiety of the Michael acceptor and the 3CP Cys-147 amide NH was also evident in the above complex. It is uncertain whether this interaction facilitates Michael addition of the cysteine to **3** or merely arises after the covalent adduct has been formed. Interestingly, two equally populated conformations were observed for two 3CP residues flanking the enzyme active site. These residues (Ser-144 and Gly-145) are believed to help stabilize the tetrahedral transition state which arises during peptide hydrolysis. One conformation, in which the Gly-145 amide NH pointed toward **3**, resembled that observed crystallographically for the corresponding residues of many trypsin-like serine proteases complexed with peptidyl inhibitors (Figure 2, shown in yellow).[19] The other involved rotation of Ser-144 so that its carbonyl moiety pointed toward the inhibitor and the Gly-145 amide NH faced the solvent. Similar alternate conformations of related residues have been observed crystallographically in other protease−inhibitor complexes,[10a,19] but the relevance of this conformational variance toward 3CP inhibitor design has not been fully evaluated.

Several other important protein−ligand interactions were observed in addition to those noted in the vicinity of the active site cysteine. The glutamine amide of **3** formed hydrogen bonds with the His-161 side chain and both the Thr-142 side chain and amide carbonyl in the bottom and on the edge of the 3CP S_1 binding pocket, respectively.[12] The inhibitor phenylalanine residue bound to 3CP in a canyon formed by the side chains of Leu-127, Ser-128, and Asn-130 on one side and His-40 on the other. In contrast, the leucine side chain of **3** was quite solvent exposed and did not make appreciable contacts with the enzyme. This observation suggested that a variety of amino acids could be tolerated at this location within the inhibitor design.[20] The N-terminal Cbz moiety of the inhibitor was situated in a hydrophobic pocket on the surface of the protein formed by the side chains of Tyr-122, Ile-125, and Phe-170. However, the interaction of this portion of **3** with 3CP appeared to be suboptimal as evidenced by significant gaps between the protein and the ligand.[21] An antiparallel β-sheet hydrogen bonding interaction was also noted

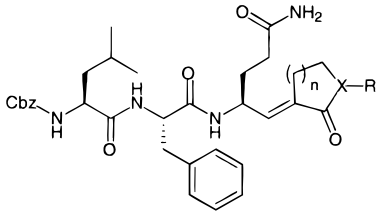
Table 3.



compd no.	X	Y	Z	prep[a]	formula[b]	k_{obs}/[I] (M^{-1} s^{-1})[c]	DTT inhib?[d]	EC_{50} (μM)[c]	CC_{50} (μM)[c]
20	H	H	H	C	$C_{38}H_{45}N_5O_6 \cdot 1.0H_2O$	6 500	no	16	>320
27	H	H	NO_2	C	$C_{38}H_{44}N_6O_8 \cdot 0.50H_2O$	12 000	no	17	>320
28	H	NO_2	H	C	$C_{38}H_{44}N_6O_8 \cdot 0.50H_2O$	29 000	no	>320	>320
29	H	$SO_2N(CH_3)_2$	H	C	$C_{40}H_{50}N_6O_8S \cdot 0.50H_2O$	28 300	no	>100	ND
30	H	SO_2Pyrrolidine	H	C	$C_{42}H_{52}N_6O_8S \cdot 0.25H_2O$	22 700	ND	>320	>320
31	H	SO_2CH_3	H	C	$C_{39}H_{47}N_5O_8S \cdot 0.75H_2O$	12 900	ND	>320	>320
32	H	H	F	C	$C_{38}H_{44}FN_5O_6 \cdot 0.50H_2O$	5 400	ND	11	>320
33	Cl	H	H	C	$C_{38}H_{44}ClN_5O_6 \cdot 0.50H_2O$	5 400	ND	56	>320
34	H	H	Cl	C	$C_{38}H_{44}ClN_5O_6 \cdot 0.50H_2O$	450	ND	5.6	56
35	H	H	Br	C	$C_{38}H_{44}BrN_5O_6$	5 800	ND	47	>320
36	H	H	OCH_3	C	$C_{39}H_{47}N_5O_7 \cdot 0.50H_2O$	3 400	ND	>320	>320
37	SO_2CH_3	H	H	C	$C_{39}H_{47}N_5O_8S \cdot 1.0H_2O$	≪1 000	ND	>320	>320
38	SCH_3	H	H	C	$C_{39}H_{47}N_5O_6S \cdot 1.8H_2O$	300	ND	ND	ND

[a] Method of preparation: C = Scheme 3. [b] Elemental analyses (C, H, N) of all compounds agreed to within ±0.4% of theoretical values. [c] Serotype 14; see the Experimental Section for assay method and error. [d] Indicates partial or complete loss of 3CP inhibitory activity after exposure of compound to 5 mM dithiothreitol for 2−3 min at 23 °C. ND = not determined.

Table 4.



compd no.	X	R	*n*	prep[a]	formula[b]	serotype[c]	k_{obs}/[I] (M^{-1} s^{-1})[d]	DTT inhib?[e]	EC_{50} (μM)[d]	CC_{50} (μM)[d]
60	O		1	A	$C_{32}H_{40}N_4O_7$		10 900	no	5.2	>320
61	O		2	A	$C_{33}H_{42}N_4O_7 \cdot 0.50H_2O$		K_i = 30 nM	ND	16	>100
62	N	$C(O)CH_3$	1	A	$C_{34}H_{43}N_5O_7 \cdot 0.50H_2O$		155 500	no	0.71	>100
						16	39 000		ND	
						2	13 000		ND	
63	N	CO_2CH_3	1	A	$C_{34}H_{43}N_5O_8 \cdot 0.75H_2O$		8 400	no	28	>100
64	N	OCH_3	1	A	$C_{33}H_{43}N_5O_7 \cdot 0.50H_2O$		800	ND	18	>100

[a] Method of preparation: A = Scheme 1. [b] Elemental analyses (C, H, N) of all compounds agreed to within ±0.4% of theoretical values. [c] Serotype 14 unless otherwise noted. [d] See the Experimental Section for assay method and error. [e] Indicates partial or complete loss of 3CP inhibitory activity after exposure of compound to 5 mM dithiothreitol for 2−3 min at 23 °C. ND = not determined.

between the leucine residue and Gly-164 (Figure 3). Similar interactions were previously observed in crystal structures of several trypsin-like serine proteases complexed with peptidyl inhibitors.[19] The two other backbone amide NHs present in **3** formed hydrogen bonds with 3CP Val-162 and Ser-128, while an additional hydrogen bond between the inhibitor Cbz carbonyl moiety and the 3CP Ser-128 amide NH was also evident (Figure 3). Thus, a total of nine hydrogen bonds involving every inhibitor amino acid residue were observed in the complex between compound **3** and HRV-2 3CP.

Analysis of the above complex suggested several possibilities for Michael acceptor modification. The Michael acceptor α-position was solvent exposed and did not appreciably contact 3CP. Inclusion of additional functionalities at this location in the inhibitor design was therefore predicted not to significantly diminish anti-3CP activity. This prediction was verified with the preparation of compounds **5**, **60**, and **62** described above. Likewise, replacement of the ethyl ester present in **3** with larger moieties was anticipated to have a minimal effect on inhibitor potency. Again, this expectation was confirmed by the synthesis of many potent 3CP inhibitors containing Michael acceptors other than an α,β-unsaturated ethyl ester (Tables 1 and 3).

Synthesis

Most of the peptide-derived Michael acceptors utilized in this study were prepared by three related methods (A, B, and C). The particular method used to synthesize a given compound is indicated in Tables 1, 3, and 4. Representative synthetic examples are described below and are detailed in the Experimental Section. Preparations of compounds which either did not involve one of the general methods described above or included additional transformations are also given in the Experimental Section (Table 1, method D).

The first synthetic route (A) is illustrated by the preparation of compound **3** (Scheme 1). *N*-α-Boc-γ-trityl-L-glutamine was transformed into aldehyde **67** by reduction of the corresponding Weinreb amide (**66**).[22,23]

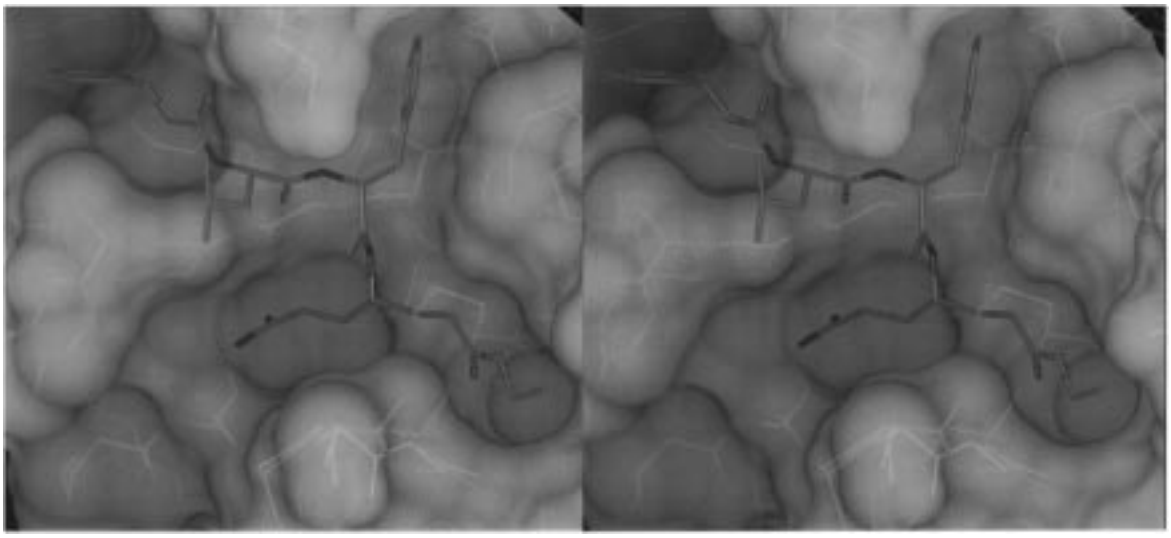


Figure 2. Stereoview of the crystal structure of **3** complexed with HRV-2 3CP. A portion of the water-accessible surface of the protein is shown in gray. An alternate conformation observed for several 3CP residues is superimposed and is shown in yellow.

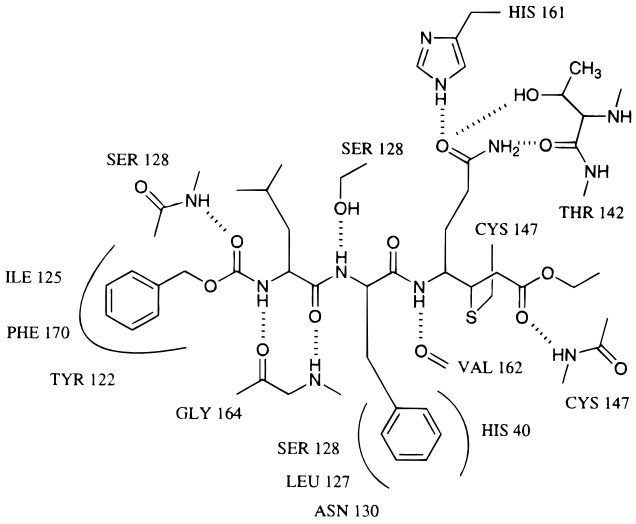


Figure 3. Schematic diagram of **3** bound in the HRV-2 3CP active site. Hydrogen bonds are represented as dashed lines, and the residues which make up the enzyme binding subsites are depicted.

The crude aldehyde thus obtained was converted to the desired Michael acceptor by reaction with the sodium enolate of triethyl phosphonoacetate to afford ethyl ester **68** in good yield. For the preparation of compounds in Tables 1 and 4 other than **3**, the desired olefination was accomplished by reaction of **67** with an appropriate phosphonate anion or phosphonium ylide.[24] Removal of the Boc protecting group from **68** under acidic conditions (HCl) and carbodiimide-mediated coupling of the resulting crude amine salt with commercially available Cbz-L-Leu-L-Phe-OH provided tripeptide **69** in moderate yield.[25] The trityl protecting group was removed by short exposure of **69** to trifluoroacetic acid (TFA) in the presence of triisopropylsilane to give compound **3** in good yield.[26] The tripeptide inhibitors prepared in this study were typically isolated as white solids by removal of the volatiles from the detritylation reaction mixture, trituration of the resulting oil with Et_2O, and subsequent filtration.

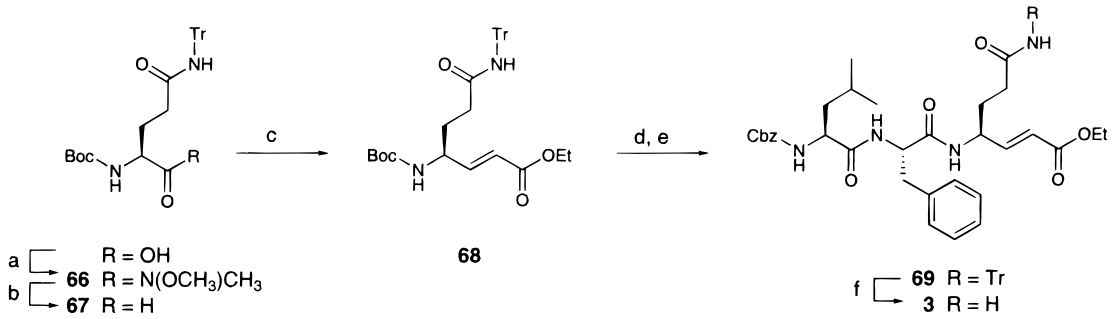
An illustration of the second preparative method (B) is provided by the synthesis of compound **5** (Scheme 2). This method allowed Michael acceptor diversification to be effected at a later synthetic step than in the preparation described above for compound **3**. Thus, *N*-α-Cbz-γ-trityl-L-glutamine was converted in several steps to aldehyde **70** by the reported literature method.[10a] Crude **70** was then subjected to a Wittig reaction employing (carbethoxyethylidene)triphenylphosphorane to provide olefin **71** after purification on silica gel. As before, the trityl protecting group was removed by short treatment of **71** with TFA to give compound **5** in moderate yield. As noted for method A, a variety of Michael acceptors could be prepared in this manner by utilization of the appropriate triphenylphosphoranes for reaction with aldehyde **70** (Table 1).[27]

The final general synthesis method (C) is depicted by the preparations of compounds **6** and **13** (Scheme 3). This method was employed to prepare esters and amides for which the appropriate phosphonoacetate or triphenylphosphorane was not commercially available. Ethyl ester **68** was carefully hydrolyzed under basic conditions to afford carboxylic acid **72** with a minimal amount of racemization.[28] This material was not purified, but was esterified with cyclopentanol to provide ester **73** in moderate yield. Again, removal of the Boc protecting group under acidic conditions and coupling of the resulting amine salt with Cbz-L-Leu-L-Phe-OH afforded the corresponding tripeptide (**74**) in moderate yield after purification on silica gel. Alternatively, carboxylic acid **72** was condensed with aniline to give amide **75** which was subsequently transformed into tripeptide **76** in a manner analogous to that used for the preparation of **74**. Compounds **6** and **13** were prepared from intermediates **74** and **76**, respectively, by brief exposure to TFA in the presence of triisopropylsilane. A variety of α,β-unsaturated esters and amides could be prepared via method C by derivitization of carboxylic acid **72** with an appropriate alcohol or amine (Tables 1 and 3).

Conclusions

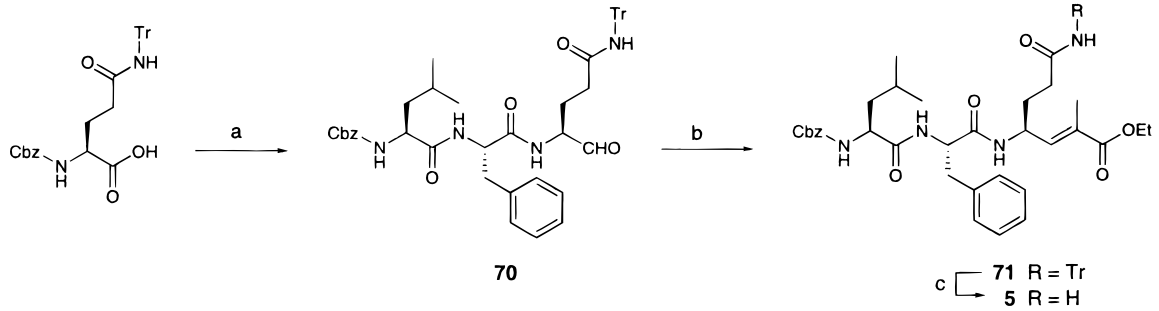
The ability of peptide-derived Michael acceptors to function as potent inhibitors of human rhinovirus 3C protease was demonstrated by the synthesis and biological evaluation of the compounds described above.

Scheme 1[a]



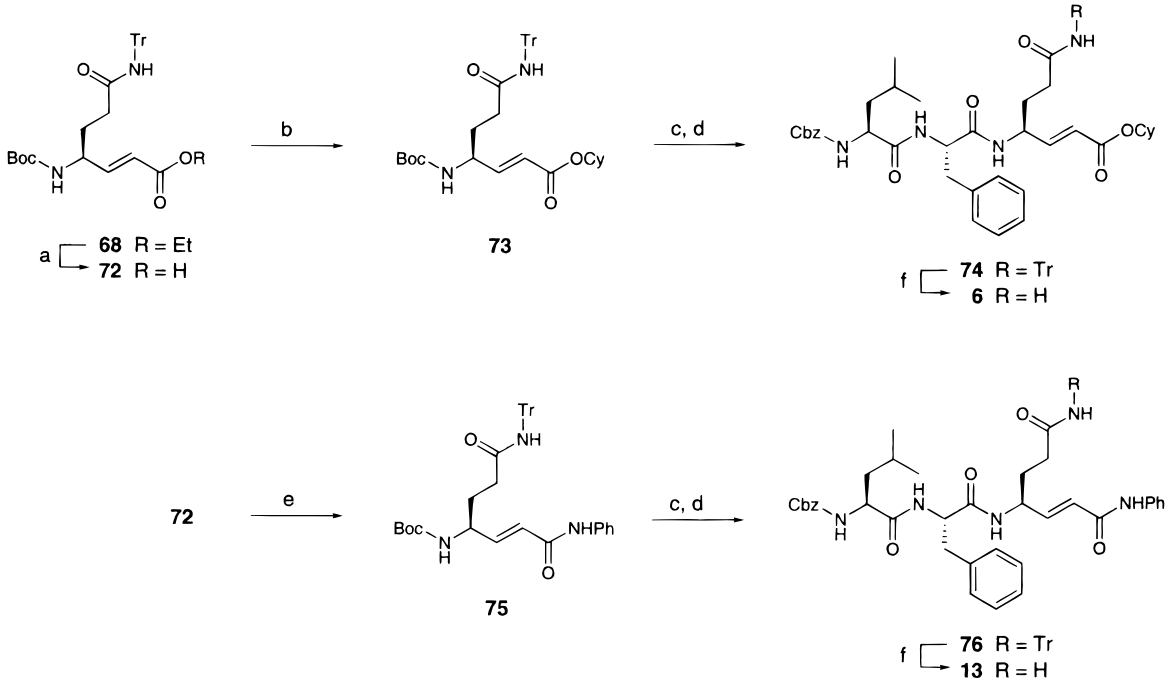
[a] Reagents and conditions (Tr = CPh_3): (a) 1.0 of equiv isobutyl chloroformate, 1.0 equiv of $HCl{\cdot}HN(OCH_3)CH_3$, 2.0 equiv of NMM, CH_2Cl_2, 0 °C, 20 min → 23 °C, 2 h, 82%; (b) 2.5 equiv DIBAL, THF, −78 °C, 4 h, 97%; (c) 1.0 equiv of $(EtO)_2POCH_2CO_2Et$, 1.0 equiv of $NaN(TMS)_2$, THF, −78 °C, 20 min, then **67**, −78 °C, 2 h → 0 °C, 10 min, 88%; (d) HCl in 1,4-dioxane, 23 °C, 3 h; (e) 1.0 equiv of Cbz-L-Leu-L-Phe-OH, 1.5 equiv of HOBT, 4.0 equiv of NMM, 1.5 equiv of EDC, CH_2Cl_2, 23 °C, 18 h, 83%; (f) 5.0 equiv of $(i\text{-}Pr)_3SiH$, 1:1 TFA:CH_2Cl_2, 23 °C, 20 min, 81%.

Scheme 2[a]



[a] Reagents and conditions (Tr = CPh_3): (a) ref 10a; (b) 1.1 equiv of $Ph_3P{=}C(CH_3)CO_2Et$, THF, 23 °C, overnight; (c) 1:10 TFA:CH_2Cl_2, 23 °C, 4 h, 35%.

Scheme 3[a]



[a] Reagents and conditions (Tr = CPh_3, Cy = cyclopentyl): (a) 2.3 equiv of NaOH, EtOH, 23 °C, 3 h, 98%; (b) 1.1 equiv of cyclopentanol, 0.10 equiv of DMAP, 1.05 equiv of EDC, CH_2Cl_2, 23 °C, overnight, 20%; (c) HCl in 1,4-dioxane, 23 °C, 2 h; (d) 1.0 equiv of Cbz-L-Leu-L-Phe-OH, 1.5 equiv of HOBT, 4.0 equiv of NMM, 1.5 equiv of EDC, CH_2Cl_2, 23 °C, overnight, 59%; (e) 1.0 equiv of isobutyl chloroformate, 2.0 equiv of NMM, 1.0 equiv of $PhNH_2$, CH_2Cl_2, 0 °C, 20 min → 23 °C, 24 h, 20%; (f) 5.0 equiv of $(i\text{-}Pr)_3SiH$, 1:1 TFA:CH_2Cl_2, 23 °C, 1 h, 56%.

These molecules irreversibly inhibited 3CP's from several HRV serotypes and exhibited antiviral activity when tested against these serotypes in cell culture. Crystallographic analysis of an enzyme−inhibitor complex confirmed the binding orientation of these compounds and suggested modifications for inhibitor design.

The use of such structural information to further improve this class of 3CP inhibitors is described in the following paper.

Experimental Section

General. All reactions were performed in septum-sealed flasks under a slight positive pressure of argon unless otherwise noted. All commercial reagents were used as received from their respective suppliers with the following exceptions. Tetrahydrofuran (THF) was distilled from sodium−benzophenone ketyl prior to use. Dichloromethane (CH_2Cl_2) was distilled from calcium hydride prior to use. Flash column chromatography[29] was performed using silica gel 60 (Merck Art 9385). ^{1}H NMR spectra were recorded at 300 MHz utilizing either a Varian UNITY*plus* 300 or a General Electric QE-300 spectrometer equipped with Techmag operating software. Chemical shifts are reported in ppm (δ) downfield relative to internal tetramethylsilane, and coupling constants are given in hertz. Infrared absorption spectra were recorded using either a MIDAC Corp. or a Perkin-Elmer 1600 series FTIR. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Melting points were determined using a Mel-Temp II apparatus and are uncorrected.

A simplified naming system employing amino acid abbreviations is used to identify some intermediates and final products. In this naming system, italicized amino acid abbreviations represent modifications at the C-terminus of that residue where the following apply: (1) acrylic acid esters are reported as "*E*" (trans) propenoates; (2) acrylamides are reported as "*E*" propenamides. The *N*,*O*-dialkylhydroxylamines required for the preparation of compounds **23** and **24** were prepared by literature methods.[30] The noncommercially available indolines utilized in the synthesis of compounds **27**−**38** were prepared by reduction of the corresponding indoles (**34**−**36**),[31] lithiation of 1-Boc-indolines (**33**, **37**, and **38**),[32] or substitution of 1-acyl-5-bromoindoline (**29**−**31**).[33] The triphenylphosphoranes required for the preparation of compounds **42**−**47** and **54**−**56** were synthesized from the corresponding α-bromo ketones,[34] while those required for inhibitors **60**−**64** were prepared by related literature methods.[35,36] The phosphonates utilized in the synthesis of compounds **48** and **49** were also prepared by literature methods.[37]

Representative Example of Preparation Method A. Synthesis of Ethyl 3-(Cbz-L-Leu-L-Phe-L-*Gln*)-(*E*)-propenoate (3). [Boc-L-(Tr-Gln)]-N(OMe)Me (66). Isobutyl chloroformate (4.77 mL, 36.8 mmol, 1.0 equiv) was added to a solution of *N*-α-Boc-γ-trityl-L-glutamine (18.7 g, 36.7 mmol, 1 equiv) and 4-methylmorpholine (8.08 mL, 73.5 mmol, 2.0 equiv) in CH_2Cl_2 (250 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 20 min, and then *N*,*O*-dimethylhydroxylamine hydrochloride (3.60 g, 36.7 mmol, 1.0 equiv) was added. The resulting solution was stirred at 0 °C for 20 min and at 23 °C for 2 h and then was partitioned between water (150 mL) and CH_2Cl_2 (2 × 150 mL). The combined organic layers were dried over Na_2SO_4 and were concentrated. Purification of the residue by flash column chromatography (gradient elution, 40 → 20% hexanes in EtOAc) provided **66** (16.1 g, 82%) as a white foam: R_f = 0.22 (50% EtOAc in hexanes); IR (cm^{-1}) 3411, 3329, 3062, 1701, 1659; ^{1}H NMR ($CDCl_3$) δ 1.42 (s, 9H), 1.63−1.77 (m, 1H), 2.06−2.17 (m, 1H), 2.29−2.43 (m, 2H), 3.17 (s, 3H), 3.64 (s, 3H), 4.73 (s, br, 1H), 5.38−5.41 (m, 1H), 7.20−7.31 (m, 15H). Anal. ($C_{31}H_{37}N_3O_5$) C, H, N.

[Boc-L-(Tr-Gln)]-H (67). Diisobutylaluminum hydride (50.5 mL of a 1.5 M solution in toluene, 75.8 mmol, 2.5 equiv) was added to a solution of **66** (16.1 g, 30.3 mmol, 1 equiv) in THF at −78 °C, and the reaction mixture was stirred at −78 °C for 4 h. Methanol (4 mL) and 1.0 M HCl (10 mL) were added sequentially, and the mixture was warmed to 23 °C. The resulting suspension was diluted with Et_2O (150 mL) and was washed with 1.0 M HCl (3 × 100 mL), half-saturated $NaHCO_3$ (100 mL), and water (100 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated to give crude **67** (13.8 g, 97%) as a white solid: mp 114−116 °C; R_f = 0.42 (50% EtOAc in hexanes); IR (cm^{-1}) 3313, 1697, 1494; ^{1}H NMR ($CDCl_3$) δ 1.44 (s, 9H), 1.65−1.75 (m, 1H), 2.17−2.23 (m, 1H), 2.31−2.54 (m, 2H), 4.11 (s, br, 1H), 5.38−5.40 (m, 1H), 7.11 (s, 1H), 7.16−7.36 (m, 15H), 9.45 (s, 1H).

Ethyl 3-[Boc-L-(Tr-*Gln*)]-(*E*)-propenoate (68). Sodium bis(trimethylsilyl)amide (22.9 mL of a 1.0 M solution in THF, 22.9 mmol, 1.0 equiv) was added to a solution of triethyl phosphonoacetate (5.59 g, 22.9 mmol, 1.0 equiv) in THF (200 mL) at −78 °C, and the resulting solution was stirred for 20 min at that temperature. Crude **67** (10.8 g, 22.9 mmol, 1 equiv) in THF (50 mL) was added via cannula, and the reaction mixture was stirred for 2 h at −78 °C, warmed to 0 °C for 10 min, and partitioned between 0.5 M HCl (150 mL) and a 1:1 mixture of EtOAc and hexanes (2 × 150 mL). The combined organic layers were dried over Na_2SO_4 and were concentrated. Purification of the residue by flash column chromatography (40% EtOAc in hexanes) provided **68** (10.9 g, 88%) as a white foam: R_f = 0.60 (50% EtOAc in hexanes); IR (cm^{-1}) 3321, 1710; ^{1}H NMR ($CDCl_3$) δ 1.27 (t, 3H, J = 7.2), 1.42 (s, 9H), 1.70−1.78 (m, 1H), 1.80−1.96 (m, 1H), 2.35 (t, 2H, J = 7.0), 4.18 (q, 2H, J = 7.2), 4.29 (s, br, 1H), 4.82−4.84 (m, 1H), 5.88 (dd, 1H, J = 15.7, 1.6), 6.79 (dd, 1H, J = 15.7, 5.3), 6.92 (s, 1H), 7.19−7.34 (m, 15H). Anal. ($C_{33}H_{38}N_2O_5$) C, H, N.

Ethyl 3-[Cbz-L-Leu-L-Phe-L-(Tr-*Gln*)]-(*E*)-propenoate (69). A solution of HCl in 1,4-dioxane (4.0 M, 20 mL) was added to a solution of **68** (1.00 g, 1.84 mmol, 1 equiv) in the same solvent (20 mL) at 23 °C. After 3 h, the volatiles were removed under reduced pressure. The residue was dissolved in CH_2Cl_2 (50 mL), and Cbz-L-Leu-L-Phe-OH (0.759 g, 1.84 mmol, 1.0 equiv), 1-hydroxybenzotriazole hydrate (HOBt, 0.373 g, 2.76 mmol, 1.5 equiv), 4-methylmorpholine (0.809 mL, 7.36 mmol, 4.0 equiv), and 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC, 0.529 g, 2.76 mmol, 1.5 equiv) were added sequentially. The reaction mixture was stirred at 23 °C for 18 h and then was partitioned between water (150 mL) and EtOAc (2 × 150 mL). The combined organic layers were dried over Na_2SO_4 and were concentrated. Flash chromatographic purification of the residue (5% CH_3OH in CH_2Cl_2) afforded **69** (1.25 g, 83%) as a white solid: mp 192−194 °C; R_f = 0.33 (5% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3295, 1696, 1678, 1655, 1519; ^{1}H NMR ($CDCl_3$) δ 0.84 (d, 3H, J = 6.5), 0.86 (d, 3H, J = 6.5), 1.24−1.32 (m, 1H), 1.28 (t, 3H, J = 7.2), 1.43−1.75 (m, 3H), 1.91−2.06 (m, 1H), 2.20−2.38 (m, 2H), 2.93−3.02 (m, 1H), 3.07−3.18 (m, 1H), 3.95−4.02 (m, 1H), 4.17 (q, 2H, J = 7.2), 4.43−4.55 (m, 2H), 4.82−4.95 (m, 2H), 5.69 (d, 1H, J = 15.7), 6.46 (d, 1H, J = 7.5), 6.60 (d, 1H, J = 8.1), 6.69 (dd, 1H, J = 15.7, 5.1), 7.09−7.38 (m, 27H). Anal. ($C_{51}H_{56}N_4O_7$) C, H, N.

Ethyl 3-(Cbz-L-Leu-L-Phe-L-*Gln*)-(*E*)-propenoate (3). Trifluoroacetic acid (20 mL) was added to a solution of **69** (1.25 g, 1.49 mmol, 1 equiv) and triisopropylsilane (1.53 mL, 7.47 mmol, 5.0 equiv) in CH_2Cl_2 (20 mL) at 23 °C, producing a bright yellow solution. The reaction mixture was stirred for 20 min at 23 °C during which time it became colorless. Carbon tetrachloride (20 mL) was added, and the volatiles were removed under reduced pressure. The residue was triturated with Et_2O (20 mL), and the resulting white solid was collected by vacuum filtration, washed with Et_2O (3 × 50 mL), and air-dried to afford **3** (0.717 g, 81%): mp 219−221 °C; R_f = 0.17 (5% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3300, 1672, 1535; ^{1}H NMR (DMSO-d_6) δ 0.78 (d, 3H, J = 6.8), 0.82 (d, 3H, J = 6.5), 1.21 (t, 3H, J = 7.0), 1.25−1.37 (m, 2H), 1.42−1.54 (m, 1H), 1.58−1.80 (m, 2H), 2.02−2.09 (m, 2H), 2.84 (dd, 1H, J = 13.2, 8.9), 2.97 (dd, 1H, J = 13.2, 5.8), 3.93−4.01 (m, 1H), 4.11 (q, 2H, J = 7.0), 4.33−4.52 (m, 2H), 4.97 (d, 1H, J = 12.3), 5.04 (d, 1H, J = 12.3), 5.64 (d, 1H, J = 15.9), 6.69 (dd, 1H, J = 15.9, 5.4), 6.76 (s, 1H), 7.13−7.37 (m, 11H), 7.43 (d, 1H, J = 7.8), 7.99 (d, 1H, J = 8.1), 8.04 (d, 1H, J = 8.1). Anal. ($C_{32}H_{42}N_4O_7$) C, H, N.

Representative Example of Preparation Method B. Ethyl 2-Methyl-3-(Cbz-L-Leu-L-Phe-L-*Gln*)-(*E*)-propenoate (5). (Carbethoxyethylidene)triphenylphosphorane (0.29 g, 0.78 mmol, 1.1 equiv) was added to a solution of crude **70**[10a] (0.54 g, 0.71 mmol, 1 equiv) in THF (14 mL) at 23 °C. After the mixture was stirred overnight, the volatiles were removed

under reduced pressure and the residue was chromatographed on silica gel (gradient elution, 0 → 2% CH_3OH in $CHCl_3$) to give **71** (0.52 g) contaminated with triphenylphosphine oxide. This material was dissolved in CH_2Cl_2 (12 mL) at 23 °C, and trifluoroacetic acid (1.2 mL) was added. After 4 h of stirring at 23 °C, the reaction mixture was concentrated and the residue was triturated with a 1:1 mixture of EtOAc and Et_2O (10 mL). The resulting white precipitate was filtered, washed with Et_2O (2 × 40 mL), and dried under vacuum to give **5** (0.15 g, 35%) as an off-white solid: mp 187−188 °C; R_f = 0.24 (5% CH_3OH in $CHCl_3$); IR (cm^{-1}) 1707, 1647, 1541, 1261; ^1H NMR (DMSO-d_6) δ 0.79 (d, 3H, J = 6.3), 0.82 (d, 3H, J = 6.6), 1.22 (t, 3H, J = 7.0), 1.29 (m, 2H), 1.43 (m, 1H), 1.61 (m, 1H), 1.75 (m, 1H), 1.79 (s, 3H), 2.02 (m, 2H), 2.83 (m, 1H), 2.92 (m, 1H), 3.99 (m, 1H), 4.13 (q, 2H, J = 7.0), 4.41 (m, 1H), 4.49 (m, 1H), 4.99 (d, 1H, J = 12.5), 5.04 (d, 1H, J = 12.1), 6.31 (d, 1H, J = 8.8), 6.73 (s, br, 1H), 7.15 (m, 6H), 7.34 (m, 5H), 7.45 (d, 1H, J = 7.7), 7.91 (d, 1H, J = 7.7), 8.04 (d, 1H, J = 8.1). Anal. ($C_{33}H_{44}N_4O_7 \cdot 0.75H_2O$) C, H, N.

Representative Example of Preparation Method C. Synthesis of Cyclopentyl-3-(Cbz-L-Leu-L-Phe-L-*Gln*)-(*E*)-propenoate (6). 3-[Boc-L-(Tr-*Gln*)]-(*E*)-propenoic Acid (72). Sodium hydroxide (4.6 mL of a 1.0 M aqueous solution, 4.6 mmol, 2.3 equiv) was added to a 23 °C solution of **68** (1.09 g, 2.10 mmol, 1 equiv) in EtOH (10 mL) dropwise via addition funnel over 20 min. The resulting white suspension was stirred at 23 °C for 3 h and then was partitioned between H_2O (100 mL) and Et_2O (100 mL). The aqueous layer was acidified to pH = 2 with 1.0 M HCl and was extracted with EtOAc (2 × 100 mL). The organic layers were dried over Na_2SO_4 and were concentrated to provide crude **72** (1.01 g, 98%) as a white solid: ^1H NMR ($CDCl_3$) δ 1.42 (s, 9H), 1.72−1.80 (m, 1H), 1.83−1.98 (m, 1H), 2.37 (t, 2H, J = 7.0), 4.22−4.37 (m, 1H), 4.86−4.89 (m, 1H), 5.85 (d, 1H, J = 15.3), 6.86 (dd, 1H, J = 15.5, 5.1), 6.92 (s, 1H), 7.18−7.32 (m, 15H).

Cyclopentyl-3-[Boc-L-(Tr-*Gln*)]-(*E*)-propenoate (73). 1-(3-(Dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (0.372 g, 1.94 mmol, 1.05 equiv) was added to a solution of **72** (0.951 g, 1.85 mmol, 1 equiv), cyclopentanol (0.185 mL, 2.04 mmol, 1.1 equiv), and 4-(dimethylamino)pyridine (0.038 g, 0.185 mmol, 0.10 equiv) in CH_2Cl_2 (30 mL) at 23 °C. The reaction mixture was stirred at 23 °C overnight and then was concentrated under reduced pressure. The residue was purified by flash column chromatography (50% EtOAc in hexanes) to provide **73** (0.215 g, 20%) as a white foam: R_f = 0.70 (50% EtOAc in hexanes); IR (cm^{-1}) 3319, 1708; ^1H NMR ($CDCl_3$) δ 1.25−1.29 (m, 2H), 1.44 (s, 9H), 1.59−1.89 (m, 8H), 2.38 (t, 2H, J = 7.2), 4.32 (s, br, 1H), 4.54−4.56 (m, 1H), 5.20−5.23 (m, 1H), 5.87 (d, 1H, J = 15.6), 6.77 (dd, 1H, J = 15.1, 4.1), 6.90 (s, br, 1H), 7.20−7.33 (m, 15H). Anal. ($C_{36}H_{42}N_2O_5 \cdot 0.50H_2O$) C, H, N.

Cyclopentyl-3-[Cbz-L-Leu-L-Phe-L-(Tr-*Gln*)]-(*E*)-propenoate (74). A 4.0 M solution of HCl in 1,4-dioxane (3.5 mL) was added to a solution of **73** (0.215 g, 0.369 mmol, 1 equiv) in the same solvent (3.5 mL) at 23 °C. After 2 h of stirring, the reaction mixture was concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 (30 mL), and Cbz-L-Leu-L-Phe-OH (0.152 g, 0.369 mmol, 1.0 equiv), 4-methylmorpholine (0.163 mL, 1.48 mmol, 4.0 equiv), 1-hydroxybenzotriazole hydrate (0.075 g, 0.554 mmol, 1.5 equiv), and 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (0.106 g, 0.554 mmol, 1.5 equiv) were added sequentially. The resulting solution was stirred overnight at 23 °C and then was partitioned between 1 M HCl (100 mL) and CH_2Cl_2 (2 × 100 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated. Purification of the residue by flash chromatography (50% EtOAc in hexanes) afforded **74** (0.191 g, 59%) as a white foam: R_f = 0.4 (50% EtOAc in hexanes); IR (cm^{-1}) 3401, 3319, 1708; ^1H NMR ($CDCl_3$) δ 0.78−0.90 (m, 6H), 1.01−1.10 (m, 1H), 1.23−1.33 (m, 1H), 1.46−1.71 (m, 9H), 1.82−1.88 (m, 1H), 2.24−2.32 (m, 2H), 2.98−3.12 (m, 4H), 3.95−4.02 (m 1H), 4.46−4.48 (m, 2H), 4.83−5.21 (m, 4H), 5.65 (d 1H, J = 15.9), 6.50 (d, 1H, J = 7.2) 6.59 (d, 1H, J = 8.1), 6.65 (dd, 1H, J = 15.9, 5.4), 7.04−7.35 (m, 25H). Anal. ($C_{54}H_{60}N_4O_7 \cdot H_2O$) C, H, N.

Cyclopentyl 3-(Cbz-L-Leu-L-Phe-L-*Gln*)-(*E*)-propenoate (6). Trifluoroacetic acid (6 mL) was added to a solution of **74** (0.150 g, 0.171 mmol, 1 equiv) and triisopropylsilane (0.175 mL, 0.854 mmol, 5.0 equiv) in CH_2Cl_2 (7 mL) at 23 °C, producing a bright yellow solution. The reaction mixture was stirred at 23 °C until it became colorless (1 h), CCl_4 (4 mL) was added, and the volatiles were removed under reduced pressure. The residue was triturated with Et_2O (10 mL), and the resulting white solid was collected by vacuum filtration, washed with Et_2O (3 × 5 mL), and air-dried to afford **6** (0.061 g, 56%) as a white solid: mp 188−190 °C; R_f = 0.5 (10% CH_3OH in $CHCl_3$); IR (cm^{-1}) 3389, 3295, 1707; ^1H NMR (acetone-d_6) δ 0.83−0.87 (m, 6H), 1.06−1.09 (m, 1H), 1.46−1.51 (m, 1H), 1.60−1.70 (m, 11H), 1.83−1.94 (m, 1H), 2.20−2.25 (m, 2H), 2.90−3.01 (m, 1H), 3.18 (dd, 1H, J = 13.9, 5.8), 4.00 (d, 1H, J = 6.8), 4.05−4.10 (m, 1H), 4.55−4.62 (m 2H), 4.97−5.16 (m, 4H), 5.76 (d, 1H, J = 15.3), 6.71 (m, 2H), 7.15−7.41 (m, 10H), 7.51 (d, 1H, J = 7.8). Anal. ($C_{35}H_{40}N_4O_7$) C, H, N.

Representative Example of Preparation Method C. Synthesis of *N*-Phenyl-3-(Cbz-L-Leu-L-Phe-L-*Gln*)-(*E*)-propenamide (13). *N*-Phenyl-3-[Boc-L-(Tr-*Gln*)]-(*E*)-propenamide (75). Isobutyl chloroformate (0.075 mL, 0.578 mmol, 1.0 equiv) was added to a solution of **72** (0.296 g, 0.575 mmol, 1 equiv) and 4-methylmorpholine (0.126 mL, 1.15 mmol, 2.0 equiv) in CH_2Cl_2 (15 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 30 min, and then aniline (0.052 mL, 0.571 mmol, 1.0 equiv) was added. The resulting suspension was stirred at 0 °C for 20 min and at 23 °C for 24 h and then was vacuum filtered through medium paper. The solid thus obtained was washed with CH_2Cl_2 (10 mL) and Et_2O (10 mL) and was air-dried to provided **75** (0.065 g, 20%) as a white solid: mp 241−243 °C; R_f = 0.31 (5% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3264, 1715, 1660; ^1H NMR (DMSO-d_6) δ 1.40 (s, 9H), 1.62−1.65 (m, 1H), 2.27−2.35 (m, 1H), 4.11 (s, br, 1H), 6.13 (d, 1H, J = 15.3), 6.64 (dd, 1H, J = 15.3, 5.9), 7.01−7.08 (m, 2H), 7.12−7.32 (m, 20H), 7.64 (d, 1H, J = 7.8), 8.62 (s, 1H). Anal. ($C_{37}H_{39}N_3O_4$) C, H, N.

***N*-Phenyl-3-[Cbz-L-Leu-L-Phe-L-(Tr-*Gln*)]-(*E*)-propenamide (76).** A solution of HCl in 1,4-dioxane (4.0 M, 6 mL) was added to a solution of **75** (0.060 g, 0.102 mmol, 1 equiv) in the same solvent (6 mL) at 23 °C. After 1 h, the volatiles were removed under reduced pressure. The residue was dissolved in CH_2Cl_2 (10 mL), and Cbz-L-Leu-L-Phe-OH (0.042 g, 0.102 mmol, 1.0 equiv), 1-hydroxybenzotriazole hydrate (0.021 g, 0.155 mmol, 1.5 equiv), 4-methylmorpholine (0.034 mL, 0.309 mmol, 3 equiv), and 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (0.029 g, 0.151 mmol, 1.5 equiv) were added sequentially. The reaction mixture was stirred at 23 °C for 26 h and then was partitioned between water (100 mL) and EtOAc (2 × 100 mL). The combined organic layers were dried over Na_2SO_4 and were concentrated. Flash chromatographic purification of the residue (5% CH_3OH in CH_2Cl_2) afforded **76** (0.049 g, 55%) as a white foam: R_f = 0.47 (10% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3278, 1642, 1536; ^1H NMR ($CDCl_3$) δ 0.82 (d, 3H, J = 11.4), 0.84 (d, 3H, J = 11.4), 1.23−1.30 (m, 1H), 1.34−1.49 (m, 2H), 1.76−1.83 (m, 1H), 1.97 (s, br, 1H), 2.28 (t, 2H, J = 7.0), 2.87−2.94 (m, 1H), 3.17−3.21 (m, 1H), 3.96−4.03 (m, 1H), 4.58 (s, br, 2H), 4.84−4.94 (m, 2H), 5.77 (d, 1H, J = 15.9), 6.53 (d, 1H, J = 7.8), 6.70−6.75 (m, 1H), 6.96−7.32 (m, 31H), 7.57 (d, 2H, J = 7.8), 7.70 (s, 1H). Anal. ($C_{55}H_{57}N_5O_6 \cdot 0.50H_2O$) C, H, N.

***N*-Phenyl-3-(Cbz-L-Leu-L-Phe-L-*Gln*)-(*E*)-propenamide (13).** Trifluoroacetic acid (4 mL) was added to a solution of **76** (0.042 g, 0.048 mmol, 1 equiv) and triisopropylsilane (0.100 mL, 0.488 mmol, 10 equiv) in CH_2Cl_2 (4 mL) at 23 °C, producing a bright yellow solution. The reaction mixture was stirred for 20 min at 23 °C, during which time it became colorless. Carbon tetrachloride (6 mL) was added, and the volatiles were removed under reduced pressure. The residue was triturated with Et_2O (6 mL), and the resulting white solid was collected by vacuum filtration, washed with Et_2O (3 × 6 mL), and air-dried to afford **13** (0.023 g, 74%): mp 240 °C dec;

R_f = 0.29 (10% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3267, 1647; ^1H NMR (DMSO-d_6) δ 0.78 (d, 3H, J = 6.2), 0.82 (d, 3H, J = 6.5), 1.29−1.38 (m, 2H), 1.46−1.48 (m, 1H), 1.73−1.75 (m, 2H), 2.07−2.12 (m, 2H), 2.84 (dd, 1H, J = 13.6, 9.2), 3.04 (dd, 1H, J = 13.6, 4.7), 3.92−4.00 (m, 1H), 4.37−4.51 (m, 2H), 4.98 (d, 1H, J = 12.5), 5.05 (d, 1H, J = 12.5), 6.13 (d, 1H, J = 15.3), 6.60 (dd, 1H, J = 15.3, 6.4), 6.77 (s, 1H), 7.04 (t, 2H, J = 7.3), 7.12−7.38 (m, 12H), 7.43 (d, 1H, J = 8.1), 7.64 (d, 2H, J = 7.8), 7.93 (d, 1H, J = 8.1), 8.14 (d, 1H, J = 8.1), 10.05 (s, 1H). Anal. ($C_{36}H_{43}N_5O_6 \cdot 0.25H_2O$) C, H, N.

Examples of Preparation Method D. Synthesis of Pyrrole 25, Nitro Compound 51, and Oxime 52. 1-(Pyrrol-1-yl)-3-(Cbz-Leu-L-Phe-L-*Gln*)-(*E*)-propenamide (25). A solution of 1-(3-pyrrolin-1-yl)-3-[Cbz-Leu-L-Phe-L-(Tr-*Gln*)]-(*E*)-propenamide (prepared utilizing general method C, 0.424 g, 0.493 mmol, 1 equiv) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (0.17 g, 0.75 mmol, 1.5 equiv) in benzene (30 mL) were stirred rapidly for 72 h at 23 °C. The volatiles were then removed under vacuum, and the residue was purified by flash column chromatography (3% CH_3OH in $CHCl_3$) to yield 1-(3-pyrrol-1-yl)-3-[Cbz-Leu-L-Phe-L-(Tr-*Gln*)]-(*E*)-propenamide (0.31 g, 73%) as a white solid: ^1H NMR (DMSO-d_6) δ 0.76 (d, 3H, J = 6.6), 0.80 (d, 3H, J = 6.3), 1.30 (m, 2H), 1.44 (m, 1H), 1.63 (m, 2H), 2.28 (m, 2H), 2.90 (m, 1H), 2.99 (m, 1H), 3.95 (m, 1H), 4.49 (m, 2H), 4.98 (q, 2H, J = 13.4), 6.35 (s, br, 2H), 6.66 (d, 1H, J = 15.8), 6.96 (dd, 1H, J = 14.9, 5.0), 7.06−7.40 (m, 25H), 7.47 (m, 3H), 8.04 (m, 2H), 8.58 (s, 1H).

This material was dissolved in CH_2Cl_2 (11 mL) and cooled to 0 °C, and trifluoroacetic acid (0.7 mL) was added. The reaction mixture was warmed to 23 °C and was stirred for 30 min. The mixture was then concentrated under reduced pressure, and the residue was dissolved in EtOAc (50 mL) and washed sequentially with 10% aqueous $NaHCO_3$ (50 mL) and brine (50 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) provided **25** (0.16 g, 74%) as a white solid: ^1H NMR (DMSO-d_6) δ 0.77 (d, 3H, J = 6.6), 0.82 (d, 3H, J = 6.3), 1.32 (m, 2H), 1.45 (m, 1H), 1.77 (m, 2H), 2.10 (t, 2H, J = 7.5), 2.89 (dd, 1H, J = 14.3, 9.7), 3.01 (dd, 1H, J = 9.7, 5.5), 3.94 (m, 1H), 4.47 (m, 2H), 5.01 (q, 2H, J = 12.9), 6.35 (s, br, 2H), 6.68 (d, 1H, J = 15.1), 6.77 (s, br, 1H), 6.96 (dd, 1H, J = 14.7, 5.2), 7.07 (s, br, 1H), 7.19 (m, 6H), 7.34 (m, 5H), 7.51 (s, br 2H), 8.05 (m, 2H). Anal. ($C_{34}H_{41}N_5O_6$) C, H, N.

Nitro Alcohol 77. Potassium fluoride (0.0014 g, 0.02 mmol, 0.04 equiv) was added to a solution of **70**[10a] (0.361 g, 0.47 mmol, 1 equiv) in a 1:1 mixture of CH_3NO_2 and *i*-PrOH (4 mL). The reaction mixture was stirred overnight at room temperature and then was concentrated under reduced pressure. The residue was purified by flash column chromatography (gradient elution, 0 → 2% CH_3OH in $CHCl_3$) to give **77** (0.32 g, 83%, 2:1 mixture of diastereomers) as a glassy solid: R_f = 0.13 (2% CH_3OH in $CHCl_3$); IR (cm^{-1}) 3500−3200 (br), 1659, 1518; ^1H NMR (DMSO-d_6, major diastereomer) δ 0.71−0.80 (m, 6H), 1.28−1.35 (m, 2H), 1.1.48−1.60 (m, 2H), 1.64−1.95 (m, 1H), 2.19−2.30 (m, 2H), 2.78−2.87 (m, 1H), 2.96−3.03 (m, 1H), 3.72−3.82 (m, 1H), 3.84−4.03 (m, 2H), 4.10−4.20 (m, 1H), 4.33−4.38 (m, 1H), 4.46−4.54 (m, 1H), 4.95 (d, 1H, J = 12.5), 5.01 (d, 1H, J = 12.5), 5.64−5.68 (m, 1H), 7.15−7.41 (m, 26H), 7.72 (d, 1H, J = 8.1), 7.97 (d, 1H, J = 6.6), 8.51 (s, 1H). Anal. ($C_{48}H_{53}N_5O_8$) C, H, N.

Vinylnitro Compound 78. Methanesulfonyl chloride (0.027 mL, 0.35 mmol, 1.0 equiv) and Et_3N (0.20 mL, 1.40 mmol, 4.0 equiv) were added sequentially to a solution of **77** (0.292 g, 0.35 mmol, 1 equiv) in CH_2Cl_2 (4 mL) at 0 °C. The reaction was allowed to warm to room temperature and was stirred overnight. Additional methanesulfonyl chloride (0.10 mL) was then added, and the reaction mixture was stirred for 2 h at 23 °C. The volatiles were then removed in vacuo, and the product was purified by flash chromatography (0.5% CH_3OH in $CHCl_3$) to afford **78** (0.11 g, 39%) as an off-white, glassy solid: R_f = 0.18 (3% CH_3OH in $CHCl_3$); ^1H NMR (DMSO-d_6) δ 0.65−0.81 (m, 6H), 1.28−1.36 (m, 2H), 1.47−1.62 (m, 1H), 1.70−1.80 (m, 2H), 2.24−2.40 (m, 2H), 2.84−2.94 (m, 2H), 3.95−4.03 (m, 1H), 4.43−4.48 (m, 2H), 4.96 (d, 1H, J = 12.9), 5.02 (d, 1H, J = 12.9), 6.86 (d, 1H, J = 13.6), 7.06 (dd, 1H, J = 13.6, 5.5), 7.13−7.32 (m, 25H), 7.67 (d, 1H, J = 7.7), 8.03 (d, 1H, J = 7.7), 8.15 (d, 1H, J = 8.5), 8.56 (s, 1H).

Nitro Compound 51. Trifluoroacetic acid (0.25 mL) was added to a solution of **78** (0.095 g, 0.12 mmol) in CH_2Cl_2 (2.5 mL), and the reaction mixture was stirred at room temperature for 6 h. The volatiles were removed under reduced pressure, and the residue was triturated with a 1:1 mixture of EtOAc/Et_2O (5 mL). The resulting white solid was filtered and washed with Et_2O (20 mL) to afford **51** (0.022 g, 33%) as a white solid: IR (cm^{-1}) 1647, 1530; ^1H NMR (DMSO-d_6) δ 0.79 (d, 3H, J = 6.6), 0.82 (d, 3H, J = 7.0), 1.28−1.30 (m, 2H), 1.47−1.53 (m, 1H), 1.67−1.82 (m, 2H), 2.04−2.09 (m, 2H), 2.89−2.92 (m, 2H), 3.97−4.02 (m, 1H), 4.41−4.48 (m, 2H), 4.97 (d, 1H, J = 12.5), 5.04 (d, 1H, J = 12.5), 6.78 (s, br, 1H), 6.88 (d, 1H, J = 13.6), 7.07 (dd, 1H, J = 13.6, 5.5), 7.14−7.34 (m, 11H), 7.44 (d, 1H, J = 7.0), 8.07 (d, 1H, J = 7.7), 8.15 (d, 1H, J = 8.1). Anal. ($C_{29}H_{37}N_5O_7 \cdot 0.85CH_2Cl_2$) C, H, N.

Oxime 52. (Triphenylphosphoranylidene)acetaldehyde (0.26 g, 0.82 mmol, 1.1 equiv) was added to a solution of **70**[10a] (0.576 g, 0.75 mmol, 1 equiv) in THF (15 mL), and the reaction was stirred at room temperature overnight. The solvent was removed under reduced pressure, and the residue was dissolved in pyridine (8 mL). Methoxylamine hydrochloride (0.064 g, 0.75 mmol, 1.0 equiv) was added, and the reaction was again stirred at room temperature overnight. The solvent was removed in vacuo, and the residue was dissolved in EtOAc (50 mL) and washed with saturated $NaHCO_3$ (50 mL), water (50 mL), and brine (50 mL). The organic layer was dried over $MgSO_4$ and concentrated. Purification of the residue by flash column chromatography (gradient elution, 0 → 1% CH_3OH in $CHCl_3$) gave intermediate **79** (0.334 g, contaminated with triphenylphosphine oxide) which was used without further purification.

Trifluoroacetic acid (1.0 mL) was added to a solution of **79** in CH_2Cl_2 (10 mL), and the reaction mixture was stirred at room temperature for 6 h. The volatiles were removed under reduced pressure, and the residue was triturated with a 1:1 mixture of EtOAc/Et_2O (20 mL). The resulting white solid was filtered and washed with Et_2O (30 mL) to afford **52** (0.104 g, 24%, mixture of oxime isomers) as a white solid: R_f = 0.10 (3% $CH_3OH/CHCl_3$); IR (cm^{-1}) 1645, 1539; ^1H NMR (DMSO-d_6, major isomer) δ 0.77−0.83 (m, 6H), 1.26−1.30 (m, 2H), 1.43−1.46 (m, 1H), 1.65−1.68 (m, 2H), 2.03−2.08 (m, 2H), 2.78−2.86 (m, 1H), 2.94−3.00 (m, 1H), 3.76 (s, 3H), 3.92−3.94 (m, 1H), 4.26−4.31 (m, 1H), 4.45−4.47 (m, 1H), 4.98 (d, 1H, J = 12.9), 5.04 (d, 1H, J = 12.5), 5.96−5.98 (m, 1H), 6.47−6.55 (m, 1H), 6.75 (s, 1H), 7.11−7.43 (m, 13H), 8.01−8.06 (m, 2H). Anal. ($C_{31}H_{41}N_5O_6$) C, H, N.

Preparation of Compound 65. A suspension of ethyl 3-[Boc-L-(Tr-*Gln*)]-(*E*)-propenoate (**68**) (0.383 g, 0.706 mmol, 1 equiv) and Pd/C (10%, 0.060 g) in EtOH (20 mL) was stirred under a hydrogen atmosphere (balloon) at 23 °C for 16 h. The reaction mixture was filtered through Celite, and the filtrate was concentrated to afford a colorless oil. This material was dissolved in 1,4-dioxane (10 mL), and HCl (4.0 M solution in 1,4-dioxane, 10 mL) was added. The resulting solution was stirred at 23 °C for 1.5 h, and then the volatiles were removed under reduced pressure. The residue was dissolved in CH_2Cl_2 (20 mL), and Cbz-L-Leu-L-Phe-OH (0.291 g, 0.705 mmol, 1.0 equiv), 1-hydroxybenzotriazole hydrate (0.114 g, 0.844 mmol, 1.2 equiv), 4-methylmorpholine (0.310 mL, 2.82 mmol, 4.0 equiv), and 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (0.162 g, 0.845 mmol, 1.2 equiv) were added sequentially. The reaction mixture was stirred at 23 °C for 21 h and then was partitioned between water (100 mL) and EtOAc (2 × 100 mL). The combined organic layers were dried over Na_2SO_4 and were concentrated. Flash chromatographic purification of the residue (3% CH_3OH in CH_2Cl_2) afforded the product tripeptide (**80**) (0.281 g, 47%) as a white foam: R_f = 0.53 (10% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3293, 1708, 1647; ^1H NMR ($CDCl_3$) δ 0.85 (t, 6H, J = 6.2), 1.24 (t, 3H, J = 7.2), 1.29−1.39 (m, 1H), 1.42−1.58 (m, 4H), 1.70−1.84 (m, 2H),

2.04−2.33 (m, 4H), 2.98 (dd, 1H, J = 13.9, 7.0), 3.12 (dd, 1H, J = 13.9, 6.7), 3.75−3.82 (m, 1H), 3.95−4.02 (m, 1H), 4.09 (q, 2H, J = 7.2), 4.45−4.53 (m, 1H), 4.85−4.94 (m, 2H), 6.17 (d, 1H, J = 9.0), 6.45 (d, 1H, J = 7.8), 7.14−7.39 (m, 27H). Anal. ($C_{51}H_{58}N_4O_7$) C, H, N.

Trifluoroacetic acid (8 mL) was added to a solution of **80** (0.270 g, 0.322 mmol, 1 equiv) and triisopropylsilane (0.330 mL, 1.61 mmol, 5.0 equiv) in CH_2Cl_2 (10 mL) at 23 °C, producing a bright yellow solution. The reaction mixture was stirred for 20 min at 23 °C during which time it became colorless. Carbon tetrachloride (6 mL) was added, and the volatiles were removed under reduced pressure. The residue was triturated with Et_2O (10 mL), and the resulting white solid was collected by vacuum filtration, washed with Et_2O (3 × 30 mL), and air-dried to afford **65** (0.171 g, 89%): mp 210−215 °C; R_f = 0.45 (10% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3267, 1689, 1643; ^1H NMR (DMSO-d_6) δ 0.78 (d, 3H, J = 6.5), 0.82 (d, 3H, J = 6.5), 1.17 (t, 3H, J = 7.2), 1.22−1.63 (m, 7H), 1.97−2.05 (m, 4H), 2.82 (dd, 1H, J = 13.5, 8.4), 2.96 (dd, 1H, J = 13.5, 6.2), 3.62 (s, br, 1H), 3.94−4.05 (m, 3H), 4.39−4.47 (m, 1H), 4.98 (d, 1H, J = 12.6), 5.05 (d, 1H, J = 12.6), 6.70 (s, br, 1H), 7.14−7.34 (m, 11H), 7.42 (d, 1H, J = 7.8), 7.64 (d, 1H, J = 8.7), 7.97 (d, 1H, J = 8.1). Anal. ($C_{32}H_{44}N_4O_7$) C, H, N.

Enzyme Assays. The general conditions of the fluorescence resonance energy transfer assay utilized to assess 3CP activity and procedures for reversible inhibitor K_i determinations are described in ref 10c. Continuous fluorometric enzyme assays were conducted using a Perkin-Elmer LS50B spectrofluorimeter with a four place motorized cuvette holder. The k_{obs}/[I] values for the irreversible inhibitors were obtained from reactions initiated by addition of 50 nM 3CP, containing 1 μM substrate and varying inhibitor concentrations. Typically, three to five concentrations were examined, and three related methods were utilized for subsequent data analysis. For reactions in which less than 20% of the substrate was processed when 100% inactivation was observed, data from the continuous assays were directly analyzed with the nonlinear regression analysis program ENZFITTER[38] to obtain first-order rate constants for enzyme inactivation at each inhibitor concentration. The slope of a graph of $k_{obs}(I)$ vs [I] was calculated using ENZFITTER and reported as k_{obs}/[I]. The error associated with this determination is less than 10% of a given value and is often less than 5%. For very fast irreversible inhibitors (k_{obs}/[I] > 400 000 M^{-1} s^{-1}), sufficient data points could not be obtained to determine k_{obs}/[I] by the above slope method. These "fast" values were therefore calculated directly from a single k_{obs} number by dividing by [I]. The error associated with this determination is less than 20% of a given value. For very slow irreversible inhibitors (k_{obs}/[I] < 1000 M^{-1} s^{-1}) and/or when >20% consumption of substrate was observed at 100% 3CP inactivation, the primary data was adjusted to account for expected changes in velocity due to changes in substrate concentration. The transformed data was used to calculate $k_{obs}(I)$ values and the k_{obs}/[I] figure was determined utilizing the slope method described above.

The sensitivity of inhibitors to nonenzymatic thiols was evaluated using reactions similar to those described in the previous paragraph, but with the addition of 5 mM dithiothreitol (DTT) to the inhibitor-containing mixture 2−3 min before the addition of enzyme. Inhibitor DTT sensitivity was indicated by a significantly reduced k_{obs} for enzyme inactivation compared to that obtained in a control reaction lacking DTT.

Antiviral Assays. All strains of human rhinovirus (HRV) were purchased from American Type Culture Collection (ATCC). HRV stocks were propagated, and antiviral assays were performed in H1-HeLa cells (ATCC). Cells were grown in Minimal Essential Medium with 10% fetal bovine serum. The ability of compounds to protect cells against HRV infection was measured by the XTT dye reduction method.[39] Briefly, H1-Hela cells were infected with HRV-14 at a multiplicity of infection (moi) of 0.08 or mock-infected with medium only. Infected or uninfected cells were resuspended at 8 × 10^5 cells per mL and incubated with appropriate concentrations of drug. Two days later, XTT/PMS was added to the test plates, and the amount of formazan produced was quantified spectrophotometrically at 450/650 nm. The EC_{50} was calculated as the concentration of drug that increased the percentage of formazan production in drug-treated, virus-infected cells to 50% of that produced by drug-free, uninfected cells. The 50% cytotoxic concentration (CC_{50}) was calculated as the concentration of drug that decreased the percentage of formazan produced in drug-treated, uninfected cells to 50% of that produced in drug-free, uninfected cells. The reported values were obtained from either a single antiviral determination or the mean of two or more experiments. To avoid false positives due to toxicity, only compounds displaying CC_{50}'s greater than 10 times the observed EC_{50}'s were considered to be truly active antiviral agents. Using the above method, the EC_{50} of a representative compound (**3**) was calculated to be 0.54 ± 0.27 μM (range 0.18−1 μM). The EC_{50} of the known antirhinoviral agent Pirodavir was similarly determined to be 0.02 ± 0.01 μM (range 0.01−0.05 μM) and was comparable to the 0.03 μM minimal inhibitory concentration value previously reported.[40]

Rat Plasma Stability Studies. Rat plasma was isolated from blood collected in vacutainers containing sodium heparin as the anticoagulant via a cardiac stick from Spraque-Dawley rats (Hilltop, Scottsdale, PA). This plasma (1:1 dilution with 100 mM potassium phosphate buffer, pH 7.4) was incubated in a final volume of 4 mL at 37 °C in a shaking water bath for 5 min. A DMSO solution of a given 3CP inhibitor (25 μM) was then added such that the amount of organic solvent in the final incubations was 0.5% (v/v). After 0, 20, 40, 60, 120, 180, and 240 min, a 200 μL aliquot of the incubate was removed and transferred to a test tube containing 2.0 mL of CH_3CN. Samples were vortexed for 30 s and subsequently centrifuged for 20 min at 2000 rpm. The supernatant (2 mL) was transferred to a clean test tube and dried under N_2 at 40 °C. The dried residue was reconstituted in 200 μL of 1:1 CH_3CN:potassium phosphate buffer (pH 3.0), and the amount of a given 3CP inhibitor present was determined by HPLC analysis at 23 °C utilizing a Hewlett-Packard 1100 HPLC equipped with a Rainin Microsorb MV C8 reverse phase column (5 μm, 4.6 mm × 150 mm) and UV detection at 210 nm. The inhibitors were eluted using a solvent gradient beginning with 25%:25%:50% CH_3OH:CH_3CN:25 mM potassium phosphate (pH 3.0) for 3 min and then increasing over 10 min to a 70%:5%:25% ratio and subsequently returning to the initial conditions over 7 min all at a flow rate of 1.0 mL/min.

Protein−Ligand Crystal Structure Determination. Serotype 2 human rhinovirus 3C protease was incubated with a 3-fold molar excess of compound **3** and the complex concentrated to a final protein concentration of 12.5 mg/mL. Equal volumes of the protein−ligand stock solution and a 2.0 M solution of ammonium sulfate buffered with 100 mM ADA pH 6.5 were mixed and allowed to incubate at 21 °C for 1 h. This solution was then passed through a 0.45 μm Centrex centrifugal filter and used immediately for crystallization experiments. Crystals were grown at 21 °C using a hanging drop vapor diffusion method in which 10 μL drops were placed on plastic coverslips and sealed over individual wells filled with 1 mL of a reservoir solution containing 2.0 M ammonium sulfate, 100 mM ADA pH 6.5, plus 2.5% (v/v) 1,4-dioxane. Crystals typically grew as large rectangular blocks but were invariably twinned. Careful use of a microscope with crossed polarizers and a dissecting needle facilitated extraction of a single crystal measuring 0.7 × 0.25 × 0.15 mm from such a binary twin. The crystallographic space group was determined to be $P2_12_12$ with a = 63.49, b = 78.56, c = 34.50 Å with one protease molecule in the crystallographic asymmetric unit.

Cu Kα X-ray diffraction data were recorded from one crystal of the serotype 2 3CP−**3** complex at 4 °C using a 9 kW rotating anode source and two Xuong-Hamlin multiwire area detector systems. These data were 90% complete to 1.97 Å resolution with R(sym)(I) = 4.65% and an average of 4.5 measurements per symmetry-unique reflection. Rotation and translation search procedures implemented in X-PLOR[41] were used to

orient and position a search model of serotype 2 3CP whose structure had been previously determined in another crystal form.[42] Refinement was initiated using the rigid body option in X-PLOR followed by simulated annealing and conjugate gradient minimization protocols using all data in the 10 to 2 Å resolution range. Atomic coordinates for compound **3** were then fit to difference electron density maps and the protein−ligand model refined. This was followed by sequential addition of ordered solvent in several cycles involving stereochemically reasonable placement of candidate waters into difference maps and subsequent refinement of the combined model. The final *R* factor was 19.1% for data between 8 and 2 Å resolution (10236 reflections with $F > 2\sigma(F)$). The root-mean-square deviations from ideal bond lengths and angles were 0.016 Å and 3.0°, respectively. The final model contained all atoms for residues 1−180 (excluding side chains for residues 12, 45, and 65) plus 104 water molecules.

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(27) Carboxylic acid **10** was prepared by olefination of **70** with (*tert*-butoxycarbonylmethylene)triphenylphosphorane followed by TFA deprotection. The attempted basic hydrolysis of **3** and related esters resulted in decomposition.

(28) The lack of extensive racemization during ester hydrolysis was inferred from the subsequent absence of significant diastereomers of the Cbz-L-Leu-L-Phe-OH coupling product.

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