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Solid-phase Synthesis of Irreversible Human Rhinovirus 3C Protease Inhibitors. Part 1: Optimization of Tripeptides Incorporating N-terminal Amides

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Abstract—The optimization of a series of irreversible human rhinovirus (HRV) 3C protease (3CP) inhibitors is described. These inhibitors are comprised of an L-Leu-L-Phe-L-Gln tripeptide containing an N-terminal amide moiety and a C-terminal ethyl propenoate Michael acceptor. Examination of approximately 500 compounds with varying N-terminal amides utilizing solid-phase synthesis and high-throughput assay techniques is described along with the solution phase preparation of several highly active molecules. A tripeptide Michael acceptor containing an N-terminal amide derived from 5-methylisoxazole-3-carboxylic acid is shown to exhibit potent, irreversible anti-3CP activity ($k_{obs}/[I] = 260{,}000\ M^{-1}\ s^{-1}$; type-14 3CP) and broad-spectrum antirhinoviral properties (average $EC_{50} = 0.47\ \mu M$ against four different HRV serotypes).

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 a series of compounds that inhibit the HRV 3C protease (3CP), a virally encoded cysteine protease essential for replication, and which exhibit antirhinoviral activity in cell culture.[3] These inhibitors contain a substrate-derived tripeptide binding determinant[4,5] that provides affinity for the target protein and a Michael acceptor which irreversibly forms a covalent adduct with the active site cysteine residue of the 3C enzyme (Fig. 1).[3] In our earlier work, we found that inclusion of an N-terminal P_4 thiocarbamate moiety in the inhibitor design significantly improved anti-3CP and antirhinoviral activity relative to similar carbamate-containing molecules (compare compounds **1** and **2**, Fig. 1).[3b] However, we were concerned that the thiocarbamate moiety might undergo facile in vivo metabolism, and we therefore sought to replace this functionality with amides which also imparted good levels of activity to the resulting inhibitors (Chart 1). Below we describe the identification of such amide moieties through the utilization of solid-phase synthesis and high-throughput assay techniques.[6]

Results and Discussion

The solid-phase synthesis of tripeptide-derived, irreversible HRV 3CP inhibitors is illustrated in Scheme 1. Although several Michael acceptor–tripeptide combinations are known to exhibit potent anti-3CP activity,[3] the ethyl propenoate Michael acceptor and L-Leu-L-Phe-L-Gln binding element were selected for this study due to their ease of synthesis. In addition, all potential 3CP inhibitors prepared on solid phase were synthesized as single entities utilizing Chiron Multipins™[7] in order to facilitate the identification of active molecules. Thus, PyBOP-mediated[8] coupling of Fmoc-protected glutamic acid derivative **3**[3b] with pin-supported Rink Amide[9] linker afforded intermediate **4**. Removal of the Fmoc protecting group from **4** by exposure to 2% DBU[10] in CH_2Cl_2 and HATU-effected[11] condensation of the resulting amine with Fmoc-L-Phe-OH gave dipeptide intermediate **5** (not shown). Treatment of **5** with 2% DBU in CH_2Cl_2 and coupling of the liberated amine with Fmoc-L-Leu-OH then provided tripeptide intermediate **6**. The purity of each coupling adduct (**4**, **5** and **6**) was evaluated by cleavage from the solid support (TFA/H_2O) and HPLC analysis of the resulting mono-, di- or tripeptide. Although such analysis indicated that intermediates **4** and **5** were produced in >90% purity, the incomplete formation of the tripeptide **6** after a single Fmoc-L-Leu-OH coupling reaction was also detected. This latter transformation was therefore repeated in order to improve the purity of **6** (88% after second coupling).

Key words: Rhinovirus; irreversible; cysteine; protease; inhibitor.
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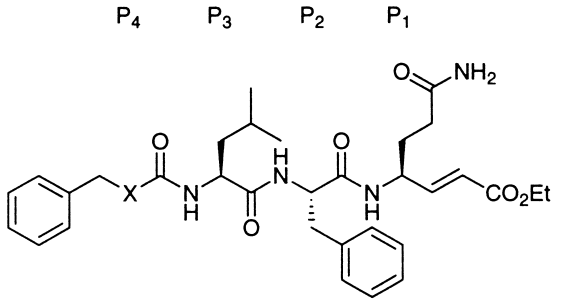


Compd.	X	k_{obs}/[I] ($M^{-1}s^{-1}$)[a]	EC_{50} (μM)[a]
1	O	25,000	0.54
2	S	280,000	0.27

Figure 1. Irreversible HRV 3CP inhibitors ([a]Serotype-14).

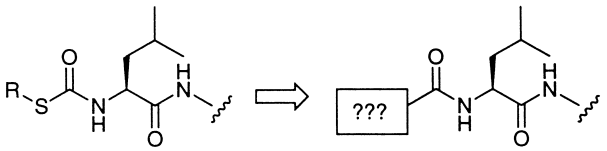


Chart 1. Design of thiocarbamate replacements.

Having assembled the tripeptide **6** on solid support, we then focussed our efforts on N-terminal amide synthesis and diversification (Scheme 1). Accordingly, the Fmoc group was removed from **6** as described above, and the newly-formed N-terminal amine was independently coupled with a variety of carboxylic acids and acid chlorides to afford approximately 500 unique intermediates **7**. Subsequent exposure of these intermediates to aqueous TFA effected cleavage from the solid support and provided the desired tripeptidyl compounds **8**. The acids and acid chlorides utilized in this study were selected from the ACD[12] based on their commercial availability, low molecular weight (< 300), and lack of highly reactive functional groups other than the desired acyl moiety (e.g. α-bromo-carboxylic acids were not included). Prior to coupling the complete set of acids and acid chlorides, several representative derivatization/cleavage reactions were performed (data not shown). HPLC analysis indicated that the tripeptide products **8** were typically obtained in the highest purities from coupling reactions utilizing acid chlorides, but some reactions employing carboxylic acids and certain acid chlorides were incomplete. Therefore, when synthesizing the entire complement of intermediates **7**, all couplings were performed twice in order to increase the purity of the desired products. A random sample (approximately 5%) of all tripeptides **8** thus prepared on solid phase was subjected to HPLC and mass spectral analysis, and several examples are presented in Table 1. In most cases, the target compounds were produced in > 50% purity and all exhibited a major peak corresponding to the correct molecular ion in the EI mass spectrum.

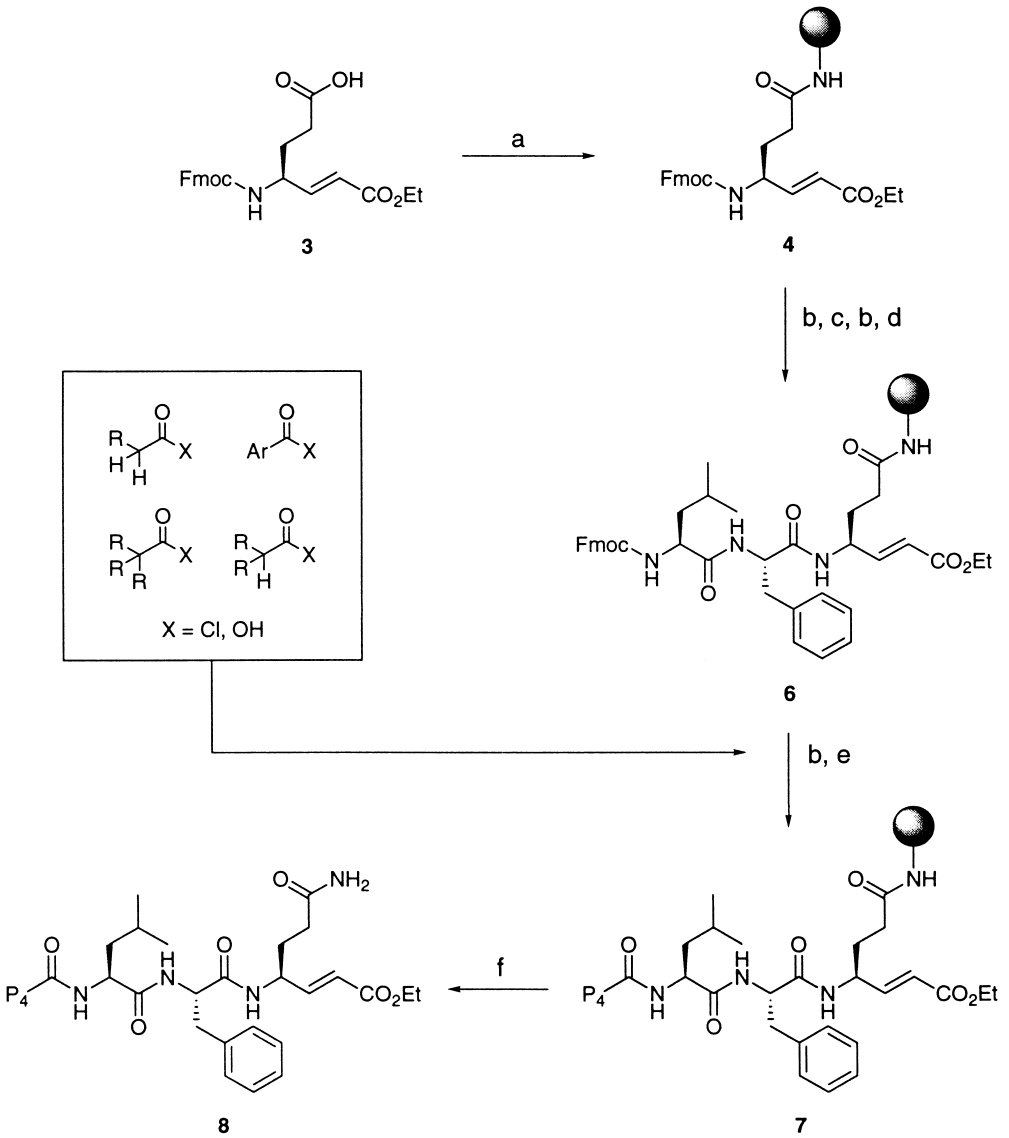
The completed series of approximately 500 derivatized tripeptide Michael acceptors (**8**) was then tested for activity against HRV-14 3CP in an assay system customized for microtiter plate use (8×12 well). As no attempt was made to quantitate the amount of a given compound produced by the above solid-phase synthesis, assay results were compared from well to well assuming a nominal product yield of 50%. Compounds **1** and **2** (Fig. 1) were included as positive controls, and assay conditions were chosen such that the inhibitory activity exhibited by **2** corresponded to the maximum measurable (100% 3CP inhibition, see Experimental). Since it was anticipated that most, if not all, tripeptides **8** would display some level of anti-3CP activity,[13] compounds were considered worthy of further examination only if their 3CP inhibition exceeded that displayed by compound **1** (75%, see Experimental). Using the above method for inhibitor evaluation, several highly-active molecules (**8a–g**, Table 2) were identified for solution-phase resynthesis and additional biological evaluation.[14,15]

The solution-phase preparation of tripeptide-derived 3CP inhibitors **8a–g** is illustrated in Scheme 2, and directly follows that previously described for the synthesis of closely related molecules.[3] Thus, Boc-protected glutamine derivative **9**[3a] was deprotected under acidic conditions (HCl) and the resulting amine salt was coupled with Boc-L-Phe-OH to afford dipeptide **10** (not shown). Acid-mediated deprotection of **10** and subsequent coupling with Boc-L-Leu-OH uneventfully provided tripeptide **11**. Removal of the N-terminal Boc moiety from **11** and derivatization of the amine salt thus obtained with an appropriate acid or acid chloride afforded the tripeptides **12a–g**. The trityl protecting groups present in **12a–g** were removed by short exposure to TFA in the presence of triisopropylsilane to give the desired compounds **8a–g** in good yields. As was described previously,[3] the tripeptide inhibitors prepared by the above solution-phase method were isolated as white solids by removal of the volatiles from the detritylation reaction mixtures, trituration of the resulting oils with Et_2O, and subsequent filtration.

The biological activities of the resynthesized compounds **8a–g** are depicted in Table 2. As expected, these molecules irreversibly inhibited HRV-14 3C protease with many exhibiting significantly greater activity than the control compound **1** (Fig. 1). Compounds **8a–g** also displayed various levels of antiviral activity without observed cytotoxicity when tested against HRV-14 in cell culture. The most-active inhibitor (**8e**) was also examined against several HRV serotypes other than 14 and, as illustrated in Table 3, exhibited broad-spectrum antiviral activity. Combination of the N-terminal isoxazole moiety present in **8e** with similar tripeptide or peptidomimetic 3CP inhibitors is thus anticipated to afford additional potent anti-3CP and antirhinoviral agents.[16]

Conclusions

The N-terminal optimization of tripeptide-derived HRV-3CP inhibitors was accomplished utilizing solid-phase synthesis and high-throughput assay techniques.



Scheme 1. Reagents and conditions: (a) 0.2 equiv solid-supported Rink-Amide linker, 2.0 equiv DIEA, 0.10 equiv HOBt, 1.0 equiv PyBOP, DMF, 23 °C, 1 h; (b) 2% DBU in CH_2Cl_2, 23 °C, 30 min; (c) 5.0 equiv Fmoc-L-Phe-OH, 10.0 equiv collidine, 5.0 equiv HATU, DMF, 23 °C, 1 h; (d) 5.0 equiv Fmoc-L-Leu-OH, 10.0 equiv collidine, 5.0 equiv HATU, DMF, 23 °C, 1 h, 2 times; (e) excess RC(O)Cl and collidine, $CHCl_3$, 23 °C, 1 h or excess RCO_2H, DIEA, HATU, DMF, 23 °C, 1 h, 2 times; (f) 95:5 TFA:H_2O, 23 °C, 1 h.

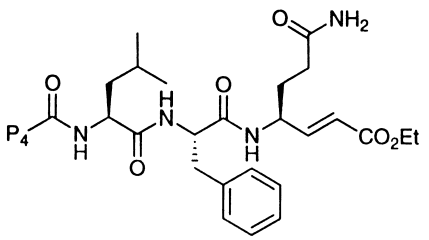
Such optimization identified a molecule (**8e**) containing an N-terminal 5-methylisoxazole-3-carboxamide as a potent 3CP inhibitor and broad-spectrum antirhinoviral agent. Incorporation of such carboxamides into other tripeptide or peptidomimetic HRV-3CP inhibitors will be described in future publications.

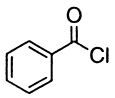
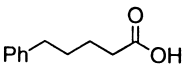
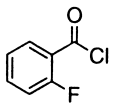
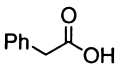
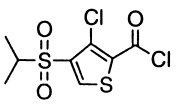
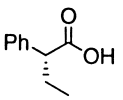
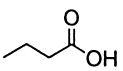
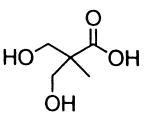
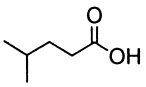
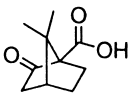
Experimental

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[17] was performed using silica gel 60 (Merck Art 9385). ^{1}H NMR spectra were recorded at 300 MHz utilizing a Varian UNITY*plus* 300 spectrometer. 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 a Perkin–Elmer 1600 series FTIR. HPLC purity analyses were conducted utilizing a Hewlett Packard Series 1100 HPLC with UV detection (222 nm) equipped with a ZORBAX RX-C18 analytical column (4.6×150 mm). Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Melting points were determined using a Mel-Temp II apparatus and are uncorrected. SynPhase™ crowns, deepwell microtiter plates (8×12), and Multipin™ stems were purchased from Chiron Technologies, San Diego, CA.

Table 1. Characterization of representative tripeptides **8** prepared on solid phase



Entry	RC(O)X	HPLC area (%)	Mass spectrum	Entry	RC(O)X	HPLC area (%)	Mass spectrum
1		78	587(MNa)	6		61	621(MH)
2		80	605(MNa)	7		93	579(MH)
3		61	711/713(MH)	8		90	607(MH)
4		51	531(MH)	9		80	599(MNa)
5		93	559(MH)	10		32	625(MH)

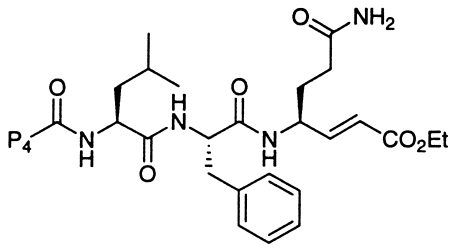
A simplified naming system employing amino acid abbreviations is used to identify intermediates and final products. When utilizing this naming system, italicized amino acid abbreviations represent modifications at the C-terminus of that residue where acrylic acid esters are reported as 'E' (*trans*) propenoates. The following abbreviations are also utilized: TFA (trifluoroacetic acid), DIEA (*N*,*N*-diisopropylethylamine), DBU (1,8-diazabicyclo[5.4.0]undec-7-ene), HOBt (1-hydroxybenzotriazole hydrate), PyBOP (benzotriazole-1-yl-oxy-*tris*-pyrrolidino-phosphonium hexafluorophosphate), HATU [*O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate], EDC [1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride].

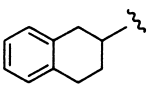
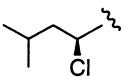
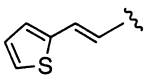
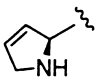
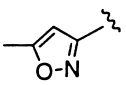
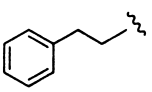
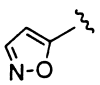
Preparation of tripeptides 8 on solid support. General solid-phase synthesis procedures.

Solid-phase synthesis was performed using SynPhase™-PS (polystyrene) O-series crowns (pins) derivatized with Fmoc-protected Rink Amide Linkers (RAM). The preparations of intermediates **4**, **5**, and **6** as well as the subsequent deprotection of **6** were performed in round bottom flasks. Intermediates **7** and final products **8** were synthesized in deepwell microtiter plates (8×12) utilizing Multipin™ stems to support the pins. The following standard purification procedures were utilized. For reactions run in round bottom flasks, the pins were washed sequentially with DMF (100 mL) and CH_2Cl_2 (100 mL) and the solvent was decanted away. The washing sequence was repeated two additional times, and the pins were then dried under vacuum to give the solid-phase intermediates. For reactions run in deepwell microtiter plates, the stems were suspended in a plastic wash bath, and the attached pins were washed sequentially with DMF (100 mL) and CH_2Cl_2 (100 mL). The washing sequence was repeated two additional times, and the pins were then air-dried to give the solid-phase intermediates.

Intermediate and product purities were determined as follows. A single pin was stirred in a mixture of TFA and H_2O (95:5, 1 mL) for 15 min at 23 °C. After removal of the pin from the reaction flask, the volatiles were evaporated under reduced pressure and the residue was dissolved in CH_3CN (1 mL). The resulting solution was then analysed by HPLC utilizing a mixture of CH_3CN, H_2O, and TFA (50%, 49.9%, and 0.1%, respectively) flowing isocratically at 1.0 mL/min as the mobile phase. Specific retention times and purities are indicated below.

Table 2. Biological activity of tripeptidyl HRV 3CP inhibitors



Compound	P_4	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)[a]	EC_{50} (μM)[a]	CC_{50} (μM)
8a[b]		95,000	1.4	> 100
8b		50,000	2.0	> 100
8c		66,000	0.55	> 100
8d		27,000	25	> 100
8e		260,000	0.25	> 100
8f		28,000	2.8	> 100
8g		47,000	1.6	> 100

[a]Serotype-14.
[b]1:1 mixture of diastereomers.

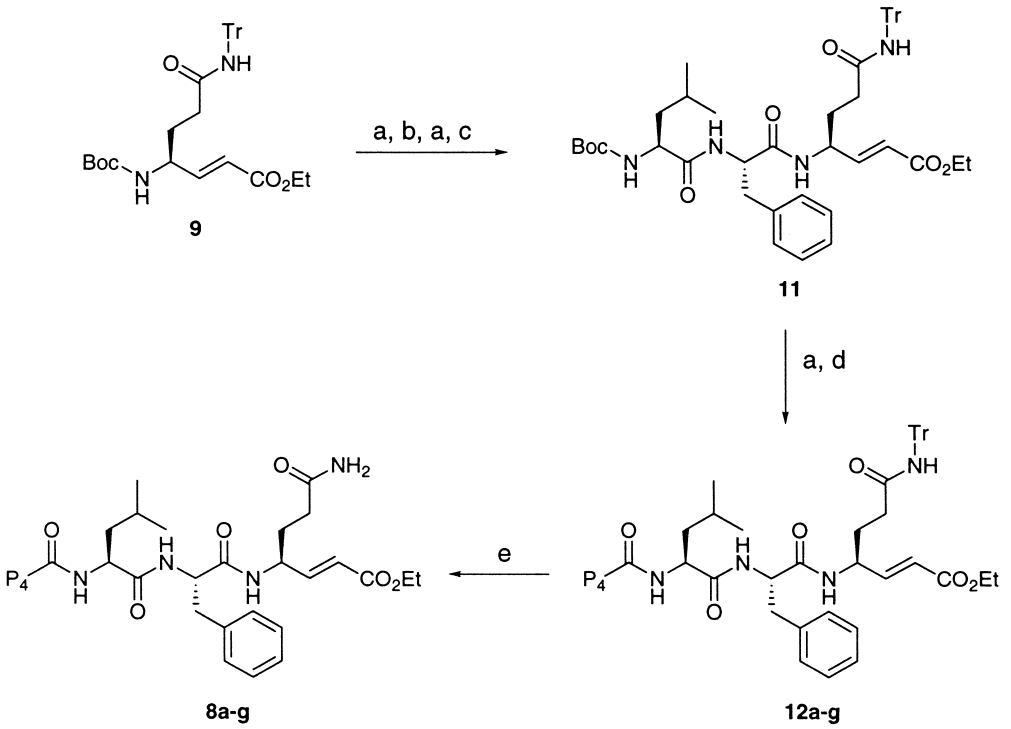
Ethyl-3-[Fmoc-L-(*N*-pin)*Gln*]-E-propenoate (4). Fmoc-Rink Amide-derivatized pins (710 pins at ~2.3 μmol/pin, ~1.63 mmol) were washed with CH_2Cl_2 and dried under vacuum, then suspended in 2% DBU in CH_2Cl_2 (100 mL) and stirred for 30 min at 23 °C. After purification by the standard procedure, the pins were suspended in DMF (100 mL) and **3**[3b] (3.45 g, 8.15 mmol, 5.0 equiv), DIEA (2.84 mL, 16.3 mmol, 10 equiv), HOBt (0.11 g, 0.815 mmol, 0.50 equiv) and PyBOP (4.24 g, 8.15 mmol, 5.0 equiv) were added sequentially. The reaction mixture was stirred for 1 h at 23 °C after which the solvent was decanted away. Standard purification then provided intermediate **4** (HPLC Anal. 4.04 min, 94% purity).

Ethyl-3-[Fmoc-L-Phe-L-(*N*-pin)*Gln*]-E-propenoate (5). Pins containing intermediate **4** (704 pins @ ~2.3 μmol/pin, ~1.62 mmol) were washed with CH_2Cl_2 and dried under vacuum. The pins were suspended in 2% DBU in CH_2Cl_2 (100 mL) and stirred for 30 min at 23 °C, then were purified by the standard procedure. The deprotected pins were resuspended in DMF (100 mL), and Fmoc-L-Phe-OH (3.13 g, 8.08 mmol, ~5.0 equiv), collidine (2.02 mL, 16.2 mmol, ~10 equiv), and HATU (3.07 g, 8.08 mmol, ~5.0 equiv) were added sequentially. The reaction mixture was stirred for 1 h at 23 °C after which the solvent was decanted away. Standard purification then provided dipeptide intermediate **5** (HPLC Anal. 6.82 min, 98% purity). Prior to the next synthetic step, the derivatized pins were treated with 5% Ac_2O in CH_2Cl_2 (100 mL) for 30 min at 23 °C in order to acylate any unreacted amine.

Ethyl-3-[Fmoc-L-Leu-L-Phe-L-(*N*-pin)*Gln*]-E-propenoate (6). Pins containing dipeptide intermediate **5** (700 pins at ~2.3 μmol/pin, ~1.61 mmol) were washed with CH_2Cl_2 and dried under vacuum. The pins were then suspended in 2% DBU in CH_2Cl_2 (100 mL) and stirred for 30 min at 23 °C. After purification by the standard procedure, the deprotected pins were resuspended in DMF (100 mL) and Fmoc-L-Leu-OH (2.84 g, 8.04 mmol, ~5.0 equiv), DIEA (2.80 mL, 16.1 mmol, ~10 equiv), and HATU (3.06 g, 8.04 mmol, ~5.0 equiv) were added sequentially. The reaction mixture was stirred for 1 h at 23 °C, after which the solvent was decanted away. Standard purification was performed and the pins were then subjected to a second Fmoc-L-Leu-OH coupling reaction using the above conditions. A second standard purification afforded tripeptide intermediate **6** (HPLC Anal. 12.81 min, 88% purity). Prior to the next synthetic step, the derivatized pins were treated with 5% Ac_2O in CH_2Cl_2 (100 mL) for 30 min at 23 °C in order to acylate any unreacted amine.

Ethyl-3-[Amide-L-Leu-L-Phe-L-(*N*-pin)*Gln*]-E-propenoate (7). Acid chloride couplings. Pins containing tripeptide intermediate **6** (697 pins at ~2.3 μmol/pin, ~1.61 mmol) were washed with CH_2Cl_2 and dried under vacuum. The pins were suspended in 2% DBU in CH_2Cl_2 (100 mL) and stirred for 30 min at 23 °C, then were purified by the standard procedure and affixed to Multipin™ stems (8×12). Solutions of an appropriate acid chloride (10% w/v acid chloride in ethanol-free $CHCl_3$, 200 μL) and collidine (10% v/v in ethanol-free $CHCl_3$, 200 μL) were added sequentially to deepwell microtiter plate wells (8×12), and the stems/pins containing the deprotected tripeptide were then placed in the wells. The assembled plates were gently shaken on a platform rotator at 23 °C for 1 h, after which the pins were purified by the standard procedure. The coupling and purification procedures were then repeated to give intermediates **7** (representative HPLC purities given in Table 1).

Ethyl-3-[Amide-L-Leu-L-Phe-L-(*N*-pin)*Gln*]-E-propenoate (7). Carboxylic acid couplings. Solutions of appropriate carboxylic acids (0.115 μM in DMF, 100 μL), DIEA (0.23 μM in DMF, 100 μL), and HATU (0.058 μM in DMF, 200 μL) were added sequentially to deepwell microtiter plate wells (8×12) and the stems/pins containing the deprotected tripeptide intermediate (prepared as described in the preceding experimental)



Scheme 2. Reagents and conditions (Tr = CPh_3): (a) HCl in 1,4-dioxane, 23 °C, 2 h; (b) 1.0 equiv Boc-L-Phe-OH, 1.5 equiv HOBt, 3.0 equiv NMM, 1.5 equiv EDC, CH_2Cl_2, 23 °C, overnight, 85%; (c) 1.2 equiv Boc-L-Leu-OH, 1.5 equiv HOBt, 3.0 equiv NMM, 1.5 equiv EDC, CH_2Cl_2, 23 °C, overnight, 87%; (d) excess RC(O)Cl and Et_3N, CH_2Cl_2, 0 °C, 30 min or excess RCO_2H, HOBt, NMM and EDC, CH_2Cl_2, 23 °C, overnight; (e) excess $(i\text{-Pr})_3SiH$, 1:1 TFA:CH_2Cl_2, 23 °C, 30 min.

Table 3. Antirhinoviral activity of compound **8e**

Serotype	EC_{50} (μM)	CC_{50} (μM)
14	0.25	> 100
1A	1.0	> 100
2	0.41	> 100
89	0.22	> 100

were then placed in the wells. The assembled plates were gently shaken on a platform rotator at 23 °C for 1 h, after which the pins were purified by the standard procedure. The coupling and purification procedures were then repeated to give intermediates **7** (representative HPLC purities given in Table 1).

Ethyl-3-(Amide-L-Leu-L-Phe-L-Gln)-E-propenoate (8). A mixture of TFA and H_2O (95:5, 400 μL) was added to deepwell microtiter plate wells (8×12) and the stems/pins containing the intermediates **7** were then placed in the wells. The assembled plates were gently shaken on a platform rotator at 23 °C for 1 h. The volatiles were removed from the plates by evaporation utilizing a Speed Vac (2 h) to provide tripeptide products **8** (representative HPLC purities given in Table 1).

Representative examples of solution phase synthesis

Preparation of (2′*R*/*S*)-ethyl-3-[(1′,2′,3′,4′-tetrahydronaphthalene-2′-carbonyl)-L-Leu-L-Phe-L-*Gln*]-E-propenoate (8a). Ethyl-3-[Boc-L-Phe-L-(Tr-*Gln*)]-E-propenoate (10). A solution of HCl in 1,4-dioxane (4.0 M, 15 mL) was added to a solution of **9**[3a] (3.26 g, 6.01 mmol, 1 equiv) in the same solvent (15 mL) at 23 °C. After 2 h, the volatiles were removed under reduced pressure. The residue was dissolved in CH_2Cl_2 (60 mL) and Boc-L-Phe-OH (1.59 g, 6.01 mmol, 1.0 equiv), HOBt (1.22 g, 9.02 mmol, 1.5 equiv), 4-methylmorpholine (1.98 mL, 18.03 mmol, 3.0 equiv), and EDC (1.73 g, 9.02 mmol, 1.5 equiv) were added sequentially. The reaction mixture was stirred at 23 °C overnight then was partitioned between water (100 mL) and CH_2Cl_2 (2×100 mL). The combined organic layers were dried over Na_2SO_4, concentrated, and the residue was purified by flash column chromatography (40% EtOAc in hexane) to afford **10** (3.55 g, 85%) as white foam: R_f = 0.52 (50% EtOAc in hexanes); IR (cm^{-1}) 3306, 1706, 1661; ^1H NMR ($CDCl_3$) δ 1.29 (t, 3H, J = 7.2), 1.38 (s, 9H), 1.65–1.76 (m, 1H), 1.87–1.99 (m, 1H), 2.25–2.27 (m, 2H), 2.94–3.01 (m, 2H), 4.14–4.26 (m, 3H), 4.48–4.53 (m, 1H), 4.95 (s, br, 1H), 5.64 (d, 1H, J = 15.8), 6.29 (d, 1H, J = 8.1), 6.64 (dd, 1H, J = 15.8, 5.4), 6.80 (s, br, 1H), 7.14–7.32 (m, 20H); Anal. calcd for $C_{42}H_{47}N_3O_6$: C, 73.13; H, 6.87; N, 6.09. Found: C, 72.94; H, 6.97; N, 6.00.

Ethyl-3-[Boc-L-Leu-L-Phe-L-(Tr-*Gln*)]-E-propenoate (11). A solution of HCl in 1,4-dioxane (4.0 M, 15 mL) was added to a solution of **10** (6.40 g, 9.28 mmol, 1 equiv) in the same solvent (15 mL) at 23 °C. After 2 h, the volatiles were removed under reduced pressure. The residue was dissolved in CH_2Cl_2 (100 mL) and Boc-L-Leu-OH (2.58 g, 11.1 mmol, 1.2 equiv), HOBt (1.88 g, 13.9 mmol, 1.5 equiv), 4-methylmorpholine (3.06 mL, 27.8 mmol,

3.0 equiv), and EDC (2.67 g, 13.92 mmol, 1.5 equiv) were added sequentially. The reaction mixture was stirred at 23 °C overnight then was partitioned between water (100 mL) and CH_2Cl_2 (2×100 mL). The combined organic layers were dried over Na_2SO_4, concentrated, and the residue was purified by flash column chromatography (2% CH_3OH in CH_2Cl_2) to afford **11** (6.46 g, 87%) as white foam: R_f = 0.40 (50% EtOAc in hexanes); IR (cm^{-1}) 3284, 1651, 1515; ^1H NMR ($CDCl_3$) δ 0.86 (d, 3H, J = 6.0), 0.89 (d, 3H, J = 6.0), 1.29 (t, 3H, J = 7.2), 1.34 (s, 9H), 1.38–1.60 (m, 3H), 1.62–1.89 (m, 1H), 1.95–1.97 (m, 1H), 2.28–2.30 (m, 2H), 3.06–3.08 (m, 2H), 3.92–3.94 (m, 1H), 4.17 (q, 2H, J = 7.2), 4.48–4.51 (m, 2H), 4.67 (m, 1H), 5.66 (d, 1H, J = 15.9), 6.51–6.57 (m, 2H), 6.69 (dd, 1H, J = 15.6, 5.1), 7.10–7.33 (m, 21H); Anal. calcd for $C_{48}H_{58}N_4O_7 \cdot 0.33H_2O$: C, 71.27; H, 7.31; N, 6.93. Found: C, 70.99; H, 7.29; N, 7.26.

(2′*R*/*S*)-Ethyl-3-[(1′,2′,3′,4′-tetrahydronaphthalene-2′-carbonyl)-L-Leu-L-Phe-L-(Tr-*Gln*)]-E-propenoate (12a). A solution of HCl in 1,4-dioxane (4.0 M, 3 mL) was added to a solution of **11** (0.218 g, 0.27 mmol, 1 equiv) in the same solvent (3 mL) at 23 °C. After 2 h, the volatiles were removed under reduced pressure. The residue was dissolved in CH_2Cl_2 (15 mL) and 1,2,3,4-tetrahydro-2-naphthoic acid (0.071 g, 0.41 mmol, 1.5 equiv), HOBt (0.055 g, 0.41 mmol, 1.5 equiv), 4-methylmorpholine (0.089 mmol, 0.81 mmol, 3.0 equiv), and EDC (0.078 g, 0.41 mmol, 1.5 equiv) were added sequentially. The reaction mixture was stirred at 23 °C overnight then was partitioned between water (50 mL) and CH_2Cl_2 (2×50 mL). The combined organic layers were dried over Na_2SO_4, concentrated, and the residue was purified by flash column chromatography (2% CH_3OH in CH_2Cl_2) to afford **12a** (0.207 g, 89%) as a white foam: R_f = 0.88 (10% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3277, 1635, 1547; ^1H NMR ($CDCl_3$) δ 0.84–0.91 (m, 6H), 1.28 (t, 3H, J = 7.2), 1.39–1.47 (m, 2H), 1.54–1.76 (m, 3H), 1.83–1.97 (m, 2H), 2.20–2.39 (m, 2H), 2.71–2.82 (m, 4H), 2.99–3.08 (m, 2H), 4.17 (q, 2H, J = 7.2), 4.24–4.25 (m, 1H), 4.48–4.55 (m, 2H), 5.68 (d, 1H, J = 15.9), 5.88–5.92 (m, 2H), 6.52–6.55 (m, 1H), 6.62–6.65 (m, 1H), 6.69 (dd, 1H, J = 15.6, 5.1), 6.70–7.32 (m, 25H); Anal. calcd for $C_{54}H_{60}N_4O_6 \cdot 0.5H_2O$: C, 74.54; H, 7.07; N, 6.44. Found: C, 74.47; H, 7.06; N, 6.46.

(2′*R*/*S*)-Ethyl-3-[(1′,2′,3′,4′-tetrahydronaphthalene-2′-carbonyl)-L-Leu-L-Phe-L-*Gln*]-E-propenoate (8a). Triisopropylsilane (0.077 mL, 0.376 mmol, 1.8 equiv) and trifluoroacetic acid (3 mL) were added sequentially to a solution of **12a** (0.185 g, 0.21 mmol, 1 equiv) in CH_2Cl_2 (3 mL) at 23 °C producing a bright yellow solution. The reaction mixture was stirred for 30 min at 23 °C during which time it became colorless. The volatiles were removed under reduced pressure, and the resulting white solid was triturated with Et_2O (10 mL), filtered, and air-dried to give **8a** (0.111 g, 85%): mp = 238–239 °C; R_f = 0.67 (10% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3289, 1654, 1544; ^1H NMR (DMSO-d_6) δ 0.81 (d, 3H, J = 6.0), 0.86 (d, 3H, J = 6.6), 1.21 (t, 3H, J = 7.2), 1.38–1.41 (m, 2H), 1.54–1.75 (m, 4H), 1.90 (m, 1H), 2.02–2.09 (m, 2H), 2.57 (m, 1H), 2.75–2.78 (m, 4H), 2.84–2.91 (m, 1H), 2.95–3.03 (m, 1H), 4.11 (q, 2H, J = 7.2), 4.20–4.27 (m, 1H), 4.39 (m, 1H), 4.43–4.50 (m, 1H), 5.66 (dd, 1H, J = 15.6, 1.5), 6.66–6.69 (m, 1H), 6.71–6.76 (m, 1H), 7.05–7.07 (m, 4H), 7.18–7.23 (m, 6H), 7.88–7.94 (m, 1H), 7.98–8.05 (m, 2H); Anal. calcd for $C_{35}H_{46}N_4O_6$: C, 67.94; H, 7.49; N, 9.05. Found: C, 67.69; H, 7.43; N, 9.00.

Preparation of ethyl-3-[(5′-methylisoxazole-3′-carbonyl)-L-Leu-L-Phe-L-*Gln*]-E-propenoate (8e). Ethyl-3-[(5′-methylisoxazole-3′-carbonyl)-L-Leu-L-Phe-L-(Tr-*Gln*)]-E-propenoate (12e). A solution of HCl in 1,4-dioxane (4.0 M, 3 mL), was added to a solution of **11** (0.216 g, 0.27 mmol, 1 equiv) in the same solvent (3 mL) at 23 °C. After 2 h, the volatiles were removed under reduced pressure. The residue was dissolved in CH_2Cl_2 (15 mL), cooled to 0 °C, and triethylamine (0.112 mL, 0.81 mmol, 3.0 equiv) and 5-methylisoxazole-3-carbonyl chloride (0.058 g, 0.40 mmol, 1.5 equiv) were added sequentially. The reaction mixture was stirred at 0 °C for 30 min, then was partitioned between water (50 mL) and CH_2Cl_2 (2×50 mL). The combined organic layers were dried over Na_2SO_4, concentrated, and the residue was purified by flash column chromatography (2% CH_3OH in CH_2Cl_2) to afford **12e** (0.199 g, 91%) as a white foam: R_f = 0.87 (10% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3286, 1650, 1541; ^1H NMR ($CDCl_3$) δ 0.86 (d, 3H, J = 5.4), 0.89 (d, 3H, J = 5.7), 1.28 (t, 3H, J = 7.2), 1.43–1.59 (m, 2H), 1.67–1.75 (m, 1H), 1.95–1.99 (m, 2H), 2.28 (t, 2H, J = 7.2), 2.41 (s, 3H), 2.97–3.04 (m, 1H), 3.06–3.13 (m, 1H), 4.17 (q, 2H, J = 7.2), 4.31–4.33 (m, 1H), 4.48–4.52 (m, 2H), 5.72 (d, 1H, J = 15.9), 6.19 (s, 1H), 6.41 (d, 1H, J = 7.5), 6.59 (d, 1H, J = 8.1), 6.71 (dd, 1H, J = 15.3, 6.0), 6.95 (d, 1H, J = 6.6), 7.09–7.21 (m, 21H); Anal. calcd for $C_{48}H_{53}N_5O_7 \cdot H_2O$: C, 69.84; H, 6.58; N, 8.63. Found: C, 69.80; H, 6.54; N, 8.57.

Ethyl-3-[(5′-methylisoxazole-3′-carbonyl)-L-Leu-L-Phe-L-*Gln*]-E-propenoate (8e). Triisopropylsilane (0.077 mL, 0.376 mmol, 1.8 equiv) and trifluoroacetic acid (3 mL) were added sequentially to a solution of **12e** (0.185 g, 0.21 mmol, 1 equiv) in CH_2Cl_2 (3 mL) at 23 °C producing a bright yellow solution. The reaction mixture was stirred for 30 min at 23 °C during which time it became colorless. The volatiles were removed under reduced pressure, and the resulting white solid was triturated with Et_2O (10 mL), filtered, and air-dried to give **8e** (0.087 g, 81%) as a white solid: mp = 223–225 °C; R_f = 0.56 (10% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3298, 1662, 1544, 1457, 1278; ^1H NMR (DMSO-d_6) δ 0.81 (d, 3H, J = 6.0), 0.85 (d, 3H, J = 6.3), 1.23 (t, 3H, J = 6.9), 1.38–1.42 (m, 1H), 1.48–1.77 (m, 4H), 2.04 (t, 2H, J = 7.2), 2.46 (s, 3H), 2.78–2.86 (m, 1H), 2.93–3.00 (m, 1H), 4.11 (q, 2H, J = 7.2), 4.36–4.54 (m, 3H), 5.63 (d, 1H, J = 15.6), 6.56 (s, 1H), 6.68 (dd, 1H, J = 15.9, 5.4), 6.76 (s, br, 1H), 7.19 (m, 6H), 8.09 (d, 1H, J = 8.1), 8.14 (d, 1H, J = 7.8), 8.58 (d, 1H, J = 7.5); Anal. calcd for $C_{29}H_{39}N_5O_7$: C, 61.15; H, 6.90; N, 12.29. Found: C, 60.98; H, 6.94; N, 12.17.

The following compounds were prepared using one of the general solution-phase synthesis procedures described above.

(2′S)-Ethyl-3-[(2′-chloro-4′-methylpentane-1′-carbonyl)-L-Leu-L-Phe-L-(Tr-*Gln*)]-E-propenoate (12b). R_f = 0.90 (10% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3296, 1654, 1524; ^1H NMR ($CDCl_3$) δ 0.84–0.94 (m, 12H), 1.29 (t, 3H, J = 7.2), 1.42–2.02 (m, 7H), 2.28 (t, 2H, J = 7.2), 2.96–3.11 (m, 2H), 3.61–3.71 (m, 1H), 3.75–3.79 (m, 1H), 4.09–4.23 (m, 3H), 4.45–4.52 (m, 2H), 5.70 (d, 1H, J = 15.6), 6.47 (d, 1H, J = 7.5), 6.59 (d, 1H, J = 8.1), 6.69 (dd, 1H, J = 15.9, 5.7), 6.75 (d, 1H, J = 7.2), 7.05–7.33 (m, 21H); Anal. calcd for $C_{49}H_{59}N_4O_6 \cdot 0.75H_2O$: C, 69.32; H, 7.18; N, 6.60. Found: C, 69.36; H, 7.15; N, 6.62.

Ethyl-3-[(3′-thiophen-2′-yl-acrylic-1′-carbonyl)-L-Leu-L-Phe-L-(Tr-*Gln*)]-E-propenoate (12c). R_f = 0.87 (10% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3290, 1654, 1526; ^1H NMR ($CDCl_3$) δ 0.85 (d, 3H, J = 5.7), 0.89 (d, 3H, J = 6.0), 1.28 (t, 3H, J = 7.2), 1.38–1.47 (m, 1H), 1.53–1.57 (m, 1H), 1.97–2.08 (m, 1H), 2.26–2.35 (m, 2H), 3.00–3.07 (m, 1H), 3.12–3.19 (m, 1H), 3.62–3.78 (m, 2H), 4.16 (q, 2H, J = 7.2), 4.25 (m, 1H), 4.46–4.53 (m, 2H), 5.75 (d, 1H, J = 15.9), 5.82 (d, 1H, J = 6.3), 5.98 (d, 1H, J = 15.6), 6.66 (d, 1H, J = 7.8), 6.73 (dd, 1H, J = 15.6, 5.1), 6.81 (d, 1H, J = 8.4), 7.02 (t, 1H, J = 3.6), 7.09–7.14 (m, 3H), 7.20–7.34 (m, 20H), 7.54 (d, 1H, J = 15.3); Anal. calcd for $C_{50}H_{54}N_4O_6S \cdot 0.5H_2O$: C, 70.81; H, 6.54; N, 6.61. Found: C, 70.50; H, 6.50; N, 6.96.

(2′S)-Ethyl-3-[(2′,5′-dihydro-1′H-pyrrole-2′-carbonyl)-L-Leu-L-Phe-L-(Tr-*Gln*)]-E-propenoate (12d). R_f = 0.88 (10% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3280, 1645, 1541; ^1H NMR ($CDCl_3$) δ 0.80 (d, 3H, J = 5.7), 0.86 (d, 3H, J = 6.0), 1.27 (t, 3H, J = 7.2), 1.47 (s, 9H), 1.75–1.86 (m, 2H), 2.00–2.07 (m, 1H), 2.26–2.41 (m, 2H), 2.88–2.96 (m, 2H), 3.44–3.50 (m, 2H), 3.62–3.71 (m, 2H), 3.76–3.80 (m, 1H), 4.16 (q, 2H, J = 7.2), 4.58 (m, 1H), 4.75 (m, 2H), 5.37 (m, 1H), 5.75–5.77 (m, 1H), 5.82 (d, 1H, J = 15.6), 6.49 (d, 1H, J = 4.8), 6.78–6.85 (m, 2H), 7.12–7.32 (m, 20H), 7.45 (s, 1H); Anal. calcd for $C_{53}H_{62}N_5O_8 \cdot 1.0H_2O$: C, 69.56; H, 7.05; N, 7.65. Found: C, 69.46; H, 6.98; N, 7.80.

Ethyl-3-[(3′-phenylpropyl-1′-carbonyl)-L-Leu-L-Phe-L-(Tr-*Gln*)]-E-propenoate (12f). R_f = 0.83 (10% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3280, 1645, 1541; ^1H NMR ($CDCl_3$) δ 0.77 (d, 3H, J = 6.0), 0.81 (d, 3H, J = 6.3), 1.28 (t, 3H, J = 7.2), 1.34–1.44 (m, 1H), 1.62 (m, 2H), 1.70–1.76 (m, 1H), 1.98–2.00 (m, 1H), 2.23–2.37 (m, 4H), 2.77 (t, 2H, J = 7.2), 2.92–2.97 (m, 1H), 3.06–3.13 (m, 1H), 4.09–4.14 (m, 1H), 4.17 (q, 2H, J = 7.4), 4.42–4.50 (m, 2H), 5.56 (d, 1H, J = 6.3), 5.72 (dd, 1H, J = 15.6, 1.5), 6.32 (d, 1H, J = 7.8), 6.62 (d, 1H, J = 7.8), 6.71 (dd, 1H, J = 15.9, 5.1), 7.08–7.12 (m, 5H), 7.18–7.31 (m, 21H); Anal. calcd for $C_{52}H_{58}N_4O_6$: C, 74.79; H, 7.00; N, 6.71. Found: C, 74.52; H, 6.97; N, 6.82.

Ethyl-3-[(isoxazole-5′-carbonyl)-L-Leu-L-Phe-L-(Tr-*Gln*)]-E-propenoate (12g). R_f = 0.76 (10% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3282, 1643, 1530; ^1H NMR ($CDCl_3$) δ 0.87 (t, 6H, J = 6.6), 1.29 (t, 3H, J = 7.2), 1.49–1.64 (m, 3H), 1.69–1.80 (m, 1H), 1.90–1.96 (m, 1H), 2.30 (t, 2H, J = 7.2), 2.92–2.96 (m, 1H), 3.02–3.09 (m, 1H), 4.17 (q, 2H, J = 7.2), 4.42–4.48 (m, 3H), 5.69 (d, 1H, J = 15.3), 6.65 (s, br, 1H), 6.66 (dd, 1H, J = 15.9, 5.4), 6.76–6.79 (m, 2H), 7.00–7.31 (m, 22H), 8.24 (s, 1H); Anal. calcd for $C_{47}H_{51}N_5O_7 \cdot 0.75H_2O$: C, 69.57; H, 6.52; N, 8.63. Found: C, 69.58; H, 6.51; N, 8.63.

(2′S)-Ethyl-3-[(2′-chloro-4′-methylpentane-1′-carbonyl)-L-Leu-L-Phe-L-Gln]-E-propenoate (8b). mp = 230–232 °C; R_f = 0.60 (10% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3288, 1640, 1544; ^1H NMR (DMSO-d_6) δ 0.81–0.89 (m, 12H), 1.21 (t, 3H, J = 7.2), 1.38–1.43 (m, 2H), 1.51–1.76 (m, 5H), 2.04 (t, 2H, J = 7.5), 2.80–2.88 (m, 1H), 2.91–2.98 (m, 1H), 4.11 (q, 2H, J = 7.2), 4.27–4.35 (m, 3H), 4.43–4.50 (m, 2H), 5.61 (d, 1H, J = 15.6), 6.69 (dd, 1H, J = 15.9, 5.4), 6.74 (s, br, 1H), 7.17–7.25 (m, 6H), 8.05 (d, 1H, J = 8.1), 8.14 (d, 1H, J = 7.8), 8.36 (d, 1H, J = 8.1); Anal. calcd for $C_{30}H_{45}ClN_4O_6 \cdot 0.75H_2O$: C, 59.39; H, 7.73; N, 9.24. Found: C, 59.37; H, 7.57; N, 9.18.

Ethyl-3-[(3′-thiophen-2′-yl-acrylic-1′-carbonyl)-L-Leu-L-Phe-L-*Gln*]-E-propenoate (8c). mp = 250–251 °C; R_f = 0.59 (10% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3288, 1654, 1543; ^1H NMR (DMSO-d_6) δ 0.80 (d, 3H, J = 6.6), 0.84 (d, 3H, J = 6.6), 1.21 (t, 3H, J = 6.9), 1.34–1.39 (m, 2H), 1.48–1.54 (m, 1H), 1.63–1.81 (m, 2H), 2.05 (t, 2H, J = 7.2), 2.81–2.89 (m, 1H), 2.98–3.04 (m, 1H), 4.07 (t, 2H, J = 6.9), 4.24–4.49 (m, 3H), 5.67 (dd, 1H, J = 15.6, 1.2), 6.47 (d, 1H, J = 15.6), 6.71 (dd, 1H, J = 15.6, 5.4), 6.77 (s, br, 1H), 7.09–7.24 (m, 7H), 7.38 (d, 1H, J = 3.0), 7.57 (d, 1H, J = 15.3), 7.60 (d, 1H, J = 4.2), 7.96 (d, 1H, J = 8.1), 8.07 (d, 1H, J = 8.1), 8.26 (d, 1H, J = 7.5); Anal. calcd for $C_{31}H_{40}N_4O_6S$: C, 62.40, H, 6.76; N, 9.39. Found: C, 62.24; H, 6.85; N, 9.35.

(2′S)-Ethyl-3-[(2′,5′-dihydro-1′*H*-pyrrole-2′-carbonyl)-L-Leu-L-Phe-L-*Gln*]-E-propenoate (8d). mp = 184–188 °C; R_f = 0.35 (10% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3279, 1657, 1560, 1202; ^1H NMR (DMSO-d_6) δ 0.87 (t, 6H, J = 7.2), 1.21 (t, 3H, J = 7.2), 1.41–1.45 (m, 2H), 1.50–1.76 (m, 3H), 2.03 (t, 2H, J = 7.8), 2.78–2.85 (m, 1H), 2.91–2.97 (m, 1H), 4.04 (m, 2H), 4.11 (q, 2H, J = 7.2 Hz), 4.34–4.41 (m, 2H), 4.43–4.50 (m, 1H), 4.97 (s, br, 1H), 5.60 (dd, 1H, J = 15.6, 1.2), 5.77–5.79 (m, 1H), 5.99–6.01 (m, 1H), 6.67 (dd, 1H, J = 15.9, 5.4), 6.78 (s, br, 1H), 7.17–7.24 (m, 7H), 8.12 (d, 1H, J = 8.1), 8.27 (d, 1H, J = 7.8), 8.74 (d, 1H, J = 8.1); Anal. calcd for $C_{29}H_{41}N_5O_6 \cdot 1.0H_2O$: C, 60.72; H, 7.55; N, 12.21. Found: C, 60.96; H, 7.34; N, 12.34.

Ethyl-3-[(3′-phenylpropyl-1′-carbonyl)-L-Leu-L-Phe-L-*Gln*]-E-propenoate (8f). mp = 245–246 °C; R_f = 0.54 (10% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3389, 1640, 1545, 1280, 1188; ^1H NMR (DMSO-d_6) δ 0.74 (d, 3H, J = 6.3), 0.80 (d, 3H, J = 6.3), 1.21 (t, 3H, J = 6.9), 1.29–1.32 (m, 2H), 1.36–1.40 (m, 1H), 1.62–1.77 (m, 2H), 2.06 (t, 2H, J = 7.5), 2.32–2.44 (m, 2H), 2.75–2.80 (m, 2H), 2.84–2.89 (m, 1H), 2.96–3.02 (m, 1H), 4.11 (q, 2H, J = 7.2), 4.18–4.22 (m, 1H), 4.38–4.45 (m, 2H), 5.62 (d, 1H, J = 15.6), 6.71 (dd, 1H, J = 15.9, 5.4), 6.76 (s, br, 1H), 7.17–7.27 (m, 11H), 7.97–7.80 (m, 3H); Anal. calcd for $C_{33}H_{44}N_4O_6$: C, 66.87; H, 7.48; N, 9.45. Found: C, 66.61; H, 7.44; N, 9.37.

Ethyl-3-[(isoxazole-5′-carbonyl)-L-Leu-L-Phe-L-*Gln*]-E-propenoate (8g). mp = 217–220 °C; R_f = 0.51 (10% CH_3OH in CH_2Cl_2); IR (cm^{-1}) 3302, 1655, 1541; ^1H NMR (DMSO-d_6) δ 0.81 (d, 3H, J = 6.0), 0.86 (d, 3H, J = 6.0), 1.21 (t, 3H J = 7.2), 1.42–1.75 (m, 5H), 2.04 (t, 2H, J = 7.2), 2.78–2.87 (m, 1H), 2.94–3.01 (m, 1H), 4.11 (q, 2H, J = 7.2), 4.37 (m, 1H), 4.41–4.52 (m, 2H), 5.64 (d, 1H, J = 15.6), 6.68 (dd, 1H, J = 15.9, 5.4), 6.76 (s, br, 1H), 7.12–7.19 (m, 7H), 8.02 (d, 1H, J = 8.1), 8.20 (d, 1H, J = 8.1), 8.74 (d, 1H, J = 1.8), 8.94 (d, 1H, J = 7.8); Anal. calcd for $C_{28}H_{37}N_5O_7$: C, 60.53; H, 6.71; N, 12.60. Found: C, 60.44; H, 6.66; N, 12.48.

Enzyme assays

The general conditions of the fluorescence resonance energy transfer assay utilized to assess 3CP activity are described in the literature.[18] 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, 3–5 concentrations were examined and data from the continuous assays were directly analysed with the nonlinear regression analysis program ENZFITTER[19] to obtain first order rate constants for enzyme inactivation at each inhibitor concentration. The slope of a graph of k_{obs}(I) versus [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%.

Enzyme assays (high-throughput)

Compounds were assayed in a 96 (8×12) well format, using the previously described[18] peptide substrate. Assays contained 2 μM (nominal) test compound, 1 μM substrate, 20 mM potassium phosphate buffer (pH 7.5), 2% DMSO and 0.2 μM HRV-14 3CP. Assays were initiated by addition of enzyme, and fluorescence was measured on a DYNEX Fluorolite 1000 plate reader prior to and 40 and 390 s after addition of enzyme. Control wells contained either 2% DMSO or 2.5 μM of the known 3CP inhibitors **1** and **2** (Fig. 1) and displayed approximately 0%, 75%, and 100% inhibition in this assay, respectively.

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.[20] Briefly, H1-HeLa cells were infected with HRV-14 at a multiplicity of infection (m.o.i.) 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 minimize false positives due to cytotoxicity, only compounds displaying CC_{50}s greater than 10× the observed EC_{50}s were considered to be active antiviral agents. Using the above method, the EC_{50} of a representative compound (**1**) was calculated to be 0.54 ± 0.27 μM (range 0.18 to 1 μM). The EC_{50} of the known antirhinoviral agent Pirodavir was similarly determined to be 0.02 ± 0.01 μM (range 0.01 to 0.05 μM) and was comparable to the 0.03 μM minimal inhibitory concentration value previously reported.[21]

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