

Isatin Compounds as Noncovalent SARS Coronavirus 3C-like Protease Inhibitors

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Abstract: A series of isatin derivatives were synthesized and tested against SARS CoV 3C-like protease. Substitutions at the N-1 and C-5 positions were examined to elucidate the differences in substrate binding sites of the rhinovirus 3C protease and SARS CoV 3C-like protease. Compound **5f** shows significant inhibition with an IC₅₀ of 0.37 μM. Further study showed that, unlike the irreversible covalent binding of isatin derivatives to human rhinovirus 3C protease, the compounds tested in this study are all noncovalent reversible inhibitors.

Severe acute respiratory syndrome (SARS), a life-threatening form of atypical pneumonia, rapidly spread from its likely origin in southern China to several other countries during late 2002 and early 2003.¹ SARS is characterized by high fever (>38 °C), malaise, rigor, headache, and nonproductive cough or dyspnea and may progress to generalized interstitial infiltrates in the lung.² The disease had a quite high mortality rate (up to 15–19%) during the initial outbreak. In early 2003, the novel human coronavirus named SARS coronavirus (SARS CoV) was identified as the causative agent of SARS.^{1a} Since SARS CoV 3C-like protease plays a very important role in the viral life cycle, it has been proposed as a key target for the structure-based drug design against SARS.³

SARS CoV 3CL protease inhibitors have been reported from both screened compound libraries^{4–9} and designed compounds based on substrate structure or active site properties.^{10–14} Their scaffolds are diverse, including C2 symmetric peptidomimetic compounds,⁴ 3-quinolinecarboxylic acid derivatives,⁵ thiophene-2-carboxylate derivatives,⁶ zinc-conjugated compounds,⁷ cinnanserin,⁸ calmodulin,⁹ keto-glutamine analogues,¹⁰ anilide,¹¹ bifunctional boronic acid compounds,¹² isatin derivatives,¹³ and etacrynic acid derivatives.¹⁴ With metal-conjugated structures, some molecules might make a covalent bond with Cys145 at the active site of SARS CoV 3CL protease.^{5–8} Some other reported peptidyl molecules have complicated scaffolds and may have low bioavailability.^{4,10,14} Till now, only a few candidates have high inhibition activity against SARS CoV 3C-like protease with IC₅₀ values below the micromolar level.

The active site of SARS CoV 3CL protease contains Cys145 and His41 to constitute a catalytic dyad, in which the cysteine residue functions as the common nucleophile in the proteolytic process.¹⁵ The catalytic domain is similar to other virus 3C proteases, such as human rhinovirus (HRV) 3C protease. Therefore, HRV 3C protease inhibitors may also be potential

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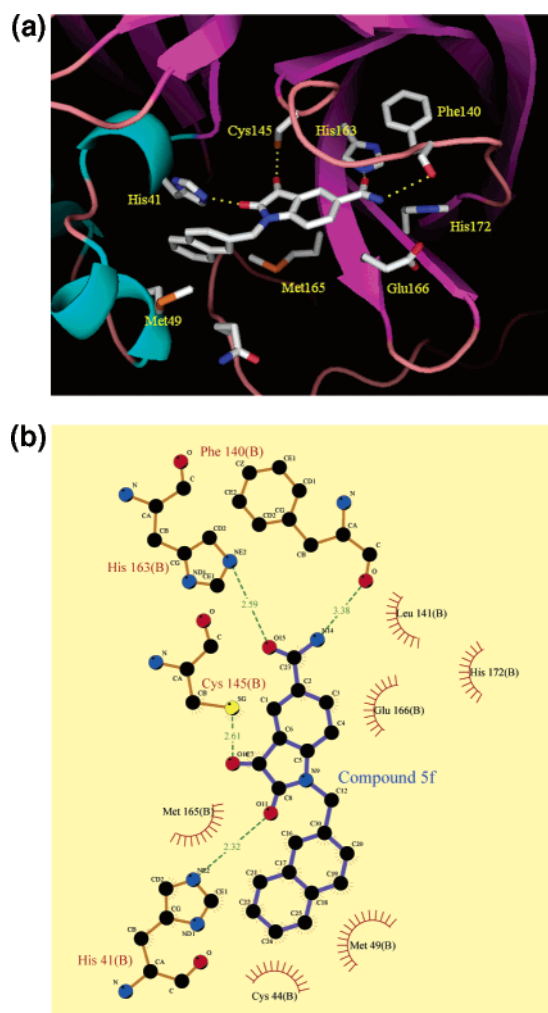


Figure 1. (a) The complex structure of compound **5f** and SARS CoV 3C-like protease from docking study. The figure was drawn by PyMOL.²¹ The protein was shown in ribbons, and the compound **5f** and interacting residues in stick (blue: nitrogen, red: oxygen, orange: sulfur). The carboxamide at C-5 makes H-bonds with Phe140 and His163, and the oxygens at C-2 and C-3 make H-bonds with His41 and Cys145. The naphthyl ring fits into the hydrophobic S2 site formed by Met 49 and Met 165. (b) The contacts of compound **5f** and SARS CoV 3C-like protease were shown by LIGPLOT.²²

inhibitors of SARS CoV 3CL protease. Chen et al. reported that N-substituted isatin derivatives which were HRV inhibitors were effective SARS CoV 3C-like protease inhibitors.¹³ They focused mainly on the isatin C-4, C-5, C-7, and N substituents, aiming at the two sites (P1, P2) that were important for HRV protease inhibitors. Most of their compounds have halogen substituents at the C-5 position, and the most active compound is an N-substituted 5-iodoisatin. As SARS CoV 3C-like protease specifically cleaves peptide substrates with Gln at the P1 site, a carboxamide group on the inhibitors may occupy the position of the Gln side chain that forms specific hydrogen bonds with Phe140 and His163. We have designed and synthesized a series of N-substituted 5-carboxamide-isatin compounds and compared their activities with 5-halogen-substituted compounds.^{13,16}

Isatin compounds were docked into the substrate binding pocket of SARS CoV 3C-like protease using the SYBYL program (Tripos, Inc.) in a similar orientation as that of the covalent binding substrate analogue as revealed in the recently

Table 1. Inhibition Activities of Isatin Derivatives against SARS CoV 3C-like Protease

name	R ₁	R ₂	IC ₅₀ or percentage (%) of inhibition at 100 μM	K _i (experimental)	K _i (theoretical)	pK _i (experimental)	pK _i (theoretical) ^a
1e	H	I	- ^b				
2a	CH ₃	I	-				
2b	CH ₃ CH ₂ CH ₂	I	25% at 100 μM				
2c	<i>n</i> -C ₄ H ₉	I	66 ± 5 μM	6.6 × 10 ⁻⁵	8.5 × 10 ⁻⁵	4.18	4.07
2d	Ph CH ₂	I	50 ± 20 μM	5.0 × 10 ⁻⁵	3.0 × 10 ⁻⁵	4.3	4.53
2f	β-C ₁₀ H ₇ CH ₂	I	1.1 ± 0.2 μM	1.1 × 10 ⁻⁶	1.2 × 10 ⁻⁶	5.96	5.93
3a	CH ₃	CO ₂ CH ₃	-				
3b	CH ₃ CH ₂ CH ₂	CO ₂ CH ₃	-				
3c	<i>n</i> -C ₄ H ₉	CO ₂ CH ₃	-				
3d	Ph CH ₂	CO ₂ CH ₃	-				
3f	β-C ₁₀ H ₇ CH ₂	CO ₂ CH ₃	-				
4a	CH ₃	CO ₂ H	-				
4b	CH ₃ CH ₂ CH ₂	CO ₂ H	-				
4c	<i>n</i> -C ₄ H ₉	CO ₂ H	-				
4d	PhCH ₂	CO ₂ H	-				
4e	H	CO ₂ H	-				
4f	β-C ₁₀ H ₇ CH ₂	CO ₂ H	46% at 100 μM				
5a	CH ₃	CONH ₂	71 ± 6 μM	7.1 × 10 ⁻⁵	2.9 × 10 ⁻⁴	4.14	3.54
5b	CH ₃ CH ₂ CH ₂	CONH ₂	25 ± 2 μM	2.5 × 10 ⁻⁵	1.6 × 10 ⁻⁴	4.6	3.79
5c	<i>n</i> -C ₄ H ₉	CONH ₂	19 ± 2 μM	1.9 × 10 ⁻⁵	1.7 × 10 ⁻⁴	4.72	3.78
5d	PhCH ₂	CONH ₂	12.5 ± 0.5 μM	1.3 × 10 ⁻⁵	3.3 × 10 ⁻⁵	4.9	4.48
5e	H	CONH ₂	-				
5f	β-C ₁₀ H ₇ CH ₂	CONH ₂	0.37 ± 0.03 μM	1.2 × 10 ⁻⁷	4.1 × 10 ⁻⁷	6.92	6.39

^a The pK_i values were calculated by SCORE program.¹⁸ ^b Inhibition less than 25% at 100 μM.

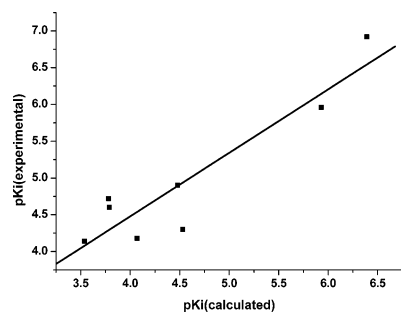
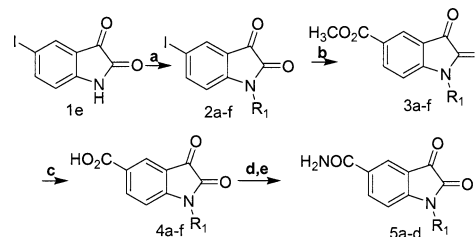


Figure 2. Calculated pK_i vs experimental pK_i. The data were fit to a straight line with an R² of 0.85.

published complex crystal structure.¹⁷ The carbonyl oxygen atoms at C-2 and C-3 make hydrogen bonds with the active site residues, His41 and Cys145. The carboxamide at C-5 holds the same position as that of the substrate P1 Gln side chain and makes H-bonds with the backbone carbonyl of Phe140 and the imidazole ring of His163. Alkyl groups at N-1 fit into the hydrophobic S2 site formed by Met 49 and Met 165. The binding orientation is similar to that in HRV-2 protease; where Thr142 is replaced by Phe140 at the P1 pocket.¹⁶ Figure 1 shows the orientation of compound **5f** in the substrate binding pocket.

We have calculated the binding affinity of the protein–ligand complexes (Table 1) using the SCORE program.¹⁸ The experimental pK_i values correlate well with the calculated pK_is (R² of 0.85, Figure 2), verifying that the complex structures built are reasonable.

Compounds **5a–d** were synthesized by a five-step synthetic route (Scheme 1).¹⁶ (1) Hydrogen at N-1 was substituted by the alkyl groups using RX. (2) 5-Iodoisatin was changed into 5-carbomethoxyisatin via bis(triphenylphosphine)palladium chloride catalysis in a carbon monoxide atmosphere. (3) The hydrolysis of the methyl ester to carboxylic acid was achieved using potassium carbonate. (4) The activated 5-carboxylic acid *N*-hydroxysuccinimide ester was made with *N*-hydroxysuccin-

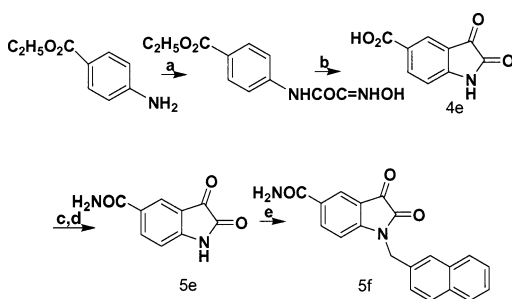
Scheme 1^a

^a Reagents and conditions: (a) NaH, DMF, R₁X; (b) CO, MeOH, DMF, Et₃N, (Ph₃P)₂PdCl₂; (c) K₂CO₃, MeOH, H₂O; (d) *N*-hydroxysuccinimide, EDC, CH₂Cl₂; (e) DMF, NH₃(g).

imide. (5) Isatin-5-carboxamides were obtained by treating 5-carboxylic acid *N*-hydroxysuccinimide ester with ammonia.

Preparation of **5f** was started from ethyl 4-aminobenzoate. First, *N*-(4-ethoxycarbonylphenyl)-2-(hydroxyimino)acetamide was obtained by reaction of ethyl 4-aminobenzoate, hydroxylamine hydrochloride, and 2,2,2-trichloroacetaldehyde monohydrate, and it was converted into isatin-5-carboxylic by treatment with concentrated sulfuric acid at 90 °C; then 1*H*-isatin-5-carboxylic acid was changed into 1*H*-isatin-5-carboxamide. Finally, **5f** was obtained by reaction of 1*H*-isatin-5-carboxamide with 2-(bromomethyl)naphthalene as shown in Scheme 2. Compound structures were confirmed by ¹H NMR, MS, and elemental analysis, and the data are consistent with previously published data.¹⁶

The inhibition activity was measured by continuous colorimetric assay using colorimetric substrate Thr-Ser-Ala-Val-Leu-Gln-pNA (HPLC purity, purchased from GL Biochemistry Ltd).¹⁹ SARS 3C-like protease was cloned and expressed in *Escherichia coli* and purified according to the published procedure.²⁰ The purified protease was stored with 5 mM DDT and 50% glycerol in Tris-HCl buffer at –20 °C. Before the inhibition test, the enzyme buffer was changed to PBS (20 mM, pH 7.4). Colorimetric measurements of the protease activity

Scheme 2^a

^a Reagents and conditions: (a) $\text{Cl}_3\text{CCH}(\text{OH})_2$, $\text{NH}_2\text{OH}\cdot\text{HCl}$, Na_2SO_4 , H_2O ; (b) H_2SO_4 ; (c) *N*-hydroxysuccinimide, EDC, CH_2Cl_2 ; (d) DMF, $\text{NH}_3(\text{g})$; (e) NaH , DMF, 2-(bromomethyl)naphthalene.

were performed in 96-well microtiter plates using a multiwell ultraviolet spectrometer (Spectra Max 190; Molecular Device). Substrate (final concentration of 200 μM) and inhibitors (final concentration 0–5.5 μM) were mixed together in 20 mM PBS buffer (pH = 7.4) in the presence of 5% (vol/vol) DMSO, and the reaction was initiated by adding SARS CoV 3C-like protease (final concentration of 1 μM). Increase in absorbance at 390 nm was measured, and the IC_{50} value was calculated by the following equation:

$$V_0/V = 1 + [I]/\text{IC}_{50}$$

where V_0 is the initial rate of the reaction without inhibitor, V is the initial rate of the reaction with the inhibitor at various concentrations, $[I]$ is the concentration of the inhibitor, and IC_{50} is the concentration resulting in 50% inhibition.

Before the reaction, the enzyme was exchanged to a fresh phosphate buffer without dithiothreitol (DTT), as DTT reacted with isatin derivatives and caused an increase in the absorbance at 390 nm. An HPLC-based enzyme activity assay was also used to verify the reaction. HPLC assay showed that the inhibition of these isatin derivatives decreased if DTT was added to the mixture. Out of the 23 compounds, 10 were found to be active at 100 μM . The IC_{50} values of 8 compounds are lower than 100 μM , the best being 0.37 \pm 0.03 μM ($K_i = 0.12$ μM , compound **5f**). Table 1 lists the inhibition activities of the compounds. The K_i values of the 8 compounds were calculated according to the literature.¹⁹ The $\text{p}K_i$ ($-\log K_i$) values are also listed in Table 1.

Compound **5f** is a potent inhibitor with an IC_{50} of 0.37 \pm 0.03 μM against SARS CoV 3C-like protease. As this compound was reported to be a covalent binding inhibitor of human rhinovirus 3C protease,¹⁶ it is interesting to know whether it will bind covalently or noncovalently to SARS CoV 3C-like protease.

SARS CoV 3C-like protease was mixed with compound **5f** and incubated at 37 $^\circ\text{C}$ under nitrogen atmosphere for 72 h. The mixture was measured by a MALDI-TOFF mass spectrometer. The protein peak remained at the same position as that in the pure enzyme, indicating that the inhibitor did not form a covalent bond with the protease (data not shown).

We also preincubated the inhibitor and the protease for 24 h at 4 $^\circ\text{C}$ and examined the enzyme activity of the solution. The activity of the protease that was mixed with the inhibitor for 24 h was the same as that of the mixture which was tested immediately after mixing. This confirms again that the protease did not change after incubation with the inhibitor for a long period of time. The UV–vis spectrum also showed little change after the protease was mixed with the inhibitor. All this evidence indicates that Cys145 did not form a covalent bond with the

Table 2. Selective Inhibition of Compound **5f** against Various Cysteine and Serine Proteases^a

enzyme	SARS 3CL	papain ^b	chymotrypsin ^c	trypsin ^d	HRV-14
IC_{50} (μM)	0.37 \pm 0.03	11 \pm 1	34 \pm 2	352 \pm 10	0.004 \pm 0.003 ¹⁶

^a The IC_{50} values were measured by HPLC except SARS 3CL protease and concentration of substrate (S01²⁰) was 400 μM . ^b The concentration of papain was 4.5 μM . ^c The concentration of chymotrypsin was 0.45 μM . ^d The concentration of trypsin was 0.23 μM .

isatin inhibitors. It is more probable that the oxygen at C-3 may form a hydrogen bond with Cys145 instead of forming a covalent bond between the carbon and sulfur atoms.

From the protein–inhibitor complex modeling study and the inhibition data, we concluded the following: (1) A hydrophobic group placed at the N-1 position can fit into the hydrophobic pocket, and large, aromatic hydrophobic groups lead to high activity. The naphthyl group also made the molecule more rigid and fit into the pocket well. (2) Carboxamide substitution at the C-5 position made the molecule three to four times more active than iodine substitution in which iodine was demonstrated to be a hydrophobic and electron affinitive group.¹³ However, the carboxylic acid and ester derivatives showed no inhibition at all, which was completely different from their binding with HRV protease.¹⁶ The carboxamide group can form hydrogen bonds with the side chain of His163 and the backbone of Phe140, while the carboxylic acid and ester can only form one hydrogen bond. The carboxylic acid and ester were also close to Glu166 and encounter strong charge repulsion, thus making them unsuitable for binding to the P1 site. (3) The C-3 oxygen formed a hydrogen bond with the side chain of Cys145 that was important for inhibition activity. If the oxygen atom was changed into other groups, such as 4-methoxyphenylimino or 1-naphthylimino, the compounds lost activity (data not shown). DTT may react with the C-3 ketone group and inactivate the compounds.¹⁶

We also tested the inhibition activity of compound **5f** against other proteases (Table 2). Compound **5f** was a more selective inhibitor against SARS CoV 3C-like protease than papain (30 times), chymotrypsin (92 times), and trypsin (950 times). As **5f** was also a potent inhibitor against HRV-14 3C protease,¹⁶ it may be used as a broad-spectrum antiviral agent.

In conclusion, a series of *N*-substituted isatin derivatives were synthesized and tested against SARS CoV 3C-like protease using a colorimetric assay and confirmed by HPLC. The compounds were shown to be noncovalent reversible inhibitors of SARS CoV 3C-like protease. The C-5 position was found to favor a carboxamide group and the N-1 position to favor large hydrophobic substituents. The lowest IC_{50} value (0.37 μM) was observed with compound **5f**, and it was selective for SARS CoV 3C-like protease over other proteases.

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Supporting Information Available: Experimental procedure for the synthesis of target compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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