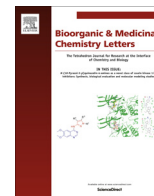




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Synthesis and evaluation of phenylisoserine derivatives for the SARS-CoV 3CL protease inhibitor



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ABSTRACT

Synthesis and evaluation of new scaffold phenylisoserine derivatives connected with the essential functional groups against SARS CoV 3CL protease are described. The phenylisoserine backbone was found by simulation on GOLD software and the structure activity relationship study of phenylisoserine derivatives gave **SK80** with an IC₅₀ value of 43 μM against SARS CoV 3CL R188I mutant protease.

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Severe acute respiratory syndrome (SARS) is a contagious respiratory disease in humans which is caused by the SARS coronavirus (SARS-CoV).^{1–3} The key enzyme in the processing of polyproteins pp1a and pp1ab, translated by the viral RNA genome of SARS-CoV is called a 3C-like protease (3CL protease). Due to its functional importance in the viral life cycle, SARS-CoV 3CL protease is considered to be an attractive target for a drug for SARS therapy. For this reason, a variety of 3CL protease inhibitors have been reported in the literature over the past decade.^{4–11} They are non-peptidyl compounds derived from natural products and/or substrate-based peptidomimetics. However, no effective therapeutic drug or vaccine has been developed to date, although many candidates targeting SARS 3CL protease have been identified. In a research program on SARS 3CL protease, we found for the first time that mature SARS 3CL protease is subject to degradation at the 188Arg/189Gln site. R188I mutant protease with high activity and stability was prepared.¹⁰ Although our group additionally found that tetrapeptide aldehyde Ac-Thr-Val-Cha-His-H (**1**) showed high inhibitory activity with an IC₅₀ value of 98 nM against SARS 3CL R188I mutant protease in 2008,¹² there are generally enzymatic digestions of peptide chains and α-proton racemization.

Next, we designed and synthesized serine derivatives focusing on the P1, P2 and P4 sites of tetrapeptide aldehyde (**1**) by the combination of docking simulation and a structure activity relationship study. As for the results, SK23 (**2**) was given as a candidate compound against SARS 3CL R188I mutant protease.¹³ In this case, the inhibitory activity of SK23 (**2**) was much lower than the peptide aldehyde inhibitor (**1**). However, SK23 (**2**) has no thiol capture unit and achieved a marked structural transformation from peptidyl compounds based on a substrate mimic strategy. Investigation of a scaffold with three essential functional groups is needed to develop small molecular inhibitors using a serine scaffold for non-peptidyl compounds (Fig. 1).

Scaffold hopping techniques have been widely applied by medicinal chemists to discover equipotent compounds with novel backbones that have improved properties.¹⁴ Scaffold hopping into four major categories, namely heterocycles replacements, ring opening or closure, peptidomimetics and topology-based hopping, has been classified. In the technique for SARS 3CL protease inhibitors, we are interested in a peptidomimetics approach based on the scaffold hopping strategy. We herein report the design, synthesis and evaluation of a new scaffold, a phenylisoserine (PIS) derivative, for SARS-CoV 3CL protease inhibitors.

We performed a variety of molecular mechanics calculations with SPARTAN from Wavefunction and docking simulations of protein interactions using GOLD from CCDC for the 1,2-hydroxyamine

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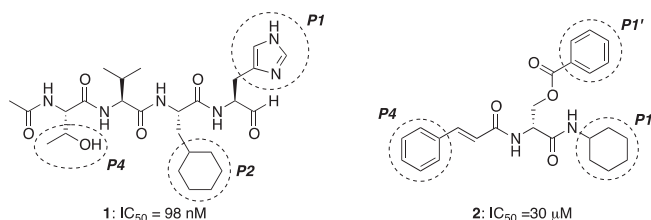


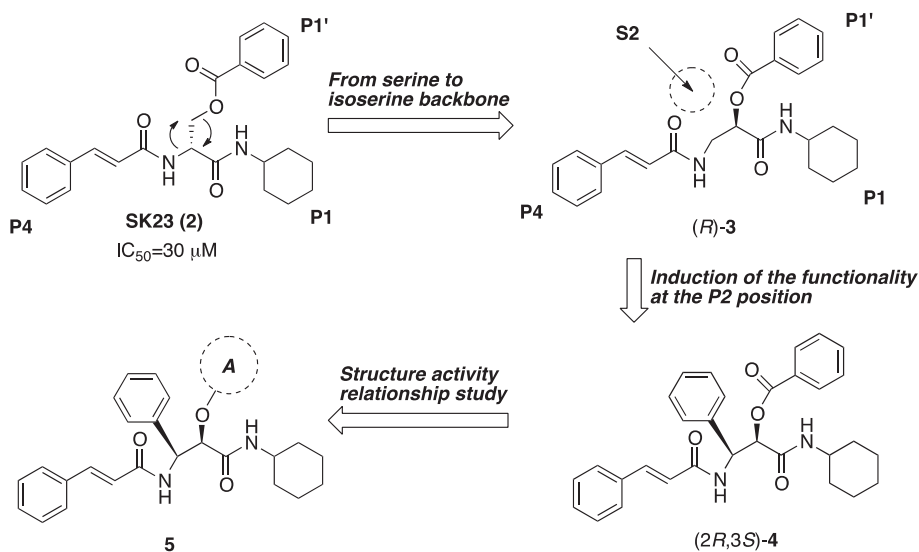
Fig. 1. Tetrapeptide aldehyde (**1**) and SK23 (**2**) for the SARS-CoV 3CL protease inhibitor.

compounds in-house. In an attempt to give good coverage of the substrate-recognition pocket of SARS 3CL R188I mutant protease (PDB code 3AW1), we found that the isoserine backbone had reasonable interactions with the mutant protease to give an isoserine derivative (**3**), which can be considered to replace the amine group at the α -position and a hydroxy group at the β -position of SK23 (**2**). Using the docking simulation of the (*R*)-isomer (**3**) with SARS 3CL protease, there was a hydrophobic space on the S2 pocket of the R188I mutant protease with the (*R*)-isomer (**3**). In the comparison with several hydrophobic functionalities, alkane, cycloalkane and aromatic rings, the phenyl group was fitted on the S2 pocket of the R188I mutant protease to obtain a (2*R*,3*S*)-PIS derivative (**4**). The docking simulations of the other three stereoisomers of (2*R*,3*S*)-**4** hardly interacted with the R188I mutant protease and therefore the (2*R*,3*S*)-isomer (**4**) as a candidate for this research was selected. The (2*R*,3*S*)-PIS derivative (**4**) has the following characteristics: (a) The substrates by nature have involved small amino acids (Ser, Ala or Gly) at the P1' position of SARS 3CL protease. However, it may be preferable to adopt the phenyl group, which was optimized as the serine-type inhibitor SK23 (**2**).¹³ (b) As with SK23 (**2**), the cyclohexyl ring may have the essential functionality of the P1 position, as well as stabilizing interactions of other positions. (c) The substrates at the P2 position are Leu, Val, Phe and Met with the hydrophobic side chains and therefore the S2 pocket is rather hydrophobic in nature. Consequently, the phenyl ring makes good contact with target regions. This is interesting in the results of docking simulations. The S2 pocket of the R188I mutant protease barely allowed the phenyl functionality of the serine-type inhibitor. (d) Although the S4 pocket prefers hydrophobic side chains (Ala, Val or Pro), aromatic derivatives on the synthetic substrates are acceptable. Especially, cinnamoyl residues play an important

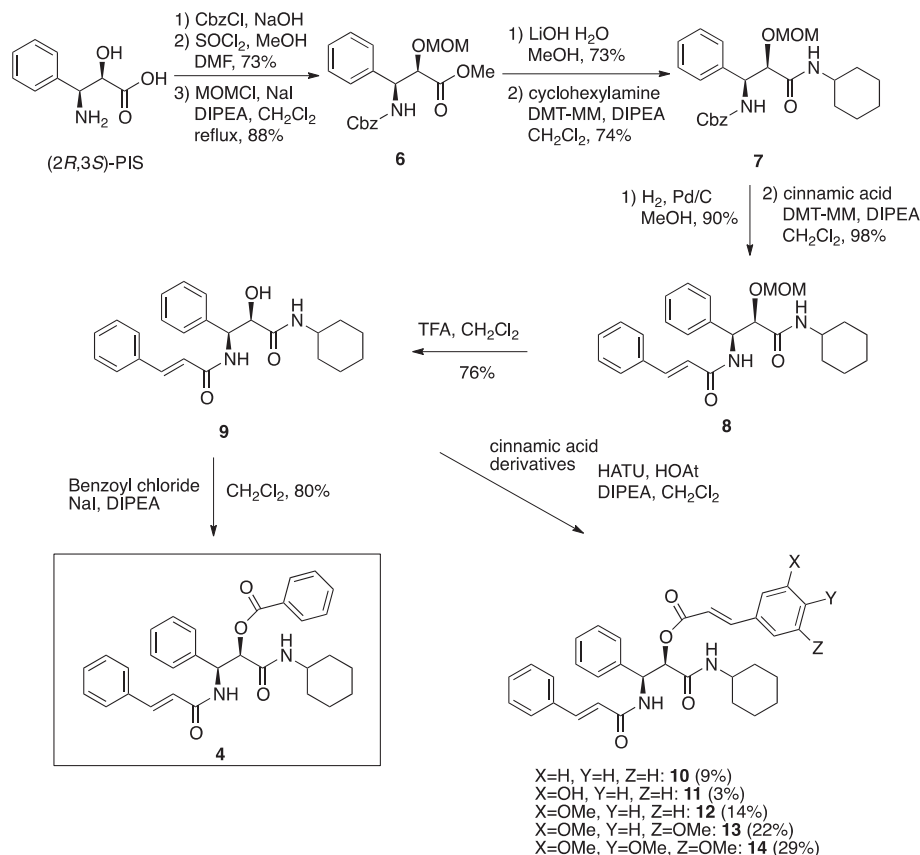
role to interact with the mutant protease. This result strongly supports those of Bai's¹⁵ and our¹³ information (Scheme 1).

As depicted in Scheme 1, the PIS derivative (**4**), designed from a computer-assisted docking simulation, was synthesized as the target molecule. Additionally, a structure activity relationship study of the PIS derivative (**4**) in terms of functionalities to interact with the corresponding P1' pocket was attempted. Functionalities of the P1' position (**A**) of **5** were designed and synthesized to evaluate its inhibitory activity against the R188I mutant (**4**). In other words, a cyclohexyl ring was essential for functionality of the P1 position on the PIS scaffold in the preliminary studies. Inhibitory activities of the synthesized PIS derivatives in absence of the cyclohexyl ring at the P2 position showed extremely weak activity against SARS 3CL R188I mutant protease ($IC_{50} > 1$ mM). In addition, cinnamoyl functionality of the P4 position was effective to maintain the inhibitory activity (Scheme 1).

The designed (2*R*,3*S*)-PIS derivative (**4**) was synthesized as shown in Scheme 2. At three step sequence for the protection of active functionalities with amine, carboxylic acid and hydroxy groups of (2*R*,3*S*)-PIS, gave the protected PIS derivative (**6**) in 64% yield over 3 steps. After the saponification of the methyl ester of **6**, coupling of the resultant carboxylic acid with cyclohexylamine by DMT-MM¹⁶/DIPEA gave the PIS amide (**7**) in 54% yield over 2 steps. Deprotection of the Cbz group of **7** by hydrogenolysis and coupling between the resultant amino group and cinnamic acid with DMT-MM/DIPEA successfully proceeded to afford the cinnamoyl derivative (**8**) in satisfactory yield. TFA-assisted deprotection of the MOM group gave the alcohol (**9**) in 76% yield. Finally, treatment of the alcohol (**9**) with BzCl/NaI/DIPEA in CH_2Cl_2 gave the target compound (**4**) in 80% yield without any problems. On the other hand, the P1' moiety of the hydroxy group of **9** was introduced by coupling with the five cinnamic acid derivatives prepared by us in the previous literature¹³ to afford **10–14** in lower yields. Thus, a variety of the coupling reagents was employed between the PIS derivative (**9**) with a large steric hindrance and cinnamic acid, it was difficult to improve the chemical yields of the corresponding ester (**10**). As a result, we selected DMT-MM/DIPEA or HATU^{17,18}/HOAt/DIPEA in CH_2Cl_2 as the coupling conditions to give the desired materials (**10**)–(**14**). Despite the poor chemical yields, it was available for the evaluation of inhibitory activities against SARS-CoV R188I mutant protease. We recognized the chemical structures for synthetic compounds clearly by ¹H and ¹³C NMRs, IR and MS spectra (Scheme 2).



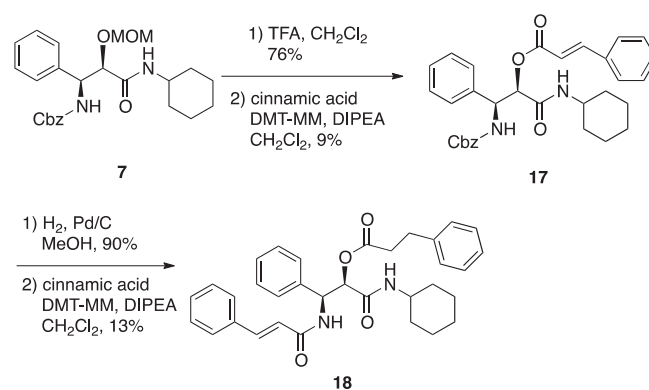
Scheme 1. Molecular design from serine to PIS template.



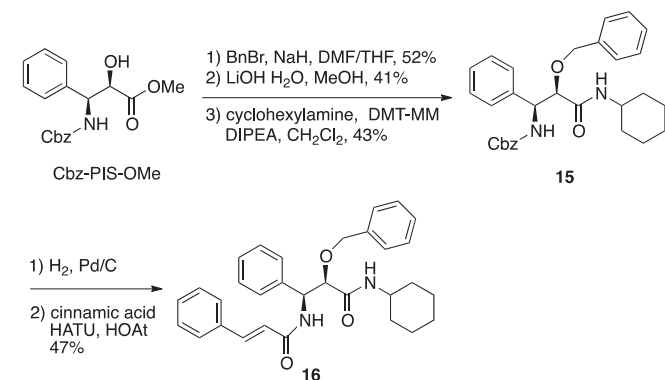
Scheme 2. Synthesis of the designed PIS compound (**4**) and its derivatives (**10**)–(**14**).

To evaluate the importance of the carbonyl group at the P1' position, the benzyl-type derivative (**16**) was prepared. Bn-protection of the hydroxy group of Cbz-PIS-OMe gave Cbz-PIS(Bn)-OMe in 52% yield. The saponification of methyl ester followed by the introduction of cyclohexylamine by DMT/MM was subjected to afford **15** in moderate yield. After deprotection of the Cbz group by hydrogenolysis, coupling with cinnamic acid was carried out to give the designed benzyl-type derivative (**16**) in 47% yield (Scheme 3).

As depicted in Scheme 4, the PIS phenylpropanoate (**18**) was prepared from **7** using the synthetic protocols as mentioned above. To investigate the importance of the flexibility of the cinnamoyl group at the P1' position, phenylpropanoate was introduced. Deprotection of the MOM group of **7** with TFA and coupling of the resultant hydroxyl group with cinnamic acid gave **17** in 9%



Scheme 4. Synthesis of the PIS derivative (**18**).




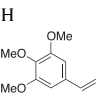







Scheme 3. Synthesis of the benzyl-type derivative (**16**).

yield. Removal of the Cbz group and coupling of cinnamic acid were carried out to give the PIS phenylpropanoate (**18**). In these cases, coupling reactions between the PIS derivatives with a large steric hindrance and cinnamic acid prove difficult to give the desired compounds in high yields (Scheme 4).

The inhibitory activity against SARS 3CL R188I mutant protease was determined by the previous procedure using a synthetic decapeptide with the S01 cleavage sequence as a substrate. Synthetic PIS derivatives with modification at the P1' position were evaluated as IC₅₀ values shown in Table 1. The PIS derivative (**4**; **SK80**) designed by docking simulations exhibited an IC₅₀ value of 43 μM (entry 1). The serine type inhibitor SK23 (**2**), which has same functionalities, showed an IC₅₀ value of 30 μM and therefore we expected that the active site of the 3CL R188I mutant protease fitted with these ligands via similar binding modes. To investigate

Table 1
Inhibitory activities of phenylisoserine derivatives.

Entry	Compound	A	ClogP ^a	tPSA ^b	IC ₅₀ ^c
1	4 SK80		6.12	84.5	43
2	16		6.25	67.4	NI
3	11		5.99	104.7	>500
4	9	H	4.17	78.4	>500
5	14		5.95	112.2	250
6	13		6.66	103.0	125
7	10		6.65	84.5	85
8	17^d		6.20	67.4	75
9	18		6.69	84.5	65
10	12		6.57	93.7	65

^a ClogP was calculated by ChemBio3D Ultra 12.0 (PerkinElmer).^b tPSA was calculated by ChemBio3D Ultra 12.0 (PerkinElmer).^c μM.^d The amino group of **17** was protected by the Cbz group.

the importance of the benzoyl carbonyl group at the P1' position, the benzyl ether (**16**) was evaluated. As a result, **16** showed no inhibitory activity (entry 2). In addition, the PIS derivative with a phenolic hydroxyl group (**11**) and the synthetic intermediate with a hydroxyl group (**9**) also showed lower inhibitory activities (IC₅₀ > 500 μM) (entries 3 and 4). These results were expected from the docking simulations and structure activity relationship study of serine type inhibitors in our previous report. Next, the PIS derivatives with modified cinnamoyl groups were evaluated. The IC₅₀ values of **14** and **13** were 250 and 125 μM, respectively (entries 5 and 6). The inhibitors containing 3,5- and/or 4-methoxy groups on a phenyl ring exhibited moderate activities. In contrast, cinnamate derivatives **10**, **17** and the phenylpropanoate derivative **18** increased the inhibitory activities to show the IC₅₀ values of 65–85 μM (entries 7, 8, 9). The CH₂O of Cbz group of **17** and the double bond of cinnamoyl group of **10** played a similar role and consequently both the phenyl rings interact with the S4 pocket to show approximately same activities. The inhibitor containing a 3-methoxy group on a phenyl ring (**12**) had 65 μM as an IC₅₀ value (entry 10). These results suggested that a planar aromatic ring and its hydrophobic functionality were essential factors to produce a reasonable 3CL protease inhibitor. Although the methoxy groups of the phenyl rings had increased infrequently inhibitory activities against SARS 3CL protease in our previous studies, there is hardly critical influence. As with SK23 (**2**), the benzoyl group at P1' position was optimized to give the inhibitor **4**. To show reasonable IC₅₀ values against SARS 3CL protease, it is important to satisfy both conditions with around 6 for ClogP values and 65–90 for tPSA. A similar tendency can also be seen in the structure activity relationship study of serine-type inhibitors (Table 1).

The binding mode of the SK80 (**4**) to SARS 3CL protease predicted by docking simulation using GOLD software is exhibited in Fig. 2. Based on these results, we decided to synthesize and evaluate inhibitory activity against SARS 3CL mutant protease. It appears to be a reasonable binding mode by the surface mode of the protease with SK80 (**4**) shown in Fig. 2A. The functionalities at the P1', P1, P2 and P4 sites of SK80 (**4**) were located on the S1', S1, S2 and S4 pockets with hydrophobic spaces, respectively. As depicted in Fig. 2B, two amide groups of SK80 (**4**) were involved in hydrogen bonding interactions with His164 and Gln189. These interactions of SK80 (**4**) with the R188I mutant protease were found to be identical to those of SK23 (**2**) with the protease. This suggests that carbonyl of the benzoyl group of the inhibitor (**4**) interacts with the active site of Cys145 and His41 of SARS 3CL protease. Interestingly, the distances of hydrogen and oxygen between the two amide groups of SK80 (**4**) as the docking simulation exhibited 2.1 and 3.1 Å, respectively. These values are reasonable to estimate the intramolecular hydrogen bonding networks. Therefore, the fixed eight-membered cyclic structure in the scaffold motif of SK80 (**4**) is proposed to locate the active site of SARS 3CL mutant protease by induced fit (Fig. 2C). In comparison with the stable conformation of SK80 (**4**) in absence of the SARS 3CL protease, a molecular mechanics calculation for conformational analysis by SPARTAN'14 was attempted (Fig. 2D). The distances of intramolecular hydrogen bonds between the corresponding two amide groups of **4** showed 2.0 and 5.3 Å, respectively. This result suggests that the conformation of **4** by the intramolecular hydrogen bond of two amide groups is partly fixed to increase the inhibitory activities against the SARS 3CL mutant protease. We conclude that the two amide groups of SK80 (**4**) interact to form inter- and intra-

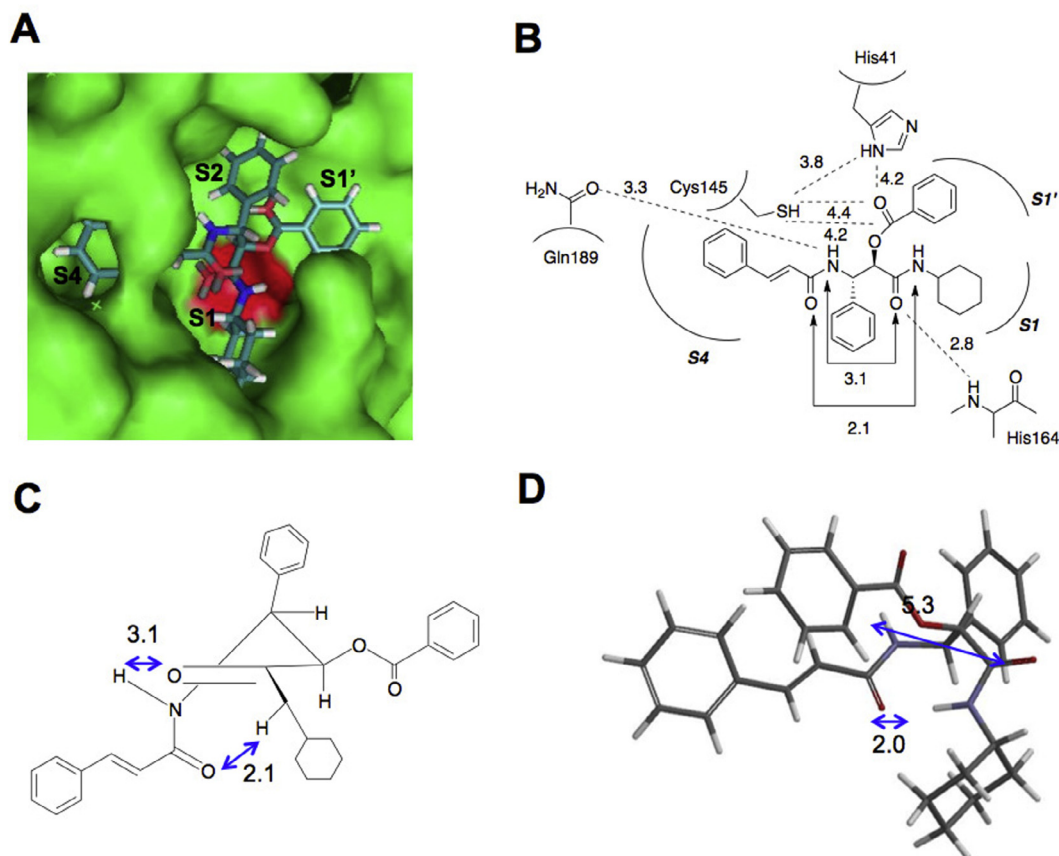


Fig. 2. Docking simulation of selected inhibitors bound to SARS 3CL protease (PDB code 3AW1) using GOLD from CCDC. Molecular graphic image shown using PyMOL from Schrödinger; oxygen (red) and nitrogen (blue) of inhibitors; Cys145 (red) of SARS 3CL protease. (A) Surface mode with **4**, (B) model of interaction with **4**, (C) Conformational analysis of **4**. (D) Conformational analysis of **4** by SPARTAN'14.

molecular hydrogen bondings and the binding of the SARS 3CL mutant protease and SK80 (**4**) using induced fit are shown (Fig. 2).

Since any protease inhibitor needs to have an orthogonal relationship between anti-virus activity and microbial activities or cytotoxicity, antibacterial and antifungal activities and cytotoxicity for SK80 (**4**) were investigated. The antifungal activities of SK80 (**4**) against *Saccharomyces cerevisiae* strain X2180-1A and *Candida viswanathii* NBRC10321 were evaluated by our assay protocols.¹⁹ In addition, we tested the bacterial activities of SK80 (**4**) against *E. coli* NBRC3972 and *Pseudomonas putida* NBRC14164 for gram-negative and *Micrococcus luteus* NBRC13867, *Bacillus subtilis* NBRC111470 and *Staphylococcus epidermidis* NBRC12993 for gram-positive. As a result, there were no inhibitory activities for any screened microbials (MICs > 800 mg/mL). The cytotoxicity of SK80 (**4**) was performed against Hela cells by a standard MTT assay using WST-8 protocols.²⁰ Although SK80 (**4**) at 1 μ M concentrations was evaluated against Hela cells RCB 0007 (5000 cells), the cell death ratio caused by these compounds was less than 10% and therefore showed practically no cytotoxicity.

In conclusion, we found a new scaffold, a phenylisoserine backbone, for a potential SARS 3CL protease inhibitor by a combination of docking simulation, chemical synthesis and ordinary assay protocols. Consequently, SK80 (**4**), which was derived from the serine-type inhibitor established by our group, was produced. Interestingly, SK80 (**4**) has intramolecular hydrogen bonds and therefore functionalities of SK80 (**4**) partly fix the trajectories to interact with the SARS 3CL protease by induced fit. It is useful information to design a non-peptidyl inhibitor of SARS 3CL protease. Our research into more suitable structures is in progress.

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A. Supplementary data

Supplementary data (additional experimental procedures, and analytical data for synthetic compounds. This material is available free of charge.) associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2017.04.056>.

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