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Geranylated flavonoids displaying SARS-CoV papain-like protease inhibition from the fruits of *Paulownia tomentosa*

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1. Introduction

Between November 2002 and July 2003 world news relayed stories of a deadly disease. The disease became known as severe acute respiratory syndrome (SARS) and it is known to have caused the deaths of 916 people. What is most staggering about this disease is that reported death rates were as high as 20% in the general population and can rise to over 50% in the elderly. Importantly, although no cases of SARS have been reported since 2004, the virus still exists in bat populations.

SARS is caused by the severe acute respiratory syndrome coronavirus (SARS-CoV), which is a single-stranded positive-sense RNA virus that replicates in the cytoplasm of infected cells. Replication is mediated by the replicase polyprotein, which is translated directly from the viral genome, and is processed by corona viral proteases present in the replicase polyprotein. Two cysteine proteases that reside within the polyprotein, a papain-like protease (PLpro) and a 3-Chymotrypsin-like protease (3CLpro), each catalyze their own release and liberation of the other nonstructural proteins from the polyprotein. These processes are all by mandatory for virus-mediated RNA replication. PLpro serves other roles because it is not only responsible for processing the viral polyprotein but can also cleave ubiquitin chains and effect deISGylation (ISGylation is involved in immune response to viruses).

SARS-CoV papain-like protease (PLpro) is an important antiviral target due to its key roles in SARS virus replication. The MeOH extracts of the fruits of the *Paulownia* tree yielded many small molecules capable of targeting PLpro. Five of these compounds were new geranylated flavonoids, tomentin A, tomentin B, tomentin C, tomentin D, tomentin E (1–5). Structure analysis of new compounds (1–5) by NMR showed that they all contain a 3,4-dihydro-2H-pyran moiety. This chemotype is very rare and is derived from cyclization of a geranyl group with a phenol functionality. Most compounds (1–12) inhibited PLpro in a dose dependent manner with IC 50’s ranging between 5.0 and 14.4 μM. All new compounds having the dihydro-2H-pyran group showed better inhibition than their parent compounds (1 vs 11, 2 vs 9, 4 vs 12, 5 vs 6). In kinetic studies, 1–12 emerged to be reversible, mixed inhibitors.

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Abbreviations: IC_{50}, the inhibitor concentration leading to 50 % activity loss; K_i, inhibition constant; K_M, Michaelis–Menten constant.

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2. Results and discussion

2.1. Structural identification of the isolated flavonoids

Starting with a methanol extract of *P. tomentosa* fruits, we purified compounds displaying PPI inhibition by activity-guided fractionation using a SARS-CoV PPIro activity assay. Compounds (1–5) emerged to be new geranylated flavonoid derivatives which we have named tomentin A (1), tomentin B (2), tomentin C (3), tomentin D (4), and tomentin E (5). As shown in Figure 1, compounds (6–12) were identified as the known extracts 3’-O-methyl-diplopin (6), 4’-O-methyl-diplopin (7), 3’-O-methyl-diplopin (8), 4’-O-methyl-diplopin (9), mimulone (10), diplope (11), and 6-geranyl-4,5,7-trihydroxy-3,5’-dimethoxyflavanone (12) by comparing our spectroscopic data (Supplementary data) and those previously reported.6–9

Compound 1 was isolated as a yellow oil with molecular formula 

\[ C_{25}H_{30}O_2 \]

established by the \[ M^+ \] ion at 442.1991 (Calcd 442.1992) in the HREIMS. \[ ^1H \] and \[ ^13C \] NMR data in conjunction with DEPT experiments indicated the presence of 25 carbon atoms, consisting of the following functional groups: 6 methylenes (sp³), 1 methine (sp³), 4 methins (sp²), 3 methyls and 11 quarternary carbons (Table 1). The analysis of degrees of unsaturation indicated a tetracyclic skeleton with two aromatic rings. According to its \[ ^13C \] and \[ ^1H \] NMR spectra, compound 1 was found to contain an sp³ methylene (diastereotopic protons: \[ H_1 \] 3.12, dd and \[ H_8 \] 2.74, dd) in the \( \alpha \)-position of the carbonyl group and an oxygenated methine (\[ H_9 \] 5.37 dd). The overall NMR characteristics suggested that 1 bears a flavone backbone. The only notable feature was that \( H-5' \) and \( H-6' \) had the same chemical shift (\[ H_9 \] 3.80) and thus were an apparent singlet (an A2 system). HMQC analysis verified that these two protons were in fact on non-equivalent carbons. The remaining proton within this ring, \( H-2' \), was also a singlet and had a chemical shift of \( \delta_H 7.04 \). The only arrangement of the ring consistent with three inequivalent proton-substituted carbons and all coupling constants 0 Hz is the proposed structure (3’,5’-disubstitution would lead to a reduction in carbon number due to symmetry and a 2’,3’-disubstitution must lead to ortho coupling between all three peaks). A characteristic hydrogen bonded proton signal of the C-5 (\( \delta_C 162.7 \)) hydroxyl group was observed at \( \delta_H 12.47 \). This is consistent with C-5OH forming a H-bond to the isolated proton at C-8 (A ring, \( \delta_H 6.03 \)). The connectivity of the C ring was affirmed by COSY correlations of C-2H and C-3H2. HMBC correlation of C-2H with aromatic ring B (principally C-1’) proved the position of the B ring relative to the C ring. The presence of the stereogenic center C-2 is responsible for the chemical inequivalence of the two methylene protons at C-3. HMBC correlation of the ketone function (\( \delta_C 197.7 \)) with C-3H2 indicated that these two groups were juxtaposed. A weak cross peak between C-3H2 and C4a confirmed that rings A and C were fused. The presence of a 2-hydroxy-2-methylpentyl moiety was deduced from proton coupling networks across H-5’/H-6’/H-7’ in the COSY spectrum and also HMBC correlations between H-7’/C-8’, C-9’ and C-10’. The presence of two methylene functions at C-1’ and C-2’ was confirmed from both a COSY connectivity between 2H-1’ and C-2, 2H-2’ and C-3, 2H-3 and C-4. 2H-2 and C-3 confirmed the position of the B ring relative to the C ring (Fig. 2). The absolute stereochemistry of 1 was elucidated as \( R \) on the basis of CD data (a positive absorption at 328 nm).6 Thus, using the above obtained spectral data, compound 1 was identified as (2S)-2-(3,4-dihydroxyphenyl)-5-hydroxy-8-(4-hydroxy-4-methylpentyl)-8-methyl-2,3,7,8-tetrahydro-2H-pyran[3,4-g]chro none-4(6H)-one and named tomentin A.

Compound 2 was a yellow oil having molecular formula 

\[ C_{27}H_{32}O_2 \]

and 11 degrees of unsaturation [HREIMS \( m/z 456.2151 \) \[ M^+ \], Calcd 456.2148)]. \[ ^1H \] and \[ ^13C \] NMR data of 2, fully assigned through 2D NMR experiments, closely resembled those of 1 (Table 1). The difference was the appearance of a 3 proton singlet at \( \delta_H 3.70 \), corresponding to methylation of one of the hydroxy groups within the pendant 3,4-dihydroxyphenyl ring (B ring). Given the broad spectral similarities between this species and compound 1, we focus on the position of the methyl group. The strongest HMBC correlation for the aryllic OCH₃ was to a quaternary carbon at 149.4 ppm (C-4’). Correlations were also observed from C-5’H to C-4’ and OCH₃ implying that the methoxy group is in the para position to the alkyl substituent. Consistent with this arrangement, C-3’ showed a strong HMBC correlation to C-2’H implying that the OH group was in the meta position to the alkyl substituent. The absolute stereochemistry of 2 was elucidated as \( S \) on the basis of the CD data (a positive absorption at 333 nm and a negative absorption at 294 nm).5 Thus, based on the above obtained spectral data, compound 2 was identified as (2S)-5-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-8-(4-hydroxy-4-methylpentyl)-8-methyl-2,3,7,8-tetrahydro-2H-pyran[3,2-g]chro none-4(6H)-one and named tomentin B.

Compound 3 a yellow oil, had the molecular formula 

\[ C_{27}H_{32}O_8 \]

[M⁺] ion at 486.2257 (Calcd 486.2254) in HREIMS. Its \[ ^1H \] and \[ ^13C \] NMR data, parsed out using 2D NMR experiments (Table 2) also closely resembled those of 1. However, there were two notable differences: firstly the B ring showed only two singlets at \( \delta_H 6.79 \) (H-2’) and \( \delta_H 6.94 \) (H-6’); there was also a new 6 proton singlet which corresponds to two methyl groups. In conjunction with the mass spectrometric data, the B ring showed characteristics of a trioxynated arene (\( R^1 = R^2 = OCH_3; R^3 = OH \)). Since H-2’ and H-6’ were
nonequivalent, the position of the lone aromatic hydroxy group must be on C-5. The signals of δC 57.2 and δH 3.87 (6H, s) were assigned as the two methoxy groups. HMBC correlation of δH 3.87 with δC 149.2 and δC 146.7 further confirmed that these two OCH₃ groups were placed on C-3′ and C-4′, respectively (Fig. 2). 3′′s absolute stereochemistry was elucidated as S on the basis of CD data (a positive absorption at 333 nm and a negative absorption at 296 nm). Thus, compound 3 was identified as (25)-5-hydroxy-
2-(5-hydroxy-3,4-dimethoxyphenyl)-8-(4-hydroxy-4-methylpentyl)-8-methyl-2,3,7,8-tetrahydropyrano[3,2-\(d\)]chromen-4(6\(g\))one named tomentin D.

The yellow oil, compound 4 had the molecular formula \(C_{27}H_{28}O_{16}\), which was established by the \([M^+]\) ion at 486.2256 (Calcld 486.2254) in the HREIMS. Based upon complete assignment of the \(^1H\) and \(^{13}C\) NMR spectra of this compound using 1D and 2D techniques, closely resembled compound 3 (Table 2). However the \(^1H\) NMR clearly showed a 2H singlet at 6.89 ppm, which was not observed in the compounds above) in this compound stems from the fact that H-5 and H-6 no longer have coincidental chemical shifts and thus are not an A2 system. The large coupling constant between H-2 and H-3 \([J = 11.6 \text{ Hz}]\) is a trans-diaxial arrangement of these two protons. This agrees with the fact that dihydroflavonols are found in nature only in the 2,3-trans configuration. The absolute stereochemistry of 5 was elucidated as 2R, 3R on the basis of CD data (a positive absorption at 332 nm and a negative absorption at 295 nm). Thus, compound 5 was identified as a (2R,3R)-3,5-dihydroxy-2-(4-hydroxy-3-methoxypyphenyl)-8-(4-hydroxy-4-methylpentyl)-8-methyl-2,3,7,8-tetrahydropyrano[3,2-\(g\)]chromen-4(6\(h\))one named tomentin E.

### Table 2

| Position | Tomentin C (\(\delta^C\)) | Tomentin D (\(\delta^C\)) | Tomentin E (\(\delta^C\)) |
|----------|--------------------------|--------------------------|--------------------------|
| 2        | 5.40, dd (2.8, 13.0)     | 1.64, dd (5.5, 10.0)     | 2.26, dd (4.8, 10.0)     |
| 3        | 4.44, s                   | 1.77, m                   | 1.77, m                   |
| 4        | 2.74, dd (2.8, 17.1)      | 2.51, dd (5.2, 10.0)     | 2.51, dd (5.2, 10.0)     |
| 5        | 1.24, s                   | 1.08, s                   | 1.08, s                   |
| 6        | 1.53, m                   | 1.37, m                   | 1.37, m                   |
| 7        | 1.46, m                   | 2.00, m                   | 2.00, m                   |
| 8        | 1.19, s                   | 1.03, s                   | 1.03, s                   |
| 9        | 0.96, s                   | 0.72, s                   | 0.72, s                   |
| 10       | 0.49, s                   | 0.49, s                   | 0.49, s                   |
| 11       | 0.37, s                   | 0.37, s                   | 0.37, s                   |
| 12       | 0.37, s                   | 0.37, s                   | 0.37, s                   |
| 13       | 0.37, s                   | 0.37, s                   | 0.37, s                   |
| 14       | 0.37, s                   | 0.37, s                   | 0.37, s                   |

\(^a\) \(^1H\)-\(^{13}C\) HMBC correlations are from the carbon(s) specified to the protons indicated.

### 2.2. In vitro assays of SARS-CoV PLpro inhibitory activity

To investigate the relative inhibitory potency of the twelve isolated compounds (1–12) against SARS-CoV PLpro, we measured SARS-CoV PLpro activity in the presence or absence of test compounds using a fluorogenic assay. The SARS-CoV PLpro (residues 1541–1855, corresponding to the protease domain) was expressed in Escherichia coli and purified by nickel affinity, ion-exchange and gel filtration chromatography. The apparent Michaelis constant \((K_m = 52 \pm 0.9 \mu M)\) was determined by plotting the initial rates normalized to enzyme concentration (25 nm) versus substrate concentration (1–104 \(\mu M)\), and fitting the hyperbolic data by non-linear regression using the Michaelis–Menten model in Sigma Plot (Fig. 3A). In SARS-CoV PLpro inhibition assays, all of the compounds tested displayed dose dependent inhibition (IC\(_{50}\) 5.0–14.4 \(\mu M)\) (Table 3). The inhibition of SARS-CoV PLpro by 2, the most potent flavonoid \((K_i = 3.5 \mu M)\), is illustrated in Fig. 3B, C and D, respectively. Plots of residual enzyme activity versus enzyme concentration at different concentrations of compound 2 gave a family straight lines with a y axis intercept of 0; this indicates that D.

With \(OCH_2 (\delta_2 3.89)\) (Fig. 2). The emergence of o-coupling (which was not observed in the compounds above) in this compound stems from the fact that H-5 and H-6 no longer have coincidental chemical shifts and thus are not an A2 system. The large coupling constant between H-2 and H-3 \([J = 11.6 \text{ Hz}]\) is a trans-diaxial arrangement of these two protons. This agrees with the fact that dihydroflavonols are found in nature only in the 2,3-trans configuration. The absolute stereochemistry of 5 was elucidated as 2R, 3R on the basis of CD data (a positive absorption at 332 nm and a negative absorption at 295 nm). Thus, compound 5 was identified as a (2R,3R)-3,5-dihydroxy-2-(4-hydroxy-3-methoxypyphenyl)-8-(4-hydroxy-4-methylpentyl)-8-methyl-2,3,7,8-tetrahydropyrano[3,2-\(g\)]chromen-4(6\(h\))one named tomentin E.

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### 2.2. In vitro assays of SARS-CoV PLpro inhibitory activity

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2 is a reversible inhibitor (Fig. 3B). The enzyme inhibition properties of target compounds were modeled using double-reciprocal plots (Lineweaver-Burk and Dixon analysis). The result shows a series of lines, which intersect to the left of the vertical axis and above the horizontal axis (Fig. 3C). This implies that increasing inhibitor concentration leads to a decrease in \( V_{max} \) and an increase in \( K_m \), indicating that compound 2 was a mixed-type inhibitor (Fig. 3D).

Thus the inhibitor binds to an allosteric site on the enzyme. The \( K_i \) values of all compounds were measured by Dixon plot (Table 3).

### Table 3

| Compound | SARS-CoV PLpro (52 nM) | IC\(_{50}\) \( (\mu M) \) | Kinetic mode \( (K_i^{b}, \mu M) \) |
|----------|-----------------------|------------------|------------------|
| 1        | 6.2 ± 0.04            | Mixed (4.8)      |
| 2        | 6.1 ± 0.02            | Mixed (3.5)      |
| 3        | 11.6 ± 0.13           | Mixed (5.0)      |
| 4        | 12.5 ± 0.22           | Mixed (13.0)     |
| 5        | 5.0 ± 0.06            | Mixed (3.7)      |
| 6        | 9.5 ± 0.10            | Mixed (6.6)      |
| 7        | 9.2 ± 0.13            | Mixed (6.3)      |
| 8        | 13.2 ± 0.14           | Mixed (7.1)      |
| 9        | 12.7 ± 0.19           | Mixed (6.9)      |
| 10       | 14.4 ± 0.27           | Mixed (7.8)      |
| 11       | 10.4 ± 0.16           | Mixed (5.1)      |
| 12       | 13.9 ± 0.18           | Mixed (8.4)      |

* All compounds were examined in a set of experiments repeated three times; error is standard deviation. IC\(_{50}\) values of compounds represent the concentration that caused 50% enzyme activity loss.

* Values of inhibition constant.

### 3. Conclusion

Although SARS is most associated with the outbreaks in 2002–2003 there have been several outbreaks since this time.\(^{11}\) Thus identification of new compounds with activity against essential SARS proteins is still of high importance. We set out to validate the medicinally used plant, *Paulownia tomentosa*, as a rich source of inhibitors of SARS-CoV PLpro. We chose to screen for inhibitors of this protease because it is a promising drug target since the enzyme is required for numerous processes in the viral life cycle. Our search led us to previously undisclosed natural products, which bear an unusual 3,4-dihydro-\(2H\)-pyran motif. This structural arrangement was found to be more effective at inhibiting SARS-CoV PLpro enzyme than the parent compounds that were cyclization precursors. This clearly underscores the importance of unearthing new or rare architectures from natural products. These novel natural products may be of general interest to the drug industry and natural product chemists.

### 4. Materials and methods

#### 4.1. General experimental procedures

Circular Dichroism (CD) spectra were measured in methanol (ca 1 mg/mL) using a Jasco J-715 CD spectropolarimeter (Gross-Umstadt, Germany) and optical rotations were measured on a Perkin–Elmer 343 polarimeter. Melting points were measured on a Thomas Scientific Capillary Melting Point Apparatus and are uncorrected. NMR spectra were recorded on a Bruker AM 500
spectrometer with TMS as an internal standard, and chemical shifts are expressed in δ values. EIMS and HREIMS were obtained on a JEOL JMS-700 mass spectrometer (JEOL, Tokyo, Japan). UV spectra were measured on a Beckman DU650 spectrophotometer Infrared (IR) spectra were recorded on a Bruker IFS66 infrared Fourier transform spectrophotometer (on KBr disks). Qualitative analyses were made using a Perkin–Elmer HPLC S200 (Perkin–Elmer, Bridgewater, USA). All purifications were monitored on commercially available glass-backed Merck precoated TLC plates and visualized under UV illumination at 254 nm and/or 366 nm or stained with 10% H2SO4 solution. Silica gel (230–400 mesh, Merck), RP-18 (log 3902, 3853, 3735, 3648, 3745, 3445, 2924, 2853, 1636, 1457 cm−1; 1H NMR and 13C NMR data, see Table 1; EIMS, m/z 442 [M]+; HREIMS, m/z 442.1991 (Calcd for C23H30O7 442.2092).

Tomentin A (1): Yellow oil; [α]23D +14 (c 0.77, MeOH); UV (MeOH) λmax (log ε) 205, 292 nm: IR (KBr) Vmax 3901, 3745, 3673, 3649, 3449, 2924, 2853, 1736, 1636, 1457 cm−1; 1H NMR and 13C NMR data, see Table 1; EIMS, m/z 442 [M]+; HREIMS, m/z 442.1991 (Calcd for C23H30O7 442.2092).

Tomentin B (2): Yellow oil; [α]23D +20 (c 0.77, MeOH); UV (MeOH) λmax (log ε) 206, 293 nm: IR (KBr) Vmax 3902, 3853, 3735, 3648, 3449, 2922, 2852, 1636, 1457, 1385 cm−1; 1H NMR and 13C NMR data, see Table 1; EIMS, m/z 456 [M]+; HREIMS, m/z 456.2151 (Calcd for C25H32O8 456.2148).

Tomentin C (3): Yellow oil; [α]23D −24 (c 0.72, MeOH); UV (MeOH) λmax (log ε) 206, 294 nm: IR (KBr) Vmax 3902, 3853, 3744, 3735, 3587, 3566, 3446, 2923, 2852, 1636, 1457 cm−1; 1H NMR and 13C NMR data, see Table 2; EIMS, m/z 486 [M]+; HREIMS, m/z 486.2257 (Calcd for C26H34O8 486.2254).

Tomentin D (4): Yellow oil; [α]23D −23 (c 0.67, MeOH); UV (MeOH) λmax (log ε) 206, 294 nm: IR (KBr) Vmax 3743, 3448, 2924, 2852, 1736, 1637, 1459, 1161, 1117 cm−1; 1H NMR and 13C NMR data, see Table 2; EIMS, m/z 486 [M]+; HREIMS, m/z 486.2256 (Calcd for C27H36O8 486.2254).

Tomentin E (5): Yellow oil; [α]23D −7 (c 0.57, MeOH); UV (MeOH) λmax (log ε) 203, 218, 295 nm: IR (KBr) Vmax 3745, 3445, 2924, 2853, 1636, 1457, 1121 cm−1; 1H NMR and 13C NMR data, see Table 2; EIMS, m/z 472 [M]+; HREIMS, m/z 472.2096 (Calcd for C27H32O8 472.2097).

4.3. Extraction and Isolation

The air-dried Paulownia tomentosa fruits (4.0 kg) were chopped, and extracted with MeOH (10 L × 3) three times at room temperature. The combined filtrate was concentrated in vacuo to yield a dark brown gum (476 g, 11.9%). The CH3OH extract was subjected to column chromatography (CC) on silica gel (10 × 40 cm, 230–400 mesh, 750 g) using a hexane to acetone gradient (50:1 → 1:1) to give 7 fractions (A–F). The purification of compounds 1–5 has not been previously reported. These five compounds together with known compounds 6–12 were isolated by a range of chromatographic methods. Characterization data of 6–12 matched with those described previously. Fraction A (4.2 g) was fractionated by silica gel flash CC employing a gradient of hexane to acetone resulting in 9 subfractions (A1–A9). Subfractions (A3–A6, 460 mg) were purified by silica gel flash CC to yield compounds 8 (19 mg), 9 (25 mg), and 12 (18 mg). Fraction B (8.9 g) was subjected to silica gel flash CC employing a gradient of CHCl3 (100%) to acetone resulting in 12 subfractions (B1–B12). Subfractions (B2–B8, 512 mg) were subjected to silica gel flash CC a employing hexane/acetone gradient (30:1 → 5:1) to give compounds 6 (21.6 mg), 7 (19.3 mg), and 10 (23.2 mg). Fraction C (1.8 g) was fractionated by silica gel flash CC employing a gradient of hexane (100%) to acetone (100%), resulting in 9 subfractions. Subfractions (C6–C8, 362 mg) were purified by reversed-phase CC (ODS-A, 12 nm, S-150 μm, YMC), and Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden) were used for column chromatography. All solvents used for extraction and isolation were of analytical grade.

4.4. Expression and purification of SARS-CoV PLpro from E. coli

The gene encoding the PLpro (945 bp) was amplified from the plasmid pSARS-REP (SARS-CoV ubani strain, Genbank AY278741) using forward primer 5′-GCCGGATCCGGTATACATATACAAAGTCTC and the reverse primer 5′-GGCCTGAGCTTTGATGTTGAGCTTTAAG (BamHI and XhoI sites are underlined). The gene was ligated into a PCR-TOPO (Invitrogen, USA) and the inserted gene was confirmed by DNA sequencing with T7 forward and T7 terminal reverse primers. Then, the correct gene fragment was transferred into a pProEX HT expression vector (Invitrogen, USA) and transformed into DH5α competent cells. The expression plasmid was constructed to carry a His6-tag followed by a TEV protease cleavage site at the N-terminus. Correct clones of the PLpro in the pProEX HT vector were identified and verified by PCR, restriction digestion with BamHI and XhoI and sequencing. The plasmid pProEx HT harbouring the PLpro gene was transformed into an E. coli strain BL21(DE3) (Novagen, Madison, WI, USA) for protein expression. A 1 ml aliquot of an overnight culture was seeded into 1000 ml of fresh LB (Luria-Bertani) medium containing 50 mg/ml ampicillin and cells were grown to OD600 nm of 0.6 at 37 °C. The cells were cooled down in ice for 30 min and protein expression was induced for 5 h with 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 30 °C. The cells were harvested by centrifugation at 6000 rpm for 6 min at 4 °C. The harvested cells were washed twice in phosphate-buffered saline. The cell pellet was used directly for purification or stored at −80 °C until use.

The cell pellet was suspended in binding buffer (50 mM NaH2PO4 pH 8.0, 500 mM NaCl, 5 mM imidazole and 5 mM β-mercaptoethanol) and cells were disrupted by sonication. After centrifugation at 15,000 rpm for 1 h, the clarified supernatant was collected, filtered (Qualitative filter paper, Advantec, Japan) and applied onto a column of Nickel Sepharose 6 Fast Flow (GE Healthcare, Sweden) beads pre-equilibrated with the binding buffer. The column was washed first with 20 column volumes of binding buffer and then with 2 column volumes of washing buffer (50 mM Tris–HCl pH 8.0, 500 mM NaCl, 30 mM imidazole). The recombinant PLpro proteins were eluted with the elution buffer.
(50 mM Tris–HCl pH 8.0, 100 mM NaCl and 300 mM imidazole). The PLpro eluted from the nickel column was further purified by ion-exchange chromatography using a salt gradient with a SOURCE 15Q column (GE Healthcare, Piscataway, NJ, USA) in 50 mM Tris–HCl pH 8.0, 150 mM NaCl and 2 mM DTT. The fractions containing PLpro were pooled, exchanged into 20 mM Tris–HCl pH 8.0 and 10 mM DTT and concentrated to a final concentration of 10 mg/ml by ultrafiltration (Microcon YM-30, Millipore Corporation, Bedford, Massachusetts, USA). The protein purity was examined by SDS–PAGE and native-PAGE. The protein concentration was determined by Bradford method (Bradford, 1976) using bovine serum albumin as the standard. The N-terminal his-tag was removed by TEV digestion for activity assay.

4.5. SARS-CoV PLpro inhibition assay

IC_{50} values for all inhibitors were determined using a 96-well plate-based assay similar to our previously reported procedures. The substrate used in the assay was the fluorogenic peptide Z-Arg-Leu-Gly-Gly-AMC (Z-RLRGG-AMC), which was purchased from ENZO Life Sciences. The substrate contains the five C-terminal residues of human ubiquitin with a C-terminal 7-amido-4-methyl-coumarin (AMC) group. Hydrolysis of the AMC-peptide bond dramatically increases the fluorescence of AMC, allowing conversion to be accurately determined. Reactions were performed in a total volume of 250 μL, which contained the following components: 20 mM Tris-buffer, pH 8.0, 10 mM DTT, 52 μM Z-RLRGG-AMC, 2% DMSO, and varying concentrations of inhibitor (0–200 μM). Reactions were initiated with the addition of PLpro to produce a final enzyme concentration of 52 nm. Reaction progress was monitored continuously on a SpectraMax M3Multi-Mode Microplate Reader (λ_{ex} = 360 nm; λ_{em} = 460 nm; gain = 40). Initial rate data were fit to the equation \( \text{vi} = \frac{[I]}{1 + [I]/IC_{50}} \) using the enzyme kinetics module of Sigma Plot (v. 9.01 Systat Software, Inc.) where \( \text{vi} \) is the reaction rate in the presence of inhibitor, \( [I] \) is the inhibitor concentration.

4.6. Statistical analysis

All measurements were made in triplicate. The results were subject to variance analysis using Sigma plot. Differences were considered significant at \( p < 0.05 \).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.03.027.

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Several references appeared incorrectly. The corrected references appear below.
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