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Structure elucidation and sulfated derivatives preparation of two α-D-glucans from *Gastrodia elata* Bl. and their anti-dengue virus bioactivities

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Abstract—The structures of two glucans, WGEW and AGEW, isolated from *Gastrodia elata* Bl. were elucidated using monosaccharide composition analysis by gas chromatography (GC), methylation analysis by gas chromatography–mass spectrometry (GC–MS) and nuclear magnetic resonance (NMR) spectroscopy. Their structures were deduced as an α-D-(1→4)-glucan with an α-(1→4) linked branch attached to O-6 branch points with different branch degrees. Their sulfate derivatives with distinct degrees of substitution (DS) were prepared. The substitution position was assigned to O-6 according to the ^{13}C NMR spectra. All sulfated derivatives showed strong anti-dengue virus bioactivities. The structure–activity relationships (SAR) between the polysaccharides and their sulfated derivatives were also investigated. Results showed that the higher the DS is, the more potent the impact on the dengue virus infection would be.

Keywords: *Gastrodia elata*; α-D-Glucan; Dengue virus; Sulfated polysaccharide

1. Introduction

As a major public health problem throughout subtropical and tropical regions, dengue viral infection is probably the most important arthropod-borne viral disease in terms of human morbidity and mortality.[1] The dengue virus is a member of the Flaviviridae family. It is one of the most important viral diseases transmitted by *Aedes aegypti* mosquitoes. Severe forms such as dengue haemorrhagic fever and dengue shock syndrome are responsible for many lethal cases.[2,3] Unfortunately, there is at present neither prophylaxis nor specific treatment to cure the disease. Thus our interest has been to find effective antiviral compounds against dengue virus.

Heparan sulfate proteoglycan (HSPG) is ubiquitously expressed on the cell surface of mammalian cells. A heparan sulfate (HS) chain attached to the core protein acts as the receptor for the virus in assisting viral infection. Recent evidence indicates that there is an intimate relationship between the structure of HS polysaccharides and their activity in promoting viral infections.[4] As for dengue virus, a highly sulfated type of HS acts as the receptor in assisting the dengue virus infection, and this function can be effectively prevented by heparin, a highly sulfated HS and suramin, a potent inhibitor of HS degradation enzyme heparanase.[5] However, heparin's anticoagulant activity is undesirable for its anti-dengue virus effect. This prompts us to discover more potent anti-dengue virus sulfated polysaccharides that

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have fewer side effects. *Gastrodia elata* Bl. is a well known and widely used traditional Chinese herb. It is usually used as an anticonvulsant, an analgesic, a sedative and an agent against general paralysis, epilepsy, vertigo and tetanus.[6] To our knowledge, neither the structure of polysaccharides isolated from this plant nor the anti-dengue virus bioactivity of their sulfated derivatives has been reported. In this study, we report the structures of two polysaccharides from *G. elata* and their anti-dengue virus activities. In addition, we also try to elucidate the structure–activity relationships among the sulfated polysaccharides.

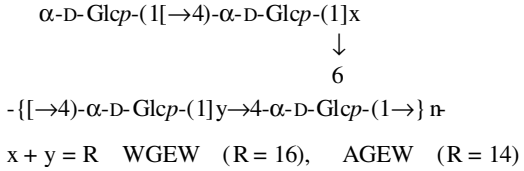
2. Results and discussion

2.1. Isolation and purification of AGEW and WGEW

The rhizomes of dried *G. elata* Bl. were defatted in 95% EtOH three times. After air drying, the residue was extracted with boiling water, and followed by 5% NaOH treatment. A water-extracted crude polysaccharide WGE and an alkali-extracted crude polysaccharide AGE were thus obtained. Using a DEAE-cellulose column, a 0.1 M NaCl elution pool of 5.1 g of WGE gave 0.6 g of WGEW, while the water elution pool of 10.0 g of AGE gave 1.8 g of AGEW (for details see Section 4). Both WGEW and AGEW showed a single symmetrical peak (data not shown), respectively, on high-performance gel-permeation chromatography (HPGPC). Thus we deduced that these were homogeneous polysaccharides.

2.2. Structural investigation of AGEW and WGEW

The mean molecular weight of AGEW and WGEW was 2.8×10^5 and 1.0×10^5, respectively (Table 1). Their specific rotations were also determined. The $[\alpha]_D^{25}$ (*c* 0.5, H_2O) of WGEW was +92.0, while the $[\alpha]_D^{25}$ (*c* 0.5, H_2O) of AGEW was +166.5 (Table 1). Monosaccharide composition analysis indicated that both WGEW and AGEW consist only of glucose.[7] Methylation analysis showed that both of them have terminal Glc (T-Glc), 1,4- and 1,4,6-linked Glc;[8] however, their molar ratios are different. The ratio of (T-Glc):1,4-:1,4,6-linked Glc in WGEW was 1:16:1, while the ratio of that in AGEW was 1:14:1. The signals in the ^{13}C NMR spectra indicated that both WGEW and AGEW share an α anomeric configuration[9] based on the fact that the anomeric carbon signals of Glc are 100.84 ppm and 102.259 ppm,[10] respectively (Fig. 1). Considering the above data, we have established the putative structure as the following:



Except for WGEW, another water-soluble α-D-glucan was obtained from WGE (2.0 g from 5.1 g WGE). Interestingly, the structural detail of this polysaccharide is nearly same as WGEW except for a low molecular size (around 4×10^3, data not shown). Based on the whole elution pools profile on DEAE-cellulose (data not shown), we find that the α-D-glucan is the main polysaccharide in *G. elata*. This can be attributed to the fact that nutrition for its growth is mostly dependent on a fungus *Armillariella mellea*.

2.3. Preparation of sulfate derivatives from AGEW and WGEW

Sulfated derivatives of WGEW and AGEW were prepared by the chlorosulfonic acid–pyridine method[11] at 25 °C and 45 °C for 4 h, respectively, using formamide as the solvent. The ratio between chlorosulfonic acid and pyridine was 1:2. After the reaction, the reactive mixture was adjusted to pH 7.8, dialyzed against 2 L supersaturated $NaHCO_3$ overnight, followed by dialyzing against 2 L deionized water three times, followed by lyophilization. WSS25 and WSS45 were prepared from WGEW, while AS25 and AS45 were prepared from AGEW at 25 °C and 45 °C, respectively. The appearance of an S=O signal near 1250 cm^{-1} and an S–O–S signal near 820 cm^{-1} in the IR spectra as shown in Figure 2 indicated that WSS25, WSS45, AS25 and AS45 were successfully sulfated.[12] This assignment was also confirmed by the ^{13}C NMR spectra where the chemical shift of C-6 was downshifted around 6 ppm. Hence, the substitution position was deduced to be exclusively at O-6 as shown in Figure 1. The degree of substitution was measured by the $BaCl_2$–gelatin method,[13] and the DS of WGEW, WSS25, WSS45, AGEW, AS25 and AS45 was 0, 0.206, 1.685, 0, 0.579 and 0.624, respectively (Table 1). The $[\alpha]_D^{25}$ (*c* 0.5, H_2O) of WGEW, WSS25, WSS45, AGEW, AS25 and AS45 was deter-

Table 1. Molecular weight, degree of substitution (DS) and specific rotation $[\alpha]_D^{25}$ of AGEW, WGEW and their sulfate derivatives

Samples	Molecular weight	$[\alpha]_D^{25}$ (*c* 0.5, H_2O)	DS[a]
WGEW	2.8×10^5	+92.0	0
WSS25	6.5×10^4	+150.0	0.206
WSS45	1.9×10^5	+142.8	1.685
AGEW	1.0×10^5	+166.5	0
AS25	1.5×10^5	+111.2	0.579
AS45	6.8×10^4	+95.0	0.624

[a] DS is calculated as $162\times\%W/(96-80\times\%W)$; $\%W$ is content of SO_4^{2-}.

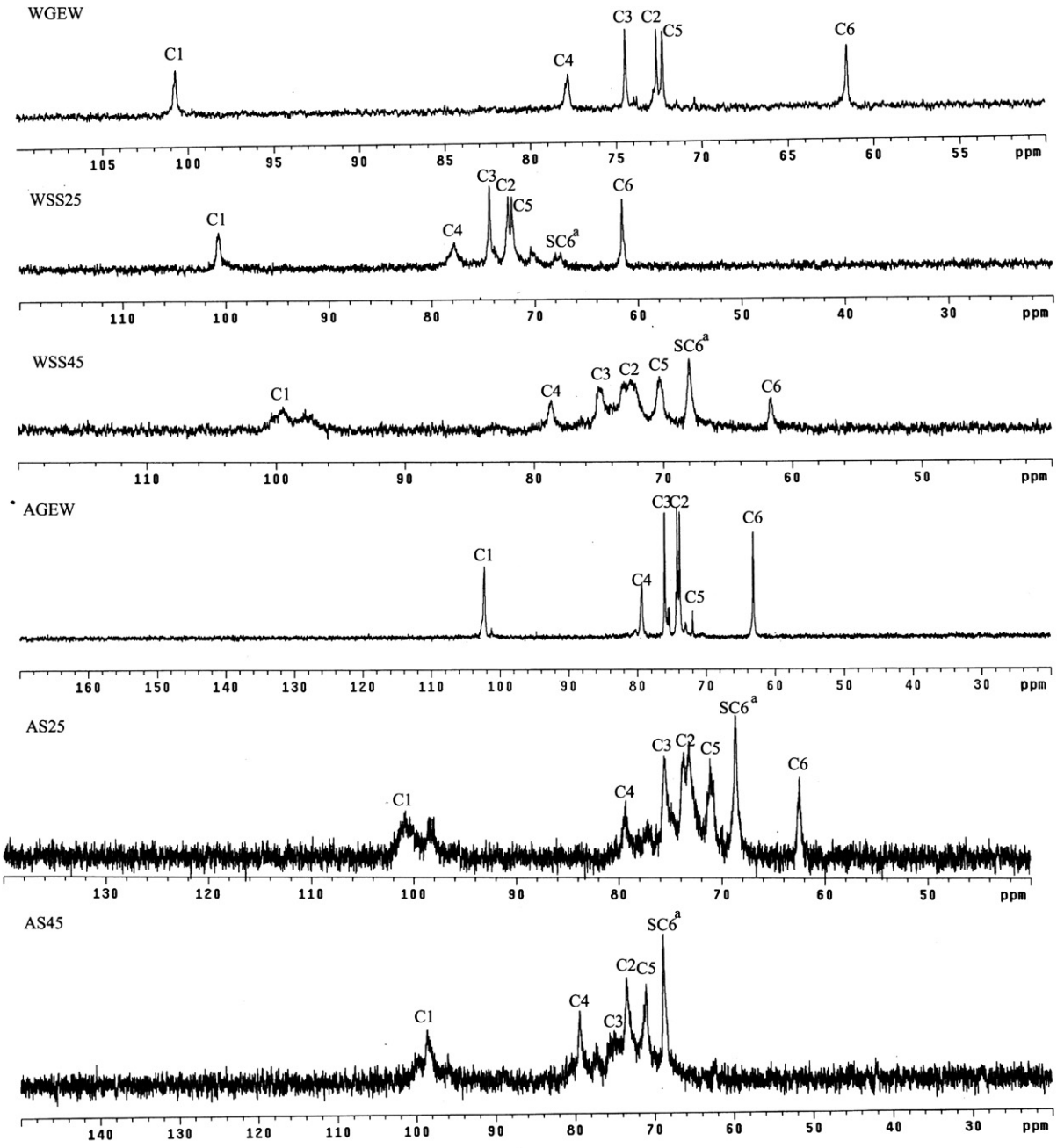


Figure 1. ^{13}C NMR spectra of AGEW, WGEW and their sulfated derivatives ($SC6^a$ indicates the sulfated carbon 6).

mined as +92.0, +150.0, +142.8, +166.5, +111.2 and +95.0, respectively (Table 1).

2.3.1. Inhibitory effect of AGEW, WGEW and their sulfate derivatives on dengue virus type 2. The antiviral activity of AGEW, WGEW and their sulfate derivatives are summarized in Table 2. When challenged with dengue virus type 2, naïve C6/36 cells showed a severe cytopathic effect (CPE),[14] presented as syncytium formation, presence of multinucleated giant cells and high mortality. WSS45 and AS45 exhibited a protective effect on dengue virus as indicated by the absence of CPE in infected C6/36 cells. The inhibitory potential of the sulfated derivatives of AGEW, WGEW with higher DS was further confirmed by a cell viability assay[15] and RT-PCR[16] that showed protection of virus-infected BHK cells and inhibition of viral RNA (Table 2). Among the six compounds tested in this bioassay, WSS45 showed significant and stronger activities with a higher selectivity index (CC_{50}/EC_{50}). These results demonstrate that the activity of polysaccharides we tested are indeed potent.

Generally, it is believed that the antiviral activity of sulfated polysaccharides increases with the molecular

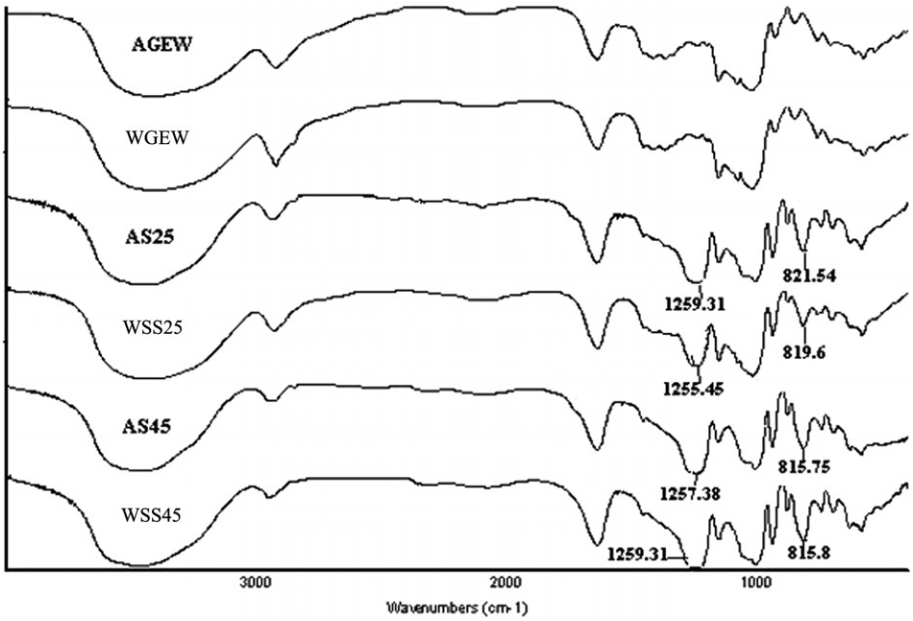


Figure 2. IR spectra of AGEW, WGEW and their sulfated derivatives.

Table 2. Inhibitory effect of AGEW, WGEW and their sulfate derivatives on dengue virus type 2

Compound (μg/mL)	CC_{50}[a]	EC_{50}[b]	IC_{50}[c]
WGEW	>200	Inactive	Inactive
WSS25	42.7 ± 5.9	20.6 ± 6.3	2.1 ± 0.4
WSS45	>10,000	10.7 ± 7.3	0.68 ± 0.17
AGEW	>200	Inactive	Inactive
AS25	>200	Inactive	Inactive
AS45	166.0 ± 4.1	6.2 ± 5.3	0.47 ± 0.18

[a] Cytotoxicity of compounds was measured by the MTT assay in BHK cells.
[b] Inhibition of virus-induced cell death was determined by MTT assay in dengue virus-infected BHK cells.
[c] Inhibition of viral load was evaluated by real-time PCR in the culture supernatant of BHK cells infected by dengue virus type 2.

weight and degree of sulfation.[17] Indeed, the anti-dengue virus activities of WGEW and its sulfated polysaccharides (WSS25 and WSS45) support a specific role for the molecular size and degree of sulfation of polysaccharides in this case. However, this is not the case for AGEW and its sulfated derivatives. These results add to accumulating evidence to support the views as pointed out in the report by Damonte et al.[18]

Previous studies demonstrated that sulfated polysaccharides such as sulfated galactomannan and sulfated polysaccharides from seaweed are potent anti-dengue virus agents.[19–21] However, sulfated polysaccharides with structures like AGEW and WGEW were presented for the first time in this study. Talarico and Damonte found that the activity of sulfated polysaccharides is dependent on the virus serotype and host cell.[20] As for the selectivity indices (CC_{50}/EC_{50}), although WSS25 and AS45 showed strong inhibitory activity on type 2 dengue virus, their selectivity indices were relatively lower compared with the reported active sulfated polysaccharide.[20] However, WSS45 was a potent inhibitor of type 2 dengue virus with a selectivity index above 1000.

3. Conclusions

In this study, we obtained two homogeneous α-D-glucans from *Gastrodia elata*, namely WGEW and AGEW, which are the main polysaccharides in *G. elata*. Both of the α-D-glucans have an α-(1→4)-linked glucosyl backbone. WGEW has an α-(1→4)-linked Glc branch attached to O-6 of every 18th residue, while in AGEW it is attached to O-6 of every 16th residue.

The substitution site for their sulfated derivatives is exclusively on O-6. Their sulfated derivatives demonstrated strong anti-dengue virus activities. However the selectivity indices of WSS25 and AS45 are relatively lower compared with the reported active polysaccharides, while WSS45 strongly inhibits type 2 dengue virus with a selectivity index above 1000. Moreover, the anti-dengue virus bioactivities positively correlate with their DS in WGEW and its sulfated derivatives. However, there are no significant structure–activity relationships between AGEW and its derivatives.

4. Experimental

4.1. Materials

Rhizomes of *G. elata* Bl. in dried slices type were purchased from the local market (Shanghai Xuhui Herb Slices Co. Ltd.). DEAE-cellulose 32 was from Whatman

Co. Ltd. Standard monosaccharides, sodium borohydride and iodidomethane were all Fluka products. Dimethyl sulfoxide was from E. Merck. All the reagents used were of analytical grade.

4.2. General methods

All evaporations were carried out at <40 °C under reduced pressure. IR spectra were determined with a Perkin–Elmer 591B spectrophotometer as KBr pellets (native polysaccharides) or Nujol films (permethylated polysacharides). Optical rotations were determined with a Perkin–Elmer 241 M digital polarimeter. GLC was conducted on a Shimadzu GC-14B instrument, equipped with a 3% OV-225-packed glass column (3.2 mm × 2 m) and an FID detector. The column temperature was kept at 210 °C for sugar analysis and at 190 °C for methylation analysis, and the carrier gas was N_2 at a flow rate of 25 mL/min. The injection and detection temperatures were 250 °C and 240 °C, respectively.

4.3. Isolation and fractionation of AGEW and WGEW

Before polysaccharide isolation, the rhizomes of *G. elata* were defatted by 95% EtOH for three times and air dried. The residue was extracted with boiling water (4 h each time) for four times. The extract was deproteinated, dialyzed, concentrated, centrifuged and lyophilized to give the crude water-soluble polysaccharide (WGE). WGE was then applied to a DEAE-cellulose column (50 × 5 cm) and eluted sequentially with deionized water, 0.1 M NaCl, and 0.2 M NaCl. The eluate was pooled according to the detection by phenol–sulfuric acid analysis. The fraction from WGE that eluted with water was named as WGEW. The residue after water extraction was dried and extracted with 5% NaOH (2 h) at 4 °C for two times. The extract was neutralized, deproteinated, dialyzed, concentrated, centrifuged and lyophilized to achieve the alkali-extracted crude polysaccharide AGE. AGE was fractioned using the same protocol as described for WGE. AGEW was obtained from the pool by water elution.

4.4. Homogeneity and molecular weight[7]

The homogeneity and molecular weight of polysaccharides were estimated using an HPGPC method,[12] which was performed on a Waters HPLC module consisting of a Model 515 pump, an RI detector (Model 2410) and a dual λ UV detector (Model 2487). The column was a serially linked combination of an Ultrahydrogel™ 2000 and an Ultrahydrogel™ 500 column. NaOAc (0.003 M) was used as the solvent for samples and as the eluent, with the flow rate kept at 0.5 mL/min. The column temperature was 30 ± 0.1 °C. The column was calibrated by reference to the MW-known T-series dextrans (T-700, T-580, T-110, T-80, T-40, T-11). Data were processed by GPC software (Millennium[32]).

4.5. Composition analysis[7]

The polysaccharide (2 mg) was hydrolyzed in 2 M TFA (2 mL) at 110 °C for 1.8 h in a sealed test tube. After evaporation to completely remove TFA, the hydrolyzate was dissolved in distilled water (2 mL) and reduced with $NaBH_4$ (30 mg) at rt for 3 h. After neutralization with HOAc and evaporation to dryness, the residue was acetylated with Ac_2O at 100 °C for 1 h. The resulting alditol acetates were subjected to GLC analysis.

4.6. NMR spectra

A sample (40 mg) was deuterium-exchanged overnight and dissolved in 0.5 mL of D_2O (99.8% D). The ^{13}C NMR spectra were measured at rt with a Bruker AM-400 NMR spectrometer. All chemical shifts are referenced to Me_4Si.

4.7. Methylation analysis[8]

Samples (5 mg) dried overnight in vacuo (P_2O_5) were dissolved in dimethyl sulfoxide (1.5 mL, dried over 4 Å molecular sieves), and methylated with the modified method of Ciucanu as described by Needs and Selvendran.[8] After the hydroxyl absorption in IR spectrum (Nujol) disappeared, the polysaccharide was depolymerized with 90% formic acid at 100 °C for 4 h, followed by hydrolysis with 2 M TFA at 100 °C for 6 h. The hydrolyzate was then converted into the partially methylated alditol acetates and analyzed by GC–MS.

4.8. Preparation of sulfated polysaccharides

Using formamide dried over 4 Å molecular sieves as the solvent, samples were prepared by the chlorosufonic acid–pyridine method[11] at 25 °C and 45 °C for 4 h. The ratio between chlorosulfonic acid and pyridine was 1:2. After the reaction, the solvent was adjusted to pH 7.8, dialyzed against 2 L of supersaturated $NaHCO_3$ overnight, then dialyzed against 2 L of deionized water three times, and lyophilized.

4.9. Cells and viruses

Aedes albopictus C6/36 cells (cloned cells of larvae of *A. albopictus*) were cultured in Eagle's minimum essential medium (MEM) (Gibco BRL, USA) supplemented with 5% fetal calf serum (FCS) (Hyclone, USA) and antibiotics (penicillin and streptomycin) at 28 °C. Baby hamster kidney cells (BHK) were cultured in MEM medium supplemented with 5% FCS and antibiotics (Penicillin and

Streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2. Dengue virus type 2 (New Guinea C strain) was kindly provided by the Guangdong Provincial Center for Disease Control and Prevention, Guang-dong, PR China. Virus was propagated in C6/36 cells at 33 °C.

4.10. Cell viability assay

Cell viability was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma–Aldrich] method.[22] Confluent BHK cultures in 96-well plates were exposed to different concentrations of the compounds, with three wells for each concentration. Then 20 μL of saline buffer containing MTT (final concentration 2.5 mg/mL) was added to each well. After 4 h of incubation at 37 °C, 100 μL lysis buffer was directly added to each well and incubated for a further 6 h before plate reading. Absorbance was measured in a microplate reader at 570 nm. The 50% cytotoxicity concentration (CC_{50}) was calculated as the compound concentration necessary to reduce cell viability by 50%.

4.11. Antiviral assays

Antiviral activity was evaluated by three methods: CPE assay in C6/36 cells,[14] MTT colorimetric assay and quantitative analysis of dengue viral RNA in BHK cells.

In the CPE assay, dengue virus type 2 was inoculated in triplicate of 96 wells plates on a confluent monolayer of C6/36 cells in the presence of the compounds. The plates were incubated at 33 °C with 5% CO_2 for 4 days and observed for the development of CPE.

In the MTT colorimetric assay, BHK cells were infected with dengue virus type 2 in the presence of different concentrations of the compounds for 4 days. The viability of the dengue virus-infected cells was determined by MTT assay.[14] The 50% effective concentration (EC_{50}) was calculated as the compound concentration required to reduce virus-induced cell death by 50%.

4.12. Reverse transcription and real-time PCR[16]

In a quantitative analysis of dengue viral RNA, the viral RNA in the cell culture supernatant was quantified by real-time RT-PCR. BHK cell monolayer grown in 96-well plates was infected with dengue virus type 2 in the presence of various concentrations of the compounds. Prior to virus quantification, cells were incubated at 37 °C for 4 days. On day 4, 50 μL of supernatant was collected by a 5-min centrifugation at 3000 rpm and subjected to viral RNA quantification. The 50% inhibitory concentration (IC_{50}) was calculated as the compound concentration required to reduce viral RNA load by 50%.

Total Viral RNA in supernatant was extracted by using the Trizol reagent (Gibco BRL, USA) according to the manufacture's instructions. The RNA pellet was resuspended in 20 μL DEPC-treated water. Prior to reverse transcription, RNA solution and antisense primers mixture were incubated at 70 °C for 5 min and chilled on ice. A 20-μL reaction mixture contained 8 μL of extracted RNA, 1 mM DV2.L1, 1 × reaction buffer (Promega, USA), 2 U MMLV reverse transcriptase (Promega, USA), 300 μM each deoxyribonucleoside (dNTP), and 5 mM Mg^{2+}. Reverse transcription was performed at 37 °C for 1 h 30 min, followed by 5 min of 94 °C to denature the reverse transcriptase.

Sequences of dengue 2 specific fluorogenic probe (DV2.P1) and a pair of flanking primers (DV2.L1 and DV2.U2) (synthesized by Shanghai Sangon Biotechnology Co., Ltd) were shown as follows: DV2.P1 (nucleotides 10,653–10,678), 5′-CTGTCTCCTCAGCATCATTCCAGGCA-3′; DV2.L1 (nucleotides 10,558–10,579), 5′-CATTCCATTTTCTGGCGTTCT-3′; DV2.U2 (nucleotides10,680–10,700), 5′-AAGGTGAGATGAAGCTGTAGTCTC-3′; DV2.P1 consists of the oligonucleotide sequence shown with a 5′-reporter dye (FAM, 6-carboxy-fluorescein) and a downstream 3′-quencher dye (TAMRA, 6-carboxy-tetramethylrhodamine).

Real-time PCR reactions were conducted in a volume of 20 μL containing 2 μL cDNA template, 1 × real-time PCR buffer (TaKaRa, Japan), 0.05 U Ex Taq HS DNA polymerase (TaKaRa, Japan), 4 mM Mg^{2+}, 400 nM dNTP, 400 nM each sense and antisense primer and 200 nM fluorogenic probe. PCR mixtures were pre-incubated at 50 °C for 1 min, then at 93 °C for 1 min, followed by 45 cycles of two-step incubations at 95 °C for 5 s and 60 °C for 1 min. Fluorescence data were collected and analyzed on a MJ Opticon Monitor 2 system.

Acknowledgments

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