

Butyrolactones and Diketopiperazines from Marine Microbes: Inhibition Effects on Dengue Virus Type 2 Replication

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ABSTRACT

Two new compounds, 4*S*,10*R*-dihydroxy-11-methyl-dodec-2-en-1,4-olide (1) (butyrolactone-type) and cyclo-(4-*trans*-6-dihydroxy-proline-D-leucine) (2) (diketopiperazine-type), as well as one known 4*S*,10-dihydroxy-10-methyl-dodec-2-en-1,4-olide (3) and three known diketopiperazines, cyclo-(L-proline-L-leucine) (4), cyclo-(4-*trans*-hydroxy-L-proline-L-leucine) (5), and cyclo-(4-*trans*-hydroxy-L-proline-L-phenylalanine) (6), were isolated from the ethyl acetate extracts of *Streptomyces gougerotii* GT and *Microbulbifer variabilis* C-03. Compounds 3, 4, 5, and 6 exhibited a significant reduction effect on dengue virus type 2 replication with EC₅₀ values of 21.2, 16.5, 12.3, and 11.2 μM, respectively.

Introduction

Dengue virus (DENV), a mosquito-borne pathogen, belongs to the genus *Flavivirus* of the Flaviviridae family and is an enveloped RNA virus containing an 11 kb positive single-strand genome [1]. DENV is dispersed across tropical and subtropical regions [2]. In 2008, more than 865 000 cases of dengue infection were reported in the Americas. Approximately 400 million people are infected with DENV and 2.5 billion people are at risk of DENV infection worldwide [3]. DENV causes various acute human diseases ranging from self-limited illnesses, such as dengue fever, to life-threatening forms, such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [4,5]. Primary DENV infection may cause severe forms of the disease; nevertheless, epidemiological studies have demonstrated that lethal DSS/DHF cases predominantly occur in either secondary heterologous infected people or in infants born to DENV-immune mother [6]. Several hypotheses have been

proposed, but the specific mechanism of this phenomenon in DSS/DHF patients remains uncertain. One hypothesis, antibody-dependent enhancement of infection theory, postulates that infection produced antibodies remain cross-reactive within different DENV serotypes without efficient neutralizing or non-neutralizing effects, enhancing virus replication into phagocytic cells by increasing vascular permeability and hemostatic disorder [7,8]. Nevertheless, serotype-specific immunity does not completely prevent serotype DENV infections; therefore, the four serotypes of DENV have presented challenges to developing a DENV vaccine [9]. Therefore, the urgent development of effective clinical therapeutic agents against DENV infection is crucial.

To explore novel bioactive compounds within marine microorganisms, we screened a series of marine-derived microbes, isolated from the deep-sea sediment collected offshore of Siaoliouciou by the gravity core, and of symbiont microbes isolated from marine invertebrates for their inhibitory effect on the expression of NS2B protease, which is crucial for virus replication. Two ex-

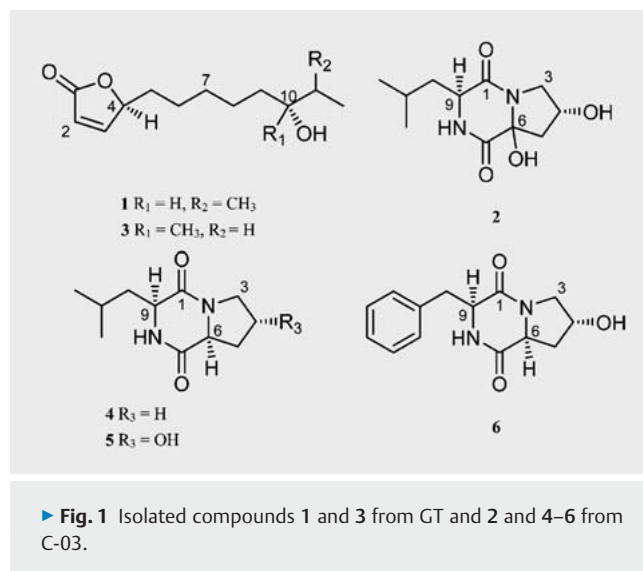
tracts of these marine bacteria were shown to inhibit the expression of NS2B protease. These marine-derived microbes were identified as *Streptomyces gougerotii* GT (Streptomycetaceae) and *Microbulifer variabilis* C-03 (Alteromonadaceae) through 16S rRNA sequencing analysis. We further studied bioactive secondary metabolites from these strains. Our paper reports two new compounds, 4*S*,10*R*-dihydroxy-11-methyl-dodec-2-en-1,4-olide (**1**) from *S. gougerotii* GT and cyclo-(4-*trans*-6-dihydroxy-proline-L-leucine) (**2**) from *M. variabilis* C-03, as well as four known compounds, 4*S*,10-dihydroxy-10-methyl-dodec-2-en-1,4-olide (**3**) [10] isolated from *S. gougerotii* GT, cyclo-(L-proline-L-leucine) (**4**) [11], cyclo-(4-*trans*-hydroxy-L-proline-L-leucine) (**5**) [12], and cyclo-(4-*trans*-hydroxy-L-proline-L-phenylalanine) (**6**) [13] from *M. variabilis* C-03 (► **Fig. 1**). The chemical structures were identified through spectroscopic methods (UV, IR, NMR, and ESI-MS). We further evaluated the anti-DENV activity of these isolates.

Results and Discussion

Compound **1** was isolated using reversed-phase HPLC with an isocratic solvent system of MeOH/H₂O (50:50) as the yellow oil with $[\alpha]_D^{25} + 44.7$ (*c* 0.35, MeOH). The HR-ESI-MS data of compound **1** showed the $[M + Na]^+$ ion at *m/z* 249.1461 (calcd. 249.1467), consistent with a molecular formula of C₁₃H₂₂O₃Na, deduced an unsaturation index of 3. The IR absorption band at 1747 cm⁻¹ indicated the presence of the carboxyl group. The ¹H NMR spectrum of **1** revealed five methine signals, five methylenes signals, and two methyl signals, while the ¹³C NMR and HSQC spectra displayed 12 resonances, including two olefinic carbons at δ_C 159.8 and 121.7, two oxygen-bearing methine carbons at δ_C 85.8 and 72.0, one methine carbon, five methylene carbons, and two methyl groups (► **Table 1**). The unsaturation index revealed that compound **1** should have a cyclic ring moiety. Downfield chemical shifts at δ_H 7.71 (H-3) and 6.12 (H-2), and a methine signal at δ_H 5.14, as well as the ¹³C resonances at δ_C 159.8, 121.7, and 85.8, supported the presence of an α,β -unsaturated γ -butyrolactone group.

The aforementioned spectral data of compound **1** were similar to those of 4*S*,10-dihydroxy-10-methyl-dodec-2-en-1,4-olide (**3**) [10], except for the oxygen-bearing methine (δ_H 3.62/ δ_C 72.0) and two doublet methyls (δ_H 1.10/ δ_C 20.3, δ_H 0.88/ δ_C 15.0) in compound **1**. In contrast to compound **3**, the terminus of an aliphatic chain of compound **1** was substituted by an isopropanyl group, which was supported by the slight but clear HMBC correlations of δ_H 3.62 (H-10) and δ_C 41.1 (C-11), 20.3 (C-12), and 15.0 (C-13).

The absolute configuration of **1** was elucidated through treatment by using (*R*)-(-)- α and (*S*)-(+)- α -methoxy- α -(trifluoromethyl)phenylacetylchloride (MTPA-Cl) to obtain the (*S*)- and (*R*)-MTPA derivatives, respectively (Mosher's method) [14]. The $\Delta\delta_{H(S-R)}$ values of methyls 12 and 13 suggested that the absolute configuration of C-10 was *R* (► **Fig. 2**). In addition, the absolute configuration of C-4 was determined to be an (*S*)-configuration according to a negative $n-\pi^*$ (239 nm) Cotton effect and a positive $\pi-\pi^*$ (200–220 nm) Cotton effect in the CD spectrum (► **Fig. 3**) [15].



Thus, compound **1** was identified as 4*S*,10*R*-dihydroxy-11-methyl-dodec-2-en-1,4-olide.

Compound **2** was obtained as a white amorphous solid with $[\alpha]_D^{25} - 130.0$ (*c* 0.30, MeOH). The HR-ESI-MS data of **2** showed the $[M + Na]^+$ ion at *m/z* 265.1159 (calcd. 265.1164), consistent with a molecular formula of C₁₁H₁₈N₂O₄Na, deduced an unsaturation index of 4. The IR absorption bands at 3394 and 1680 cm⁻¹ indicated the presence of the hydroxy and ketone groups, respectively.

The ¹H NMR spectrum of **2** revealed the three methine signals, three methylenes, and two methyls, while the ¹³C NMR and HSQC spectra revealed the presence of 11 ¹³C resonances, including two carbonyl carbons at δ_C 170.8 and 169.7, two nitrogen-bearing carbons at δ_C 57.0 and 53.6, two oxygen-bearing carbons at δ_C 88.1 and 68.0, one methine carbon, two methylene carbons, and two methyls (► **Table 2**).

The HMBC correlations of δ_H 1.93 and 1.66 (H₂-10)/ δ_C 57.0 (C-9), 45.8 (C-11), 23.5 (C-13), and 21.7 (C-12), of δ_H 1.81 (H-11)/ δ_C 45.8 (C-10), 23.5 (C-13), and 21.7 (C-12), of δ_H 0.98 (Me-13)/ δ_C 45.8 (C-10), 45.8 (C-11), and 21.7 (C-12), and of δ_H 0.95 (Me-12)/ δ_C 45.8 (C-10), 45.8 (C-11), and 23.5 (C-13), as well as the carbonyl carbon at δ_C 169.7, indicated the presence of a leucine moiety. Additionally, the HMBC correlations of δ_H 3.74 and 3.55 (H₂-3)/ δ_C 68.0 (C-4), and 46.5 (C-5), and of δ_H 2.47 and 2.39 (H₂-5)/ δ_C 68.0 (C-4), and 53.6 (C-3), as well as the carbonyl carbon at δ_C 170.8, were implied as the presence of a proline moiety substituted with a hydroxyl at the C-4 position, which was similar to that of compound **5** [12]. Moreover, the HMBC correlations of H-3b (δ_H 3.55) and H₂-5 (δ_H 2.47 and 2.39) with a quaternary carbon at δ_C 88.1 suggested that the oxygen-bearing carbon (δ_C 88.1) was located on the C-6 of a pyrrolidinyl group. Comparing the spectral data (e.g., NMR data and specific rotation values) of cyclo-(4-*trans*-hydroxy-L-proline-D-leucine) [16], cyclo-(L-proline-L-leucine) [17] and cyclo-(L-proline-D-leucine) [16], compound **2** was identified as cyclo-(4-*trans*-6-dihydroxy-proline-L-leucine).

► **Table 1** ^1H (400 MHz) and ^{13}C NMR (100 MHz) data of **1** and **3** in CD_3OD [δ (ppm), J (Hz)].

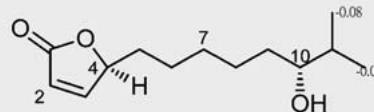
Position	1			3		
	δ_{C}		δ_{H} (J in Hz)	δ_{C}		δ_{H} (J in Hz)
1	176.4*	qC		176.0	qC	
2	121.7	CH	6.12, dd, $J = 5.5, 1.6$	121.7	CH	6.12, dd, $J = 5.5, 1.6$
3	159.8	CH	7.71, d, $J = 5.5$	159.8	CH	7.71, d, $J = 5.5$
4	85.8	CH	5.14, dd, $J = 7.1, 5.9$	85.7	CH	5.14, dd, $J = 7.0, 5.9$
5	34.2	CH_2	1.82, m, H-5a 1.64, m, H-5b	34.2	CH_2	1.80, m, H-5a 1.63, m, H-5b
6	26.2	CH_2	1.42, m	26.2	CH_2	1.48, m
7	30.9	CH_2	1.40, m	31.3	CH_2	1.40, m
8	28.4	CH_2	1.33, m	42.2	CH_2	1.43, m
9	33.6	CH_2	1.33, m, H-9a 1.10, m, H-9b	24.8	CH_2	1.36, m
10	72.0	CH	3.62, m	73.6	qC	
11	41.1	CH	1.42, m	35.1	CH_2	1.46, m
12	20.3	CH_3	1.10, d, $J = 6.3$	8.7	CH_3	0.88, t, $J = 7.4$
13	15.0	CH_3	0.88, d, $J = 7.0$	26.4	CH_3	1.11, s

* ^{13}C resonance was not measured in the ^{13}C NMR experiment, but was identified in the HMBC experiment indirectly.

The known compounds, cyclo-(L-proline-L-leucine) (**4**), cyclo-(4-*trans*-hydroxy-L-proline-L-leucine) (**5**), and cyclo-(4-*trans*-hydroxy-L-proline-L-phenylalanine) (**6**), were identified by comparing their NMR and MS data with the data reported in relevant literature [11–13].

To investigate the inhibitory effects of the ethyl acetate extracts of *S. gougerotii* GT and *M. variabilis* C-03 on DENV2 replication, the two respective extracts were treated at indicated concentrations in DENV2-infected Huh-7 cells for 3 days. Total cell lysates were collected and subjected to Western blotting with specific antibodies. Both *S. gougerotii* GT and *M. variabilis* C-03 extracts dose-dependently reduced DENV2 replication in protein levels (Fig. 1 S, Supporting Information). Furthermore, we treated each pure compound isolated from these extracts at various concentrations in the DENV2-infected Huh-7 cells for 3 days to investigate their inhibitory effects on DENV2 replication. As displayed in ► Table 3, although new compounds **1** and **2** showed no inhibitory effects on DENV2 replication, compounds **3**–**6** significantly reduced DENV replication and showed selectivity indices (SI, $\text{CC}_{50}/\text{EC}_{50}$) at 4.3, 5.9, 7.4, and 8.9, respectively. On the other hand, a previous study indicated that butyrolactones exhibit anti-fouling functions [18]. According to a similar antifouling assay, neither compound **1** nor **3** inhibited larval settlement of barnacle *Amphibalanus amphitrite* at a concentration of $10\text{ }\mu\text{g/mL}$ (Table 1 S, Supporting Information).

In summary, we isolated six compounds from the ethyl acetate extracts of *S. gougerotii* GT and *M. variabilis* C-03, namely two γ -butyrolactones (**1**, **3**) and four diketopiperazines (**2**, **4**–**6**). Among them, compounds **1** and **2** are new compounds. By identifying the absolute configuration of the chiral center in **1** by using Mosher's method and CD spectra, we confirmed the absolute configuration of the γ -butyrolactone-derived compound **1** as 4*S* and 10*R*. We in-

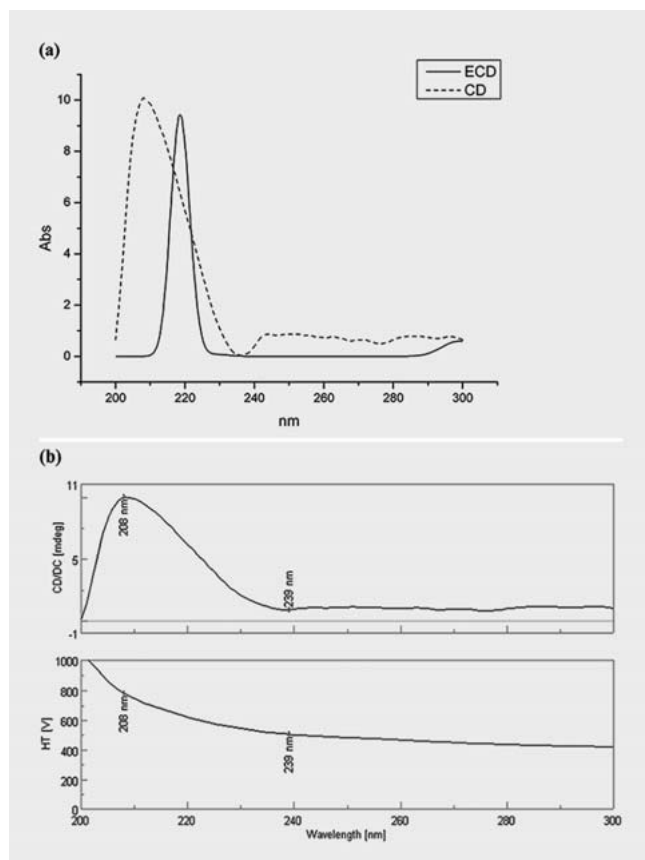
► **Fig. 2** $\Delta\delta_{\text{H(S-R)}}$ values of methyls **12** and **13** of **1**.

ferred the leucine unit in compound **2** to be L-form by comparing the specific rotation with relevant literature data. In addition, the polarity of both types of isolates from marine micribes would be a key factor for their antivirus activity. Thus, compounds **3**, **4**, **5**, and **6** exhibited noteworthy activity regarding antivirus DENV replication.

Materials and Methods

General experimental procedures

Specific rotations were measured on a JASCO P2000 optical rotations spectrometer. UV spectra were recorded on a Hitachi U-3210 UV-VIS spectrophotometer. Circular dichroism was obtained on a JASCO J-815 CD spectrophotometer and IR spectra were taken on a JASCO FT/IR-4100 spectrophotometer. Infrared spectra involved using potassium bromide (KBr) salt tablets as a background with the solvent CHCl_3 , the number of microwave spectroscopy units of the wavenumber (cm^{-1}). Both 1D and 2D NMR spectra were recorded using Bruker 300 and Varian Unity 400 FT NMR spectrometers. Coupling constants (J) are shown in Hz.



► **Fig. 3** (a) Experimental CD spectrum of **1** in MeOH and the calculated ECD spectrum of the (4S,10R) analog. (b) Experimental CD spectrum of **3** in MeOH.

ESI-MS/MS were obtained using a Bruker amaZon SL system and HRESI-MS were recorded on a Thermo LTQ Orbitrap XL mass spectrometer. The column chromatography was used with Sephadex LH-20 (Amersham Biosciences) and silica gel; the analytical TLC was used with precoated silica gel plates (Merck, silica gel 60 F254). The HPLC was taken on a Hitachi L-2420 UV-VIS detector and a Hitachi L-2455 PDA detector, both of which were deployed with a Discovery® C18 5 μ m (250 \times 4.6 mm i.d.) of analytical and semipreparative C18 5 μ m (250 \times 10 mm i.d.) columns for preparative purposes.

Materials

S. gougerotii GT was isolated from marine sediment offshore of Siaoiliouciou at sea levels, -555 meters (22°09'41.1''N, 120°07'13.8''E), by using the gravity core. *M. variabilis* C-03 was separated from *Palythoa tuberculosa* in the intertidal zone of Wanlitong. The voucher strains (MB-SG-GT and MB-MV-C-03) were deposited in the Department of Marine Biotechnology and Resources, Kaohsiung, Taiwan. The bacteria strains were identified according to 16S rRNA sequence analysis by using the BLAST comparison.

Extraction and isolation

S. gougerotii GT was cultured in 2500 plates with marine agar at 27°C for 4 days. The marine agar with the bacteria was then scraped and cut into pieces, and immersed in EtOAc (12 L) at room temperature for 1 day (24 h). The EtOAc was then filtered and vacuumed by using a rotary evaporator to obtain the deep-yellow crude EtOAc extract of *S. gougerotii* GT (total 1692.0 mg). The extract was partitioned by *n*-hexane and MeOH (ratio 1:1) to yield *n*-hexane (23.4 mg) and MeOH (1649.6 mg) layers. The MeOH layer was subject to reversed-phase (RP-18) column chromatography (5 \times 120 cm) with a gradient solvent system of MeOH/H₂O (50: 50, 5 L) and pure MeOH (6 L) to obtain 21 fractions. We further separated Fr. GT-12 (20.4 mg) through RP-HPLC (Discovery C18, 250 \times 10 mm, 1.0 mL/min, MeOH/H₂O, 50: 50) to yield **1** (3.5 mg, t_R = 54.0 min) and **3** (3.0 mg, t_R = 58.0 min). *M. variabilis* C-03 was cultured in 1056 plates with marine agar at 25°C for 2 days. Following the GT extract step, a deep-yellow crude EtOAc extract of *M. variabilis* C-03 was obtained (total 1820.0 mg). The extract was partitioned by *n*-hexane and MeOH (ratio 1:1) to yield *n*-hexane (447.2 mg) and MeOH (1327.5 mg) layers. The MeOH layer was subject to Sephadex LH-20 column chromatography (5 \times 120 cm) with an isocratic solvent system of pure MeOH (6 L) to obtain 10 fractions. Fr. C-03-5 (545.6 mg) was further separated through silica gel column chromatography (3 \times 75 cm, silica gel, 230–400 mesh), eluted with the gradient solvent system from pure *n*-hexane (1 L), *n*-hexane/CHCl₃ (10:1, 2:1, 1:1, each for 500 mL), pure CHCl₃ (1.5 L), and CHCl₃/MeOH (50:1, 10:1 and 4:1 each for 500 mL) to pure MeOH (2 L) to obtain 20 subfractions. Moreover, the subfraction C-03-5-12 was purified through silica gel column chromatography eluted with the gradient solvent system from pure *n*-hexane (500 mL), *n*-hexane/CHCl₃ (10:1, 2:1, and 1:1 each for 250 mL), pure CHCl₃ (500 mL), and CHCl₃/MeOH (50:1, 30:1, 10:1 and 4:1 each for 250 mL) to pure MeOH (1 L) to yield **4** (5.4 mg, in CHCl₃/MeOH, 30:1). The subfraction C-03-5-15 was purified through silica gel column chromatography eluted with the gradient solvent system from pure *n*-hexane (250 mL), *n*-hexane/CHCl₃ (10:1, 2:1, and 1:1 each for 250 mL), pure CHCl₃ (500 mL), and CHCl₃/MeOH (50:1, 30:1, 15:1 and 4:1 each for 250 mL) to pure MeOH (1 L) to yield **5** (5.4 mg, in CHCl₃/MeOH, 15:1). The subfraction C-03-5-16 was purified through RP-HPLC (Discovery C18, 250 \times 10 mm, 1.5 mL/min, MeCN/H₂O (0.1% TFA), 40: 60) to yield **2** (2.0 mg, t_R = 14.1 min). Fraction C-03-6 (458.0 mg) was separated through silica gel column chromatography (3 \times 75 cm, Silica gel, 230–400 mesh), eluted with the gradient solvent system from pure *n*-hexane (500 mL), *n*-hexane/CHCl₃ (10:1, 2:1, and 1:1 each for 500 mL), pure CHCl₃ (1 L), and CHCl₃/MeOH (50:1, 30:1 and 10:1, each for 500 mL) to pure MeOH (1 L) to obtain 14 subfractions. The subfraction C-03-6-8 was purified through RP-HPLC [Discovery C18, 250 \times 10 mm, 1.5 mL/min, MeCN/H₂O (0.1% TFA), (40:60)] to yield **6** (1.1 mg, t_R = 21.1 min).

Isolates

4S,10R-Dihydroxy-11-methyl-dodec-2-en-1,4-olide (1): C₁₃H₂₂O₃, yellow oil; [α]_D²⁵ + 44.7 (c 0.3, MeOH); UV (MeOH) λ_{max} (log ϵ) 211.0 (3.89) nm; IR (KBr) ν_{max} : 1747 cm⁻¹; ¹H NMR (CD₃OD, 400 MHz) and ¹³C NMR (CD₃OD, 100 MHz) see ► **Table**

1; $[M + Na]^+ m/z$ 249.32 (100); HRFABMS m/z 249.1461 $[M + Na]^+$ (calcd. for $C_{13}H_{22}O_3Na^+$, 249.1461).

Cyclo-(4,6-*trans*-dihydroxy-proline-L-leucine) (2): $C_{11}H_{18}N_2O_4$, colorless crystalline solid; $[\alpha]_D^{25} -130.0$ (c 0.30, MeOH); UV (MeOH) λ_{max} (log ϵ) 253.0 (3.25), 205.0 (3.89) nm; IR (Neat) ν_{max} : 3394, 1680 cm^{-1} ; 1H NMR (CD_3OD , 400 MHz) and ^{13}C NMR (CD_3OD , 100 MHz) see ► Table 2; $[M + Na]^+ m/z$ 265.09 (100); HRFABMS m/z 265.1159 $[M + Na]^+$ (calcd. for $C_{11}H_{28}N_2O_4Na$, 265.1159).

4S,10-Dihydroxy-10-methyl-dodec-2-en-1,4-olide (3): $C_{13}H_{22}O_3$, yellow oil; $[\alpha]_D^{25} +12.8$ (c 0.8, MeOH); UV (MeOH) λ_{max} (log ϵ) 207.0 (3.93) nm; IR (KBr) ν_{max} : 1746 cm^{-1} ; 1H NMR (CD_3OD , 400 MHz) and ^{13}C NMR (CD_3OD , 100 MHz) see ► Table 1; $[M + Na]^+ m/z$ 249.15 (100).

Preparation of (R)- and (S)-MTPA derivatives [14]

The tested compounds (each 1.0 mg) were dried in two vials under vacuuming overnight, soluted using deuterated pyridine (300 μL), and then (S)-(+)- α - or (R)-(-)- α -methoxy- α -(trifluoromethyl) phenylacetyl chloride (5 μL) was injected into each vial. (R)-MTPA and (S)-MTPA ester derivatives were generated in each vial, separately. The vials were stored overnight at room temperature to complete the reaction before NMR measurements were taken.

Density functional theory (DFT) calculations

A combination of M06-2X/6-31+G* basis sets was used for geometric optimization and vibrational frequency analysis [19,20]. The electronic circular dichroism (ECD) spectrum was simulated at the same theoretical level by applying the time-dependent density functional theory (TDDFT) approach. All calculations were performed using the Gaussian 09 program [21].

Western blotting

Huh-7 cells were attached in 24-wells plates with a concentration of 5×10^4 cells/well. After 12–16 h of incubation, DENV2 16881, the second serum type of DENV, was used to infect Huh-7 cells at a multiplicity of infection of 0.2 for 2 h. The infected cells were washed with PBS and then refreshed with a fresh culture medium containing DMSO or test compounds at various concentrations. After 72 h of incubation, the effects of the test compound on DENV replication were analyzed according to a Western blotting assay with anti-NS2B (1:4000; GeneTex) and anti-GAPDH antibodies (1:10000; GeneTex), and the signal was detected using an enhanced chemiluminescence (ECL) detection kit (PerkinElmer). Ribavirin (purity > 99%, Sigma-Aldrich) was used as the positive control.

Cytotoxicity assay

Huh-7 cells were attached in 96-well plates with a concentration of 5×10^3 cells/well. After 12–16 h of incubation, the test compounds were treated in 96-well plates at various concentrations for 3 days. The cytotoxicity of the test compounds was measured by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium assay (Promega) according to the manufacturer's instructions. The absorbance

► Table 2 1H (400 MHz) and ^{13}C NMR (100 MHz) data of 2 in CD_3OD [δ (ppm), J (Hz)].

Position	2	
	δ_C	δ_H (J in Hz)
1	169.7	qC
3	53.6	CH ₂ 3.55, dd, $J = 12.3, 6.4$, H-3a 3.74, dd, $J = 12.3, 6.4$, H-3b
4	68.0	CH 4.41, m
5	46.5	CH ₂ 2.39, dd, $J = 14.0, 6.1$, H-5a 2.47, dd, $J = 14.0, 6.1$, H-5b
6	88.1	qC
7	170.8	qC
9	57.0	CH 3.90, dd, $J = 9.9, 4.9$
10	45.8	CH ₂ 1.93, ddd, $J = 18.1, 8.7, 4.9$, H-10a 1.66, ddd, $J = 18.1, 8.7, 5.0$, H-10b
11	25.5	CH 1.81, m
12	21.7	CH ₃ 0.95, d, $J = 6.4$
13	23.5	CH ₃ 0.98, d, $J = 6.4$

► Table 3 Anti-DENV activity of compounds from *S. gougerotii* GT and *M. variabilis* C-03.

Compound	EC ₅₀ ^a (μM)	CC ₅₀ ^b (μM)	SI ^c
1	N. D.	> 100	N. D.
2	N. D.	> 100	N. D.
3	21.2	91.2	4.3
4	16.5	97.2	5.9
5	12.3	91.2	7.4
6	11.2	> 100	> 8.9
Ribavirin	12.5	56.3	4.5

^a EC₅₀: the concentration of the compound at which DENV RNA replication of Huh-7 cells decreased by 50% was determined. ^b CC₅₀: the concentration of the compound at which cell viability of Huh-7 cells decreased by 50% was determined. ^c Values of the selective index were CC₅₀/EC₅₀. N. D.: not detected. All results are expressed as the mean \pm S. E. M. of three independent experiments. Student's t-test was used for statistical analyses; p values < 0.05 were considered significant.

of the sample was detected at 490 nm by a 550 BioRad plate-reader (Bio-Rad). Ribavirin was used as the positive control.

Compounds 1–6 were repurified through reversed-phase HPLC before the bioassay test (purity > 99%). All results are expressed as the mean \pm S. E. M. of three independent experiments. Student's t-test was used for statistical analyses; p values < 0.05 were considered significant.

Supporting information

The inhibition of marine microbes extracts on DENV replication and anti-fouling assay, 1D and 2D selective NMR spectra of new compounds, as well as the fully assigned NMR data of the known

compounds, are available and published on the journal homepage at <http://www.thieme-connect.de/ejournals/toc/plantamedica>.

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Conflict of Interest

There is no conflict of interest.

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