

Article

Chemical Constituents and Bioactivities of *Clinacanthus nutans* Aerial Parts

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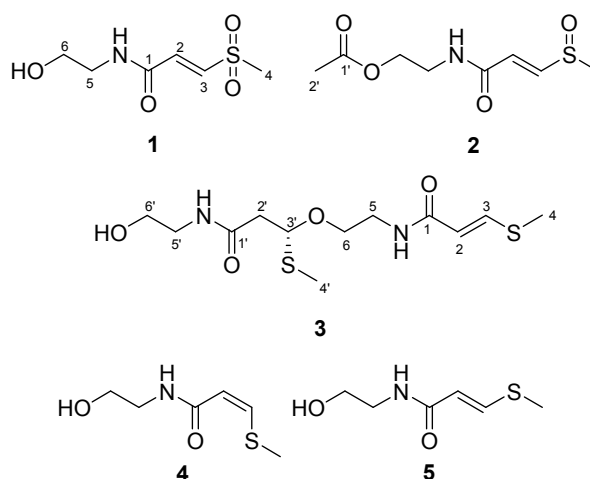
Abstract: Four new sulfur-containing compounds, named clinamides A-C (**1–3**), and 2-*cis*-entadamide A (**4**), were isolated together with three known compounds from the bioactive ethanol extract of the aerial parts of *Clinacanthus nutans*. These secondary metabolites possess sulfur atoms and acrylamide functionalities. The structures of the isolated components were established by interpretation of their spectroscopic data, especially 1D and 2D NMR.

Keywords: *Clinacanthus nutans*; sulfur-containing compound

1. Introduction

The genus *Clinacanthus* (family Acanthaceae) consists of two species. *Clinacanthus nutans* is a small shrub about one meter tall native to tropical Asia [1]. In Malaysia and Thailand, this plant has been claimed to be a useful folk medicine in the treatment of skin rashes, snake bites, herpes simplex virus (HSV), and varicella-zoster virus (VZV) lesions [2]. Actually, extracts from the leaves of *C. nutans* have been reported to possess analgesic, anti-inflammatory, and antiviral activities against VZV and HSV-2 [3]. In previous phytochemical studies, a series of flavonoids, steroids, triterpenoids, cerebrosides, glycolglycero-lipids, glycerides, sulfur-containing glycosides were isolated from this plant [1,2,4]. Among those natural products, glycolglycerolipids and digalactosyl diglycerides exhibited antiviral and anti-HSV activity, respectively [4]. In our bioactive screening, the 80% ethanol extract showed anti-inflammatory, anti-dengue virus, and immune-modulating activity. Those results implied that the active compounds were contained at the 80% ethanol extract. Thus, a phytochemical investigation of *C. nutans* was carried out. Herein, we describe the isolation and structural elucidation of four new sulfur-containing compounds, clinamides A-C (**1–3**) and 2-*cis*-entadamide A (**4**) (Figure 1).

Figure 1. The structures of clinamides A-C (**1–3**), 2-*cis*-entadamide A (**4**), and entadamide A (**5**).



2. Results and Discussion

Four new compounds **1–4** and three known compounds, entadamide A (**5**) [5], entadamide C [6], and *trans*-3-methylsulfinyl-2-propenol [7] were isolated from the ethanolic extract of the aerial parts of *C. nutans*. The known compounds were identified by comparison of their physical and spectroscopic data to those reported in the literature. The structures of the new compounds were established by interpretation of their spectroscopic data, especially 2D NMR.

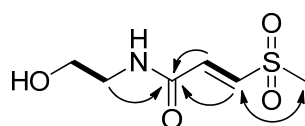
Clinamide A (**1**) was obtained as a pale yellow oil and was assigned the molecular formula, C₆H₁₁NO₄S, as deduced from HRESIMS (*m/z* 216.0308 [M+Na]⁺). The IR spectrum showed strong absorption bands at 3380 cm⁻¹ (OH), 1668 cm⁻¹ (NH–C=O), 1555 cm⁻¹ (C=C), and 1302 and 1135 cm⁻¹ (O=S=O). The ¹H-NMR data of **1** (Table 1) exhibited one methylsulfonyl signal [δ_H 3.08 (s)] [7], two methylene signals [δ_H 3.41 (t, *J* = 5.7 Hz) and 3.65 (t, *J* = 5.7 Hz)], and a *trans*-disubstituted double bond [δ_H 7.01 (d, *J* = 15.0 Hz)] and 7.43 (d, *J* = 15.0 Hz)]. The ¹³C-NMR (Table 2), DEPT and HSQC spectra of **1** showed six carbon signals, consisting of one methylsulfonyl group (δ_C 42.4), one oxymethylene group (δ_C 61.2), one methylene group (δ_C 43.4), one disubstituted olefin group (δ_C 136.3 and 140.1), and one amide carbonyl group (δ_C 164.6). These signature characteristics were used to identify **1** as an entadamide-type compound. The COSY spectrum of **1** (Figure 2) exhibited two proton spin systems of H-2 (δ_H 7.01)/H-3 (δ_H 7.43) and H-5 (δ_H 3.41)/H-6 (δ_H 3.65). In the HMBC spectrum, the H-2, H-3 and H-5 showed correlations to C-1 (δ_C 164.6), which indicated the presence of the double bond, the amide group and two methylene groups were connected. In addition, the key NOESY correlation between H-4 (δ_H 3.08) and H-3 indicated the methylsulfonyl group at C-3. These NMR data of **1** were closely similar to dambullin [8], except for the absence of an aromatic moiety. On the basis of the above discussion, the configuration of **1** was established as shown.

Table 1. ¹H-NMR spectroscopic data of compounds **1–4** (**1** and **2** in CD₃OD, **3** and **4** in CDCl₃, 400 MHz).

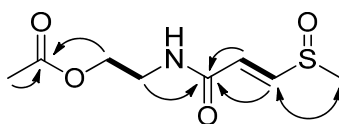
NO.	1	2	3	4
2	7.01 (d, 15.0)	6.68 (d, 15.0)	5.78 (d, 14.8)	5.80 (d, 10.0)
3	7.43 (d, 15.0)	7.63 (d, 15.0)	7.62 (d, 14.8)	6.83 (d, 10.0)
4	3.08 (s)	2.76 (s)	2.33 (s)	2.35 (s)
5	3.41 (t, 5.7)	3.54 (t, 5.4)	3.65 (m)	3.46 (t, 5.0)
			3.38 (m)	
6	3.65 (t, 5.7)	4.17 (t, 5.4)	3.88 (m)	3.73 (t, 5.0)
			3.52 (m)	
2'		2.05 (s)	2.80 (dd, 15.0, 9.8)	
			2.70 (dd, 15.0, 3.2)	
3'			4.87 (dd, 9.8, 3.2)	
4'			2.06 (s)	
5'			3.52 (m)	
			3.38 (m)	
6'			3.74 (m)	

Table 2. ^{13}C -NMR spectroscopic data of compounds **1–4** (**1** and **2** in CD_3OD , **3** and **4** in CDCl_3 , 100 MHz).

NO.	1	2	3	4
1	164.6 (s)	165.2 (s)	165.1 (s)	167.4 (s)
2	136.3 (d)	129.0 (d)	115.9 (d)	114.9 (d)
3	140.1 (d)	147.9 (d)	142.6 (d)	147.8 (d)
4	42.4 (q)	39.9 (q)	14.5 (q)	19.4 (q)
5	43.4 (t)	39.8 (t)	38.9 (t)	42.3 (t)
6	61.2 (t)	63.8 (t)	67.5 (t)	62.4 (t)
1'		172.7 (s)	171.0 (s)	
2'		20.7 (q)	43.3 (t)	
3'			81.4 (d)	
4'			10.2 (q)	
5'			42.2 (t)	
6'			62.1 (t)	

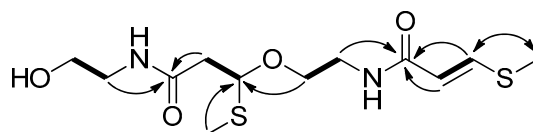
Figure 2. COSY (bold lines), key HMBC (arrows), and NOESY (left right arrow) correlations of **1**.

Clinamide B (**2**) was assigned a molecular formula of $\text{C}_8\text{H}_{13}\text{NO}_4\text{S}$ by HRESIMS (m/z 242.0461 $[\text{M}+\text{Na}]^+$). The UV spectrum exhibited absorption maximum at 247 nm. The IR spectrum showed bands at 1732 cm^{-1} ($\text{C}=\text{O}$), $1,660\text{ cm}^{-1}$ ($\text{NH}-\text{C}=\text{O}$), and $1,031\text{ cm}^{-1}$ ($\text{S}=\text{O}$). In the ^1H -NMR spectrum (Table 1), a pair of *trans*-disubstituted olefinic protons [δ_{H} 6.68 (d, $J = 15.0$ Hz) and 7.63 (d, $J = 15.0$ Hz)] were found as similar to the case of **1**. Besides, the proton spectrum showed a methylsulfinyl group [δ_{H} 2.76 (s)], a methyl group [δ_{H} 2.05 (s)], and two methylene groups [δ_{H} 3.54 (t, $J = 5.4$ Hz) and 4.17 (t, $J = 5.4$ Hz)]. The ^{13}C -NMR (Table 2) spectrum revealed signals for an amide group as well as **1**. In addition, the ^{13}C -NMR and DEPT spectra exhibited one amide carbon group (δ_{C} 172.7), two methine groups (δ_{C} 129.0 and 147.9), two methylene groups (δ_{C} 39.8 and 63.8), and two methyl groups (δ_{C} 20.7 and 39.9). These signals were similar to entadamide C, suggesting that they possess the same skeleton. Compound **2** was found to have an additional acetyl group, when compared to entadamide C. This acetoxy group was assigned at C-6 by the HMBC correlations from both H-2' (δ_{H} 2.05) and H-6 (δ_{H} 4.17) to C-1' (δ_{C} 172.7) (Figure 3). Therefore, structure **2** was assigned to clinamide B.

Figure 3. COSY (bold lines), key HMBC (arrows), and NOESY (left right arrow) correlations of **2**.

Clinamide C (**3**) was isolated as pale yellow oil, and exhibited the molecular formula $C_{12}H_{22}N_2O_4S_2$ by HRESIMS. The UV spectrum exhibited absorption maxima at 228 nm and 270 nm. The IR spectrum showed absorption bands at $3,296\text{ cm}^{-1}$ (OH), $1,644\text{ cm}^{-1}$ (NH–C=O), and $1,582\text{ cm}^{-1}$ (C=C). The $^1\text{H-NMR}$ data (Table 1) showed two methylthio signals [$\delta_{\text{H}} 2.06$ (s) and 2.33 (s)], and a *trans*-disubstituted double bond [$\delta_{\text{H}} 5.78$ (d, $J = 14.8$ Hz) and 7.62 (d, $J = 14.8$ Hz)]. The $^{13}\text{C-NMR}$ (Table 2), DEPT and HSQC spectra exhibited signals for 12 carbons, including two methylthio groups ($\delta_{\text{C}} 10.2$ and 14.5), five methylene groups ($\delta_{\text{C}} 38.9, 42.2, 43.3, 62.1,$ and 67.5), one disubstituted olefin ($\delta_{\text{C}} 115.9$ and 142.6), and two amide carbonyl groups ($\delta_{\text{C}} 165.1$ and 171.0). The partial NMR spectroscopic data of **3** were close to those of entadamide A (**5**) [5]. The COSY spectrum (Figure 4) of **3** indicated the presence of fragments of H-2/H-3, H-5/H-6, H-2'/H-3', and H-5'/H-6'. The connectivity between two amide groups and above fragments were linked by HMBC correlations of H-2/C-1, H-3/C-1, H-5/C-1, H-4/C-3, H-2'/C-1', H-5'/C-1', H-3'/C-4', H-4'/C-3', and H-6/C-3'. The optical rotation value of **3** (-10.7) was opposite to the synthetic compound (*S*)-4-methoxy-4-(phenylthio)-2-butanone ($+53.9$). Thus, the relative configuration of C-3' was proposed to be R^* on the basis of its optical rotation result. According to the above interpretation, the structure of **3** was established as clinamide C.

Figure 4. COSY (bold lines), key HMBC (arrows), and NOESY (left right arrow) correlations of **3**.



The HRESIMS indicated a molecular formula $C_6H_{11}NO_2S$ for compound **4**, identical to that of **5**. The ^1H and $^{13}\text{C-NMR}$ data of **4** were similar to those of **5**, suggesting that they should be geometric isomers. The $^1\text{H-NMR}$ spectrum of **4** exhibited the signals of a *cis*-double bond at [$\delta_{\text{H}} 5.80$ (d, $J = 10.0$ Hz) and 6.83 (d, $J = 10.0$ Hz)]; however, a *trans*-one at [$\delta_{\text{H}} 5.85$ (d, $J = 14.8$ Hz) and 7.56 (d, $J = 14.8$ Hz)] were found in that of **5**. Thus, the structure of 2-*cis*-entadamide A (**4**) was determined as shown.

The results of the biological activity were discussed in the Conclusion section and the bioactivity tables were put in supporting information.

3. Experimental Section

3.1. General Procedures

Optical rotations were measured with a JASCO DIP-370 digital polarimeter. The IR spectra were measured on a Mattson Genesis II spectrometer. ^1H and $^{13}\text{C-NMR}$ spectra were recorded on Varian Unity Inova-600, Varian Unity Plus-400, or Varian Gemini 2000-200 NMR spectrometers. Chemical shifts are reported in parts per million (δ), and coupling constants (J) are expressed in Hertz. LRESIMS were measured on a VG Biotech Quattro 5022 mass spectrometer. HRESIMS were measured on a Bruker Daltonics APEX II mass spectrometer. Silica gel 60 (Merck, 230–400 mesh) and Sephadex LH-20 were used for column chromatography, while TLC analysis was carried out on silica gel GF₂₅₄ pre-coated plates with detection using 50% H_2SO_4 followed by heating on a hot plate. HPLC was

performed with Hitachi L-7100 series HPLC with a Bischoff RI detector and Shimadzu LC-10AT series HPLC with a SPD-10A UV-vis detector or a photodiode array detector. ODS (Hypersil[®], 250 × 10 mm) columns were applied for HPLC separation.

3.2. Plant Material

The aerial parts of *Clinacanthus nutans* were collected in Taichung, Taiwan, in November 2010, and identified by Ming-Hong Yen. The samples were deposited at the Graduate Institute of Natural Products, Kaohsiung, Taiwan.

3.3. Extraction and Isolation

The aerial parts of *C. nutans* (2.3 kg) were collected freshly, cut into small pieces (*ca.* 0.5–1.0 cm thickness), and air-dried for three days. After drying, the materials were soaked and extracted with ethanol (10 L) at room temperature for three times, and then concentrated under reduced pressure to afford a crude extract (283.0 g). This crude extract was partitioned between EtOAc and H₂O (1:1) to obtain an EtOAc-soluble layer. After evaporating the organic solvent, the EtOAc residue (51.1 g) was partitioned between *n*-hexane–EtOH–H₂O (5:4:1) to afford an 80% ethanol extract. The 80% ethanol extract (14.6 g) was subjected to passage over a silica gel column using gradient elution of CH₂Cl₂–MeOH (1:0 to 5:1) to furnish fractions 1–20. Fraction 13 (618.5 mg) was separated on a Sephadex LH-20 column (5 × 55 cm) eluted with EtOAc–CH₂Cl₂–MeOH (1:1:6) to afford fraction 13-5 (298.0 mg), which was further chromatographed on a silica gel column (3 × 25 cm; CH₂Cl₂–MeOH, 20:1) to furnish subfractions 13-5-1 to 13-5-10. Compound **6** (16.0 mg) was obtained by separation of subfraction 13-5-8.9 (25.5 mg) on preparative TLC eluting with CH₂Cl₂–MeOH (6:1). Subfraction 13-5-4 (149.6 mg) was chromatographed by preparative TLC using CH₂Cl₂–MeOH (8:1) for elution, and further purified by reverse-phase HPLC (270 nm; Hypersil[®], 250 × 10 mm) with MeOH–H₂O (35:65) to yield **3** (3.8 mg; flow rate: 2 mL/min; *R_t* 11.5 min). Then the subfraction was eluted with MeOH–H₂O (40:60) to yield **4** (6.8 mg; flow rate: 2 mL/min; *R_t* 8 min) as well as **5** (99.9 mg; flow rate: 2 mL/min; *R_t* 10 min). Fraction 15 (1035.1 mg) was purified on a Sephadex LH-20 column (5 × 55 cm) using EtOAc–CH₂Cl₂–MeOH (1:1:6) as eluting solvent to afford nine subfractions. Subfraction 15-5 (296.0 mg) was applied to a silica gel column (3 × 25 cm) eluted with CH₂Cl₂–MeOH (30:1) to give ten subfractions. Compound **2** was obtained by separation of subfraction 15-5-4 (40.1 mg) on preparative TLC eluting with CH₂Cl₂–MeOH (10:1) and further purification by reverse-phase HPLC (244 nm; Hypersil[®], 250 × 10 mm) with MeOH–H₂O (43:57) (0.8 mg; flow rate: 2 mL/min; *R_t* 7.5 min). Subfraction 15-5-7 (29.1 mg) was subjected to preparative TLC with CH₂Cl₂–MeOH (15:1) and further purified by reverse-phase HPLC (245 nm; Hypersil[®], 250 × 10 mm) with MeOH–H₂O (25:75) to yield **7** (14.2 mg; flow rate: 2 mL/min; *R_t* 9 min) and with MeOH–H₂O (15:85) to yield **1** (2.2 mg; flow rate: 2 mL/min; *R_t* 13 min).

Clinamide A (**1**): Pale yellow oil; IR (neat): ν_{\max} 3380, 2927, 1668, 1555, 1302, 1135 cm⁻¹; ¹H-NMR (CD₃OD, 400 MHz) and ¹³C-NMR (CD₃OD, 100 MHz) spectroscopic data, see Tables 1 and 2, respectively; HRESIMS: *m/z* 216.0308 [M+Na]⁺ (calcd. for C₆H₁₁NO₄SNa, 216.0306).

Clinamide B (2): White amorphous powder; IR (neat): ν_{\max} 2918, 1732, 1031 cm^{-1} ; $^1\text{H-NMR}$ (CD_3OD , 400 MHz) and $^{13}\text{C-NMR}$ (CD_3OD , 100 MHz) spectroscopic data, see Tables 1 and 2, respectively; HRESIMS: m/z 242.0461 $[\text{M}+\text{Na}]^+$ (calcd. for $\text{C}_8\text{H}_{13}\text{NO}_4\text{SNa}$, 242.0463).

Clinamide C (3): Pale yellow oil; $[\alpha]_{\text{D}}^{25}$ -10.7 (c 1.0, MeOH); IR (neat): ν_{\max} 3296, 2924, 1644, 1582 cm^{-1} ; $^1\text{H-NMR}$ (CDCl_3 , 400 MHz) and $^{13}\text{C-NMR}$ (CDCl_3 , 100 MHz) spectroscopic data, see Tables 1 and 2, respectively; HRESIMS: m/z 345.0903 $[\text{M}+\text{Na}]^+$ (calcd. for $\text{C}_{12}\text{H}_{22}\text{N}_2\text{O}_4\text{S}_2\text{Na}$, 345.0905).

2-cis-Entadamide A (4): Pale yellow oil; IR (neat): ν_{\max} 3306, 2924, 1644, 1583 cm^{-1} ; $^1\text{H-NMR}$ (CDCl_3 , 400 MHz) and $^{13}\text{C-NMR}$ (CDCl_3 , 100 MHz) spectroscopic data, see Tables 1 and 2, respectively; HRESIMS: m/z 184.0409 $[\text{M}+\text{Na}]^+$ (calcd. for $\text{C}_6\text{H}_{11}\text{NO}_2\text{SNa}$, 184.0408).

3.4. Anti-Inflammatory Assay

Human neutrophils were collected by means of Ficoll centrifugation and dextran sedimentation. Assessments of superoxide anion generation and elastase release were carried out according to known procedures [9,10]. In brief, superoxide anion production was assayed by monitoring the superoxide dismutase-inhibitable reduction of ferricytochrome *c*. Elastase release experiments were executed using MeO-Suc-Ala-Ala-Pro-Valp-nitroanilide as the elastase substrate. In the *in vitro* anti-inflammatory bioassay, the inhibitory effects on the generation of superoxide anion and the release of elastase by activated neutrophils were used as indicators. For significant activity of pure compounds, an inhibition rate $\geq 50\%$ is required.

3.5. Anti-Dengue Virus Assay

Naïve Huh-7 cells were obtained from American type culture collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 1% non-essential amino acids, and 1% antibiotic-antimycotic in a 5% CO_2 in air atmosphere at 37 °C. Huh-7 cells were seeded at 24 wells plate at density 5×10^4 cells/well for 12–16 h and then infected with DENV-2, strain 16681 at a multiplicity of infection (MOI) of 0.2 for 2 h at 37 °C. Cells were washed with PBS and then re-fed with DMEM-10% FBS medium containing test compounds. Cells were harvested at 72 h post-infection on a western blot assay with anti-DENV NS2B (1:2000; Abcam, Cambridge, MA, USA) or anti-GAPDH antibody (1:10000; GeneTex, Irvine, CA, USA), a loading control. The signal was detected using an ECL detection kit (PerkinElmer, Shelton, CT, USA). Ribavirin served as positive control [11].

3.6. Immune-Modulating Assay

To examine the inhibitory effect of *Lactobacillus casei* on IgE production, splenocyte obtained from ovalbumin (OVA)-primed BALB/c mice were re-stimulated *in vitro* with the same antigen in the presence of heat-killed *L. casei*. The effect of this bacterium on helper (Th) phenotype development was also examined with native T cells from T cell receptor-transgenic mice [12].

4. Conclusions

The plant material and extracts of *C. nutans* have now become popular commercial products in Thailand and Malaysia, even available through Internet markets worldwide, and dubitatively used in anticancer and anti-cardiovascular diseases, making the active components from this plant an important issue to be understood. Our current study enhances the bioactivity and chemical diversity of this plant material. In bioactive screening, the 80% ethanol extract showed anti-inflammatory, anti-dengue virus and immune-modulating activity. For the study of anti-inflammatory activity, the extract at 10 µg/mL had the strongest elastase release inhibitory effect at 68.33%. Moreover, this extract displayed moderate anti-dengue virus activity in the IC₅₀ 31.04 µg/mL. In an immune-modulating experiment, using 0.1 µg/mL of 80% ethanol extract led to up-regulation of IFN-γ. On the other hand, using high concentration of 80% ethanol extract (100 µg/mL) led to down-regulation of IFN-γ exhibiting immune-modulating activity. Unfortunately, the new compounds isolated so far did not showed any targeted activity.

Supplementary Materials

Supplementary materials can be accessed at: <http://www.mdpi.com/1420-3049/19/12/20382/s1>.

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Author Contributions

Yang-Chang Wu and Fang-Rong Chang designed the whole experiment and contributed to manuscript preparation; Shu-Fen Tu analyzed data and wrote the manuscript; Rosa Huang Liu provided the plant materials; Yuan-Bin Cheng revised the manuscript and the format; Yu-Ming Hsu, Ying-Chi Du, and Mohamed El-Shazly analyzed the data and performed data acquisition.

Conflicts of Interest

The authors declare no conflict of interest.

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