Zoanthus spp.

Ecdysones from *Zoanthus* spp. with inhibitory activity against dengue virus 2

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ABSTRACT

Bioassay-guided fractionation of an ethanolic extract of *Zoanthus* spp. collected in Taiwan has resulted in the isolation of one new ecdysone, zoanthone A (**1**), along with thirteen known compounds (**2–14**). The structures of these compounds were determined by spectroscopic methods, especially 2D NMR analyses. The in vitro antiviral activities of all isolated ecdysones (**1–14**) against dengue virus type 2 (DENV-2) were evaluated using DENV infectious system. New compound (**1**) exhibited potent antiviral activity ($EC_{50} = 19.61 \pm 2.46 \mu M$) with a selectivity index (CC_{50}/EC_{50}) value of 36.7. The structure-activity relationships of isolated ecdysones against DENV-2 were concluded. Molecular docking information of **3** and NS5 polymerase was performed either.

Keywords: Ecdysone; *Zoanthus*; Dengue virus type 2; Structure-activity relationships;

Molecular docking

Dengue virus (DENV) is an arbovirus transmitted by mosquito of the genus *Aedes*. Over 2.5 billion people living in the tropical and subtropical areas are susceptible to dengue virus. Infection of the four serotypes of DENV (DENV-1–4) gives rise to dengue fever, dengue hemorrhage fever, and dengue shock syndrome, which leads to a 20% death rate. Because this mosquito-borne disease has become a considerable global burden, there is an urgent need to develop its remedy. The DENV genome encodes three structural (C, prM and E) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins.¹ NS5 is the largest and the most conserved protein, comprising a N-terminal methyltransferase domain for the viral RNA cap structure^{2,3} and a C-terminal RNA-dependent RNA polymerase (RdRp) domain which is essential for RNA replication and represents an attractive anti-DENV target.^{4,5} In our preliminary antiviral screening, the ethanol extract of *Zoanthus* spp. collected in the east coast of Taiwan showed inhibitory activity against DENV-2, the most infective and epidemic DENV in Asia. Therefore, this animal material was chosen for natural product study.

Sea anemone of the genus *Zoanthus* (family Zoanthidae) is widely distributed in the intertidal zones of subtropical or tropical region. This marine invertebrate are commonly cultured in aquarium because of its fascinating fluorescence. In previous natural product studies, fatty hydrocarbons,⁶ ecdysones,⁷ alkaloids,^{8–15} and

sphingolipids¹⁶ were purified as the major components of *Zoanthus* spp.. Ecdysone, steroidal hormone with a 14 α -hydroxy-7-en-6-one chromophore, was frequently isolated from invertebrates and plants. Except for the moulting function toward arthropod, these type of steroids are regarded for their pesticidal,¹⁷ antidepressive,¹⁸ hepatoprotective,¹⁹ immunoprotective,²⁰ and antiarrhythmic²¹ activities. Herein, the separation and structural elucidation of one new and thirteen known compounds (Figure 1) from the ethanolic extract of *Zoanthus* spp. are described. In addition, the *in vitro* anti-DENV-2 on NS5 polymerase activity and a docking study of DENV NS5 RdRp domain with the isolated ecdysteroids are performed.

The animal materials of *Zoanthus* spp. were collected in east Taiwan and extracted with 95% ethanol. After removing solvent, the residue was partitioned between H₂O and EtOAc to yield an organic extract. A series of column chromatography of the EtOAc extract resulted in the isolation of one new ecdysone (**1**), together with thirteen known compounds, viticosterone E (**2**),²² ajugasterone C (**3**),²³ 20-hydroxyecdysone (**4**),²² α -ecdysone (**5**),²² 24(28)-dehydromakisterone A (**6**),²⁴ 20-hydroxyecdysone 3-acetate (**7**),²⁵ ponasterone A (**8**),²⁶ 2-deoxyecdysterone (**9**),²² pterosterone (**10**),²⁷ *epi*-inokosterone (**11**),²⁸ inokosterone (**12**),²⁸ turkesterone (**13**),²² and dihydropoststerone (**14**).²⁹ The structures of all isolates were determined

by their spectroscopic data.

Figure 1. Structures of ecdysones **1–14** from *Zoanthus* spp..

Zoanthone A (**1**), $[\alpha]_D^{25}$ 47 (c 0.05, MeOH), had a molecular formula of $C_{29}H_{46}O_9$ and seven indices of hydrogen deficiency, as deduced from HRESIMS (m/z 561.3034 $[M+Na]^+$) and ^{13}C -NMR spectra. The IR absorption bands of **1** revealed the presence of hydroxy (3382 cm^{-1}), ketone (1654 cm^{-1}), and C-O (1026 cm^{-1}) functionalities. The Mass, IR and UV absorption (239 and 215 nm) of **1** suggested that **1** possesses an ecdysone framework. The 1H NMR data of **1** (Table 1) clearly indicated the presences of five tertiary methyls (δ 1.29, 1.34, 1.41, 1.48, and 1.63), one olefinic methine (δ 6.32), four oxymethines (δ 3.85, 4.25, 4.59, and 4.61), and one *O*-acetyl unit (δ 1.95). In the ^{13}C NMR (Table 1) and DEPT spectra of **1**, twenty nine

carbon signals were classified into one carbonyl (δ 203.9), one ester carbonyl (δ 170.2), one olefinic methine (δ 122.3), one olefinic quaternary carbon (δ 164.2), four oxygenated methines (δ 68.2, 68.4, 68.9, and 77.4), three aliphatic methines (δ 42.8, 50.0, and 52.5), two aliphatic quaternary carbons (δ 39.5, and 48.1), three oxygen-bearing quaternary carbons (δ 76.8, 82.3, and 84.2), seven aliphatic methylenes (δ 21.5, 26.9, 31.9, 32.9, 39.3, 39.9, and 44.2), and six methyls (δ 18.9, 20.2, 21.5, 24.9, 26.1, and 26.3). The above data accounted for three indices of hydrogen deficiency, which implied that **1** has a tetracyclic ring system.

Table 1¹H and ¹³C NMR Data of **1** in C₅D₅N^a

position	δ _H , mult (<i>J</i> in Hz)	δ _C , type	HMBC (¹ H- ¹³ C)
1	2.01, t (7.8) 3.49, dd (12.5, 4.1)	39.9, CH ₂	2, 3, 5, 9, 10
2	4.59, m	68.4, CH	
3	4.25, br s	68.2, CH	
4	1.78, m 2.06, dt (9.1, 2.1)	32.9, CH ₂	
5	3.06, t (8.5)	52.5, CH	
6		203.9, C	
7	6.32, d (2.3)	122.3, CH	5, 9, 14
8		164.2, C	
9	3.90, dd (8.6, 2.4)	42.8, CH	8, 11
10		39.5, C	
11	4.61, m	68.9, CH	
12	2.72, dd (12.4, 6.3) 3.07, d (14.2)	44.2, CH ₂	9, 11, 13, 14, 18 11, 13, 18
13		48.1, C	
14		84.2, C	
15	1.83, m 2.23, m	31.9, CH ₂	
16	2.18, m 2.47, m	21.5, CH ₂	
17	3.09, t (9.8)	50.0, CH	
18	1.29, s	18.9, CH ₃	12, 13, 14, 17
19	1.34, s	24.9, CH ₃	1, 5, 9, 10
20		76.8, C	
21	1.63, s	21.5, CH ₃	17, 20, 22
22	3.85, dd (10.2, 5.5)	77.4, CH	
23	1.69, m 1.86, m	26.9, CH ₂	
24	2.14, m 2.41, dd (12.0, 5.0)	39.3, CH ₂	25, 26, 27
25		82.3, C	
26	1.48, s	26.3, CH ₃	24, 25, 27
27	1.41, s	26.1, CH ₃	24, 25, 26
OAc-25	1.95, s	170.2, C 20.2, CH ₃	

^a ¹H and ¹³C NMR data (δ) were measured at 400 and 150 MHz, respectively; Chemical shifts are in ppm

In the COSY spectrum, correlations of H₂-1 (δ 2.01 and 3.49)/H-2 (δ 4.59)/H-3 (δ 4.25)/H₂-4 (δ 1.78 and 2.06)/H-5 (δ 3.06), H-9 (δ 3.90)/H-11 (δ 4.61)/H₂-12 (δ 2.72 and 3.07), and H₂-15 (δ 1.83 and 2.23)/H₂-16 (δ 2.18 and 2.47)/H-17 (δ 3.09) were observed. This three proton sequences were connected by the key HMBC correlations of Me-19 (δ 1.34)/C-1 (δ 39.9), C-5 (δ 52.5), C-9 (δ 42.8), C-10 (δ 39.5) and Me-18 (δ 1.29)/C-12 (δ 44.2), C-13 (δ 48.1), C-14 (δ 84.2), C-17 (δ 50.0). In addition, HMBC correlations of H₂-4/C-6 (δ 203.9), H-9/C-8 (δ 164.2), and H-7 (δ 6.32)/C-5, C-9, C-14 were used to establish the 7-en-6-one tetracyclic ring system of **1** (Figure 2). The HMBC correlation of H₃-21 (δ 1.63)/C-17, C-20 (δ 76.8), C-22 (δ 77.4), Me-26 (δ 1.48)/C-24 (δ 39.3), C-25 (δ 82.3), C-27 (δ 26.1), together with the COSY correlations of H-22 (δ 3.85)/H₂-23 (δ 1.69 and 1.86)/H₂-24 (δ 2.14 and 2.41) indicated the presence of side chain C-20 to C-27 and the connection between C-17 and C-20. The *O*-acetyl group was assigned at C-25 by virtue of the similar C-25 chemical shift of **1** and **2**.¹⁷ On the basis of those definitive 2D NMR analyses, the planar structure of **1** was established.

Figure 2. COSY (bold bond) and selected HMBC (arrow) correlations of **1**.

The stereochemistry of **1** was determined by its NOESY correlations (Figure 3) and by comparing its proton chemical shift with congeners. The NOESY correlations of H₃-19/H-5 suggested that the A-B ring fusion of **1** should be *cis*. The hydroxy groups attached at C-2 and C-3 was both assigned on the β -face according to the NOESY correlations between H-3 and H-2. The NOESY cross-peaks of H-19/H-11/Me-18 revealed that these protons are on the β -face. In addition, the presence of NOESY correlations of H-17/Me-21 and H-2/H-9 proved the α -orientation of these protons. On the basis of the similar proton chemical shift of **1** and cyasterone (structure was determined by X-ray single crystallographic analysis),³⁰ the configurations of C-20 and C-22 were both determined to be *R*. Therefore, structure of **1** was determined and a trivial name zoanthone A was given.

Figure 3. Key NOESY (left right arrow) correlations of **1**.

All isolated ecdysones were evaluated for their antiviral and cytotoxic activities. In the antiviral assay against dengue virus 2 by infectious system, compounds **1** and **3** were seen to be the most active substances with the EC₅₀ value of $19.61 \pm 2.46 \mu\text{M}$ and $10.05 \pm 2.37 \mu\text{M}$, respectively. The other ecdysones demonstrated moderate to

weak activities of DENV-2 inhibition. In a half maximal cytotoxicity test against Huh-7 cells, all tested compound showed no activity ($CC_{50} > 700 \mu\text{M}$). In addition, the most active compound **3** were also tested for its EC_{50} value against DENV-1 ($15.70 \pm 2.36 \mu\text{M}$), DENV-3 ($9.48 \pm 0.24 \mu\text{M}$), and DENV-4 ($12.15 \pm 1.22 \mu\text{M}$). These results suggested **3** is active for all dengue virus serotypes.

According to our anti-DENV-2 results (Table 2), the following structure-activity relationships can be observed and illustrated in Figure 4:

- (1) In comparison of compounds **4** and **9**, the absence of a hydroxy group at C-2 is considerably decreases the EC_{50} value.
- (2) Activity of compound **7**, when compared to that of **4**, shows that *O*-acetyl group at C-3 can decrease activity.
- (3) The EC_{50} values of compounds **1** and **3** are less than that of **2**, which suggest the presence of an α -hydroxy group at C-11 can increase the anti-DENV activity.
- (4) The close EC_{50} values of compounds **4** and **5** imply the presence of a hydroxy group at C-20 may not influence the activity.
- (5) Compound **14** has no activity, so we can deduce that missing the side chain of C-23 to C-27 can markedly decrease activity.
- (6) Presence of a hydroxy group at C-27 can strongly decrease anti-DENV-2 activity, as seen from compounds **12** is inactive and compound **11** has weak activity.

Table 2

Anti-DENV-2 Activity of isolated compounds

Compound	EC ₅₀ (μM) ^a	CC ₅₀ (μM) ^b	SI ^c
1	19.61 ± 2.46	>700	>36.7
2	39.76 ± 2.14	>700	>17.6
3	10.05 ± 2.37	>700	>69.7
4	53.55 ± 2.46	>700	>13.1
5	54.28 ± 3.19	>700	>12.9
6	>100	>700	-
7	87.34 ± 4.54	>700	>8.0
8	>100	>700	-
9	>100	>700	-
10	NT ^d	NT	-
11	79.37 ± 1.18	>700	>8.8
12	>100	>700	-
13	38.15 ± 2.40	>700	>18.3
14	>100	>700	-
2CMC ^e	11.20 ± 0.3	94.75 ± 4.15	>8.5

^aHalf maximal effective concentration. ^bHalf maximal cytotoxicity concentration. ^cSelectivity index, CC₅₀/EC₅₀. ^dNot tested. ^e2'-C-methylcytidine, positive control.

Figure 4. Structure-activity relationship studies for the effect of ecdysteroids against DENV-2. The structural modifications leading to EC_{50} value changes is indicated by arrows (↓). The most active compound **3** was used for the SAR description.

In order to further understand the correlations of dengue virus polymerase and the active ecdysones, a computational protein–ligand binding simulation was carried out. A resemble conformation was observed in different DENV and West Nile virus RdRps,³¹ containing fingers, palm, and thumb subdomains in right handed topology. Therefore, X-ray crystallographic of RdRp (PDB code 2J7U) of DENV NS5^{32,33} was adopted in our binding study. In silico docking calculations of NS5 RdRp with ajugasterone C (**3**) were illustrated in Figure 5, suggesting active compounds arrest DENV RNA replication by blocking the viral polymerase channel. Which findings corresponds to a current report, a synthetic non-nucleoside compound acts as an RdRp inhibitor by binding to an allosteric pocket in DENV RNA template tunnel.³⁴

Figure 5. 3D model of overall complex of ecdysone-DENV RdRp entry represented in hydrophobicity surface (left) and ribbons (right). Compound **3** was chosen for illustration.

In the docking results (Figure S7) for ajugasterone C (**3**), three hydrogen bonds between C-6=O and charged NH residue of Arg729, OH-2 and backbone NH of Trp795, as well as OH-20 and NH at imidazole His798 were observed. It is noted that **3** changed its pose and had four H-bonds of C-6=O/Ser710, OH-2 and OH-3/Asp533, and OH-20/Ile797 while the grip box size was shrunk (data not shown). To compare Zoanthone A (**1**) with **3**, compound **1** cross-linked to RdRp by the same hydrogen bonding and possessed an extra linking between OAc-25 and NH₂ of Asn609. According to the docking results of selected compounds (Table S1), carbonyl group at C-6 and hydroxyl at C-2 are not only characteristics in ecdysone skeleton but also essential for their bioactivities. Furthermore, molecular modeling studies of these

protease-ligand complexes also ascertained the influence of hydroxy groups in ecdysone, revealing the following. Intermolecular hydrogen bond of OH-2 and OH-3 enhance the inhibition ability and are formed with backbone NH of Trp795 and OH of Thr794, respectively; and finally, OH-22 at the side chain link to the carbonyl substituents of Asp663 and/or Asp664, giving the bioactivity benefit. The information corresponded to the above structure activity relationship analyses.

Phytoecdysone has the function of insect repellent and is regarded as a biomarker in many primitive plants such as ferns and gymnosperms. Zoanthid, a primitive invertebrate adapting to the above water environment, was found to secrete a series of ecdysones in our natural product investigation. This finding confirmed the ecological function of secondary metabolite usually acts as chemical defense substance. Besides, *in vitro* anti-dengue virus type 2 activity of ecdysones was first discovered. By the assistance of SAR and molecular docking analyses, the plausible action mechanism between ecdysone and DENV-2 NS5 polymerase was successfully proposed. Our findings not only demonstrate the structural diversity of marine natural products, but especially provide further insight into ecdysteroid as an anti-dengue virus agent.

Acknowledgments

This work was supported by grants from ministry of science and technology of Taiwan (MOST103-2628-B-037-001-MY3 award to Y.-B. C.; MOST

103-2320-B-037-005-MY2 award to F.-R. C.), national health research institutes (NHRI-EX103-10241BI), and in part from the grant from Chinese medicine research center, China medical university (the ministry of education, the Aim for the Top University Plan). This study is also supported partially by Kaohsiung medical university (Aim for the Top Universities Grant, grant No. KMU-TP104H03, and KMU-TP104E40), ministry of health and welfare of Taiwan (MOHW105-TDU-B-212-134005), and health and welfare surcharge of tobacco products.

The RCSB PDB (<http://www.rcsb.org/pdb/home/home.do>), an information portal to 112968 biological macromolecular structures, is funded by a grant (DBI-1338415) from the National Science Foundation, the National Institutes of Health, and the US Department of Energy. AutoDock 4 (<http://autodock.scripps.edu/>) is free and is available under the GNU General Public License. AutoDock is Copyright© 1989-2015 The Scripps Research Institute. UCSF Chimera (<https://www.cgl.ucsf.edu/chimera/>) is a highly extensible molecular modeling system. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics, funded by the National Institutes of Health (NIGMS P41-GM103311).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at

http://

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