New Cytotoxic Cardenolide Glycoside from the Seeds of *Cerbera manghas*

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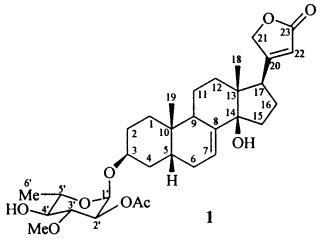
A new cytotoxic cardenolide glycoside, 3β-*O*-(2′-*O*-acetyl-α-L-thevetosyl)-14β-hydroxy-7-en-5β-card-20(22)-enolide, (7,8-dehydrocerberin), together with five known cardenolides, 17β-neriifolin, deacetyltanghinin, tanghinin, cerberin and 2′-*O*-acetyl-cerleaside A were isolated from the seeds of *Cerbera manghas* L. Their structures were elucidated by 1D- and 2D-NMR techniques as well as UV, IR and mass spectral data. 7,8-Dehydrocerberin, deacetyltanghinin and tanghinin exhibited cytotoxic activities against oral human epidermoid carcinoma (KB), human breast cancer cell (BC) and human small cells lung cancer (NCI-H187).

Key words *Cerbera manghas*; Apocynaceae; cardenolide; cytotoxicity

Cerbera is a mangrove plant belonging to the Apocynaceae and distributed widely in the coastal areas of South East Asia and Indian Ocean. Only 2 species, *Cerbera odollam* C. F. Gaertn. and *Cerbera manghas* L., are found in Thailand. The two species can be differentiated through the color of the corolla, fruit-shape and color of the ripe fruit. *C. odollam* has a yellow-eye white corolla with a spherical to ovoid-shape fruit and green when ripe whereas *C. manghas* has a red-eye white corolla with an oblong or ellipsoid-shape fruit and reddish when ripe.[1)] In the preceding paper, we isolated 2′-*O*-acetyl-cerleaside A, 17β-neriifolin, cerberin, cerleaside A and 17α-neriifolin from the seeds of *C. odollam*.[2)] As a continuation of our study on this genus, we isolated a new cytotoxic cardenolide glycoside (**1**), along with five known cardenolides, 17β-neriifolin (**2**),[3)] deacetyltanghinin (**3**),[4)] tanghinin (**4**),[4)] cerberin (**5**)[4)] and 2′-*O*-acetyl-cerleaside A (**6**)[2)] from a methylene chloride extract of the seeds of *C. manghas*. The structures of the compounds were elucidated by comparison of their physical and spectral data with reported values.

Compound **1** was obtained as a white solid, and its molecular formula was determined as $C_{32}H_{46}O_9$ by HR-FAB-MS ($[M+1]^+$ *m/z* 575.3205, calcd 575.3220). The IR spectrum showed the presence of hydroxyl (3461 cm^{-1}) and carbonyl (1745, 1716 cm^{-1}) groups. The UV spectrum (λ_{max} 217 nm) suggested an α,β-unsaturated γ-lactone.[5)] ^{1}H-NMR spectral data (Table 1) showed signals of cardenolide framework such as methylene protons at C-21 (δ 4.82, 4.99, each dd, J=18.0, 1.5 Hz), an olefinic proton at C-22 (δ 5.92, br t, J=1.5 Hz) and a methine proton at C-17 (δ 2.84, dd, J=9.5, 6.0 Hz). The ^{1}H- and ^{13}C-NMR spectral data indicated the presence of one sugar molecule according to an anomeric proton at δ 5.06 (d, J=3.5 Hz) for H-1′ connected to an anomeric carbon at δ 93.8 from the HMQC experiment. The connectivity of all protons of the sugar moiety was assigned by the COSY spectrum and comparison with 2′-*O*-acetyl-L-thevetose, the sugar moiety of cerberin (**5**).[4)] Clear separations of the proton signals were shown at δ 4.65 (1H, dd, J=10.0, 3.5 Hz, H-2′), 3.59 (1H, t, J=10.0 Hz, H-3′), 3.22 (1H, t, J=10.0 Hz, H-4′), 3.80 (1H, m, H-5′), 1.27 (3H, d, J=6.5 Hz, H-6′) and 3.59 (3H, s, 3′-OMe). Two methyl singlets at δ 0.80 and 0.86 were assigned to 3H-18 and 3H-19, respectively. One oxymethine proton at δ 3.83 (m) was assigned to H-3. Comparison of its ^{1}H-NMR spectral data with those of cerberin (**5**)[4)] revealed their close structural similarity, except that **1** showed an olefinic proton at δ 5.80 (br d, J=5.5 Hz). From the



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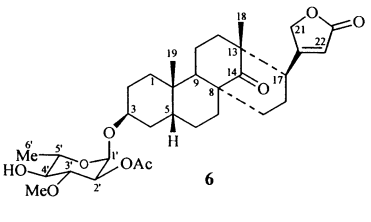

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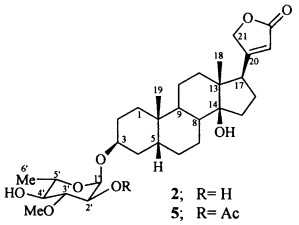
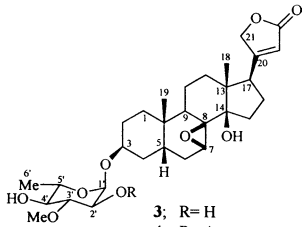


2; R= H
5; R= Ac



3; R= H
4; R= Ac

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Table 1. ^{1}H, ^{13}C and HMBC Spectral Data of Compound **1** (500, 125 MHz in $CDCl_3$)

No.	^{13}C	DEPT	^{1}H	HMBC ($^{1}H \rightarrow ^{13}C$)
1	30.3	CH_2	1.51 (2H, m)	—
2	27.4[a)]	CH_2	[c)]	—
3	72.2	CH	3.83 (1H, m)	C-1, C-5 and C-1′
4	31.1	CH_2	1.29 (1H, m), 1.31 (1H, m)	—
5	34.7	CH	1.71 (1H, m)	—
6	29.9	CH_2	2.43 (1H, m), 1.60 (1H, m)	—
7	117.9	CH	5.80 (1H, br d, J=5.5 Hz)	C-5, C-6 and C-14
8	138.7	C	—	—
9	33.9	CH	2.27 (1H, m)	—
10	—	C	—	—
11	28.5	CH_2	1.58 (2H, m)[d)]	C-8 and C-13
12	39.2	CH_2	1.54 (2H, m)[d)]	C-14 and C-18
13	50.8	C	—	—
14	85.2	C	—	—
15	39.8	CH_2	[c)]	—
16	27.7[a)]	CH_2	[c)]	—
17	50.3	CH	2.84 (1H, dd, J=9.5, 6.0 Hz)	C-12, C-14, C-20, C-21 and C-22
18	16.0	CH_3	0.80 (3H, s)	C-12, C-14 and C-17
19	24.8	CH_3	0.86 (3H, s)	C-1 and C-5
20	174.5[b)]	C	—	—
21	73.4	CH_2	4.82 (1H, dd, J=18.0, 1.5 Hz) 4.99 (1H, dd, J=18.0, 1.5 Hz)	C-17, C-20 and C-22
22	117.7	CH	5.92 (1H, br t, J=1.5 Hz)	C-17, C-20 and C-21
23	174.3[b)]	C	—	—
1′	93.8	CH	5.06 (1H, d, J=3.5 Hz)	C-3, C-3′ and C-5′
2′	74.3	CH	4.65 (1H, dd, J=10.0, 3.5 Hz)	C-3′, 2′-C=O and C-4′
3′	80.8	CH	3.59 (1H, t, J=10.0 Hz)	C-4′, C-5′ and 3′-OMe
4′	75.3	CH	3.22 (1H, t, J=10.0 Hz)	C-3′, C-5′ and C-6′
5′	67.0	CH	3.80 (1H, m)	—
6′	17.6	CH_3	1.27 (3H, d, J=6.5 Hz)	C-4′ and C-5′
2′-OAc	20.9	CH_3	2.09 (3H, s)	2′-C=O
2′-C=O	170.2	C	—	—
3′-OMe	60.5	CH_3	3.59 (3H, s)	C-3′

a, *b*) Assignment with the same superscripts may be interchanged. *c*) The chemical shifts of proton resonated at δ 1.25—2.15. *d*) Type of proton deduced by HMQC.

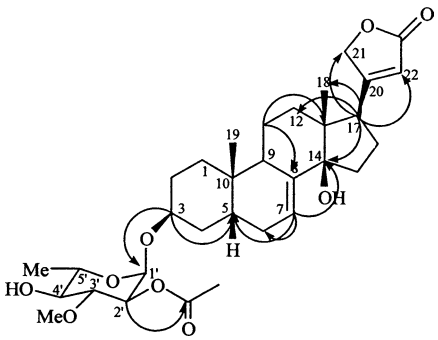

Fig. 1. HMBC Correlation of Compound **1**

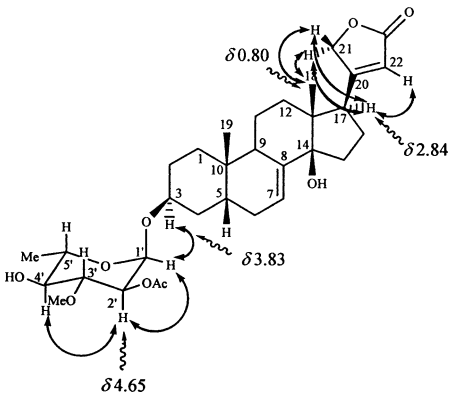

Fig. 2. Selected NOE experiments of compound **1**

HMQC experiment, this olefinic proton was connected to an olefinic carbon at δ 117.9. The location of the double bond was deduced to be a Δ^7 from HMBC correlations (Fig. 1, Table 1), in which correlations of H-7 (δ 5.80) were observed with C-5 (34.7), C-6 (29.9) and C-14 (85.2); of H-11 (δ 1.58) with C-8 (138.7) and C-13 (50.8). Additional correlations of H-17 (δ 2.84) were observed with C-12 (39.2), C-14 (85.2), C-20 (174.5), C-21 (73.4) and C-22 (117.7); and of H-3 (δ 3.83) with C-5 (34.7) and C-1′ (93.8), indicating that the α,β-unsaturated γ-lactone was located at C-17 (50.3) and the sugar moiety was located at C-3 (72.2), respectively. The H-2′ (δ 4.65) showed correlation with carbonyl carbon of acetate group (170.2), indicating that the acetate group was located at C-2′.

The relative stereochemistry of compound **1** was supported from NOE correlations (Fig. 2) of H-17 (δ 2.84) with 2H-21 and H-22, and of 3H-18 (δ 0.80) with 2H-21 and H-22 but no correlation with H-17. These observations, together with the chemical shift of H-17 at δ 2.84 (dd, J=9.5, 6.0 Hz) were in agreement with 17β-cardenolide.[6] Furthermore, the results of strong enhancements of H-1′ and H-4′ upon irradiation of H-2′ (δ 4.65) and of H-1′ upon irradiation of H-3 (δ 3.83), together with a small coupling constant of H-1′ (δ 5.06, J=3.5 Hz) revealed that the sugar was 2′-*O*-

Table 2. Cytotoxic Activity of Compounds **1**, **3** and **4**

Compound	Cell lines[a)]		
	KB	BC	NCI-H187
1	1.75	0.0006	16.7
3	0.05	1.48	0.1
4	1.29	0.77	2.3

a) Results are expressed as ED_{50} values (μg/ml); activity: <5 strong, 5—20 moderate, 20—50 weak, >50 inactive. Key to cell lines used: KB=oral human epidermoid carcinoma; BC=human breast cancer cells; NCI-H187=human small cells lung cancer.

acetyl-α-L-thevetose.[3,4] The carbon chemical shifts were conclusively assigned on the basis of ^{13}C-NMR, DEPT and HMQC experiments and by comparison with cerberin (**5**). Therefore, compound **1** was determined as 3β-*O*-(2′-*O*-acetyl-α-L-thevetosyl)-14β-hydroxy-7-en-5β-card-20(22)-enolide, (7,8-dehydrocerberin).

Compounds **1**, **3** and **4** were evaluated against a panel of human tumor cell lines. Compounds **3** and **4** exhibited significant cytotoxic effects with ED_{50} values in the general range of 0.05—2.3 μg/ml, whereas compound **1** was strongly active against KB and BC cell lines but moderately active against NCI-H187 cell line (Table 2).

Experimental

General Experimental Procedures Melting points were determined on an Electrothermal melting point apparatus. UV spectra were measured with a SPECORD S 100 (Analytikjena) and a UV-160A spectrophotometers (Shimadzu). The IR spectra were measured with a FTS FT-IR Perkin Elmer spectrophotometer. The ^{1}H- and ^{13}C-NMR spectra were recorded using 500 MHz Varian UNITY INOVA Spectrometer in $CDCl_3$. Chemical shifts were recorded in part per million (δ) in $CDCl_3$. HR-FAB-MS was performed using a Thermofinnigan MAT 95 XL mass spectrometer. The $[\alpha]_D$ values were determined with an Autopol II automatic polarimeter. Column chromatography (CC) was carried out on silica gel 100. Precoated plates of silica gel 60 F_{254} (Merck) were used for analytical purposes.

Plant and Material Seeds of *Cerbera manghas* L. were collected from Faculty of Pharmaceutical Science, Prince of Songkla University, Songkhla, Thailand, in March 2002. A voucher specimen (No. 0012281) was deposited in the Herbarium of the Department of Biology, Faculty of Science, Prince of Songkla University, Songkhla, Thailand.

Extraction and Isolation Seeds from fresh fruits of *C. manghas* (300 g) were extracted with methylene chloride (2×2.5 l, for 5 d each) at room temp. The mixture was filtered and concentrated under reduced pressure to give white solid (16.96 g) and crude methylene chloride extract as a yellow oil (29.39 g). The white solid obtained was further purified by chromatography on silica gel using hexane as eluent and increasing polarity with diethyl ether and MeOH to give seven fractions. Fraction F2 (0.640 g) was purified by CC with MeOH–CH_2Cl_2 (1 : 19, v/v) to give two subfractions. Subfraction F2a (0.420 g) was purified by CC using acetone–CH_2Cl_2 (1 : 10, v/v) to give **6** (0.003 g). Subfraction F2b (0.122 g) was purified by prep. TLC using diethyl ether–hexane (7 : 3, v/v) to give **1** (0.010 g), **4** (0.055 g) and **5** (0.008 g). Fraction F4 (1.900 g) was purified by CC using gradient elution of diethyl ether–MeOH to give **2** (0.055 g) and **3** (0.017 g).

Biological Evaluation The cytotoxic assay employed the colorimetric method.[7] Ellipticine, the reference substance, exhibited activity toward KB, BC and NCI-H187 cell lines with IC_{50} ranges of 0.3—0.6 μg/ml.

3β-*O*-(2′-*O*-Acetyl-α-L-thevetosyl)-14β-hydroxy-7-en-5β-card-20(22)-enolide (**1**): White solid, mp 103—105 °C. IR (KBr) cm^{-1}: 3461 (OH) and 1745, 1716 (C=O). UV λ_{max} (MeOH) nm (log ε): 217 (4.04). HR-FAB-MS ($[M+1]^+$) *m/z* 575.3205 (Calcd for $C_{32}H_{47}O_9$: 575.3220). $[\alpha]_D^{26}$ −166.1° (*c*=0.024, $CHCl_3$). ^{1}H-NMR (500 MHz in $CDCl_3$) and ^{13}C-NMR (125 MHz in $CDCl_3$) see Table 1.

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