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Spectacular modification of Gambogic acid on microwave irradiation in methanol: Isolation and structure identification of two products with potent anti-tumor activity

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ABSTRACT

Treatment of gambogic acid with methanol in acidic condition under microwave irradiation led to the formation of two new products bearing spectacular A ring systems which were different from reported *Garcinia* natural products. The chemical structures of these two compounds were elucidated by extensive NMR and MS spectroscopic analysis as well as crystallographic study. Both of the two products could significantly inhibit the growth of various tumor cell lines in vitro with IC_{50} values at μM level through apoptotic pathway.

Gamboge resin from *Garcinia hanburyi* tree in Southeast Asia was reported in traditional folk medicine records to possess many interesting biological profiles, including anti-viral, anti-inflammatory, and anti-infective activities. Gambogic acid (GA, CAS No. 2752-65-0) is one of the representative ingredients secreted from the gamboge resin.[1] The chemical structure of GA has been well established by NMR spectroscopic analysis[2,3] and crystallographic studies.[4] GA contains the same unique 4-oxa-tricyclo[4.3.1.0^{3,7}]dec-2-one scaffold (the so called caged ring system) as other natural products isolated from *Garcinia* genus.[5] Biological evidences indicated that GA could significantly inhibit the growth of human cancer cells and prevent tumor angiogenesis and metastasis without affecting normal cells both in vitro and in vivo.[6–9] Now GA has been approved to enter Phase II clinical trials in China.

During recent years, total synthesis and SAR studies of GA as well as other *Garcinia* natural products have been springing up vigorously due to their specific caged scaffold and striking bioactivities.[7,10–13] Previous structure modifications of GA mainly focused on the 6-hydroxyl group, 9,10-double carbon bond of α,β-unsaturated ketone, 30-carboxyl group and the isopentenyl groups.[7,10,11] However, the A ring modified GA derivatives were rarely reported so far since it does not possess any directly modifiable site. Therefore, it is highly desirable to expand the molecular diversity of A ring system to explore SAR information and develop novel GA derivatives. In this Letter, two GA derivatives with neo-type A ring systems that differed from traditional *Garcinia* natural products were generated during microwave irradiation in acidic condition (Scheme 1). Details of the structure resolution, mechanism elucidation and biological evaluation for these new compounds were reported herein.

The reaction was conducted under microwave irradiation (200 W, 80 °C, 5 min) using small amount of H_2SO_4 as acid and methanol as both the solvent and reactant. Two products, encoded **1** and **2**, were obtained and purified by silica column chromatography (eluent, PE:EA = 8:1). ^{1}H NMR, ^{13}C NMR and 2D NMR analysis showed that both **1** and **2** maintained the same bridged ring system as GA. Subsequently, the structure of **1** was confirmed by single crystal X-ray analysis[14] (Fig. 1). Then the structure of **2** was identified by comparing NMR data with **1**. In accordance with previously reported microwave assisted esterification methodology,[15] the esterification of GA's carboxylic acid group was achieved under the microwave condition, whereas the isopentenyl group was cyclized with the alkene on the A ring forming a new bridged ring skeleton (compound **1**) or a new six-membered ring structure (compound **2**).

Since this reaction was unprecedented and the A ring systems in **1** and **2** were different from reported *Garcinia* natural

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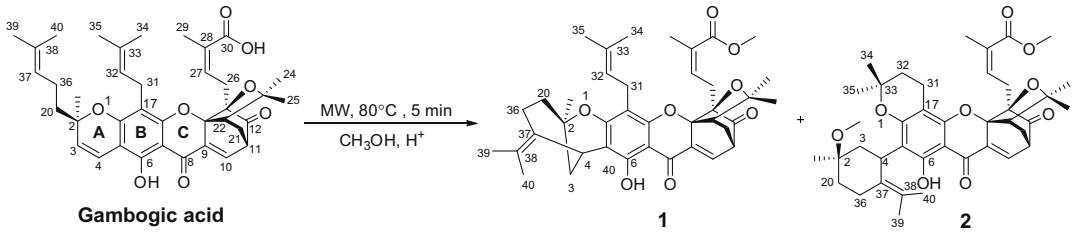


Scheme 1. The formation of products **1** and **2**.

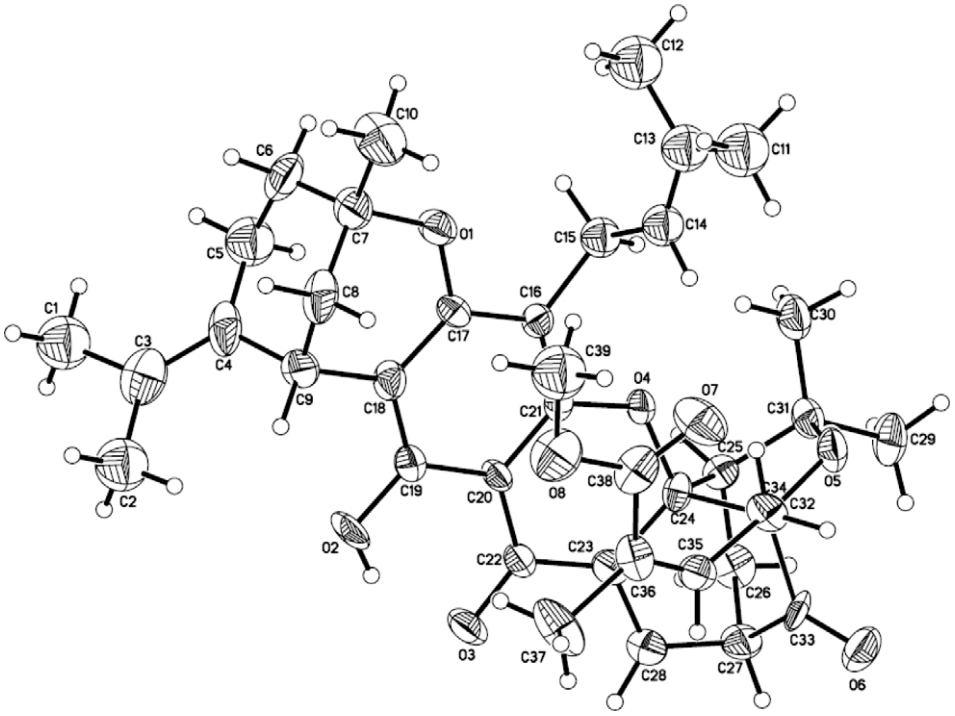


Figure 1. X-ray structure of compound **1**.

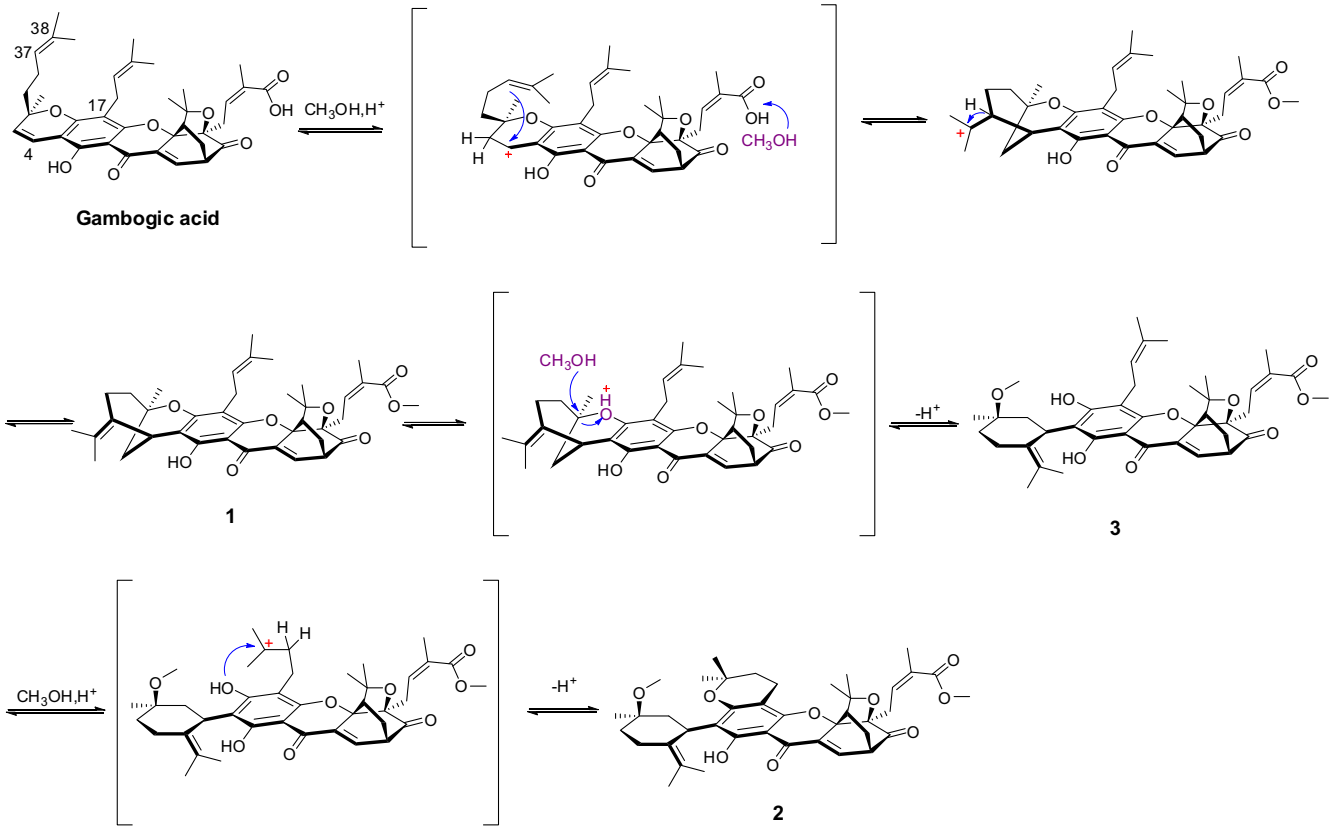
products,[13] the reaction condition were investigated. As shown in Table 1, compounds **1** and **2** were not formed without microwave irradiation (entries **1** and **2**). The product ratio of **1** and **2** was neither proportional to the reaction time nor related to the degree of acidity (entries **3**–**5**). Subsequently, pure **1** and **2**, respectively, were subjected to the same microwave conditions in the presence of methanol and acid. The results showed that **1** and **2** existed in equilibrium amount without the formation of other products after microwave heating. All the evidences suggested the thermodynamic control of the product distribution.

On the basis of the above investigations, a possible mechanism of the reactions was proposed to explain the formation of **1** and **2** (Scheme 2). Presumably, in the presence of acid, the alkene of the A ring was protonated, which led to the formation of a carbocation on the 4-position. This reaction is probably facilitated by the fact that the carbocation formed is benzylic and that there are two electron-donating groups on C-17, and C-6 of the same phenyl ring, which helps to stabilize the positive charge. Then attack of this carbocation by the π-electrons of the double bond between C37 and C38 led to the formation of the new bridged ring of compound **1**.

Table 1
The attempted reaction conditions and their corresponding yields of **1** and **2**

Entry	Conditions			Yields[a]		GA recovery (mg)
	Temperature (°C)	Acids/solvent	Time	Compd **1** (%)	Compd **2** (%)	
1	rt	H_2SO_4/CH_3OH	48 h	0	0	185
2	80	H_2SO_4/CH_3OH	6 h	0	0	180
3	MW, 80	H_2SO_4/CH_3OH	5 min	49	28	20
			10 min	50	28	15
			15 min	50	27	15
			30 min	50	27	18
4	MW, 80	CH_3SO_2H/CH_3OH	5 min	47	40	8
5	MW, 80	p-CH_3-$PhSO_2H/CH_3OH$	5 min	46	37	10

[a] In each entry, 200 mg GA was used.



Scheme 2. Proposed mechanism for the formation of **1** and **2**.

The esterification of the carboxylic acid group can be independent of the cyclization event. As for the step leading to the formation of compound **2**, the O atom of the **1** bridged ring can be protonated, facilitating an S_N2 attack by methanol, resulting in ring cleavage and intermediate **3**. Protonation of the isopentenyl group on 17-position and the subsequent formation of a carbocation initiate the second cyclization thus resulting for the formation of compound **2**.

Compounds **1** and **2** were evaluated for their in vitro anti-tumor effects against four human cancer cell lines including human hepatocellular liver carcinoma HepG2 cell line, human gastric carcinoma BGC-803 cell line, human stomach carcinoma SGC-7901 cell line, human mammary carcinoma MCF-7 cell line using MTT assay.[16] Interestingly, after incubation for 48 h, both **1** and **2** showed comparable anti-proliferative activities against these tumor cell lines to GA and methyl gambogate, the methyl-esterified derivative of GA (Table 2).[7] HepG2 cell line was chosen for further investigation since both **1** and **2** showed better anti-proliferative activities than GA and methyl gambogate in this cell line. Cell morphological observations were subsequently performed under an inverted-microscope. Under the light microscope (×200), incubation of 1.25 μM, 2.5 μM and 5 μM of compound **1** and 0.5 μM, 1 μM and 2 μM of compound **2**, respectively, in HepG2 cells for 24 h led to severe distortion or elongation, membrane blebbing and cell shrinkage. With the gradient enhancement of drug concentration, a large proportion of cells became round in shape and necrosed, while the untreated cells displayed normal shape and clear skeleton under the same conditions (Fig. 2A and B). Under the fluorescence microscope (×400), HepG2 cells treated with indicated concentration of **1** and **2** for 24 h displayed the morphological features of early apoptotic cells, such as bright nuclear condensation identified by DAPI staining, and the apoptotic body appeared significantly with the increased concentration of **1** and **2** (Fig. 2C and D).

Table 2
The in vitro anti-tumor effects of compounds **1** and **2**[a]

Cell lines	IC_{50} (μM)			
	1	**2**	GA	Methyl gambogate[b]
HepG2	1.96	1.53	2.40	2.53
BGC-803	1.45	2.01	1.54	1.24
SGC-7901	2.15	1.51	1.47	0.79
MCF-7	1.88	1.24	0.50	0.52

[a] The IC_{50} values were determined from eight different concentrations of compounds at two fold dilutions, and were the means of two separate experiments.
[b] Methyl gambogate was synthesized according to Ref. 7.

The explorations of **1** and **2** on apoptosis induction were performed using Annexin-V/propidium iodide (PI) double staining assay on flow cytometry instrument. For compound **1**, the percentage of apoptotic cells (Annexin V^+/PI^- staining) in the control group was 5.3%. After incubation of 1.25 μM, 2.5 μM and 5 μM of **1** for 24 h, the early and median apoptotic cells (lower right section of fluorocytogram) were increased to 5.7%, 13.6% and 20.7%, respectively, while the late apoptotic and necrotic cells (upper right section of fluorocytogram) were increased slightly (Fig. 3A). For compound **2**, the percentage of apoptotic cells in the control group was 3.5%. After incubation of 0.5 μM, 1 μM and 2 μM of **2** for 24 h, the early and median apoptotic cells were increased to 4.3%, 12.9% and 35.0%, respectively, whereas the late apoptotic and necrotic cells were increased slightly (Fig. 3B). These results suggested that **1** and **2** could induce cell death mainly via apoptosis rather than necrosis. Further explorations of the apoptotic induction mechanism of **1** and **2** on the expression level of apoptosis related proteins were performed. After incubation with 1.25 μM, 2.5 μM and 5 μM of compound **1** for 24 h, the expression level of bcl-2 and

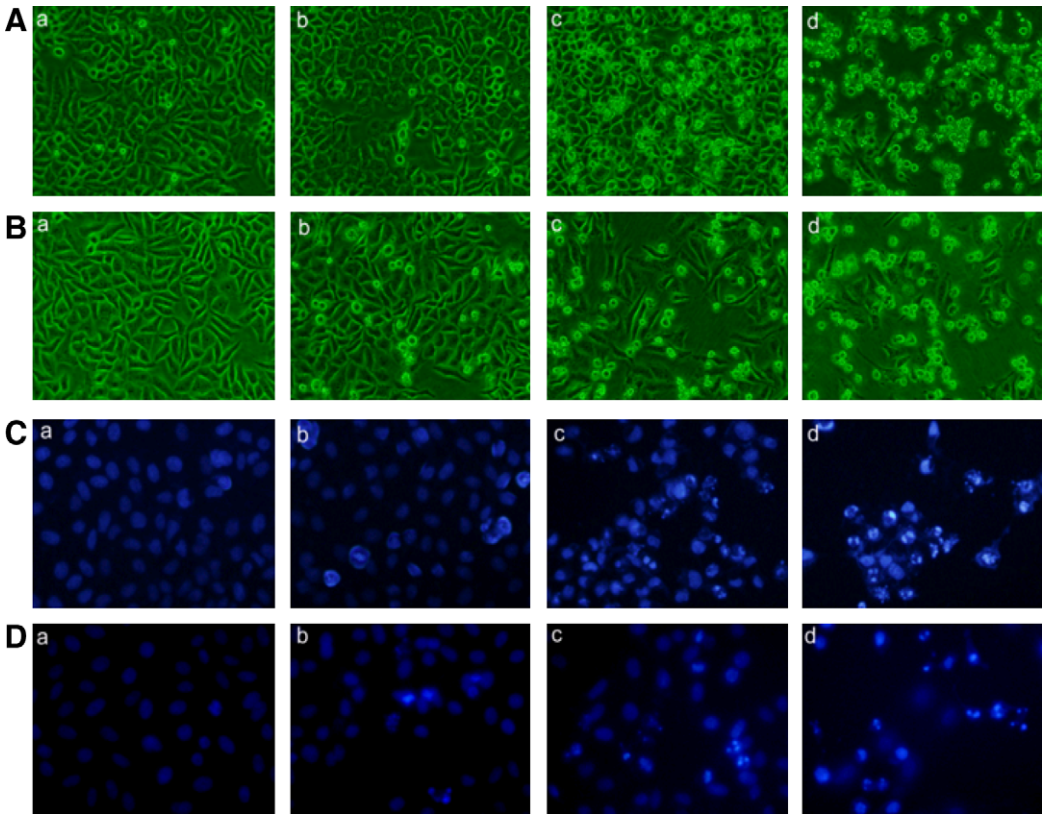


Figure 2. Cell morphological assessment of compounds **1** and **2** under light microscope instrument (A, B) and under fluorescence microscope instrument (C, D) on HepG2 cells. (A) Compound **1**: (a) control group; (b) 1.25 μM of **1**; (c) 2.5 μM of **1**; (d) 5 μM of **1**. (B) Compound **2**: (a) control group; (b) 0.5 μM of **2**; (c) 1 μM of **2**; (d) 2 μM of **2**. (C) Compound **1**: (a) control group; (b) 1.25 μM of **1**; (c) 2.5 μM of **1**; (d) 5 μM of **1**. (D) Compound **2**: (a) control group; (b) 0.5 μM of **2**; (c) 1 μM of **2**; (d) 2 μM of **2**.

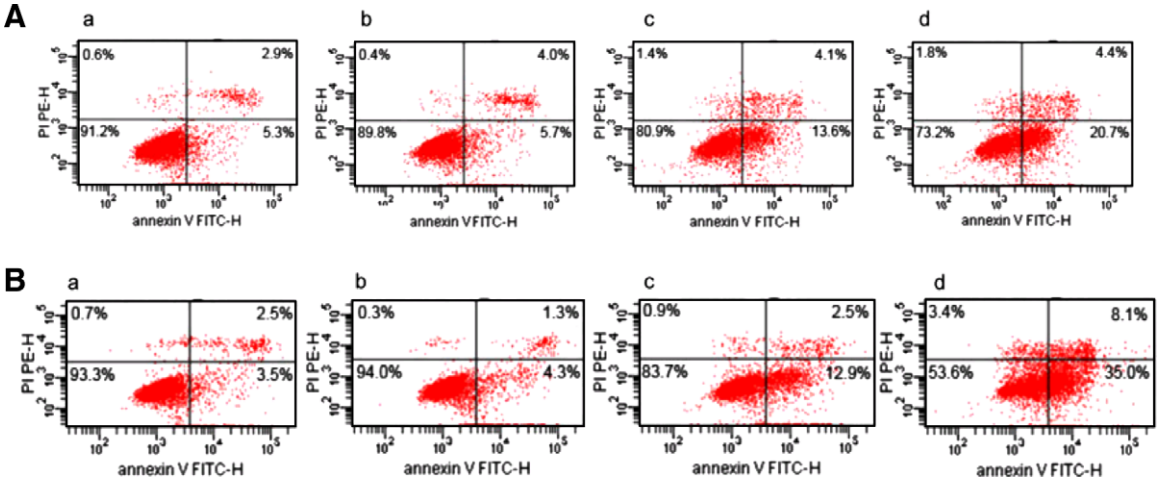


Figure 3. Fluorescence-activated cell sorter analysis of compounds **1** and **2** on HepG2 cells by Annexin-V/PI staining. Upper left: dead cells; upper right: late apoptotic cells; lower left: fully viable cells; lower right: early apoptotic cells. (A) Compound **1**: (a) control group; (b) 1.25 μM of **1**; (c) 2.5 μM of **1**; (d) 5 μM of **1**. (B) Compound **2**: (a) control group; (b) 0.5 μM of **2**; (c) 1 μM of **2**; (d) 2 μM of **2**.

pro-caspase-3 were decreased significantly in HepG2 cells, while the protein level of bax had no significant change compared with control (Fig. 4A). For compound **2**, being treated with 0.5 μM, 1 μM and 2 μM concentration, the expression level of bax were increased, while the protein level of bcl-2 had no obvious change. Meanwhile, the expression level of pro-caspase-3 was decreased, accompanied by the increasing expression of active form caspase-3 (Fig. 4B). The results indicated that after treatment with **1** and **2**, the general ratio of apoptosis suppressor protein bcl-2 and activator protein bax was increased, and pro-caspases-3 was transformed to its active caspase-3 form, all of which indicated the initiation of apoptosis. Therefore, it was concluded that **1** and **2** could inhibit cancer cell growth through apoptotic pathway.

In summary, a novel intramolecular cyclization reaction of gambogic acid under microwave irradiation was reported. Two new products named **1** and **2** with new types of ring systems which could not be obtained without microwave heating were obtained. A possible mechanism was proposed by deep investigation of the

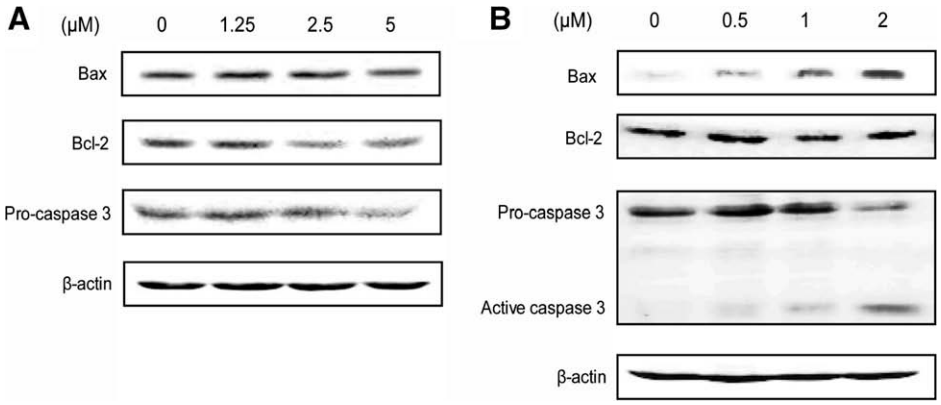


Figure 4. Effects of compounds **1** and **2** on the expression of apoptotic related proteins of HepG2 cells. (A) Compound **1**; (B) compound **2**.

reaction condition and the structures of **1** and **2**. This new reaction indicated that the specific 4-oxa-tricyclo[4.3.1.$0^{3,7}$]dec-2-one ring system can tolerate acidic conditions under microwave heating, thus make it possible to extend the application of the microwave reaction to other *Garcinia* natural products. The preliminary biological studies identified that these two compounds with novel A ring systems could inhibit tumor cell growth through apoptotic pathway. Their disparities in the mechanistic studies also precipitated further investigations.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.03.021.

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