Stability and Cytotoxicity of Gambogic Acid and Its Derivative, Gambogoic Acid

Quan-Bin Han,[a] Susan Cheung,[b] Joseph Tai,[b] Chun-Feng Qiao,[a] Jing-Zheng Song,[a] and Hong-Xi Xu*,[a]

[a] *Chinese Medicine Laboratory, Hong Kong Jockey Club Institute of Chinese Medicine; Hong Kong, P. R. China: and* [b] *Departments of Pathology and Pediatrics, Center for Complementary Medicine Research, Children's and Family Research Institute, University of British Columbia; Vancouver, BC, V5Z4H4 Canada.*

Received August 5, 2005; accepted September 20, 2005

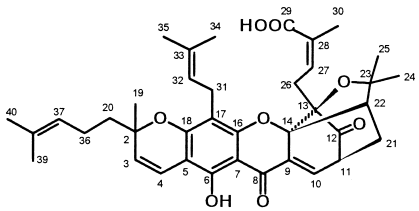
In this study, the stability of gambogic acid (GA), a polyprenylated xanthone with potent cytotoxicities against various cancer cell lines, was evaluated under several experimental conditions including addition of acids, alkalis and organic solvents. GA was stable when dissolved in acetone, acetonitrile, and chloroform, even when acids were added. However, a new derivative was produced after GA was stored in the methanol solution for a week at room temperature. The addition of alkalis could increase the rate of this chemical transformation. This derivative was determined to be gambogoic acid (GOA) by the HPLC-MS comparison with the known compound. GOA was proposed to be the product of neuclophilic addition of methanol to the olefinic bond at C-10 of GA. Furthermore, when these two compounds were tested for their cytotoxicity, GOA showed significantly weaker inhibitory effects than GA. It was therefore deduced that the α,β-unsaturated carbonyl moiety at C-10 contributed to the cytotoxicity of gambogic acid.

Key words *Garcinia hanburyi*; gambogic acid; gambogoic acid; stability; cytotoxicity

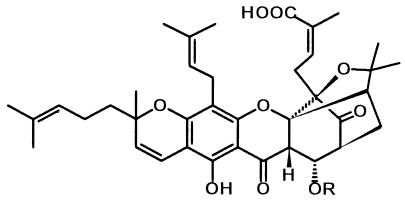
Gambogic acid (GA, CAS No. 2752-65-0) is the principal active component of gamboge, the resin from various *Garcinia* species including *G. morella* and *G. hanburyi*. Many modern pharmaceutical studies are focused on its extensive and potent cytotoxicity.[1—7] It has been developed as an anti-tumor drug for clinical usage by intravenous (IV) injection in China.

Polyprenylation to the xanthone skeleton made GA's structure very complicated, with five prenyl groups and six asymmetric centres. The structural determination was incomplete until the (*R*)-absolute configuration at C-2 in GA was confirmed recently by X-ray crystallographic analysis of its pyridine salt.[8—11] The complex stereochemistry also increased the difficulty of isolation and separation. Phytochemical investigations of these xanthone derivatives have been carried out ever since GA was discovered at the end of 19th century. Up to now, only less than twenty xanthones have been reported from *G. hanburyi*. To explore more active compounds, chemical synthesis methods were used recently to modify the carboxylic to acidamide group on GA.[12]

GA and its derivatives exhibited poor resolution in normal TLC and CC on silica gel, unless weak alkali such as triethylamine was added into the eluting solution. However, GA could be degraded in strong alkali such as NaOH to produce another new derivative, garcinolic acid.[13] In the reverse-phase high performance liquid chromatography (RP-HPLC) analyses, GA presented as a broad peak which could be well sharpened by the addition of some acid, *e.g.* 1% acetic acid. To find an effective chromatographic method for isolating more active xanthones from gamboges, the stability of GA in different chromatographic conditions was studied. Several extraction and isolation conditions such as adding acids, alkalis and organic solvents were compared using RP-HPLC method. As a result, GA was found to be stable in acetone, acetonitrile, chloroform and other acids, but unstable in methanol. A newly produced derivative was found in the methanol solution of GA that was stored for a week at room temperature. By adding alkalis, this rate of chemical transformation was increased. The derivative was identified as gambogoic acid (GOA) by analysis of its spectroscopic data including MS, 1D and 2D NMR spectra. In order to compare the anti-tumor activities of GA and GOA, further biological studies were conducted. This paper describes the results of the stability and cytotoxicity of GA and its derivative, gambogoic acid.



gambogic acid



	R
gambogoic acid	CH_3
gambogoic acid B	CH_2CH_3

MATERIALS AND METHODS

Materials and Reagents The gamboges resin (0.1 g) of *Garcinia hanburyi* was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP), P. R. China. A voucher specimen (CMS-0283) was deposited in the Herbarium of Hong Kong Jockey Club Institute of Chinese Medicine, Hong Kong, China. GA was isolated from the resin by the authors using preparative

* To whom correspondence should be addressed. e-mail: xuhongxi@hkjcicm.org

HPLC. Methanol, acetone, acetonitrile, acetic acid, formic acid, phosphoric acid, trifluoroacetic acid, triethylamine, and chloroform of HPLC grade were purchased from IL, U.S.A. Analytical grade ammonia solution was purchased from BDH, England. Distilled water was prepared using MILLI-Q SP reagent water system, and was distilled twice before use.

Analytical Conditions HPLC analysis was carried out on an Agilent 1100 series and an Alltima-C_{18} column (4.6× 250 mm, 5 μ) at room temperature. The mobile phase was acetonitrile/0.1% acetic acid (90 : 10) at a flow rate of 1.0 ml/min. The UV detection wavelength was set at 360 m.

Sample Preparation for Stability Tests 1.0 mg of gamboges was accurately weighed and dissolved in different extraction solvents (5.0 ml) as listed in Table 1. In tests 1—4, the sample solution was stored for a week before testing. In tests 5—11, the extraction was completed after refluxing for 1 h. The extract was filtered through a 0.2 μm Millipore filter unit (Advantec, Japan). 10.0 μl of this solution was injected into the HPLC system for analysis.

Tumor Cells and Culture Conditions[14] MCF7 and K562 cells were cultured in Dulbecco's minimal essential (DMEM) medium; Jurkat cells were cultured in RPMI 1640 medium; HL60 cells were cultured in Iscove's medium and MDA-MB-468 cells were cultured in L15 medium. The culture media was supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 50 μg/ml gentamycin. All cultures were maintained in a humidified 5% CO_2 incubator at 37 °C except for MDA-MB-468, which was maintained in an incubator with air intake only. Cells were sub-cultured every 3—4 d to maintain logarithmic growth and were allowed to grow for 24 h before use.

Cell Proliferation Assay[14] For testing, tumor cells were cultured in 96-well plates. Starting cell numbers were 5×10^4 cells per well for Jurkat, 2.5 (104 cells per well for K562, 104 cells for MCF7 and MDA-MB-468. For HL60 cells, the starting cell number was 10^5 cells per well in 24-well plates in order to coordinate with the cell differentiation assay. After the cells have stabilized overnight, triplicate (duplicate for HL60) samples of cells were treated with culture medium containing 0.1, 0.2, 0.5, 0.75, 1, 2.5 μg/ml of GA or GOA diluted from a 5 mg/ml in DMSO. Cell counting was set at 2 d for Jurkat and K562, 3 d for HL60, 4 d for MCF7, and 5 d for MDA-MB-468 after the addition of the test compound to allow at least two generations of cell proliferation. Cell numbers were determined by hemocytometer counting and viability was assessed by trypan blue dye exclusion test. Cell counts in samples treated with the test compounds were normalized to percent of control. The means and S.E.M.s were then calculated. Experiments were repeated 3 times for Jukat, K562, HL60 and MCF7 cell lines and 2 times for MDA-MB-468 cell line.

Table 1. Extract Solvents Tested for Stability of GA

Tests	Extract solvents
1.	Acetone
2.	Acetonitrile
3.	Chloroform
4.	Methanol
5.	Acetone/1% acetic acid 1 : 1
6.	Acetonitrile /1% acetic acid 1 : 1
7.	Acetone/1% formic acid 1 : 1
8.	Acetone/1% trifluoroacetic acid 1 :1
9.	Acetone/1% phosphoric acid 1 : 1
10.	Methanol/chloroform/triethylamine 1 : 8 : 1
11.	Methanol/chloroform/ammonia solution 1 : 8 : 0.5

RESULTS

Tests 1—4 were first performed. The extraction by acetone, acetonitrile, and chloroform presented the same chromatographic pattern in the parallel HPLC analyses (Fig. 1A). It was interesting that the methanol extraction showed one new peak in the chromatogram, which eluted close behind that of GA (Fig. 1B). The results of HPLC-ESIMS showed its molecular ion peak at *m/z* 660 which was exactly the same as that of gambogoic acid (GOA), a derivative from methanol addition onto GA. This new peak was further identified to be GOA by HPLC comparison with the known reference compound whose structure was determined by the spectral analysis of its 1D and 2D NMR data (Fig. 1E). Tests 5—11 were performed to evaluate the effects of acids and alkalis on this chemical reaction. Interestingly, in all the acidified extracts, no GOA peak was observed (Fig. 1A). However, in those basified, the GOA peak was present in significant amount (Fig. 1B). These results indicated that acids had no effect on the production of this new derivative, but alkalis could greatly increase the addition reaction.

Using five tumor cell lines including Jurkat, K562, MDA-MB-468, HL60, and MCF7 cell lines, a comparison was made between the newly produced derivative GOA and GA to see which has greater cytotoxicity. As shown in Table 2, both GA and GOA showed potent cytotoxicity against these tested tumor cell lines with IC_{50} ranged from 0.17—0.33 μg/

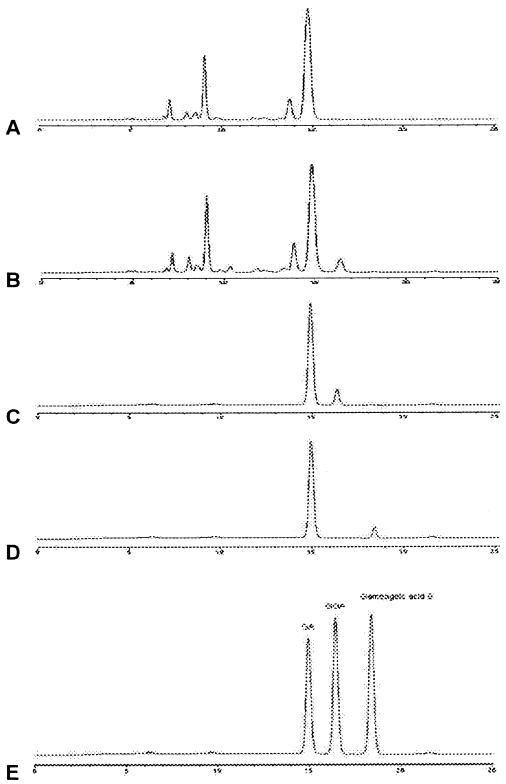


Fig. 1. Representative HPLC Chromatograms of Stability Tests

(A) Tests 1—3, and 5—9; (B) Tests 4, and 10; (C) GA+methanol; (D) GA+ethanol; (E) reference standards: GA, GOA, and gambogoic acid B.

Table 2. The Inhibitory Effects of GA and GOA against Jurkat, K562, HL60, MCF7, and MDA-MB-468 Cell Lines

	Jurkat	K562	HL60	MCF7	MDA-MB-468
GA	0.32±0.03	0.28±0.04	0.17±0.03	0.24±0.02	0.33±0.05
GOA	0.71±0.03	0.65±0.07	0.83±0.1	0.47±0.02	0.54±0.02

IC_{50}, mg/ml±S.E.M.

ml, and 0.47—0.83 μg/ml, respectively. However, it is important to note that GA consistently exhibited significantly stronger activities than its derivative on all five human tumor cell lines tested.

DISCUSSION

Storage of GA in methanol for a week at room temperature produced a new derivative GOA. The origin of this new derivative was proposed as shown in Fig. 2. The negative ion MeO^- existed in the methanol solution. It was easy for this negative ion to attack the β-olefinic carbon of the α,β-unsaturated carbonyl moiety at C-10 in the structure of GA. Therefore, this was a typical nucleophilic addition. This chemical reaction would happen at a low rate in pure methanol solution because of insufficient negative ion MeO^-. It required a few days for a small percentage of GA (about 7%) to be transformed into GOA in the methanol solution. The addition of alkalis induced faster production of MeO^-, which increased the nucleophilic addition reaction. This deduction well explains the fact that not only β-oriented methoxyl derivative like GOA, but also α-oriented isomorreollin and morreolic acid were isolated from gamboges.[4)]

According to this deduction, ethanol was considered to be another candidate to provide the nucleophilic ions. This reaction was repeated using a pure sample of GA instead of gamboges, together with methanol, and ethanol, respectively. As it was proposed, GA could transform into GOA in methanol solution (Fig. 1C), and could also generate another new derivative in ethanol solutions (Fig. 1D). The new derivative was similarly identified as gambogoic acid B, which was the addition product of ethanol. In view of our new findings, it is therefore suggested that no alcohol should be used in the processing of gamboges. If used, it is necessary to shorten the storage time of GA and gamboges in the alcohol solutions. To avoid this addition reaction, alkali should be strictly excluded from the alcohol solution.

The key structural difference between GA and GOA was the α,β-unsaturated carbonyl moiety at C-10. In the structure of GOA, the methoxy group at C-10 was assigned in α-orientation, as those of other reported analogues like isomorreolin B and morreolic acid.[4)] Different from these two analogues, however, H-9 was β-orientated. In the NOESY spectrum of GOA, the NOEs among H-9, H-10, and H-21 rather than that between H-9 and H-11α were displayed clearly. The preponderant stereochemistry at C-9 and C-10 could be deduced by the related surrounding structural groups. Because there is a big group near the β-orientation of C-10, the methoxy group tends to α-substitution without trammel. After the methoxy group was located as α-substitution, H-9 accordingly had to be β-orientated for the same reason.

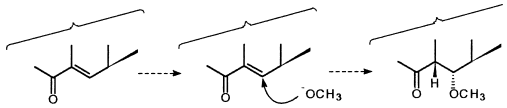


Fig. 2. Proposed Transformation from GA to GOA

The α,β-unsaturated carbonyl moiety was also the origin of the considerable differences of their antitumor activities. As deduced in the previous report,[3)] one cytotoxic mechanism consistent with the bioassay data was nucleophilic attack at position C-10, which resulted from the presence of the α,β-unsaturated carbonyl moiety. There are many examples to support the cytotoxic effects by the α,β-unsaturated carbonyl group. For instance, hundreds of cytotoxic *ent*-kaurane diterpenoids have been isolated from *Isodon* plants with most of them containing an α,β-unsaturated carbonyl group. Those containing two such moieties showed stronger cytotoxicity,[15)] while those without this moiety were inactive.[16)] Although GOA was weaker than GA, its bioactivities were still noticeable. Therefore, apart from the α,β-unsaturated carbonyl group, there must be other bioactive structural centres responsible for the cytotoxicity of GA and its derivatives. Further study on the structure–activity relationship is necessary to find more bioactive moieties in GA and its derivatives.

Acknowledgments This research is funded by the Hong Kong Jockey Club Charities Trust.

REFERENCES

1) Guo Q. L., Zhao L., You Q. D., Wu Z. Q., Gu H. Y., *Chin. J. Nat. Med.*, **2**, 106—110 (2004).
2) Liu W. Y., Feng F., You Q. D., Zhang Z. X., *Jiangsu J. Pharm. Clin. Res.*, **11**, 16—18 (2003).
3) Lin L. J., Lin L. Z., Pezzuto J. M., Cordell G. A., *Magn. Reson. Chem.*, **31**, 340—347 (1993).
4) Asano J., Chiba K., Tada M., Yoshi T., *Phytochemistry*, **41**, 815—820 (1996).
5) Guo Q. L., You Q. D., Wu Z. Q., Yuan S. T., Zhao L., *Acta Pharmacol. Sin.*, **25**, 769—774 (2004).
6) Zhao L., Guo Q. L., You Q. D., Wu Z. Q., *Biol. Pharm. Bull.*, **27**, 998—1003 (2004).
7) Wu Z. Q., Guo Q. L., You Q. D., Zhao L., Gu H. Y., *Biol. Pharm. Bull.*, **27**, 1769—1774 (2004).
8) Wu Z. Q., Guo Q. L., You Q. D., Zhao L., *Chin. J. Nat. Med.*, **1**, 99—102 (2003).
9) Ollis W. D., Ramsay M. V. J., Sutherland I. O., Mongkolsuk S., *Tetrahedron*, **21**, 1453—1470 (1965).
10) Cardillo G., Merlini L., *Tetrahedron Lett.*, **8**, 2529—2530 (1967).
11) Weakley T. J. R., Cai S. X., Zhang H. Z., Keana J. F. W., *J. Chem. Crystallogr.* **31**, 501—505 (2001).
12) Zhang H. Z., Kasibhatia S., Wang Y., Herich J., Guastella J., Tseng B., Drewe J., Cai S. X., *Bioorg. & Med. Chem.*, **12**, 309—317 (2004).
13) Liu W. Y., Feng F., Chen Y. S., You Q. D., Zhao S. X., *Chin. J. Nat. Med.*, **2**, 75—77 (2004).
14) Tai J., Cheung S., Chan E., Hasman D., *J. Ethnopharm.*, **93**, 9—19 (2004).
15) Sun H. D., Xu Y. L., Jiang B., "Diterpenoids from Isodon Species," Sciences Press, Beijing, 2001, p. 94.
16) Han Q. B., Li M. L., Li S. H. Mou Y. K., Lin Z. W., Sun H. D., *Chem. Pharm. Bull.*, **51**, 790—793 (2003).