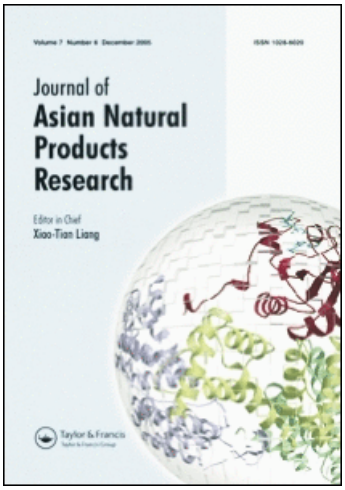


Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

Studies on the preparation, crystal structure and bioactivity of ginsenoside compound K

W. Zhou[a]; M. -Q. Feng[a]; J. -Y. Li[a]; P. Zhou[a]
[a] Department of Biosynthetic Drugs, School of Pharmacy, Fudan University, Shanghai, China

To cite this Article Zhou, W. , Feng, M. -Q. , Li, J. -Y. and Zhou, P.(2006) 'Studies on the preparation, crystal structure and bioactivity of ginsenoside compound K', Journal of Asian Natural Products Research, 8: 6, 519 — 527
To link to this Article: DOI: 10.1080/10286020500208600
URL: http://dx.doi.org/10.1080/10286020500208600

Journal of Asian Natural Products Research, Vol. 8, No. 6, September 2006, 519–527

Studies on the preparation, crystal structure and bioactivity of ginsenoside compound K

W. ZHOU, M.-Q. FENG, J.-Y. LI and P. ZHOU*

Department of Biosynthetic Drugs, School of Pharmacy, Fudan University, 138 YiXueYuan Road, Shanghai 200032, China

(Received 12 December 2004; in revised form 25 January 2005; in final form 14 March 2005)

Microbial transformation of *Panax notoginseng* saponins (PNS) using *Aspergillus niger* afforded, as the main metabolite, ginsenoside compound K (20-*O*-β-glucopyranosyl-20(*S*)-protopanaxadiol). Its structure was determined spectroscopically and by X-ray analysis, and this is the first time the crystal structure of ginsenoside has been reported. In comparison with ginsenoside Rb1, the pro-drug for this metabolite, compound K exhibits potent cytotoxic activity against tumor cell lines. The mean concentrations of compound K needed to inhibit the proliferation of cells by 50% (IC_{50}) were 12.7, 11.4, 8.5 and 9.7 μM for mouse high-metastatic melanoma (B16-BL6), human hepatoma (HepG2), human myeloid leukemia (K562) and human high-metastatic lung carcinoma (95-D) cell lines, respectively. The data show that ginsenoside compound K is a good antitumor drug candidate.

Keywords: Microbial transformation; Ginsenoside compound K; X-ray analysis; Cytotoxic activity

1. Introduction

Ginseng (the roots of *Panax ginseng* C. A. Mayer) is frequently used as an orally taken drug in the traditional medicine of Asian countries. The major active components of ginseng are ginsenosides, glycosides containing an aglycone (protopanaxadiol or protopanaxatriol) with a dammarane skeleton. Thus far, ginsenosides have been reported to be radioprotecive, immunomodulatory, antiviral and antioxidant, and to affect the nervous system and reproductive performance. Several studies using ginsenosides have also reported antitumor effects, particularly the inhibition of tumor-induced angiogenesis and the prevention of tumor invasion and metastasis [1].

While poor absorption of some major ginsenosides, such as Rb1 and Rg1, has also been observed, pharmacokinetic studies have demonstrated that orally administered ginsenosides pass through the stomach and small intestine, without decomposition by either gastric juice or liver enzymes, into the large intestine, where ginsenosdies are metabolized by colonic bacteria [2]. Recently, novel ginsenoside metabolites formed by the human

*Corresponding author. E-mail: pzhou@shmu.edu.cn

Journal of Asian Natural Products Research
ISSN 1028-6020 print/ISSN 1477-2213 online © 2006 Taylor & Francis
http://www.tandf.co.uk/journals
DOI: 10.1080/10286020500208600

intestinal bacteria have been found. Ginsenoside compound K (C–K), which does not occur naturally in ginseng, is the main metabolite of ginsenosides Rb1, Rb2 and Rc produced by intestinal bacteria (see figure 3) [3] and most likely the major form of protopanaxadiol saponins absorbed from the intestine [4]. The hypothesis that C–K may be the active metabolite responsible for the anticarcinogenic effect of ginseng saponins has prompted several groups to investigate the pharmacological effects of C–K. Wakabayashi *et al*. have reported that the antimetastatic effects of ginseng saponins are mediated by this metabolite [5]. Lee *et al*. have also found that C–K possesses chemopreventive and chemotherapeutic potential, as it showed antigenotoxic and anticlastogenic activity induced by benzo[α]pyrene [6].

Biotransformation of *Panax notoginseng* saponins (PNS) to prepare compound K was first attempted in the present work utilizing a fungal system instead of intestinal bacteria. In order to obtain C–K on a large scale at a low cost, we established a new and rapid technological process which was much better than the traditional method of ginsenoside isolation. The compound was characterized by mass and NMR spectroscopy. The relative configuration, conformation and crystal structure were elucidated by X-ray crystallography, and may help to clarify the mechanism of its bioactivity and explain the 3D-QSAR. This is the first report of the crystal structure of ginsenoside. Moreover, we examined the antitumor effect of compound K and compared it with that of Rb1 and cyclophosphamide in four cancer cell lines.

2. Results and discussion

Three hundred microorganisms collected from soil were screened to identify organisms capable of metabolizing PNS. TLC (figure 1) indicated that several organisms were capable of transforming the saponins into compound K. One strain, Z229, identified as *Aspergillus niger*, was selected for scale-up studies, as it showed the highest transformation ability. Furthermore, the strain was mutagenized by UV and the fermentation conditions were optimized using the orthogonal test, resulting in an enhancement of the rate of transformation to 35% according to HPLC analysis (figure 2).

In order to isolate and purify C-K efficiently on a large scale for manufacture and pharmacological research, we established a new method to produce C-K at low cost. First, considering that the polarity of C-K is much less than those of other ginsenosides, extraction was completed with EtOAc to remove most of the hydrosoluble saponins and pigments. Then the EtOAc extract was chromatographed on a silica gel column and eluted with $CHCl_3$–MeOH–EtOAc–H_2O = 2:1:4:1 (lower phase) to afford C-K (>90% purity). This mobile phase produced a very good separation result, and is much more convenient and more suitable for the large-scale preparation of C-K than the traditional method of gradient elution using a mixture of chloroform and methanol.

Recrystallization afforded colorless needles, suitable for X-ray analysis. Information from this, coupled with ^{1}H- and ^{13}C-NMR and mass spectral data, enabled us to establish the structure as compound K (figure 3).

Compound K crystallizes in the monoclinic space group $P2_1$. The packing adopted in space presents a symmetrical motif, with connections by hydrogen bonds. Each unit cell contains two symmetrical compound K molecules and four H_2O molecules (O17, O18, O19 and O20) connected to each other by hydrogen bonds [O_5–$H_{5(...}O_{14}$, O_6–$H_{6...}O_{18}$

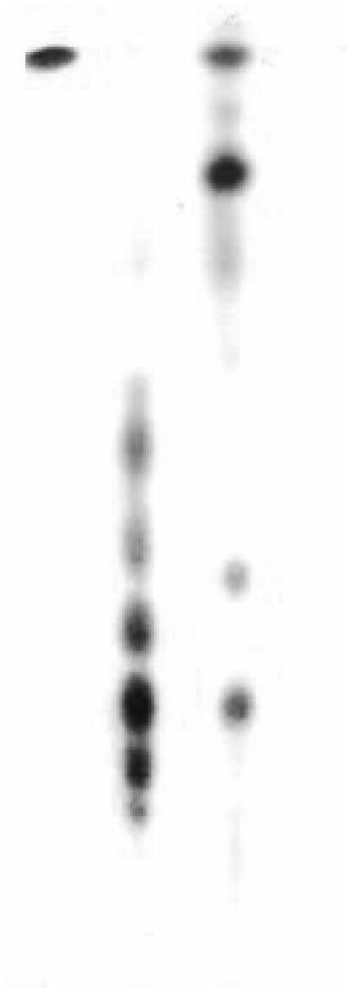


Figure 1. TLC analysis. TLC profiles of C-K (1), PNS (2) and biotransformed PNS (3); TLC plate, silica gel H60; developing solvent, $CHCl_3$–MeOH–H_2O (65:35:10, v/v, lower phase); detection reagent, H_2SO_4/C_2H_5OH (1:9, v/v), with heating at 110°C for 5 min.

$(-x + 1, y + 1/2, -z + 1)$, O_7–$H_{7\ldots}O_{10}$ $(x, y + 1, z)$, O_9–$H_{9\ldots}O_5$ $(-x + 1, y-1/2, -z + 2)$, O_{13}–$H_{13\ldots}O_{18}$ $(-x + 1, y + 1/2, -z + 1)$, O_{15}–$H_{15\ldots}O_{17}$ $(-x + 1, y + 1, -z + 1)$].

The crystal structure of compound K established by X-ray diffraction analysis is shown in figure 4. The structure consists of a glucopyranosyl group and a tetracyclic aglycone. The glucopyranosyl group E is a β-D-glucosyl group. The tetracyclic aglycone is a dammarane-type triterpenoid composed of one five-membered ring D and three six-membered rings A, B and C. The conformations of rings A, B and C are the chair form, but that of ring C is highly distorted, and they are connected through a *trans* ring-junction. The conformation of ring D is an envelope form and ring D is connected by a *trans* ring-junction to ring C. Furthermore, the results clearly establish a double bond between C_{24} and C_{25} with a bond length of 1.267(15) Å. The relative configuration of C_{20} is *S*. There are many research reports concerning the structures of ginsenosides, but no X-ray data for any ginsenoside has ever been reported. Our results clearly show the beautiful crystal structure of C-K, and its relative

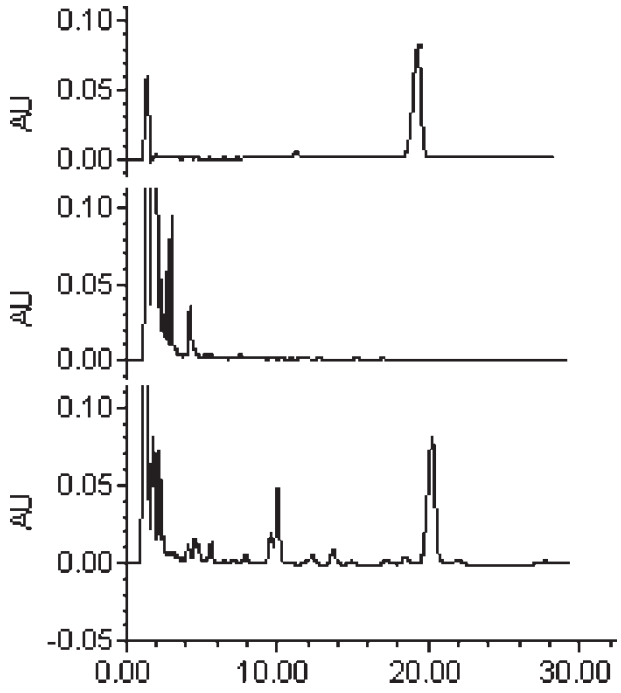


Figure 2. HPLC analysis. HPLC chromatograms of C-K (*a*), PNS (*b*) and biotransformed PNS (*c*). Peak 1 is C-K; column, C_{18} (Waters Symmetry, 4.6 × 150 mm, 5 μm); mobile phase, *V*(acetonitrile):*V*(water) = 48:52; flow rate, 1 ml/min; detection wavelength, 203 nm.

configuration and conformation have been elucidated by X-ray crystallography for the first time.

Ginseng has traditionally been used for the prevention and treatment of various diseases, including cancer. Among the diverse constituents of ginseng, ginsenosides are regarded as the principal pharmacoactive components. C-K is a novel ginseng saponin metabolite formed by intestinal bacteria after oral administration of ginseng extract to humans and rats [3]. However, the mechanism by which C-K exerts its cytotoxic activity on tumor cells is mostly unknown. In the present study, we investigated the anti-proliferative activity of C-K with

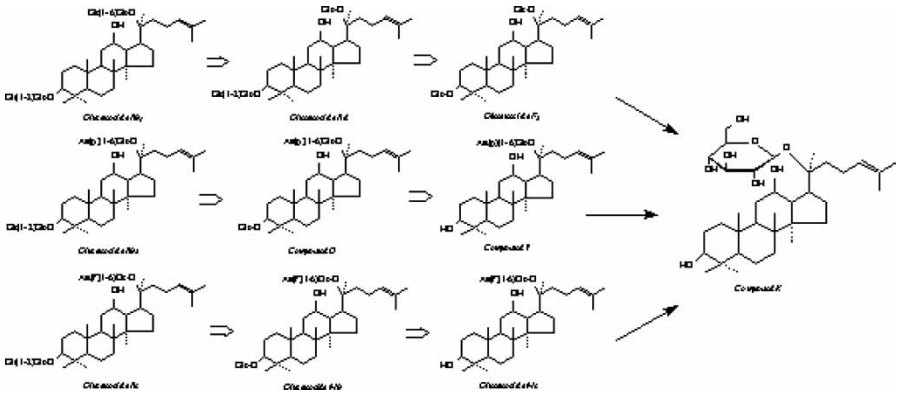


Figure 3. Suggested pathway for the formation of compound K.

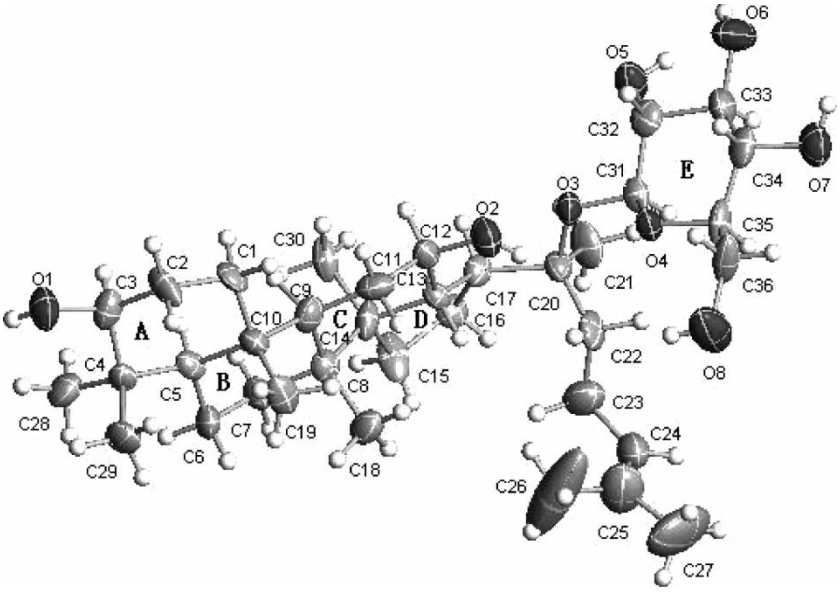


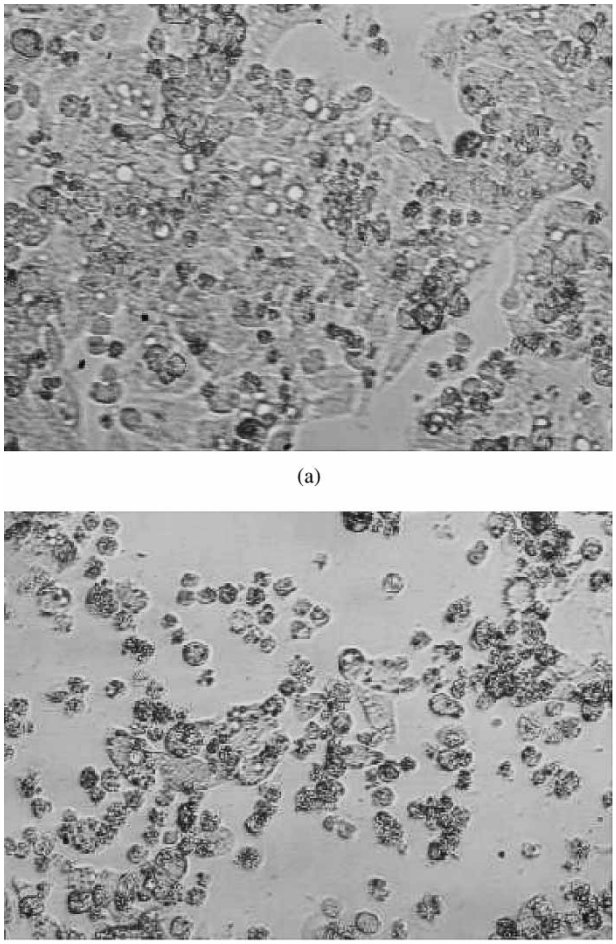
Figure 4. The crystal structure of compound K.

respect to four cancer cell lines—mouse high-metastatic melanoma (B16-BL6), human hepatoma (HepG2), human myeloid leukemia (K562) and human high-metastatic lung carcinoma (95-D) cell lines. We found that co-incubation of tumor cells with C-K at concentrations ranging from 5 to 80 μM resulted in a time- and concentration-dependent inhibition of tumor cell proliferation and that the IC_{50} values were nearly equal to that of cyclophosphamide, which is one of the most effective antitumor agents for the treatment of many types of cancer. The mean concentrations of compound K needed to inhibit the proliferation of the cells by 50% (IC_{50}) are shown in table1 compared with those of Rb1 and cyclophosphamide. When tumor cells were cultured for 24 h with C-K (10 μM), marked morphological changes were observed compared with the untreated control, and swelling of the cells was observed (figure 5). In contrast, incubation with ginseoside Rb1 (200 μM) did not affect the morphology of the tumor cells or their proliferation. Therefore, apoptosis might be the mode of cell death induced by C-K, which leads to antitumor and antimetastatic activity. All of these results suggest that ginsenosides are natural pro-drugs which can be transformed into active forms by intestinal bacteria, and it is not the intact ginsenosides, but their intestinal bacterial metabolites, that are indeed the primary active principles.

In conclusion, the present results demonstrate that ginsenoside compound K inhibits the proliferation of tumor cells in a time- and concentration-dependent manner, and, in addition,

Table 1. Cytotoxicity of C-K with respect to tumor cell lines.

Compound	*IC_{50} (μM)*			
	B16-BL6	*HepG2*	*K562*	*95-D*
Rb1	>200	>200	>200	>200
C-K	12.7 ± 1.3	11.4 ± 1.0	8.5 ± 0.9	9.7 ± 1.1
Cyclophosphamide	7.4 ± 0.7	3.2 ± 0.3	12.3 ± 0.9	7.8 ± 0.6



(a)

(b)

Figure 5. Apoptotic morphology of the cells untreated (*a*) or treated with 10 μM C-K (*b*) with × 100 original magnification.

induces apoptotic cell death. Taken together with the antimetastatic, antigenotoxic and apoptosis-inducing activities of C-K, this compound may have potential clinical use in the prevention of cancer on the basis of a C-K preparation using a fungal biotransformation system. The potential value of this compound will be further emphasized by the discovery of new activities and clarification of the mechanism of action from the crystal structure analysis.

3. Experimental

3.1 General experimental procedures

^{1}H- and ^{13}C-NMR spectra were obtained on a Mercury plus 400 spectrometer using TMS as internal standard. The mass spectrum was obtained on an Agilent LC-MS spectrometer. Optical rotation was measured with a Jasco P-1020 digital polarimeter. The X-ray analysis

was obtained using a Bruker Smart Apex-CCD. The optical density was measured at 550 nm on a Molecular Device ν max microplate reader.

3.2 Substrate material

Panax notoginseng saponins (PNS) were purchased from HongYun Biotechnology Co., YunNan, China. The main ginsenosides are Rb1 (30%), Rb2 (5%), Rb3 (40%), Rg1 (2%) and a small quantity of Rc, Rd and Re ($<1\%$).

3.3 Microorganisms and culture media

The 300 microorganisms used for screening were obtained from soil where ginseng grew. Fermentation experiments were carried out in a medium consisting of corn extracts, 25 g; glucose, 30 g; $(NH_4)_2SO_4$, 1 g per liter of distilled water.

3.4 Biotransformation of panax notoginseng saponins

Preliminary screening was performed in conical flasks (250 ml) containing 30 ml of medium. After 72 h cultivation on a rotary shaker (150 r.p.m.), the substrate was added to the medium from a stock solution of 30 mg/ml in water at a final concentration of 2 mg/ml. After 48 h incubation, the broth was centrifuged and the cells were extracted with EtOH for TLC and HPLC analysis to select the best microorganism. Substrate controls were composed of sterile medium to which the substrate was added and incubated without microorganisms. Culture controls consisted of fermentation blanks in which the strains were grown under identical conditions but without adding the substrate. Scale-up fermentations were performed under the same conditions with three 5 l flasks, containing 1 l of medium and 5 g of substrate each. HPLC analyses were performed using a Waters C_{18} column (4.6 $\times$ 150 mm, 5 μm) with acetonitrile–water (48:52) as mobile phase at a flow rate of 1.0 ml/min and detection was at 203 nm. The regression equation of C-K was determined to be $Y = 5459777X - 69010$ ($r = 0.9998$). According to the HPLC analysis, about 1.86 g of C-K was obtained after biotransformation, while the quantity of Rb1 and Rb2 in the substrate was $3 \times 5 \times 35\% = 5.25$ g, so the rate of transformation was about 35.4%.

3.5 Isolation and purification of ginsenoside compound K

The combined fermentation broth was centrifuged and the cells were extracted with EtOH for 48 h. After evaporation of the solvent, a suspension of the resulting EtOH extract in H_2O was extracted with EtOAc. The combined EtOAc layers were concentrated to dryness *in vacuo*, and the residue (3 g) was chromatographed on silica gel to afford compound K (300 mg) (eluted with $CHCl_3$–MeOH–EtOAc–H_2O = 2:1:4:1, lower phase). Further purification was then performed on recrystallization from acetonitrile–water.

3.6 Ginsenoside compound K

Fine colorless needles (CH_3CN–H_2O = 85:15); mp 162–164°C; [alpha; $]_D^{22}$ + 43.1 (*c* 0.248, MeOH); EI-MS, *m/z* 645 $[M + Na]^+$. Its ^1H (400 MHz, pyridine-d_5) and ^{13}C (125 MHz,

pyridine-d_5) data are virtually identical to those reported by Koizumi [7]. ^{1}H-NMR δ 5.22 (1H, t, $J = 7.1$ Hz, H-24), 5.17 (1H, d, $J = 7.8$ Hz, H-31), 3.93 (1H, ddd-like, H-12-OH), 3.4 (1H, dd, $J = 10.5$, 5.1 Hz, H-3-OH), 1.62 (3H, s, H-21), 1.57 (6H, s, H-26, 27), 1.22 (3H, s, H-28), 1.03 (3H, s, H-29), 0.97 (3H, s, H-30), 0.93 (3H, s, H-18), 0.87 (3H, s, H-19), 0.79 (1H, d, $J = 11.0$ Hz, H-5); ^{13}C-NMR δ 130.8 (C, C-25), 125.9 (CH, C-24), 98.4 (CH, C-31), 83.5 (C, C-20), 79.6 (CH, C-33), 78.6 (CH, C-35), 78.3 (CH, C-3), 75.4 (CH, C-32), 72.0 (CH, C-34), 70.5 (CH, C-12), 63.3 (CH_2, C-36), 56.8 (CH, C-5), 52.0 (CH, C-17), 51.9 (C, C-14), 50.8 (CH, C-9), 50.0 (CH, C-13), 40.6 (C, C-8), 40.1 (C, C-4), 39.9 (CH_2, C-1), 37.9 (C, C-10), 36.7 (CH_2, C-22), 35.7 (CH_2, C-7), 31.6 (CH_2, C-15), 31.4 (CH_2, C-11), 29.3 (CH_3, C-28), 28.9 (CH_2, C-2), 27.3 (CH_2, C-16), 26.4 (CH_3, C-26), 23.9 (CH_2, C-23), 23.0 (CH_3, C-21), 19.5 (CH_2, C-6), 18.5 (CH_3, C-27), 18.1 (CH_3, C-30), 17.1 (CH_3, C-18 or C-29), 16.7 (CH_3, C-19).

3.7 X-ray crystal structure analysis of compound K

A crystal (colorless needle) of compound K with dimensions $0.508 \times 0.106 \times 0.094$ mm was selected for data collection on a Bruker Smart Apex CCD diffractometer with graphite-monochromated Mo Kα radiation ($\lambda = 0.71073$ Å). A total of 20 757 reflections were collected in the range $1.99 \leq \theta \leq 25.99°$at 293(2) K, of which 13 762 were unique reflections with $I > 2\sigma(I)$ and were used in the subsequent refinements (R(int) = 0.1129). $C_{36}H_{64}O_8{\cdot}2H_2O$, monoclinic space group $P2_1$ with $a = 15.992(3)$ Å, $b = 11.960(19)$ Å, $c = 20.127(3)$ Å, $\alpha = 90°$, $\beta = 101.85°$, $\gamma = 90°$, $V = 3767.5(11)$ Å^3, $\rho_c = 1.162$ Mg/m^3 for $Z = 4$ and $fw = 658.89$, $F(000) = 1448$. All non-hydrogen atoms were refined anisotropically in a full-matrix least-squares refinement process with the SHELXL-97 [8] software package. The final standard residual R value for the fluoro-model was 0.0898 for the observed data (13 762 reflections) and 0.2348 for all data (20 757 reflections). The corresponding Sheldrick R values were $\omega R2 = 0.1749$ and 0.2435, respectively. A final difference Fourier map showed significant residual electron density, the largest difference peak and hole being 0.335 and -0.274 e/Å^3, respectively.

3.8 Antiproliferation effects of compound K in vitro

3.8.1. Chemicals. 3-(4,5-Dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich Chemical Company (MO, USA). Ginsenoside Rb1 and cyclophosphamide were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). C-K, ginsenoside Rb1 and cyclophosphamide were dissolved in DMSO to make a stock solution and diluted with culture medium to the desired concentration and used immediately.

3.8.2. Cell lines. Mouse high-metastatic melanoma B16-BL6, human myeloid leukemia K562, human hepatoma HepG2 and human high-metastatic lung carcinoma 95-D were obtained from the laboratory of Professor Liu (State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology).

3.8.3. Cell culture. Cell culture. The cells were cultured in DMEM medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 0.03% L-glutamine (Gibco) at 37°C in 5% CO_2.

3.8.4. MTT assays. The cell growth inhibitory effect of C-K and Rb1 was determined using the MTT assay [9]. Ginsenoside compound K and Rb1 were dissolved in DMSO. Cells undergoing exponential growth were suspended in fresh medium at a concentration of 3×10^4 cells/ml and inoculated in 96-well plates (Costar, USA) in a volume of 100 μl/well. Cells were stabilized by incubation for 24 h at 37°C and 100 μl aliquots of each drug were then added to each well. The plate was incubated at 37°C for 40 h. For the assay, 10 μl of MTT (5 mg/ml) was added to each well and the plate was incubated for 4 h at 37°C. The supernatant was aspirated, 150 μl DMSO was added and mixed thoroughly to dissolve the formazan crystals. The optical density was measured at 550 nm on a microplate reader. Each experiment was performed in triplicate. Antitumor activity was evaluated using IC_{50} determined by nonlinear regression analysis.

3.8.5. Observation of morphological changes. HepG2 cells in DMEM containing 10% FBS were seeded into 96-well plates (Costar, USA) and cultured for 24 h. C-K (10 μM) was added to the cell culture and the cellular morphology was observed using phase contrast microscopy (COIC, China) after 24 h.

Acknowledgements

This project was supported by the Shanghai SK Research and Development Foundation (No. 2003001-S). We sincerely thank Professor Jie Sun of the Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences (CAS) for assistance with the X-ray crystallography. We are grateful to Professor Jian-Wen Liu of the State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology for the cell cultivation and bioactivity tests.

References

[1] K. Sato, M. Mochizuki, I. Saiki, Y.C. Yoo, K. Samukawa, I. Azuma. *Biol. Pharm. Bull.*, **17**, 635 (1994).
[2] S.R. Ko, Y. Suzuki, K.J. Choi, Y.H. Kim. *Biosci. Biotechnol. Biochem.*, **64**, 2739 (1999).
[3] M. Karikura, T. Miyase, H. Tanizawa, T. Tanizawa, Y. Takino. *Chem. Pharm. Bull.*, **39**, 2357 (1991).
[4] H. Hasegawa, J.H. Sung, Y. Benno. *Planta Med.*, **63**, 436 (1997).
[5] C. Wakabayashi, H. Hasegawa, J. Murata, I. Saiki. *Oncol. Res.*, **9**, 411 (1997).
[6] B.H. Lee, S.J. Lee, J.H. Hur, S. Lee, J.H. Sung, J.D. Huh, C.K. Moon. *Planta Med.*, **64**, 500 (1998).
[7] H. Koizumi, S. Sanada, Y. Ida. *J. Shoji. Chem. Pharm. Bull.*, **30**, 2393 (1982).
[8] G.M. Sheldrick. *SHELXTL97: Program for Refinement of Crystal Structures*, University of Göttingen, Göttingen (1997).
[9] A. Monks, D. Scudiero, P. Skehan, R. Shoemaker, K. Paull, D. Vistica, C. Hose, J. Langley, P. Cronise, A.V. Wolff, M.G. Goodrich, H. Campbell, J. Mayo, M. Boyd. *J. Natl. Cancer*, **83**, 757 (1991).