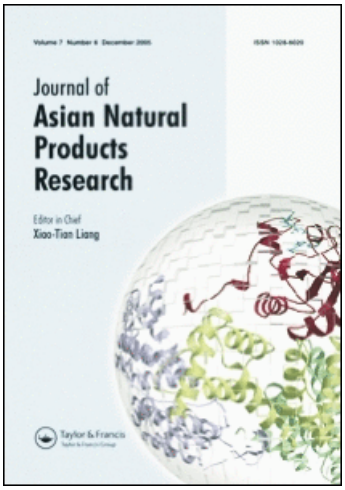

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

Hillasides A and B, two new cytotoxic triterpene glycosides from the sea cucumber *Holothuria hilla* Lesson

JUN Wu[a]; YANG-HUA Yi[b]; HAI-FENG Tang[c]; HOU-MING Wu[d]; ZHEN-RONG Zhou[e]
[a] Ningbo Biomedical Research Institution, Ningbo, China [b] College of Pharmacy, Research Center for Marine Drugs, Second Military Medical University, Shanghai, China [c] Department of Pharmacy, Xijing Hospital, Fourth Military Medical University, Xi'an, China [d] State Key Laboratory of Bioorganic and Natural Products Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Science, Shanghai, China [e] College of Lifesciences, Jiangxi Normal University, Nanchang, China

To cite this Article Wu, JUN , Yi, YANG-HUA , Tang, HAI-FENG , Wu, HOU-MING and Zhou, ZHEN-RONG(2007) 'Hillasides A and B, two new cytotoxic triterpene glycosides from the sea cucumber *Holothuria hilla* Lesson', Journal of Asian Natural Products Research, 9: 7, 609 — 615
To link to this Article: DOI: 10.1080/10286020600882676
URL: http://dx.doi.org/10.1080/10286020600882676

Journal of Asian Natural Products Research, Vol. 9, No. 7, October–November 2007, 609–615

Hillasides A and B, two new cytotoxic triterpene glycosides from the sea cucumber *Holothuria hilla* Lesson

JUN WU†‡¶, YANG-HUA YI†*, HAI-FENG TANG§, HOU-MING WU‖ and ZHEN-RONG ZHOU#

†College of Pharmacy, Research Center for Marine Drugs, Second Military Medical University, Shanghai 200433, China
‡Postdoctor Workstation of Zhejiang University, Hanzhou 310008, China
¶Ningbo Biomedical Research Institution, Ningbo 315201, China
§Department of Pharmacy, Xijing Hospital, Fourth Military Medical University, Xi'an 710001, China
‖State Key Laboratory of Bioorganic and Natural Products Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Science, Shanghai 200032, China
#College of Lifesciences, Jiangxi Normal University, Nanchang 330022, China

(Received 15 February 2006; revised 29 May 2006; in final form 1 June 2005)

Two new triterpene glycosides, hillasides A (**1**) and B (**2**), were isolated from the sea cucumber *H. hilla* Lesson, together with one known glycoside holothuria B (**3**). Their structures were deduced by extensive spectral analysis and chemical evidences. The presence of conjugated double bonds [22*E*,24-diene] in the aglycone of **1** is a rare structural feature among sea cucumber glycosides. The two glycosides showed significant cytotoxicity against eight human tumour cell lines (A-549, MCF-7, IA9, CAKI-1, PC-3, KB, KB-VIN and HCT-8) with IC_{50} in the range of 0.1–3.8 μg/ml.

Keywords: *Holothuria hilla*; Triterpene glycosides; Cytotoxicity; Hillaside A; Hillaside B

1. Introduction

Triterpene glycosides are the predominant secondary metabolites of holothurians and are responsible for their general toxicity. These glycosides have been reported to have a wide spectrum of biological effects, including antifungal, cytotoxic, hemolytic, and immunomodulatory activities [1]. More than 100 of these glycosides have been described, and the majority are usually lanosterol type triterpenes with an 18(20) lactone and a sugar chain linked to the C-3 of the aglycon [2]. There are about 500 species of sea cucumbers in China. *Holothuria hilla* Lesson (family Holothuriidae) is widely distributed in the South China Sea especially the offshore waters of Dongshan Island, Fujian Province, China [3]. As a part of our ongoing investigation on bioactive constituents from echinoderms [4–7], we studied the bioactive triterpene glycosides of this sea cucumber. In this paper, we report the isolation and

*Corresponding author. Email: wu919067@126.com

Journal of Asian Natural Products Research
ISSN 1028-6020 print/ISSN 1477-2213 online © 2007 Taylor & Francis
http://www.tandf.co.uk/journals
DOI: 10.1080/10286020600882676

structural elucidation of two new triterpene glycosides named hillasides A (**1**) and B (**2**), as well as their potential cytotoxicity against eight human tumour cell lines.

2. Results and discussion

Glycoside **1** was positive to Liebermann–Burchard and Molisch test. Its molecular formula was determined as $C_{35}H_{52}O_8$ from the pseudomolecular ion peaks at $m/z = 623.3558$ $[M + Na]^+$ in positive-ion mode HRESI-MS and at $m/z = 599$ $[M - H]^-$ in the negative-ion mode ESI-MS. The IR spectrum showed the presence of hydroxyl (3478 cm^{-1}), lactone carbonyl (1751 cm^{-1}) and olefinic (1637 cm^{-1}) groups. An examination of the ^1H NMR and ^{13}C NMR spectra of **1** suggested the presence of a triterpenoid aglycone with seven methyls, three olefinic bonds and one lactone carbonyl group, together with an monosaccharide chain. The assignments of the NMR signals associated with the aglycone moiety showed a close similarity to those reported for 16β-acetoxy-holosta-7,22*E*,24-triene-3β,17α-diol, the aglycone of intercedenside C (**4**), isolated from the sea cucumber *Mensamaria intercedens* [4] (figure 1). The position of double

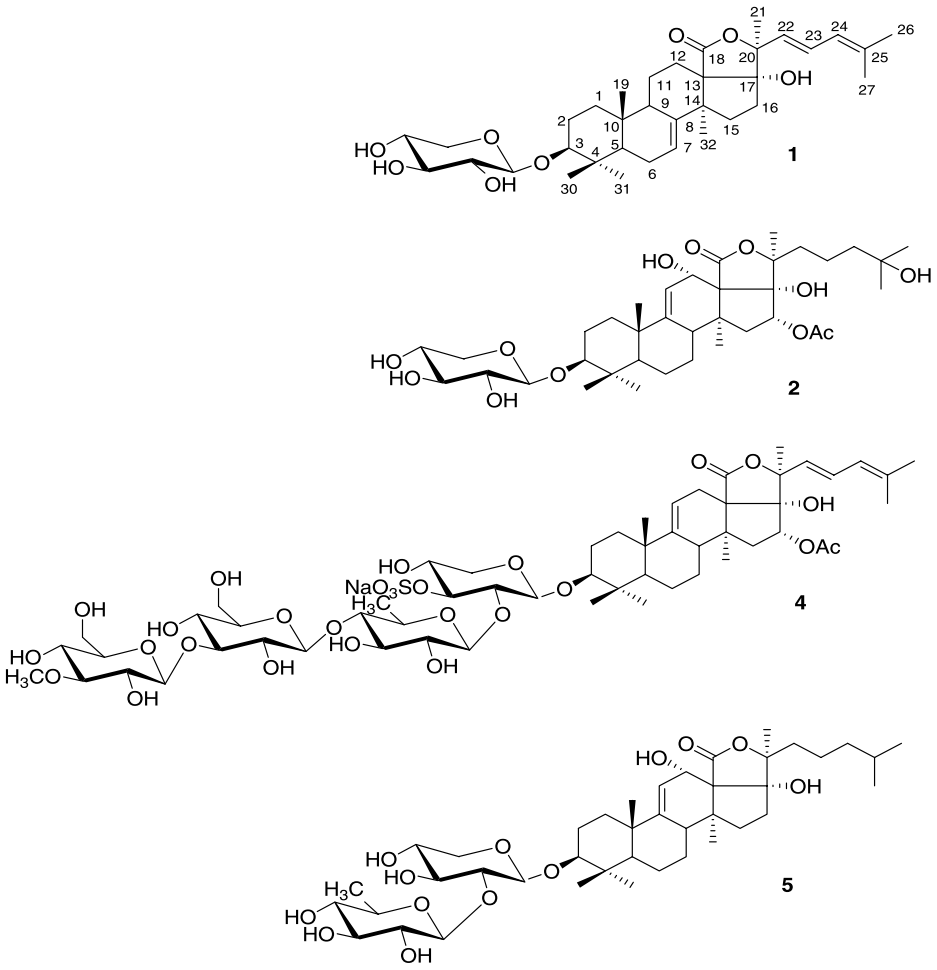

Figure 1. Structures of compounds **1**, **2** and **4**, **5**.

bond at $\Delta^{7(8)}$ was deduced from the NMR signals at $\delta = 145.8$ (C-8), 120.1 (C-7); $\delta = 5.57$ (1H, br s, H-7) together with the analysis of the TOCSY and HMBC experiments. In the TOCSY spectrum, three protons [$\delta = 5.35$ (H-11) and 2.42 (2H, H-12)] and four protons [$\delta = 1.08$ (H-5), 2.06 (2H, H-6) and 5.54 (H-7)] comprised a three-spin and a four-spin system, respectively, and the HMBC spectrum showed cross peaks between H-5/C-7, H-7/C-9, H-11/C-8, H-11/C-10, H-11/C-13, H-19/C-9 and H-32/C-8. The TOCSY spectrum of **1** indicated that three olefinic protons [$\delta = 6.24$ (d, $J = 12.8$ Hz, H-22), 5.60 (t, $J = 12.8$ Hz, H-23) and 6.27(d, $J = 12.8$ Hz, H-24)] comprised a three-spin system; correspondingly, a conjugated double bond (22*E*,24-diene) should be present in the aglycone side chain. The *E* stereochemistry of the Δ^{22} double bond was deduced from the large coupling constant for H-22 with H-23 (12.8 Hz). This conclusion was also confirmed by HMBC correlations between H-22 and C-17, C-20, C-21; H-23 and C-20, C-24, C-25; H-24 and C-25, C-26, C-27. Similarly to **4**, the quaternary carbon signal at $\delta = 89.8$ also indicated the presence of a hydroxyl group at C-17 and this was confirmed from the TOCSY, DQFCOSY, and HMBC spectra, too. The presence of a β configuration of anomeric proton for sugar unit in **1** was deduced from the ^{13}C NMR and ^{1}H NMR spectra, which showed an anomeric carbon resonance and an anomeric proton with coupling constant (*J* value) 7.6 Hz. This sugar moiety was confirmed to be D-xylose (Xyl) by acidic hydrolysis followed by GC-MS analysis of the corresponding aldononitrile peracetate and by comparing the GC retention time of the corresponding trimethylsilylated hydrolysate with those of the authentic samples prepared in the same manner [6]. The ^{1}H NMR and ^{13}C NMR signals attributable to the sugar unit were assigned by the 2D NMR experiments and the data indicated that D-xylose is in its pyranose form. In the HMBC spectra, a correlation between H-1 of xylose and C-3 of the aglycone indicated that Xyl was connected to C-3 of the aglycone. On the basis of the data discussed above, the structure of **1** was determined as 3-*O*-β-D-xylopyranosyl-holosta-7,22*E*,24-triene-3β,17α-diol and named hillaside A. The presence of two conjugated double bonds in the aglycone is a rare structural feature among sea cucumber glycosides.

The molecular formula of **2** was analysed for $C_{37}H_{58}O_{12}$ by ^{13}C NMR and HRESI-MS. The ^{13}C NMR data of the aglycon moiety was closely similar with those reported for holosta-9(11)-ene-3β,12α,17α-triol, the aglycon of bivittoside A (**5**) [10], from which **2** differed only by the replacement of a methylene ($\delta = 38.6$, C-16) by the signals of an acetoxy group ($\delta = 170.8$, 20.5) and a methine ($\delta = 73.4$) and downfield or upfield shifts of the signals due to neighbouring carbons (figure 1). The location of the acetoxy group at C-16 was deduced from the chemical shift of the H-16 signal (δ 6.24), which showed coupling to signals at $\delta = 1.88$ (H-15α), 1.44 (H-15β) in the TOCSY spectrum, and correlation with the carbonyl signal at $\delta = 170.8$ in the HMBC spectrum. The 16β configuration of the acetoxy group was confirmed by correlation between H-15α and H-16α ($\delta = 1.88$ and 6.26) in the NOESY spectrum and from the coupling constants for H-16 with H-15α (8.4 Hz) and H-15β (7.6 Hz) [4]. And an extra hydroxyl group linked to C-25 was confirmed by correlation between H-26 and C-25, H-27 and C-25, H-23 and C-25, H-24 and C-25 in the HMBC spectrum. Comparison of the NMR data of **2** with those of **1** suggested that **2** bore the same β-D-xylose residue linked to C-3 of aglycone as that of **1**. This was confirmed by the chemical evidence. Hence, **2** was defined as 16β-acetoxy-3-*O*-β-D-xylopyranosyl-holosta-9(11)-ene-3β,12α,17α,25β-tetraol and named hillaside B.

The *in vitro* cytotoxicity of **1** and **2** against human tumour cell lines A-549, MCF-7, IA9, CAKI-1, PC-3, KB, KB-VIN and HCT-8 was evaluated and the results (tables 1 and 2)

Table 1. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) data (δ value, J in Hz) for the glycosides **1** and **2** in C_5D_5N—D_2O (4:1)[†].

Position	***1***		***2***	
	δ_H	δ_C	δ_H	δ_C
1	1.37 m, 1.84 m	36.4	1.38 m, 1.86 m	36.5
2	1.89 m, 2.08 m	27.0	1.92 m, 2.10 m	27.2
3	3.12 dd (4.2, 15.6)	88.3	3.15 dd (4.0, 11.6,)	88.8
4		40.1		40.1
5	1.08 m	51.0	0.97 d (10.4)	52.8
6	2.06 m	23.5	1.69 m, 1.52 m	23.3
7	5.57 br s	120.1	1.46 m, 1.70 m	21.2
8	3.36	145.8	3.27 d (11.6)	41.0
9		47.3		153.9
10		39.7		39.8
11	5.35 m	22.1	5.54 d (4.8)	115.5
12	2.42 m, 2.86 dd (8.4, 13.6)	28.6	4.86 d (4.8)	71.7
13		57.6		58.9
14		48.9		45.9
15	1.82 m, 1.42 m	34.8	1.88 m, 1.44 m	36.8
16	2.38 dd (4.0, 9.2), 2.91 dd (7.2, 14.4)	35.6	6.24 dd (7.6, 8.4)	73.4
17		89.8		89.7
18		174.5		174.4
19	1.36 s	22.3	1.75 s	22.5
20		86.7		87.4
21	1.71 s	18.9	1.93 s	22.5
22	6.24 d (12.8)	129.5	4.22 m	80.7
23	5.60 t (12.8)	118.8	1.43 m	28.1
24	6.27 d (12.8)	124.2	1.25 m	38.4
25		136.4		81.3
26	1.19 s	28.7	0.77 s	28.6
27	1.17 s	27.5	0.76 s	27.4
30	1.05 s	16.7	1.02 s	16.7
31	1.24 s	28.0	1.22 s	28.0
32	1.64 s	21.0	1.58 s	20.3
CH_3**C**OO				170.8
C$\mathbf{H_3}$COO			2.00 s	20.5
Xyl				
1	4.67 d (7.6)	105.6	4.68 d (7.6)	105.5
2	4.04 m	78.0	3.96 d (8.8)	76.4
3	3.98 m	73.4	4.21 d (8.4)	73.7
4	4.00 m	69.1	4.00 m	70.7
5	3.65 m, 4.18 m	62.3	3.72 m, 4.32 m	66.8

[†] Assignments aided by DQFCOSY, TOCSY, HMQC, HMBC and NOESY experiments.

showed that both glycosides exhibited significant cytotoxicity against the eight cell lines with IC_{50} values in the range of 0.1–3.8 μg/ml. Based on these initially promising results, hillasides A and B merit further study as potential antitumour agents.

3. Experimental

3.1 General experimental procedures

Melting points were determined on a XT5-XMT apparatus and are uncorrected. Optical rotations were measured with a Perkin–Elmer MC-241 polarimeter. IR spectra were recorded on a Perkin–Elmer 683 infrared spectrometer. NMR spectra were recorded on a

Table 2. Cytotoxic activity of **1** and **2** against human tumour cell lines *in vitro* (IC_{50}, μg/ml)[†].

Cell line	***1***	***2***	*HCP*[‡]
A-549	2.55 ± 0.42	3.14 ± 0.57	0.84 ± 0.05
MCF-7	3.80 ± 0.63	2.68 ± 0.29	0.90 ± 0.04
IA9	0.10 ± 0.02	0.26 ± 0.07	0.02 ± 0.01
CAKI-1	1.79 ± 0.15	2.28 ± 0.20	0.75 ± 0.04
PC-3	3.05 ± 0.89	3.48 ± 1.03	1.46 ± 0.19
KB	3.62 ± 0.65	3.75 ± 0.39	1.79 ± 0.37
KB-VIN	3.67 ± 0.58	3.78 ± 0.42	3.80 ± 0.55
HCT-8	1.14 ± 0.22	2.84 ± 0.36	0.82 ± 0.09

[†] The data represent the mean ± SE of three independent experiments in which each compound concentration was tested in three replicate wells.
[‡] 10-hydroxycamptothecine (HCP) as positive control.

Varian Inova-600 spectrometer with TMS as internal standard using C_5D_5N—H_2O (4:1) as solvent. ESI-MS and HRESI-MS were acquired using a Micromass Quattro mass spectrometer. GC and GC-MS were performed on a Finnigan Voyager apparatus using a DB-5 column (0.25 mm × 30 m) for analysis of aldononitrile peracetates with an initial temperature of 150°C for 2 min and then temperature programming to 300°C at a rate of 15°C/min, or an L-Chirasil-Val column (0.32 mm × 25 m) for analysis of trimethylsilylated sugars with an initial temperature of 100°C for 1 min and then rising to 180°C at the rate of 5°C/min. HPLC was carried out on an Agilent 1100 liquid chromatograph equipped with a refractive index detector using a Zorbax 300 SB-C_{18} column (9.4 × 250 mm). Column chromatography was performed on silica gel H (10–40 μm, Qingdao Marine Chemical Inc.). Fractions were monitored by TLC on precoated Si gel $HSGF_{254}$ ($CHCl_3$/EtOAc/MeOH/H_2O, 4:4:2.5:0.5) or RP-C_{18} plates (MeOH/H_2O, 1:1) and spots were visualised by spraying with 10% H_2SO_4/EtOH solution, followed by heating.

3.2 Animal material

Specimens of *Holothuria hilla* were collected at a depth of 3–30 m by a fishery bottom trawler from offshore waters of Dongshan island in the South China Sea in May 2003 and identified by Professor Yu-Lin Liao (Qingdao Oceanic University, Qingdao, Sandong Province, China). A voucher specimen (No. HYSC-2003-06) is deposited in our laboratory.

3.3 Extraction and isolation

The sea cucumbers (7 kg, dried weight) were cut into pieces and extracted with refluxing ethanol. The extract was concentrated, the residue was suspended in H_2O and then partitioned successively with dichloromethane and *n*-BuOH. The *n*-BuOH extract (crude glycoside-containing mixture, 75.2 g) was chromatographed over silica gel (2000 g), eluted with $CHCl_3$/MeOH/H_2O (8:2:1 to 6.5:3.5:1, lower phase, 10,000 ml each) and divided into four major fractions (A–D) based on TLC analysis. Fraction D (3.9 g) mainly contained triterpene glycosides and was chromatographed on silica gel eluted with $CHCl_3$/MeOH/H_2O (7.6:2.4:1) to yield a mixture of glycosides (fraction D-a) and a fraction (D-b) containing mixture of more polar glycosides. Fraction D-a was purified by HPLC using MeOH/H_2O (45:55) as the mobile phase with a flow rate of 1.9 ml/min to give the pure glycosides **1**

(25 mg, $t_R = 23.4$ min) and **2** (18 mg, $t_R = 26.1$ min). Fraction D-b was purified by HPLC using MeOH/H_2O (50:50) with a flow rate of 1.9 ml/min to give the pure glycoside **3** (44 mg, $t_R = 27.7$ min).

3.3.1 Hillaside A (1). Colourless amorphous powder, mp 210–211°C, $[\alpha]_D^{20}$ −21.0 (*c* 0.5, MeOH). IR (KBr): $\nu_{max} = 3478, 1751, 1637\,cm^{-1}$. ^1H NMR and ^{13}C NMR, see table 1. ESI-MS (+) mode: *m/z* = 623 $[M + Na]^+$; (−) mode: *m/z* = 599 $[M - H]^-$; HRESI-MS (+) mode: *m/z* = 623.3558 $[M + Na]^+$ (calcd for $C_{35}H_{52}O_8Na$, 623.3560).

3.3.2 Hillaside B (2). Colourless amorphous powder, mp 222–223°C, $[\alpha]_D^{20}$ −27.5 (*c* 0.2, MeOH). IR (KBr): $\nu_{max} = 3419, 1739, 1632\,cm^{-1}$. ^1H NMR and ^{13}C NMR, see table 1. ESI-MS (+) mode: *m/z* = 717 $[M + Na]^+$, 1141 $[2M + Na]^+$, 585 $[M - Xyl + Na + H]^+$, 658 $[M - Oac + Na]^+$; (−) mode: *m/z* = 693 $[M - H]^-$; HRESI-MS (+) mode: *m/z* = 717.3823 $[M + Na]^+$ (calcd for $C_{37}H_{58}O_{12}Na$, 717.3826).

3.3.3 Holothuria B (3). Colourless amorphous powder, $[\alpha]_D^{20}$ −13.8 (*c* 0.5, MeOH). ^1H NMR and ^{13}C NMR data are identical with the literature values [8].

3.4 Acid hydrolysis of 1 and 2

Both glycosides (3 mg) were hydrolysed with 1 ml 2 mol/L trifluoroacetic acid at 120°C for 2 h, the corresponding aldononitrile peracetates and the trimethylsilylated hydrolysate were prepared, respectively, according to a procedure previously reported [6,9]. The aldononitrile peracetates were analysed by GC-MS with a DB-5 column using standard aldononitrile peracetates as reference samples. The trimethylsilylated hydrolysate was analysed by GC using an L-Chirasil-Val column. Peaks of the hydrolysates were detected at 10.74, 11.79 min (**1**) and 10.75, 11.79 min (**2**), respectively. Retention times for authentic samples after being treated simultaneously with 1-(trimethylsilyl)-imidazole in pyridine were 10.75 and 11.79 min (D-xylose), 10.78 and 11.86 min (L-xylose), 14.47 min (D-glucose), and 14.40 min (L-glucose), respectively.

3.5 Bioassays

The cytotoxicity against eight human tumour cell lines (A-549, MCF-7, IA9, CAKI-1, PC-3, KB, KB-VIN and HCT-8) were evaluated by sulforhodamine B (SRB) protein assay [9]. Dose-response curves were plotted for the samples and the IC_{50} values were calculated as the concentrations of the test saponins resulting in 50% reduction of absorption compared to the control cells. The data represented the mean ± SE of three independent experiments in which each compound concentration was tested in three replicate wells. The anticancer agent 10-hydroxycamptothecine (HCP) was used as reference compound.

Acknowledgements

This investigation was supported by a grant from Shanghai Foundation for New Drug Research awarded to Yang-HuaYi (02DZ19101) and a grant from the National Cancer Institution, awarded to K.H.L.(NIH, CA17625). We wish to thank Professor Yu-Lin Liao of Qingdao Oceanic University for taxonomic identification of the sea cucumber.

References

[1] H.D. Chludil, C.C. Muniain, A.M. Seldes, M.S. Maier. *J. Nat. Prod.*, **65**, 860 (2002).
[2] V.A. Stonik, G.B. Elyakov. *Bioorganic Marine Chemisty*, pp. 43–88, Springer, Berlin (1988).
[3] Y.L. Liao. *Chinese Fauna Echinodermata Holothuroidea*, p. 157, Science Press, Beijing (1997).
[4] Z.R. Zou, Y.H. Yi, H.M. Wu, J.H. Wu, C.C. Liaw, K.H. Lee. *J. Nat. Prod.*, **66**, 1055 (2003).
[5] Z.R. Zou, Y.H. Yi, H.M. Wu, X.S. Yao, L.J. Du, J.H. Wu, C.C. Liaw, K.H. Lee. *J. Nat. Prod.*, **68**, 540 (2005).
[6] H.F. Tang, Y.H. Yi, L. Li, P. Sun, S.Q. Zhang, Y.P. Zhao. *Planta Med.*, **71**, 458 (2005).
[7] H.F. Tang, Y.H. Yi, L. Li, P. Sun, S.Q. Zhang, Y.P. Zhao. *J. Nat. Prod.*, **68**, 337 (2005).
[8] J. Rodriguez, R. Castro, R. Riguera. *Tetrahedron*, **47**, 4753 (1991).
[9] P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica. *J. Natl. Cancer Inst.*, **82**, 1107 (1990).
[10] I. Kitagawa, M. Kobayashi, M. Hori, Y. Kyogoku. *Chem. Pharm. Bull.*, **37**(1), 61 (1989).