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Annonaceous Acetogenins from the Leaves of *Annona Montana*

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Abstract—A novel Annonaceous acetogenin, montanacin F, with a new type of terminal lactone unit, was isolated from the leaves of *Annona montana*. Its structure was determined on the basis of spectral evidences and chemical methods, and a possible biosynthetic pathway was discussed. In addition, the cytotoxicity of montanacin F was evaluated in vitro against Lewis lung carcinoma (LLC) tumor cell lines. Furthermore, the previously isolated cytotoxic acetogenin annonacin against LLC was examined for in vivo antitumor activity with LLC tumor cells.

Introduction

The Annonaceous acetogenins are promising new antitumor and pesticidal agents, which are found only in the plant family Annonaceae. Chemically, they are derivatives of long-chain fatty acids.[1] *Annona montana* (Annonaceae) is a medium-sized tree, mainly distributed in neo-tropic regions including Hainan Island of southern of China. It was reported that an infusion of the plant leaves growing in Trinidad is used for the treatment of influenza and insomnia.[2] Literature survey showed that about eighteen Annonaceous acetogenins have been isolated from fruits and leaves of the plant.[2–7] Our continuing investigation of the same plant led to isolation of an additional novel acetogenin, montanacin F (**1**), which possesses a new type of terminal lactone unit. Its structure was determined by spectral analysis including 1D and 2D NMR and Mosher ester methodology.

Most of acetogenins are reported to possess cytotoxic activity. Annonacin, which has been isolated from various plants,[8] was reported to possess cytotoxicity against VERO (ED_{50}: 1×10^{-4} μg/mL) and KB cells (ED_{50}: 1×10^{-2} μg/ml).[9–10] The in vivo antitumor activity of uvaricin, bullatacin, rolliniastatin-1, isorolliniastatin-1 and so on have also been reported.[11–13] However, the toxicity of some acetogenins was too great to prove antitumor activity.[12,14] In this paper, the in vitro cytotoxic activity against LLC of montancin F and in vivo antitumor activity with LLC tumor cells in mice are also presented.

Results and Discussion

Montanacin F (**1**) was obtained as a whitish waxy solid, which showed a quasi-molecular ion peak at m/z 617 $[M+Na]^+$ in FABMS spectrum. A molecular formula of $C_{35}H_{62}O_7$ was determined by HRFABMS, which gave a mass of m/z 617.4399 (calcd for $C_{35}H_{62}O_7Na$: 617.4389). The UV absorption at λ_{max}^{MeOH} 207 and 217 nm and IR absorption at ν_{max}^{KBr} 1760 cm^{-1} indicated the presence of an α, β-unsaturated γ-lactone (Fig. 1), which was further confirmed by ^1H NMR data (δ 7.38, H-3; 4.99, H-4) and ^{13}C NMR data (δ 173.4, C-1; 126.6, C-2; 152.2, C-3; 81.9, C-4) (Table 1). Up to 1998, over 350 Annonaceous acetogenins have been isolated from 37 species and four types of terminal lactone unit were reported.[1] Comparison of NMR data of the terminal lactone unit in **1** with those of known ketolactone revealed similarities structurally,[8] except a double bond lying between C-2 and C-3 in **1**. In HMBC spectrum, ^1H signal at δ 3.46 (2H, s, H-33), correlated with ^{13}C signals at δ 173.4 (C-1), 126.6 (C-2), 152.2 (C-3), 203.1 (C-34) and 30.3 (C-35), confirming the presence of a terminal α,β-unsaturated γ-lactone substituted by an acetonyl group at the α position as shown in Figure 2.

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The existence of three OH groups in **1** was evidenced by successive losses of H_2O from $[M-CH_3]^+$ in the EIMS and NMR resonances due to oxygen-bearing carbons at δ 71.6, 71.8, and 74.3, which correlated with proton signals at δ_H 3.81 (1H), 3.59 (1H), and 3.39 (1H), respectively. The mono-THF ring with usual flanking OH groups on each side was indicated by ^1H signals (Table 1) at δ 3.39 (H-15) and 3.81 (H-20) and ^{13}C signals (Table 1) at δ 74.3 (C-15) and 71.6 (C-20). The ring was placed between C-15 and C-20 based on the EIMS fragments at *m*/*z* 185, 309 and 315 (Fig. 3). A third hydroxyl group attached along with aliphatic chain could be identified by a multiplet at δ 3.59 in ^1H NMR and a signal at δ 71.8 in ^{13}C NMR spectra. The EIMS peaks at *m*/*z* 43, 73 and 225 suggested that this hydroxyl group should be located at C-29.

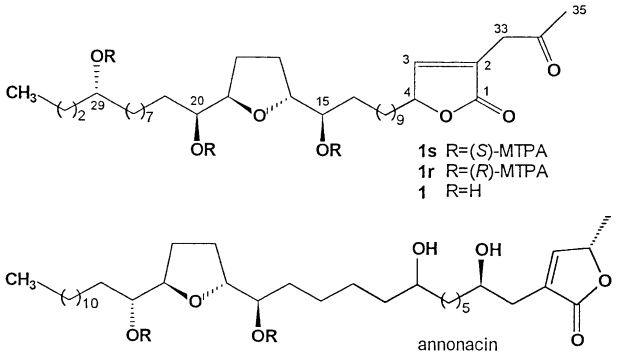

Figure 1. Structures of montanacin F (**1**) and (*S*)- and (*R*)-MTPA esters (**1s**, **1r**) and annonacin.

Table 1. ^1H (400 MHz) and ^{13}C NMR (100 MHz) spectral data of **1** and its (*S*)- and (*R*)-Mosher esters[a]

No.	^{13}C[b]	^1H[c]			Δδ
	1	**1**	**1s**	**1r**	$\delta_S-\delta_R$
1	173.4 s				
2	126.6 s				
3	152.2 d	7.38 d (1.4)			
4	81.9 d	4.99 m			
5–13	22.7–38.8	1.26–2.41 m			
14	33.2 t	1.41 m	1.55	1.50	+0.05
15	74.3 d	3.39 m	4.96	4.95	*R*
16	83.2 d	3.81 m	3.91	3.75	+0.16
17	28.6 t	1.57 m, 1.98 m	1.39, 1.81	1.49, 1.86	−(0.10, 0.05)
18	25.3 t	1.81 m, 1.90 m	1.62, 1.68	1.74, 1.83	−(0.12, 0.15)
19	82.2 d	3.88 m	3.94	3.98	−0.04
20	71.6 d	3.81 m	5.20	5.26	*S*
21	32.5 t	1.41 m	1.52	1.56	−0.04
22–28	22.7–38.8	1.26–2.41 m			
29	71.8 d	3.59 m	5.01	5.03	*S*
30–31	22.7–38.8	1.26–2.41 m			
32	14.1 q	0.88 t (6.8)	1.27	1.28	−0.01
33	38.8 d	3.46 s	0.81	0.88	−0.07
34	203.1 s	—			
35	30.3 q	2.26 s			

[a]Data recorded in $CDCl_3$.
[b]Multiplicities were determined by DEPT techniques.
[c]Coupling constants in Hz are given.

Relative stereochemistry of the THF ring and flanking hydroxyl groups were assigned either as *threo*/*trans*/*erythro* or *erythro*/*trans*/*threo* from C-15 to C-20 based on ^1H and ^{13}C NMR data, which were consistent with those of model compounds.[15–17] According to Fujimoto's models and the NMR data of squamolstatin A,[18] in the case of *threo*/*trans*/*erythro* from C-15 to C-20, the carbon signals of C-14 and C-21 will be at δ 33.2 and 32.5, otherwise the chemical shifts of C-14 and C-21 might be shifted. Based on a similarity of NMR data of **1** to the model compounds,[15–18] the relative stereochemistries from C-15 to C-20 were determined as *threo*/*trans*/*erythro*.

The (*S*)- and (*R*)- α-methoxy-α-trifluoromethyl-phenylacetic acid (MTPA) esters of 1 were prepared as **1s** and **1r**. Careful examination of ^1H NMR and ^1H–^1H COSY of these two derivatives (Table 1) was used to establish the absolute configuration of the carbinol centers. In *per*-MTPA ester of **1**, the two phenyl rings on the MTPA esters flanking the THF ring take the same orientation, causing H-16, 17, 18, and 19 to receive both shielding and deshielding effects simultaneously. However, it was anticipated that the chemical shift of H-14 was influenced by the MTPA group at C-15, while much less by that at C-20. With this assumption, the data was interpretable and the absolute stereochemistry at C-15 was assigned as *R* configuration, and thus the absolute stereochemistry at C-20 was consequently defined as *S*. The ^1H NMR chemical shift data of **1s** and **1r** demonstrated negative signs for $\Delta\delta_{1s-1r}$ of H-32 and H-33, indicating the *S* configuration at C-29. The absolute stereochemistry at C-4 remained undetermined due to the lack of a sufficient amount of the sample.

As mentioned above, the acetogenins discovered so far possess four types of terminal lactone units, which are γ-methyl γ-lactone, *cis* or *trans* ketolactone, γ-hydroxy-γ-methyl γ-lactone and β-hydroxy-γ-methyl γ-lactone.[1] The terminal lactone unit in **1** was determined as α-acetonyl-α, β-unsaturated γ-lactone as a new type in acetogenins. We speculated a possible biosynthetic

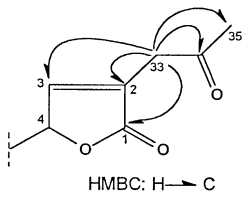

Figure 2. Main HMBC principles in α,β-unsaturated γ-lactone substituted by a propane group.

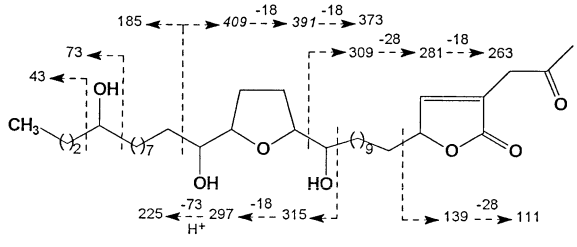

Figure 3. Diagnostic EIMS fragment ions (*m*/*z*) of montanacin F (1); loss of H_2O is indicated by (−18 amu), ions not observed are in italics.

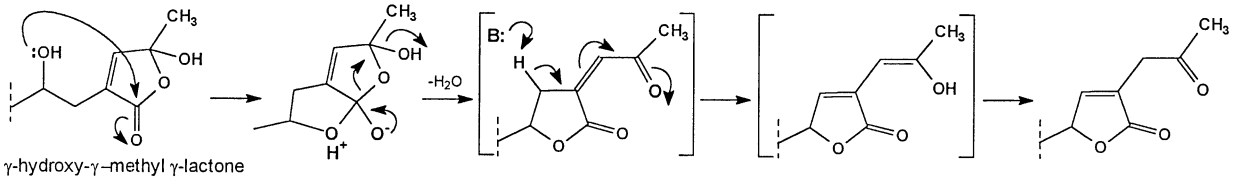

Figure 4. Possible biosynthetic pathway of the terminal lactone unit in **1**.

pathway as shown in Figure 4 to formation of the new type of terminal lactone unit in 1 from a related acetogenin with γ-hydroxy-γ-methyl γ-lactone.

The in vitro cytotoxicity against LLC tumor cell line of montanacin F was evaluated, which showed potent activity with ED_{50} value at 0.083 μg/mL (positive control: adriamycin, ED_{50}: 0.15 μg/mL). In the previous paper, we reported some cytotoxic acetogenins against LLC and Meth-A tumor cell lines, in which annonacin exhibited the most potent cytotoxicity (ED_{50}: 0.012 μg/ml) against LLC tumor cell lines. Thus, the in vivo antitumor activity of annonacin with LLC tumor cells in BDF-1 mice was examined. When the mice were treated for 2 weeks, annonacin showed an antitumor activity with a T/C value of 57.9% at a dose of 10 mg/kg (Table 2 and Fig. 5), comparable to that of positive control adriamycin (T/C: 54.6% at a dose of 2 mg/kg). The in vivo antitumor activity of uvaricin, bullatacin, rolliniastatin-1, isorolliniastatin-1 and so on have been reported.[11–13] However, if antitumor activity is defined as activity against a tumor in an animal model without undue toxicity towards the host, there are only a few published results relating to this criterion.[14] In the present animal experiment, none of the six mice in the group administered annonacin was dead, while three and five of the six mice in the control and standard groups were survived, respectively. This suggested that annonacin was less toxic in mice. On considering the antitumor activity and toxicity, annonacin might be used as a lead to develop a potential anticancer agent.

Experimental

Optical rotations were measured in MeOH solutions using a JASCO DIP-360 automatic polarimeter at 25 °C. UV spectra were taken on a Shimadzu UV-2200 UV–vis spectrophotometer (Kyoto, Japan) in MeOH solution. ^1H and ^{13}C spectra were taken on JNA-LAA 400 WB-FT (^1H, 400 MHz; ^{13}C, 100 MHz; JEOL) spectrophotometer with tetramethylsilane (TMS) as an internal standard. EI-MS and FAB-MS spectra were measured on a Finnigan MAT-95, and HR-FABMS were obtained with a VG Autospec-3000.

Plant material

Leaves of *A. montana* were collected in 1997 from Hainan Province, China and the species was identified by Prof. Renzhou Yang, South China Institute of Botany, Chinese Academy of Sciences. A voucher speciman (No. SIMM-AM-970702) was deposited in the Herbarium of Shanghai Institute of Materia Medica.

Tumor cells

Mouse lung carcinoma (LLC) was purchased from RIKEN Cell Line Bank (Tsukuba, Japan). The cells were maintained as monolayer cultures in RPMI 1640 medium supplemented with 7% fetal bovine serum, sodium bicarbonate (2 g), penicillin G (100,000 units) and streptomycin (100 mg).

Extraction and isolation

The air-dried leaves (3 kg) were powdered and percolated with 95% EtOH (10 L×3) for three days. The EtOH extract was evaporated in vacuo to give 200 g of a solid residue. The residue was suspended in H_2O and then shaken with CH_2Cl_2 to give H_2O and CH_2Cl_2 layers. The CH_2Cl_2 layer was evaporated to dryness and the residue (120 g) was suspended in 90% MeOH and shaken with hexane. The MeOH layer was evaporated to give 85 g of a solid residue, which was chromatographed on columns of silica gel, using gradients of CH_2Cl_2–MeOH (CH_2Cl_2, 49:1, 19:1, 9:1, each 4 L), to give fractions A, B, C and D. The pooled fraction B (12.3 g) was again

Table 2. Antitumor activity of annonacin against LLC implanted s.c. in mouse

Day	Control			Annonacin, 10 mg/kg			Adriamycin, 2 mg/kg		
	Tumor size[a]	W[b]	SM[c]	Tumor size	W	SM	Tumor size	W	SM
8	999±120	16.8	6/6	244±42	16.0	6/6	382±88	15.8	6/6
10	1593±238	17.5	6/6	637±105	16.6	6/6	815±128	16.1	6/6
12	2358±343	18.6	6/6	1024±91	17.8	6/6	1447±143	16.6	6/6
14	3693±412	19.0	5/6	1984±150	18.2	6/6	2233±213	16.5	6/6
16	5378±435	20.0	4/6	2947±286	19.7	6/6	2648±161	17.1	6/6
18	7053±661	20.5	3/6	4086±362	20.6	6/6	3849±339	18.1	5/6
				T/C: 57.9%			T/C: 54.6%		

[a]Standard±SEM.
[b]Weight of mouse.
[c]Survival mice.

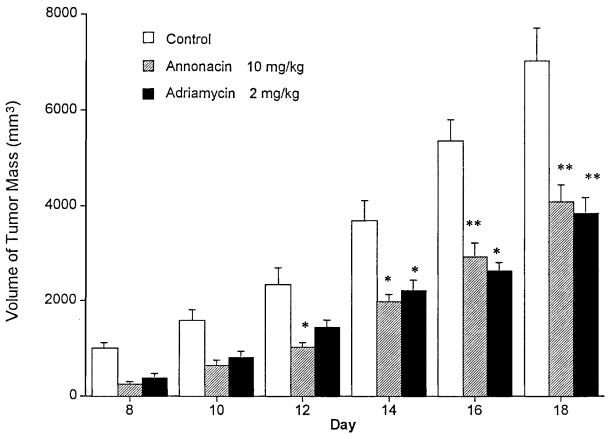

Figure 5. In vivo antitumor activity of Annonacin against LLC tumor cells. Statistical significance: $*P<0.05$, $**P<0.001$, vs control. Points, mean±SEM (Annonacin: 10 mg/kg, Adriamycin: 2 mg/kg).

subjected to repeated column chromatography (CH_2Cl_2 with increasing amounts of MeOH), and then further purified on columns of RP-18 (eluted with 85% aq MeOH) and Sephadex LH-20 (eluted with 95% aq MeOH) to give **1** (3.2 mg).

Preparation of *S*- and *R*- α-methoxy-α-trifluoromethylphenylacetic acid (MTPA) esters

(+)- or (−)-MTPA-Cl (20 μL) in dry pyridine–CCl_4 (0.4 and 0.3 mL, respectively) was sequentially added via syringe to a solution of acetogenin (1 mg) in CH_2Cl_2 (0.4 mL). The reaction mixture was then shaken and allowed to stand at room temperature until the reaction was complete as judged from no more formation of crystalline pyridine hydrochloride. Excess 3-dimethylamino-1-propylamine (18 μL) was added, and the mixture was diluted with ether after allowing to stand for 5 min. The ether layer was washed with cold 1 N HCl, cold saturated Na_2CO_3, and saturated NaCl, and then dried over $MgSO_4$. The filtered ether solution was evaporated in vacuo to give a residue, and the NMR spectrum was taken of this residue.

Physical and spectroscopic properties

Montanacin F (1). A white waxy, $[\alpha]_D^{25}$ +4.4 °(*c* 0.05, MeOH); UV (MeOH) λ_{max} 207 nm (log ε 3.87), 217 nm (log ε 3.77); IR ν_{max}^{KBr}3354 (OH), 2923, 2840, 1760 (C=O), 1710 (C=O), 1590, 1402, 1360, 1121, 1080, 820 cm^{-1}; FAB-MS: *m/z* 617 $[M+Na]^+$, HR-FABMS: *m/z* 617.4399 $[M+Na]^+$ for $C_{35}H_{64}O_7Na$ (calcd 617.4389); EI–MS: *m/z* 579 (5), 561 (13), 543 (18), 525 (8), 373 (7), 349 (15), 333 (50), 321 (15), 309 (5), 307 (11), 315 (13), 297 (12), 282 (100), 263 (16), 245 (19), 225 (30), 139 (6), 111 (13), 73 (5); ^1H, ^{13}C NMR, ^1H NMR of **1s** and **1r** (see Table 1).

Cytotoxicity assay

LLC cells were cultured with a RPMI 1640 medium containing 7% fetal bovine serum (FBS). For sulforhodamine B (SRB) assay,[19] the cells were cultured in a RPMI 1640 medium containing 7% FBS. A portion of the cell suspension (4–50,000 cells/ml) in the culture medium was inoculated to each well of 96-well microtiter plates. One day after plating, time zero control plate was made. Compounds were directly treated, and the cells were incubated for further 48 h in a humidified 5% CO_2 atmosphere at 37C. The cells were fixed with 50 μL of 50% trichloroacetic acid (TCA) solution for 1 h at 4 °C and the plates were washed five times with tap water and air-dried. A 50 μl of SRB solution (0.4% in 1% acetic acid) was added and staining was done at room temperature for 30 min. Residual dye was washed out with 1% acetic acid and air-dried. To each well, Tris buffer solution (10 mM, pH 10.5) was added. Optical density (OD) was measured with a microtiter plate reader at 540 nm. Growth inhibition was calculated that the OD of the treated well was subtracted with the OD at a time-zero (Tz) plate and divided by a calculated value of untreated control. 50% Growth inhibition of (ED_{50}) was calculated by Probit method.[20]

Animal

Specific pathogenic-free female BDF-1 mice, purchased from Japan SLC, Inc. (Shizuoka, Japan), used at 4 weeks of age, weighing 14–16 g. The animals were fed with a commercial pellet chow (Clea Japan Inc., Tokyo, Japan) in a temperature-controlled room at 25±2 °C and water ad libitum.

Tumor transplantation

LLC cells were maintained in cell culture. A suspension of 5×10^5 cells in 0.2 mL 0.9% NaCl solution was inoculated subcutaneously into the left flank of mice for the subcutaneous tumor assay.[21]

Drug

Annonacin was dissolved in 0.1 mL of 0.9% NaCl solution, and administered intraperitoneally once daily for consecutive 2 weeks to mice. Control animals were given 0.1 mL of a 0.9% NaCl solution by ip injection.

Estimation

Tumors were measured on each alternate day using a vernier caliper from the initiation of treatment to the time when gross ulceration of the tumor was developed in control mice. The tumor size was calculated as:

$$\text{tumor vol. } (mm^3) = 0.5 \times a \times b^2$$

where *a* is the longest diameter and *b* is the shortest diameter.[22] The effects by treatments were represented by:

$$T/C\ (\%) = \left(\begin{array}{c}\text{mean value of treated group/}\\ \text{mean value of control group}\end{array}\right) \times 100$$

Statistical analysis

The significance of differences between the experimental groups was calculated by Dunnett's *t*-test; $P < 0.05$ was considered significant.

Acknowledgements

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References and Notes

1. Alali, F. Q.; Liu, X. X.; McLaughlin, J. L. *J. Nat. Prod.* **1999**, *62*, 504.
2. Mootoo, B. S.; Ali, A.; Khan, A.; Reynolds, W. F.; McLean, S. *J. Nat. Prod.* **2000**, *63*, 807.
3. Jossang, A.; Dubaele, B.; Cav, A.; Bartoli, M. H.; Bériel, H. *Tetrahedron Lett.* **1990**, *13*, 1861.
4. Jossang, A.; Dubaele, B.; Cav, A.; Bartoli, M. H.; Bériel, H. *J. Nat. Prod.* **1991**, *54*, 967.
5. Wu, Y. C.; Chang, F. R.; Chen, K. S.; Liang, S. C.; Lee, M. R. *Heterocycles* **1994**, *38*, 1475.
6. Wang, L. Q.; Nakamura, N.; Meselhy, M. R.; Hattori, M.; Zhao, W. M.; Cheng, K. F.; Yang, R. Z.; Qin, G. W. *Chem. Pharm. Bull.* **2000**, *48*, 1109.
7. Wang, L. Q.; Li, Y.; Min, B. S.; Nakamura, N.; Qin, G. W.; Li, C. J.; Hattori, M. *Planta Med.* **2001**, *67*, 847.
8. Cavé, A., Figadère, B., Laurens, A. and Cortes, D. In *Progress in the Chemistry of Organic Natural Products*; Herz, W., Kirby, G. W., Moore, R. E., Steglich, W., Tamm, Ch. (Eds.); Elsevier: 1997; p 81
9. Myint, S. H.; Cortes, D.; Laurens, A.; Hocquemiller, R.; Leboeuf, M.; Cavé, A.; Cotte, J.; Québo, A. M. *Phytochemistry* **1989**, *30*, 3335.
10. Roblot, T.; Laugel, T.; Leboeuf, M.; Cavé, A.; Laprévote, O. *Phytochemistry* **1993**, *34*, 281.
11. Jolad, S. D.; Hoffmann, J. J.; Schram, K. H.; Cole, J. R.; Tempesta, M. S.; Kriek, G. R.; Bates, R. B. *J. Org. Chem.* **1982**, *47*, 3151.
12. Holschneider, C. H.; Johnson, M. T.; Knox, R. B.; Rezai, A.; Ryan, W. J.; Montz, F. *J. Caner Chemother. Pharmacol.* **1994**, *34*, 166.
13. Ahammadsahib, K. I.; Hollingworth, R. M.; McGovren, J. P.; Hui, Y. H.; McLaughlin, J. L. *Life Sci.* **1993**, *53*, 1113.
14. Suffness, M.; Newman, D. J.; Snader, K. Discovery and Development of Antineoplastic Agents from Natural Sources; In *Bioorganic Marine Chemistry*; Springer:Berlin/Heidelberg, 1989; Vol. 3.
15. Woo, M. H.; Zeng, L.; Ye, Q.; Gu, Z. M.; Zhao, G. X.; McLaughlin, J. L. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1135.
16. Liu, X. X.; Alali, F. Q.; Pilarinou, E.; McLaughlin, J. L. *Phytochemistry* **1999**, *50*, 815.
17. Liu, X. X.; Alali, F. Q.; Pilarinou, E.; McLaughlin, J. L. *J. Nat. Prod.* **1998**, *61*, 620.
18. Fujimoto, Y.; Murasaki, C.; Shimada, H.; Nishioka, S.; Kakinuma, K.; Singh, S.; Singh, M.; Gupta, Y. K.; Sahai, M. *Chem. Pharm. Bull.* **1994**, *42*, 1175.
19. Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1107.
20. Wu, L.; Smythe, A. M.; Stinson, S. F.; Mullendore, L. A.; Monks, A.; Scudiero, D. A.; Paull, K. D.; Koutsoukos, A. D.; Rubinstein, L. V.; Boyd, M. R.; Shoemaker, R. H. *Cancer Res.* **1992**, *52*, 3029.
21. Kato, T.; Sato, K.; Kakinuma, H.; Matsuda, Y. *Cancer Res.* **1994**, *54*, 5143.
22. Geran, R. I.; Greenberg, N. H.; MacDonald, M. M.; Schumacher, A. M.; Abbott, B. *J. Cancer Chemother. Rep* **1972**, *3*, 1.