JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

# Suppression of the SOS-Inducing Activity of Mutagenic Heterocyclic Amine, Trp-P-1, by Triterpenoid from *Uncaria sinensis* in the *Salmonella typhimurium* TA1535/pSK1002 *Umu* Test

MITSUO MIYAZAWA,* YOSHIHARU OKUNO, AND KOJI IMANISHI

Department of Applied Chemistry, Faculty of Science and Engineering, Kinki University Kowakae, Higashiosaka-shi, Osaka 577-8502, Japan

The methanol extract from *Uncaria sinensis* showed a suppressive effect on *umu* gene expression of the SOS response in *Salmonella typhimurium* TA1535/pSK1002 against the mutagen 3-amino-1,4-dimethyl-5H-pyrido[4,3*b*]indole (Trp-P-1), which requires liver metabolizing enzymes. The methanol extract from *U. sinensis* was re-extracted with hexane, $CH_2Cl_2$, BuOH, and water, respectively. $CH_2Cl_2$ extract showed a suppressive effect. A suppressive compound **1** in $CH_2Cl_2$ extract was isolated by $SiO_2$ column chromatography. Compound **1** was identified as ursolic acid by IR, electron ionization EI-MS, and NMR spectroscopy. Suppressive effects of ursolic acid (**1**) and its derivatives, methyl ursolate (**1M**), acetylursolic acid (**1A**), and methyl acetylursolate (**1MA**), were determined in the *umu* test. These compounds suppressed 61.3, 37.7, 71.5, and 37.8% of the Trp-P-1-induced SOS response at a concentration of 0.4 $\mu$mol/mL, respectively. The $ID_{50}$ values of compounds **1** and **1A** were 0.17 and 0.20 $\mu$mol/mL. In addition, these compounds were assayed with the activated Trp-P-1. Suppressive effects on activated Trp-P-1 were decreased as compared with those of Trp-P-1.

**KEYWORDS:** ***Uncaria sinensis*; Rubiaceae; ursolic acid; SOS response; *umu* test**

## INTRODUCTION

Several sort-term tests for screening environmental mutagens and carcinogens have been developed and are widely used in many laboratories (*1*, *2*). The Ames test is a convenient method for evaluating mutagenic activity (*1*), and several pieces of evidence have suggested that the mutagenic activity of a number of chemicals be correlated well with the carcinogenic activity so far reported (*3*, *4*).

The SOS response appears to be induced by an alteration in DNA synthesis, either directly by DNA damage blocking to the replication fork or indirectly by antibiotic, such as novobiocin, that inhibits DNA synthesis. The *umu* test system was developed to evaluate the genotoxic activity of a wide variety of environmental carcinogens and mutagens, using the expression of one of the SOS genes to detect DNA damaging agents (*5*, *6*). The results of this test are in agreement with the results of the Ames test and may be more useful with respect to simplicity, sensitivity, and rapidity (*7*).

*Uncaria sinensis* is a plant that belongs to the family Rubiaceae and is commonly known as Chotoko in Japanese. The plant is used in traditional Peruvian medicine for the treatment of cancer, arthrits, gastritis, cytostatic, contraceptive, antiinflammatory, and certain epidemic diseases, as an aqueous extract of the bark (*8*). The plants of the genus *Uncaria* (*sinensis*) are well-known for their rich content of alkaloids and tannins, triterpenoid saponins. A number of alkaloids displaying a pronounced enhancement of phagocytosis were isolated as well as quinovic acid glycosides with antiviral activity (*9*, *10*). Also, various biological activities have been described for triterpenoid saponins (*11*). A strong antitumor activity of triterpenoid saponins against Ehrich carcinoma ascite was reported (*12*) in our search for new naturally occurring suppressive compounds of SOS inducing activity in plants, which have a history of safe use as Chinese crude drugs (*13*−*15*). We found that the methanol extract of *U. sinensis* exhibited suppressive effects of the SOS inducing activity of Trp-P-1. In this paper, we report the identification of the suppressive compound in *U. sinensis* and its structure−activity relationship.

## MATERIALS AND METHODS

**General Procedure.** Gas chromatography (GC) was performed on a Hewlett Packard 5890 gas chromatograph equipped with a flame ionization detector (FID). GC-MS was performed on a Hewlett Packard 5972 Series mass spectrometer interfaced with a Hewlett Packard 5890 gas chromatograph fitted with a column (HP-5MS, 30 m × 0.25 mm i.d.). IR spectra were determined with a Perkin-Elmer 1760-x infrared Fourier transform spectrometer. NMR spectra ($\delta$, $J$ in hertz) were recorded on a JEOL GSX 270 NMR spectrometer. Tetramethylsilane (TMS) was used as the internal reference ($\delta$ 0.00) for $^1$H NMR spectra measured in $CDCl_3$.

* To whom correspondence should be addressed. Tel: +81-6-6721-2332. Fax: +81-6-6727-4301. E-mail: miyazawa@apch.kindai.ac.jp.

10.1021/jf035430y CCC: $30.25 
Published on Web 02/18/2005

**Materials.** The commercially available air-dried rhizome of *U. sinensis* was purchased from Yamada Yakken Co. (Osaka, Japan). The rhizomes for use as a crude drug were cultivated in China. Trp-P-1 was purchased from Wako Pure Chemical Co. (Osaka, Japan). S9 (supernatant of 9000 g) and coenzyme, NADPH, NADH, and G-6-P were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan).

***Umu* Test.** The *umu* test is based upon the abilities of carcinogens and mutagens to introduce expression of an *umu* gene in *Salmonella typhimurium* TA1535/pSK1002, in which a plasmid (pSK1002) carrying a fused gene (*umuC-lacZ*) had been introduced. The SOS response appears to be induced by an alteration in DNA synthesis, either directly by DNA damage blocking to the replication fork or indirectly by antibiotics, such as novobiocin, that inhibits DNA synthesis. The SOS regulatory system is controlled in part by the interplay of two proteins—the lexA protein, which represses asset of unlinked genes during normal cell growth, and the recA protein, which is required in vivo for inactivation of lexA protein after treatments that derepress the system by DNA damaging its metabolism (*16*, *17*). The SOS inducing potency is estimated by the measurement of the level of *umu* operon expression in terms of cellular $\beta$-galactosidase activity.

Briefly, an overnight culture of the tester bacterial strain was in TGA broth (1% bactotryptone, 0.5% NaCl, and 0.2% glucose; supplemented with 50 mg/L ampicillin) and incubated at 37 °C until the bacterial density at 600 nm reached 0.25−0.30. The culture was divided into 2.1 mL portions in test tubes. The test compound (50 $\mu$L, diluted in DMSO), S9 metabolizing enzyme instead of 0.1 M phosphate buffer mixture (300 $\mu$L, pH 7.4), and Trp-P-1 (50 $\mu$L, 40 $\mu$g/mL in DMSO) were added to each tube. After 2 h of incubation at 37 °C with shaking, the culture was centrifuged to collect cells, which were resuspended in 2.5 mL of PBS; the cell density was read at 600 nm with one portion (1.0 mL) of the suspension. Using the other portion (0.25 mL), the level of $\beta$-galactosidase activity in the cell was assayed by the method of Miller (*18*).

**Preparation of Activated Trp-P-1.** The preparation of activated Trp-P-1 was carried out according to the method of Arimoto et al. (*19*).

**Purification and Identification of the Suppressive Compound from *U. sinensis*.** The dry powder of *U. sinensis* was refluxed with methanol for 12 h to give a methanol extract (5 kg). This extract was suspended in water and partitioned between hexane, dichloromethane, butanol, and water successively. Each soluble extract was concentrated under reduced pressure to give hexane (12.8 g), dichloromethane (83.4 g), butanol (112.1 g), and water (122.7 g) extracts. To purify the compound responsible for suppression of SOS inducing activity, these extracts were subjected to the *umu* test. The $CH_2Cl_2$ extracts showed a suppressive effect, whereas the other extracts did not. The $CH_2Cl_2$ extract was repeatedly fractionated by $SiO_2$ column chromatography using the *umu* test as a guide, and suppressive compound **1** (196 mg) was isolated (**Figure 1**). Compound **1** was identified as ursolic acid by IR, GC-MS, and NMR spectroscopy.

**Methylation of Compound 1.** The methylated derivative of **1** (compound **1M**) was obtained by reaction with excess diazomethane in diethyl ether.

**Acetylation of Compounds 1 and 1M.** Pyridine (0.5 mL) was added to a solution of compound **1** (20 mg) in acetic anhydride (5 mL), and the solution was refluxed for 3 h. The product was treated with 5% HCl and 5% $NaHCO_3$ solution and extracted with $CH_2Cl_2$, and the solvent was evaporated. The residue was separated by silica gel column chromatography, and the acetate **1A** (19 mg) was obtained. Similarly, compound **1M** (20 mg) was acetylated to give the acetate **1MA** (21 mg).

**Suppressive Compound 1 and Derivatives.** *Compound **1***. Compound **1** was obtained as a white crystal; mp 238.4−240.2 °C. EI-MS (70 eV): *m*/*z* (rel int.) 456 ([M]$^+$, 6), 438 (3), 410 (6), 248 (100), 203 (25), 189 (10), 133 (20), 119 (11), 105 (9). IR $\gamma_{max}^{KBr}$ (cm$^{-1}$): 3432, 2926, 1695, 1457, 1387. The specific rotation, the $^1$H NMR, and the $^{13}$C NMR were measured with methyl ester. Compound **1** was identified as ursolic acid (3$\beta$-hydroxy-urs-12-en-28-oic acid) (*20*, *21*).

*Compound **1M***. Compound **1M** was a white crystal; mp 172.1−173.0 °C. EI-MS (70 eV): *m*/*z* (rel int.) 470 ([M]$^+$, 10), 452 (6), 410 (6), 262 (100), 203 (59), 133 (28), 119 (16), 105 (11). IR $\gamma_{max}^{KBr}$ (cm$^{-1}$): 3525, 2926, 1725, 1456, 1386; $[\alpha]^{23}$d +49.8° ($CHCl_3$; *c* 1.0). $^1$H NMR and the $^{13}$C NMR corresponded with those of methyl ursolate (*20*−*22*). Compound **1M** was identified as methyl ursolate (3$\beta$-hydroxyurs-12-en-28-oic acid methyl ester) from these spectra data and physical properties.

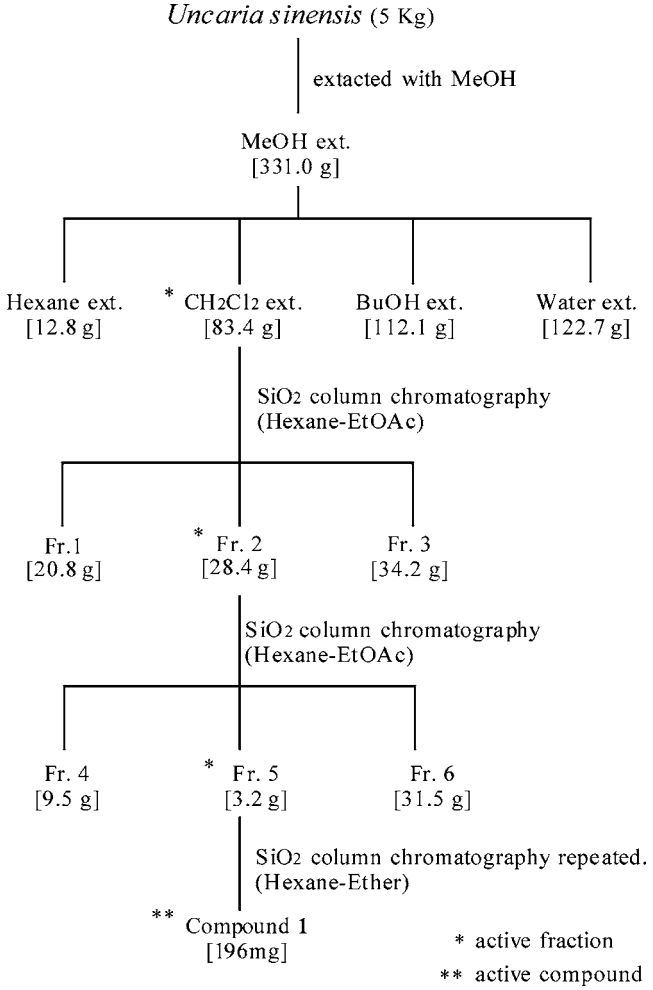



**Figure 1.** Isolation scheme of suppressive compound from *U. sinensis*.

*Compound **1A***. Compound **1A** was a white crystal; mp 242.7−244.1 °C. EI-MS (70 eV): *m*/*z* (rel int.) 498 ([M]$^+$, 10), 483 (1), 452 (4), 438 (5), 248 (100), 203 (31), 190 (21), 133 (22), 119 (11), 105 (8). IR $\gamma_{max}^{KBr}$ (cm$^{-1}$): 2927, 1736, 1697, 1455, 1371, 1244, 1027; $[\alpha]^{23}$d +71.2° ($CHCl_3$; *c* 1.0). The $^1$H NMR and the $^{13}$C NMR were identified with those of ursolic acid (*20*, *21*, *23*−*25*). Compound **1A** was identified as acetyl ursolic acid [3$\beta$-(acetyloxy)-urs-12-en-28-oic acid] from these spectra data and physical properties.

*Compound **1MA***. Compound **1MA** was a white crystal; mp 243.2−244.8 °C. EI-MS (70 eV): *m*/*z* (rel int.) 512 ([M]$^+$, 7), 497 (2), 452 (9), 437 (2), 262 (100), 249 (19), 203 (60), 189 (20), 133 (26), 119 (12), 107 (7). IR $\gamma_{max}^{KBr}$ (cm$^{-1}$): 2940, 2924, 1733, 1730, 1456, 1371, 1243, 1202, 1025; $[\alpha]^{23}$d +56.4° ($CHCl_3$; *c* 1.0). The $^1$H NMR and the $^{13}$C NMR were identified with those of ursolic acid (*20*−*22*, *25*, *26*). Compound **1MA** was identified as acetyl ursolic acid [3$\beta$-(acetyloxy)-urs-12-en-28-oic acid methyl ester] from these spectra data and physical properties.

## RESULT AND DISCUSSION

The methanol extract of *U. sinensis* was fractionated to search for suppressive compound using the *umu* test as a guide. To prepare the suppressive compound, fractionation of the methanol extract was carried out as described in **Figure 1**. Suppressive compound **1** (196 mg) was isolated and identified as ursolic by spectral comparison and physical constants with literature data.

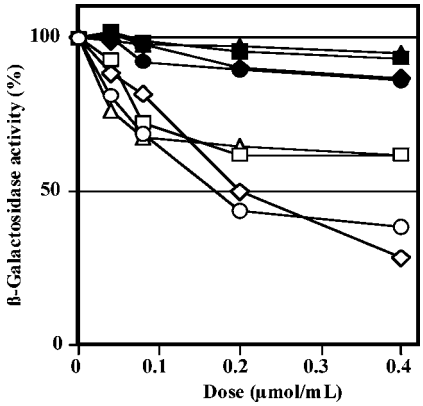
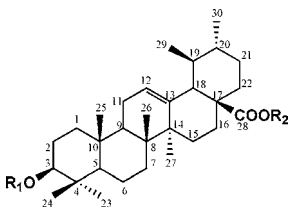



**Figure 2.** Suppressive effects of Trp-P-1- and Act. Trp-P-1-induced SOS response by compounds **1**, **1M**, **1A**, and **1MA**. Key: (○) effect of **1** on Trp-P-1; (□) effect of **1M** on Trp-P-1; (◇) effect of **1A** on Trp-P-1; (△) effect of **1MA** on Trp-P-1; (●) effect of **1** on Act.Trp-P-1; (■) effect of **1M** on Act.Trp-P-1; (◆) effect of **1A** on Act.Trp-P-1; (▲) effect of **1MA** on Act.Trp-P-1. Trp-P-1 (40 $\mu$g/mL in DMSO) was added at 50 $\mu$L. Act.Trp-P-1 (10 $\mu$g/mL in DMSO) was added at 100 $\mu$L.

The suppressive effects of compounds **1** and its esterified compounds, such as in **1M**, **1A**, and **1MA**, were determined in the *umu* test. As shown in **Figure 2**, these compounds exhibited inhibition of the SOS inducing activity of Trp-P-1. Compounds **1**, **1M**, **1A**, and **1MA**, respectively, suppressed 61.3, 37.7, 71.5, and 37.8% of the SOS inducing activity at a concentration of 0.4 $\mu$mol/mL. The $ID_{50}$ values of **1** and **1A** were 0.17 and 0.20 $\mu$mol/mL, respectively. In addition, the suppressive effects of these compounds on activated Trp-P-1 induced SOS response were determined. These compounds did not exhibit the inhibitory effects (**Figure 2**).

The suppressive compound of SOS inducing activity in *U. sinensis* was clearly identified as ursolic acid (**1**). This compound had a suppressive effect *umu* gene expression of the SOS response in *S. typhimurium* TA1535/pSK1002 against Trp-P-1, which requires liver metabolizing enzymes. As shown in **Figure 2**, compounds **1** and **1A** exhibited a greater suppressive effect on the SOS inducing activity of Trp-P-1 than compounds **1M** and **1MA.** The suppressive effect of **1A** is similar to that of **1**. These results were indicated that free carboxyl group at the C-17 position is an important factor for suppressive effect on *umu* genes expression of the SOS response in S. *typhimurium* TA1535/pSK1002 against Trp-P-1. The structure−activity relationship of ursolic acid and its esters has been investigated. Chuha et al. reported the trypanocidal activity of compounds **1**, **1M**, and **1A** (*27*). In that study, free carboxyl and a hydroxy




| | $R_1$ | $R_2$ |
|---|---|---|
| 1 | H | H |
| 1M | H | Me |
| 1A | Ac | H |
| 1MA | Ac | Me |

group were important factors for activity. Lee et al. have also reported that ursolic acid showed a significant cytotoxicity in the human tumor cell lines, but that of esters was decreased (*21*). Taking these results together, we consider that free carboxyl group is an important factor for suppressive effect. On the other hand, these compounds did not show the suppressive effect against activated Trp-P-1 in the *umu* test. It may be expected that the suppressive effect of Trp-P-1 be due to the inhibition of metabolic activation by S9. Previously, ursolic acid have been isolated from *Eriobotrya japonica* and *Ligustrum lucidum* as an antimutagenic compound (*28*, *29*). Ursolic acid exhibited the potent antimutagenic activity against AfB1 and benzo[*a*]pyrane using *S. typhimurium* TA100/TA98 Ames test. However, structure−activity relationships attend esterification for exhibition of suppression of SOS inducing activity, and the metabolic activation of S9 was not investigated. In summary, this research suggested that the suppressive compound in *U. sinensis* was primarily ursolic acid, and free carboxyl group is an important factor for activity.

## LITERATURE CITED

(1) Ames, B. N.; McCann, J.; Yamasaki, E. Method for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. *Mutat. Res.* **1975**, *31*, 347−363.

(2) Kada, T. Recent reserch on enviromental mutagens. *Nippon Nougeikagaku Kaishi* **1981**, *55*, 597−605.

(3) McCann, J.; Choi, E.; Yamasaki, E.; Ames, B. N. Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 5135−5139.

(4) Shugimura, T.; Sato, S.; Nagao, M.; Yahagi, T.; Matsushima, T.; Seino, Y.; Takeuchi, M.; Kawachi, T. Japan Scientific Societies Press: Tokyo, **1976**; pp 191−215.

(5) Oda, Y.; Nakamura, S.; Oki, I. Evaluation of the new system (*umu*-test) for the detection of enviromental mutagens and carcinogens. *Mutat. Res*. **1985**, *147*, 219−229.

(6) Nakamura, S.; Oda, Y.; Shimada, T. SOS-inducing activity of chemical carcinogens in *Salmonella typhimurium* TA1535/pSk1002: Examination with 151 chemicals. *Mutat. Res*. **1987**, *192*, 239−246.

(7) Reifferscheid, G.; Heil, J. Validation of the SOS/*umu* test using test results of 486 chemicals and comparison with the Ames test and carcinogenicity data. *Mutat. Res.* **1996**, *369*, 129−145.

(8) Keplinger, K. *Cytostatic, Contraceptive and Antiinflammatory Agents from Uncaria tomentosa. PTLC Int. Appl. WO 8201* **1982**, *130*, Austria.

(9) Cerri, R.; De Simone, F.; Pizza, C. New quinovic acid glycosides from *Uncaria tomentosa*. *J. Nat. Prod.* **1988**, *50*, 257−261.

(10) Aquino, R.; De Simone, F.; Pizza, C.; Coti, C.; Stein, M. L. Plant metabolites. Structure *in vitro* activity of quiovic acid glycosides from *Uncaria tomentosa* and Guettarda. *J. Nat. Prod.* **1989**, *52*, 679−685.

(11) Hiller, H.; Hostettmann, K.; Lea, K. *Biologically Active Natural Products*; Oxford Science Publications: 1987; Vol. 167.

(12) Nagamoto, N.; Noguchi, H.; Hokawa, A.; Nakata, K.; Namba, K.; Nishimura, M.; Matsui, M.; Mizuno, M. Antitumor constituents from bulbs of *Crocosmia crocosmiiflora*. *Planta Med.* **1988**, *43*, 305−307.

(13) Miyazawa, M.; Kinoshita, H.; Okuno, Y. Antimutagenic activity of sakuranetin from *Prunus jamasakura. J. Food Sci.* **2003**, *68*, 52−56.

(14) Miyazawa, M.; Hisama, M. Antimutagenic activity of flavonoids from *Chrysanthemum morifolium. Biosci., Biotechol., Biochem.* **2003**, *67*, 2091−2099.

(15) Miyazawa, M.; Hisama, M. Antimutagenic activity of phenylpropanoids from Clove(*Syzygium aromaticum*). *J. Agric. Food Chem.* **2003**, *51*, 6413−6422.

(16) Walker, C. G. Mutagenisis and inducible responses to deoxyribonucleic acid damage in *Escherichia coli*. *Microbiol. Rev.* **1984**, 60−93.

(17) Little, J. W.; Mount, D. W. The SOS regulatory system of *Escherichia coli*. *Cell* **1982**, *29*, 11−22.

(18) Miller, J. H. *Experiments in Molecular Genetics*; Cold Spring Harbor Labolatory: Cold Spring Harbor, NY, 1972; pp 352−355.
(19) Arimoto, S.; Ohara, Y.; Namba, T.; Negishi, T.; Hayatsu, H. Inhibition of the mutagenicity of amino acid pyrolysis products by hemin and other biolpgical pyrrole pigments. *Biochem. Biophys. Res. Commun.* **1980**, *92* (2), 662−668.
(20) Tkachev, A. V.; Denisov, A. Y.; Gatilov, Y. V.; Bagryanskaya, I. Y.; Shevtsov, S. A.; Rybalova, T. V. Stereochemistry of hydrogen peroxide-acetic acid oxidation of ursolic acid and related compounds. *Tetrahedron* **1994**, *50*, 11459−88.
(21) Lee, K. H.; Lin, Y. M.; Wu, T. S.; Zhang, D. C.; Yamagishi, T.; Hayashi, T.; Hall, I. H.; Chang, J. J.; Wu, R. Y.; Yang, T. H. Antitumor agents. LXXXVIII. The cytotoxic principles of *Prunella vulgaris*, *Psychotria serpens*, and *Hyptis capitata*: ursolic acid and related derivatives. *Planta Med.* **1988**, *54*, 308−11.
(22) Bhandari, S. P. S.; Garg, H. S.; Agrawal, P. K.; Bhakuni, D. S. Ursane triterpenoids from Nepeta eriostachia. *Phytochemistry* **1990**, *29*, 3956−3958.
(23) Tkachev, A. V.; Denisov, A. Y. Oxidative decarboxylation by hydrogen peroxide and a mercury(II) salt: A simple route to nor-derivatives of acetyloleanolic, acetylursolic and dehydroabietic acids. *Tetrahedron* **1994**, *50*, 2591−2598.
(24) Hota, R. J.; Bpuji, M. Triterpenoids from the resin of *Shorea robusta*. *Phytochemistry* **1993**, *32*, 466−468.
(25) Talapatra, S. K.; Sarkar, A. C.; Talapatra, B. Terpenoid and related compounds. Part XV111. Two pentacyclic triterpenes from *Rubia cordifolia*. *Phytochemistry* **1981**, *20*, 1923−1927.
(26) Santos, G. G.; Alves, J. C. N.; Rodilla, J. M. L.; Duarte, A. P.; Lithgow, A. M.; Urones, J. G. Terpenoids and other constituents of Eucalyptus globulus. *Phytochemistry* **1997**, *44*, 1309−1312.
(27) Cunha, W. R.; Martins, C.; Ferreira, D. S.; Crotti, A. E. M.; Lopes, N. P.; Albuquerque, S. *In vitro* trypanocidal activity of triterpenes from *Miconia* Spiecies. *Planta Med.* **2003**, *69*, 470−472.
(28) Young, H.-S.; Chung, H.-Y.; Lee, C.-K.; Park, K.-Y.; Yokozawa, T.; Oura, H. Ursolic acid inhibits aflatoxin B1-induced mutagenicity in a *Salmonella* assay system. *Biol. Pharm. Bull.* **1994**, *17*, 990−992.
(29) Niikawa, M.; Hayashi, H.; Sato, T.; Nagase, H.; Kito, H. Isolation of substances from glossy privet (*Ligustrum lucidum* Ait.) inhibiting the mutagenicity of benzo[*a*]pyrene in bacteria. *Mutat. Res.* **1993**, *319*, 1−9.

**Received for review December 5, 2003. Revised manuscript received January 11, 2005. Accepted January 13, 2005.**

JF035430Y