New Steroidal Glycosides from the Fruits of *Tribulus terrestris*

Erdal Bedir[†,‡] and Ikhlas A. Khan*[,†,§]

National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences, and Department of Pharmacognosy, School of Pharmacy, University of Mississippi, University, Mississippi 38677, and Department of Pharmacognosy, Faculty of Pharmacy, Hacettepe University, 06100-Ankara, Turkey

Received July 17, 2000

Three new steroidal saponins (**1**−**3**) were isolated from the fruits of *Tribulus terrestris*. Their structures were assigned by spectroscopic methods (IR, HRESIMS, 1D- and 2D-NMR) as 26-*O*-*β*-D-glucopyranosyl-(25*S*)-5*β*-furost-20(22)-en-3*β*,26-diol-3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-*β*-D-glucopyranoside (**1**), 26-*O*-*β*-D-glucopyranosyl-(25*S*)-5*β*-furost-20(22)-en-3*β*,26-diol-3-*O*-α-L-rhamnopyranosyl-(1→2)-[*β*-D-glucopyranosyl-(1→4)]-*β*-D-galactopyranoside (**2**), and 25(*S*)-5*β*-spirostan-3*β*-ol-3-*O*-α-L-rhamnopyranosyl-(1→2)-[*β*-D-glucopyranosyl-(1→4)]-*β*-D-galactopyranoside (**3**). Compound **3** showed cytotoxicity against a human malignant melanoma cell line (SK-MEL).

The genus *Tribulus* comprises approximately 20 species which grow as shrubs in subtropical areas around the world. The fruits of *T. terrestris* Linn. (Zygophyllaceae) are traditionally used primarily in decoctions or infusions in cases of spermatorrhea, phosphaturia, and disease of the genitourinary system, as well as kidney, liver, and eye diseases in Ayurvedic and Chinese traditional medicine.[1,2] Earlier investigations performed on *Tribulus* species resulted in the isolation of steroidal saponins, lignanamides, alkaloids, and flavonoids.[3−9] As part of our ongoing analyses and fingerprinting of crude and medicinal plants, and our search for active compounds from natural sources, we initiated a phytochemical study of a commercially available ethanolic extract of *T. terrestris*. Here we report the isolation and characterization of three new steroidal glycosides, tribulosaponin A (**1**) and B (**2**), and isoterrestrosin B (**3**). Isoterrestrosin B (**3**) showed mild cytotoxicity against a human malignant melanoma cell line (SK-MEL).

Three steroidal-type glycosides were isolated and purified by a combination of chromatographic methods from the fruits of *T. terrestris*. Compound **1** ($C_{51}H_{84}O_{21}$) gave a quasimolecular ion peak at *m*/*z* 1056.5483 for $[M + Na]^+$ in its HRESIMS. The NMR data of **1** were consistent with **1** being a steroidal glycoside.[10] The ^{1}H NMR spectrum of **1** displayed signals for one secondary methyl proton (*δ* 1.05) and three tertiary Me groups (*δ* 0.70, 1.08, and 1.64) in the aglycon moiety. Resonances of anomeric protons were observed in the low-field region at *δ* 4.83 (d, *J* = 7.3 Hz, H-1′), 4.86 (d, *J* = 7.3 Hz, H-1‴′), 5.88 (br s, H-1‴), and 6.55 (br s, H-1″) for **1**, indicative of the presence of a tetraglycosidic structure. Full assignments of the proton and carbon signals of the aglycon part of **1** were secured from its ^{1}H−^{1}H DQF-COSY, TOCSY, and HMQC spectra. The resonances assigned to the sapogenol moiety were in good agreement with anemarrhenasaponin IV, possessing furost-20(22)-ene-3,26-diol as the aglycon, which was glycosylated at C-3 and C-26.[11] The glycosylation shifts observed for these carbons (*δ* 76.3, d, C-3; 75.4, t, C-26) suggested that **1** was a bisdesmosidic saponin.

On acid hydrolysis, **1** yielded D-glucose and L-rhamnose. A combination of DQF-COSY, TOCSY, and HMQC experiments allowed unambiguous assignment of all sugar signals in **1** and identified the sugar moieties as two terminal α-L-rhamnopyranosyl units, one terminal *β*-D-glucopyranosyl unit, and one 2,4-disubstituted *β*-D-glucopyranosyl unit.[9] The sugar substituent at C-3 was identified from the following evidence: Key correlation peaks in the HMBC spectrum of **1** were observed between H-1′ (*δ* 4.83) of the glucose and C-3 (*δ* 76.3) of the aglycon, and between H-1″ (*δ* 6.55) of the rhamnose and C-2′ of the disubstituted glucose at C-3, and between H-1‴ (*δ* 5.88) of the second rhamnose and C-4′ (*δ* 77.1) of the same glucose moiety, revealing the presence of a trisaccharide unit at C-3. Long-range correlation between the anomeric proton of the second glucose moiety at *δ* 4.86 (H-1‴′) and C-26 (*δ* 75.4) confirmed the glycosylation at C-26.

The relative stereochemistry of **1** was resolved by a combination of 2D-NOESY data and comparison with analogous compounds. The ^{13}C NMR spectrum of **1** showed C-5 at *δ* 36.2, C-9 at *δ* 40.5, and C-19 at *δ* 24.0, characteristic of 5*β*-steroidal sapogenins.[10,12] Cross-peaks observed in the NOESY spectrum between H-5 and H_3-19 confirmed the proposed stereochemistry. The chemical shift and coupling constants of H-26a (*δ* 3.49, dd, *J* = 7.1 and 9.1 Hz) were comparable to those of analagous compounds with 25(*S*) configuration.[7] Consequently, the structure of **1** was elucidated as 26-*O*-*β*-D-glucopyranosyl-(25*S*)-5*β*-furost-20-(22)-en-3*β*,26-diol-3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-*β*-D-glucopyranoside, and it was named tribulosaponin A.

HRESIFTMS of **2** showed an ion peak for $[M + Na]^+$ at *m*/*z* 1072.5305, in agreement with the molecular formula $C_{51}H_{84}O_{22}$. Detailed examination of 1D- and 2D-NMR spectra of **2** and comparison with those of **1** showed their considerable structural similarity. The differences consisted only in the signals of the sugar chain which was attached at C-3 of the sapogenol moiety. Acid hydrolysis followed by TLC analysis showed the presence of D-glucose, D-galactose, and L-rhamnose. The assignment of the sugar moieties was resolved by a combination of DQF-COSY, TOCSY, and HMQC data, which indicated the presence of two terminal *β*-D-glucopyranosyl units, one terminal α-L-rhamnopyranosyl unit, and a 2,4-disubstituted-*β*-D-galactopyranoside unit. Direct evidence for the sugar sequence and the linkage sites was derived by an HMBC experiment. On the basis of these results, the structure of tribulosaponin B (**2**) was elucidated as 26-*O*-*β*-D-glucopyranosyl-(25*S*)-

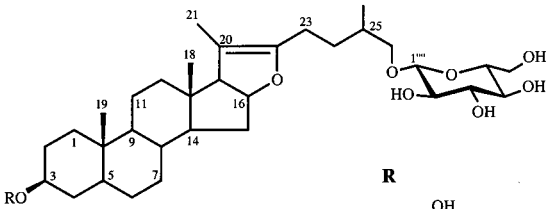
* To whom correspondence should be addressed. Tel./Fax: (662) 915-7821. E-mail: rikhan@olemiss.edu.
† National Center for Natural Products Research.
‡ Department of Pharmacognosy, Hacettepe University.
§ Department of Pharmacognosy, University of Mississippi.

10.1021/np000353b CCC: $19.00
Published on Web 10/26/2000

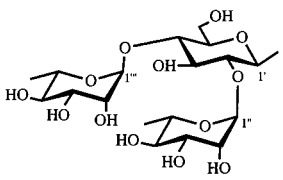
5β-furost-20(22)-en-3β,26-diol-3-*O*-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-β-D-galactopyranoside.

Compound **3** ($C_{45}H_{74}O_{17}$) showed a peak at *m/z* 909.4930, $[M + Na]^+$, in the HRESIFTMS. The ^1H and ^{13}C NMR spectra of **3** showed resonances typical of a 25(*S*)-5β-spirostane-type sapogenol glycosylated at C-3.[10,12] The spectrum clearly showed three anomeric proton signals (δ 4.76, 5.32, and 5.53) in the downfield region, indicative of a triglycosidic structure. On comparison of the ^1H and ^{13}C NMR spectra of **3** with those of **2**, the signals due to the sugar moieties at C-3 were superimposable, indicating that they have the same trisaccharide chain at C-3. 2D-NMR data of **3** also supported the proposed structure. On the basis of the above data, the structure of **3** was established as 25(*S*)-5β-spirostan-3β-ol-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside. Since the 5α isomer of **3**, terrestrosin B, was earlier reported from *T. terrestris*,[4] we named compound **3** isoterrestrosin B.

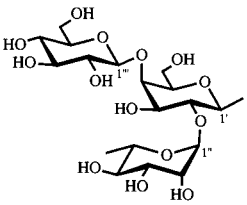
Cytotoxicity of compounds **1**−**3** was evaluated against SK-MEL, KB, BT-549, and SK-OV-3 cancer cell lines and VERO normal cell line. Only compound **3** exhibited cytotoxicity against SK-MEL, with an IC_{50} value of 6.7 μg/mL. None of the compounds were active in antimicrobial and antiprotozoal assays.

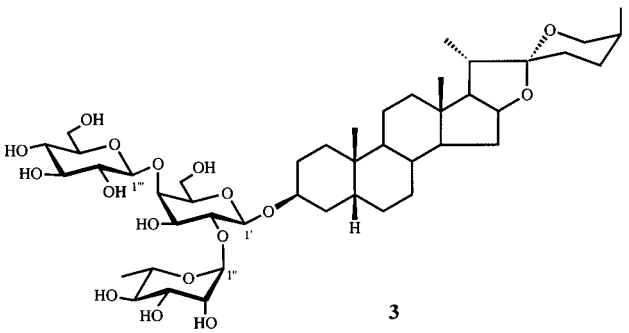


1



2





3

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Jasco DIP-370 polarimeter using a sodium lamp operating at 589 nm. IR spectra were recorded with an ATI Mattson Genesis Series FTIR spectrophotometer. 1D- and 2D-NMR spectra were obtained on a Bruker Avance DRX 500 FT spectrometer operating at 500 and 125 MHz, respectively. The chemical shift values are reported as parts per million (ppm) units relative to tetramethylsilane (TMS) for ^1H and ^{13}C, and coupling constants are in hertz (in parentheses). For ^{13}C NMR spectra, multiplicities were determined by a DEPT experiment. HRESIFTMS were obtained on a Bruker BioApex FT-MS in ESI mode.

Chromatographic Conditions. TLC: precoated Si 250F plates (Baker); developing system: $CHCl_3$−MeOH−H_2O (61:32:7 or 70:30:3); visualization: 50% H_2SO_4; column chromatography: silica gel 230−400 mesh (Merck).

Plant Material. An ethanolic extract of *T. terrestris* fruits was provided by USA NutraSource, Inc., 1300 Industrial Rd., Unit 16, San Carlos, CA 94070, lot number UN-TT-991024. This sample was obtained from China, and a voucher specimen is deposited at USA NutraSource, Inc.

Extraction and Isolation. The EtOH extract (10 g) was dissolved in H_2O and subjected to vacuum-liquid chromatography using reversed-phase material (C_{18}), employing H_2O, H_2O−MeOH (90:10 → 10:90), and MeOH as the eluents to give 10 fractions (A−J). Fraction I (225 mg) was subjected to column chromatography (silica gel, 50 g), eluted with $CHCl_3$−MeOH (85:15) and $CHCl_3$−MeOH−H_2O mixtures (80:20:1; 80:20:2), to give compound **3** (117 mg). Fraction H (400 mg) was chromatographed on a silica gel column (60 g), eluted with $CHCl_3$−MeOH (85:15) and $CHCl_3$−MeOH−H_2O mixtures (80:20:1; 80:20:2, and 70:30:3), yielding compounds **1** (19 mg) and **2** (145 mg).

Tribulosaponin A (**1**): white powder, $[\alpha]^{25}_D$ −73° (*c* 0.004, MeOH); IR (KBr) ν_{max} 3404, 2948, 2395, 2340, 1649, 1455 cm^{-1}; ^1H NMR data of aglycon part (500 MHz, C_5D_5N) δ 4.86 (1H, H-16), 4.24 (1H, H-3), 4.06 (1H, dd, J = 8.0, 11.5 Hz, H_a-26), 3.49 (1H, dd, J = 7.1, 9.1 Hz, H_b-26), 2.49 (1H, d, J = 10.0 Hz), 1.64 (3H, s, Me-21), 1.08 (3H, s, Me-19), 1.05 (3H, d, J = 6.5 Hz, Me-27), 0.70 (3H, s, Me-18); sugar portions 6.55 (1H, br s, H-1″), 5.88 (1H, br s, H-1‴), 4.86 (1H, d, J = 7.3, H-1⁗), 4.83 (1H, d, J = 7.6, H-1′), 1.75 (3H, d, J = 6.1, H_3-6‴), 1.61 (3H, d, J = 6.1, H_3-6″); ^{13}C NMR data of aglycon (125 MHz, C_5D_5N) δ 152.5 (C-22), 103.0 (C-20), 84.8 (C-16), 76.3 (C-3), 75.4 (C-26), 64.9 (C-17), 55.0 (C-14), 44.1 (C-13), 40.5 (C-9), 40.3 (C-12), 37.4 (C-8), 35.4 (x2) (C-5 and C-10), 34.6 (C-15), 33.8 (C-25), 31.6 (C-24), 31.1 (×3) (C-1, C-4, and C-7), 27.0 (×2) (C-2 and C-6), 24.0 (C-19), 23.8 (C-23), 21.5 (C-11), 17.3 (C-27), 14.6 (C-18), 12.0 (C-21); Glu-I 102.0 (C-1′), 78.7 (C-2′), 78.6 (C-3′), 77.1 (×2) (C-4′ and C-5′), 61.9 (C-6′); Rha-1 101.6 (C-1″), 74.0 (C-4″), 72.9 (C-3″), 72.6 (C-2″), 69.6 (C-5″), 18.5 (C-6″); Rha-2 102.4 (C-1‴), 74.1 (C-4‴), 72.9 (C-3‴), 72.8 (C-2‴), 70.5 (C-5‴), 18.8 (C-6‴); Glu-II 105.3 (C-1⁗), 78.7 (C-3⁗), 78.6 (C-5⁗), 75.3 (C-2⁗), 71.8 (C-4⁗), 63.0 (C-6⁗); HRESIFTMS 1033.5636 $[M]^+$, 1056.5483 $[M + Na]^+$ (calcd for $C_{51}H_{84}O_{21}$ 1033.2153).

Tribulosaponin B (**2**): white powder, $[\alpha]^{25}_D$ −34° (*c* 0.004, MeOH); IR (KBr) ν_{max} 3385, 2918, 2359, 1733, 1456, 1376 cm^{-1}; ^1H NMR data of aglycon part (500 MHz, C_5D_5N) δ 4.85 (1H, H-16), 4.23 (1H, H-3), 4.07 (1H, dd, J = 8.0, 11.5 Hz, H_a-26), 3.49 (1H, dd, J = 7.1, 9.1 Hz, H_b-26), 2.49 (1H, d, J = 10.0 Hz, H-17), 1.65 (3H, s, Me-21), 1.04 (3H, d, J = 6.3 Hz, Me-27), 0.98 (3H, s, Me-19), 0.70 (3H, s, Me-18); sugar portions 5.90 (1H, br.s, H-1″), 5.45 (1H, d, J = 7.6, H-1‴), 4.87 (1H, d, J = 7.0, H-1⁗), 4.84 (1H, d, J = 7.7, H-1′), 1.75 (3H, d, J = 6.1, H_3-6‴), 1.61 (3H, d, J = 6.1, H_3-6″); ^{13}C NMR data of aglycon (125 MHz, C_5D_5N) δ 152.3 (C-22), 103.5 (C-20), 84.5 (C-16), 75.3 (C-3), 75.2 (C-26), 64.6 (C-17), 54.7 (C-14), 43.8 (C-13), 40.1 (×2) (C-9 and C-12), 36.7 (C-8), 35.2 (×2) (C-5 and C-10), 33.6 (×2) (C-15 and C-25), 31.4 (C-24), 30.8 (C-1), 30.7 (C-4) 26.8 (×3) (C-2, C-6, and C-7), 24.0 (C-19), 23.6 (C-23), 21.3 (C-11), 17.1 (C-27), 14.4 (C-18), 11.8 (C-21); Gal 101.8 (C-1′), 82.7 (C-4′), 77.3 (C-2′), 77.0 (C-5′), 76.9 (C-3′), 61.0 (C-6′); Rha 102.3 (C-1″), 73.9 (C-4″), 72.7 (C-3″), 72.4 (C-2″), 70.1 (C-5″), 18.4 (C-6″); Glu-I 105.6 (C-1‴), 78.5 (C-5‴), 78.4 (C-3‴), 75.2 (C-2‴), 71.6 (C-4‴), 62.8 (C-6‴); Glu-II 105.1 (C-1⁗), 78.4 (C-3⁗), 77.8 (C-5⁗), 76.3 (C-2⁗), 71.8 (C-4⁗) 62.8 (C-6⁗); HRESIFTMS *m/z* 1049.5611 $[M]^+$, 1072.5305. $[M + Na]^+$ (calcd for $C_{51}H_{84}O_{22}$ 1049.2147).

Isoterrestrosin B (**3**): white powder, $[\alpha]^{25}_D$ −140° (*c* 0.004, MeOH); IR (KBr) ν_{max} 3384, 2922, 2359, 1732, 1652, 1455, 1376 cm^{-1}; ^1H NMR data of aglycon part (500 MHz, C_5D_5N) δ 4.53

(1H, H-16), 4.24 (1H, H-3), 4.07 (1H, dd, J = 8.8, 5.3 Hz, H_a-26), 3.30 (1H, dd, J = 5.0, 9.8 Hz, H_b-26), 2.49 (1H, d, J = 10.0 Hz, H-17), 1.08 (3H, d, J = 6.6, Me-21), 0.99 (3H, d, J = 6.9, Me-27), 0.85 (3H, s, Me-19), 0.73 (3H, s, Me-18); sugar portions 5.53 (1H, br s, H-1″), 5.32 (1H, d, J = 7.6, H-1‴), 4.76 (1H, d, J = 7.7, H-1′), 1.61 (3H, d, J = 6.1, H_3-6″); ^{13}C NMR data of aglycon (125 MHz, C_5D_5N) δ 110.0 (C-22), 81.4 (C-16), 75.3 (C-3), 65.2 (C-26), 62.5 (C-17), 56.3 (C-14), 42.4 (C-20), 40.8 (C-13), 40.2 (C-12), 40.1 (C-9), 36.2 (C-5), 35.4 (C-8), 35.1 (C-10), 31.9 (C-15), 30.6 (C-1), 30.4 (C-4) 27.3 (C-25), 26.7 (C-7), 26.5 (×2) (C-2 and C-6), 26.1 (C-23), 25.9 (C-24), 23.9 (C-19), 21.0 (C-11), 16.5 (C-18), 16.1 (C-27), 14.7 (C-21); Gal 100.7 (C-1′), 80.5 (C-4′), 77.6 (C-2′), 76.6 (C-5′), 76.4 (C-3′), 61.0 (C-6′); Rha 102.2 (C-1″), 73.5 (C-4″), 72.2 (C-3″), 72.1 (C-2″), 70.1 (C-5″), 18.1 (C-6‴); Glu 104.2 (C-1‴), 78.3 (C-5‴), 77.7 (C-3‴), 76.1 (C-2‴), 71.7 (C-4‴), 62.7 (C-6‴); HRESIFTMS m/z 887.4930 $[M + H]^+$, $[M + Na]^+$ 909.4688 (calcd for $C_{45}H_{74}O_{17}$ 887.0723).

Acid Hydrolysis of 1−3. A solution of each compound (5 mg) in 2 N HCl (1 mL) was refluxed for 3 h. The reaction mixture was extracted with EtOAc. After separating the organic layer, the aqueous phases was neutralized with $NaHCO_3$ and lyophilized. The lyophilized residue was dissolved in pyridine (0.2 mL) and analyzed by TLC in EtOAc−*n*-BuOH−H_2O (20:70:10, v/v), together with the authentic sugar samples.

Cytotoxicity Assay. Compounds were evaluated for cytotoxic activity against four cancer cell lines: SK-MEL, KB, BT-549, and SK-OV-3, obtained from American Type Culture Collection (ATCC, Rockville, MD). Neutral red staining was used to determine the number of viable cells after treatment with compounds.[13] The cytotoxicity assay was run at two different levels: (i) a primary assay for initial natural product screening at a single concentration and (ii) a secondary assay for retesting active substances at three to six concentrations. The cytotoxicity results were compared with results from normal mammalian VERO cell testing with the same substances in order to determine level of cytotoxicity. The assay is based on the fact that viable cells attached to the bottom of the plate will accumulate neutral red dye in their lysosomes, and it will remain inside the cell while the liquid supernatant is washed away with warm saline. A subsequent addition of 2-propanol will lyse the cells, releasing the dye into solution; hence, a higher number of viable cells in a well will yield a greater absorbance and an increase in reddish hue. *Day 1*: 25 000 cells were seeded into each well of a culture-treated 96-well microtiter plate and allowed to incubate for 24 h. *Day 2*: Diluted test samples were added in duplicate to the plate. Final test concentrations were 10, 3.3, and 1.1 μg/mL for pure compounds, while extracts were initially tested at 100 μg/mL, and then follow-up secondary testing of active extract was performed at 100, 33, and 11 μg/mL. The plates were then allowed to incubate 48 h. *Day 4*: Media was poured from the plates, and neutral red dye was added. After 90 min of incubation, the excess dye was poured and each well was washed with warm saline. The cells were then lysed by adding a 0.33% HCl in 2-propanol solution. Absorbance was measured at dual wavelength settings of 490 and 630 nm. Corresponding growth inhibition numbers were calculated and graphed. For secondary assays, IC_{50}'s were determined from logarithmic graphs of growth inhibition values.

Acknowledgment. We are grateful to Dr. Chuck Dunbar for conducting the HRESIFTMS analysis. We also thank Mr. Frank Wiggers for his assistance in obtaining the 2D-NMR spectra. This work was supported in part by the United States Department of Agriculture, ARS Specific Cooperative Research Agreement No. 58-6408-7-012.

References and Notes

(1) Davis, P. H.; *Flora of Turkey and East Aegean Islands*; University Press: Edinburgh, 1970; Vol. 4, pp 49−254.
(2) Capoor, L. D. *Handbook of Ayurvedic Medicinal Plants*; CRC Press: Boca Raton, FL, 1990; pp 325−326.
(3) Mahato, S. B.; Sahu, P. N.; Ganguly, A. N.; Miyahara, K.; Kawasaki, T. *J. Chem. Soc., Perkin Trans. 1* **1981**, 2405−2410.
(4) Yan, W.; Ohtani, K.; Kasai, R.; Yamasaki, K. *Phytochemistry* **1996**, *42*, 1417−1422.
(5) Xu, Y. X.; Chen, H. S.; Liu, W. Y.; Gu, Z. B.; Liang, H. Q. *Phytochemistry* **1998**, *49*, 199−201.
(6) Wu, G.; Jiang, S.; Jiang, F.; Zhu, D.; Wu, H.; Jiang, S. *Phytochemistry* **1996**, *42*, 1677−1681.
(7) Wang, Y.; Ohtani, K.; Kasai, R.; Yamasaki, K. *Phytochemistry* **1997**, *45*, 811−817.
(8) Li, J. X.; Shi, Q.; Xiong, Q. B.; Prasain, J. K.,; Tezuka, Y.; Hareyama, T.; Wang, Z. T.; Tanaka, K.; Namba, T.; Kadota, S. *Planta Med*. **1998**, *64* , 628−631.
(9) Wu, T. S.; Shi, L. S.; Kuo, S. C. *Phytochemistry* **1999**, *50*, 1411−1415.
(10) Agrawal, P. K.; Jain, D. C.; Gupta, R. K.; Thakur, R. S. *Phytochemistry* **1985**, *24*, 2479−2496.
(11) Saito, S.; Nagase, S.; Ichinose, K. *Chem. Pharm. Bull*. **1994**, *42*, 2342−2345.
(12) Tori, K.; Seo, S.; Terui, Y.; Nishikawa, J.; Yasuda, F. *Tetrahedron Lett*. **1981**, *22*, 2405−2408.
(13) Borenfraund, E.; Abich, H. B.; Martin-Alguachil, N. *Toxic. in Vitro* **1988**, *2*, 1−6.

NP000353B