Natural Product Sciences
20(2) : 76-79 (2014)

Cytotoxic Ergosterol Derivatives from the Mushroom *Naematoloma fasciculare*†

Ki Hyun Kim[1,]*, Sang Un Choi[2], Hyung Jun Noh[3], Okpyo Zee[4], and Kang Ro Lee[4]

[1]*Natural Product Research Laboratory, School of Pharmacy, Sungkyunkwan University, Suwon 440-746, Korea*
[2]*Research Institute of Chemical Technology, Teajeon 305-600, Korea*
[3]*Department of Herbal Crop Research, National Institute of Horticultural & Herbal Science, RDA, Eumseoung 369-873, Korea*
[4]*School of Pharmacy, Sungkyunkwan University, Suwon 440-746, Korea*

Abstract – In our ongoing search for structurally interesting and biologically active metabolites from Korean wild mushrooms, bioassay-guided fractionation and a chemical investigation of the MeOH extracts of the fruiting bodies of the mushroom *Naematoloma fasciculare* resulted in the isolation of three ergosterol derivatives, (22*E*,24*R*)-ergosta-7,22-diene-3*β*,5*α*,6*β*,9*α*-tetrol (**1**), (22*E*,24*R*)-5*α*,8*α*-epidioxyergosta-6,22-diene-3*β*-ol 3-*O*-*β*-D-glucopyranoside (**2**), and (22*E*,24*R*)-5*α*,8*α*-epidioxyergosta-6,9,22-triene-3*β*-ol 3-*O*-*β*-D-glucopyranoside (**3**). The structures of **1 - 3** were determined by comparison of their spectroscopic and physical data with reported values. The isolated steroid derivatives **1** and **3** were reported for the first time from this mushroom. Compounds **1 - 3** were tested for their cytotoxic activities against four human cancer cell lines (A549, SK-OV-3, SK-MEL-2, and HCT-15).

Keywords – *Naematoloma fasciculare*, Strophariaceae, ergosterol derivatives, cytotoxicity

Introduction

In our ongoing search for structurally interesting and biologically active metabolites from Korean wild mushrooms,[1-6] we have collected scores of endemic Korean mushroom species in the mountainous areas during the hot humid summer and prepared MeOH extracts of the mushrooms for anti-tumoral screening tests. Among the collected wild mushrooms, we found that the extract of the mushroom *Naematoloma fasciculare* (Strophariaceae) displayed a significant cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines in our screening test. The bitter poisonous mushroom *N. fasciculare* (Japanese name: Nigakuritake), which is widely distributed worldwide, grows on the stumps of old trees in tufts.[7] Previous phytochemical studies on this mushroom resulted in the isolation of diverse metabolites (ergosterols, triterpenoids, and sesquiterpenoids),[7-14] particularly, the isolation of lanostane triterpenoids as the toxic components of this mushroom.[9-13] In addition, pharmacological studies with lanostane triterpenoids reported to have antimicrobial and calmodulin inhibitory activities.[9-13] Recently, we reported the isolation of four new lanostane triterpenoids, together with 11 known compounds, and their antiproliferative and anti-inflammatory activities.[14] Our ongoing search for active constituents from this mushroom *N. fasciculare* led to the isolation of three ergosterol derivatives (**1 - 3**) (Fig. 1). Their structures were determined by comparison of their spectroscopic and physical data with reported values. Compounds **1 - 3** were tested for their cytotoxic activities against four human cancer cell lines (A549, SK-OV-3,

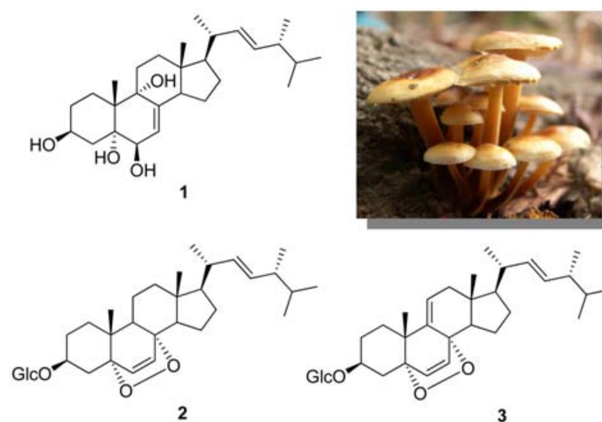

Fig. 1. The chemical structures of **1 - 3** and the mushroom *N. fasciculare*.

†Dedicated to Prof. Okpyo Zee of the Sungkyunkwan University for his leading works on Natural Products Research.

*Author for correspondence
Ki Hyun Kim, Natural Product Research Laboratory, School of Pharmacy, Sungkyunkwan University, Suwon 440-746, Korea
Tel: +82-31-290-7700; E-mail: khkim83@skku.edu

SK-MEL-2, and HCT-15). We describe here the isolation, structures, and cytotoxic activities of these steroid derivatives.

Experimental

General experimental procedures – Optical rotations were measured on a JASCO P-1020 polarimeter. IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer. NMR spectra were recorded on a Varian UNITY INOVA 500 NMR spectrometer operating at 500 MHz (^{1}H) and 125 MHz (^{13}C), respectively. ESIMS spectra were recorded on a Micromass QTOF2-MS. Semi-preparative HPLC was performed using a Gilson 306 pump with a Shodex refractive index detector. Chromatographic separation was performed on Econosil RP-18 10 µ column (250 × 10 mm i.d.). Silica gel 60 (Merck, 230 - 400 mesh) and RP-C_{18} silica gel (Merck, 230 - 400 mesh) were used for column chromatography. Silica Waters Sep-Pak Vac 6 cc and C_{18} Waters Sep-Pak Vac 6 cc cartridges were also used for column chromatography. TLC was performed using Merck pre-coated Silica gel F_{254} plates and RP-18 F_{254s} plates. Spots were detected on TLC under UV light or by heating after spraying with anisaldehyde-sulfuric acid.

Mushroom materials – Fresh fruiting bodies of *N. fasciculare* were collected at Donggureung, Guri of GyeongGi-do, Korea, in August, 2009. A voucher specimen (SKKU-2009-08) of the mushroom was authenticated by one of the authors (K.H.K.) and was deposited at the herbarium of the School of Pharmacy, Sungkyunkwan University, Korea.

Extraction and isolation – Air-dried and powdered *N. fasciculare* fruiting bodies (88 g) were extracted twice with 80% aqueous MeOH (each 2.0 L × 3 days) at room temperature and filtered. The filtrate was evaporated under vacuum to obtain a crude MeOH extract (9.4 g), which was suspended in distilled water and then successively partitioned with *n*-hexane, $CHCl_3$, and *n*-BuOH, yielding 900 mg, 1.8 g, and 2.8 g of residues, respectively. Each fraction was evaluated for cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines using the SRB bioassay. We selected the $CHCl_3$-soluble fraction for the current phytochemical study, since the $CHCl_3$-soluble fraction was the most cytotoxic against the tested tumor cell lines. The $CHCl_3$-soluble fraction (1.8 g) was separated by a silica gel (230 - 400 mesh) column chromatography [65 g, 1.5 × 32 cm, eluted with $CHCl_3$-MeOH, 20:1 (1.8 L) and 4:1 (1.0 L)] to yield 10 fractions (Fr. A, 20:1, 0.3 L; Fr. B, 20:1, 0.3 L; Fr. C, 20:1, 0.3 L; Fr. D, 20:1, 0.3 L; Fr. E, 20:1, 0.3 L; Fr. F, 20:1, 0.3 L; Fr. G, 4:1, 0.2 L; Fr. H, 4:1, 0.2 L; Fr. I, 4:1, 0.2 L; and Fr. J, 4:1, 0.4 L). Fraction F (115 mg) was subjected to fractionation with passage over a C_{18} Waters Sep-Pak Vac 6 cc (80% aq. MeOH) to give 13 subfractions (F1 – F13).

Subfraction F11 (10 mg) was purified by semi-preparative HPLC (95% aq. MeOH) with a Shodex refractive index detector, using an Econosil RP-18 10 µ column (250 × 10 mm i.d.), to yield compound **1** (6 mg). Subfraction F12 (7 mg) was purified by separation with semi-preparative HPLC (95% aq. MeOH) to give compound **3** (4 mg). Compound **2** (5 mg) was obtained from subfraction F13 (12 mg) by separation with semi-preparative HPLC (98% aq. MeOH).

(22*E*,24*R*)-Ergosta-7,22-diene-3*β*,5*α*,6*β*,9*α*-tetrol (1) – A white powder, ESI-MS *m*/*z*: 447.4 $[M + H]^+$; ^{1}H NMR (pyridine-d_5, 500 MHz): δ 0.69 (3H, s, H-18), 0.84 (3H, d, *J* = 6.8 Hz, H-26), 0.85 (3H, d, *J* = 6.8 Hz, H-27), 0.94 (3H, d, *J* = 6.8 Hz, H-28), 1.06 (3H, d, *J* = 6.8 Hz, H-21), 1.60 (3H, s, H-19), 4.43 (1H, d, *J* = 4.0 Hz, H-6), 4.81 (1H, m, H-3), 5.21 (2H, m, H-22, H-23), 5.82 (1H, d, *J* = 4.0 Hz, H-7). ^{13}C NMR (pyridine-d_5, 125 MHz): δ 143.04 (C-8), 136.2 (C-22), 132.2 (C-23), 121.3 (C-7), 78.7 (C-9), 75.0 (C-5), 73.8 (C-6), 67.3 (C-3), 56.3 (C-17), 51.3 (C-14), 44.2 (C-13), 43.1 (C-24), 42.0 (C-4), 41.3 (C-10), 40.7 (C-20), 36.0 (C-12), 33.3 (C-25), 32.4 (C-2), 29.1 (C-1), 28.5 (C-11), 28.2 (C-16), 23.5 (C-15), 22.4 (C-19), 21.4 (C-21), 20.1 (C-27), 19.8 (C-26), 17.6 (C-28), 12.1 (C-18).

(22*E*,24*R*)-5*α*,8*α*-Epidioxyergosta-6,22-diene-3*β*-ol 3-*O*-*β*-D-glucopyranoside (2) – An amorphous powder, ESI-MS *m*/*z*: 613.2 $[M + Na]^+$; ^{1}H NMR (pyridine-d_5, 500 MHz): δ 0.77 (3H, s, H-18), 0.79 (3H, s, H-19), 0.87 (3H, d, *J* = 6.8 Hz, H-26), 0.88 (3H, d, *J* = 6.8 Hz, H-27), 0.97 (3H, d, *J* = 6.8 Hz, H-28), 1.03 (3H, d, *J* = 6.8 Hz, H-21), 4.46 (1H, m, H-3), 5.19 (1H, dd, *J* = 15.4, 7.5 Hz, H-22), 5.27 (1H, dd, *J* = 15.4, 7.5 Hz, H-23), 6.23 (1H, d, *J* = 8.5 Hz, H-6), 6.51 (1H, d, *J* = 8.5 Hz, H-7), Glc; 4.94 (1H, d, *J* = 7.5 Hz, H-1'), 4.05 (1H, dd, *J* = 9.0, 7.7 Hz, H-2'), 4.25 (1H, dd, *J* = 9.0, 8.8 Hz, H-3'), 4.32 (1H, dd, *J* = 9.3, 8.8 Hz, H-4'), 3.86 (1H, ddd, *J* = 9.3, 4.9, 2.5 Hz, H-5'), 4.41 (1H, dd, *J* = 11.9, 4.9 Hz, H-6'a), 4.50 (1H, dd, *J* = 11.9, 2.5 Hz, H-6'b). ^{13}C NMR (pyridine-d_5, 125 MHz): δ 136.1 (C-6), 136.0 (C-22), 132.3 (C-23), 131.0 (C-7), 103.0 (C-1'), 82.0 (C-5), 79.3 (C-8), 78.6 (C-5'), 78.3 (C-3'), 75.3 (C-2'), 73.8 (C-3), 71.5 (C-4'), 62.7 (C-6'), 56.3 (C-17), 52.0 (C-14), 51.8 (C-9), 44.7 (C-13), 43.0 (C-24), 40.1 (C-20), 39.5 (C-12), 37.4 (C-10), 35.1 (C-1), 34.6 (C-4), 33.3 (C-25), 29.0 (C-2), 29.0 (C-16),

23.6 (C-15), 21.1 (C-11), 21.1 (C-21), 20.2 (C-26), 19.9 (C-27), 18.1 (C-19), 17.9 (C-28), 13.0 (C-18).

(22*E*,24*R*)-5*α*,8*α*-Epidioxyergosta-6,9,22-triene-3*β*-ol 3-*O*-*β*-D-glucopyranoside (3) – An amorphous powder, $[\alpha]_{25}^{D}$: +5.6° (*c* 2.0, MeOH). IR ν_{max} cm^{-1}: 3400, 1060, 1040. ESI-MS *m/z*: 589.7 $[M + H]^+$; ^1H NMR (pyridine-d_5, 500 MHz): δ 0.74 (3H, s, H-18), 0.86 (6H, d, *J* = 6.8 Hz, H-26, H-27), 0.95 (3H, d, *J* = 6.9 Hz, H-28), 0.99 (3H, d, *J* = 6.6 Hz, H-21), 1.05 (3H, s, H-19), 4.49 (1H, m, H-3), 5.17 (1H, dd, *J* = 15.4, 8.5 Hz, H-22), 5.26 (1H, dd, *J* = 15.4, 7.7 Hz, H-23), 5.44 (1H, dd, *J* = 6.0, 1.9 Hz, H-11), 6.31 (1H, d, *J* = 8.5 Hz, H-6), 6.67 (1H, d, *J* = 8.5 Hz, H-7), Glc; 4.93 (1H, d, *J* = 7.7 Hz, H-1'), 4.04 (1H, dd, *J* = 9.0, 7.7 Hz, H-2'), 4.24 (1H, dd, *J* = 9.0, 8.8 Hz, H-3'), 4.31 (1H, dd, *J* = 9.3, 8.8 Hz, H-4'), 3.84 (1H, ddd, *J* = 9.3, 4.7, 2.5 Hz, H-5'), 4.40 (1H, dd, *J* = 11.9, 4.7 Hz, H-6'a), 4.46 (1H, dd, *J* = 11.9, 2.5 Hz, H-6'b). ^{13}C NMR (pyridine-d_5, 125 MHz): δ 143.7 (C-9), 136.1 (C-23), 135.2 (C-6), 132.4 (C-22), 131.0 (C-7), 119.3 (C-11), 103.0 (C-1'), 82.7 (C-5), 78.6 (C-3'), 78.4 (C-8), 78.3 (C-5'), 75.3 (C-2'), 73.7 (C-3), 71.5 (C-4'), 62.6 (C-6'), 55.9 (C-17), 48.6 (C-14), 43.8 (C-13), 43.0 (C-24), 41.3 (C-12), 40.2 (C-20), 38.5 (C-10), 33.8 (C-4), 33.3 (C-25), 33.0 (C-1), 30.0 (C-2), 29.0 (C-16), 25,4 (C-19), 21.3 (C-15), 20.9 (C-21), 20.1 (C-27), 19.8 (C-26), 17.8 (C-28), 13.1 (C-18).

Cytotoxicity testing – The cell lines used were A549 (non-small cell lung adenocarcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and HCT-15 (colon adenocarcinoma). These cancer cell lines such as A549, SK-OV-3, SK-MEL-2, and HCT-15 cells were provided by the National Cancer Institute (NCI). A SRB bioassay was used to determine the cytotoxicity of each compound against the cell lines mentioned above.[15] The assays were performed at the Korea Research Institute of Chemical Technology. Doxorubicin was used as a positive control. Doxorubicin cytotoxicity against the A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines was IC_{50} 0.02, 0.01, 0.01, and 0.13 μM, respectively.

Results and Discussion

Compound **1** was isolated as a white powder. The ESI-MS (*m/z* 447 $[M + H]^+$) and ^{13}C NMR spectral data of **1** gave a molecular formula of $C_{28}H_{46}O_4$. The ^{13}C NMR spectrum demonstrated 28 carbon signals comprising six methyls, eight methylenes, nine methines, and five quaternary carbons as indicated in DEPT experiments. The signals for six methyl groups at δ 0.69 (s), 0.84 (d, *J* = 6.8 Hz), 0.85 (d, *J* = 6.8 Hz), 0.94 (d, *J* = 6.8 Hz), 1.06 (d, *J* = 6.7 Hz), and 1.60 (s) in the ^1H NMR spectrum implied a sterol nucleus. Four of the carbon signals at δ 67.3, 73.8, 75.0, and 78.7 in the ^{13}C NMR spectrum were assigned to C-3, C-6, C-5, and C-9, respectively, bearing hydroxyl groups. One typical proton signal at δ 4.81 (m) for H-3α indicated the presence of a 3β-hydroxyl group. The proton signal at δ 4.43 (d, *J* = 4.0 Hz) assigned to H-6α inferred the presence of a 6β-hydroxyl group. One tertiary and one quaternary carbon signals at δ 121.3 and 143.0, respectively, were attributable to C-7 and C-8. The olefinic proton at δ 5.82 (d, *J* = 4.0 Hz) was assigned to H-7 based on chemical shift and coupling constant with H-6α. The remaining tertiary carbon signals at δ 132.2 and 136.2 were characteristic of a double bond of C-22/C-23 in the ergosterols. On the basis of the further comparison with reported values,[16] the structure of **1** was determined to be (22*E*,24*R*)-ergosta-7,22-diene-3*β*,5*α*,6*β*,9*α*-tetrol.

Compound **2** was obtained as an amorphous powder, and the molecular formula was established as $C_{34}H_{54}O_8$ on the basis of ESI-MS (*m/z* 613.2 $[M + Na]^+$). The ^1H NMR spectrum showed the presence of the four secondary methyl signals at δ 0.87 (d, *J* = 6.8 Hz), 0.88 (d, *J* = 6.8 Hz), 0.97 (d, *J* = 6.8 Hz), 1.03 (d, *J* = 6.8 Hz), two singlet methyls at δ 0.77 (s), 0.79 (s), two sets of olefinic protons at δ 5.19 (dd, *J* = 15.1, 7.5 Hz), 5.27 (dd, *J* = 15.1, 7.3 Hz), and 6.23 (d, *J* = 8.5 Hz), 6.51 (d, *J* = 8.5 Hz), and one anomeric proton at δ 4.94 (d, *J* = 7.3 Hz). Its ^{13}C NMR spectrum contained 34 signals, of which 28 were assigned to a steroid moiety and six signals due to a hexose unit. The sugar group was determined to be glucose by the comparison of chemical shifts values with reported values.[17] The vicinal coupling constant (7.3 Hz) between H-1' and H-2' for glucose indicated that this sugar occurred in **2** as the β-anomer. The *R* configuration at C-24 was determined by comparison of chemical shift values as in the case of **1**, and the stereochemistry of C-5/8 peroxide was established by comparison of ^{13}C NMR data with literature data.[17] Accordingly, the structure of **2** was elucidated as (22*E*,24*R*)-5*α*,8*α*-epidioxyergosta-6,22-diene-3*β*-ol 3-*O*-*β*-D-glucopyranoside.[17,18]

Compound **3** was isolated as an amorphous powder, and the molecular ion at *m/z* 589.7 $[M + H]^+$ in its ESI-MS and 34 carbon signals in its ^{13}C NMR spectrum suggested a molecular formula of $C_{34}H_{52}O_8$. Analysis of the ^1H NMR and ^{13}C NMR spectra revealed that compound **3** also possesses a 3β-hydroxyl group, glucose with the anomeric center of β-configuration, 5*α*,8*α*-peroxide ring, and 22*E*,24*R*-methyl-ergostane-type side chain.[18] Comparison of the spectral features of **3** with those of **2** suggested the close relationship of two structures. The only difference

Table 1. Cytotoxicity of compounds **1 - 3** using the SRB bioassay

Compound	IC_{50} (μM)[a]			
	A549	SK-OV-3	SK-MEL-2	HCT-15
1	10.83	12.16	10.39	13.20
2	14.21	15.11	9.01	17.49
3	15.42	18.26	12.96	19.32
Doxorubicin[b]	0.02	0.01	0.01	0.13

[a] IC_{50} value of compounds against each cancer cell line, which was defined as the concentration (μM) that caused 50% inhibition of cell growth *in vitro*.
[b] Doxorubicin as a positive control.

was the assignment of additional trisubstituted double bond (143.7 (C-9) and 119.3 (C-11)) to C-9/C-11 in **3**. The stereochemistry of C-5/8 peroxide was determined to be same as compound **2** by comparison of NMR data with those of **2**. Compound **3** was, therefore, identified as (22*E*,24*R*)-5α,8α-epidioxyergosta-6,9,22-triene-3β-ol 3-*O*-β-D-glucopyranoside by the further comparison with published data.[19] To the best of our knowledge, the isolated steroid derivatives **1** and **3** were reported for the first time from this mushroom.

Compounds **1 - 3** were evaluated for their cytotoxic activities against the A549, SK-OV-3, SK-MEL-2, and HCT-15 human cancer cell lines using the SRB bioassay.[15] All isolated ergosterol derivatives showed moderate cytotoxic activity against the four human cancer cell lines (Table 1).

Acknowledgments

The authors thank Drs. E. J. Bang, S. G. Kim, and J. J. Seo at the Korea Basic Science Institute for the NMR and MS spectra measurements.

References

(1) Kim, K. H.; Choi, S. U.; Park, K. M.; Seok, S. J.; Lee, K. R. *Arch. Pharm. Res*. **2008**, *31*, 579-586.
(2) Kim, K. H.; Choi, S. U.; Lee, K. R. *Chem. Lett*. **2009**, *38*, 894-895.
(3) Kim, K. H.; Park, K. M.; Choi, S. U.; Lee, K. R. *J. Antibiot*. **2009**, *62*, 335-338.
(4) Kim, K. H.; Noh, H. J.; Choi, S. U.; Park, K. M.; Seok, S. J.; Lee, K. R. *J. Antibiot*. **2010**, *63*, 575-577.
(5) Kim, K. H.; Noh, H. J.; Choi, S. U.; Park, K. M.; Seok, S. J.; Lee, K. R. *Bioorg. Med. Chem. Lett*. **2010**, *20*, 5385-5388.
(6) Kim, K. H.; Choi, S. U.; Lee, K. R. *Lipids* **2012**, *47*, 593-599.
(7) Shiono, Y.; Matsuzaka, R.; Wakamatsu, H.; Muneta, K.; Murayama, T.; Ikeda, M. *Phytochemistry* **2004**, *65*, 491-496.
(8) Kim, D. S.; Baek, N. I.; Oh, S. R.; Jung, K. Y.; Lee, I. S.; Kim, J. H.; Lee, H. K. *Arch. Pharm. Res*. **1997**, *20*, 201-205.
(9) Ikeda, M.; Niwa, G.; Tohyama, K.; Sassa, T.; Miura, Y. *Agric. Biol. Chem*. **1977**, *41*, 1803-1805.
(10) Ikeda, M.; Sato, Y.; Izawa, M.; Sassa, T.; Miura, Y. *Agric. Biol. Chem*. **1977**, *41*, 1539-1541.
(11) Kubo, I.; Matsumoto, A.; Kozuka, M.; Wood, W. F. *Chem. Pharm. Bull*. **1985**, *33*, 3821-3825.
(12) Suzuki, K.; Fujimoto, H.; Yamazaki, M. *Chem. Pharm. Bull*. **1983**, *31*, 2176-2178.
(13) Takahashi, A.; Kusano, G.; Ohta, T.; Ohizumi, Y.; Nozoe, S. *Chem. Pharm. Bull*. **1989**, *37*, 3247-3250.
(14) Kim, K. H.; Moon, E.; Choi, S. U.; Kim, S. Y.; Lee, K. R. *J. Nat. Prod*. **2013**, *76*, 845-851.
(15) Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; MaMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst*. **1990**, *82*, 1107-1112.
(16) Yue, J. M.; Chen, S. N.; Lin, Z. W.; Sun, H. D. *Phytochemistry* **2001**, *56*, 801-806.
(17) Takaishi, Y.; Uda, M.; Ohashi, T.; Nakano, K.; Murakami, K.; Tomimatsu, T. *Phytochemistry* **1991**, *30*, 4117-4120.
(18) Shi, X. W.; Li, X. J.; Gao, J. M.; Zhang, X. C. *Chem. Biodivers*. **2011**, *8*, 1864-1870.
(19) Yoshikawa, K.; Ikuta, M.; Arihara, S.; Matsumura, E.; Katayama, S. *Chem. Pharm. Bull*. **2001**, *49*, 1030-1032.

Received May 2, 2014
Revised May 30, 2014
Accepted May 31, 2014