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# The isolation of secondary metabolites and in vitro potent anti-cancer activity of clerodermic acid from *Enicosanthum membranifolium*

Mai Efdi,[a] Tomohiro Itoh,[b] Yukihiro Akao,[b] Yoshinori Nozawa,[b] Mamoru Koketsu[c,*] and Hideharu Ishihara[a]

[a]*Department of Chemistry, Faculty of Engineering, Gifu University, Gifu 501-1193, Japan*
[b]*Gifu International Institute of Biotechnology, 1-1 Naka-Fudogaoka, Kakamigahara, Gifu 504-0838, Japan*
[c]*Division of Instrumental Analysis, Life Science Research Center, Gifu University, Gifu 501-1193, Japan*

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**Abstract**—Four compounds were isolated from *Enicosanthum membranifolium*. The structures of the compounds were confirmed by spectroscopic data. Their structures were determined as *N-trans*-feruloyltyramine, *R*-(−)-mellein, clerodermic acid, and salicifoline chloride as a quaternary alkaloid compound. The structures of *R*-(−)-mellein and salicifoline chloride were confirmed by using X-ray diffraction. Clerodermic acid was shown to induce potent apoptosis against human leukemia HL60 cells.


## 1. Introduction

Annonaceae is a large family comprising ca. 130 genera and more than 2300 species. Economically the family is of appreciable importance as a source of edible fruits, oils, soap, and raw material for perfumery.[1] The structural diversity of this class of natural products, aside from their large range of biological activities, such as cytotoxic, anti-tumoral, and pesticidal effects, has stimulated phytochemical studies of some genera of this family. For example, some species of Goniothalamus have been investigated and the predominant isolates have been acetogenins, styryl lactones, and isoquinoline-derived alkaloids with significant cytotoxic, insecticidal, and antimicrobial effects.[2–4] A new triterpene, suberosol, as an anti-HIV principle was isolated from *Polyalthia suberosa* and some antimicrobial compounds such as 16-oxocleroda-3, 13E-dien-15-oic acid, kolavenic acid, and 16β-hydroxycleroda-3,13-dien-15,16-olide were obtained from *Polyalthia longifolia*.[5,6] It is clear that the family now requires thorough phytochemical investigations in search of medicinally important as well as chemically interesting compounds.

We have collected some species belonging to annonaceae family in Rimbo Panti forest in West Sumatra-Indonesia, one of them is *Enicosanthum membranifolium*. Preliminary phytochemical screening result showed that the plant contains interesting secondary metabolites such as phenolic, steroid, terpenoid, and alkaloid compounds. On the basis of literature survey there is no scientific information regarding phytochemical and pharmacological investigation of this species.

Herein we report the isolation of secondary metabolites and the biological study of isolated compounds.

## 2. Results and discussion

The methanol extract (369 g) of branches of *E. membranifolium* Sinclair was fractionated with *n*-hexane, ethyl acetate, and *n*-butanol, successively. The ethyl acetate fraction was repeatedly subjected to column chromatography on silica gel column chromatography, HPLC, and/or recrystallization. Compound **1** was isolated from *n*-hexane fraction. Compounds **2** and **3** were isolated from the ethyl acetate fraction. And compound **4** was

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* Corresponding author. Tel./fax: +81 293 2619; e-mail: koketsu@gifu-u.ac.jp

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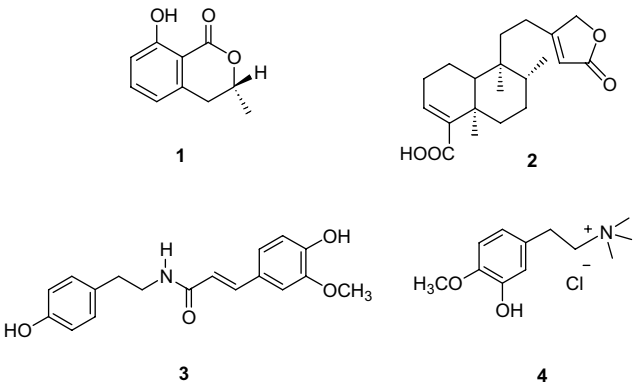
isolated from *n*-butanol fraction. The structures of the compounds **1**–**4** were analyzed by using IR, $^{1}H$, $^{13}C$ NMR and MS. Finally, the structures were confirmed by comparison with the reference data, and they were identified as *R*-(−)-mellein (**1**),[7,8] clerodermic acid (**2**), *N*-*trans*-feruloyltyramine (**3**), and salicifoline chloride (**4**). All of these compounds were isolated for the first time from this plant.




(*R*)-(−)-Mellein (**1**), which is one of pentaketide, has been shown to possess an impressive array of biological activities such as pheromone, bactericidal, fungicidal, and algicidal properties.[9–11] The structure of **1** was studied by using X-ray analysis.[12] Salicifoline chloride [systematic name: 2-(3-hydroxy-4-methoxyphenyl)-*N*,*N*,*N*-trimethylethanaminium chloride] (**4**) was isolated from *n*-butanol fraction. Some compounds having the same name, salicifoline, were also found as natural products but their structures are different, for instance the salicifoline isolated from *Euphorbia salicifolia* is a tricyclic diterpene involving 5–8–8 fused ring system.[13,14] The compound **4** has been reported to have a curare-like action on the extirpated rectus abdominis muscles of frogs and on the sciatic skeletal muscle of rats in situ.[15–17] The structure of **4** was studied by using X-ray analysis.[18] *N*-*trans*-Feruloyltyramine (**3**) was isolated from flowering plants and has been reported to show stimulatory effect on insulin secretion in rat pancreatic RIN-5F cells.[19–23] Clerodermic acid (**2**) is one of clerodane diterpenoid compounds.[24–26] Many clerodane compounds have been reported to have anti-cancer activity. For example, bucidarasins A–C showed potent cytotoxicity against human tumor cell lines with $IC_{50}$ values of 0.5–1.9 μM.[27] Laetiaprocerines A–D and laetianolide A showed cytotoxicity toward a human tumor cell line MCF7.[28] Caseamembrins G–L were cytotoxic against human oral epidermoid (KB), cervical epitheloid (HeLa), and liver (Hep59T/VGH) carcinoma cell lines.[29] Caseamembrins A, C, E showed the activities against human prostate (PC-3) and hepatoma (Hep3B) cancer cells with $IC_{50}$ values below 3 μM.[30] Caseamembrols A and B were also cytotoxic against human prostate (PC-3) cancer cells.[31] These reports stimulated us to investigate biological significance of the compounds isolated in this study. Four compounds **1**–**4** were tested for growth inhibitory effect on HL60 cells. Among them, clerodermic acid showed the strongest activity. Therefore, clerodermic acid was further

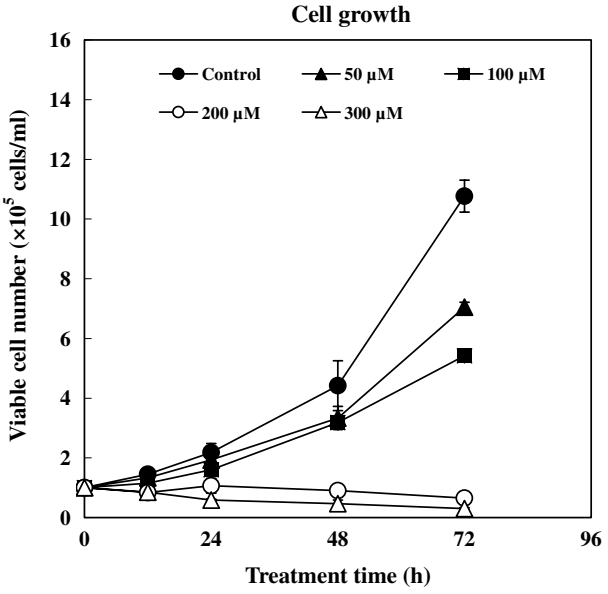



**Figure 1.** Effect of clerodermic acid (**2**) on cell growth of the human leukemia cell line HL60.1 × $10^5$ cells/well were cultured in 24-well plate dishes, to which DMSO alone or 50, 100, 200, and 300 μM clerodermic acid were added at 24 h after plating the cells. Viable cells were counted by Trypan-blue dye exclusion test at 12, 24, 48, and 72 h and data are expressed as means ± SD from three different experiments.

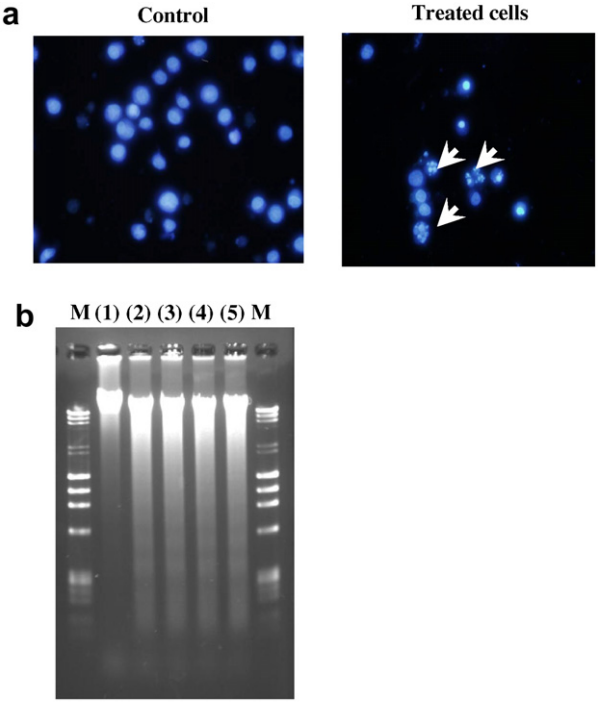



**Figure 2.** Apoptosis induced by clerodermic acid (**2**) in human leukemia HL60 cells. (a) Morphological examination of clerodermic acid-treated cells was performed by Hoechst 33342 nuclear staining (treated with 200 μM of clerodermic acid for 24 h, ×400). (b) Nucleosomal DNA fragmentation was detected by 2% agarose electrophoresis. Lane 1, untreated control, Lanes 2, 3, 4, and 5, cells treated with 50, 100, 200, and 300 μM of clerodermic acid for 24 h (M, DNA size marker).

studied to define its activity against the HL60 cells. As shown in Figure 1, viable cell number gradually decreased in a concentration-dependent manner after treatment with the clerodermic acid. The $IC_{50}$ of clerodermic acid was 68 μM. It was shown that its cytotoxic effect resulted from apoptosis, which was assessed by the typical morphologic changes characteristic of apoptosis, such as nuclear condensation and fragmentation with Hoechst 33342 in the cells treated with clerodermic acid (Fig. 2a), and the nucleosomal DNA ladder formation (Fig. 2b).

To examine mechanisms of clerodermic acid-induced apoptosis in HL60 cells, we have tested the effect of pan-caspase inhibitor, Z-VAD-FMK on apoptosis induced by clerodermic acid. HL60 cells were pre-incubated with 100 μM of Z-VAD-FMK for 1 h and then treated with 200 μM of clerodermic acid.

As shown in Figure 3, clerodermic acid-induced apoptosis was not prevented even by 100 μM of Z-VAD-FMK, suggesting that clerodermic acid-induced apoptosis was probably executed through caspase-independent pathway. Thus, clerodermic acid-induced growth inhibition observed in HL60 cells was mainly due to caspase-independent apoptosis.

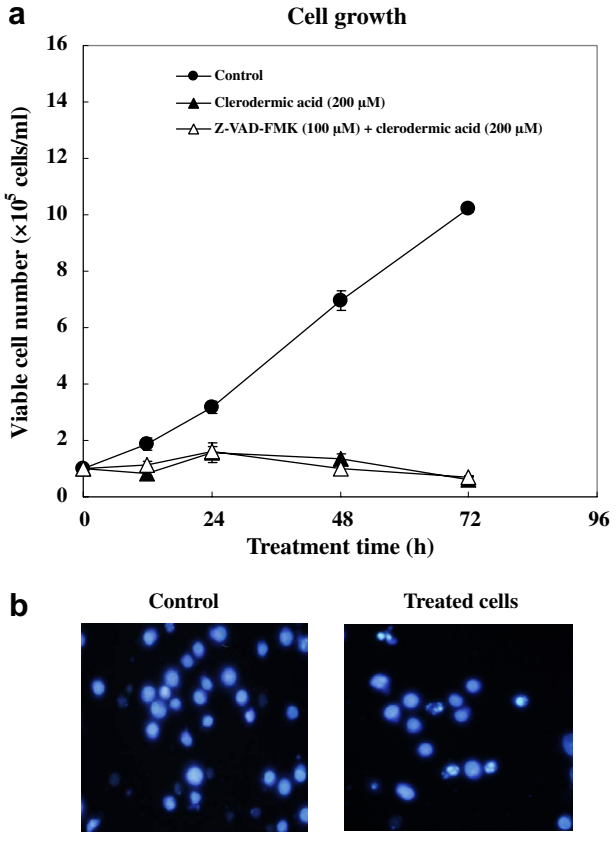



**Figure 3.** Effect of pan-caspase inhibitor (Z-VAD-FMK 100 μM) on clerodermic acid (**2**)-induced apoptosis. (a) Viable cell number was measured by Trypan-blue dye exclusion test. Data were expressed as means ± SD from three different experiments. (b) Morphological changes by Hoechst 33342 nuclear staining at 24 h after Z-VAD-FMK, and clerodermic acid-treatment.

## 3. Conclusion

The present study showed that *E. membranifolium*, one member of annonaceae family, produces secondary metabolites, one of which exhibited apoptosis-inducing activity. The clerodermic acid (**2**) may be of potential merit for the design of a range of novel semi-synthetic and synthetic compounds as anti-cancer medicinal agents in the future.

## 4. Experimental

### 4.1. Isolation procedure

Air-dried branches of *E. membranifolium* Sinclair (14 kg) were finely ground and macerated at room temperature in methanol. The mixture was subsequently filtered and concentrated in vacuo to give methanol extract (369 g). The methanol extract was partitioned successively with *n*-hexane, ethyl acetate, and *n*-butyl alcohol.

The *n*-hexane extract (64 g) was fractionated on a silica-gel 60 N (Kanto Chemical Co., Ltd., 40–50 μm) column chromatography (∅6 mm × 500 mm, 700 g), eluted with *n*-hexane-ethyl acetate and acetone in turn. This fractionation process gave ten fractions (fractions A–J). Fraction C (6 g) was subjected to column chromatography (silica-gel 60, Merck Ltd., 70–230 mesh ASTM, 240 g), eluted with $CHCl_3$–acetone. Fractions having the same $R_f$ values were combined and seven fractions (C1–C7) were obtained. Fraction C1 (270 mg) was repeatedly separated by column chromatography on silica-gel 60 (Kanto chemical Co., Ltd., 40–50 μm, 20 g) and eluted with *n*-hexane–$CHCl_3$–acetone (5:2:1) to give four fractions (C1a–C1d). Purification of fraction C1a (80 mg) by high-performance liquid chromatography (Wakosil-II 5C18 HG Prep, ∅20 mm × 250 mm, Wako Pure Chemical Industries, Ltd., Tokyo) and PTLC (preparative thin-layer chromatography) [1 mm layer thickness, *n*-hexane–$CHCl_3$–acetone (5:2:1)] afforded *R*-(−)-mellein (**1**) (12.0 mg).

The ethyl acetate extract (53 g) was fractionated on a silica-gel 60 (Kanto Chemical Co., Ltd., 40–50 mm) column (∅6 mm × 500 mm, 700 g), eluted with $CHCl_3$-acetone and methanol in turn. This fractionation process gave twelve fractions (fractions A to L). Fraction G (2.68 g) was separated by flash column chromatography on silica gel (105 g) using mobile phase *n*-hexane, *n*-hexane-ethyl acetate, and acetone in turn. The eluates were combined based on TLC analysis that afforded 10 fractions (Ga to Gj). Fraction Gb (310 mg) was further purified with PTLC (eluent $CHCl_3$–acetone = 8:2) to give clerodermic acid (**2**) (85 mg). Fraction I (3.5 g) was separated by flash column chromatography on silica gel (130 g) using mobile phase *n*-hexane, *n*-hexane-ethyl acetate, and acetone in turn. The eluates were combined based on TLC analysis to give 5 fractions (Ia to Ie). Fraction Ic (260 mg) was further purified by high-performance liquid chromatography (Wakosil-II 5C18 HG Prep, ∅20 mm × 250 mm, Wako Pure Chemical

Industries, Ltd., Tokyo) to give *N-trans*-feruloyltyramine (**3**) (56 mg).

The *n*-butyl alcohol extract (79 g) was dissolved in MeOH (500 ml). Then it was acidified with 5% acetic acid (500 ml) and extracted with EtOAc. The acidic aqueous solution was made alkaline with $NaHCO_3$ and extracted with *n*-butyl alcohol (5 × 500 ml) to yield a crude alkaloid mixture (40 g) which was fractionated by a silica gel 60 (Kanto Chemical Co., Ltd., 40–50 μm) column chromatography (∅6 mm × 500 mm, 500 g) and eluted with acetone–methanol (stepwise). This fractionation processing gave six fractions (A–F). Salicifoline chloride (**4**) (800 mg) was crystallized spontaneously from fraction C (1.6 g) as colorless prismatic crystals.

### 4.2. Data of isolated compounds

**4.2.1. *R*-(−)-Mellein (1).** Mp: 55–56 °C. $[\alpha]_D = -85.2$ (c. 0.0027, MeOH, 295 K). $^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 1.53 (3H, d, $J = 6.3$ Hz), 2.93 (2H, d, $J = 7.4$ Hz), 4.73 (1H, m), 6.69 (1H, d, $J = 8.0$ Hz), 6.89 (1H, d, $J = 8.0$ Hz), 7.41 (1H, $J = 8.0$ Hz), 11.0 (1H, s). $^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ 20.9, 34.8, 76.9, 108.4, 116.4, 118.0, 136.3, 139.5, 162.4, 170.1.

**4.2.2. Clerodermic acid (2).** Mp: 161–162 °C; $^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 0.80 (3H, s), 0.82 (3H, d, $J = 5.7$ Hz), 1.10–1.18 (1H, m), 1.12–1.31 (1H, m), 1.26 (3H, s), 1.40–1.63 (5H, m), 1.64–1.73 (1H, m), 2.11–2.22 (3H, m), 2.27–2.39 (2H, m), 2.41–2.48 (1H, m), 4.74 (2H, d, $J = 2$ Hz), 5.85 (1H, t, $J = 2$ Hz), 6.85 (1H, t, $J = 4$ Hz). $^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ 15.86, 17.43, 18.14, 20.44, 22.20, 27.06, 27.36, 35.37, 35.67, 36.27, 37.53, 38.74, 46.66, 73.04, 115.08, 139.84, 141.37, 170.78, 172.26, 173.98. EIMS *m*/*z*: 314 ($M^+ - H_2O$).

**4.2.3. *N-trans*-Feruloyltyramine (3).** Mp: 92–93 °C. $^1$H NMR (500 MHz, $CD_3OD$): $\delta$ 2.75 2H, t, $J = 7.2$ Hz) 3.46 (2H, t, $J = 7.2$ Hz), 3.86 (3H, s), 6.40 (1H, d, $J = 15.5$ Hz), 6.72 (2H, d, $J = 8.6$ Hz), 6.78 (1H,d, $J = 8.6$ Hz), 7.00 (1H, dd, $J = 2.3$, 8.6 Hz), 7.04 (2H, d, $J = 8.6$ Hz), 7.09 (1H, d, $J = 2.3$ Hz), 7.43 (1H, d, $J = 15.5$ Hz). $^{13}$C NMR (125 MHz, $CD_3OD$): $\delta$ 35.8, 42.5, 56.3, 111.5, 116.3, 116.5, 118.6, 123.3, 128.0, 130.7, 131.3, 142.1, 149.4, 150.2, 156.9, 169.2. EIMS *m*/*z*: $[313]^+$.

**4.2.4. Salicifoline chloride (4).** Mp: 260-261 °C. $^1$H NMR (500 MHz, $D_2O$): $\delta$ 3.02–3.08 (2H, m), 3.19 (9H, s), 3.50–3.56 (2H, m), 3.88 (3H, s), 6.87–6.90 (2H, m), 7.02–7.05 (1H, m). $^{13}$C NMR (125 MHz, $D_2O$): $\delta$ 28.3, 53.14, 53.17, 53.2, 56.2, 67.3, 113.1, 116.2, 121.3, 129.0, 145.2, 146.7.

### 4.3. Assay for cytotoxic activity

**4.3.1. Cell culture and treatment.** Human M2-type leukemia cell line HL60 was provided by RIKEN Cell Bank (Tsukuba, Ibaraki, Japan). HL 60 cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Isolated compounds dissolved in DMSO were added to the cell culture with final concentration of DMSO (<0.1%) that showed no significant effect on the growth and differentiation of HL60 cells (data not shown). Viable cell number was measured by trypan-blue dye exclusion test using a Burker-Turk type cell count chamber.

**4.3.2. Morphological change in HL60 cells.** For the morphological examination of cell death, the cells were stained with Hoechst 33342 (Calbiochem, Sandiego, CA, USA). Hoechst 33342 was added to the cultured medium at a concentration of 5 μg/ml. After incubation for 30 min, the collected cells were washed with phosphate-buffered saline (PBS) and then observed under a fluorescence microscope, Olympus BX-50 (Olympus, Tokyo, Japan).

**4.3.3. DNA extraction and agarose gel electrophoresis.** The cultured cells were treated with clerodermic acid. The control cells were treated with DMSO alone. The cells were collected and washed with PBS. Lysis buffer (100 mM) Tris–HCl (pH 7.4), 5 mM EDTA, 200 mM NaCl, 0.2% SDS, 200 μg/ml Proteinase K (Takara Bio Co., Ltd., Ohtsu, Shiga, Japan) was added to the cell pellet and incubated at 55 °C for 3 h. After incubation, DNA was extracted with phenol/chloroform from the cell lysate. DNA was precipitated with ethanol and dissolved with Tris–EDTA buffer. RNase A (Sigma) was added to the DNA solution at a final concentration of 20 μg/ml. DNA (3 μg) was analyzed by electrophoresis on 2% agarose gel.

## References and notes


1. Leboueuf, M.; Cavé, A.; Bahaumik, P. K.; Mukherjee, M.; Mukherjee, R. *Phytochemistry* **1982**, *21*, 2783–2813.
2. Zafra-Polo, M. C.; Figadére, B.; Gallardo, T.; Tormo, J. R.; Cortes, D. *Phytochemistry* **1998**, *48*, 1087–1117.
3. Bermejo, A.; Blázquez, M. A.; Rao, K. S.; Cortes, D. *Phytochemistry* **1998**, *47*, 1375–1380.
4. Omar, S.; Chee, C. L.; Ahmad, F.; Ni, J. X.; Jaber, H.; Huang, J.; Nakatsu, T. *Phytochemistry* **1992**, *31*, 4395–4397.
5. Li, H. Y.; Sun, N. J.; Kashiwada, Y.; Sun, L.; Snider, J. V.; Cosentino, L. M.; Lee, K. H. *J. Nat. Prod.* **1993**, *56*, 1130.
6. Rashid, M. A.; Hossain, M. A.; Hasan, C. M.; Reza, M. S. *Phytother. Res.* **1996**, *10*, 79–81.
7. Mori, K.; Gupta, A. K. *Tetrahedron* **1985**, *41*, 5295–5299.
8. Dimitriadis, C.; Gill, M.; Harte, M. F. *Tetrahedron Asymmetry* **1997**, *8*, 2153–2158.
9. Hoeller, U.; Koenig, G. M.; Wright, A. D. *J. Nat. Prod.* **1999**, *62*, 114–118.
10. Sun, C. M.; Toia, R. F. *J. Nat. Prod.* **1993**, *56*, 953–956.
11. Tan, R. X.; Zou, W. X. *Nat. Prod. Rep.* **2001**, *18*, 448–459.
12. Efdi, M.; Koketsu, M.; Ebihara, M.; Ishihara, H., *Acta Crystallogr., Sect. E: Struct. Rep. Online* **2006**, *E62*, o2135–o2137. CCDC 610755 for **1** contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.
13. Hohmann, J.; Evanics, F.; Dombi, G.; Szabó, P. *Tetrahedron Lett.* **2001**, *42*, 6581–6584.

14. Estevez-Braun, A.; Estevez-Reyes, R.; Gonzalez, A. G. *Tetrahedron* **1994**, *50*, 5203–5210.
15. Inoue, K. *Nippon Yakurigaku Zasshi* **1957**, *53*, 797–820.
16. Shimamoto, K.; Inoue, K.; Ogiu, K. *Jpn. J. Pharmacol.* **1958**, *7*, 135–156.
17. Song, W.; Gui, J.; Zhang, G. *Fitoterapia* **1990**, *61*, 245–247.
18. Efdi, M.; Koketsu, M.; Ebihara, M.; Ishihara, H. *Acta Crystallogr., Sect. E: Struct. Rep. Online* **2006**, *E62*, o2993–o2994, CCDC 613599 for **4** contains the supplementary crystallographic data for this paper.
19. Hussain, S. F.; Gozler, B.; Samma, M.; Gozler, T. *Phytochemistry* **1982**, *21*, 2979–2980.
20. Martin-Tanguy, J.; cabanne, F.; Perdrizet, E.; Martin, C. *Phytochemistry* **1978**, *17*, 1927.
21. Yoshihara, T.; Takamatsu, S.; Sakamura, S. *Agric. Biol. Chem.* **1978**, *42*, 623–627.
22. Sakakibara, I.; Katsuhara, T.; Ikeya, Y.; Hayashi, K.; Mitsuhashi, H. *Phytochemistry* **1991**, *30*, 3013–3016.
23. Nomura, E.; Kashiwada, A.; Hosoda, A.; Nakamura, K.; Morishita, H.; Tsuno, T.; Taniguchi, H. *Bioorg. Med. Chem.* **2003**, *11*, 3807–3813.
24. Ortega, A.; Cardenas, J.; Gage, D. A.; Maldonado, E. *Phytochemistry* **1995**, *39*, 931–933.
25. Achari, B.; Chaudhuri, C.; Saha, C. R.; Dutta, P. K.; Pakrashi, S. C. *Phytochemistry* **1990**, *29*, 3671–3673.
26. Pandey, R.; Verma, R. K.; Gupta, M. M. *Phytochemistry* **2005**, *66*, 643–648.
27. Hayashi, K.; Nakanishi, Y.; Bastow, K. F.; Cragg, G.; Nozaki, H.; Leea, K.-H. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 345–348.
28. Jullian, V.; Bonduelle, C.; Valentin, A.; Acebey, L.; Duigou, A.-G.; Prévost, M. -F. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 5065–5070.
29. Shen, Y.-C.; Lee, C. L.; Khalil, A. T.; Cheng, Y.-B.; Chien, C.-T.; Kuo, Y.-H. *Helv. Chim. Acta* **2005**, *88*, 68–77.
30. Shen, Y.-C.; Wang, C.-H.; Cheng, Y.-B.; Wang, L.-T.; Guh, J.-H.; Chien, C.-T.; Khalil, A. T. *J. Nat. Prod.* **2004**, *67*, 316–321.
31. Shen, Y.-C.; Wang, L.-T.; Wang, C.-H.; Khalil, A. T.; Guh, J.-H. *Chem. Pharm. Bull.* **2004**, *52*, 108–110.