J Nat Med (2009) 63:331–334
DOI 10.1007/s11418-009-0323-y

NOTE

A new amide from the leaves and twigs of *Litsea auriculata*

Hitoshi Tanaka · Saya Yatsuhashi · Tadashi Yasuda · Masaru Sato · Eiji Sakai · Chen Xiao · Hiroko Murata · Jin Murata

Received: 12 November 2008 / Accepted: 21 January 2009 / Published online: 24 February 2009

Abstract A new amide, *N-trans*-sinapoylmethoxytyramine (**1**), along with three known amides (**2**–**4**) and two known flavonol 3-*O*-rhamnosides (**5** and **6**), were isolated from the leaves and twigs of *Litsea auriculata*. The structures of the isolated compounds (**1**–**6**) were characterized on the basis of spectroscopic analyses, and their cytotoxic activity against HeLa cells was estimated.

Keywords *Litsea auriculata* · Lauraceae · Leaves · Twigs · Amides · HeLa cell

H. Tanaka (✉) · S. Yatsuhashi
Faculty of Pharmacy, Meijo University,
Yagoto, Tempaku-ku, Nagoya 468-8503, Japan
e-mail: hitoshi@ccmfs.meijo-u.ac.jp

T. Yasuda · M. Sato
Departments of Periodontology and Oral Pathology,
Asahi University School of Dentistry,
1851 Mizuho, Gifu 501-0296, Japan

E. Sakai
Gifu Pharmaceutical University,
Mitahora-higashi, Gifu 502-8585, Japan

C. Xiao
Kunming Institute of Botany, Chinese Academy of Sciences,
132 Lanhei Rd, Heilongtan,
650204 Kunming, Yunnan, China

H. Murata
Faculty of Pharmaceutical Sciences,
Setsunan University, Nagaotoge-cho, Hirakata,
Osaka 573-0101, Japan

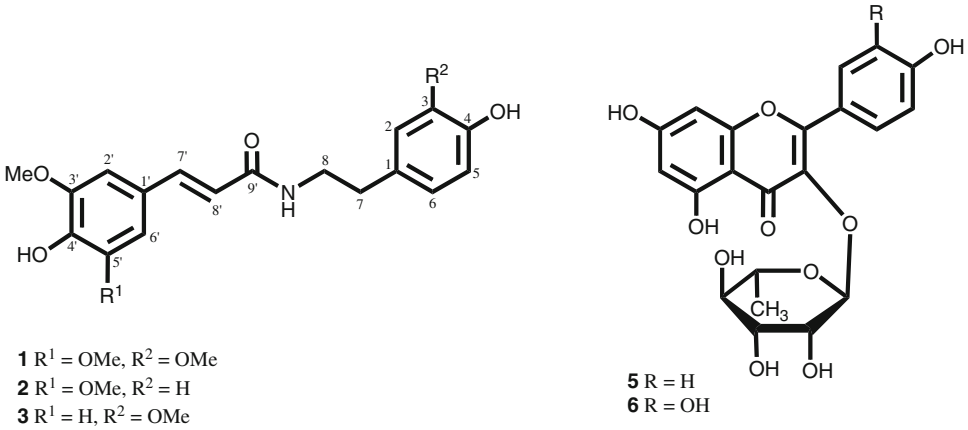
J. Murata
Botanical Gardens, Graduate School of Science,
The University of Tokyo, Hakusan, Bunkyo-ku,
Tokyo 112-0001, Japan

Introduction

The genus *Litsea* (Lauraceae) comprises nearly 200 species, which are widely found in tropical and subtropical Asia, North America, and subtropical South America. The *Litsea* plants have been reported to have a significant cytotoxic activity against human tumor cells [1], and recently, two *Litsea* plants (*Litsea lii* var. *nunkao-tahangensis* and *Litsea akoensis* Hayata) exhibited cytotoxic activity against human cancer cell lines, including human breast adenocarcinoma, non-small-cell lung cancer, and glioblastoma cell lines [2, 3]. The description of the cytotoxic compounds prompted us to begin studying the constituents of *Litsea auriculata* Chien & Cheng, whose phytochemical investigation has not been completed to date. *L. auriculata* is found in the southeast region of the People's Republic of China as a deciduous tree with a height typically of 20 m. The root bark and fruits of this plant are used medicinally as a tapeworm eliminator, and the leaves are also utilized for the treatment of muscular injuries [4]. We now describe the isolation and structural elucidation of a new amide, *N-trans*-sinapoylmethoxytyramine (**1**), along with three known amides (**2**–**4**) and two known flavonol 3-*O*-rhamnosides (**5** and **6**), from the leaves and twigs of *L. auriculata*. We also report the cytotoxic activities of the isolates (**1**–**6**) (Fig. 1) against HeLa cells.

Results and discussion

The MeOH extract of the leaves and twigs of *L. auriculata* was subjected to separation as described in the Extraction and isolation section. Repeated column chromatography afforded a new amide **1**, along with five known compounds



1 R^1 = OMe, R^2 = OMe
2 R^1 = OMe, R^2 = H
3 R^1 = H, R^2 = OMe
4 R^1 = H, R^2 = H

5 R = H
6 R = OH

Fig. 1 Structures of compounds **1**–**6** isolated from *Litsea auriculata*

2–**6**. The five known isolates were identified as *N-trans*-sinapoyltyramine (**2**) [5], *N-trans*-feruloylmethoxytyramine (**3**) [6], *N-trans*-feruloyltyramine (**4**) [7, 8], afzelin (kaemferol-3-*O*-α-L-rhamnoside) (**5**) [9], and quercitrin (quercetin-3-*O*-α-L-rhamnoside) (**6**) [9] by comparison of their spectroscopic data with those described in the literature. In the reported ^{13}C-NMR spectral data (in CD_3OD) of the known amides **2** [5] and **4** [8], several erroneous assignments have been found, and the revised ^{13}C-NMR signals are reported in this paper. Compounds **2**–**6** were isolated from *L. auriculata* for the first time.

Compound **1** was obtained as a colorless oil, whose molecular formula, $C_{20}H_{23}NO_6$, was determined by its high-resolution electron ionization–mass spectrometry (HREI-MS) spectrum ($[M]^+$ *m/z* 373.1525). The IR spectrum showed the presence of hydroxyl (3,500 cm^{-1}) and amide carbonyl (1,660 cm^{-1}) functions. The UV spectrum showed several absorption maxima in the same regions as **2**. The ^{1}H-NMR spectrum showed two aromatic protons at δ 6.84, two methoxyl groups at δ 3.87, and two olefinic protons at δ 6.43 and 7.42 (each 1H, d, J = 15.9 Hz) on a sinapoyl acid moiety, as well as three aromatic protons in an ABX-type signal at δ 6.67 (dd, J = 7.6, 1.5 Hz), 6.72 (d, J = 7.6), and 6.82 (d, J = 1.5 Hz), one methoxyl group at δ 3.83, and two coupled triplets of methylene protons at δ 2.77 and 3.49 (each 2H, t, J = 7.2 Hz) on a methoxytyramine moiety. A downfield doublet at δ 7.42 (J = 15.9 Hz) was assigned to the C-7′ olefinic proton on the sinapoyl acid moiety, revealing its *trans* coupling with the C-8′ olefinic proton, which appeared as a doublet at δ 6.43 (J = 15.9 Hz). The locations of the two methoxyl groups at the C-3′ and C-5′ positions on the sinapoyl acid moiety were determined by the HMBC experiment (Fig. 2), which showed correlations between OMe-3′ and C-3′, and OMe-5′ and C-5′. The further assignment of the methoxyl groups was obtained by the NOESY spectrum (Fig. 2), indicating NOE interactions between OMe-3′ and H-2′, and OMe-5′ and H-6′. The placement of the other methoxyl group at the C-3 position on the methoxytyramine moiety was established from both the HMBC technique (correlation between OMe-3 and C-3) and the NOESY data (NOE interaction between OMe-3 and H-2). Thus, compound **1** was characterized as *N-trans*-sinapoylmethoxytyramine (*N-trans*-sinapoyl-3-*O*-methyldopamine).

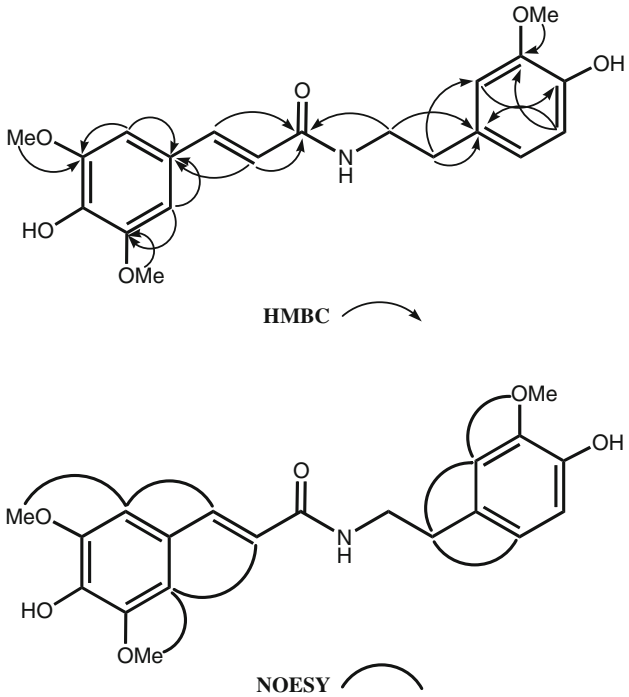


Fig. 2 Key HMBC and NOESY correlations of compound **1**

The cytotoxic activity of the isolates (**1**–**6**) against HeLa cells is summarized in Table 1. The isolated compounds, with the exception of compound **4**, reduced the number of HeLa cells in a dose-dependent manner in the concentration range of 6.25–50 μg/ml. Compounds **1**–**3** (12.5–50 μg/ml) exhibited almost identical cytotoxic potency, and the

Table 1 Viability of HeLa cells stimulated by compounds **1–6**

Compound	(μg/ml)			
	6.25	12.5	25	50
1	87.0[a]	55.8	38.5	33.3
2	66.8	53.4	38.6	32.4
3	66.8	53.4	38.5	32.3
4	116.6	119.4	114.0	96.7
5	85.0	77.1	74.1	52.1
6	84.4	78.5	65.2	45.2
Adriamycin[b]	84.2	55.6	37.1	2.0

[a] Viability is expressed as percentage versus control cell number
Values are the mean of three different determinations ($n = 3$)

[b] Positive control (final concentration: 0.0625, 0.125, 0.25, and 0.5 μg/ml)

cell number was reduced by 67–68% at 50 μg/ml. Compounds **5** and **6** stimulated HeLa cells to decrease the cell number by 48 and 55% (50 μg/ml), respectively. In contrast, compound **4** slightly increased the cell number in the concentration range of 6.25–25 μg/ml. The cytotoxic potency of the amides (**1–3**) against HeLa cells was stronger than that of the flavonol 3-*O*-rhamnosides (**5** and **6**). Tetra- and pentasubstituted oxygenation amides (**1–3**) showed cytotoxic activity against HeLa cells, though the less oxygenated amide (**4**) exhibited no cytotocity.

Experimental

General procedures

The IR spectra were measured using a JASCO IR-810 spectrophotometer, and the UV spectra were obtained using a Beckman DU-530 spectrophotometer. The MS spectra were recorded using a JEOL JMS-SX 102A spectrometer. The ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were measured by using a JEOL ECA-500 spectrometer. Column chromatography was performed using Merck silica gel (230–400 mesh). Spots on TLC plates (Merck silica gel 60 F_{254}) were detected with a UV lamp or by spraying with 50% H_2SO_4 followed by heating.

Plant material

The leaves and twigs of *L. auriculata* were collected from Kunming in the Yunnan Province, People's Republic of China, in August 2007, and identified by one of the authors (J.M.). The voucher specimen (No. 070829) was deposited in the Department of Natural Product Chemistry, Faculty of Pharmacy, Meijo University.

Extraction and isolation

The dried leaves and twigs of *L. auriculata* (1.8 kg) were cut into small pieces and extracted with MeOH (3 × 20 l, 72 h) at room temperature. The extract was evaporated under reduced pressure that produced a residue (155.6 g). The residual oil was suspended in H_2O (5 l) and then partitioned successively with the same volume of *n*-hexane, CH_2Cl_2, and EtOAc to afford the *n*-hexane (33.0 g), CH_2Cl_2 (28.2 g), and EtOAc (7.7 g) fractions, respectively. The CH_2Cl_2 extract (28.2 g) was subjected to silica gel column chromatography (CC) eluted with *n*-hexane/$CHCl_3$ (1:20, 1 l), $CHCl_3$ (1 l), $CHCl_3$/acetone (40:1→20:1→10:1→5:1→1:1, 1 l each volume), and $CHCl_3$/MeOH (10:1, 1 l) to give 15 fractions (A_1–A_{15}). Fractions A_{13}–A_{15} (1.42 g) were subjected to silica gel CC eluted with benzene/EtOAc (1:1→1:2→1:5, 7 ml each volume) to afford 123 fractions (B_1–B_{123}). Fractions B_{44}–B_{55} (141 mg) were purified by silica gel CC eluted with $CHCl_3$/acetone (5:1) to give **4** (8.5 mg). Fractions B_{56}–B_{71} (246 mg) were chromatographed over silica gel eluted with benzene/EtOAc (1:1→1:2, 1 ml each volume) to give 100 fractions (C_1–C_{100}). Fractions C_{35}–C_{55} (89 mg) were purified by silica gel CC eluted with $CHCl_3$/acetone (5:1) to afford **3** (5.2 mg). Fractions C_{56}–C_{85} (77 mg) were subjected to silica gel CC repeatedly eluted with $CHCl_3$/acetone (5:1) to afford **2** (24 mg). Fractions B_{72}–B_{90} (130 mg) were purified by silica gel CC eluted with *n*-hexane/acetone (2:1→1:1) followed by $CHCl_3$/acetone (5:1→2:1) to give **1** (11 mg). The EtOAc extract (7.7 g) was separated by silica gel CC eluted with $CHCl_3$/MeOH (5:1→2:1→1:1) to give the crude compound **5** (858 mg) and the crude compound **6** (4.0 g). A portion of the crude compound **5** (338 mg) was rechromatographed over silica gel eluted with $CHCl_3$/MeOH (5:1) to give the pure compound **5** (174 mg), and the crude **6** (343 mg) was purified by silica gel CC eluted with $CHCl_3$/MeOH (5:1) to afford the pure compound **6** (141 mg).

The unambiguous assignment of all the ^{1}H- and ^{13}C-NMR signals of the isolates (**1–4**) was accomplished by analyses of the ^{1}H–^{1}H COSY, NOESY, HMQC, and HMBC spectra.

N-trans-Sinapoylmethoxytyramine (**1**). Colorless oil; UV λ_{max} nm (MeOH) (log ε): 319 (4.38), 290 sh (4.15), 234 (4.46), 224 sh (4.40), 203 (4.67); IR (KBr) ν_{max} cm^{-1}: 3,500, 1,660, 1,610, 1,520; ^{1}H-NMR (CD_3OD) δ: 2.77 (2H, t, J = 7.2 Hz, H-7), 3.49 (2H, t, J = 7.2 Hz, H-8), 3.83 (3H, s, MeO-3), 3.87 (6H, s, MeO-3′, MeO-5′), 6.43 (1H, d, J = 15.9 Hz, H-8′), 6.67 (1H, dd, J = 7.6, 1.5 Hz, H-6), 6.72 (1H, d, J = 7.6 Hz, H-5), 6.82 (1H, d, J = 1.5 Hz, H-2), 6.84 (2H, s, H-2′, H-6′), 7.42 (1H, d, J = 15.9 Hz, H-7′); ^{13}C-NMR (CD_3OD) δ: 169.1 (C-9′), 149.5 (C-3′, C-5′), 149.0 (C-3), 146.1 (C-4), 142.2 (C-7′), 139.0 (C-4′),

132.0 (C-1), 127.2 (C-1′), 122.3 (C-6), 119.2 (C-8′), 116.2 (C-5), 113.5 (C-2), 106.4 (C-2′, C-6′), 56.8 (MeO-3′, MeO-5′), 56.4 (MeO-3), 42.5 (C-8), 36.2 (C-7); EI-MS *m/z* (relative intensity): 373 ($[M]^+$, 14), 308 (54), 293 (100), 223 (29), 220 (12), 207 (22), 178 (13), 150 (32), 147 (15); HREI-MS *m/z*: 373.1525 ($[M]^+$, calculated for $C_{20}H_{23}NO_6$: 373.1524).

N-trans-Sinapoyltyramine (**2**). Colorless oil; ^{13}C-NMR (CD_3OD) δ: 169.0 (C-9′), 156.9 (C-4), 149.4 (C-3′, C-5′), 142.2 (C-7′), 138.8 (C-4′), 131.3 (C-1), 130.7 (C-2, C-6), 127.2 (C-1′), 119.2 (C-8′), 116.3 (C-3, C-5), 106.4 (C-2′, C-6′), 56.8 (MeO-3′, MeO-5′), 42.5 (C-8), 35.8 (C-7); EI-MS *m/z* (relative intensity): 343 ($[M]^+$, 67), 222 (96), 207 (100), 192 (16), 175 (18), 120 (22), 107 (23); HREI-MS *m/z*: 343.1409 ($[M]^+$, calculated for $C_{19}H_{21}NO_5$: 343.1418).

N-trans-Feruloyltyramine (**4**). Colorless oil; ^{13}C-NMR (CD_3OD) δ: 169.2 (C-9′), 156.9 (C-4), 149.9 (C-4′), 149.3 (C-3′), 142.0 (C-7′), 131.3 (C-1), 130.7 (C-2, C-6), 128.3 (C-1′), 123.2 (C-6′), 118.7 (C-8′), 116.5 (C-5′), 116.3 (C-3, C-5), 111.5 (C-2′), 56.4 (MeO-3′), 42.5 (C-8), 35.8 (C-7); EI-MS *m/z* (relative intensity): 313 ($[M]^+$, 34), 194 (34), 193 (59), 192 (59), 177 (100), 145 (27), 120 (22), 107 (11), 89 (8); HREI-MS *m/z*: 313.1308 ($[M]^+$, calculated for $C_{18}H_{19}NO_4$: 313.1313).

Cell culture

HeLa cells (American Type Culture Collection, Manassas, VA) were cultured at 37°C in DMEM supplemented with 10% fetal calf serum and antibiotics (culture medium). The subcultures between the third to sixth passages were used for the experiments.

Method for cytotoxicity assay

Subconfluent HeLa cells were cultured for 3 days on 96-well tissue culture plates in the culture medium. The cells were then incubated in the same medium without fetal calf serum. After 12 h, the medium was exchanged and compounds **1**–**6** were added to the mixture to give a final concentration of 6.25–50 μg/ml. After 48 h incubation at 37°C in 5% CO_2, the conditioned medium on the 96-well plates was collected and assayed for cell viability (Cell counting kit-8, Dojin Chemical Laboratory, Kumamoto, Japan) [10, 11]. Three different determinations were run for all compounds. Adriamycin (positive control) was added to the medium, providing final concentrations of 0.0625, 0.125, 0.25, and 0.5 μg/ml, and assayed as described above.

References

1. Cheng H-I, Lin W-Y, Duh C-Y, Lee K-H, Tsai I-L, Chen I-S (2001) New cytotoxic butanolides from *Litsea acutivena*. J Nat Prod 64:1502–1505
2. Wang T-A, Cheng M-J, Lee S-J, Yang C-W, Chang H-S, Chen I-S (2008) Secondary metabolites from the leaves of *Litsea lii* var nunkao-tahangensis. Helv Chim Acta 91:1036–1044
3. Chang S-Y, Cheng M-J, Kuo Y-H, Lee S-J, Chang H-S, Chen I-S (2008) Secondary metabolites from the stem bark of *Litsea akoensis* and their cytotoxic activity. Helv Chim Acta 91:1156–1165
4. Jiangsu New Medical College (1977) Dictionary of traditional Chinese medicines. Shanghai Science and Technology Press, Shanghai, p 339
5. Chaves MH, Roque NF (1997) Amides and lignanamides from *Porcelia macrocarpa*. Phytochemistry 46:879–881
6. Cutillo F, D'Abrosca B, DellaGreca M, Marino CD, Golino A, Previtera L, Zarrelli A (2003) Cinnamic acid amides from *Chenopodium album*: effects on seeds germination and plant growth. Phytochemistry 64:1381–1387
7. Okuyama T, Shibata S, Hoson M, Kawada T, Osada H, Noguchi T (1986) Effect of oriental plant drugs on platelet aggregation; III. Effect of chinese drug "Xiebai" on human platelet aggregation. Planta Med 52:171–175
8. Lajide L, Escoubas P, Mizutani J (1995) Termite antifeedant activity in *Xylopia aethiopica*. Phytochemistry 40:1105–1112
9. Fossen T, Larsen Å, Kiremire BT, Andersen ØM (1999) Flavonoids from blue flowers of *Nymphaéa caerulea*. Phytochemistry 51:1133–1137
10. Tominaga H, Ishiyama M, Ohseto F, Sasamoto K, Hamamoto T, Suzuki K, Watanabe M (1999) A water-soluble tetrazolium salt useful for colorimetric cell viability assay. Anal Commun 36:47–50
11. Miyamoto T, Min W, Lillehoj HS (2002) Lymphocyte proliferation response during *Eimeria tenella* infection assessed by a new, reliable, nonradioactive colorimetric assay. Avian Dis 46:10–16