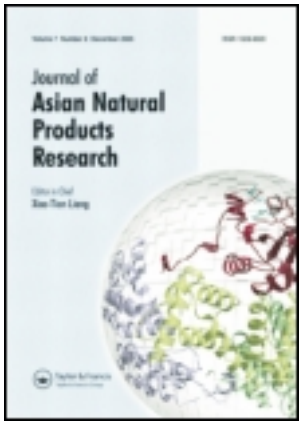

Journal of Asian Natural Products Research

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/ganp20

Two new amides with cytotoxic activity from the fruits of *Piper longum*

Priyanka Mishra [a], Sadhna Sinha [a], Santosh Kumar Guru [b], Shashi Bhushan [b], R.A. Vishwakarma [b] & Sabari Ghosal [a]

[a] Department of Biotechnology, Amity Institute of Biotechnology, Amity University, Noida, 201303, India

[b] Division of Cancer Pharmacology, Indian Institute of Integrative Medicine, Canal Road, Jammu, 180001, India

Available online: 28 Jan 2011

To cite this article: Priyanka Mishra, Sadhna Sinha, Santosh Kumar Guru, Shashi Bhushan, R.A. Vishwakarma & Sabari Ghosal (2011): Two new amides with cytotoxic activity from the fruits of *Piper longum* , Journal of Asian Natural Products Research, 13:02, 143-148

To link to this article: http://dx.doi.org/10.1080/10286020.2010.546789

Journal of Asian Natural Products Research
Vol. 13, No. 2, February 2011, 143–148

Two new amides with cytotoxic activity from the fruits of *Piper longum*

Priyanka Mishra[a], Sadhna Sinha[a], Santosh Kumar Guru[b], Shashi Bhushan[b], R.A. Vishwakarma[b] and Sabari Ghosal[a]*

[a]*Department of Biotechnology, Amity Institute of Biotechnology, Amity University, Noida 201303, India;* [b]*Division of Cancer Pharmacology, Indian Institute of Integrative Medicine, Canal Road, Jammu 180001, India*

(*Received 30 July 2010; final version received 7 December 2010*)

Bioassay-guided fractionation of the fruits of *Piper longum* afforded two new minor amides, piperlongimin A (**2**) [2*E*-*N*-isobutyl-hexadecenamide] and piperlongimin B (**4**) [2*E*-octadecenoylpiperidine] together with five known compounds with moderate cytotoxic activity. The structures were elucidated on the basis of spectroscopic evidences. All these compounds inhibited cell proliferation of human leukemia, HL-60 cell lines, and displayed major apoptosis-inducing effects.

Keywords: *Piper longum*; alkylamide; piperlongimin A; piperlongimin B; cytotoxic; HL-60

1. Introduction

Piper longum Linn. (Piperaceae), a slender aromatic climber of the genus *Piper*, is widely grown in tropical and subtropical India, Nepal, Bangladesh, Myanmar, Srilanka, and Malay Peninsula [1]. *Piper* species has high economical, medicinal, and commercial importance which might be the reason for wide research interest in the genus. Previous phytochemical studies have led to the isolation of a number of compounds, including long-chain esters, amides, alkaloids, lignans, terpenes, steroids, flavones, and flavanones. Some of the extracts of the plant are reported to be antibacterial, anti-inflammatory, insecticidal, tumoricidal, and cytotoxic agents [2]. However, there are very few systematic investigations to uncover cytotoxic compounds from the extracts. As a part of our general program of research, aiming to explore cytotoxic compounds from Indian medicinal plants, we have chemically investigated the fruits of *P. longum* by bioassay-guided fractionation. This has resulted to the isolation of two new monoenoic amides named piperlongimin A and piperlongimin B in a very small quantity from the complex mixture. Although, there are reports of a number of di- and polyunsaturated aliphatic amides, very few monoenoic structures have been reported to date. In addition, five known compounds namely 1-(3,4-methylenedioxyphenyl)-1*E*-tetradecene (**1**), 2*E*,4*E*-*N*-isobutyl-octadecenamide (**3**), 2*E*,4*E*-*N*-isobutyl-dodecenamide (**5**), guineensine (**6**), and piperine (**7**) were also isolated from *n*-hexane fraction (Figure 1). All the constituents showed medium cytotoxic activity on HL-60 cell lines, a cell line particularly suited to examine cell proliferation, cell cycle, and apoptotic events [3].

*Corresponding author. Email: sabarighosal@gmail.com

ISSN 1028-6020 print/ISSN 1477-2213 online

DOI: 10.1080/10286020.2010.546789
http://www.informaworld.com

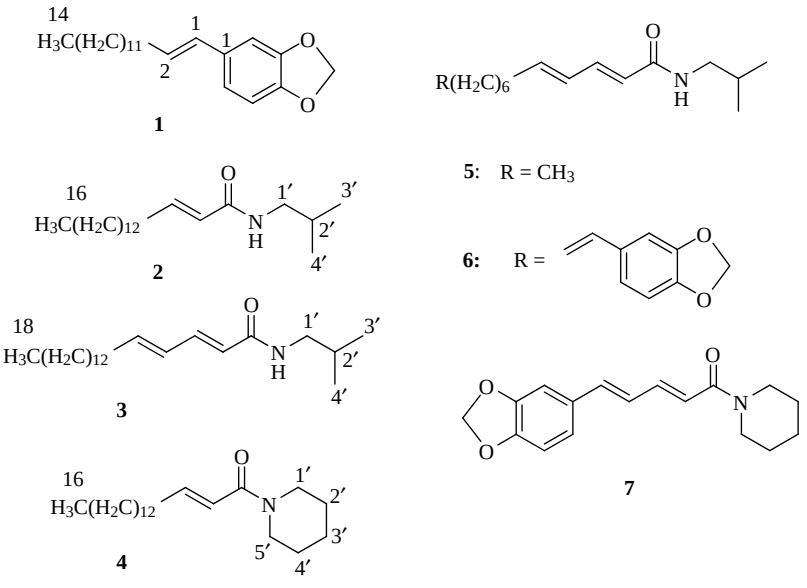

Figure 1. Chemical structures of compounds **1**–**7**.

2. Results and discussion

The methanol extract of the dried fruits of *P. longum* (2.5 kg) was fractionated into *n*-hexane, dichloromethane, ethyl acetate, and aqueous fractions, respectively. Cytotoxic screening of all these fractions by MTT-based *in vitro* assay on HL-60 cell lines resulted in the identification of *n*-hexane fraction as the most active fraction. A combination of a series of column chromatography and reversed phase HPLC of the active fraction afforded two new amides, viz. **2** and **4** with IC_{50} values of 14 and 15 μg/ml, respectively.

Piperlongimin A (**2**) was obtained as a pale yellow oil. HR-ESI-MS showed $[M + H]^+$ at *m/z* 310.3109, which suggested that the molecular formula of the compound was $C_{20}H_{39}NO$. The alkylamide moiety in the compound was established by the characteristic IR bands at 3300, 3080, 1660, and 1540 cm^{-1} while the monoenoic structure was indicated by the UV absorption maximum at 211 nm [4] and IR band at 980 cm^{-1}. In the ^1H NMR spectrum, characteristic signals at δ 5.41 (1H, br s, —NH—), 3.15 (2H, apparent t, $J = 6.6$ Hz, H-1′), 1.84–1.73 (1H, m, H-2′), and 0.92 (6H, d, $J = 6.9$ Hz, Me-3′ and Me-4′) indicated the presence of an isobutyl amide moiety in the compound. In addition, signals for the *trans*-olefinic protons of an α-β unsaturated carbonyl system appeared at δ 5.74 (1H, d, $J = 15.3$ Hz, H-2) and 6.91 and 6.78 (1H, m, H-3), an allylic methylene at δ 2.17–2.13 (br q, H-4), a terminal methyl at δ 0.88 (3H, t, $J = 6.6$ Hz, H-16) along with 11 methylene groups at δ 1.23 (22H, br s, H-5 to H-15). In concurrence with the ^1H NMR spectral data, the ^{13}C NMR spectrum showed an amide carbonyl at δ 166.3, two *trans*-olefinic carbons at δ 144.7 and 125.0, one methine carbon at δ 46.9, one terminal methyl at δ 14.7, two germinal methyl groups at δ 20.9, along with 13 methylene carbons. Analysis of the COSY spectrum of the compound showed cross peaks due to three-bond ^1H–^1H coupling between H-2 to H-3, H-1′ to N-H, H-1′ to H-2′, H-2′ to H-3′, and H-4′ and H-4 to H-5. These correlations also indicated the presence of a *trans*-olefinic α-β unsaturated isobutyl amide moiety in the molecule. Thus, on the basis of ^1H, ^{13}C and 2D NMR spectral analysis, the

structure of **2** was elucidated as 2*E*-*N*-isobutyl-hexadecenamide as shown in Figure 1.

Piperlongimin B (**4**) was obtained as a pale yellow oil. The molecular formula of $C_{21}H_{39}NO$ was established by HR-ESI-MS at *m/z* 322.3110 $[M + H]^+$. The diagnostic IR bands for an unsaturated carbonyl amide appeared at 1610, 1550, and 980 cm^{-1}. The monoenoic structure was further supported by the appearance of UV absorption maximum at 216 nm. The ^1H and ^{13}C NMR spectra of **4** were very close to those of **2**; differences were found in the appearance of signals due to a piperidine ring [δ 3.67 and 3.58 (each 2H, br s, H-1′ and H-5′), 1.65 and 1.57 (6H, br s, H-2′, H-3′ and H-4′); δ_C 46.5 and 42.7 (C-1′ and C-5′), 26.2 and 25.3 (C-2′ and C-4′) and 24.2 (C-3′)] in the place of the signals of isobutyl moiety. The *trans*-olefinic protons conjugated to a carbonyl appeared at δ_H 6.21 (H-2) and 6.83–6.78 (H-3) and the corresponding carbons appeared at δ_C 122.9 (C-2) and 144.4 (C-3). The down field shift of H-2 was in consistence with a 2*E*-monoenoylpiperidine moiety [5]. The assignment of ^1H and ^{13}C NMR spectral data were further confirmed by the HSQC correlations. Accordingly, the structure of compound **4** was elucidated as 2*E*-octadecenoylpiperidine as shown in Figure 1.

The structures of the known compounds were identified as 1-(3,4-methylenedioxyphenyl)-1*E*-tetradecene (**1**) [6,7], 2*E*,4*E*-*N*-isobutyl-octadecenamide (**3**) [8], 2*E*,4*E*-*N*-isobutyl-dodecenamide (**5**) [9], guineensine (**6**) [10], and piperine (**7**) by comparison of their ^1H NMR and MS data with those reported in the literature.

A critical SAR study of compounds **2**–**6** revealed that **6**, containing a terminal 3,4-methylenedioxystyryl moiety, was more potent with an IC_{50} value of 5 μg/ml than the rest of the compounds possessing a terminal methyl group (Table 1). This suggests that isobutyl amides containing a long alkyl chain linked to a methylenedioxy phenyl moiety may play an important role in bioactivity. Cell cycle analysis of all constituents at a concentration of 30 μg/ml by flow cytometry showed a sharp increase in hypodiploid sub-GO DNA fraction of HL-60 cells in the range of 20–80% (Figure 2). Furthermore, the cell cycle G2/M phase was not affected indicating that they do not cause any mitotic block.

Table 1. Cytotoxicity of compounds **1**–**7** against HL-60 cell lines.

Compound	Cytotoxicity (HL-60) IC_{50} (μg/ml)[a]
1	18
2	14
3	10
4	15
5	11
6	05
7	12

Note: [a]Media without test solution were considered as control (IC_{50} of control 100 μg/ml).

3. Experimental

3.1 General experimental procedures

UV spectra were recorded on a Shimadzu UV-1650 PC spectrophotometer. IR (KBr) spectra were recorded using a Nicolet model Protégé 460 spectrophotometer. ^1H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ solution with Bruker DRX 300 MHz spectrometer using TMS as internal standard. The chemical shift values are reported in ppm (δ) units and the scalar coupling constants (*J*) are in Hz. HR-ESI-MS were performed on a Waters 1525 LCT Micromass Spectrometer. Column chromatography was performed with silica gel 60 (Merck, Darmstadt, Germany), while aluminum sheets (200 μm, 20 × 20 cm) precoated with silica gel 60 GF_{254} (Merck) were used for analytical TLC and glass plates precoated with silica gel 60 GF_{254} (Merck, Germany; 250 μm, 5 × 10 cm) were used for preparative TLC. HPLC was performed on Waters LC system including a 600 pump and 2998 photodiode

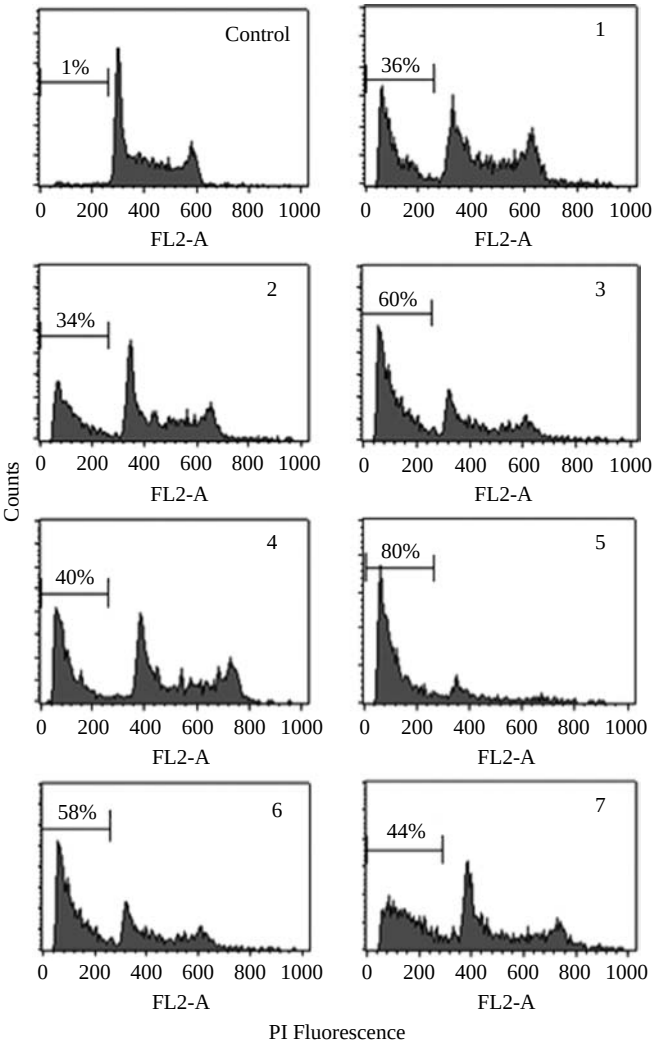

Figure 2. Effects of **1**–**7** on sub-G0 DNA fraction of cell cycle.

array detector. RP-18 analytical column (5 μm, 3.9 × 300 mm i.d.) and HPLC grade solvents were used. The spots were visualized under UV light (254 and 366 nm) followed by spraying with Dragendorff's reagent.

3.2 Plant material

The fruits of *P. longum* were procured from a registered vendor in New Delhi. A voucher specimen (SG/021/2006) was authenticated by Dr M. P. Sharma, Department of Botany, Hamdard University, New Delhi and has been deposited in the herbarium of Amity Institute of Biotechnology, Amity University, Noida, India.

3.3 Extraction and isolation

The air-dried fruits (2.5 kg) of *P. longum* were extracted with MeOH–H_2O (9:1, 3.5 l × 3 × 24 h) followed by H_2O (2 l) at room temperature and were concentrated under reduced pressure. Concentrated

methanol extract was suspended in water and successively partitioned with *n*-hexane (3.5 l, 40 g), dichloromethane (3.5 l, 43 g), ethyl acetate (2.5 l, 32 g), and aqueous (25 g) fractions. Thirty-nine grams of the *n*-hexane fraction on open column chromatography (5.7 × 85 cm) were eluted with *n*-hexane and a 10% stepwise gradient of EtOAc afforded nine fractions (F-1 to F-9) after solvent distillation under vacuum. Chromatography of F-1 (5 g) over silica gel 100–200 (2 × 70 cm) with *n*-hexane–EtOAc, 100:0 eluent afforded subfractions 1–5 and 90:10 eluent produced subfractions 6–14, respectively. Subfractions 6–9 upon concentration afforded **1** as a semi-solid white precipitate. All attempts to separate compounds from F-2 (2 g) by column chromatography were unsuccessful, as the R_f of constituting three compounds, **2**–**4** were very close. Preparative TLC of F-2 (*n*-hexane–EtOAc; 7:3; double run) afforded **4** (6 mg) and a less polar mixture, containing **2** and **3** in a nearly equal proportion. Reversed phase HPLC of the mixture with CH_3CN-H_2O (70:30, 30 min, 1 ml/min) afforded **2** (4 mg) and **3** (5 mg) at t_R 11 and 17 min, respectively. Chromatography of F-3 (4 g) with *n*-hexane–EtOAc (90:10) afforded a pale yellow precipitate of **5**. Furthermore, chromatography of F-5 (6 g) with *n*-hexane–EtOAc, 90:10 as eluent produced **6**, while 70:30 eluent afforded light yellow crystals of **7** in excess.

3.3.1 *Piperlongimin A (**2**)*

Pale yellow liquid. UV (MeOH) λ_{max}: 211 nm; IR (KBr) ν_{max}: 3300, 3080, 2950, 1660, and 1540, and 980 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 6.91 and 6.78 (1H, m, H-3), 5.74 (1H, d, $J = 15.3$ Hz, H-2), 5.41 (br s, —NH—), 3.15 (2H, d, $J = 6.6$ Hz, H-1′), 2.17–2.13 (2H, m, H-4), 1.84–1.73 (1H, m, H-2′), 1.26–1.23 (22H, br s, H-5 to H-15), 0.92 (6H, d, $J = 6.9$ Hz, H-3′ and H-4′), 0.88 (3H, t, $J = 6.6$ Hz, H-16); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 166.3 (C-1), 144.7 (C-3), 125.0 (C-2), 46.9 (C-1′), 32.7 (C-4), 32.0 (C-14), 29.5-29.2 (C-5 to C-13), 28.8 (C-2′), 23.4 (C-15), 20.9 (C-3′ and C-4′), 14.7 (C-16). HR-ESI-MS: *m/z* 310.3109 $[M + H]^+$ (calcd for $C_{20}H_{40}NO$, 310.3112), 332.2928 $[M + Na]^+$ (calcd for $C_{20}H_{39}NONa$, 332.2930).

3.3.2 *Piperlongimin B (**4**)*

Pale yellow liquid, UV (MeOH) λ_{max}: 216 nm; IR (KBr) ν_{max}: 1657, 1610, 1550, and 980 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 6.83–6.78 (1H, m, H-3), 6.21 (1H, d, $J = 15.0$ Hz, H-2), 3.67, 3.58 (each 2H, br s, H-1′ and H-5′), 2.18–2.15 (2H, m, H-4), 1.65 and 1.57 (6H, br s, H-2′, H-3′ and H-4′), 1.24 (22H, br s, H-5 to H-15), 0.88 (3H, t, $J = 6.3$ Hz, H-16); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 166.2 (C-1), 144.4 (C-3), 122.9 (C-2), 46.5, 42.7 (C-1′ and H-5′), 32.1 (C-4), 31.5 (C-14), 29.5-28.9 (C-5 to C-13), 26.2, 25.3 (C-2′ and H-4′), 24.2 (C-3′) 22.3 (C-15), 13.7 (C-16). HR-ESI-MS: *m/z* 322.3110 $[M + H]^+$ (calcd for $C_{21}H_{40}NO$, 322.3112).

3.4 *Assay for cytotoxicity*

Human leukemia HL-60 cell line was procured from NCCS Pune, India. Cells were grown in RPMI-1640 medium supplemented with 10% FCS, penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (0.3 mg/ml), pyruvic acid (0.11 mg/ml), 0.37% $NaHCO_3$, and 50 μM of 2-mercaptoethanol.

3.4.1 *MTT assay*

Test samples were aseptically dissolved in DMSO and were added at a further 100 μl/well concentration to give a final concentration of 5, 10, 30, and 100 μg/ml. Cells from adherent HL-60 cultures were grown in 96-well plates and were exposed to

various dilutions of individual test samples for 48 h. Thereafter, 20 μl of MTT solution (2.5 mg/ml) was added to each well and incubated at 37°C for 2 h. The plates were centrifuged and the supernatant was discarded, while the MTT–formazan crystals were dissolved in 100 μl DMSO. The OD was measured at 570 nm with reference wavelength of 620 nm [11] and cytotoxicity for each compound was expressed as IC_{50} values.

3.4.2 Cell cycle analysis

For cell cycle analysis, cells (1×10^6/ml) were fixed in cold 70% alcohol in PBS buffer and were treated with test samples at 30 μg/ml concentration for 24 h. The protocol applied for cytotoxicity and cell cycle analysis were the same as for a triterpenediol from *Boswellia serrata* [12].

Acknowledgements

The authors acknowledge DST and CSIR, Government of India for financial support, Dr A.K. Chauhan, Founder President, Amity University, for his continuous encouragement and Prof. P.L. Majumder, formerly of the University of Calcutta, Kolkata, for his helpful discussions.

References

[1] J.A. Parrotta, *Healing Plants of Peninsular India* (CABI Publishing, New York, 2001), p. 571.
[2] S. Kumar, P. Arya, C. Mukherjee, B.K. Singh, N. Singh, V.S. Parmar, A.K. Prasad, and B. Ghosh, *Biochemistry* **44**, 15944 (2005).
[3] S.J. Collins, *Blood* **70**, 1233 (1987).
[4] R. Bauer, P. Remiger, and H. Wagner, *Phytochemistry* **28**, 505 (1989).
[5] V.S. Parmar, R. Sinha, N.A. Shakil, O.D. Tyagi, P.M. Boll, and A. Wengel, *Indian J. Chem.* **32B**, 392 (1993).
[6] K. Likhitwitayawuid, *Tetrahedron* **43**, 3689 (1987).
[7] S.N. Upadhyay, R.A. Vishwakarma, S. Ghosal, S. Shukla, C. Bose, and A. Kamra, US patent no. 5,962,515 (1999).
[8] T. Morikawa, H. Matsuda, I. Yamaguchi, Y. Pongpiriyadacha, and M. Yoshikawa, *Planta Med.* **70**, 152 (2004).
[9] K.L. Dhar and M.L. Raina, *Planta Med.* **28**, 505 (1973).
[10] S.K. Koul, S.C. Taneja, V.K. Agarwal, and K.L. Dhar, *Phytochemistry* **27**, 3523 (1988).
[11] S. Bhushan, J. Singh, M.J. Rao, A.K. Saxena, and G.N. Qazi, *Nitric oxide* **14**, 72 (2006).
[12] S. Bhushan, A. Kumar, F. Malik, S.S. Andotra, V.K. Sethi, I.P. Kaur, S.C. Taneja, G.N. Qazi, and J. Singh, *Apoptosis* **12**, 1911 (2007).