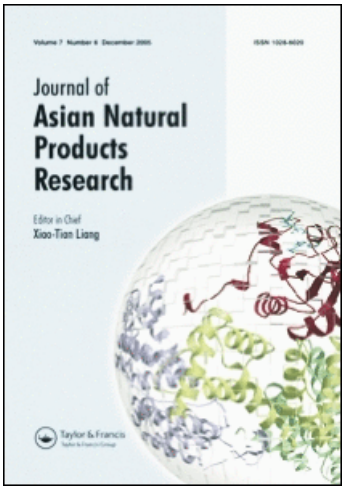

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

The metabolites of a mangrove endophytic fungus, *Penicillium thomi*

G. Chen[a]; Y. Zhu[a]; H. -Z. Wang[a]; S. -J. Wang[a]; R. -Q. Zhang[a]
[a] Department of Biological Science and Biotechnology, Tsinghua University, Beijing, China

To cite this Article Chen, G. , Zhu, Y. , Wang, H. -Z. , Wang, S. -J. and Zhang, R. -Q.(2007) 'The metabolites of a mangrove endophytic fungus, *Penicillium thomi*', Journal of Asian Natural Products Research, 9: 2, 159 — 164
To link to this Article: DOI: 10.1080/10286020500480423
URL: http://dx.doi.org/10.1080/10286020500480423

Journal of Asian Natural Products Research, Vol. 9, No. 2, March 2007, 159–164

The metabolites of a mangrove endophytic fungus, *Penicillium thomi*

G. CHEN, Y. ZHU, H.-Z. WANG, S.-J. WANG and R.-Q. ZHANG*

Department of Biological Science and Biotechnology, Tsinghua University, Beijing 100084, China

(Received 23 January 2005; revised 18 October 2005; in final form 3 November 2005)

The chemical constituents research of the fermentation of *Penicillium thomi* separated from the root of *Bruguiera gymnorrhiza* led to the isolation of a new compound, 4′,5-dihydroxy-2,3-dimethoxy-4-(hydroxypropyl)-biphenyl (**1**) and 11 known compounds. The structures of isolated compounds were determined by spectroscopic and chemical analysis. Their cytotoxic effects against three human cancer cell lines (A549, HepG2 and HT29) were also investigated.

Keywords: Metabolites; Mangrove endophytic fungus; *Penicillium thomi*; 4′,5-Dihydroxy-2,3-dimethoxy-4-(hydroxypropyl)-biphenyl; Cytotoxic effect

1. Introduction

Marine microorganisms have been proven to be rich sources of bioactive secondary metabolites, and numerous compounds with potent biological activities and unique chemical structures have been isolated [1]. The mangrove habitat has proved to be a rich source of new fungal species, and forms the second largest ecological sub-group of marine fungi. Recently, research on the secondary metabolites of mangrove endophytic fungi has led to the isolation of many new compounds with different bioactivities [2–4].

In this paper, we report the metabolites of a mangrove endophytic fungus, which was separated from the root of *Bruguiera gymnorrhiza* and identified as *Penicillium thomi*. Cytotoxicity guided fractionation and chromatographic separation led to the isolation of a new biphenyl derivative, 4′,5-dihydroxy-2,3-dimethoxy-4-(hydroxypropyl)-biphenyl (**1**) and 11 known compounds β-sitosterol (**2**), cholesterol (**3**), ergosta-7,22-diene-3β,5α,6β-triol (**4**), uracil (**5**), cyclo-(Ala-Gly) (**6**), cyclo-(Pro-Gly) (**7**), cyclo-(Ala-Pro) (**8**), (±)-1-monopalmitin (**9**), succinic acid (**10**), *N*-(2-hydroxy-4-methoxyphenyl)acetamide (**11**), and dibutylphthalate (**12**). The structures of isolated compounds were determined by

*Corresponding author. Email: rqzhang@mail.tsinghua.edu.cn

Journal of Asian Natural Products Research
ISSN 1028-6020 print/ISSN 1477-2213 online © 2007 Taylor & Francis
http://www.tandf.co.uk/journals
DOI: 10.1080/10286020500480423

spectroscopic and chemical analysis. Their cytotoxic effects against three human cancer cell lines (A549, HepG2 and HT29) were also investigated.

2. Results and discussion

The ethyl acetate extract of the fermentation broth of the fungus, which showed cytotoxic activity against A549 cell line, was repeatedly chromatographed on silica gel using a gradient elution from chloroform to ethyl acetate.

Compound **1** was obtained as colourless needles and gave a positive reaction with $FeCl_3$ reagent. HRESI-MS gave the molecular ion at *m/z* 304.1309 $[M]^+$, indicating a molecular formula $C_{17}H_{20}O_5$. The IR spectrum showed absorption bands for hydroxyl group (3413 cm^{-1}) and benzene skeleton (1600, 1517, 1447 cm^{-1}) that were confirmed by ^{13}C NMR spectrum.

The ^1H NMR spectrum of compound **1** exhibited two D_2O-exchangeable phenolic protons at δ 8.11 and 8.25, together with two carbon signals at δ 151.2 and 156.5 in ^{13}C NMR spectrum, from which two hydroxyl groups could be identified in the structure (table 1). Four aromatic protons (AA′BB′) at δ 7.38 (2H, d, $J = 8.2$ Hz), 6.80 (2H, d, $J = 8.2$ Hz) were observed, indicating a *para*-substituted benzene. The aromatic proton at δ 6.56 (1H, s) suggested another benzene moiety in the molecule. In addition, two methoxy group signals were also observed at δ 3.71 (3H, s) and 3.67 (3H, s). In the ^{13}C NMR spectrum, 12 aromatic carbon signals showed a biphenyl structure characteristic [5,6]. The signals appearing at δ 55.8 and 60.8 belong to two methoxy groups, which were assigned by the correlations between δ 3.71 with δ 55.8 and δ 3.67 with δ 60.8 in the HMQC spectrum. Moreover, the signals at δ 29.0, 34.1 and 64.2 indicate a hydroxypropyl group in the molecule, which were also confirmed by DEPT and HMQC spectra.

In the HMBC spectrum, correlations were observed between δ_H 7.38 and δ_C 130.0, 156.5; δ_H 6.80 and δ_C 114.8, 128.9; δ_H 8.25 and δ_C 156.5, from which one hydroxyl group can be attached to the C-4′ position, and the other carbon signals in this *para*-substituted benzene

Table 1. NMR data of compound **1** in DMSO-d_6.

Position	δ_H	δ_C
1	–	126.8
2	–	140.8
3	–	147.5
4	–	124.5
5	–	151.2
6	6.56 (1H, s)	108.4
1′	–	128.9
2′, 6′	7.38 (2H, d, $J = 8.2$ Hz)	130.0
3′, 5′	6.80 (2H, d, $J = 8.2$ Hz)	114.8
4′	–	156.5
1″	3.30 (2H, t, $J = 6.5$ Hz)	34.1
2″	1.74 (2H, m)	29.0
3″	3.64 (2H, m)	64.2
2-OCH_3	3.67 (3H, s)	60.8
3-OCH_3	3.71 (3H, s)	55.8
5-OH	8.11 (1H, s)	–
4¢-OH	8.25 (1H, s)	–

could also be assigned. Since the aromatic proton at δ 6.56 showed HMBC correlation with δ 128.9, which belongs to the C-1′ position, this aromatic proton could be identified at the C-6 position. Two methoxy protons (δ 3.67 and 3.71) correlated with δ 140.8 and 147.5, respectively in HMBC, from which the positions of the methoxy groups in the molecule were determined. Moreover, the hydroxyl signal at δ 8.11 showed HMBC correlations with δ 151.2 and 108.4, so it could be attached to the C-5 position. On the basis of the correlations between H-6 with δ 124.5, 140.8, 151.2, H-1″ with δ 124.5, 147.5, 151.2 in the HMBC spectrum, two methoxy groups (δ 60.8 and 55.8) and the hydroxypropyl group should locate at C-2, C-3 and C-4 positions, respectively.

A further NOE experiment was also carried out, since there was no enhancement of H-6 by irradiation of the two methoxy groups, and H-2′ showed enhancement by irradiation of δ 3.67, the methoxy (δ 60.8) should locate at C-2 and the other methoxy (δ 55.8) should locate at C-3, respectively. On the basis of the above evidence, the structure of compound **1** can be identified as 4′,5-dihydroxy-2,3-dimethoxy-4-(hydroxypropyl)-biphenyl (figure 1).

Eleven known compounds, β-sitosterol (**2**), cholesterol (**3**), ergosta-7,22-diene-3β,5α,6β-triol (**4**) [7], uracil (**5**) [8], cyclo-(Ala-Gly) (**6**) [9], cyclo-(Pro-Gly) (**7**) [10], cyclo-(Ala-Pro) (**8**) [11], (±)-1-monopalmitin (**9**) [12], succinic acid (**10**), *N*-(2-hydroxy-4-methoxyphenyl)acetamide (**11**) [13] and dibutylphthalate (**12**) [14], were also isolated and identified by comparison of their spectral data and TLC behaviour with those of the authentic samples and reported spectroscopic data.

Twelve isolated compounds were evaluated for their cytotoxic effects against A549, HepG2 and HT29 cancer cell lines by using MTT assay and cyclophosphamide as positive control (table 2). Among them, compounds **1**, **6**–**8**, and **12** exhibited cytotoxicity against different cell lines and gave IC_{50} values in the range 8.9–20.1 μM. On the other hand, compounds **2**–**5**, **9**–**11** displayed no cytotoxicity effects against these cancer cell lines (>50 μM). It has been reported that cyclic dipeptide compounds exhibit anticancer effects [15,16], and this was also observed in this study.

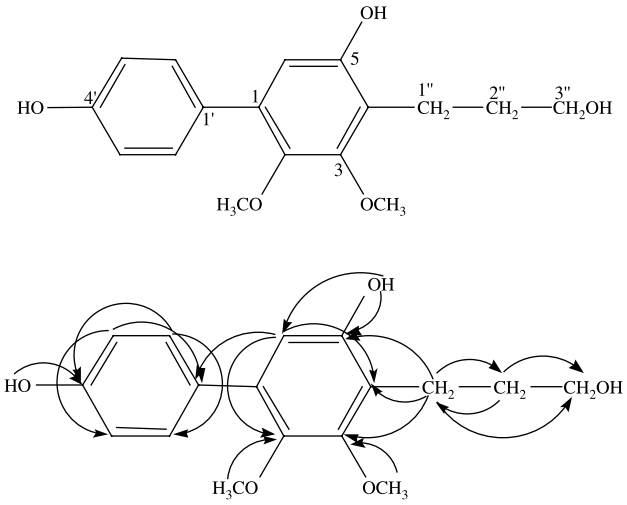

Figure 1. Structure and key HMBC correlations of compound **1**.

Table 2. Cytotoxicity of compounds **1**, **6**–**8**, **12** against A549, HepG2 and HT29 cancer cell lines.

	Growth inhibition constant (IC_{50})[a] (μM)		
Compound	*A549*	*HepG2*	*HT29*
Cyclophosphamide[b]	7.5 ± 1.8	5.7 ± 2.1	4.1 ± 1.6
1	10.1 ± 2.6	12.2 ± 2.3	8.9 ± 1.9
6	18.1 ± 2.4	9.5 ± 1.4	10.3 ± 2.0
7	17.6 ± 1.6	>50	10.8 ± 1.1
8	9.6 ± 1.6	13.6 ± 2.8	20.1 ± 2.3
12	17.3 ± 1.6	15.2 ± 1.3	11.1 ± 1.7

[a]IC_{50} is defined as the concentration that resulted in a 50% decrease in cell number and the results are means ± standard deviation of three independent replicates.
[b]Positive control substance.

3. Experimental

3.1 General experimental procedures

A Perkin–Elmer Spectrum GX instrument was used for IR spectrum experiments. NMR spectra were recorded on Bruker-ARX-400 spectrometer. HRESI-MS was recorded on a Bruker APEX II FT-ICR MS spectrometer. The chromatographic silica gel (200–300 mesh) was provided by Qingdao Ocean Chemical Factory. Sephadex LH-20 was purchased from Amersham Pharmacia Biotech. TLC analysis was performed on silica gel 60 F_{254} (Merck). All other chemicals and solvents used in this study were of reagent grade.

3.2 Fungal strain

A strain of the fungus *Penicillium thomi* was isolated from the root of *Bruguiera gymnorrhiza* in Guang Xi, and is stored at the Department of Biological Science and Biotechnology, Tsinghua University, Beijing, China.

3.3 Culture conditions

Starter cultures were maintained on Czapek agar. Plugs of agar supporting mycelial growth were cut and transferred aseptically to an Erlenmeyer flask (250 ml) containing 100 ml liquid medium (sucrose 30 g/L, $NaNO_3$ 3 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, KCl 0.5 g/L, $K_2HPO_4 \cdot 3H_2O$ 1.5 g/L, $FeSO_4 \cdot 7H_2O$ 0.06 g/L, pH 7.5) that was sterilised at 121°C for 30 min. The flask was then incubated at 25°C on a rotary shaker (120 rpm) for 3 days. The mycelium was subsequently aseptically transferred to Erlenmeyer flasks (500 ml) containing 200 ml of the same liquid medium. The flasks were then incubated at 25°C on a rotary shaker (120 rpm) for 5 days.

3.4 Extraction and isolation of metabolites

The growth culture (40 L) was filtered through cheesecloth. The culture broth was subsequently concentrated and then extracted with ethyl acetate and *n*-butanol for 3 times. Both extracts were concentrated by rotary evaporation and combined. After *in vitro* cytotoxicity screening using A549 cell line, ethyl acetate extract, which showed a cytotoxic

effect, was subjected to silica gel chromatography eluting with mixtures of chloroform and acetone (30:1, 10:1, 3:1, 1:1), to obtain fractions A–E. Among these, fractions C and D exhibited in vitro cytotoxic activity against A549 cell line.

Fraction A was chromatographed on silica gel using mixtures of petroleum and ethyl acetate (30:1, 10:1, 3:1) to give β-sitosterol (**2**, 13.2 mg) and cholesterol (**3**, 8.9 mg).

Fraction B was subjected to Sephadex LH-20 chromatography eluting with MeOH–H_2O (1:1) to give ergosta-7,22-diene-3β,5α,6β-triol (**4**, 21.3 mg) and (±)-1-monopalmitin (**9**, 10.9 mg).

Fraction C was chromatographed on silica gel using mixtures of petroleum and ethyl acetate (10:1, 5:1, 2:1) to obtain cyclo-(Ala-Gly) (**6**, 8.6 mg), cyclo-(Pro-Gly) (**7**, 12.1 mg), cyclo-(Ala-Pro) (**8**) and *N*-(2-hydroxy-4-methoxyphenyl)acetamide (**11**, 17.8 mg).

Fraction D was further chromatographed on Sephadex LH-20 eluting with MeOH–H_2O (2:1) to afford dibutylphthalate (**12**, 7.8 mg), 4′,5-dihydroxy-2,3-dimethoxy-4-(hydroxypropyl)-biphenyl (**1**, 12.1 mg) and uracil (**5**, 9.1 mg).

Fraction E was purified by Sephadex LH-20 eluting with MeOH/H_2O (2:1) to give succinic acid (**10**, 12.5 mg).

3.4.1 Compound 1. Colourless needles (12.1 mg), mp 205–207°C. IR (KBr) ν_{max} (cm^{-1}): 3413, 1600, 1517, 1447; HRESI-MS: *m/z* 304.1309 $[M]^+$ (calcd for $C_{17}H_{20}O_5$, 304.1311); ^1H NMR (DMSO-d_6, 400 MHz), ^{13}C NMR (DMSO-d_6, 100 MHz) data: see table 1.

3.4.2 Compounds 2–12. The ^1H NMR and ^{13}C NMR data were identical with those published in the literature.

3.5 Cell cultures

The human pulmonary adenocarcinoma cell line A549 and hepatic carcinoma cell line HepG2 were cultured in DMEM medium (high glucose), pH 7.3. Colorectal carcinoma cell line HT29 was cultured in PRIM-1640 medium, pH 7.3. Both cell cultures were supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% foetal calf serum (FCS). Cells were grown at 37°C under 5% CO_2 atmosphere. Culture media were changed every 2–3 days. When they reached confluence, cells were dissociated by 0.05% trypsin/0.02%EDTA and re-plated at 1:5 dilutions.

3.6 MTT assay

Cancer cells were seeded onto 96-well microtitre plates at 6×10^3 cells per well, and were pre-incubated for 24 h at 37°C. The medium was replaced with 180 μl fresh medium containing different concentrations of each isolated compound. The cells were then incubated at 37°C for 48 h, then 20 μl of MTT was added to each well. After incubation in 37°C for 4 h, the supernatants were removed and the formazan crystals were dissolved by adding 200 μl DMSO. The plate was then read on a microplate reader at 490 nm to evaluate the effects of the test compounds on cell growth. Experiments were conducted in triplicate.

References

[1] D.J. Faulkner. *Nat. Prod. Rep.*, **18**, 1 (2001).
[2] M. Isaka, C. Suyarnsestakorn, M. Tanticharoen. *J. Org. Chem.*, **67**, 1561 (2002).
[3] Y. Lin, X. Wu, S. Feng, G. Jiang, J. Luo, S. Zhou, L.L.P. Vrijmoed, E.B.G. Jones, K. Krohn, K. Steingrover, F. Zsila. *J. Org. Chem.*, **66**, 6252 (2001).
[4] H. Li, Y. Lin, J. Yao, L.L.P. Vrijmoed, E.B.G. Jones. *J. Asian Nat. Prod. Res.*, **6**, 185 (2004).
[5] W.B. Wysocki, C. Lester, A.B. Attygalle, G. Hrazdina. *Phytochemistry*, **50**, 231 (1999).
[6] G. Chen, S. Xu, H. Wang, R. Zhang. *J. Asian Nat. Prod. Res.*, **7**, 265 (2005).
[7] V. Piccialli, D. Sica. *J. Nat. Prod.*, **50**, 915 (1987).
[8] D. Yu, J. Yang. *Handbook of Analytical Chemistry (7)*, H. Ren, H. Tian (Eds.), p. 907, Chemical Industry Press, Beijing (2002).
[9] S. Nakatsuka, H. Miyazaki, T. Goto. *Tetrahedron Lett.*, **21**, 2817 (1980).
[10] W. Qin, Q. Kong, Z. Fan. *Chin. Trad. Herb. Drugs*, **15**, 10 (1984).
[11] Y. Gong. *^{13}CNMR Chemical Shifts of Natural Organic Compounds*, M. Wang (Ed.), p. 49, Yunnan Science and Technology Press, Kunming (1986).
[12] D.S. Jang, M. Cuendet, H.H. Fong, J.M. Pezzuto, A.D. Kinghorn. *J. Agric. Food Chem.*, **52**, 2218 (2004).
[13] Y. Zhang, T. Wang, X. Jiang, Y. Pei, X. Qi, H. Hua, B. Feng. *Chin. J. Med. Chem.*, **11**, 333 (2001).
[14] M.E. Savard, J.D. Miller, L.A. Blais. *Mycopathologia*, **127**, 19 (1994).
[15] S.C. Brauns, P. Milne, R. Naude, V.M. Van. *Anticancer Res.*, **24**, 1713 (2204).
[16] Y. Lin, S. Zhou. *Marine Microorganisms and their Metabolites*, Y. Yang (Ed.), pp. 416–417, Chemical Industry Press, Beijing (2003).