Available online at www.sciencedirect.com

SCIENCE DIRECT®

Phytochemistry 67 (2006) 728–734

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Dimeric progestins from rhizomes of *Ligusticum chuanxiong*

L.S. Lim [1], P. Shen [1], Y.H. Gong, E.L. Yong *

Department of Obstetrics and Gynecology, Yong Loo Lin School of Medicine, National University Hospital,
National University of Singapore, Lower Kent Ridge Road, 119074, Republic of Singapore

Received 16 September 2005; received in revised form 17 January 2006
Available online 3 March 2006

Abstract

Five dimeric phthalides were isolated from rhizomes of *Ligusticum chuanxiong* and their structures deduced based on spectral data. All compounds and their parent extracts were assessed for progesterone-like activity using a progesterone receptor driven reporter-gene bioassay. Among all the compounds, riligustilide, displayed weak progesterone-like activity ($EC_{50} \sim 81\ \mu M$), whereas, (3*Z*′)-(3a′*R*,6′*R*,3*R*,6*R*,7*R*)-3,8-dihydro-6.6′,7.3a′-diligustilide (M_r: 382, $EC_{50} \sim 90$ nM), was found to be a potent and specific activator of the progesterone receptor. Levistolide A, although having a very similar plenary structure, was inactive indicating the importance of stereochemistry of chiral centers and flexibility of butylidene side chain for progestogenic activity. These bioactive phthalides and their parent extracts ($EC_{50} \sim 8\ \mu g/ml$) may have utility for treatment of conditions requiring progesterone action.

Keywords: *Ligusticum chuanxiong*; Umbelliferae; Bioassay-guided isolation and structure elucidation; Dimeric phthalides; 3,8-Dihydro-diligustilide (**1**); 4,5-Dehydro-diligustilide (**2**); Levistolide A (**3**); Tokinolide B (**4**); Riligustilide (**5**)

1. Introduction

The dried rhizome of *Ligusticum chuanxiong* Hort., family Umbelliferae, is a very common crude drug in traditional Chinese, Japanese and Korean folk medicines (Li, 1991; Kee, 1999; Suk et al., 1974). Extracts of *L. chuanxiong* have been used, alone or in combination, for its "utero-tonic" effects and is prescribed for menstrual disorders such as amenorrhoea and dysmenorrhoea (Pharmacopoeia, 1997). Major fractions of *L. chuanxiong* are essential oils (monomeric and dimeric phthalides), alkaloids (tetramethylpyrazine), phenolic acids (ferulic acid, coniferylferulate), polysaccharides, and proteins (Zhang et al., 2003). Progesterone is a steroid hormone with an essential role in human reproduction. In the uterus, the hormone transforms endometrium from a proliferative to a secretory phase, and together with estradiol maintains endometrial integrity in the luteal phase of the menstrual cycle and in pregnancy (Li et al., 2004). Progesterone acts by binding specifically to the ligand binding domain of the progesterone receptor (PR), a member of the nuclear/steroid receptor superfamily of transcription factors (O'Malley, 2005), to activate expression of genes in target cells. Progestins, compounds with biological activity similar to progesterone, are approved for use in contraception, menopause hormone replacement therapy, and in gynecological conditions such as endometriosis, endometrial hyperplasia and endometrial cancer (Sitruk-Ware, 2004).

We hypothesize that Chinese medicinal herbs with "utero-tonic" properties may act through PR-signaling pathways. Screening of these herbs for progestogenic activity resulted in the identification of *L. chuanxiong*, whose hydrophobic fractions exhibited potent progestogenic activity. Fractionation of the extract resulted in isolation and structural characterization of five phthalides. We deduced the structure and determined the biological activities of 3,8-dihydro-diligustilide, a potent progestin with a unique dimeric structure.

---

* Corresponding author. Tel.: +65 6772 4261; fax: +65 6779 4753.
E-mail address: obgyel@nus.edu.sg (E.L. Yong).

[1] Joint first authors.

0031-9422/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2006.01.024

2. Results and discussion

2.1. Screening of Chinese medicinal herbs for progestogenic activity

Thirteen herbs described by contemporary (Pharmacopoeia, 1997) and ancient texts (Li, 1991) as having "uterotonic" properties, or prescribed for "menstrual support" were examined for progestogenic activity with the PR-driven reporter gene assay (Fig. 1a). EtOH extracts of the dried rhizome of *L. chuanxiong* demonstrated strongest progestogenic activity, reaching 80% of that observed with progesterone (100 nM) and had an EC_{50} of 8 μg/ml (Fig. 1b). The bioactivity of *L. chuanxiong* extract was specific to the PR, and the extract was at least 200-fold less active on other related members of the steroid receptor family like androgen, estrogen and glucocorticoid receptors. Bioactivity of *L. chuanxiong* extract, like progesterone itself, can be competed by the specific PR antagonist RU486, indicating that the bioactive compounds bind to the same canonical PR ligand binding domain as the native ligand (Fig. 1c).

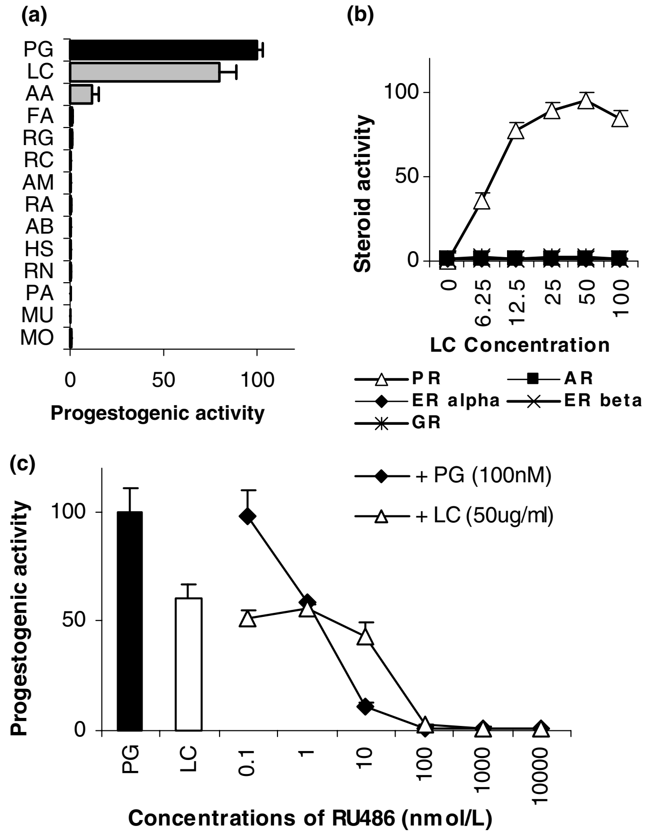


Fig. 1. Identification and characterization of the progestogenic properties of extracts from *L. chuanxiong*. (a) Screening of herbal extracts for progestogenic activity. Progestogenic activity was expressed as a percentage of progesterone (PG) at 100 nM. LC, *L. chuanxiong*; AA, *Folium artemisiae argyi*; FA, *Fructus aurantii*; RG, *Radix glycyrrhizae*; RC, *Rhizoma corydalis*; AM, *Rhizoma atractylodis marcocephalae*; RA, *Radix astragali*; AB, *Radix achyranthis bidentatae*; HS, *Herba schizonepetae*; RN, *Rhizoma seu radix notopterygii*; PA, *Radix peaoniae alba*; MU, *Concha margaritifera usta*; MO, *Cortex magnoliae officinalis*. (b) Effect of *L. chuanxiong* extract on PR, GR, AR, ERα- and ERβ-mediated transcription. Hormonal effects of *L. chuanxiong* (μg/ml) were expressed as percentage of that observed with of saturating doses of natural ligands for PR (progesterone, 100 nM), AR (dihydrotestosterone, 1 nM), ERα and ERβ (estradiol, 1 nM) and GR (hydrocortisone, 1 nM). (c) Effect of the specific progesterone antagonist, RU486, on activity of *L. chuanxiong* (LC). Indicated doses of RU 486 were added to cells exposed to progesterone (PG) or LC and progestogenic activity measured as in (b). Experiments were performed at least twice and representative figures are shown. All data points (means ± SEM) were in triplicate.

2.2. Chemical characterization

EtOH extracts of *L. chuanxiong* were subjected sequentially to solvent/solvent partition, medium-pressure liquid chromatographic (MPLC)/reverse phase C18 separation, high speed counter-current chromatography, and preparative HPLC to yield **1**, **2**, **3**, **4**, and **5** (Fig. 2).

Compound **1** was isolated as a colorless powder from a bioactive HPLC subfraction. Its APCIMS spectrum (positive mode), similar to a known dimeric phthalide senkyunolide P (Naito et al., 1991) demonstrating a molecular ion peak at *m*/*z* 383 [$(M+H)^+$], consistent with the molecule formula of $C_{24}H_{30}O_4$. The MS/MS spectrum of the molecular ion *m*/*z* 383 presented two strong product ions at *m*/*z* 193 and *m*/*z* 191, both with the same intensity. The MS/MS/MS spectrum of product ion *m*/*z* 193, presented ions at *m*/*z* 175 ($-H_2O$), 147 ($-H_2O$, –CO), 137 ($-C_4H_8$), 105 ($-H_2O$, –CO, $-C_3H_6$) and 91 ($-H_2O$, –CO, $-C_4H_8$); while the MS/MS/MS spectrum of product ion *m*/*z* 191, similar to *Z*-ligustilide (98% purity, from ChromaDex), presented ions at *m*/*z* 173, 145, 135, 117 and 91. It is tempting to speculate that such a phthalic dimer may be formed with sedanenolide (MW 192) (Lin et al., 1998) and liguistilide (MW 190) (Lin et al., 1998) through Diels–Alder cycloaddition (Kaouadji et al., 1983). Compared to 6.6′,7.3a′-diligustilide (levistolide A) (Naito et al., 1991) which has three olefinic signals resonating at

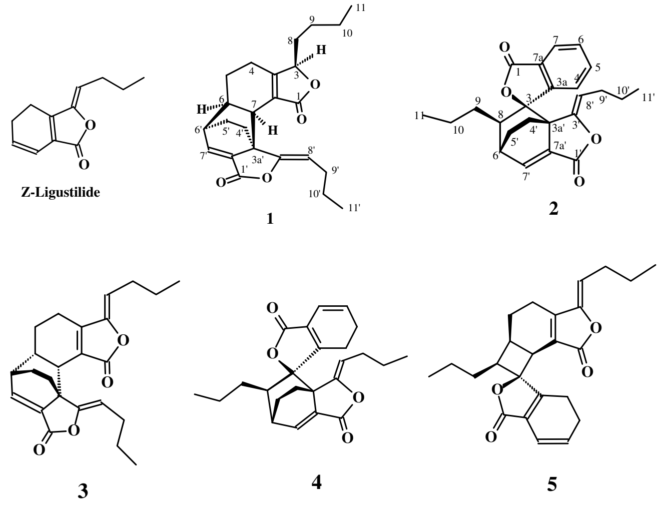
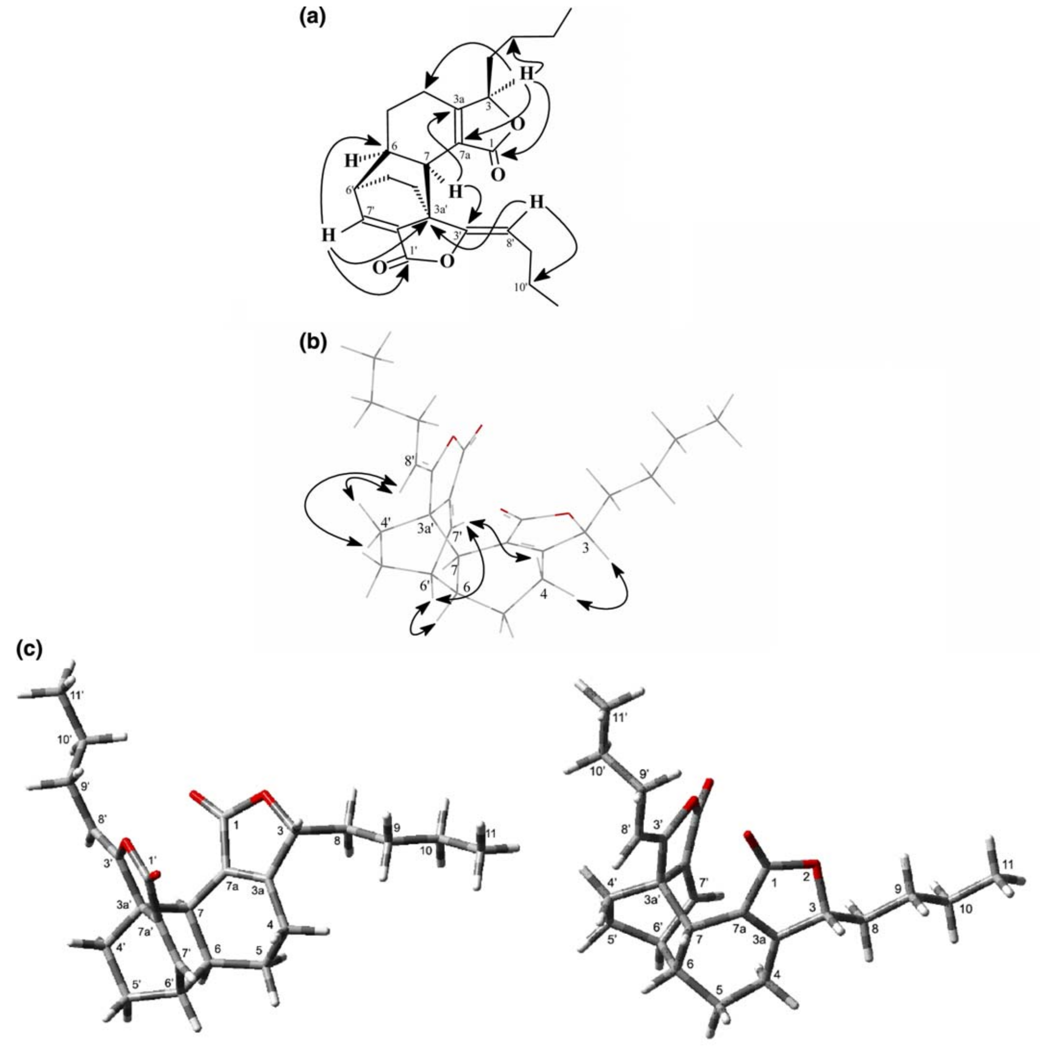


Fig. 2. Phthalides from *L. chuanxiong*.

δ_H 5.07, *t*, H-8; δ_H 5.00, *t*, H-8′; and δ_H 7.35, *d*, H-7′, the ^{1}H NMR spectrum of **1** only showed the signals of two olefinic protons (δ_H 7.33, 1H, *d*, $J = 6.5$ Hz, H-7′; and δ_H 5.00, 1H, *t*, $J = 7.85$ Hz, H-8′). Additional signals appeared at δ_H 4.80 (1H, *m*) and between δ_H 1.10–1.36. The ^{13}C NMR and HMQC spectra showed a CH carbon signal at δ_C 81.3 and CH_2 signal at δ_C 26.3 and no carbon signals were demonstrated at around δ_C 154.9 and 112.1 (levistolide A) (Naito et al., 1991), suggesting **1** may be a hydrogenated derivative of 6.6′,7.3a′-diligustilide. The proton signals at δ_H 5.00 and 7.33 were assigned to the olefinic proton of butylidene residue (H-8′) and the olefinic proton (H-7′) of benzofuranone ring, respectively. This was achieved by combining the analysis of 1D and 2D NMR spectra of **1**, in particular, coupling constants (*J*), peak splitting profiles (*t* and *d*), COSY correlations with neighbor proton signals (H-8′ with H-9′ at δ_H 2.13–2.23; H-7′ with H-6′ at δ_H 2.98), and crossing peaks in its HMBC spectrum (H-8′ with C-10′, 3a′; H-7′ with C-1′,C-3a′,C-5′,C-6) (Fig. 3a). If hydrogenation occurred on the double bond between 3′ and 8′, the typical C-3′ signals would appear at around δ_C 84.3 (3′*R*) and 90 (3′*S*) (Rios et al., 1998). However, the ^{13}C NMR spectra of **1** did not show such a carbon signal at the above two areas. Based on the above analysis, 1D and 2D NMR data (HMQC, COSY, HMBC and NOESY), and comparison with the literatures (Naito et al., 1991; Rios et al., 1998; Delgado et al., 1988), the planar structure of **1** was suggested to be 3,8-dihydro-6.6′,7.3a′-diligustilide.

If **1** was predicted to be a 3,8-dihydrogenated levistolide A, **1** would have a stereochemistry of C-3a′*S*,6′*S*,6*S* and 7*S*, and the configuration of C-3 would be *R* or *S*. The H-3 signal of a related compound (3*S*)-3,8-dihyro-diligustilide has been reported to resonate at δ_H 4.54 (Delgado et al., 1988). However, the H-3 signal of **1** was shifted down to δ_H 4.80 in its 1H spectrum, and the NOEs in the NOESY spectrum of **1**, were observed between H-3/H-4 *eq* (δ_H 1.38), H-7′ (δ_H 7.33)/H-4 *ax* (δ_H 2.04), H6′ (δ_H



1a **1b**

Fig. 3. (a) Selected HMBC correlations for **1**. (b) Selected NOESY correlations for **2**. (c) Model **1a** and **1b**.

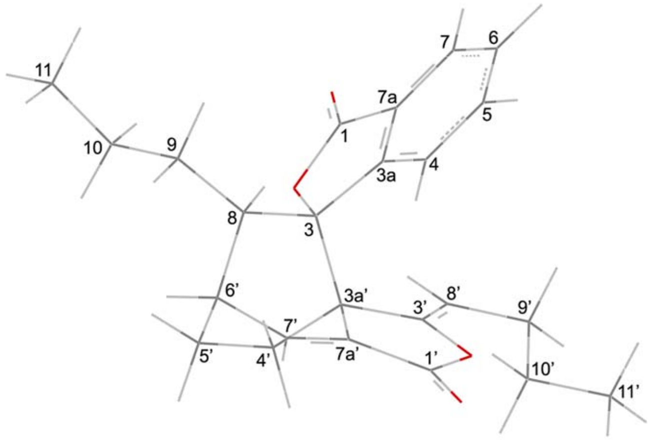
2.98)/H-7′, and H-7 (δ_{H} 3.21)/H-6 (δ_{H} 2.54) (Fig. 3b), but not between H-3/H-4 *ax*, indicating that **1** is neither of 3*S* nor 3*R*-3,8-dihydro-levistolide A (model 1a) (Fig. 3c), and may have different stereochemistry on C-3a′, 6′, 6 and 7 (model 1b) (Fig. 3c). As a Diels–Alder cycloaddition can result in either *endo* or *exo* product as cited in our models of 1a (*endo*) or 1b (*exo*). In model 1b, H-3 could only have NOE with H-4 *eq*, but not H-4 *ax*, and the latter could show NOE with H-7; the NOEs between H-8′/H-4′ (*ax* and *eq*), H-8′/H-7, and H-6′/H-6 would be the same as model 1a. Generally, model 1b was consistent with our NMR data (1D and 2D), and the relative stereochemistry and structure of the progestogenic compound **1** was suggested to be (3*Z*′)-(3a′*R*,6′*R*,3*R*,6*R*,7*R*)-3,8-dihydro-6.6′,7.3a′-diligustilide (Fig. 2, Table 1). Crystal X-ray diffraction would have to be conducted to provide absolute stereochemistry.

From the same fraction, compound **2** was isolated as colorless powder with a molecular ion peak at *m*/*z* 379 [(M+H)$^{+}$] in its APCIMS spectrum (positive mode). MS/MS/MS spectrometry and NMR analysis indicated the molecular formula to be $C_{24}H_{26}O_{4}$. The existence of a benzene ring in the molecule was indicated by the four olefinic signals resonating at δ_{H} 7.09 (1H, *d*, $J = 7.4$ Hz), 7.56 (1H, *t*, $J = 7.9$ Hz), 7.48 (1H, *t*, $J = 7.9$ Hz) and 7.79 (1H, *d*, $J = 7.9$ Hz) in the downfield of the ^{1}H NMR spectrum, and the COSY correlations between δ_{H} 7.09/δ_{H} 7.56, δ_{H} 7.56 and δ_{H} 7.48, δ_{H} 7.48 and δ_{H} 7.79. In addition, another two olefinic proton signals (δ_{H} 4.56, dd, $J = 1.9$, 6.9 Hz; δ_{H} 7.68, *d*, $J = 6.5$ Hz), two methyl proton signals (δ_{H} 0.79, 3H, *t*, $J = 7.4$ Hz; δ_{H} 0.55, 3H, *t*, $J = 7.4$ Hz) together with two carboxyl carbon signals (δ_{C} 169.5; δ_{C} 164.2) suggested that **2** could be a phthalic dimer formed by 3-butylidenephthalide (Lin et al., 1998) and liguistilide through Diels–Alder cycloaddition (Kaouadji et al., 1983). The olefinic signal at δ_{H} 4.56 and δ_{H} 7.68 were later assigned to H-8′ and H-7′, by their respective COSY correlation with neighboring proton H-9′ (δ_{H} 1.72–1.77, 1H, *m*, δ_{H} 1.81–1.88, 1H, *m*), H-6′ (δ_{H} 3.16, 1H, *br.d*), and HMBC crossing peaks (H-8′ with C-3a′ at δ_{C} 51.1, C-10′ at δ_{C} 22.1; H-7′ with C-1′ at δ_{C} 164.2, C-3a′, C-8 at δ_{C} 47.2, and C-4′ at δ_{C} 27.4). In the NOESY spectrum of **2**, NOE between H-8′ and H-4′ax (δ_{H} 2.52–2.58, 1H, *m*) indicated the *Z* form of butylidene residue; NOEs between H-8 (δ_{H} 1.92–1.96, 1H, *m*) and H-4 (7.09), H-7′ and H-6′, suggested the *exo* (rather than *endo*) orientation of Diels–Alder adduct, as depicted in model **2a** (Fig. 4). Further crystal X-ray information would be needed to elucidate the absolute configurations of the chiral centers and support our hypothesis predicted in **2a**. Based on the above analysis, the structure and relative stereochemistry of **2** was suggested as (3*Z*′)-(3*S*,8*R*,3a′*S*,6′*R*)-4,5-dehydro-3.3a′,8.6′-diligustilide (Fig. 2, Table 1).

Another three phthalide dimers (**3**, **4** and **5**) (Fig. 2) were isolated and identified as levistolide A, tokinolide B and riligustilide, respectively, based on physical properties and comparisons with published spectroscopic data (Delgado et al., 1988; Naito et al., 1991; Zhang et al., 2003).

Table 1
^{1}H NMR and ^{13}C NMR data of compounds **1** and **2** ($CDCL_{3}$, δ in ppm, *J* in Hz)

C/H number	Compound **1**		Compound **2**	
	δ_{C}	δ_{H}	δ_{C}	δ_{H}
1	171.9		169.5	
3	81.3	4.80 (1H, *m*)	88.6	
3a	167.8		152.5	
4	31.1	2.04 (1H, *m*), 1.29–1.40 (1H, *m*)	121.0	7.09 (1H, *d*, $J = 7.4$)
5	29.2	1.50–1.60 (1H, *m*), 1.85–1.93 (1H, *m*)	134.8	7.56 (1H, *t*, $J = 7.9$)
6	38.2	2.54 (1H, *m*)	129.5	7.48 (1H, *t*, $J = 7.9$)
7	41.0	3.21 (1H, *m*)	125.2	7.79 (1H, *d*, $J = 7.9$)
7a	127.6		126.2	
8	26.3	1.10–1.21(1H, *m*), 1.23–1.35 (1H, *m*)	47.2	1.92–1.96 (1H, *m*)
9	26.2	1.22–1.34 (2H, *m*)	28.9	1.46–1.51 (1H, *m*), 1.36–1.38 (1H, *m*)
10	22.4	1.20–1.39 (2H, *m*)	21.1	1.18–1.22 (1H, *m*), 0.99–1.02 (1H, *m*)
11	13.9	0.93 (3H, *t*, $J = 7.3$)	14.0	0.79 (3H, *t*, $J = 7.4$)
1′	165.0		164.2	
3′	150.4		148.4	
3a′	47.4		51.1	
4′	31.0	1.27–1.51 (1H, *m*), 2.05 (1H, *m*)	27.4	2.52–2.58 (1H, *m*), 1.36–1.38 (1H, *m*)
5′	25.5	1.31 (1H, *m*), 1.89 (1H, *m*)	17.2	2.04–2.17 (1H, *m*), 1.36–1.38 (1H, *m*)
6′	41.6	2.98 (1H, *m*)	37.2	3.16 (1H, *br.d*)
7′	142.0	7.33 (1H, *d*, $J = 6.5$)	145.3	7.68 (1H, *d*, $J = 6.5$)
7a′	134.4		133.2	
8′	108.8	5.00 (1H, *t*, $J = 7.85$)	107.2	4.56 (1H, td, $J = 6.9$, 1.9)
9′	27.4	2.13–2.23 (2H, *m*)	26.7	1.72–1.77 (1H, *m*), 1.81–1.88 (1H, *m*)
10′	22.3	1.37–1.51 (2H, *m*)	22.1	0.97–1.00 (1H, *m*)
11′	13.8	0.93 (3H, *t*, $J = 7.4$)	13.2	0.55 (3H, *t*, $J = 7.4$)



2a

Fig. 4. Model **2a**.

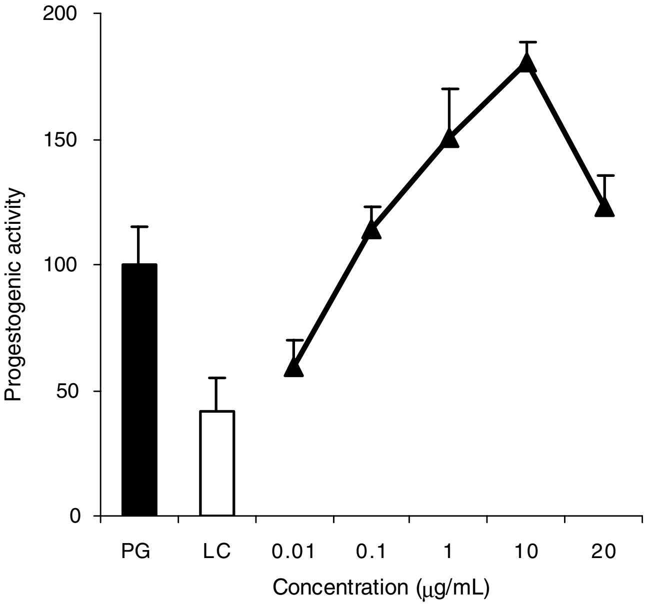


Fig. 5. Progestogenic activity of 3,8-dihydro-diligustilide. Cells were exposed to increasing concentrations of 3,8-dihydro-diligustilide and progesterone activity measured as described in methods and expressed as a percentage of progesterone (PG) 100 nM. All data points (means ± SEM) were in triplicate. LC, EtOH extract of *L. chuanxiong* (50 µg/ml).

2.3. Characterization of progestogenic activity

Phthalide dimers have been isolated as natural active ingredients of Umbelliferae plants for more than 20 years (Delgado et al., 1988; Kaouadji et al., 1983; Kaouadji et al., 1986; Kobayashi and Mitsuhashi, 1987; Naito et al., 1991), When we tested the progestogenic activity of LC exacts prepared with various methods, including soaking (room temperature) and refluxing (heating at boiling point of EtOH) herbal materials with ethanol, although the yield and activity profile varied a bit, the active components are the same as analyzed by LC/MS/MS, indicating **1**–**5** occurred as racemates in nature (see Fig. 5).

Among the five pure phthalides, **1** showed the most potent progestogenic activity (Table 2). Compound **1** is present in less than 0.02% of the crude extract, but had maximal activity that was 180% of 100 nM progesterone (Fig. 3). Unlike known progestins, **1** is non-steroidal, had a unique dimeric isobenzofuranone skeleton and is a dimer of the common *L. chuanxiong* phthalide, *Z*-ligustilide (Fig. 2). Compound **1** was highly potent with an EC_{50} of 91 nM comparable to standard progestogenic lead compounds (Table 2). In comparison, riligustilide (**5**) (Fig. 2) was much less potent (EC_{50} of 81 µM) and efficacious (maximal activity 15% that of 100 nM progesterone) (Table 2). Interestingly, levistolide A (**3**), although having a similar plenary structure, was inactive (Table 2), indicating the importance for stereochemistry of the chiral centers and flexibility of butylidene side chain at position C3 for binding to PR ligand binding domain. Tokinolide B (**4**) and compound **2** did not display any progestogenic properties and their activities were similar to vehicle (Table 2).

3. Conclusions

Although *L. chuanxiong* and its extracts have centuries of documented use for gynecological conditions (Li, 1991), its mechanisms of action were unclear. Our studies indicate that its "utero-tonic" effects could be mediated through PR-signaling mechanisms. Active fractions of the

Table 2
Compounds isolated from *L. chuanxiong* and their bioactivities compared to reference progestins

	M_r	Structure	E_{max} (% of 100 nM progesterone)	EC_{50}
L. chuanxiong (EtOH extract)		Mixture	95	7 µg/ml
3,8-Dihydro-diligustilide (**1**)	382	Dimer	180	91 nM
4,5-Dehydro-diligustilide (**2**)	378	Dimer	NSA	NSA
Levistolide A (**3**)	380	Dimer	NSA	NSA
Tokinolide B (**4**)	380	Dimer	NSA	NSA
Riligustilide (**5**)	380	Dimer	15	81 µM
3-Butylpthalide	190	Monomer	NSA	NSA
Progesterone	314	Phenanthrene ring	177	3 nM
6-Imidazol-1-yl-1-isopropyl-1*H*-benzoimdazole-2-thiol[a]	258	Benzimidazole-2-thione	93	53 nM

E_{max}: maximal steroidal activity; NSA, no significant activity.

[a] Synthetic compound (Dong et al., 2004).

herb exerted strong ($EC_{50} \sim 8$ μg/ml) and specific progestogenic activity due to the presence of 3,8-dihydro-diligustilide (**1**) and riligustilide (**5**). To our knowledge, 3,8-dihydro-diligustilide (**1**) and riligustilide (**5**) are the first progestins of botanical origin to be characterized. 3,8-Dihydro-diligustilide had a unique dimeric structure, was potent with EC_{50} in the nanomolar range, and had maximal activity comparable to progesterone itself. In comparison riligustilide, had an EC_{50} that was 1000-fold weaker and peak activity that was an order of magnitude lower. Further studies are required to determine whether 3,8-dihydro-diligustilide or its derivatives have any selective progesterone modulatory properties (Chabbert-Buffet et al., 2005) with favorable risk benefit profiles (Koh and Sakuma, 2004). Such compounds are required for hormone replacement therapy and other progesterone-dependent gynecological conditions such as menopause, contraception, menstrual bleeding and endometrial cancer (Turgeon et al., 2004). The discovery of these bioactive phthalides suggests the possibility that consumption of these compounds, their parent herb, or its hydrophobic essential oil fractions, may confer benefits for conditions requiring progesterone action.

4. Experimental

4.1. General experimental procedures

MPLC separations were performed using a reverse phase C18 column (LiChrospher C18, MERCK, 5 μm, 100 Å, 10 × 23 cm), a Diol column (LiChrospher Diol, MERCK, 5 μm, 100 Å, 3.6 × 23 cm) and MPLC system (Buchii, Flawil, Switzerland). High-speed counter-current chromatography was done using a CCC-Model 100 system (Pharma-Tech Research Corp., Baltimore, MA) with Hexane as stationary phase and EtOH/water as mobile phase. Preparative HPLC was done using a Hypersil ODS C18 column (150 mm × 21.2 mm, 5 μm) and Jasco preparative HPLC system (JASCO PU-1586 intelligent Prep. Pump and MD-1510 Multiwavelength Detector, Japan); NMR spectra were obtained on a Bruker instrument equipped with a 5 mm ^{1}H and ^{13}C probe operating at 500 and 125 MHz, respectively, with TMS as internal standard. LC/MS study was performed on Agilent ion trap 1100 LC/MSD (Agilent, USA) system with a Cadenza CD-C18 column (150 × 2 mm, Silvertone Sciences, Philadelphia, PA, USA). Chemical structures were drawn with ChemWindow (Bio-Rad Laboratory, USA), and stereochemistry models were generated with Gauss View 2.1 (Gaussion, Inc., USA).

4.2. Plant materials

Rhizomes of *L. chuanxiong*, were purchased from a commercial retailer, Eu Yan Sang International, Singapore. Voucher specimens (SBG-LC-040720) were deposited in the The Singapore Herbarium, Botanic Gardens, National Parks Board, Republic of Singapore. Herbs were extracted with EtOH and screened for progestogenic activity at a final concentration of 50 μg/ml in HeLa cells.

4.3. Measurement of steroidogenic activity

Plasmid constructs encoding the PR, androgen receptor (AR), and glucocorticoid receptor (GR) and HRE2-Luc, containing two tandem copies of the consensus hormone-response element from the human aminotransferase gene driving the luciferase reporter has previously been described (Loy et al., 2003). Transient transfections for plasmid encoding PR, AR, GR and estrogen receptors (ER) were performed as previously described (Wang et al., 2001). Plasmids encoding PR, GR or AR were co-expressed with HRE2-Luc in HeLa cells to measure respective hormonal activities. Estrogenic activity was measured by expressing ERα and ERβ in the presence of the estrogen-responsive MMTV-ERE-Luc reporter gene. To increase the assay sensitivity, HeLa cells were pre-exposed to charcoal-stripped serum in phenol free RPMI for at least 7 days prior to performing the assay. After transfection, the media was replaced with fresh hormone-depleted media containing the desired concentrations of test compounds. Luciferase induction responses for each treatment group were expressed as fold-increase compared to vehicle treated cells. All data points represented the means ± SEM of at least three replicates. To ensure comparability between assays, results were expressed as a percentage of progesterone (100 nM).

4.4. Bioassay-guided extraction and isolation

Raw rhizomes of *L. chuanxiong* (10 kg) were ground and refluxed with 100% EtOH and 1 kg crude extract was obtained. Then the EtOH extract was suspended in water and partitioned with dichloromethane (DCM) to give 400 g of non-aqueous layer (DCM layer). As the dichloromethane fraction exhibited strong progestogenic activity, it was subjected to MPLC extraction with a dry-pack C18 column eluting with MeOH and water. Fractions eluted with 70–90% MeOH, had highest progestogenic activity, and this procedure was repeated 10 times to collect sufficient material (1.2 g) for another MPLC separation (600 mg) and high speed counter-current chromatographic extraction (600 mg). One hundred and twenty fractions were collected from the next MPLC Diol column chromatography by a mixture of *iso*-PrOH and hexane (5–95%, 2.4 L total over flow rate of 20 ml/min) and only fraction 25–28 was tested to be active. Fractions 25–28 was further purified by a preparative C18 HPLC column under gradient conditions of MeOH in water (60–90%). HPLC fraction 10 was tested to have the most potent progestogenic activity and yielded Compounds **1** (0.8 mg) and**2** (0.5 mg). Compounds **3** (2 mg) and **4** (1 mg) were isolated from HPLC fraction 12, which was non-active. Six fractions (C-fractions) were collected from counter-current chromatographic fractionation, dried and analyzed for progestogenic activity. From C-fraction 9 (50 mg), nice white needles (compound **5**) were crystallized (15 mg) and later characterized as riligustilde. C-fraction 8 (20 mg) showed the highest progestogenic activity (20 mg) and was subjected to preparative HPLC using a C18 column with a mobile phase comprising of 60–90% MeOH and a flow rate 5 ml/min over 90 min. Four subfractions were collected with maximum bioactivity residing in subfraction 3 (5 mg). Sub-fraction 3 was dried subjected to a further LC/MS analysis under an isocratic mode of 65% MeOH:35% water with a flow rate 0.2 ml/min. Two peaks were baseline separated and a colorless powder (peak I) with strong progestogenic activity was eventually isolated at >90% purity (0.5 mg), which is the same compound **1** as obtained from preparative HPLC separation. Peak II (0.9 mg) did not show any progestogenic activity and later was assigned to be compound **2** as obtain from preparative HPLC separation.

Acknowledgements

We are deeply grateful to Dr. Mark Butler for his help in the isolation of progestogenic compounds for *L. chuanxiong* extract. Supported by NMRC/03may047 from National Medical Research Council of Singapore and NUS Academic Research Fund, Ministry of Education, Singapore. ELY is a BMRC Clinician-Scientist Investigator.

References

Chabbert-Buffet, N., Meduri, G., Bouchard, P., Spitz, I.M., 2005. Selective progesterone receptor modulators and progesterone antagonists: mechanisms of action and clinical applications. Hum. Reprod. Update 11, 293–307.

Delgado, G., Reza-Garduno, R.G., Toscano, R.A., Bye, R., Linares, E., 1988. Secondary metabolites from the roots of *Ligusticum porteri* (Umbelliferae). X-ray structure of *z*-6.6′,7.*S*a′-diligustilide. Heterocycles 27, 1305–1312.

Dong, Y., Roberge, J.Y., Wang, Z., Wang, X., Tamasi, J., Dell, V., Golla, R., Corte, J.R., Liu, Y., et al., 2004. Characterization of a new class of selective nonsteroidal progesterone receptor agonists. Steroids 69, 201–217.

Kaouadji, M., Reutenauer, H., Chulia, A.J., Marsura, A., 1983. (*Z*,*Z*′) diligustilide, nouveau phtalide dimere isole de *Ligusticum wallichii* franch. Tetrahedron Lett. 24, 4677–4678.

Kaouadji, M., Pachtere, F.D., Pouget, C., Chulia, A.J., 1986. Three additional phthalide derivatives, an epoxymonomer and two dimers, from *Ligusticum wallichii* rhizomes. J. Nat. Prod. 49, 872–877.

Kee, C.H., 1999. The Pharmacology of Chinese Herbs, second ed. CRC Press, New York, pp. 94.

Kobayashi, M., Mitsuhashi, H., 1987. Studies on the constituents of Umbelliferae plants. XVII. Structrues of three new ligustilide derivatives from *Ligusticum wallichii*. Chem. Pharm. Bull. 35, 4789–4792.

Koh, K.K., Sakuma, I., 2004. Should progestins be blamed for the failure of hormone replacement therapy to reduce cardiovascular events in randomized controlled trials? Arterioscler. Thromb. Vasc. Biol. 24, 1171–1179.

Li, S.C., 1991. Compendium of Materia Medica [Bai Hua Ben Cao Gang Mu]. Xue Yuan Publishing House, Beijing, pp. 817.

Li, X., Lonard, D.M., O'Malley, B.W., 2004. A contemporary understanding of progesterone receptor function. Mech. Ageing Dev. 125, 669–678.

Lin, L.Z., He, X.G., Lian, L.Z., King, W., Elliott, J., 1998. Liquid chromatographic-electrospray mass spectrometric study of the phthalides of *Angelica sinensis* and chemical changes of *z*-ligustilide. J. Chromatogr. A 810, 71–79.

Loy, C.J., Sim, K.S., Yong, E.L., 2003. Filamin-a fragment localizes to the nucleus to regulate androgen receptor and coactivator functions. Proc. Natl. Acad. Sci. USA 100, 4562–4567.

Naito, T., Katsuhara, T., Niitsu, K., Ikeya, Y., Okada, M., Mitsuhashi, L.H., 1991. Phthalide dimers from *Ligusticum chuangxiong* hort. Heterocycles 32, 2433–2442.

O'Malley, B.W., 2005. A life-long search for the molecular pathways of steroid hormone action. Mol. Endocrinol. 19, 1402–1411.

Pharmacopoeia of the People's Republic of China, 1997. English edition, vol. 1. Chemical Industry Press, Beijing.

Rios, Y.M., Delgado, G., Toscano, R.A, 1998. Chemical reactivity of phthalides. Relay synthesis of diligustilide, rel-(3′*R*)-3′,8′-dihyrodiligustilide and wallichilide. Tetrahedron 54, 3355–3366.

Sitruk-Ware, R., 2004. Pharmacological profile of progestins. Maturitas 47, 277–283.

Suk, K.T., Nitta, A., Konoshima, M., 1974. Studies on origin of Japanese chuanxiong. Iv. *Ligusticum and Tilingia*. Yakugaku Zasshi 94, 1257–1264.

Turgeon, J.L., McDonnell, D.P., Martin, K.A., Wise, P.M., 2004. Hormone therapy: physiological complexity belies therapeutic simplicity. Science 304, 1269–1273.

Wang, Q., Lu, J., Yong, E.L., 2001. Ligand- and coactivator-mediated transactivation function (af2) of the androgen receptor ligand-binding domain is inhibited by the cognate hinge region. J. Biol. Chem. 276, 7493–7499.

Zhang, X., Xiao, H., Xu, Q., Li, X., Wang, J., Liang, X., 2003. Characterization of phthalides in *Ligusticum chuanxiong* by liquid chromatographic-atmospheric pressure chemical ionization-mass spectrometry. J. Chromatogr. Sci. 41, 428–433.