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藁本内酯的稳定性及其主要转化产物研究

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摘 要:藁本内酯是川芎和当归的主要有效成分,其化学结构的不稳定性限制了它的进一步研究和临床应用。本实验通过色谱方法对其室温静置一个月后的产物进行制备,得到了14个藁本内酯的转化产物,并通过ESI-MS、NMR等光谱分析法确定了其结构,包括一个新化合物7′-carboxyl-wallichilide(1)。利用LC-MS峰面积归一化法,确定了藁本内酯及其转化产物的相对含量,阐明了藁本内酯的主要转化产物为其二聚体。

关键词:藁本内酯;稳定性;转化产物

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Study on the Stability of Ligustilide and Its Main Transformation Products

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Abstract: Ligustilide is one of main active components from Rhizoma Chuanxiong and Radix Angelicae Sinensis. However, the instability of ligustilide limits its further research and application in clinics. In this study, the transformation products of ligustilide were prepared, after one month at room temperature, by conventional chromatography and spectroscopic methods. Fourteen transformation products were obtained and elucidated, which included a new dimerphthalide, namely, 7′-carboxyl-wallichilide (1). The relative contents of ligustilide and its fourteen transformation products were determined by area normalization using LC-MS. The results showed that ligustilide dimers were the main transformation products of ligustilide.

Key words: ligustilide; stability; transformation products

Introduction

Ligustilide (Fig. 1) was isolated from the root of *Ligusticum acutilobum* Sieb. et Zucc. for the first time by Mitsuhashi *et al.* in 1960 [1], and they further elucidated its chemical structure in the following year [2]. Since then, ligustilide has demonstrated a wide range of pharmacological activities against migraines, oxidative damage and cardiovascular disease. Existing research has shown ligustilide to significantly protect the brain from damage induced by transient forebrain cerebral ischemia because of its antioxidant and anti-apoptotic properties [3]. Vasorelaxation activities of ligustilide were determined in contractions to various contractile agents in rat-isolated aorta [4]. Moreover, ligustilide also possesses antinociceptive and anti-inflammatory properties, and therefore has potential to be developed into an effective drug for the treatment of various pain syndromes, including primary dysmenorrhoea [5].

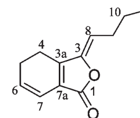

Fig. 1 Chemical structure of ligustilide

Until now, it is clear that ligustilide is one of the main effective components from Rhizoma Chuanxiong and Radix Angelicae Sinensis. However, the instability of ligustilide limits its further research and application in clinics. Ligustilide is an unsaturated phthalide with a 3-butenyl group, and thus likely to isomerize to other sim-

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ilar phthalides by isomerization reactions [6]. Stability studies on ligustilide showed a decrease from 99.48% to 41.97%, when stored at room temperature for 15 days and none detected after another 15 days [6]. Studies also showed that the content of ligustilide in Rhizoma Chuanxiong herbs also decreased significantly when dried at 60 ℃ or under direct sunlight [7].

Gas chromatography-mass spectrometry (GC-MS) has previously been employed to measure the stability of ligustilide [8]. With reference to this literature, we studied the transformation products of ligustilide. Using GC-MS, 19 transformation products were selected and cross-referenced with online NIST databases. Strikingly, some discrepancies arose. For example, the database suggested that one of the transformation products was 5,7,8-trimethyl-2-chromanone ($C_{12}H_{14}O_2$, M = 190.1); however, when we compared our data with reference substances, we noticed it was butylphthalide that had the same molecular weight. Therefore, GC-MS alone may not provide credible or comprehensive analysis results, and a considerable portion of transformation products cannot be confirmed based on limited database and reference substances.

Based on the above reasons, in this study we investigated the transformation products of ligustilide by conventional chromatography and spectral identification methods. Ligustilide was dissolved in 90% aqueous methanol and placed at room temperature for one month. It was then subjected to column chromatography on silica gel, RP-18 and Sephadex LH-20, using various solvent systems that afforded fourteen compounds, which included: 7′-carboxyl-wallichilide (**1**), a new ligustilide dimmer, seven known ligustilide dimmers and six known ligustilide analogues. By comparing physical and spectroscopic data with the reported data, the known compounds were identified as: Z-butylidenephthalide (**2**)[9], butylphthalide (**3**)[10], senkyunolide A (**4**)[11], 3′,6,8′,3a-diligustilide (**5**)[12], chuanxiongnolide A (**6**)[13], levistolide A (**7**)[14], Z-3′,8′,3′a,7′a-tetrahydro-6,3′,7′,7′a-diligustilide-8′-one (**8**)[15], tokinolide B (**9**)[16], exo-Z,Z′-3a,7′a,7,3′a- diligustilide (**10**)[17], Z-6,8′,7,3′-diligustilide (**11**)[18], Z-6-hydroxy-7-methoxy- dihydroligustilide (**12**)[19], senkyunolide I (**13**)[20] and senkyunolide H (**14**)[20].

Materials and Methods

General Procedure

Optical rotation was measured with a Perkin Elmer 341 polarimeter (Perkin-Elmer, Beaconsfield, UK). The UV spectrum was conducted on a Shimadzu UV-1800 spectrometer (Shimadzu, Kyoto, Japan). The FT-IR spectra were obtained on a Bruker Vector 22 spectrometer (Bruker Optik GmBH, Ettlingen, Germany) with KBr pellets. ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker DRX-400 NMR spectrometer (Bruker BioSpin GmBH, Rheinstetten, Germany) using TMS as an internal standard. ESI-MS data were obtained on a Q-TOF micro mass spectrometer. HR-ESI-MS data was recorded on a Q-TOF micro mass spectrometer (Waters, Millford, MA, USA). Materials for column chromatography were silica gel (100-200 mesh; Huiyou Silica Gel Development Co. Ltd. Yantai, China), silica gel H (10-40 μm; Yantai, China), Sephadex LH-20 (40-70 μm; Amersham Pharmacia Biotech AB, Uppsala, Sweden), and YMC-gel ODS-A (50 μm; YMC, Allentown, PA, USA). Preparative TLC (0.4-0.5 mm) was conducted with glass precoated silica gel GF_{254} plates (Yantai, China). GC-MS: Thermo Focus DSQ (Texas, USA), GC Column: VF-5S (30 mm × 0.25 mm × 0.25 mm). HPLC-MS: HPLC system (Thermo finnigan, San Jose, CA, USA), Finnigan LCQ DECA XP plus ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA), LC Column: ZORBAX Eclipse XDB-C_{18} reversed phase column (Agilent, Palo Alto, CA, USA), ZORBAX Eclipse XDB-C_{18} guard column (Agilent, Palo Alto, CA, USA).

Plant material

Ligusticum chuanxiong Hort. was purchased from Shanghai Kangqiao Chinese herbal pieces Co., Ltd. and identified as *Ligusticum chuanxiong* Hort. by Prof. Baokang Huang of Second Military Medical University, Shanghai. A voucher specimen was deposited in the Herbarium of the Shanghai University of Traditional Chinese Medicine (No. 200705-03).

Extraction and isolation

The air-dried and powdered rhizoma of *Ligusticum chuanxiong* Hort. (2.5 kg) was extracted by CO_2 su-

percritical fluid extraction. This procedure gave 100 g of volatile oil, and the volatile oil was subjected to column chromatography on silica gel (100-200 mesh, 200 g), eluted successively with gradient $CHCl_3$-MeOH (100:0-100:5) mixtures of increasing polarity and got 10 g ligustilide (purity ≥98%). We dissolved it in 90% methanol aqueous solution, and stored it at room temperature for one month. Transformation products were then subjected to column chromatography on silica gel (100-200 mesh, 20 g), eluted successively with gradient $CHCl_3$-MeOH (100:0-100:50) mixtures of increasing polarity and separated into six fractions (Fr_1-Fr_6). Fr_2 (3.7 g) was chromatographed on silica gel (100-200 mesh, 8 g) with petroleum ether-EtOAc (100:0, 100:2, 100:5, 100:10, 100:20, 100:50) followed by Sephadex LH-20 with MeOH, which yielded compound **2** (74 mg), **3** (57 mg), **4** (17 mg) and **5** (37 mg), respectively. Fr_3 (2.1 g) was purified by Sephadex LH-20 with MeOH to furnish compound **6** (59 mg) and **1** (15 mg). Fr_4 (2.3 g) was chromatographed on silica gel with gradient $CHCl_3$-MeOH (50:1-50:10) followed by Sephadex LH-20 with MeOH to give compound **7** (17 mg), **8** (27 mg), **9** (9 mg), **10** (21 mg) and **11** (11 mg). Fr_5 (2 g) was chromatographed on ODS with MeOH-H_2O (35:65, 45:55, 55:45, 65:35, 85:15, 100:0) and purified by Sephadex LH-20 with MeOH to give compound **12** (55 mg), **13** (60 mg) and **14** (16 mg).

Compound **1**: Colorless crystalline needles, $[\alpha]_D^{18}$: -1.47 (c = 0.068, MeOH); UV (MeOH) λ_{max} (log ε) nm: 204.0 (0.533), 274.5 (0.231); IR (KBr) cm^{-1}: 3431, 2958, 2933, 2873, 1768, 1728, 1639, 1437, 1281, 1095, 962, 737; ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) ($CDCl_3$) see Table 1; positive HR-ESI-MS Found 395.1471, calcd. 395.1481 for $C_{21}H_{24}O_6Na$ $[M+Na]^+$.

LC-MS analysis

The analysis was performed with a Surveyor HPLC system equipped with a quaternary gradient pump for mass spectrometry, an autosampler and a PDA detector. The products were separated on a ZORBAX Eclipse XDB-C_{18} reversed phase column (4.6 × 150 mm, 5 μm), which was coupled to a ZORBAX Eclipse XDB-C_{18} guard column. The products were eluted with a gradient system consisting of acetonitrile (A) and 0.1% formic acid in water. The linear gradient elution program was as follows (T/B%): 0/85, 10/80, 40/47, 60/0. The detection wavelength was set at 280 nm. The mobile phase flow rate was 1 mL/min and column temperature was maintained at 30 ℃.

MS analysis was performed using a Finnigan LCQ DECA XP plus ion trap mass spectrometer equipped with an electrospray ion (ESI) source, operated in the positive ion mode. The optimized parameters were as follows: electrospray needle voltage, 5 kV; sheath gas flow, 32 arbitrary unit; auxiliary nitrogen gas flow, 10 arbitrary unit; capillary voltage, 21 V; heated capillary temperature of 300 ℃. The relative collision energy for CID was adjusted to 40% of the maximum to acquire satisfactory product ion spectra. Data acquisition was performed in the full-scan, selective ions monitoring (SIM) and MS^n modes, in the range of m/z 100-1000. All operations were controlled by Xcalibur software version 1.2 (Finnigan).

Results and Discussion

Compound **1** was obtained as colorless crystalline needles. The molecular formula was inferred as $C_{21}H_{24}O_6$ based on the HR-ESI-MS (m/z 395.1471 $[M+Na]^+$). The ^{13}C NMR spectrum of 1 showed the presence of 21 carbon atoms comprised of two methyl groups, which included one methyl group bearing an oxygen atom, six methylenes, three methines, one tetrasubstituted carbon, two trisubstituted double bonds, one tetrasubstituted double bond and three carbonyl groups. The ^1H NMR spectrum exhibited signals due to a methyl group at δ_H 0.93 (3H, t, J = 10.0 Hz)], a methyl ester group at δ_H 3.65 (3H, s) and two olefinic protons at δ_H 5.08 (1H, t, J = 10.4 Hz) and 6.84 (1H, d, J = 11.6 Hz). In fact, the NMR spectral data of 1 (Table 1) were very similar to those of a known compound, wallichilide [21], except for the occurrence of signals due to a butyl group at C-7′ in wallichilide. Therefore, the structure of 1 was determined as 7′-carboxyl-wallichilide (Fig. 2), which was further confirmed by HSQC, ^1H-^1H COSY, HMBC (Fig. 3) and

NOESY experiments.

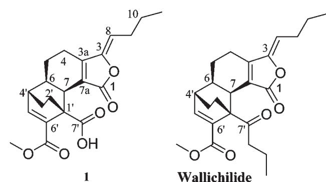

Fig. 2 Structures of compound 1 and wallichilide

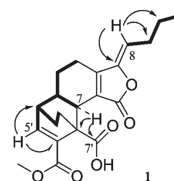

Fig. 3 Key HMBC (→) and ^{1}H-^{1}H COSY (—) correlations of compound 1

Table 1 ^{1}**H NMR and** ^{13}**C NMR data of wallichilide and compound 1 (** $CDCl_3$ **)**

Carbon	Wallichilide δ_C	Wallichilide δ_H (J in Hz)	1 δ_C	1 δ_H (J in Hz)
1	168.7		169.3	
3	149.0		148.4	
3a	153.5		153.9	
4	19.0	2.23 m	19.0	2.22 m
		2.12 m		2.15 m
5	25.8	1.88 m	28.4	1.83 m
		1.55 m		1.63 m
6	38.4	2.50 m	40.0	3.34 m
7	38.5	3.32 m	37.5	2.47 d (6.8)
7a	127.5		127.1	
8	110.7	5.06 t (7.8)	111.2	5.08 t (10.4)
9	28.0	2.31 m	27.8	2.31 m
10	22.5	1.46 m	22.4	1.46 m
				1.29 m
11	13.7	0.93 t (7.8)	13.8	0.93 t (10.0)
1′	57.4		50.6	
2′	27.9	2.13 m	27.2	1.89 m
		1.63 m		1.36 m
3′	28.5	1.92 m	29.5	2.21 m
		1.40 m		1.73 m
4′	38.2	2.59 m	38.4	2.69 m
5′	138.8	6.70 d (6.9)	140.4	6.84 d (11.6)
6′	138.0		136.3	
7′	208.1		175.9	
8′	39.7	3.07 m		
		2.55 m		
9′	28.5	1.65 m		
10′	22.5	1.33 m		
11′	14.0	0.92 t (7.3)		
COOMe	51.4	3.59 s	51.8	3.65 s
COOMe	169.3		168.0	

The relative contents of ligustilide and the fourteen transformation products were determined by area normalization using LC-MS. Fifteen peaks were structurally identified comparing their retention times and molecular weights with those of reference standards (Table 2). The results showed ligustilide was almost undetectable. Instead, ligustilide dimers that included levistolide A (14.38%) and 7′-carboxyl-wallichilide (13.49%) were the main transformation products. The results suggested that ligustilide was more likely to form dimers through Diels-Alder reactions at different positions, such as at the 3-, 6- and 7- positions. At the same time, a small number of monomeric transformation products such as senkyunolide I (4.02%) and butylphthalide (0.79%) were formed through hydrolysis, dehydrogenation and oxidation reactions.

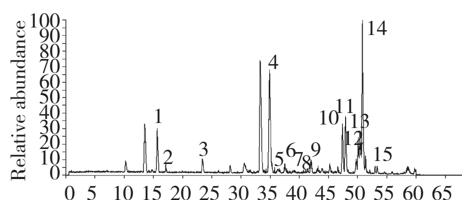

Fig. 4 LC-MS total ion chromatogram of transformation products of ligustilide

Table 2 Retention time (T_R) and relative contents of tansformation products detected by LC-MS

Peak	T_R (min)	Identification results	Peak area (%)
1	15.68	Senkyunolide I	4.02
2	17.13	Senkyunolide H	0.33
3	23.41	Z-6-hydroxy-7-methoxy- dihydroligustilide	1.30
4	34.84	7′-carboxyl-wallichilide	13.49
5	34.98	Senkyunolide A	0.29
6	37.44	Butylphthalide	0.79
7	41.36	Ligustilide	0.30
8	41.50	Chuanxiongnolide A	0.21
9	42.07	Butylidenephthalide	1.25
10	47.38	Tokinolilde B	4.40
11	47.89	Z-3′,8′,3′a,7′a-tetrahydro-6,3′,7′,7′a-diligustilide-8′-one	5.60
12	50.09	3′,6,8′,3a-diligustilide	3.09
13	50.58	Z-6,8′,7,3′-diligustilide	3.05
14	50.84	Levistolide A	14.38
15	53.29	exo-Z,Z′-3a,7′a,7,3′a- diligustilide	0.54

Our experiments and previous studies from other researchers [6,7] have revealed ligustilide to be highly unstable. In particular, there are many opportunities for ligustilide to isomerize to various transformation products during post-harvest drying, processing, storage, water decoction and ethanol extraction procedures. Therefore, in many cases, not only ligustilide but also its transformation products play an important role when Rhizoma Chuanxiong and Radix Angelicae Sinensis used in clinics.

References

1 Mitsuhashi H, Nagai U, Muramatsu T, *et al*. Studies on the constituents of Umbelliferae plants. II. Isolation of the active principles of ligusticum root. *Chem Pharm Bull*, 1960, 8: 243-245.

2 Mitsuhashi H, Nagai U, Muramatsu T. Studies on the constituents of Umbelliferae Plants. Ⅲ. Structure of Ligustilide. *Chem Pharm Bull*, 1961, 9: 115-119.

3 Kuang X, Yao Y, Du JR, *et al*. Neuroprotective role of Z-ligustilide against forebrain ischemic injury in ICR mice. *Brain Res*, 2006, 1102: 145-153.

4 Chan SSK, Cheng TY, Lin G. Relaxation effects of ligustilide and senkyunolide A, two main constituents of *Ligusticum chuanxiong*, in rat isolated aorta. *J Ethnopharmacol*, 2007, 111: 677-680.

5 Du JR, Yu Y, Ke Y, *et al*. Ligustilide attenuates pain behavior induced by acetic acid or formalin. *J Ethnopharmacol*, 2007, 112: 211-214.

6 Li GS, Ma CJ, Liu XY, *et al*. Studies on the stability of ligustilide and the analysis of its isomerized products by LC-MS. *Chin Tradit Herbal Drugs*, 2000, 32: 581-584.

7 Li SL, Yan R, Tam YK, *et al*. Post-harvest alteration of the main chemical ingredients in *Ligusticum chuanxiong* HORT. *Chem Pharm Bull*, 2007, 55: 140-144.

8 Lin LZ, He XG, Lian LZ, *et al*. Liquid chromatographic-electrospray mass spectrometric study of the phthalides of *Angelica sinensis* and chemical changes of Z-ligustilide. *J Chromatogr A*, 1998, 810: 71-79.

9 Luo YM, Pan JG, Ding KP, *et al*. Anticonvulsive constituents in the essential oil of *Chuanxiong*. *Chin Tradit Herb Drugs*, 1996, 27: 456-457.

10 Wang PS, Gao XL, Fukuyama Y, *et al*. Studies on the constituents of *Ligusticum chuanxiong* HORT. *Chin Herbal Med*, 1985, 16: 137-138.

11 Naito T, Katshuara T, Niitsu K, *et al*. Two phthalides from *Ligusticum chuanxiong*. *Phytochemistry*, 1992, 31: 639-642.

12 Kobayashi M, Mitsuhashi H. Studies on the constituents of Umbelliferae plants. XVIII. Structures of three new ligustilide derivatives from *Ligusticum wallichii*. *Chem Pharm Bull*, 1987, 35: 4789-4790.

13 Li YH, Peng SI, Zhou Y, *et al*. Two new phthalides from *Ligusticum chuanxiong*. *Planta Med*, 2006, 72: 652-656.

14 Tsuchida T, Kobayashi M, Kaneko K, *et al*. Studies on the constituents of Umbelliferae plants. XVI. *Chem Pharm Bull*, 1987, 35: 4460-4464.

15 Su DM, Yu SS, Qin HL. New dimeric phthalide derivative from *Angelica sinensis*. *Acta Pharm Sin*, 2005, 40: 141-144.

16 Kaouadji M, Pachtere FD, Pocget C, *et al*. Three additional phthalide derivatives, an epoxymonomer and two dimmers, from *Ligusticum Wallichii* rhizomes. *J Nat Prod*, 1986, 49: 872-877.

17 Beatriz QG, Ricardo FJ, Antonio C, *et al*. Phytocyclodimers from Z-ligustilide. experimental results and FMO analysis. *Tetra Lett*, 2005, 46: 3003-3006.

18 Zhang L, Liu YP, Li W, *et al*. Study on chemical constituents of *Ligusticum chuanxiong* and content determination of ligustilide. *Chin Pharm*, 2010, 21: 1381-1383.

19 Deng S, Chen SN, Yao P, *et al*. Serotonergic activity-guided phytochemical investigation of the roots of *Angelica sinensis*. *J Nat Prod*, 2006, 69: 536-541.

20 Kobayashi M, Fujita M, Mitsuhashi H. Studies on the constituents of Umbelliferae plants. XV. Constituents of cnidium officinale: ocurrence of pregendone, coniferulate and hydroxy phthalides. *Chem Pharm Bull*, 1987, 35: 1427-1433.

21 Wang PS, Gao YX, Yoshiyasu F, *et al*. Phthalides from the rhizome of *Ligusticum Wallichii*. *Phytochemistry*, 1984, 23: 2033-2038.