Absolute Configuration of Antifibrotic (+)-Episesamin Isolated from *Lindera obtusiloba* BLUME

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Dedicated to Prof. Dr. Hans Brockmann, Bielefeld, on the occasion of his 75th birthday

Fractionation of a 70% ethanolic extract from twigs of *Lindera obtusiloba* BLUME (Japanese spicebush, Tohaku) yielded five fractions of different polarity. The antifibrotic activity within the chloroform phase was best assessed by an *in vitro* bioassay using rat hepatic stellate cell (HSC) proliferation and their autocrine transforming growth factor beta (TGF-β) expression as sensitive fibrosis-associated read out. Chromatography of the chloroform extract on Sephadex LH-20 or liquid-liquid extractions yielded a crystalline compound as an active principle, which was identified from NMR and ESI-MS analyses, its melting point, and its optical rotation as (7*S*,7´*R*,8*R*,8´*R*)-3,4:3´,4´-bis(methylenedioxy)-7,9´:7´,9-diepoxy-lignane [(+)-episesamin]. X-Ray diffraction confirmed the structure and provided, for the first time, directly its absolute configuration. (+)-Episesamin blocked proliferation and the profibrotic autocrine TGF-β expression HSC without significant cytotoxicity.

Key words: TGF-β, Liver Fibrosis, X-Ray Structure

Introduction

In traditional Korean medicine, an aqueous extract of the plant *Lindera obtusiloba* has been used for centuries to treat inflammation, improve blood circulation, and prevent liver damage (Yook, 1989). Recently, we demonstrated antifibrotic, antioxidative, anti-inflammatory, and antiadipogenic activity of this extract (Rühl *et al.,* 2009; Freise *et al.,* 2009). Freise *et al.* (2011) documented that the *L. obtusiloba* extract suppresses growth and attenuates insulin-like growth factor-1 receptor signaling and NF-κB activity in human liver cancer cell lines.

The aim of the present study was to isolate biologically active compounds from the *L. obtusiloba* extract and elucidate their absolute configuration. Here, for the first time, we report the isolation and identification of the absolute configuration of (+)-episesamin [**(+)–1**] from the *L. obtusiloba* extract. By applying (+)-episesamin in *in vitro* bioassays with activated hepatic stellate cells (HSC), the main target cell in the fibrotic liver (Rühl *et al.*, 2009), we suggest **(+)–1** to be an active antifibrotic principle of the extract.

Material and Methods

General

The Sephadex LH-20 (Pharmacia, Uppsala, Sweden) column chromatography (15 cm x 2.5 cm) was performed with methanol as solvent (flow, 1 mL/min). Alumina plates with fluorescence indicator (λ = 254 nm) for thin layer chromatography (tlc) were from Merck (Darmstadt, Germany). All solvents were from Merck, and of analytical grade.

(+) ESI mass spectra were recorded on an Orbitrap Velos instrument (Thermoscientific, Bremen, Germany). The optical rotation was recorded on

a Perkin Elmer (Waltham, MA, USA) MC 241 polarimeter (d = 10 cm) with chloroform as solvent. ^{1}H (1D and 2D COSY, TOCSY, ROESY and NOESY) and ^{13}C (1D and 2D HMQC and HMBC) NMR spectra were recorded on either Bruker (Rheinstetten, Germany) DPX 300, ARX 400 or DMX 600 NMR spectrometers, respectively, locked to the major deuterium resonance of the solvent. The samples were dissolved in chloroform-d_3 to achieve final volumes of 700 μL. Measurements were carried out at 300 K with mixing times of 110 ms for the TOCSY and 500 ms for the ROESY and NOESY spectra. ^{13}C chemical shifts are given in ppm relative to tetramethylsilane, but were determined relative to the residual signals of the solvent (^{13}C, 77.05 ppm).

Plant material

Stems and twigs of *Lindera obtusiloba* BLUME (taxonomy: Phylum Magnoliophyta; class: Magnoliatae; order: Laurales; family: Lauraceae Juss.; genus: *Lindera* Thunb.) were collected in Iksan City, South Korea, in September 2007, and identified by Prof. Ki Y. Kim, University of Wonkwang, Iksan City, South Korea. A voucher specimen (34797) has been deposited at the herbarium of the Department of Botany, University of Chonnam, South Korea.

Isolation of (+)-episesamin

Stems and twigs from the shrubs (4.3 kg) of *L. obtusiloba* were stirred overnight with 20 L of 70% ethanol at 60 °C. The concentrated lyophilized crude extract (375.64 g) was partitioned between *n*-hexane/water (1:1, v/v), and gave a dried *n*-hexane extract of 38.82 g. The NMR spectra (not shown) indicated that this fraction contained the cytotoxic obtusilactones isolated from *Lindera benzoin* (Anderson *et al*., 1992), from *Lindera communis* (Tsai *et al*., 2002), and from *Aiouea trinervis* (a Lauraceae as well) (Garcez *et al.,* 2005). Chloroform was added to the remaining water layer and, after intensive extraction and evaporation of the solvent, 23.73 g of a crude extract were isolated. The water layer was further extracted with ethyl acetate to give an extract of 2.58 g, and finally with *n*-butanol to give an extract of 112.23 g. At the end, the water phase was lyophilized to give 168.26 g of extract. About 100 mg of the chloroform extract were dissolved in 2 mL methanol/chloroform (1:1, v/v) with the aid of an ultrasonic system and filtered through a paper tissue. This solution was separated using a Sephadex LH-20 column and methanol as solvent. After the first 50 mL, the eluate was fractionated into 4 fractions, 30 mL each, of which the last one showed at 254 nm a single tlc spot at R_f = 0.5 [*tert*.-butyl-methylether (TBME)/*n*-hexane (1:1, v/v)] consisting of a single molecular species as indicated by the corresponding ^{1}H NMR spectrum (not shown), while the other fractions contained complex mixtures.

Alternatively, the lyophilized chloroform extract (1 g) was dissolved in 20 mL chloroform in an 100-mL flask and *n*-hexane (50 mL) was added. To precipitate material the flask was left overnight at 8 °C, and after filtration the organic phase was evaporated to a waxy material (ca. 100 mg), to which 20 mL of TBME were added. Spontaneous crystallization took place generating slight yellow to white needles. After recrystallization from TBME/*n*-hexane (1:1, v/v), 45 mg of pure colourless needles with a melting point of 120 °C and optical rotation of $[\alpha]_D^{25}$ = + 120° (c = 0.5, $CHCl_3$) were obtained. ^{1}H, ^{13}C homo- and ^{1}H-^{13}C heteronuclear NMR spectra were taken, only the ^{13}C data ($CDCl_3$) are given (Table I). High-resolution electrospray ionization mass spectrometry (ESI-MS) analysis revealed a molecular mass of 377.0997 for $C_{20}H_{18}O_6Na$.

In vitro bioassay

Cell cultures were performed, and the effects of **(+)–1** on the proliferation, cytotoxicity, and the autocrine mRNA expression of TGF-β were determined as described by Rühl *et al.* (2009).

Statistical analysis

One way ANOVA/Tukey tests were realized using SigmaStat for Windows (version 2.03; Systat, San Jose, CA, USA). $P < 0.05$ was considered significantly different.

Results and Discussion

Isolation and structure elucidation

The isolation of (+)-episesamin [**(+)–1**] was straight forward. Following ethanolic (70%) extraction of the lyophilized aqueous extract of stems and twigs of *L. obtusiloba* compounds were distributed between water, the less polar solvents *n*-hexane, chloroform, ethyl acetate, and finally

Table I. ^{13}C NMR data of sesamin, episesamin, and **(+)–1** in $CDCl_3$.

C	Sesamin[a]	Episesamin[a]	(+)–1
8		54.8	54.7
	54.2		
8´		50.4	50.2
9		71.0	70.9
	71.5		
9´		69.7	69.7
7		87.7	87.7
	85.6		
7´		82.1	82.1
1		135.6	135.1
	143.9		
1´		132.6	132.3
		146.8	146.8
3, 3´	146.9	147.4	147.4
4, 4´	147.7	147.9	147.9
		148.2	148.2
2, 2´	106.3	106.6	106.4
5, 5´	108.0	106.7	106.6
		108.3	108.2
		118.9	118.7
6, 6´	119.1		
		119.6	119.6
O-CH_2-O	100.9	101.1	101.0; 101.1

[a] Data taken from Pelter *et al.* (1976).

n-butanol. The strongest antiproliferative activity and lowest cytotoxicity against HSC was found for the chloroform fraction (data not shown) from which **(+)–1** could be isolated by gel chromatography using Sephadex LH-20 or more easily by liquid-liquid extraction and crystallization with TBME.

The NMR (^1H,^{13}C and ^1H-^{13}C heteronuclear spectra) data (given for ^{13}C NMR only, see Table I), melting point, and rotation values were in accordance with data for **(+)–1** from Pelter *et al*. (1976). The determined value from the high-resolution ESI mass spectrum (M_R 377.0997) correlates with the calculated value of 377.0996 for $C_{20}H_{18}O_6Na$, the $[M + Na]^+$ ion of **(+)–1**. Based on the X-ray data collected after Cu-K_α radiation (for several reasons – summarized by the Chemical Crystallography Lab of the University of Oklahoma – Cu-K_a radiation is necessary for the determination of the absolute configuration of small molecules: http://xrayweb.chem.ou.edu/notes/xray.html) the absolute configuration of the *L. obtusiloba* isolate could be unequivocally assigned to: 7*S*,7´*R*,8*R*,8´*R* (Fig. 1). The structure

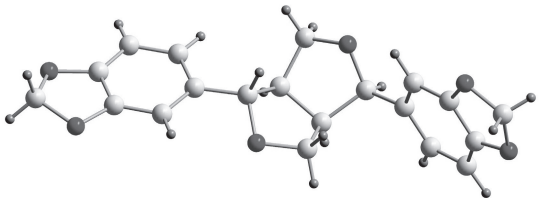

Fig. 1. Crystal structure of (+)-episesamin (absolute configuration). The dimensions of the cell were determined as: a = 9.6098(24) Å, b = 5.5343(17) Å, c = 15.6464(36) Å, and β = 103.86(0)°, its volume is V = 807.91(42) Å^3.

was solved in the monoclinic space group $P2_1$, which is in contrast to Li *et al.* (2005), who found the crystals of asarinin [synonym for (+)-episesamin] to be triclinic. None of six published X-ray analyses of sesamin and episesamin provides direct proof of their absolute configurations, but of their relatives only: Baures *et al.* (1992), II´in *et al.* (1994), Parmar *et al.* (1998), Li *et al.* (2005), Macias and Zubia (1992), Hsieh *et al.* (2005). Mata *et al.* (1998) showed an X-ray structure of asarinin obtained by Cu-K_α radiation and mentioned a deposition of the X-ray data at the Cambridge Crystallographic Data Centre (CCDC), but no number was given, and the data were not found to contain any studies of Mata *et al*. In addition, from the paper it was not clear whether episesamin exhibited a positive value of optical rotation or not, but the depicted X-ray structure points to (–)-asarinin. Our analysis determined the dimensions of the cell as: a = 9.6098(24) Å, b = 5.5343(17) Å, c = 15.6464(36) Å, β = 103.86(0)°, and volume V = 807.91(42) Å^3.

All data of interest were deposited at the Cambridge Crystallographic Data Centre (CCDC) under the code VUKBUY03. CCDC VUKBUY03 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via: www.ccdc.cam.ac.uk/data_request/cif, or by e-mailing: data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

To our knowledge, this is the first direct proof of the absolute configuration of **(+)–1** being 7*S*, 7´*R*, 8*R*, and 8´*R* which accords to the results of Freudenberg and Sidhu (1961) (Fig. 2). They proved that the bridgehead carbon atoms in (+)-sesamin and the pinoresinol groups have the same absolute configuration, which was known

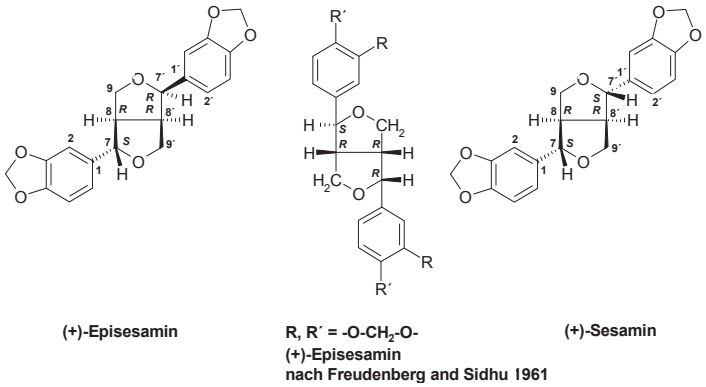

Fig. 2. Structural formula of (+)-episesamin and (+)-sesamin.

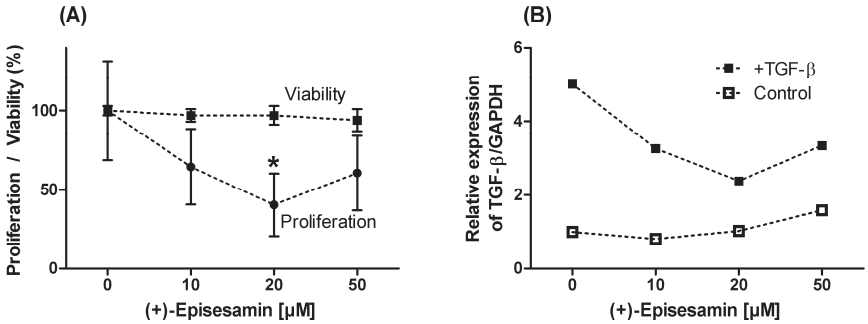

Fig. 3. Effects of (+)-episesamin on proliferation and autocrine TGF-β expression of hepatic stellate cells. (A) Rat hepatic stellate cells (HSC) were treated with 2 ng/mL TGF-β ± (+)-episesamin. Proliferation was determined by [^{3}H]-thymidine incorporation. Effects of (+)-episesamin on viability were assessed by trypan blue staining and manual counting of viable cells (n = 5). (B) Autocrine mRNA expression of the profibrotic master cytokine TGF-β/GAPDH was quantified by TaqMan® qPCR. *P < 0.05.

from chemical correlation of the degradation product of guaiaretic acid with (–)-2-methyl-butanol (Carnmalm, 1956); the latter has been chemically correlated with D-glucose (Freudenberg and Hohmann, 1953), and the absolute configurations of the ether carbon atoms were deduced from Freudenberg's "rule of shift".

Several approaches towards the total chemical synthesis of the optically active sesamins have been published (Kise *et al.*, 2002; Takano *et al.*, 1988; Enders *et al.*, 2002; Banerjee and Roy, 2005), but only the synthesis of racemic episesamin has been reported (Takano *et al.*, 1993; Aldous *et al.*, 1999; Samizu and Ogasawara,1995).

Antifibrotic activity of (+)-episesamin

Antifibrotic effects of **(+)–1** were tested using the rat HSC cell line CFSC, an established model cell line for testing antifibrotic activity *in vitro* (Rühl *et al.*, 2009). (+)-Episesamin (20 µM) reduced *de novo* DNA synthesis in activated HSC by about 50% without significant cytotoxicity (Fig. 3A). In parallel, the effects of (+)-episesamin on the autocrine induction of TGF-β in the HSC were investigated. Here, a concomitant treatment of the cells with 15 µM **(+)–1** and TGF-β resulted in half-maximum levels of TGF-β expression compared to the control with TGF-β alone (Fig. 3B).

These data demonstrate a biological activity of isolated **(+)–1** and suggest that (+)-episesamin has the potential to ameliorate the process of fibrogenesis by counteracting the autocrine loop of the profibrotic master cytokine TGF-β. Thus, (+)-episesamin might represent an antifibrotic principle within the total extract from *Lindera obtusiloba*.

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