Arch Pharm Res Vol 30, No 11, 1392-1397, 2007

Archives of Pharmacal Research
http://apr.psk.or.kr

Antioxidant and Antiatherogenic Activity of *cis*-Hinokiresinol from *Trapa pseudoincisa*

Myoung-Chong Song, Hye-Joung Yang, Myun-Ho Bang, Dae-Keun Kim[1], Tae-Sook Jeong[2], Jong-Pyung Kim[3], and Nam-In Baek

Graduate School of Biotechnology & Plant Metabolism Research Center, Kyung Hee University, Suwon 446-701, Korea, [1]Department of Pharmacy, Woosuk University, Jeonju 565-701, Korea, [2]National Research Laboratory of Lipid Metabolism & Atherosclerosis, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Korea, and [3]Laboratory of Antioxidant, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Korea

(Received May 25, 2007)

cis-Hinokiresinol, also known as (+)-nyasol, was isolated for the first time from an aquatic herbaceous plant, *Trapa pseudoincisa* NAKAI, via silica gel and octadecyl silica gel column chromatographies. The chemical structure was determined via analyses of the spectroscopic data, including NMR, MS and IR. *cis*-Hinokiresinol was also found to exhibit antioxidant and antiatherogenic activities. The IC_{50} values for the scavenging activities of *cis*-hinokiresinol on ABTS cation and superoxide anion radicals were 45.6 and 40.5 μM, respectively. The IC_{50} values for the inhibitory effects on Lp-PLA_2, hACAT1, hACAT2 and LDL-oxidation were 284.7, 280.6, 398.9 and 5.6 μM, respectively.

Key words: *Trapa pseudoincisa*, *cis*-Hinokiresinol, ABTS, Superoxide, Lp-PLA_2, hACAT, LDL-oxidation

INTRODUCTION

Trapa pseudoincisa NAKAI (Hydrocaryaceae) is an aquatic annual herb found in Korea, Japan and China, which has been used as a remedy of several diseases, including quadriplegia, diarrhea and gastric ulcers (Jung and Shin, 1990). Prior analytical results have shown ergosta-4,6,8(14),22-tetraen-3-one, 22,23-dihydrostigmast-4-en-3,6-dion and β-sitosterol from *T. bispinosa* (= *T. pseudoincisa*) to be effective antitumor steroids against ascites sarcoma (Irikura *et al.*, 1972). During an investigation on the biologically active constituents from natural sources, *T. pseudoincisa* was found to possess antioxidant qualities (Kim *et al.*, 1997). *cis*-Hinokiresinol, a norneolignan, was isolated from *T. pseudoincisa*, as the principal component manifested antioxidant activity. Hinokiresinol has previously been found only in terrestrial plants; this paper identifies an aquatic plant as a source of hinokiresinol for the first time. Herein, the isolation and identification of *cis*-hinokiresinol from an aquatic plan source is described, with several biological activities evaluated, including inhibitive effects against human acyl-CoA: cholesterol acyltransferase (hACAT-1), hACAT-2 and oxidized low-density lipoprotein (LDL), as well as a lipoprotein-associated phospholipase A_2 (Lp-PLA_2) as an antiatherogenic agent, and radical scavenging activities for the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) free cation and superoxide anion radicals as an antioxidant.

Correspondence to: Nam-In Baek, Graduate School of Biotechnology, Kyung Hee University, Suwon 446-701, Korea
E-mail: nibaek@khu.ac.kr

MATERIALS AND METHODS

Plant materials

The *Trapa pseudoincisa* NAKAI was collected from a stream in Sunchang province, Korea, in September, 2005, and identified by Prof. Dae-Keun Kim, Woosuk University, Jeonju, Korea. A voucher specimen (KHU05092501) was deposited at the Laboratory of Natural Products Chemistry, Kyung Hee University, Suwon, Korea.

Instrumentation

The optical rotation was measured on a JASCO P-1010 digital polarimeter (Jasco, Tokyo, Japan). The IR spectrum was run on a Perkin Elmer spectrum One FT-IR spectro-

meter (Perkin Elmer, Norwalk, U.S.A.). EIMS and FABMS were recorded on a JEOL JMS 700 (JEOL, Tokyo, Japan). ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were obtained on a Varian Unity Inova AS 400 FT-NMR spectrometer (Varian, California, U.S.A.).

Isolation of hinokiresinol

Whole *Trapa pseudoincisa* NAKAI plants (1.1 kg) were extracted three times, for 5 h at 50°C, with 2 L of 80% aqueous MeOH (2 L × 3). The extracts were partitioned with water (1 L) and EtOAc (1 L × 2). The EtOAc extract (30 g) was subjected to silica gel column chromatography (c.c.) (6 × 12 cm), gradient eluted with *n*-hexane:EtOAc (10:1→7:1→5:1→3:1→1:1) and $CHCl_3$:MeOH (10:1→7:1→5:1→3:1→MeOH), and monitored by thin layer chromatography (TLC), yielding 24 fractions (TIE1 to TIE24). TIE13 [2.1 g, Ve/Vt (elution volume/total volume) 0.62-0.71] was purified using octadecyl silica gel (ODS) c.c. (3.5 × 10 cm) with MeOH:H_2O (1:1→3:1) as the eluent, ultimately producing compound **1** [79 mg, Ve/Vt 0.35-0.43; R_f 0.25 on ODS TLC in MeOH:H_2O (2:1)].

cis-Hinokiresinol (1)

Reddish oil; $[\alpha]_D$: +112° (*c* 0.4, CH_2Cl_2); IR_f (KBr, cm^{-1}): 3600, 3400 (br), 1610, 1250; EIMS *m/z* (%): 252 [M^+, $C_{17}H_{16}O_2$, 100], 238 [M^+-CH_2, 37], 158 (74), 107 (97); FABMS *m/z* 253.2 $[M+H]^+$; ^{1}H-NMR (400 MHz, $CDCl_3$, δ ppm): 7.14 (2H, d, *J*=8.6 Hz, H-2',6'), 7.10 (2H, d, *J*=8.6 Hz, H-2",6"), 6.78 (2H, d, *J*=8.6 Hz, H-3',5'), 6.77 (2H, d, *J*=8.6 Hz, H-3",5"), 6.50 (1H, d, *J*=11.2 Hz, H-1), 5.98 (1H, ddd, *J*= 16.8, 10.2, 5.8 Hz, H-4), 5.66 (1H, dd, *J*=11.2, 10.0 Hz, H-2), 5.16 (1H, dd, *J*=16.8, 1.6 Hz, H-5a), 5.13 (1H, dd, *J*= 10.2, 1.6 Hz, H-5b), 4.47 (1H, dd, *J*=10.0, 5.8 Hz, H-3); ^{13}C-NMR (100 MHz, $CDCl_3$, δ ppm): 154.4 (C-4'), 153.9 (C-4"), 140.6 (C-4), 135.4 (C-1"), 131.6 (C-2), 129.9 (C-2', 6'), 129.7 (C-1'), 128.8 (C-2", 6"), 128.5 (C-1), 115.6 (C-3", 5"), 115.1 (C-3', 5'), 115.0 (C-5), 46.9 (C-3).

ABTS cation radical preparation and reduction

ABTS was dissolved in water to a concentration of 7 mM, with the radical cation ($ABTS^{\bullet+}$) produced by reacting the ABTS stock solution with 2.45 μM potassium persulfate (final concentration) and allowing the mixture to stand in a dark environment at room temperature (Roberta *et al.*, 1999). After the addition of 1.0 mL of the diluted ABTS radical cation solution ($A_{734\,nm}$=0.700) to 10 μL *cis*-hinokiresinol in ethanol and mixing the solution for 6 min, the absorbance was measured using an ELISA reader (Molecular devices Co. U.S.A.) (Lee *et al.*, 2003).

Superoxide radical scavenging activity

Superoxide radical scavenging activity of compound **1** was assayed using a modified irradiated riboflavin/EDTA/Nitroblue tetrazolium (NBT) system (Beauchamp and Fridovich, 1971). The assay mixture consisted of 140 mL of 0.03 mM riboflavin, 1 mM EDTA, 0.6 mM methionine and 0.03 mM NBT solution, in 50 mM potassium phosphate buffer (pH 7.8) and 10 mL *cis*-hinokiresinol solution, which included the test and reference compounds (α-tocopherol and trolox) at various concentrations in MeOH, as well as MeOH as a control. The photoinduced reactions for the generation of the superoxide anion were performed in an aluminum foil-lined box with two 20 W fluorescent lamps. The distance between the reactant and the lamps was adjusted to attain an illumination intensity of 1000 lux. The reactant was illuminated at 25°C for 7 minutes. The photochemically reduced riboflavin generated the superoxide anion, which reduced NBT to blue formazan. The reduction of NBT was measured by the change in the absorbance at 570 nm before and after the irradiation, using a microplate reader (Yoo *et al.*, 2006).

hACAT1 and hACAT2 activity assay

Microsomal fractions of Hi5 cells harboring baculovirally-expressed hACAT-1 or hACAT-2, and rat liver microsomes were used as enzyme sources. The activities of hACAT-1 and hACAT-2 were determined via the method developed by Brecher and Chan (Brecher and Chan, 1980), with slight modifications (Jeong *et al.*, 2004; Lee *et al.*, 2001). The reaction mixture, which contained 4 μL of microsomes (8 mg/mL protein) and 20 μL of 0.5-M potassium phosphate buffer (pH 7.4), with 10-mM dithiothreitol, 15 μL of bovine serum albumin (fatty acid free, 40 mg/mL), 2 μL of cholesterol in acetone (20 μL/mL, added last), 41 μL of water and 10 μL of the test sample, in a total volume of 92 μL, was preincubated for 20 min at 37°C, with brief vortexing and sonication. The reaction was initiated by the addition of 8 μL of [1-^{14}C] oleoyl-CoA solution (0.05 μCi, final conc. 10 μM). After 25 min of incubation at 37°C, the reaction was halted via the addition of 0.1 mL isopropanolheptane (4:1; v/v). A mixture of 0.6 mL heptane and 0.4 mL of 0.1 M potassium phosphate buffer (pH 7.4), also containing 2 mM dithiothreitol, was subsequently added. This solution was mixed, allowed to phase separate under gravity for 2 min and cholesterol oleate recovered from the upper heptane phase (total volume 0.9-1.0 mL). The radioactivity in 100 μL of the upper phase was then measured in a liquid scintillation vial containing 3 mL of a scintillation cocktail (Upoluma, Lumac Co.), using a liquid scintillation counter (1450 Microbeta Trilux Wallac Cy, Turku.. Finland). The background values were determined via the preparation of heat-inactivated or normal insect cell lysate microsomes. The background value normally fell within 200-250 cpm at 8000 cpm for the ACAT reaction. The ACAT activity was expressed in units, as defined by: cholesteryl oleate prnol/min/rng protein.

Low-density lipoprotein isolation and oxidation assay

Plasma was obtained from fasted healthy normalipidemic volunteers. The LDL was isolated using a standard procedure, with a slight modification (Havel *et al.*, 1955). The thiobarbituric acid-reactive substance (TBARS) assay of Buege and Aust (Buege and Aust, 1978) was used, with a slight modification. Thus, an LDL solution (250 μL, 50-100 μg of protein) in 10 mM PBS (pH 7.4) was supplemented with 10 μM $CuSO_4$. The oxidation was performed in a screw-capped 5 mL glass vial, at 37°C, in a shaking water bath. After 4 h incubation, the reaction was terminated by the addition of 1 mL of 20% trichloroacetic acid. Following precipitation, 1 mL of 0.67% thiobarbituric acid (TBA), in 0.05 M NaOH, was added, with the mixture vortexed and heated for 5 min at 95, cooled on ice, and centrifuged for 2 min at 1000 g. The optical density of the malondialdehyde (MDA) produced was measured at 532 nm. A calibration curve was constructed using an MDA standard prepared in tetramethoxypropane.

Measurement of Lp-PLA2 activity

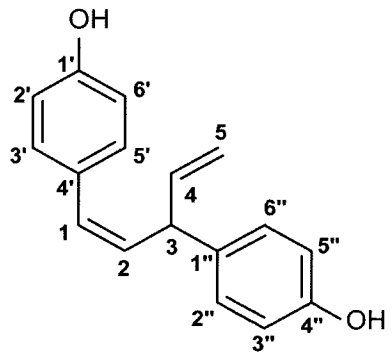
The activity of Lp-PLA_2, which is also known as platelet-activating factor acetylhydrolase (PAF-AH), was measured using a previously described modified method (Lee *et al.*, 2005; Jeong *et al.*, 2006). Ten milliliters of [3H]PAF (50 mM) and whole plasma were incubated for 60 s at 37°C, in a final volume of 200 mL (human LDL). The reaction was stopped by vortexing with 600 mL of $CHCl_3$:MeOH (2:1) and $CHCl_3$, with the aqueous layers were separated by centrifugation. The aqueous layer was subsequently removed (250 mL) and vortexed with a further 250 mL of $CHCl_3$. The aqueous layer was again removed, with the [3H]acetate concentration determined using a scintillation counter. The inhibitory effect of *cis*-hinokiresinol was determined by mixing whole plasma (190 mL) and 2 mL of a DMSO solution, with preincubation of the compound, at 37°C for 10 min, prior to running the enzyme assay, as outlined above (Boyd *et al.*, 2000).

RESULTS AND DISCUSSION

Hinokiresinol (1)

Compound **1**, obtained as reddish oil, showed absorbance bands due to the phenolic alcohol (3600, 3400(br) cm^{-1}) and olefine (1610, 1250 cm^{-1}) in the IR spectrum. The molecular ion peak was recoreded [M^+, $C_{17}H_{16}O_2$] at *m/z* 252 (100), with 238 [M^+-CH_2, 37], 158 (74), 107 (97) in the EIMS, and $[M+H]^+$ at *m/z* 253.2 in the FABMS. In the ^{1}H-NMR spectrum (400 MHz, $CDCl_3$), olefine methine signals of two *para*-substituted benzene rings {δ_H 7.14 (H-2',6'), δ_H 7.10 (H-2",6"), δ_H 6.78 (H-3',5'), δ_H 6.77 (H-3",5")}, exomethylene proton signals {δ_H 5.16 (H-5a), δ_H 5.13 (H-5b)} and an olefine methine proton signal (δ_H 5.98, H-4), as well as two other olefine methine signals at δ_H 6.50 (H-1) and δ_H 5.66 (H-2), with a *cis* coupling constant of 11.2 Hz, were observed, as was a sp3 methine signal δ_H 4.47 (H-3). In the ^{13}C-NMR spectrum (100 MHz, $CDCl_3$), 17 signals, comprising sixteen olefine carbons and one sp3 methine signal (δ_C 46.84), were detected. In the olefine carbon region, four olefine carbon signals from eight methine carbons {δ_C 129.9 (C-2',6'), δ_C 128.8 (C-2",6"), δ_C 115.4 (C-3",5"), δ_C 115.1 (C-3',5')} and four olefine quaternary carbon signals {δ_C 154.4 (C-4'), δ_C 153.9 (C-4"), δ_C 135.4 (C-1"), δ_C 129.7 (C-1')} from two *para*-substituted benzene rings, three olefine methine carbon signals {δ_C 140.6 (C-4), δ_C 131.6 (C-2), δ_C 128.5 (C-1)} and an exomethylene carbon signal {δ_C 115.0 (C-5)} were observed. These data were directly compared with those found in the literature (Tsui and Brown, 1996; Jeong *et al*, 1999). The data comparison showed compound **1** to be a *cis*-hinokiresinol (Fig. 1).

Hinokiresinol has two geometric isomers, these being the *cis*- and *trans*-configurations. The former isomer is also referred to as (+)-nyasol (Minami *et al.*, 2000) and the latter as hinokiresinol. While *cis*-hinokiresinol has been isolated from herbaceous plants, such as *Asparagus cochinchinensis* (Tsui and Brown, 1996) and *Anemarrhena asphodeloides* (Jeong *et al.*, 1999; Lee and Ryu, 1999; Park *et al.*, 2003) in Liliaceae, and *Hypoxis nyasica* in Hypoxidaceae (Galeffi *et al.*, 1987; Marini-Bettolo *et al.*, 1985; Messana *et al.*, 1989); *trans*-hinokiresinol has primarily been isolated from wood plants, such as *Chamaecyparis obtuse* (Hirose *et al.*, 1965) and *Libocedrus yateensis* (Erdtman and Harmatha, 1979) in Cupressaceae, and *Agathis australis* (Enzell *et al.*, 1967a, 1967b) and *Araucaria angustiflia* (Willfor *et al.*, 2003) in Araucariaceae. This paper presents, for the first time, the isolation of hinokiresinol from an aquatic plant. *cis*-Hinokiresinol, (+)-nyasol, has been reported to exhibit a number of biological activities,



cis-hinokiresinol (**1**)

Fig. 1. Chemical structure of compound **1** (*cis*-hinokiresinol) isolated from *Trapa pseudoincisa*

including a bovine uterine estrogen receptor (Minami *et al.*, 2000) and an antiplasmodial (Skytte *et al*, 2006), which causes hyaluronidase inhibition (Jeong *et al.*, 1999, 2003), and antiallergic activity, and also effects the inhibition of β-hexosaminidase release and pro-inflammation (Bae *et al.*, 2006).

Antioxidant activities

cis-Hinokiresinol exhibited scavenging activity against ABTS cation and superoxide anion radicals, with IC_{50} values of 45.6 and 40.5 μM, respectively. Some well known antioxidants were also evaluated for the activity; the IC_{50} value of vitamin E on the ABTS radical cation was 25 μM, and those of BHA and Trolox on the superoxide anion radical were 22.8 and 940 μM, respectively. *T. pseudoincisa* has also been reported to show high scavenging activity against the 1,1-diphenly-2-picryl hydrazyl (DPPH) free radical generating system (Kim *et al.*, 1997). Thus, *cis*-hinokiresinol could be a principal contributor to the radical scavenging activity displayed by *T. pseudoincisa*.

Antiatherogenic activities

The inhibitory activity of *cis*-hinokiresinol on LDL-oxidation (IC_{50}=5.6 μM) was almost equivalent to that of probucol (IC_{50}=2.0 μM), a well known and much more potent inhibitor than those of other active components obtained from natural sources (Ahn *et al.*, 2001; Kim *et al.*, 2005b). Additionally, with regard to the inhibitory effects against hACAT1, hACAT2 and Lp-PLA_2, *cis*-hinokiresinol showed IC_{50} values of 280.6, 398.9 and 284.7 μM, respectively, all of which were relatively low compared to some other well known inhibitors; for example, the IC_{50} values of oleic acid anilide (Roth *et al.*, 1992; Kim *et al.*, 1994) against hACAT1 and hACAT2 were 0.32 and 0.30 mM, and that of SB402564 (Jeong *et al.*, 2004) against Lp-PLA_2 was 6.1 nM. However, many other inhibitors from natural sources (Kim *et al.*, 2005a; Yu *et al.*, 2003) have shown activities no higher than *cis*-hinokiresinol.

Some agents that show scavenging activities toward radical ions also show inhibitory activities toward LDL-oxidation (Jeong *et al.*, 2004), hACAT and Lp-PLA_2 (Kim *et al.*, 2005a). Lp-PLA_2 has also been suggested as a pro-inflammatory agent, which can be detected in the macrophages of atherosclerotic lesions in both hyperlipidemic humans and rabbits (Hakkinen *et al.*, 1999). Therefore, the inhibition of Lp-PLA_2 activity has been highlighted as a key component in the prevention and treatment of atherosclerosis. In this study, the *cis*-hinokiresinol isolated from *T. pseudoincisa* was demonstrated to exert inhibitory effects against the ABTS cation radical, superoxide anion radical, hACAT1, hACAT2, LDL oxidation and Lp-PLA_2 activities. The relative paucity of naturally occurring ABTS, superoxide and LDL-oxidation inhibitors renders this discovery rather more important than initially expected.

In conclusion, the findings of this study suggest that *T. pseudoincisa,* as well as its isolated compound, *cis*-hinokiresinol, may prove useful in the treatments of hypercholesterolemia and atherosclerosis.

ACKNOWLEDGEMENTS

This work was supported by the SRC program of MOST/KOSEF (R11-2000-081), through the Plant Metabolism Research Center, KyungHee University, and via a grant from the BioGreen 21 Program, Rural Development Administration, Republic of Korea.

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