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Structure-Activity Relationships of Gagaminine and Its Derivatives on the Inhibition of Hepatic Aldehyde Oxidase Activity and Lipid Peroxidation

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In order to determine the structure-activity relationships for antioxidative effects of gagaminine, a steroidal alkaloid isolated from the roots of *Cynanchum wilfordi* (Asclepiadaceae), two derivatives identified as sarcostin and penupogenin were prepared from gagaminine by hydrolysis and reduction. These compounds were evaluated for the inhibitory effects on the aldehyde oxidase activity and on lipid peroxidation *in vitro*. Furthermore, their effects were compared with those of gagaminine and the related compounds, cinnamic acid and nicotinic acid. The results of this study prove that the cinnamoyl group in the structure of gagaminine is critical in inhibition of the aldehyde oxidase activity while the nicotinoyl group may be necessary for anti-lipid peroxidation of the compound.

Key words : *Cynanchum wilfordi*, Gagaminine, Sarcostin, Penupogenin, Antioxidant, Structure-activity relationship

INTRODUCTION

The highly reactive oxygen species (ROS), such as superoxide anion and hydroxyl radical, are known to be generated by aldehyde oxidase and xanthine oxidase and induce lipid peroxidation in cell membrane (Muller, 1992). Therefore, the inhibition of these enzymes may reduce the production of ROS. When ROS are generated *in vivo,* many antioxidants come into play in order to protect cell membrane against the progress of lipid peroxidation (Halliwell, 1995). The numerous attempts in recent years have been performed and are in progress in order to search more active antioxidants from synthetic compounds and also from natural sources.

In connection with this view we have previously reported the antioxidative activities of the alkaloid fraction (Lee, 1995) and gagaminine (Lee *et al.*, 1996) isolated for the first time by us from the alkaloid fraction of the roots of *Cynanchum wilfordi.* This medicinal plant has been used as folk remedies in Korea for a long time for prevention and treatment of various geriatric diseases involved in vascular disorder, diabetes mellitus, ischemia-induced diseases, and aging progress. It has been considered that the lipid peroxidation damage is involved in againg (Harman, 1969) and some pathological disorders such as atherosclerosis (Glavind *et al.*, 1952).

Gagaminine revealed a potent inhibitory effect (IC_{50}= 0.8 μM) on the activity of aldehyde oxidase (EC. 1.2.3.1), one of molybdenum containing cytosolic oxidation enzymes as well as xanthine oxidase. Gagaminine exhibited a much higher activity than those of KCN and pyridoxal (IC_{50}=1.5 μM and 246 μM, respectively) which are well known aldehyde oxidase inhibitors (Lee *et al.*, 1996).

Gagaminine inhibited not only the aldehyde oxidase activity, but significantly ameliorated lipid peroxidation in a dose-dependent manner, although its antioxidative activity was somewhat lower than those of well-known natural antioxidants, ascorbic acid and α-tocopherol (Lee *et al.*, 1996).

As a part of our continuing study (Lee *et al.*, 1994, 1996) for searching for more active antioxidants and to obtain information about structure-activity relationships for gagaminine, we have chemically modified the structure of gagaminine, thereby obtaining two natural compounds. Their antioxidative activities were compared with those of gagaminine and the related com-

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pounds, cinnamic acid and nicotinic acid.

MATERIALS AND METHODS

General

The plant material was provided by a local drug-store in Youngchon, Korea, and was identified by Prof. Byung-Soo Kang, College of Oriental Medicine, Dong-guk University, Kyongju, Korea. A voucher specimen is deposited in the Herbarium of this University. The enzyme source was prepared from male Wistar rats, weighing 200 to 250 g. Animals were given water only for 16 hours before experiments. Malondialdehyde (MDA), sodium dodecyl sulfate (SDS), *N*-methylnicotinic acid amide (NMN), 2-thiobarbituric acid (TBA), *trans*-cinnamic acid, and nicotinic acid were purchased from Sigma Chemical Co.(St. Louis, MO, USA). All other chemicals were of the highest analytical grade available. Melting points were measured on the Electrothermal IA9100 without correction. FT-IR spectra were recorded on the Bomem MB 100-10. ^{1}H-NMR spectra were obtained on Varian XL-200 (200 MHz) in $CDCl_3$ solution with TMS as an internal standard. UV spectra were measured on the Shimadzu UV-2001S. TLC were done on Kieselgel 60 F_{254} aluminum sheets.

Isolation of gagaminine (1)

The methanol extract of the dried powder of the roots of Cynanchum wilfordi (6 kg) was treated by a conventional method to give an alkaloid extract (2.5 g). Gagaminine was isolated and purified from the alkaloid fraction by silica gel (230~400 mesh, Merck) column chromatography using $CHCl_3$/MeOH 9:1 as a solvent to afford pale yellow crystals (0.5 g). R_f 0.32 ($CHCl_3$/MeOH 9:1). The Liebermann-Burchard test for steroid skeleton: positive (bluish green). The Meyer test for alkaloidal moiety: postive. Physical and spectral data: see ref. (Lee *et al.*, 1996).

Preparation of the derivatives of gagaminine

Sarcostin (2): Gagaminine **1** (0.16 mmol) was hydrolyzed with 2 ml of methanolic KOH solution (5%) under reflux for 2 h. After filteration of the reaction mixture, water was added and the solvent was evaporated. The water phase was extracted several times with n-butanol, which was washed with water and removed *in vacuo*. The remaining residue was recrystallized with acetone to give colourless needles, which was identified as sarcostin 2 by comparison with the spectral data of the authentic compound (Shimizu *et al.*, 1968). Yield: 75%. Liebermann-Burchard test: positive (bluish green). Meyer test: negative. mp. 148~151°C (first mp.)/254~257°C (final mp.) (confer 150°C/250~255°C) (Fukuoka *et al.*, 1969).

Penupogenin (3): The solution of 0.185 g (0.3 mmol) of gagaminine **1** in 5 ml of absolute tetrahydrofuran was added dropwise to the suspension of 0.01 g (0.3 mmol) of $LiAlH_4$ in 8 ml of absolute tetrahydrofuran under gentle reflux. After the reaction under reflux for 1 h, the reaction mixture was diluted with cold water, followed by addition of 10% sulfuric acid for the degradation of excess $LiAlH_4$. The organic phase was washed with saturated NaCl and concentrated to give colourless needles from ether, which was identified as penupogenin 3 by comparison with the physical and spectral data of authentic compound (Warashina *et al.*, 1995). Yield: 52%. Liebermann-Burchard test: positive (bluish green). Meyer test: negative.

Preparation of the enzyme source

Male rats were killed under anesthesia by exsanguination from the abdominal aorta. The liver was exhaustively perfused with ice-cold saline through the portal vein until uniformly pale, immediately removed and weighed. After trimmed and minced, the pieces of liver were homogenized in 4 volume of ice-cold 0.1 M potassium phosphate buffer (pH 7.4). The homogenate was spun at 10,00×g for 20 min. The supernatant was collected and further centrifuged at 105,000×g for 60 min. The resultant cytosolic fraction was used as the enzyme source for aldehyde oxidase and xanthine oxidase assays.

Enzyme assays

The aldehyde oxidase activity was measured according to the known method (Branzoli *et al.*, 1974). Samples of the cytosolic fraction (0.2 ml) were incubated with 1.0 μmole of N-methylnicotinic acid amide (NMN) in 0.2 ml of 0.075 M potassium phosphate buffer (pH 7.5) at 37°C with shaking. The reaction mixtures were routinely incubated for 30 min, then the conversion of NMN to 6-pyridone was measured at 300 nm on a Shimadzu 1201 spectrophotometer.

The xanthine oxidase activity was aerobically determined by measuring the rates of uric acid formation without NAD^+ from xanthine sodium salt. The xanthine dehydrogenase activity was assayed by measuring spectrophotometrically the amount of uric acid formed from xanthine sodium salt with NAD^+ as electron acceptor (Stripe *et al.*, 1969).

Measurement of lipid peroxidation

Lipid peroxidation of liver tissues was measured by the formation of MDA according to the known procedure (Ohkawa *et al.*, 1979). The test was performed at 37°C in a capped tube containing 0.2 ml homo-

genate in 0.1 M potassium phosphate buffer (pH 7.4), 0.5 ml of distilled water, and 0.5 ml of 30% trichloroacetic acid. The incubated reaction mixture was centrifuged at 3,000 rpm for 3 min. The supernatant was added to an equal volume of aqueous 0.67% TBA, heated to boiling point for 60 min, and cooled to room temperature. The level of lipid peroxides was expressed in terms of MDA equivalents (nmoles MDA/g of tissue) by measuring the absorbance at 532 nm.

Protein assay and statistical analysis

Protein concentration was determined by the Lowry method (Lowry *et al.*, 1951) using bovine serum albumin as a standard. Statistical analysis was performed by Student's t-test.

RESULTS AND DISCUSSION

Preparation of the derivatives of gagaminine

For preparation of the test compounds gagaminine **1** was modified by alkaline hydrolysis and metal hydride reduction. Firstly, **1** was treated with 5% methanolic KOH solution under reflux to cleave both ester-linkages to furnish colourless needles which were identified as sarcostin **2** (Fig. 1). **1** has already been reported to yield two kinds of monoesters and **2** on mild alkaline hydrolysis using K_2CO_3 in methanol solution (Yamagishi *et al.*, 1972). However, we have obtained **2** only by the above method with 75% yield. **2** is known to exist in some Asclepiadaceae plants (Shimizu *et al.*, 1968). Secondly, **1** was reduced with $LiAlH_4$ (one equivalent) in anhydrous tetrahydrofuran to give colourless needles (52% yield) which was identified as penupogenin **3**, a mono-cinnamic ester of sarcostin **2** (Fig. 1). This compound has earlier been isolated from *Cynanchum caudatum* (Mitsuhashi *et al.*, 1962).

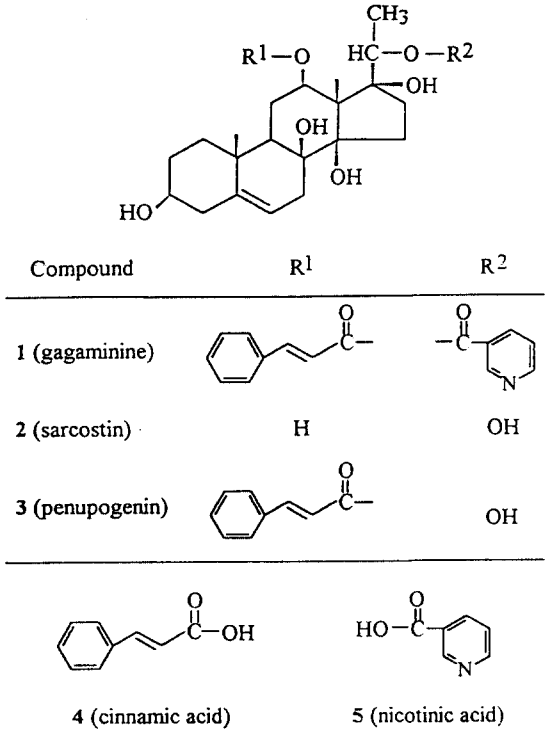

Fig. 1. Chemical structures of the tested compounds.

Inhibition on the aldehyde oxidase activity and lipid peroxidation

In the previous report (Lee *et al.*, 1996) we have found that gagaminine **1**, a steroidal alkaloid from the roots of *Cynanchum wilfordi* (Asclepiadaceae), exerts a potent inhibitory effect on the activity of aldehyde oxidase (EC 1.2.3.1) and restrains significantly the formation of lipid peroxides *in vitro*. In order to find more active antioxidants than **1** and to obtain information about structure-activity relationships, some modifications were carried out. We focussed initially on the effects of the ester moieties-cinnamoyl and nicotinoyl groups-in the antioxidative activities of **1**.

As shown in Fig. 2, gagaminine **1** inhibits the aldehyde oxidase activity in a dose-dependent manner, exhibiting a high inhibitory effect (0.021 nmoles of 6-pyridone/mg protein/min) at 0.05 mg/ml. At the same concentration, the inhibitory effects of cinnamic acid **4**, one of the degradative products of **1**, and penupogenin **3**, a mono-cinnamic ester of **1**, revealed respectively 0.177 and 0.182 nmoles of 6-pyridone/mg protein/min. The inhibitory effect of **4** on the aldehyde oxidase activity has not yet been reported. In contrast, sarcostin **2** which lacks a cinnamoyl group and nicotinic acid 5 derived from the degradation of **1** showed practically no inhibition of the enzyme activity. These results indicate that the cinnamoyl group is regarded as being important in the inhibitory effect of 1 on the aldehyde oxidase activity. It would be sug-

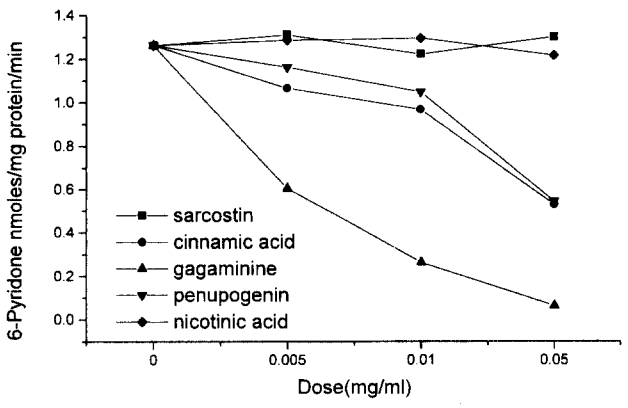

Fig. 2. Effects of the tested compounds on the aldehyde oxidase activity *in vitro*.

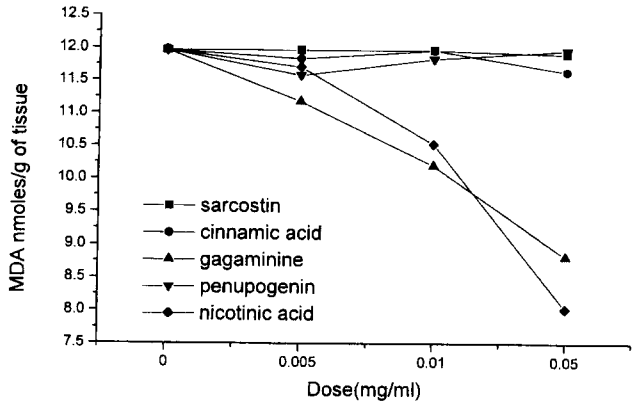

Fig. 3. Effects of the tested compounds on lipid peroxidation *in vitro*.

gested that the cinnamoyl group may bind well to the active site of aldehyde oxidase with competitive inhibition, because a gagaminine-containing alkaloid fraction showed such inhibitory effect (Lee *et al.*, 1994).

In the next step, in order to know the structure-activity relationships of the prevention of lipid peroxidation of gagaminine **1**, above five compounds were tested for their ability to suppress the formation of lipid peroxides *in vitro*. As shown in Fig. 3, **1** and **5** suppressed strongly the hepatic lipid peroxidation to the extent similar to that observed with MDA, a secondary product of lipid peroxidation, at 0.05 mg/ml, while sarcostin **2** and penupogenin **3** which do not contain the nicotinoyl group as well as cinnamic acid **4** did not show any significant activity. These results suggest that the nicotinoyl group is necessary for the activity of anti-lipid peroxidation of **1**.

Table I. Effect of gagaminine **1**, cinnamic acid **4** and nicotinic acid **5** on the hepatic xanthine oxidase activity

Dose (mg/ml)	compound	Specific activity[a]		Type conversion ratio (%)[b]
		Type O	Type D+O	
0		0.37 ± 0.02	2.92 ± 0.13	12.6
0.005	1	0.37 ± 0.03	2.93 ± 0.11	12.6
	4	0.33	2.72	12.1
	5	0.36	2.84	12.7
0.01	1	0.36 ± 0.03	2.91 ± 0.17	12.4
	4	0.38	2.51	15.1
	5	0.36	3.00	12.0
0.05	1	0.37 ± 0.02	2.89 ± 0.21	12.8
	4	0c	0c	-
	5	0.36	2.92	12.3
0.1	1	0.35 ± 0.03	2.91 ± 0.18	12.0
	4	0[c]	0c	-
	5	0.31	2.75	11.3

Values are mean±S.E. for 3 separate experiments.
[a]uric acid nmoles/mg protein/min.
[b]Type conversion ratio (%) was calculated from Type O/Type D+O × 100.
[c]Cinnamic acid **4** inhibited completely the activity at 0.04 mg/ml.

In contrast to the strong inhibitory effect on aldehyde oxidase, gagaminine **1** showed interestingly no significant change in the activity of xanthine oxidase (EC 1.2.3.2) which is also a molybdenum-containging cytosolic enzyme as well as aldehyde oxidase (Table I). We have compared the activity of **1** on xanthine oxidase with those of cinnamic acid and nicotinic acid. As shown in Table I, at low concentrations all three compounds exhibited no effect on the enzyme activity. However, at higher concentrations (over 0.04 mg/ml) cinnamic acid inhibited completely the xanthine oxidase activity.

Although the structure and function of xanthine oxidase are similar to those of aldehyde oxidase, above results indicate that **1** has much higher affinity for aldehyde oxidase than xanthine oxidase and **5** has no affinity for both oxidases in contrast to the proper affinities of **4**. The inhibitory effects of **4** on both enzyme activities will be further investigated.

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