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Effect of Hispidulin 7-O-neohesperidoside on Lipid Peroxidation in Rat Liver and NMR Assignment

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Abstract – The full NMR assignment of hispidulin 7-O-neohesperidoside (1) isolated from *Cirsium japonicum* var. *ussuriense* was made with the aid of 2D correlation NMR techniques such as HMQC and HMBC. To investigate detoxification of bromobenzene-induced hepatic lipid peroxidation by compound 1, hepatic lipid peroxide level and the activities of enzymes responsible for production and removal of epoxide were studied. The level of lipid peroxide elevated by bromobenzene was significantly reduced by compound 1. This compound administered daily over one week before intoxication with bromobenzene did not affect the activities of aminopyrine N-demethylase, aniline hydroxylase, glutathione S-transferase. Epoxide hydrolase activity was decreased significantly by bromobenzene, which was restored to the control level by pretreatment of persicarin. The results suggest that the bromobenzene-induced hepatic lipid peroxidation by compound 1 is reduced by enhancing the activity of epoxide hydrolase, an enzyme removing bromobenzene epoxide.

Key words – hispidulin-7-O-neohesperidoside; bromobenzene-induced hepatic lipid peroxidation; epoxide hydrolase.

In the previous study,[1] a novel flavone diglycoside, hispidulin 7-O-neohesperidoside (1) was isolated from the whole plants of *Cirsium japonicum* var. *ussuriense* Kitamura (Compositae), which has been used for the remedy of inflammation as a folkloric medicine in Korea. As part of our continuing search for anti-lipoperoxidative compound from natural products,[2] the effect of compound 1 on the formation of hepatic lipid peroxide has been investigated. In addition, the ^{1}H- and ^{13}C-NMR signals of this compound have been fully assigned by utilizing ^{1}H-^{1}H COSY, HMQC and HMBC experiments.

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MATERIALS AND METHODS

Isolation of compound and instrument – Hispidulin 7-O-neohesperidoside was isolated from whole plants of *C. japonicum* var.

ussuriense as in reported previously.[1] ^{1}H-(400 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded on Bruker model AMX 400 spectrometer with TMS as internal standard.

Animals - Male Sprague-Dawley rats of three groups were used for the study. Animals were fed with commercial standard rat diet and water *ad lib.*, and maintained at 20±2℃ and with the illumination of a 12 hour light/dark cycle. Animals were orally administered daily with 10 mg/kg of compound 1 for one week. Then bromobenzene (460 mg/kg) was i.p. injected with 12 hours interval for final two days. Sample was dissolved in 1% Tween 80 solution. Control group was given with 0.2 ml of 1% Tween 80 solution per 200 g. The animals were starved overnight before sacrificed in order to reduce the variation of hepatic metabolism.

Preparation of enzyme source - Animals were sacrificed by exsanguination from the abdominal aorta under anesthesia with CO_2 gas. The liver exhaustively perfused with ice-cold normal saline through the portal vein until uniformly pale and immediately removed and weighed. After trimmed and minced, the piece of liver homogenized with four volumes of ice cold 0.1 M potassium phosphate buffer (pH 7.5) solution. The homogenate was centrifuged at 600×g for 10 minutes. The pellet discarded and supernatant was recentrifuged at 10,000×g for 20 minutes. The supernatant was further centrifuged at 105,000×g for 60 minutes. The resulting supernatant, cytosolic fraction, used as the enzyme sources of glutathion S-transferase, and the microsomal fraction was used as the enzyme sources of aniline N-demethylase, aniline hydroxylase and epoxide hydrolase.

Analytical methods - The thiobarbituric acid (TBA) reactive substance in the liver was measured as a marker of lipid peroxidation by the method of Ohkawa *et al.*[3] The 0.4 ml of 10% liver homogenate in 0.9% NaCl was added to 1.5 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 20% acetate buffer (pH 3.5) and 1.5 ml of 0.8% TBA solution. The mixture was heated at 95℃ for 1 hour. After cooling, 5.0 ml of n-butanol-pyridine (15:1) was added for extraction, and the absorbance of the n-butanol-pyridine layer at 532 nm was measured for the determination of TBA reactive substance. Aminopyrine N-demethylase activity[4] was assayed by measuring the production of formaldehyde formed by the demethylation of aminopyrine. The reaction mixture consisted of 300-400 μg microsomal protein, 0.1 M Na^+/K^+ phosphate buffer (pH 7.5) and 2.0 mM aminopyrine in a total volume of 2.0 ml. The mixture was preincubated for 3 min at 37℃ and 0.5 mM NADPH was added to initiate the reaction. After 30 minutes the reaction was stopped by the addition of 0.5 ml of 15% $ZnSO_4$ and saturated Ba $(OH)_2$, and cooled at room temperature. After centrifugation at 1,000×g for 10 minutes, 1 ml of the supernatant was mixed with 5 ml of Nash reagent. Then the tubes were capped and heated at 60℃ for 30 minutes. After cooling in tap water, the absorbance was read at 415 nm versus a water blank. Aniline hydroxylase activity[5] was assayed by determining p-aminophenol formation from aniline. The basic incubation system was same as described above, except that 1 mM aniline was used as the substrate. The reaction was initiated by the addition of 0.5 mM NADPH. After shaking for 30 min at 37 ℃, the reaction was terminated by the addition of 0.5 ml of 20% trichloroacetic acid. The mixture was centrifuged at 1,000×g for 10 minutes and 1 ml of the supernatant

was added to 1 ml of 0.2 N NaOH containing 2% phenol. After mixing, 1 ml of 10% Na_2CO_3 was added. After 20 minutes at room temperature the absorbance was read at 640 nm against a water blank. Glutathione S-transferase activity[6] was assayed by conjugated glutathione 2,4-dinitrobenzene formation from 1-chloro-2,4-dinitrobenzene. The reaction mixture consisted of 100 μl cytosol fraction, 0.1 M potassium phosphate buffer (pH 6.5), 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM glutathione in a total volume of 3.5 ml. The mixture was incubated at 25℃ for 2 minutes. The measurement was carried out by spectrometric changes at maximal absorbance wavelength (340 nm) per unit time and calculated with molar extinction coefficient (9.6 mM^{-1} cm^{-1}). The activity was expressed as formed product nmole per mg protein per minute. Epoxide hydrolase activity[7] was measured spectrophotometically by monitoring the rate of transstilbene oxide (TSO) decreasing at 229 nm as described previously. The reaction mixture consisted of 100-200 g microsomal protein and 3.0 mM TSO in 50 M potassium phosphate buffer (pH 7.0) in a total volume of 3.0 ml. The mixture was incubated for 20 minutes at 37℃. Protein content[8] was determined by using bovin serum albumin as a standard. The statistical differences between the experimental group were analyzed with Student's t-test.

RESULTS AND DISCUSSION

In the HMBC spectrum of compound 1, the proton-signal at δ3.87 ($-OCH_3$) showed long-range correlation with the carbon-signal at δ132.9 (C-6), indicating that the methoxyl group is located at the C-6 position (Table I). The proton-signal at δ5.43 (H-1″) showed correlation with the signal at δ155.9 (C-7) while the proton-signal at δ5.37 (H-1‴) showed correlation with the signals at δ76.0 (C-2″), indicating a glucose and a rhamnose are located at C-7 position of flavone aglycone and C-2 position of glucose, respectively. The chelated hydroxyl proton at δ6.94 (H-3) showed cross peaks because of long-range coupling with the three quarternary carbons at δ164.4 (C-2), 105.9 (C-4a) and 121.1(C-1′), and the proton-signal at δ7.13 (H-8) showed correlations with the signals at δ132.9 (C-6), 155.9 (C-7), 105.9 (C-4a) and 152.6 (C-8a). On the other hand,

Table I. ^1H, HMQC and HMBC NMR data of hispidulin 7-O-neohesperidoside in DMSO-d_6

position	H(mult., J-Hz)	Correlated C-atom HMQC	Correlated C-atom HMBC (H→C)
2		164.4	
3	6.94 (s)	102.7	2, 4a, 1′
4		182.4	
5		152.1	
6		132.9	
7		155.9	
8	7.13 (s)	94.5	6, 7, 4a, 8a
4a		105.9	
8a		152.6	
1′		121.1	
2′	8.03 (d, 8.8)	128.7	2, 4′
3′	7.04 (d, 8.8)	116.1	1′
4′		161.5	
5′	7.04 (d, 8.8)	116.1	1′
6′	8.03 (d, 8.8)	128.7	2, 4′
glucosyl 1″	5.43 (d, 7.7)	98.0	7
2″	3.72 (dd, 8.9, 7.7)	76.0	
3″	3.63 (dd, 8.9, 8.9)	77.7	
4″	3.34 (dd, 8.9, 8.9)	69.9	
5″	3.57-3.60	77.3	
6″	3.57-3.60, 3.79-3.85	60.6	
rhamnsoyl 1‴	5.37 (d, 1.1)	100.1	2″
2‴	3.79-3.85	70.5	
3‴	3.48 (dd, 9.4, 3.1)	70.6	
4‴	3.29 (dd, 9.4, 9.4)	72.1	
5‴	3.79-3.85	68.7	
6‴	1.21 (d, 6.1)	18.2	
$-OCH_3$	3.87 (s)	60.4	6

Table II. Effects of hispidulin 7-O-neohesperidoside (1) on the hepatic lipid peroxide (LPO), aminopyrine N-demethylase (AD), aniline hydroxylase (AH), cytosolic glutathione S-transferase (GST) and epoxide hydrolase (EH) activities in bromobenzene-treated rats

Group	Dose (mg/kg)	LPO[a]	AD[b]	AH[c]	GST[d]	EH[e]
Control	0	21.3±3.22	4.80±0.63	0.47±0.09	270.3±10.2	11.6 ±2.07
Bromobenzene (BB)	460 (i.p.)	41.3±4.72**	6.10±0.89	0.98±0.13**	283.4±12.4	5.21±0.79**
Compound 1 +BB	10 (p.o.)	35.7±6.23*	7.00±0.62**	1.06±0.23**	290.6±20.3	7.43±0.97*

The values are mean±S.D. in five rats. Significantly different from the control value: *p<0.05, *p<0.01. [a]unit: malondialdehyde nmole/g of tissue. [b]unit: p-aminophenol nmole/mg protein/min. [c]unit: HCHO-nmole/mg protein/min. [d]unit: conjugated 1,2-dichloro-4-nitrobenzene. [e]unit: TSO nmole/mg protein/min.

the aromatic protons at δ8.03 (H-2′ and H-6′) showed long-range correlations with the carbons at δ102.7 (C-2) and 161.5 (C-4′), and the one at δ7.04 (H-3′ and H-5′) did so with that at δ121.1 (C-1′), respectively. Thus the attachment locations of the methoxyl group, the glycosidic linkages of disaccharide, and the connectivities of quarternary carbons in compound 1 were analyzed with the aid of HMBC experiment.

Compound 1 orally administered daily with 10 mg/kg for one week, significantly reduced the level of lipid peroxides induced by bromobenzene (Table II). To determine the mechanism of the protective effect of compound 1, the activities of enzymes responsible for production and removal of epoxide, aminopyrine N-demethylase, aniline hydroxylase, glutathione S-transferase and epoxide hydrolase were investigated. Activities of microsomal aminopyrine N-demethylase and aniline hydroxylase in liver increased significantly by bromobenzene injection. Pretreatment of compound 1 did not show any effect on the increases of the microsomal enzyme activities by bromobenzene. And no significant differences were observed in the activity of glutathione S-transferase by bromobenzene and compound 1. Epoxide hydrolase activity decreased significantly by the treatment of bromobenzene. However, the enzyme activity was restored in liver of rats given with compound 1. Epoxides inhibit the activities of various enzymes[9,10] and act as a mutagen. They are often associated with cancer, aging and metabolic diseases. Bromobenzene, a xenobiotic liver toxin, is converted to bromobenzene 3,4-oxide by mixed function oxidase system in the liver. Bromobenzene 3,4-oxide, an epoxide acting as a liver toxin, is metabolized to a nontoxic bromobenzene 3,4-dihydrodiol by epoxide hydrolase. The bromobenzene 3,4-oxide can also be converted to bromobenzene glutathione by the action of glutathione S-transferase and excreted thereafter.

In conclusion, the results speculate on a protective effect of compound 1 from hepatic lipid peroxidation induced by bromobenzene. It is thought to be via enhancing the activity of epoxide hydrolase, an enzyme removing bromobenzene epoxide, rather than through acting to epoxide-producing system.

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