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ORIGINAL PAPER

Murine metabolism and absorption of lancemaside A, an active compound in the roots of *Codonopsis lanceolata*

Noriko Komoto · Makoto Ichikawa · Sanae Ohta · Daisuke Nakano · Takeshi Nishihama · Mitsuyasu Ushijima · Yukihiro Kodera · Minoru Hayama · Osamu Shirota · Setsuko Sekita · Masanori Kuroyanagi

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Abstract Lancemaside A, a triterpenoid saponin isolated from the roots of *Codonopsis lanceolata*, has been reported to ameliorate the reduction of blood testosterone levels induced by immobilization stress in mice. In the present study, we investigated the metabolism and absorption of lancemaside A in mice. After oral administration of lancemaside A at 100 mg/kg body weight, the unmetabolized compound appeared rapidly in plasma ($t_{max} = 0.5$ h). Lancemaside A has a low bioavailability (1.1%) because of its metabolism by intestinal bacteria and its poor absorption in the gastrointestinal tract. Furthermore, we identified four metabolites from the cecum of mice after oral administration of lancemaside A: codonolaside II, echinocystic acid, echinocystic acid 28-*O*-*β*-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl ester, and echinocystic acid 28-*O*-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl ester. Among these metabolites, codonolaside II and echinocystic acid were detected in plasma, and their t_{max} values were 4 and 8 h, respectively. These findings should be helpful for understanding the mechanism of the biological effect of lancemaside A.

Keywords Lancemaside A · Metabolism · *Codonopsis lanceolata* · Codonolaside II · Echinocystic acid · Bioavailability

N. Komoto (✉) · M. Ichikawa · S. Ohta · D. Nakano · T. Nishihama · M. Ushijima · Y. Kodera · M. Hayama
Healthcare Research Institute, Wakunaga Pharmaceutical Company, Ltd., 1624 Shimokotachi, Kodacho, Akitakata, Hiroshima 739-1195, Japan
e-mail: komoto_n@wakunaga.co.jp

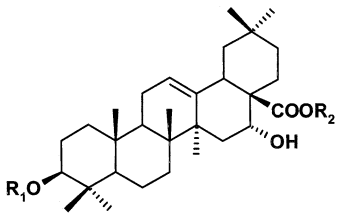
O. Shirota · S. Sekita
Laboratory of Pharmacognosy and Natural Product Chemistry, Faculty of Pharmaceutical Sciences at Kagawa Campus, Tokushima Bunri University, 1314-1 Shido, Sanuki, Kagawa 769-2193, Japan

M. Kuroyanagi
Department of Life Sciences, Faculty of Life and Environmental Sciences, Prefectural University of Hiroshima, 562 Nanatsuka, Shobara, Hiroshima 727-0023, Japan

Introduction

The roots of *Codonopsis lanceolata* Trautv. (Campanulaceae) have been used as a traditional medicine for their antitussive, expectorant, and antidotal effects. Recently, the root extracts have been found to inhibit the reduction of blood testosterone levels induced by immobilization stress in mice [1]. Moreover, a supplement containing the root extracts has been reported to ameliorate partial androgen deficiency of the aging male (PADAM)-like symptoms as well as the total testosterone levels in middle-aged men with PADAM [2]. It has been previously reported that the components in the roots of *C. lanceolata* include saponins [3–7] and phenylpropanoid glycosides [8]. Among them, lancemaside A (**1**, Fig. 1), a 3,28-bidesmosidic triterpenoid saponin, has been reported to ameliorate the reduction of blood testosterone levels induced by immobilization stress in mice [8]. Therefore, **1** is expected to be useful for the prevention of and as a complementary therapy for PADAM symptoms associated with the lowering of blood testosterone levels.

It is known that the bioavailability of saponins is low because of their poor oral absorption and gastrointestinal metabolism [9, 10]. Some of saponins are transformed to their deglycosylated compounds by intestinal bacteria, and the resulting compounds exhibit biological activities [11, 12]. This suggests that **1** may not itself be responsible



Compound	R_1	R_2
1	β-GlcA	β-Xyl-(1→3)-β-Xyl-(1→4)-α-Rha-(1→2)-α-Ara
2	H	β-Xyl-(1→3)-β-Xyl-(1→4)-α-Rha-(1→2)-α-Ara
3	H	H
4	H	β-Xyl-(1→4)-α-Rha-(1→2)-α-Ara
5	H	α-Rha-(1→2)-α-Ara

Fig. 1 Chemical structures of lancemaside A (**1**) and its metabolites (**2–5**). β-GlcA, β-D-glucuronopyranosyl; α-Ara, α-L-arabinopyranosyl; α-Rha, α-L-rhamnopyranosyl; β-Xyl, β-D-xylopyranosyl

for its biological activities. However, no data are available on the absorption and metabolism of **1**.

In the present study, we investigated the pharmacokinetics of **1** in mice. The metabolism of **1** in the gastrointestinal tract was evaluated by using liquid chromatography–mass spectrometry (LC–MS) with an electrospray ionization (ESI) source. In addition, **1** and its metabolites were identified by using LC–MS with multiple-stage mass spectrometry (MS^n), which is extensively applied to the online analysis of saponins [13–15]. As shown in Fig. 1, four metabolites, namely, codonolaside II (**2**), echinocystic acid (**3**), echinocystic acid 28-*O*-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl ester (**4**), and echinocystic acid 28-*O*-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl ester (**5**), were identified from the cecum of mice after oral administration of **1**. We also established an LC–MS method to determine the concentration of **1** and its gastrointestinal metabolites, **2** and **3**, in mouse plasma, and then investigated the pharmacokinetics of **1** in mice.

Materials and methods

Chemicals and reagents

Lancemaside A (**1**), lancemaside E (used as the internal standard, IS), and foetidissimoside A were isolated from the roots of *C. lanceolata* [6, 16]. The IS solution was prepared at a concentration of 0.05 μg/ml in ethanol. Saline (0.9% NaCl injectable solution) was obtained from Fuso Pharmaceutical Co., Ltd. (Osaka, Japan). Acetonitrile for LC–MS was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). β-Glucuronidase (type H-5, 654200 U/g) was obtained from Sigma (St. Louis, MO, USA). All other chemicals of reagent grade were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and Wako Pure Chemical Industries, Ltd. Water purified with a Milli-Q Labo system (Millipore, Bedford, MA, USA) was used for mobile phase preparation.

Preparation of standard compounds **2–5**

Codonolaside II (2)

Compound **1** (50 mg) was dissolved in 40 ml of 0.5 mol/l sodium acetate buffer (pH 5.0), and β-glucuronidase (21 mg) was then added to the mixture, followed by incubation for 6 h at 37°C. After incubation, the mixture was applied to a Diaion HP-20 (Mitsubishi Chemical Co., Tokyo, Japan) column, and the column was washed with 10% methanol, followed by elution with methanol. The methanol eluate was concentrated in vacuo. The residue was purified by silica gel column chromatography with chloroform–methanol–water (40:10:1). Finally, 25 mg of codonolaside II (**2**) was isolated and identified by comparison of the NMR spectroscopic data with those reported in the literature [5]. The ^{13}C-NMR data of **2** are shown in Table 1.

Echinocystic acid (3)

Compound **1** (80 mg) was dissolved in 80 ml of acetate buffer (pH 5.0), and β-glucuronidase (40 mg) was then added to the mixture, followed by incubation for 6 h at 37°C. After incubation, the mixture was applied to a Diaion HP-20 column, and the column was washed with 30% methanol, followed by elution with methanol. The methanol eluate was concentrated in vacuo. The residue was dissolved in 3 ml of methanol, and 12 ml of 3% potassium hydroxide was then added to the mixture, followed by heating at 80°C for 2 h. The mixture was added to 50% methanol (40 ml) and applied to a Diaion HP-20 column, and the column was washed with 40% methanol, followed by elution with methanol. The methanol eluate was concentrated in vacuo. The residue was purified by silica gel column chromatography with chloroform–methanol (20:1). Finally, 9 mg of echinocystic acid (**3**) was isolated and identified by comparison of the NMR spectroscopic data with those reported in the literature [17]. The ^{13}C-NMR data of **3** are shown in Table 1.

*Compounds **4** and **5***

Foetidissimoside A (47 mg) was dissolved in 40 ml of acetate buffer (pH 5.0), and β-glucuronidase (21 mg) was then added to the mixture, followed by incubation for 6 h at 37°C. After the incubation, the mixture was applied to a Diaion HP-20 column, and the column was washed with 30% methanol, followed by elution with methanol. The methanol eluate was concentrated in vacuo. The residue

Table 1 ^{13}C-NMR chemical shifts of lancemaside A (**1**) and its metabolites (**2**–**5**) in pyridine-d_5

Carbon	**1**	**2**	**3**	**4**	**5**	Carbon	**1**	**2**	**4**	**5**
1	38.7	39.1	39.0	39.1	39.1	Glucuronic acid				
2	26.6	28.1	28.2	28.1	28.1	1	107.2			
3	89.0	78.2	78.1	78.1	78.1	2	75.5			
4	39.5	39.4	39.4	39.4	39.4	3	78.2			
5	55.8	55.9	55.9	55.9	55.9	4	73.4			
6	18.3	18.8	18.8	18.9	18.8	5	77.9			
7	33.4	33.5	33.6	33.5	33.5	6	172.8			
8	39.9	40.0	39.9	40.0	40.0	Arabinose				
9	47.1	47.3	47.3	47.3	47.3	1	93.4	93.4	93.7	93.5
10	36.9	37.4	37.4	37.4	37.4	2	75.1	75.3	75.0	75.2
11	23.7	23.8	23.8	23.8	23.8	3	69.4	69.5	70.3	70.1
12	122.8	122.8	122.4	122.8	122.8	4	65.8	65.8	66.3	66.1
13	144.3	144.4	145.1	144.4	144.5	5	62.7	62.7	63.4	63.1
14	42.0	42.1	42.1	42.1	42.1	Rhamnose				
15	36.1	36.1	36.2[a]	36.2	36.2	1	100.9	101.0	101.1	101.5
16	74.0	74.0	74.8	74.0	73.9	2	71.8	71.8	71.9	72.4
17	49.5	49.5	48.9	49.5	49.6	3	72.7	72.7	72.7	72.6
18	41.2	41.2	41.5	41.3	41.3	4	83.3	83.4	83.6	73.9
19	47.0	47.1	47.3	47.2	47.2	5	68.4	68.5	68.6	70.5
20	30.9	30.9	31.0	30.9	30.9	6	18.3	18.4	18.4	18.5
21	35.9	36.0	36.1[a]	36.0	36.0	Xylose (inner)				
22	32.1	32.1	32.9	32.1	32.1	1	106.1	106.1		
23	28.1	28.8	28.7	28.8	28.7	2	74.9	74.9		
24	16.9	16.6	16.6	16.6	16.6	3	87.0	87.1		
25	15.6	15.7	15.7	15.8	15.8	4	68.9	69.0		
26	17.5	17.6	17.5	17.6	17.6	5	66.8	66.8		
27	27.1	27.1	27.2	27.1	27.1	Xylose (terminal)				
28	175.9	175.9	180.0	175.9	175.9	1	106.0	106.0	106.9	
29	33.2	33.2	33.3	33.2	33.2	2	75.3	75.3	76.0	
30	24.7	24.8	24.7	24.7	24.7	3	78.1	78.2	78.6	
						4	70.9	70.9	71.0	
						5	67.3	67.3	67.4	

Chemical shifts are given in δ values (ppm), with tetramethylsilane as an internal standard

[a] Values may be interchanged

was purified by silica gel column chromatography with chloroform–methanol–water (40:10:1). Finally, **4** (25 mg) and **5** (6 mg) were isolated and identified by high-resolution (HR)-ESI–MS (Micromass Q-Tof micromass spectrometer, Waters Corp., Milford, MA, USA) and NMR (JNM-ECP500 spectrometer, JEOL, Tokyo, Japan) including ^{1}H-NMR, ^{13}C-NMR, HMQC, and HMBC.

Animals

Six-week-old male ddY mice (SLC, Shizuoka, Japan) were purchased. The mice were acclimated for 1 week in a laboratory animal facility maintained at constant temperature (23 ± 3°C) and humidity (50 ± 20% relative humidity) with a 12-h light/dark cycle and free access to food and water, before the experiments were performed. All animal treatments in this study were approved by the Institutional Animal Care and Use Committee of Wakunaga Pharmaceutical Co., Ltd.

LC–MS conditions

LC–MS analysis was carried out by coupling a Shimadzu HPLC system (LC-20A Prominence, Shimadzu, Kyoto, Japan) to a ThermoElectron LTQ linear ion trap mass spectrometer (ThermoElectron, San Jose, CA, USA) fitted with an ESI source. HPLC separation was performed on a 100 × 2 mm (3 μm) YMC-Pack Pro C18 RS column

(YMC, Kyoto, Japan). The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile at a flow rate of 0.2 ml/min. Gradient elution was used, with the initial mobile phase at 34% B, ramped to 100% B at 3.2 min, and held until 4.2 min. At the end of the gradient program, the eluent composition was brought to the initial condition in 0.1 min, and the column was equilibrated for 5.7 min before the next injection. The column temperature was maintained at 40°C, and the injection volume was 5 µl. The mass spectrometric conditions were as follows: electrospray voltage, 4.5 kV; heated capillary temperature, 350°C; sheath gas (nitrogen gas) pressure, 35 arbitrary units; auxiliary gas (nitrogen gas) pressure, 5 arbitrary units. Identification of metabolites of **1** in the cecum was carried out by using LC–MS with MS^n at a collision energy of 30%. LC–MS analysis for determination of **1**–**3** in plasma was performed in selective reaction monitoring (SRM). Three different time segments were used: (1) from 0 to 3.5 min for **1** and IS, (2) from 3.5 to 5.2 min for **2**, and (3) from 5.2 to 7 min for **3**, respectively. The SRM detection setup for **1** was *m/z* 1189 → 647; for IS it was *m/z* 1351 → 809; for **2** it was *m/z* 1013 → 469, 471, 541, and 923; and for **3** it was *m/z* 471 → 407, 409, and 425. The isolation width of the precursor ion was set at 3 *m/z* for **1**, **2**, and IS, and at 2 *m/z* for **3**, respectively, and the collision energy was set at 30%.

Determination of lancemaside A and its metabolites in cecum

Compound **1** (100 mg/kg body weight) was administered orally to three mice that had been fasted for 16 h. At 8 h after administration, the animals were exsanguinated by decapitation, and their ceca were rapidly extirpated and frozen in liquid nitrogen. The frozen cecum samples were homogenized with a 15-fold volume of methanol by using a pestle and mortar. The mixture was centrifuged at 11000*g* for 5 min, and the supernatant was analyzed by LC–MS to determine **1** and its metabolites.

Administration of lancemaside A and collection of plasma samples

Compound **1** was administered to mice in a single dose of 100 mg/kg body weight for oral administration (p.o.) and 1 mg/kg body weight for intravenous administration (i.v.). Compound **1** was dissolved in distilled water at 5 mg/ml for oral administration and in saline at 0.2 mg/ml for intravenous administration. Mice were fasted for 16 h prior to administration. Blood samples were collected by decapitation in a heparin-containing tube at 0.5, 1, 2, 4, 8, and 24 h after oral administration and at 2, 5, 10, 20, 30, 60, and 120 min after intravenous administration. The blood was centrifuged at 1000*g* for 10 min, and plasma was collected and stored at −35°C until analysis.

Plasma sample preparation for LC–MS analysis

A plasma sample (40 µl) was transferred to a 1.5-ml glass tube, and 900 µl of the IS solution was added. The mixture was shaken for 20 s and then centrifuged at 11000*g* for 5 min. The supernatant was dried, and the residue was dissolved in 100 µl methanol and then centrifuged at 11000*g* for 5 min. The supernatant was analyzed by LC–MS to determine **1**–**3**.

Validation of the analytical method for the determination of lancemaside A and its metabolite in plasma

Specificity was assessed by analyzing three different sources of plasma. Chromatograms were visually inspected for interfering chromatographic peaks from endogenous substances. To examine the linearity of **1**–**3**, calibration standards with ten concentrations were prepared and assayed. The final concentration ranges were 11–11363 ng/ml for **1**, 8–7819 ng/ml for **2**, and 6–5513 ng/ml for **3**. A calibration curve was derived from the peak area ratio (analyte/IS) versus the known concentrations of the calibration samples. A weighting of $1/x^2$ (where x is the concentration of a given standard) was used for curve fitting. To evaluate the accuracy and precision of the method, recovery experiments ($n = 3$) were carried out by adding different concentrations of **1**–**3** to plasma. The added concentrations were 11–5681 ng/ml for **1**, 8–3909 ng/ml for **2**, and 6–2756 ng/ml for **3**. The accuracy was expressed in terms of relative error, and the precision was evaluated in terms of the relative standard deviation (RSD). The limit of detection (LOD) was determined as the analyte concentration that yielded a signal equal to three times the noise level.

Pharmacokinetic analysis

Pharmacokinetic parameters were calculated by using the MOMENT computer program [18]. The peak plasma concentration (C_{max}) and time to reach C_{max} (t_{max}) after oral administration were read directly from the experimental data. The total area under the plasma concentration–time curve from time zero to infinity (AUC_{0-inf}) was calculated by the trapezoidal rule extrapolation method. The elimination rate constant (K_{el}) was calculated from the slope of the line by regression analysis of the log plasma concentration versus time plot, and the half-life ($t_{1/2\beta}$) was calculated as $0.693/K_{el}$. The bioavailability (F) was calculated from the following

equation: $F(\%) = \{[AUC_{0\text{–inf}}\ (p.o.)][dose\ (i.v.)]\}/\{[AUC_{0\text{–inf}}\ (i.v.)][dose\ (p.o.)]\} \times 100\%$.

Results and discussion

Identification of lancemaside A metabolites in cecum

Intestinal flora plays an important role in the metabolism of glycosides in humans and animals. Metabolic reactions such as deglycosylation and deglucuronidation occur during passage through the gastrointestinal tract, especially in the small intestine. To study the intestinal metabolism of lancemaside A (**1**), the cecum sample from mice at 8 h after oral administration of **1** was analyzed by LC–MS in the negative ion mode. Five peaks (peaks 1–5) were observed in the chromatogram of the cecum sample (Fig. 2). The MS^n data of their peaks are summarized in Table 2. Peak 1 was identified as lancemaside A (**1**) on the basis of its retention time and mass fragmentation pattern in comparison with those of authentic compounds. The mass spectra of peaks 2 (4.2 min), 4 (4.3 min), and 5 (5.3 min) showed the ions to be at *m/z* 1013, 881, and 749, respectively, corresponding to the loss of 176 Da (glucuronic acid), 308 Da (glucuronic acid and xylose), and 440 Da (glucuronic acid and two xyloses) from **1**. The mass spectrum of peak 3 (5.8 min) showed the ions to be at *m/z* 471, corresponding to the aglycone (echinocystic acid) moiety of **1**. In MS^n spectra (Table 2), the *m/z* 471 ions from peaks 1–5 produced the same product ions, at *m/z* 425, 409, and 407, with similar intensities, and this fragmentation pattern was similar to that reported previously for echinocystic acid moiety [19]. The data suggest the presence of an echinocystic acid moiety in the structure of these compounds.

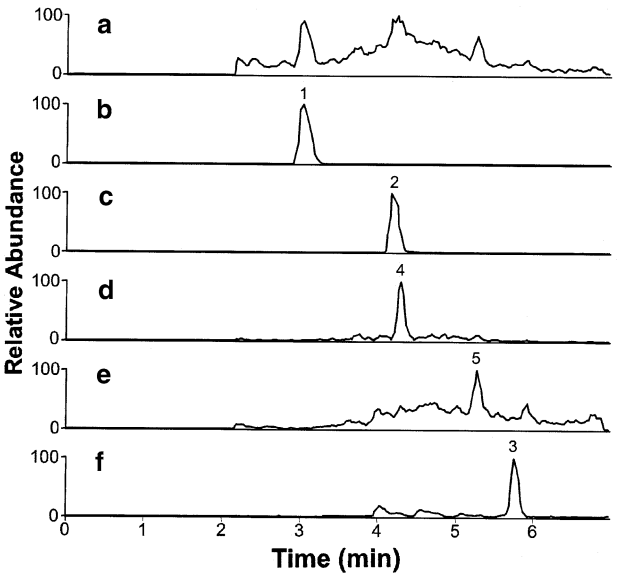


Fig. 2 Representative chromatograms of a cecum sample at 8 h after oral administration of lancemaside A. **a** Total ion chromatogram, mass chromatograms at **b** *m/z* 1189, **c** *m/z* 1013, **d** *m/z* 881, **e** *m/z* 749 + 795, **f** *m/z* 471. Peak *1*, lancemaside A; peaks *2–5*, metabolites of lancemaside A

On the basis of these results, the presumed metabolites **2**–**5**, corresponding to peaks 2–5 (Fig. 2), were synthesized enzymatically and chemically and identified by NMR and MS. The structures of **2** and **3** were determined to be codonolaside II and echinocystic acid, respectively, by comparison with the NMR spectroscopic data from previous reports [5, 17]. The structures of the new compounds **4** and **5** were determined based on their spectroscopic data, including MS, 1D- and 2D-NMR analyses. The molecular formula of **4** was assigned as $C_{46}H_{74}O_{16}$, determined from its pseudomolecular ion $[M–H]^-$ at *m/z* 881.4925 (calcd for $C_{46}H_{73}O_{16}$: 881.4899) in the HR-ESI–MS spectrum. The ^{1}H-NMR spectrum of **4** showed the presence of seven singlet methyl groups [δ_H 0.95 (3H, s), 1.03 (3H, s), 1.04 (3H, s), 1.15 (6H, s), 1.22 (3H, s), 1.81 (3H, s)], one doublet methyl group [δ_H 1.78 (3H, d, $J = 5.5$ Hz)], and three anomeric protons [δ_H 5.20 (1H, d, $J = 7.5$ Hz), 5.85 (1H, s), 6.48 (1H, d, $J = 3.5$ Hz)]. The ^{13}C-NMR spectrum of **4** showed the presence of three anomeric carbons (δ_c 93.7, 101.1, 106.9), as shown in Table 1. These data, together with correlations observed in the HMQC and HMBC spectra, indicated the absence of a xylose moiety in **2**. Thus the structure of **4** was determined to be echinocystic acid 28-*O*-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl ester. The molecular formula of **5** was assigned as $C_{41}H_{66}O_{12}$, which was determined from its pseudomolecular ion $[M–H]^-$ at *m/z* 749.4486 (calcd for $C_{41}H_{65}O_{12}$: 749.4476) in the HR-ESI–MS spectrum. The ^{1}H-NMR spectrum of **5** showed the presence of seven singlet methyl groups [δ_H 0.97 (3H, s), 1.03 (6H, s), 1.14 (3H, s), 1.16 (3H, s), 1.22 (3H, s), 1.81 (3H, s)], one doublet methyl group [δ_H 1.72 (3H, d, $J = 6.0$ Hz)], and two anomeric protons [δ_H 5.84 (1H, s), 6.55 (1H, d, $J = 2.5$ Hz)]. The ^{13}C-NMR spectrum of **5** showed the presence of two anomeric carbons (δ_c 93.5, 101.5), as shown in Table 1. These data, together with correlations observed in the HMQC and HMBC spectra, indicated the absence of one xylose moiety present in **4**. Thus, the structure of **5** was determined to be echinocystic acid 28-*O*-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl ester. In the negative ion LC–MS analysis, the metabolite peaks (peaks 2–5) observed in the cecum sample after oral administration of **1** had retention times and mass spectra that were identical to those of the synthetic compounds **2**–**5**, and thus the structures of the metabolites were determined to be codonolaside II (**2**), echinocystic acid (**3**), echinocystic acid 28-*O*-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl ester (**4**),

Table 2 MS^n spectra in the negative ion mode of lancemaside A and its metabolites detected in the cecum at 8 h after oral administration of lancemaside A

Peak[a]	Retention time (min)	Isolated *m/z*	MS^n type	Major product ions (*m/z*)
1 (Lancemaside A)	3.0	1189 $[M–H]^-$	MS^2	647 (100)[b]
		647	MS^3	629 (43), 571 (57), 525 (25), 471 (100), 407 (30)
		471	MS^4	425 (21), 409 (30), 407 (100)
		407	MS^5	391 (100), 377 (46)
2 (Codonolaside II)	4.2	1013 $[M–H]^-$	MS^2	995 (31), 923 (72), 541 (36), 471 (53), 469 (100)
		469	MS^3	337 (100)
		471	MS^3	425 (25), 409 (35), 407 (100)
		407	MS^4	391 (100), 377 (35)
3 (Echinocystic acid)	5.8	471 $[M–H]^-$	MS^2	425 (26), 409 (44), 407 (100)
		407	MS^3	391 (100), 377 (46)
4	4.3	881 $[M–H]^-$	MS^2	471 (100)
		471	MS^3	425 (33), 409 (45), 407 (100)
		407	MS^4	391 (100), 377 (45)
5	5.3	749 $[M–H]^-$	MS^2	471 (100)
		471	MS^3	425 (22), 409 (37), 407 (100)
		407	MS^4	391 (100), 377 (39)
		795 $[M+HCOO]^-$	MS^2	749 (15), 539 (100)

[a] Peak numbers are the same as in Fig. 2

[b] Relative abundances (%) are given in parentheses

and echinocystic acid 28-*O*-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl ester (**5**), as shown in Fig. 1.

It is known that saponins are metabolized in the gastrointestinal tract into their deglycosylated form. Akao et al. [20] reported that ginsenoside Rb_1 is enzymatically metabolized into compound K as a result of the hydrolysis of the glycosidic linkage by rat intestinal bacteria. Glycyrrhizin has been shown to be hydrolyzed by glucuronidase of intestinal bacteria to glycyrrhetinic acid, an active principal aglycone [11, 21]. Therefore, our data suggest that orally administered **1** was metabolized into its deglycosylated metabolites **2**–**5** by the digestive enzymes in the gastrointestinal tract. In this metabolic pathway, the ester linkage at C-28 of **1** was hydrolyzed to a carboxylic acid. To our knowledge, this is the first report of the hydrolysis of the carboxyl ester bond of saponin occurring in the gastrointestinal tract.

LC–MS method to quantify lancemaside A and its metabolites in plasma

Lancemaside A (**1**) and its metabolites, **2** and **3**, in plasma were quantified by LC–MS analysis, which has high specificity and selectivity. The ESI spectra mainly showed the deprotonated molecule ($[M–H]^-$) of **1** (*m/z* 1189), **2** (*m/z* 1013), **3** (*m/z* 471), and IS (*m/z* 1351). The product ions at *m/z* 647 for **1**, *m/z* 469, 471, 541 and 923 for **2**, *m/z* 407, 409 and 425 for **3**, and *m/z* 890 for IS in the MS/MS experiments of the deprotonated molecule were monitored in SRM mode in order to quantify them. Typical SRM chromatograms of a blank plasma sample spiked with **1** at 284 ng/ml, **2** at 195 ng/ml, **3** at 138 ng/ml, and IS at 1203 ng/ml are shown in Fig. 3. The retention times of **1**–**3**, and IS were 3.1, 4.2, 5.8, and 2.6 min, respectively.

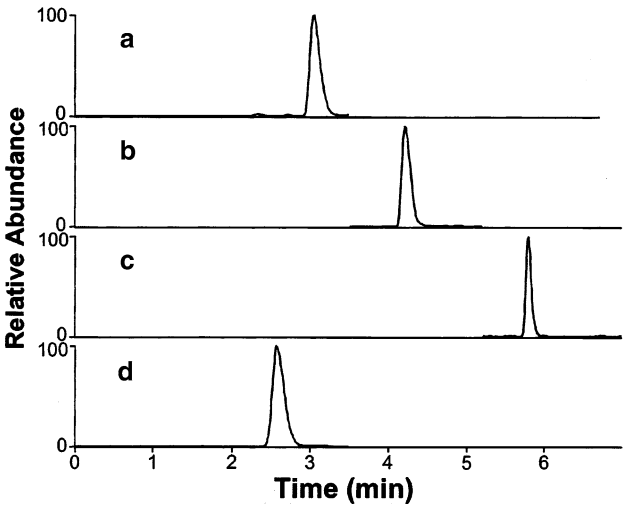


Fig. 3 Selective reaction monitoring chromatograms of rat plasma sample spiked with lancemaside A, codonolaside II, echinocystic acid, and lancemaside E (internal standard). The product ions were monitored at **a** *m/z* 1189 → 647 for lancemaside A, **b** *m/z* 1013 → 469, 471, 541, and 923 for codonolaside II, **c** *m/z* 471 → 407, 409, and 425 for echinocystic acid, and **d** *m/z* 1351 → 809 for lancemaside E

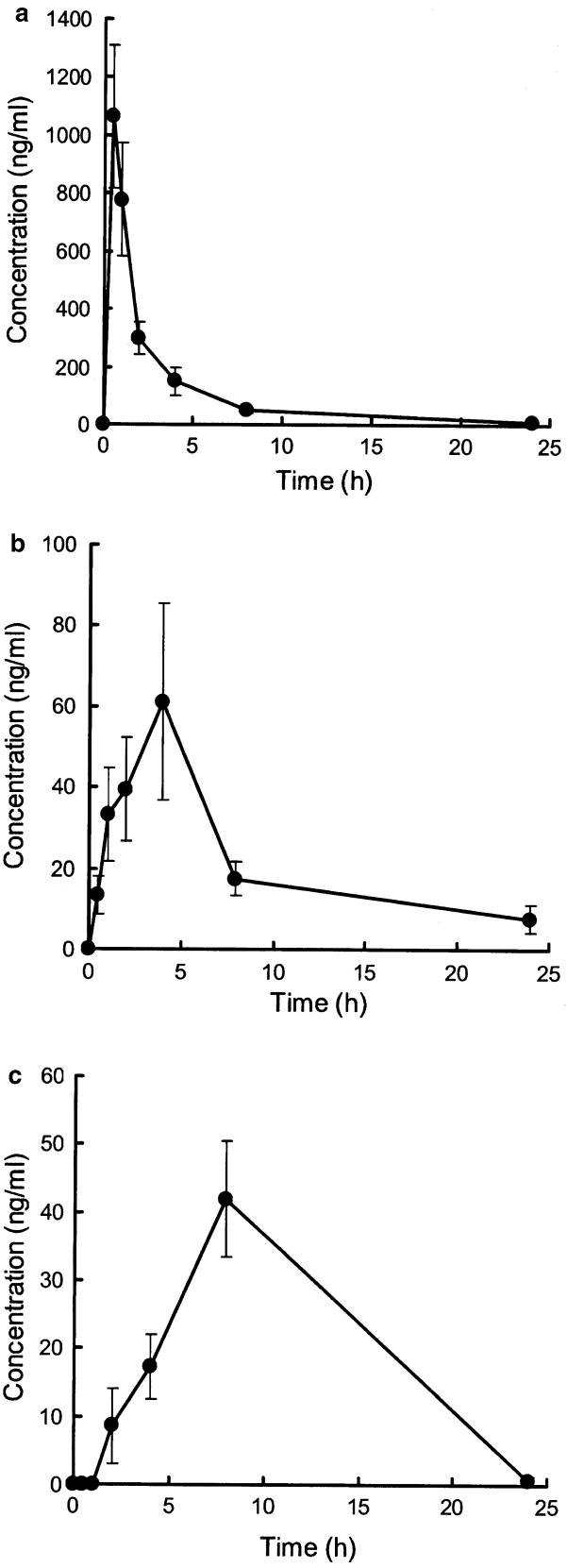


Fig. 4 Plasma concentration–time profiles of **a** lancemaside A, **b** ▶ codonolaside II, and **c** echinocystic acid following oral administration of lancemaside A at 100 mg/kg to mice. *Each point* represents the mean ± SE ($n = 9$ or 10)

Validation of the LC–MS method to quantify lancemaside A and its metabolites in plasma

Specificity was assessed by analyzing blank plasma from three different sources. No endogenous interfering sources were observed on the chromatograms obtained from blank plasma at the retention times of **1–3** and IS. The calibration curves of **1–3** in the respective spiked samples had correlation coefficients of 0.999, 0.990, and 0.997, respectively. Accuracy and precision were determined by using three replicates of the spiked samples. The relative error ranged from −8 to 3% for **1**, from −13 to 12% for **2**, and from −16 to 7% for **3**, whereas intraday RSD did not exceed 14% for **1**, 15% for **2**, and 13% for **3**. The LOD was 3 ng/ml for **1** and **3**, and 2 ng/ml for **2**.

Study of lancemaside A pharmacokinetics

The plasma concentration–time profiles after oral administration of lancemaside A (**1**) at 100 mg/kg are shown in Fig. 4a, and some relevant pharmacokinetic parameters are listed in Table 3. Compound **1** was rapidly absorbed after oral administration, and the concentration in plasma showed peaks at 0.5 h. The values of C_{max}, $t_{1/2\beta}$, and AUC_{0-inf} were 1063 ng/ml, 5.02 h, and 2634 ng h/ml, respectively. After intravenous administration at 1 mg/kg, the plasma level of **1** declined rapidly (Fig. 5), and the concentration–time curve exhibited a biphasic decline. The $t_{1/2\beta}$ and AUC_{0-inf} values were 0.54 h and 2359 ng h/ml, respectively. The bioavailability (F) of **1** was 1.1%. It is known that saponins are very poorly absorbed following oral administration to animals. Liang et al. [10] reported the bioavailability of tubeimoside I was only 0.23% in rats, and Li et al. [22] reported the bioavailability of ginsenoside Rg1, Rb1, and Rd to be 15.62, 0.28, and 0.34%, respectively, in rats. Moreover, after oral administration of glycyrrhizin to rats, the unmetabolized compound was not detected in plasma, but glycyrrhetic acid, which is converted by intestinal bacteria, was detected [21]. Therefore, our data indicated that the poor bioavailability of **1** might be due to its low absorption from the gastrointestinal tract and/or its metabolism by intestinal bacteria. After oral administration of **1** to mice, deglycosylated metabolites **2** and **3** were detected in the plasma after 0.5 and 2 h, respectively, and the concentration in plasma showed peaks

Table 3 Pharmacokinetic parameters of lancemaside A, codonolaside II, and echinocystic acid in mice following oral (p.o., 100 mg/kg) and intravenous (i.v., 1 mg/kg) administration of lancemaside A

	Lancemaside A		Codonolaside II	Echinocystic acid
	p.o.	i.v.	p.o.	p.o.
t_{max} (h)	0.5		4	8
C_{max} (ng/ml)	1063		61	42
$t_{1/2\beta}$ (h)	5.02	0.54	7.83	–
AUC_{0-inf} (ng h/ml)	2634	2359	590	494
F (%)	1.1			

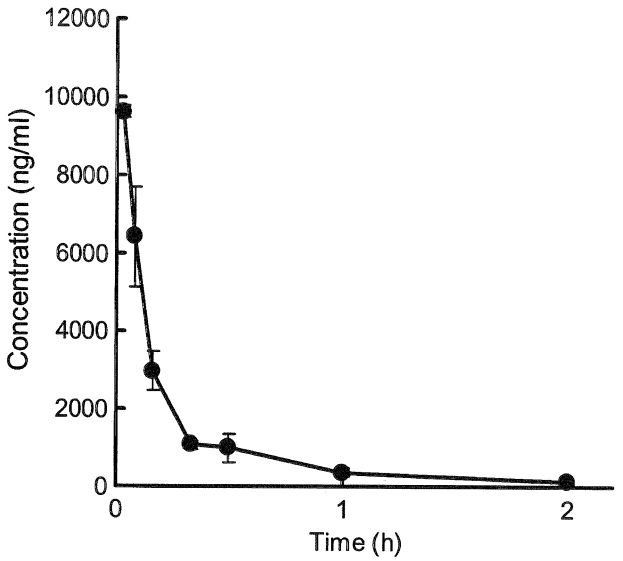


Fig. 5 Plasma concentration–time profiles of lancemaside A following intravenous administration of lancemaside A at 1 mg/kg body weight to mice. *Each point* represents mean ± SE ($n = 4$)

at 4 and 8 h, respectively (Table 3). Our results suggest that metabolites **2** and **3** may be involved in the biological effects when compound **1** was administered orally to mice. In addition, metabolite **2** was detected in both the small intestine and cecum, while **3** was detected only in the cecum (data not shown). Our data indicated that **1** was metabolized to **2** in the upper gastrointestinal tract and then further metabolized to **3** in the cecum. On the other hand, metabolites **4** and **5**, identified in the cecum, were not detected in plasma at any time.

In conclusion, the present study investigated the metabolism and absorption of lancemaside A (**1**), which is considered to be one of the biologically active compounds present in the roots of *C. lanceolata*. After oral administration of **1** to mice, the unmetabolized compound appeared rapidly in plasma, and its low bioavailability (1.1%) was due to deglycosylation by the intestinal bacteria and to poor absorption in the upper gastrointestinal tract. Furthermore, four deglycosylated metabolites, **2–5**, in the cecum were identified, and metabolites **2** and **3** were detected in plasma. We speculate that metabolites **2** and **3** may contribute to the biological effects following oral administration of **1**. Thus, our findings should be helpful for understanding the mechanism of the biological effects of **1**.

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