Characterization of In Vivo Metabolites of Toad Venom Using Liquid Chromatography–Mass Spectrometry

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Abstract

The structures of in vivo metabolites of marinobufotoxin (marinobufagin 3-suberoylarginine ester), marinobufagin, or bufalin which are typical components of toad venom widely used as the traditional Chinese drug, *Ch'an Su*, are confirmed using authentic samples based on their liquid chromatography–mass spectrometric behavior. A rat is orally administered 2 mg of the previously mentioned components of toad venom, the serum is collected 30 min after the administration, extracted, and then characterized. Marinobufotoxin is hydrolyzed and further epimerized into 3-epimarinobufagin, but marinobufagin 3-hemisuberate is not detected. After the administration of marinobufagin, 3-epimarinobufagin is detected in both the male and female rats, but marinobufagin 3-sulfate is formed only in the female rats. Bufalin is metabolized to 3-epibufalin, which is found to undergo further conjugation resulting in its 3-glucuronide. Furthermore, 3-epibufalin 3-sulfate is formed only in the female rats.

Introduction

The Chinese drug, *Ch'an Su*, which is prepared from toad venom, has been widely used for the treatment of congestive heart failure and other cardiac diseases. The cardiotonic effect of *Ch'an Su* is mostly due to bufadienolide, which is classified into bufogenin and bufotoxin (1). In a previous paper from this series, we have clarified the in vitro metabolic pathway (rat or human) of bufotoxin (marinobufotoxin: marinobufagin 3-suberoylarginine ester) and bufogenin (marinobufagin and bufalin), which are representative components of toad venom. The results showed that marinobufotoxin was enzymatically hydrolyzed to form marinobufagin followed by epimerization at the 3-position, but not marinobufagin 3-hemisuberate. Marinobufagin was metabolized to its sulfate and a new phase I metabolite of bufalin, telocinobufagin, which was hydroxylated at the 5β-position, was obtained from the incubation mixture using human liver as the enzyme source (2).

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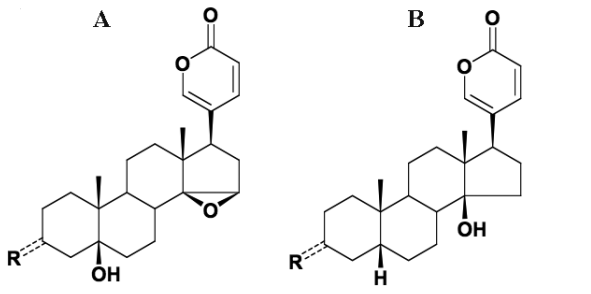
In this study, we have clarified the in vivo metabolites of marinobufotoxin, marinobufagin and bufalin in rat serum (Figure 1). The structures of these metabolites were confirmed using authentic samples based on their liquid chromatographic–mass spectrometric (LC–MS) behavior.

Experimental

Materials, reagents and animals

Marinobufotoxin, marinobufagin 3-hemisuberate, marinobufagin, 3-epimarinobufagin, marinobufagin 3-sulfate, 3-epimarinobufagin 3-sulfate, bufalin, 3-epibufalin, bufalin 3-sulfate,



A	R
marinobufotoxin	α-H, β-OCO$(CH_2)_6$COArgOH
marinobufagin 3-hemisuberate	α-H, β-OCO$(CH_2)_6$COOH
marinobufagin	α-H, β-OH
3-epimarinobufagin	α-OH, β-H
marinobufagin 3-sulfate	α-H, β-OS

B	R
bufalin	α-H, β-OH
3-epibufalin	α-OH, β-H
bufalin 3-sulfate	α-H, β-OS
bufalin 3-glucuronide	α-H, β-OG
3-epibufalin 3-sulfate	α-OS, β-H
3-epibufalin 3-glucuronide	α-OG, β-H

S: SO_3H G: Glucuronic acid

Figure 1. Structures of bufotoxin and bufogenin. Marinobufotoxin and related compounds (A) and bufalin and related compounds (B).

and 3-epibufalin 3-sulfate were obtained or synthesized as described in a previous paper (2). Marinobufagin 3-glucuronide and bufalin 3-glucuronide were prepared from marinobufagin and bufalin using a known method (3), respectively. These glucuronides were not obtained in the pure form based on their high-performance liquid chromatographic (HPLC) behavior, but their structures were confirmed using liquid chromatoraphy–mass spectrometry and enzymatic hydrolysis as described below.

Pooled rat liver microsome was purchased from Xenotech LLC (Lenex, KS). β-Glucuronidase from *Esherichia coli* and uridine 5'-diphosphoglucuronic acid (UDPGA) were purchased from Nacalai Tesque (Kyoto, Japan). Strata-X (60 mg, 3 mL) cartridges were obtained from Shimadzu GLC (Shimadzu, Kyoto), and successively conditioned with AcOEt (2 mL), MeOH (2 mL) and H_2O (2 mL), prior to use. All other reagents were of analytical grade and commercially available. Wistar strain rats (male and female, 7 weeks old) were obtained from Japan S.L.C. (Hamamatsu, Japan).

Apparatus

HPLC was carried out using a Waters 600s controller (Waters, Milford, MA) equipped with a 616 pump and 486 tunable absorbance detector (UV 300 nm) (Waters). A Symmetry C_{18} column (5 µm; 150 mm × 4.6 mm i.d.) (Waters) and Chromolith Performance RP-18e column (100 mm × 4.6 mm i.d.) (Merck, Darmstadt, Germany) were used at the flow rate of 1.0 mL/min and 40°C.

The LC–MS system consisted of a Shimadzu LC-10AT chromatograph (Shimadzu) coupled with an API 2000 triple-stage quadrupole mass spectrometer (Applied Biosystems, Foster City, CA).

The metabolites of marinobufotoxin, and marinobufagin and phase II metabolites of bufalin were analyzed in the selected ion monitoring (SIM) mode and/or selected reaction monitoring (SRM) mode of electrospray ionization (ESI) in the positive or negative ion mode. A Symmetry C_{18} column (5 µm; 150 mm × 2.1 mm i.d.) (Waters) and a J'sphere ODS-L80 column (4 µm; 150 mm × 2.0 mm i.d.) (YMC, Kyoto) were used at the flow rate of 0.2 mL/min and 40°C. The ionization conditions were as follows: declustering potential, ± 80 V; entrance potential, ± 10 V; focusing potential, ± 350 V; curtain gas, 20 psi; turbo gas temperature, 500°C; ion source gas 1 (nitrogen), 70 psi; ion source gas 2 (nitrogen), 80 psi; interface heater, on. Nitrogen was used as the collision gas in the SRM mode with a collision energy of –55 eV (3-epibufalin 3-sulfate) and –60 eV (marinobufagin 3-sulfate, 3-epibufalin 3-glucuronide). The monitoring ions were as follows: marinobufotoxin, *m/z* 713.2; marinobufagin 3-hemisuberate, *m/z* 555.0; marinobufagin and 3-epimarinobufagin, *m/z* 401.1; monohydroxylates of marinobufagin and 3-epimarinobufagin, *m/z* 417.0; marinobufagin 3-sulfate and 3-epimarinobufagin 3-sulfate, *m/z* 479.0; marinobufagin 3-glucuronide and 3-epimarinobufagin 3-glucuronide, *m/z* 575.2; bufalin 3-sulfate and 3-epibufalin 3-sulfate, *m/z* 465.0; bufalin 3-glucuronide and 3-epibufalin 3-glucuronide, *m/z* 561.0. The precursor and monitoring ions of the conjugates of bufogenin were as follows: marinobufagin 3-sulfate, *m/z* 479.0 and 96.9; 3-epibufalin 3-sulfate, *m/z* 465.0 and 96.9.

Bufalin and its phase I metabolites were analyzed in the SIM mode of atmospheric pressure chemical ionization (APCI) and in the positive ion mode. A Chromolith Performance RP-18e column was used at the flow rate of 1.0 mL/min and 40°C. The ionization conditions were as follows: declustering potential, 80 V; entrance potential, 10 V; focusing potential, 350 V; curtain gas, 40 psi; ion source gas 1 (nitrogen), 70 psi; ion source gas 2 (nitrogen), 15 psi; vaporizer temperature, 450°C; interface heater, on. The monitoring ions of bufalin and its phase I metabolites were as follows: bufalin and 3-epibufalin, *m/z* 387.2; monohydroxylates of bufalin and 3-epibufalin, *m/z* 403.0.

In vivo studies of bufadienolide

All rats were fasted overnight before the experiment. After fasting, marinobufotoxin, marinobufagin, or bufalin [2 mg each suspended in dimethylsulfoxide (0.1 mL), Tween 80 (0.2 mL) and saline (0.7 mL)] was orally administered to the rats. After 30 min, the blood was immediately collected after decapitation, left at 4°C for 3 h, then centrifuged at 1500 × *g* (4°C, 15 min) to produce the serum (1–1.5 mL). On the other hand, the vehicle was orally administered to the rats. The serum was separated and

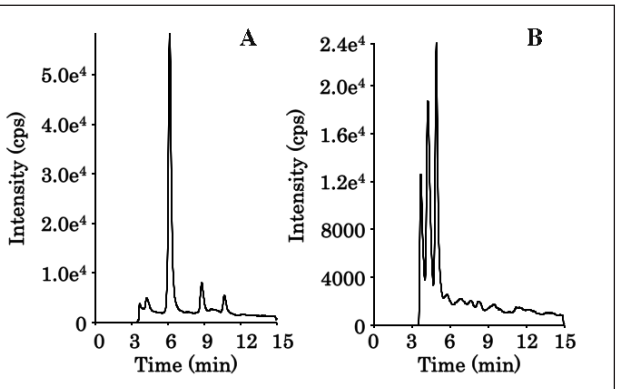


Figure 2. LC–ESI-MS chromatograms of marinobufotoxin metabolites: SIM (*m/z* 401.1) of 3-epimarinobufagin (A) SIM (*m/z* 417.0) of monohydroxylates of genins (B). LC conditions: Symmetry C18 (5 µm; 150 mm × 2.1 mm i.d.), MeCN-5mM HCO_2NH_4 (pH 5.0 adjusted with HCO_2H) (1:2, v/v).

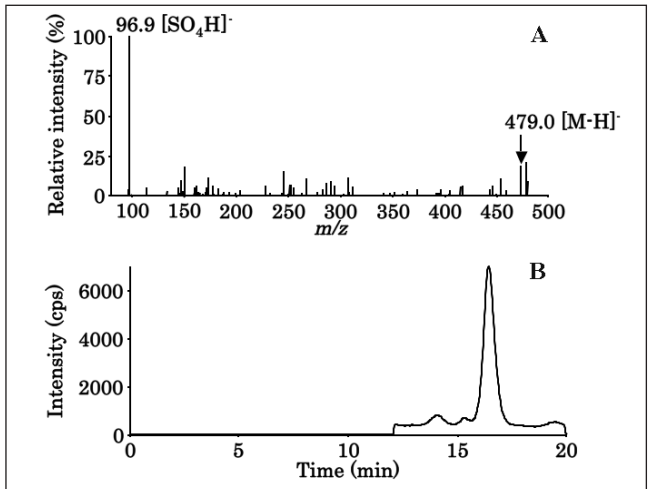


Figure 3. MS–MS spectrum and LC–ESI-MS chromatogram of marinobufagin 3-sulfate. MS–MS spectrum of authentic sample (precursor ion: *m/z* 479.0, MS range: *m/z* 80–500) (A) SIM (*m/z* 479.0) of serum sample (B). LC conditions: J'sphere ODS-L80 (4 µm; 150 mm × 2.0 mm i.d.), MeOH-5 mM HCO_2NH_4 (pH 5.0 adjusted with HCO_2H) (2:3, v/v).

stored at –20°C until used as the control serum.

Serum (0.5 mL) was added to the solution of MeCN–AcOH (94:6, v/v; 0.5 mL) and subjected to centrifugation at 4°C for 15 min. The obtained supernatant was evaporated to dryness using an N_2 gas stream. The residue was diluted with H_2O (2 mL), and then passed through a Strata-X cartridge. After washing with H_2O (2 mL), the unconjugated steroid was eluted with AcOEt (2 mL), and then the conjugated one was eluted with MeOH (2 mL). The organic layer was evaporated to dryness using an N_2 gas stream. The residue was dissolved in 50% MeOH (0.15 mL), part of which was subjected to an LC–MS(–MS) analysis.

The recovery rates (mean, n = 2) during the pretreatment were examined using the control serum (0.5 mL/tube): marinobufagin (10 ng/tube), 69%; marinobufagin 3-sulfate (20 ng/tube), 60%; marinobufagin 3-glucuronide (20 ng/tube), 47%.

General procedure of treatment with β-glucuronidase

The substrate (< 10 µg) was dissolved in MeOH (0.15 mL). This solution (50 µL) was then added to the assay mixture (total volume 1 mL) containing β-glucuronidase (1400 unit/mL) in 0.1M AcONa–AcOH buffer (pH 5.0). The assay mixture was incubated at 37°C for 1 h. After incubation, the reaction was terminated by the addition of AcOEt (1 mL). After extraction with AcOEt, the organic layer was washed with H_2O and evaporated to dryness using an N_2 gas stream. The residue was dissolved in 50% MeOH (50 µL), part of which was subjected to the HPLC analysis.

Structural confirmation of synthetic marinobufagin 3-glucuronide and bufalin 3-glucuronide

The structures of the synthetic marinobufagin 3-glucuronide and bufalin 3-glucuronide as described above were confirmed by LC–ESI-MS. Marinobufagin 3-glucuronide: [Symmetry C_{18} (5 µm; 150 mm × 2.1 mm i.d.), MeOH–5 mM HCO_2NH_4 (pH 5.0 adjusted with HCO_2H) (2:3, v/v), t_R 6.2 min]. MS: *m/z* 577.3 $[M+H]^+$, 559.3 $[M–H_2O+H]^+$, 365.2 [M–glucuronic acid–$2H_2O$+H]$^+$. Bufalin 3-glucuronide: [Symmetry C_{18} (5 µm; 150 mm × 2.1 mm i.d.), MeOH-5 mM HCO_2NH_4 (pH 5.0 adjusted with HCO_2H) (4:5, v/v), t_R 7.1 min]. MS: *m/z* 563.2 $[M+H]^+$, 387.2 [M–glucuronic acid+H]$^+$, 369.2 [M–glucuronic acid–H_2O+H]$^+$, 351.2 [M–glucuronic acid–$2H_2O$+H]$^+$.

These glucuronides were subjected to hydrolysis using β-glucuronidase that produced the respective genins [HPLC, marinobufagin: Symmetry C_{18} (5 µm, 150 mm × 4.6 mm i.d.), MeOH–H_2O (5:4, v/v), t_R 7.2 min; bufalin: Chromolith Performance RP-18e, MeOH-H_2O (5:4, v/v), t_R 5.7 min].

Preparation of 3-epibufalin 3-glucuronide using rat liver microsome

3-Epibufalin [2 µg/MeOH (50 µL)] was added to the assay mixture (total volume 0.5 mL) containing 0.5 mg protein of the rat liver microsome in *Tris*-HCl buffer (pH 7.4, 50 mM) containing EDTA (1 µL), $MgCl_2$ (5mM), 0.05% Triton X-100 and UDPGA (4mM). The assay mixture was then incubated at 37°C for 2 h. After the incubation, the reaction was terminated by heating (80°C) for 5 min. After centrifugation at 1500 x *g* for 10 min, the supernatant was passed through a Strata-X cartridge. After washing with H_2O (2 mL) and AcOEt (2 mL), the conjugate was eluted with MeOH (2 mL). The MeOH was evaporated to dryness using an N_2 gas stream. The residue was dissolved in 50% MeOH (0.15 mL), part of which was subjected to the HPLC and/or LC–MS(–MS) analysis. [HPLC, Symmetry C_{18} (5 µm; 150 mm × 4.6 mm i.d.), MeOH-5 mM HCO_2NH_4 (pH 5.0 adjusted with HCO_2H) (4:5, v/v), t_R 10.5 min]. [LC–MS(–MS), Symmetry C_{18} (5 µm, 150 mm × 2.1 mm i.d.), MeOH-5 mM HCO_2NH_4 (pH 5.0 adjusted with HCO_2H) (4:5, v/v), t_R 9.4 min: MS: *m/z* 563.1 $[M+H]^+$, 387.2 [M–glucuronic acid+H]$^+$, 369.2 [M–glucuronic acid–H_2O+H]$^+$, 351.2 [M–glucuronic acid–$2H_2O$+H]$^+$. The negative ion mode showed the specific fragment patterns of the glucuronide (4). MS: *m/z* 561.0 $[M–H]^–$, 113.0 [glucuronic acid–CO_2–H_2O]$^–$, 84.9 [glucuronic acid–CO–CO_2–H_2O]$^–$, 74.9 [glucuronic acid–2CO–CO_2]$^–$.

In order to confirm the structure, the obtained glucuronide was subjected to hydrolysis using β-glucuronidase that produced 3-epibufalin [HPLC, Chromolith Performance RP-18e, MeOH-H_2O (5:4, v/v), t_R 6.1 min].

Results

The characterization of the in vivo metabolites of bufadienolide, bufotoxin, and bufogenin, was done using LC–MS(–MS).

Characterization of *in vivo* metabolite of marinobufotxin

The representative LC–ESI-MS chromatograms of rat serum that received 2 mg of marinobufotoxin are shown in Figure 2. 3-Epimarinobufagin was detected as the main metabolite (*ca*. 75 ng/mL; mean, *n*=2) [Symmetry C_{18} (5 µm; 150 mm × 2.1 mm i.d.), MeCN-5 mM HCO_2NH_4 (pH 5.0 adjusted with HCO_2H) (1:2, v/v), t_R 6.2 min (Figure 2A); MeOH-5 mM HCO_2NH_4 (pH 5.0 adjusted with HCO_2H) (5:4, v/v), t_R 4.7 min]. In addition, the monohydroxylates of marinobufagin or 3-epimarinobufagin were detected in the LC–MS chromatogram (Figure 2B). On the other hand, marinobufagin [LC–ESI-MS: Symmetry C_{18} (5 µm; 150 mm × 2.1 mm i.d.), MeCN-5 mM HCO_2NH_4 (pH 5.0 adjusted with HCO_2H) (1:2, v/v), t_R 12.1 min (Figure 2A)], marinobufagin 3-hemisuberate [LC–ESI-MS: Symmetry C_{18} (5 µm; 150 mm × 2.1 mm i.d.), MeOH-5 mM HCO_2NH_4 (pH 5.0 adjusted with HCO_2H) (4:3, v/v), t_R 17.0 min] and the substrate [Symmetry C_{18} (5 µm; 150 mm × 2.1 mm i.d.), MeOH-5 mM HCO_2NH_4 (pH 5.0 adjusted with HCO_2H) (4:3, v/v), t_R 7.3 min] were not detected.

Characterization of in vivo metabolite of marinobufagin

While 3-epimarinobufagin (*ca.* 125 ng/mL; mean, n = 2) and the monohydroxylates of marinobufagin or 3-epimairnobufagin were detected as phase I metabolites, the substrate, marinobufagin, was not detected in the rat serum that received 2 mg of marinobufagin. Although marinobufagin 3-glucuronide was not detected in the sera of both the male and female rats, marinobufagin 3-sulfate (*ca.* 9 ng/mL; mean, n = 2) was detected in that of the female rats, which was confirmed using LC–ESI-MS and LC–MS–MS analyses [marinobufagin 3-glucuronide: Symmetry C_{18} (5 µm; 150 mm × 2.1 mm i.d.), MeOH-5 mM HCO_2NH_4 (pH 5.0 adjusted with HCO_2H) (2:3, v/v), t_R 6.2 min; marinobufagin

3-sulfate and 3-epimarinobufagin 3-sulfate: J'sphere ODS-L80 (4 µm; 150 mm × 2.0 mm i.d.), MeOH-5 mM HCO_2NH_4 (pH 5.0 adjusted with HCO_2H) (2:3, v/v), marinobufagin 3-sulfate (t_R 16.5 min), 3-epimarinobufagin 3-sulfate (t_R 15.3 min) (Figure 3)]. Although the authentic 3-epimarinobufagin 3-glucuronide was not available, the corresponding peak was not detected near at the t_R for that of marinobufagin 3-glucuronide in the LC–ESI-MS chromatogram.

Characterization of *in vivo* metabolite of bufalin

While 3-epibufalin (*ca.* 950 ng/mL; mean, n = 2) and the monohydroxylates of bufalin or 3-epibufalin were detected as the metabolites of bufalin, the substrate was not detected in the rat serum that received 2 mg of bufalin. [LC–APCI-MS: MeOH–H_2O (12:13), bufalin (t_R 13.0 min), 3-epibufalin (t_R 14.2 min)].

Bufalin 3-glucuronide was not obtained, but 3-epibufalin 3-glucuronide (< 1 ng/mL; mean, n = 2) was obtained from both the male and female rats, which was confirmed by comparison to an authentic sample obtained from an in vitro experiment. [LC–ESI-MS: Symmetry C_{18} (5 µm; 150 mm × 2.1 mm i.d.), MeCN-5 mM HCO_2NH_4 (pH 5.0 adjusted with HCO_2H) (7:25, v/v), t_R 8.0 min; MeOH-5 mM HCO_2NH_4 (pH 5.0 adjusted with HCO_2H) (4:5, v/v), t_R 9.4 min (Figure 4)],

3-Epibufalin 3-sulfate (*ca.* 6 ng/mL, mean, n = 2) was only detected in the female rats [LC–ESI-MS(–MS): MeOH-5 mM HCO_2NH_4 (pH 5.0 adjusted with HCO_2H) (4:5, v/v), t_R 11.8 min]. However, bufalin 3-sulfate, which was produced more easily than 3-epibufalin 3-sulfate from the in vitro experiment (2), was not detected in both the male and female rats [LC–ESI-MS(–MS): MeOH-5 mM HCO_2NH_4 (pH 5.0 adjusted with HCO_2H) (4:5, v/v), t_R 10.0 min], and the phenomenon was also observed in serum obtained from rat 15 min after the administration of bufalin.

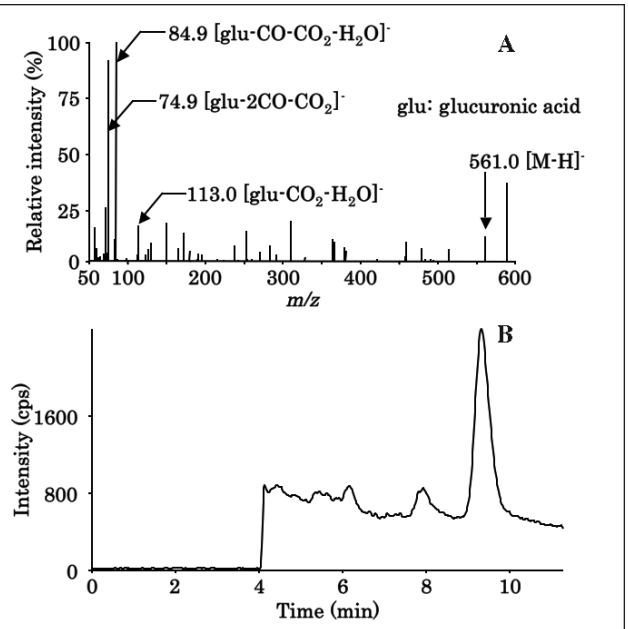


Figure 4. MS–MS spectrum and LC–ESI-MS chromatogram of 3-epibufalin 3-glucuronide. MS–MS spectrum of authentic sample (precursor ion: *m/z* 561.0, MS range: *m/z* 50–600) (A). SIM (*m/z* 561.0) of serum sample (B). LC conditions: Symmetry C18 (5 µm; 150 mm × 2.1 mm i.d.), MeOH-5 mM HCO_2NH_4 (pH 5.0 adjusted with HCO2H) (4:5, v/v).

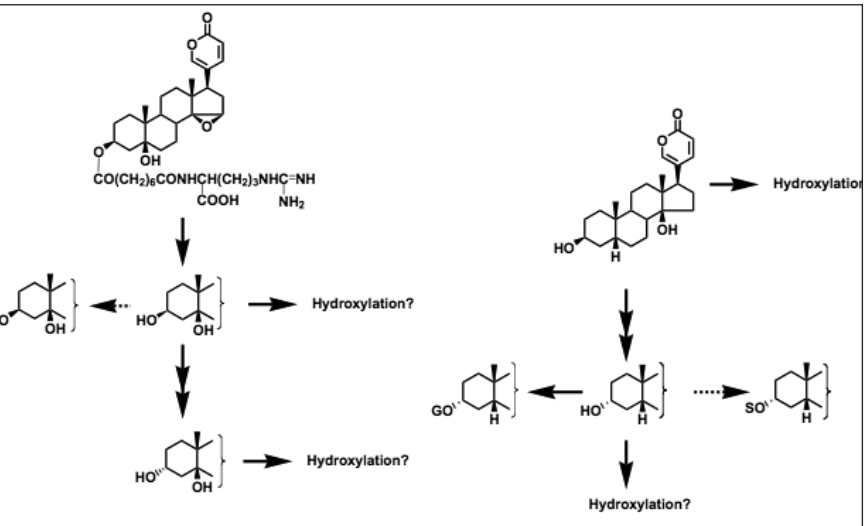


Figure 5. Proposed in vivo metabolic pathway of marinobufotoxin and bufalin in rat female rat.

Discussion

The utilization of LC–MS methodologies to elucidate the structures of metabolites of toad venom is very effective not only in vitro (2) but also in vivo experiments as described above. The proposed in vivo metabolic pathway of bufadienolide in rats is summarized in Figure 5. Marinobufotoxin was hydrolyzed to form marinobufagin, but not 3-hemisuberate, and accordingly produced 3-epimarinobufagin and the monohydroxylates of marinobufagin or those of 3-epimarinobufagin. The structures of these monohydroxlates were not confirmed due to the limited amount of the samples. However, these data showed that the hydrolysis of marinobufotoxin preferentially occurred at the ester (-CO_2R) group rather than at the amide (-CONH-) group, and suggested that marinobufagin 3-hemisuberate hardly contributed to the metabolism of marinobufotoxin.

After the administration of marinobufagin, 3-epimarinobufagin together with some phase I metabolites were detected. The structures of the latter metabolites were not clarified due to the limited amounts of the samples. In addition, marinobufagin also produced its 3-sulfate only in the female rat, while the glucuronide of marinobufagin or that of 3-epimarinobufagin was not detected. These data are consistent with the results that the female rat has a 6–8 times higher sulfotransferase activity than the male rat (5).

Bufalin produced 3-epibufalin as the main metabolite. In addition, some monohydroxylates of bufalin or 3-epibufalin were also detected, but we could not

identify these metabolites due to the limited amounts of the samples. In a previous paper, we clarified telocinobufagin as a phase I metabolite of bufalin using the human or rat liver microsome (2), but we could not detect the compound in the rat serum. In fact, the production of telocinobufagin in the rat liver microsome was much less than that obtained from the human liver microsome. It is known that the enzymatic activity of the rat 3α-hydroxysteroid dehydrogenase is approximately 5 times higher than that of humans (6). Therefore, we proposed that bufalin produced telocinobufagin, which was then immediately transformed into 3-epitelocinobufagin, therefore we could not detect telocinobufagin. 3-Epitelocinobufagin was not available, so we could not prove the above hypothesis. On the other hand, bufalin produced 3-epibufalin 3-glucuronide in both the male and female rats and 3-epibufalin 3-sulfate in the female rats as phase II metabolites. We have reported that bufalin 3-sulfate was predominately produced rather than 3-epibufalin 3-sulfate in the rat liver cytosol (2), so the above result was in conflict with the in vitro study. Although the t_{max} of bufalin was reported to be 15 min (7), bufalin 3-sulfate was also not detected in the serum obtained from the rat 15 min after the administration of the substrate. This discrepancy may be attributable to the following two reasons (i.e., the phase I reaction of bufalin was much faster than the sulfation, so bufalin 3-sulfate was poorly produced, or bufalin produced its 3-sulfate which was then excreted into the bile acid). The other one is that bufalin was easily excreted into the bile acid after absorption, and the metabolites of bufalin in the bile acid are supposed to be highly-polar compounds such as glucuronides (8).

By the way, none of the parent compounds used in this study was detected in the rat serum. It is suggested that after absorption, bufadienolide is easily metabolized and excreted which results in a low accumulation. It is reported that the accumulation of bufalin and cinobufagin in the rat was much less than that of digitoxin [digitoxigenin *tris*(digitoxoside)], which is one of the representative cardiac glycosides (7). In the digitoxin metabolism, cleavage of digitoxigenin *bis*(digitoxiside) to produce digitoxigenin *mono*(digitoxoside) was considered to be the rate-limiting step (9), which may be attributable to the high accumulation of the cardiac glycoside. On the other hand, marinobufotoxin was easily hydrolyzed, and immediately underwent a further biotransformation that resulted in detoxification (10,11), which may cause the low accumulation of the toad venom.

Conclusion

Marinobufotoxin was easily hydrolyzed to form marinobufagin and then underwent the phase I reaction to produce 3-epimarinobufagin etc. The formed marinobufagin was also subjected to the phase II metabolism to produce the 3-sulfate. Bufalin was metabolized to 3-epibufalin followed by the phase II metabolism in serum. Further investigations of the urinary and biliary metabolites are necessary in order to clarify the in vivo metabolic pathway of bufadienolide.

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