Characterization of in vitro metabolites of toad venom using high-performance liquid chromatography and liquid chromatography - Mass spectrometry

Characterization of *in vitro* metabolites of toad venom using high-performance liquid chromatography and liquid chromatography-mass spectrometry*

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Short title: *In vitro* metabolites of toad venom

Original paper

* This paper is dedicated to emeritus Professor Toshio Nambara (Tohoku University, Sendai, Japan) on the occasion of his 80th birthday.

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ABSTRACT

The characterization of the *in vitro* metabolites of toad venom, which has been widely used as a traditional Chinese drug, Ch'an Su, has been completed. Toad venom contains bufotoxin (such as marinobufotoxin; marinobufagin 3-suberoylarginine ester) and bufogenin (such as marinobufagin, bufalin) as the main cardiac steroids. An *in vitro* experiment using the rat or human liver cytosolic fraction disclosed that marinobufotoxin produced marinobufagin, but not its 3-hemisuberate. Marinobufagin was subjected to the enzyme reaction using the rat or human liver microsomal fraction together with NADPH and NAD, which produced 3-dehydromarinobufagin and 3-epimarinobufagin. Marinobufagin produced its 3-sulfate upon treatment with the rat or human liver cytosolic fraction and 3'-phosphoadenosine 5'-phosphosulfate. Bufalin was also subjected to the above enzyme reactions and showed almost the same results except for the result that the hydroxylation has occurred at 5β-position. On the other hand, small amounts of marinobufagin 3-glucuronide were obtained only by treatment with the human liver microsomal fraction and uridine 5'-diphosphoglucuronic acid. The structures of these metabolites were confirmed using authentic samples regarding their high performance liquid chromatographic behavior and/or liquid chromatography-mass spectrometry analysis.

KEYWORDS: toad venom, bufotoxin, bufogenin, *in vitro* metabolite, high performance liquid chromatography, liquid chromatography-mass spectrometry

INTRODUCTION

Digitalis glycosides, represented by digoxin and digitoxin having a five-member lactone ring at the 17β-position of the steroid (cardenolide), have been commonly used for the treatment of congestive heart failure and other cardiac diseases. The metabolic fates of these glycosides have already been clarified (Volp and Lage, 1978; Gault *et al.,* 1982; Ohta *et al.,* 1984). On the other hand, toad venom has also been widely used as a traditional Chinese drug, Ch'an Su, where the bufotoxin and bufogenin having a six-member lactone ring at the 17β-position of the steroid (bufadienolide) are the main cardiac steroids (Shimada *et al.*, 1977). The metabolic fate of bufogenin (such as bufalin and cinobufagin) has been studied, and the 3-epimer has been obtained *via* the 3-dehydro compound (Tóma *et al.*, 1987; Shimada *et al.*, 1989; Zhang *et al.*, 1990; Zhang *et al.*, 1991; Shimizu and Morishita, 1996) together with the other hydroxylated compounds, but the position of the introduced hydroxy group still remains unclear (Tóma *et al.*, 1991). Furthermore, the metabolic pathway of bufotoxin (bufogenin 3-suberoylarginine ester) and the phase II metabolites of bufogenin have not been clarified. The metabolism of toad venom, especially bufotoxin, has been poorly investigated in spite of the historic age of Ch'an Su as shown above.

In this study, we have clarified the *in vitro* metabolites of the representative bufotoxin, marinobufotoxin (marinobufagin 3-suberoylariginine ester), together with its genin, marinobufagin (Shimada *et al.*, 1977) (Fig. 1a). In comparison, those of the representative bufogenin, bufalin, were also studied (Fig. 1b). The structures of these metabolites were confirmed using authentic samples regarding their high performance liquid chromatographic behavior and/or liquid chromatography-mass spectrometry (LC/MS) analysis.

EXPERIMENTAL

Animals and chemicals. Wistar strain rats (male, 7weeks old) were obtained from Japan SLC (Hamamatsu, Japan). 3'-Phosphoadenosine 5'-phosphosulfate (PAPS) and uridine 5'-diphosphoglucuronic acid (UDPGA) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Nacalai Tesque (Kyoto, Japan), respectively. Marinobufotoxin, marinobufagin 3-hemisuberate, marinobufagin, bufalin and telocinobufagin were obtained from the Japanese toad venom (Shimada *et al.*, 1977). 3-Epibufalin and 3-dehydrobufalin (bufalone) were donated by Dr. Kamano (Kanagawa University,

Yokohama, Japan). Strata-X (60 mg, 3 mL) cartridges were obtained from Shimadzu GLC (Shimadzu), and successively conditioned with MeOH (2 mL) and H_2O (2 mL), prior to use.

Apparatus. High performance liquid chromatography (HPLC) was carried out using a Waters 600s controller (Waters, Milford, MA, USA) 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) was used at a flow rate of 1.0 mL/min at 40℃ unless otherwise stated. An LC/MS system, which consisted of a Shimadzu LC-10AT chromatograph (Shimadzu) coupled with an API 2000 triple-stage quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA), was operated with electrospray ionization (ESI) in the positive-ion mode. A Symmetry C_{18} column (5 μm; 150 mm×2.1 mm i.d.) (Waters) was used at a flow rate of 0.2 mL/min at 40℃. The ionization conditions were as follows: declustering potential, 30 V; entrance potential, 12 V; curtain gas, 20 psi; turbo gas temperature, 500℃; ion source gas 1, 70 psi; ion source gas 2, 80 psi; interface heater, on. The ^{1}H-NMR spectra were obtained using a JEOL JNM-EX270 (270 MHz) or -GSX500 (500 MHz) spectrometer. $CDCl_3$ and Me_4Si were used as the solvent and the internal standard, respectively.

Synthesis of authentic samples. 3-Epimarinobufagin and 3-dehydromarinobufagin were synthesized in our laboratories according to the known procedure (Pettit and Kamano, 1972). ^{1}H-NMR (3-epimarinobufagin) δ (500 MHz): 0.78 (3H, s, H-18), 0.93 (3H, s, H-19), 3.52 (1H, s, H-15α), 4.03 (1H, brs, H-3β), 6.25 (1H, d, *J*=10.5 Hz, H-23), 7.77 (1H, dd, *J*=10.5, 3.0 Hz, H-22). (3-dehydromarinobufagin) δ (270 MHz): 0.82 (3H, s, H-18), 1.04 (3H, s, H-19), 3.54 (1H, s, H-15α), 6.27 (1H, d, *J*=10.5 Hz, H-23), 7.77 (1H, dd, *J*=10.5, 3.0 Hz, H-22). The sulfates of bufogenin and its 3-epimer were synthesized in our laboratories according to the known procedure (Shimada *et al.*, 1977). LC/ESI-MS [MeOH-5 mM HCO_2NH_4 (pH 5.0 adjusted with HCO_2H) (2:3)] marinobufagin 3-sulfate, t_R 15.1 min, *m/z* 481.2 $[M+H]^+$, 3-epimarinobufagin 3-sulfate, t_R 13.5 min, *m/z* 481.2 $[M+H]^+$, bufalin 3-sulfate, t_R 14.7 min, *m/z* 467.1 $[M+H]^+$.

Enzyme sources. The pooled human liver microsomal and cytosolic fractions were purchased from Xenotech LLC (Lenex, KS, USA). Rat liver tissues were homogenized in three volumes of 0.25 M sucrose and the homogenate was centrifuged at 9,000*g* for 20 min. The supernatant was further centrifuged at 105,000*g* for 60 min. The 9,000*g* and 105,000*g* supernatants were used as the rat liver S9 fraction and the rat liver cytosolic

fraction, respectively. The precipitate obtained from the centrifugation at 105,000*g* was used as the rat liver microsomal fraction. The protein concentration was measured by the method of Bradford using bovine serum albumin as the standard (Bradford, 1976).

Enzymatic hydrolysis of marinobufotoxin. Marinobufotoxin (up to 100 μM final concentration in the incubation medium, from a stock solution in methanol) was added to the assay mixture (total volume of 1.0 mL) containing 1 mg protein of the rat or human liver cytosolic fraction in Tris-HCl buffer (pH 7.4, 50 mM) containing EDTA (2 mM). The assay mixture was incubated at 37℃ for 3 h. After the incubation, the reaction was terminated by the addition of ethyl acetate (2 mL). After extraction with ethyl acetate, the ethyl acetate layer was washed with H_2O and evaporated to dryness under a N_2 gas stream. The residue was dissolved in methanol (100 μL) and subjected to HPLC.

Analysis of phase I metabolites of bufogenin. Marinobufagin or bufalin (up to 100 μM final concentration in the incubation medium, from a stock solution in methanol) was added to the assay mixture (total volume of 0.5 mL) containing 1 mg protein of the rat or human liver microsomal fraction in Tris-HCl buffer (pH 7.4, 50 mM) containing NAD and NADPH (each 100 μM). The assay mixture was incubated at 37℃ for 3 h. After the incubation, the reaction was terminated by the addition of ethyl acetate (2 ml). After extraction with ethyl acetate, the ethyl acetate layer was washed with H_2O and evaporated to dryness under a N_2 gas stream. The residue was dissolved in methanol (50 μL) and subjected to HPLC.

Sulfation assay. Marinobufagin, 3-epimarinobufagin or bufalin (up to 50 μM final concentration in the incubation medium, from a stock solution in methanol) was added to the assay mixture (total volume of 0.5 mL) containing 0.5 mg protein of the rat or human liver cytosolic fraction in Tris-HCl buffer (pH 7.4, 50 mM) containing 1,4-dithiothreitol (DTT, 50 μM), $MgCl_2$ (5 mM) and PAPS (149 μM). The assay mixture was incubated at 37℃ for 2 h. After the incubation, the reaction was terminated by heating for 5 min. After centrifugation at 1,500*g* for 10 min, the supernatant was passed through a Strata-X cartridge. After washing with H_2O (2 mL) and ethyl acetate (2 mL), the conjugate was eluted with methanol (2 mL). The methanol layer was evaporated to dryness under a N_2 gas stream. The residue was dissolved in methanol (50 μL) and subjected to HPLC and/or LC/MS analysis. The authentic marinobufagin

3-sulfate (*ca.* 1 μg/tube) was quantitatively recovered (103%, n=2).

Glucuronidation assay. Marinobufagin or 3-epimarinobufagin (up to 50 μM final concentration in the incubation medium, from a stock solution in methanol) was added to the assay mixture (total volume of 0.5 mL) containing 0.5 mg protein of the rat or human liver microsomal fraction in Tris-HCl buffer (pH 7.4, 50 mM) containing EDTA (1 μM), $MgCl_2$ (5 mM), 0.05% Triton X-100 and UDPGA (4 mM). The assay mixture was incubated at 37℃ for 2 h. After the incubation, the reaction was terminated by heating for 5 min. After centrifugation at 1,500*g* for 10 min, the supernatant was passed through a Strata-X cartridge. After washing with H_2O (2 mL) and ethyl acetate (2 mL), the conjugate was eluted with methanol (2 mL). The methanol phase was evaporated to dryness under a N_2 gas stream. The residue was dissolved in methanol (50 μL) and subjected to the LC/MS analysis.

RESULTS AND DISCUSSION

The characterization of *in vitro* metabolites of toad venom, bufotoxin and bufogenin, has been done using HPLC and/or LC/MS. The approximate conversion and recovery rates were calculated using an authentic sample regarding its UV absorption intensity at 300 nm in HPLC/UV.

Enzymatic hydrolysis of marinobufotoxin

Marinobufotoxin was subjected to enzymatic hydrolysis using the rat or human liver cytosolic fraction. The incubation mixture was extracted with ethyl acetate. It was reported that marinobufagin and marinobufagin 3-hemisuberate was obtained from incubation mixture using hog pancreas lipase as an enzyme source by ethyl acetate extraction (Shimada *et al.*, 1977). About 10% of the marinobufotoxin produced marinobufagin, which was confirmed using an authentic sample regarding its HPLC chromatographic behavior [MeOH-0.5% AcONa (pH 5.0 adjusted with AcOH)(5:4), marinobufagin (t_R 5.8 min), marinobufotoxin (t_R 6.8 min); MeCN-0.5% AcONa (pH 5.0 adjusted with AcOH)(2:3), marinobufagin (t_R 5.6 min)], but marinobufagin 3-hemisuberate has not been obtained [MeOH-0.5% AcONa (pH 5.0 adjusted with AcOH)(5:3), t_R 9.0 min]. Even if the incubation time was reduced to ten min, marinobufagin 3-hemisuberate has not been obtained. The treatment of marinobufotoxin with the rat liver S9 fraction or microsomal fraction gave almost the

same results. These data showed that the enzyme reacted at the ester (-COOR) group rather than at the acid amide (-CONH-) group.

Analysis of phase I metabolites of bufogenin

Marinobufagin was subjected to an enzymatic reaction using the rat liver microsomal fraction in the presence of NAD and NADPH. The residue obtained from the incubation mixture was subjected to HPLC using MeCN-H_2O (1:2) as the mobile phase. 3-Dehydromarinobufagin (t_R 8.4 min) and 3-epimarinobufagin (t_R 5.1 min) were confirmed using authentic samples regarding their chromatographic behavior together with the substrate (t_R 11.7 min). These were also confirmed by inclusion chromatography containing γ-cyclodextrin (CD)[MeOH-H_2O (1:1) containing γ-CD (4 mM); 3-epimarinobufagin (t_R 5.6 min), 3-dehydromarinobufagin (t_R 6.3 min), marinobufagin (t_R 7.2 min)] (Fig. 2a). The treatment of marinobufagin with the human liver microsomal fraction gave almost the same result. The treatment of 3-dehydromarinobufagin with the rat liver microsomal fraction in the presence of NAD and NADPH as described above gave not only 3-epimarinobufagin (*ca.* 20%) but also marinobufagin (*ca.* 12%).

The representative bufogenin, bufalin, was also subjected to the reaction using the rat or human liver microsomal fraction to produce the 3-epimer together with bufalone [HPLC, Chromolith Performance RP-18e (100 x 4.6 mm i.d.; Merck, Darmstadt, Germany), MeOH-H_2O (12:13); 3-epibufalin (t_R 14.0 min), bufalone (t_R 11.5 min), bufalin (t_R 12.6 min)]. These data are compatible with our previous results using the rat liver homogenate in the presence of NADPH and NAD (Shimada *et al.*, 1989). About 30% of bufalin produced the new metabolite, telocinobufagin (Fig. 1b), upon treatment with the human liver microsomal fraction in the presence of co-factors, which was confirmed using an authentic sample regarding its HPLC chromatographic behavior [MeOH-H_2O (11:9) containing γ-CD (2 mM), t_R 8.9 min; Chromolith Performance RP-18e, MeOH-H_2O (12:13) t_R 7.9 min (Fig. 2b); Develosil 60-5 (5 μm, 250 x 4.6 mm i.d.; Nomura, Seto, Japan), AcOEt-isopropanol (95:5), t_R 7.1 min]. It was reported that digitoxigenin (cardenolide) having a five-member lactone ring instead of the six-member one (bufadienolide) was metabolized to the 5β-hydroxylated metabolite (periplogenin) upon treatment with the rabbit liver homogenate (Bulger and Stohs, 1973), which is compatible to the above results. On the other hand, about 1% of bufalin produced telocinobufagin upon treatment with the rat liver microsomal fraction in the presence of co-factors and the main metabolite was 3-epimer (about 90% from the substrate). These different data obtained from rat and human demonstrated the differences in

different species.

All of the above data showed that bufogenin having the 3β-hydroxy-5β-structure is enzymatically metabolized to the inactive metabolite having the 3α-hydroxy-5β-structure (Nambara *et al.*, 1972) together with hydroxylation such as at 5β-position to give telocinobufagin.

Sulfation assay. Marinobufagin or 3-epimarinobufagin was treated with the rat or human liver cytosolic fraction together with PAPS. After the incubation, the reaction mixture was passed through a Strata-X cartridge. The substrate was washed out with ethyl acetate, and the conjugate was eluted with methanol. The residue obtained from the incubation mixture was subjected to HPLC and LC/MS analysis. Marinobufagin produced the 3-sulfate which was confirmed using an authentic sample regarding its HPLC chromatographic behavior [MeOH-5 mM HCO_2NH_4 (pH 5.0 adjusted with HCO_2H) (2:3); t_R 9.1 min] (Fig. 3a) and LC/ESI-MS analysis (Fig. 3b). However, the 3-epimarinobufagin did not produce the respective 3-sulfate, which was confirmed using an authentic sample regarding its HPLC chromatographic behavior [MeOH-5 mM HCO_2NH_4 (pH 5.0 adjusted with HCO_2H) (2:3); t_R 7.1 min]. Bufalin was also subjected to the sulfation reaction to produce the sulfate, which was confirmed using an authentic sample regarding its chromatographic behavior [HPLC, MeOH-5 mM HCO_2NH_4 (pH 5.0 adjusted with HCO_2H) (2:3); t_R 12.5 min] (Fig. 4a) and LC/ESI-MS analysis (Fig. 4b).

Glucuronidation assay. Marinobufagin or 3-epimarinobufagin was treated with the rat or human liver microsomal fraction in the presence of UDPGA. The residue obtained from the incubation mixture was subjected to an LC/ESI-MS analysis [MeOH-5 mM HCO_2NH_4 (pH 5.0 adjusted with HCO_2H) (2:3), t_R 9.2 min]. Marinobufagin produced small amounts of 3-glucuronide (less than 1 %) only by treatment with the human liver microsomal fraction (Fig. 5). The respective glucuronide has not been obtained under the other conditions.

All the above data suggested the following *in vitro* metabolic pathway of marinobufotoxin using the rat or human liver as an enzyme source as shown in Fig. 6. Marinobufotoxin was hydrolyzed to marinobufagin, which was transformed into the 3-epimer possibly *via* the 3-dehydro compound. Instead of glucuronidation, sulfation mainly occurred on marinobufagin to produce the marinobufagin 3-sulfate as the phase II metabolite of bufogenin. The data showed that bufogenin is enzymatically metabolized to the biologically inactive sulfate (Shimada *et al.*, 1985). The representative bufogenin, bufalin, showed the similar metabolic pathway as marinobufagin. But it is noteworthy

that telocinobufagin was obtained from bufalin upon treatment with the human and rat liver microsomal fraction in the presence of NADPH and NAD, which means hydroxylation occurred at 5β-position like the metabolic pathway of digitoxigenin (Bulger and Stohs, 1973).

Further experiments including the *in vivo* one are now in progress in our laboratories in order to clarify the metabolic pathway of bufotoxin and bufogenin.

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Figure captions

Fig. 1. Structures of bufotoxin and bufogenin
a) marinobufotoxin and related compounds b) bufalin and related compounds

Fig. 2. HPLC chromatograms of phase I metabolites of bufogenin
Conditions; column, a) Symmetry C_{18} (5 μm; 150 mm x 4.6 mm i.d.) b) Chromolith Performance RP-18e (100 x 4.6 mm i.d.); detection, UV 300 nm; flow rate, 1 mL/min; solvent, a) MeOH-H_2O (1:1) containing γ-CD (4 mM) b) MeOH-H_2O (12:13); Substrate, a) marinobufagin b) bufalin; Enzyme source, a) rat liver microsomal fraction b) human liver microsomal fraction
a) 1. 3-epimarinobufagin 2. 3-dehydromarinobufagin 3. marinobufagin
b) 1. telocinobufagin 2. bufalone 3. bufalin 4. 3-epibufalin

Fig. 3. HPLC chromatogram (a) and LC/ESI-MS spectrum (b) of marinobufagin 3-sulfate obtained from incubation mixture
Conditions; solvent, MeOH-5 mM $HCOO_2NH_4$ (pH 5.0 adjusted with HCOOH)(2:3) a) column, Symmetry C_{18} (5 μm; 150 mm x 4.6 mm i.d.); detection, UV 300 nm; flow rate, 1 mL/min b) column, Symmetry C_{18} (5 μm; 150 mm x 2.1 mm i.d.); flow rate, 0.2 mL/min; t_R 15.1 min
Substrate, marinobufagin Enzyme source, rat liver cytosolic fraction

Fig. 4. HPLC chromatogram (a) and LC/ESI-MS spectrum (b) of bufalin 3-sulfate obtained from incubation mixture
Conditions; solvent, MeOH-5 mM $HCOO_2NH_4$ (pH 5.0 adjusted with HCOOH)(2:3) a) column, Symmetry C_{18} (5 μm; 150 mm x 4.6 mm i.d.); detection, UV 300 nm; flow rate, 1 mL/min b) column, Symmetry C_{18} (5 μm; 150 mm x 2.1 mm i.d.); flow rate, 0.2 mL/min; t_R 14.7 min
Substrate, bufalin Enzyme source, rat liver cytosolic fraction

Fig. 5. LC/ESI-MS spectrum of marinobufagin 3-glucuronide obtained from incubation mixture
Conditions; a) column, Symmetry C_{18} (5 μm; 150 mm x 2.1 mm i.d.); flow rate, 0.2 mL/min; solvent, MeOH-5 mM $HCOO_2NH_4$ (pH 5.0 adjusted with HCOOH)(2:3); t_R 9.2 min
Substrate, marinobufagin Enzyme source, human liver microsomal

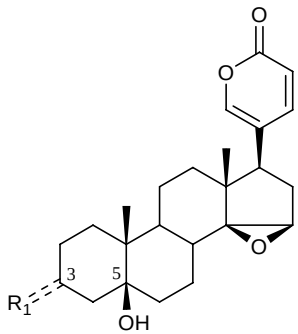
fraction

Fig. 6. Proposed *in vitro* metabolic pathway of marinobufotoxin using rat or human liver as an enzyme source

----▶ human liver only

Fig. 1. K. Shimada *et al.*

a)



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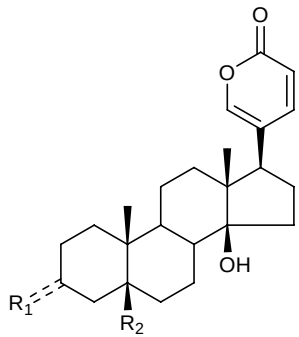

	R_1
marinobufotoxin	α-H, β-OCO$(CH_2)_6$COArgOH
marinobufagin	α-H, β-OH
3-dehydromarinobufagin	O
3-epimarinobufagin	α-OH, β-H
marinobufagin 3-sulfate	α-H, β-OS
3-epimarinobufagin 3-sulfate	α-OS, β-H
marinobufagin 3-glucuronide	α-H, β-OG

	R_1	R_2
bufalin	α-H, β-OH	H
bufalone	O	H
3-epibufalin	α-OH, β-H	H
bufalin 3-sulfate	α-H, β-OS	H
telocinobufagin	α-H, β-OH	OH

S: SO_3H G: Glucuronic acid

Fig. 2. K. Shimada *et al.*

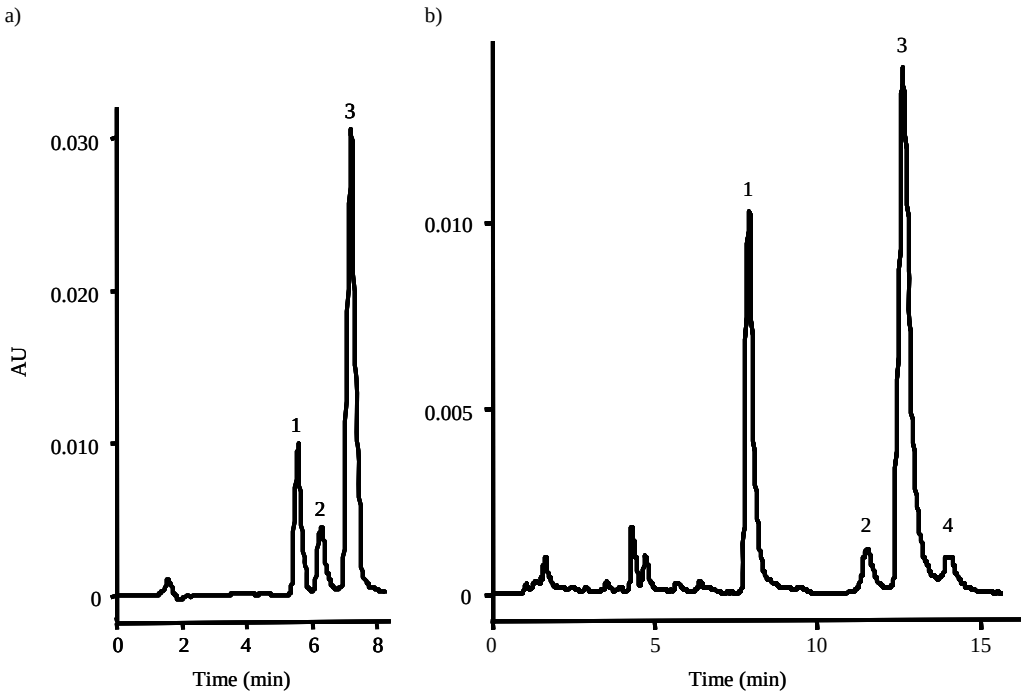

Fig. 3. K. Shimada *et al*.

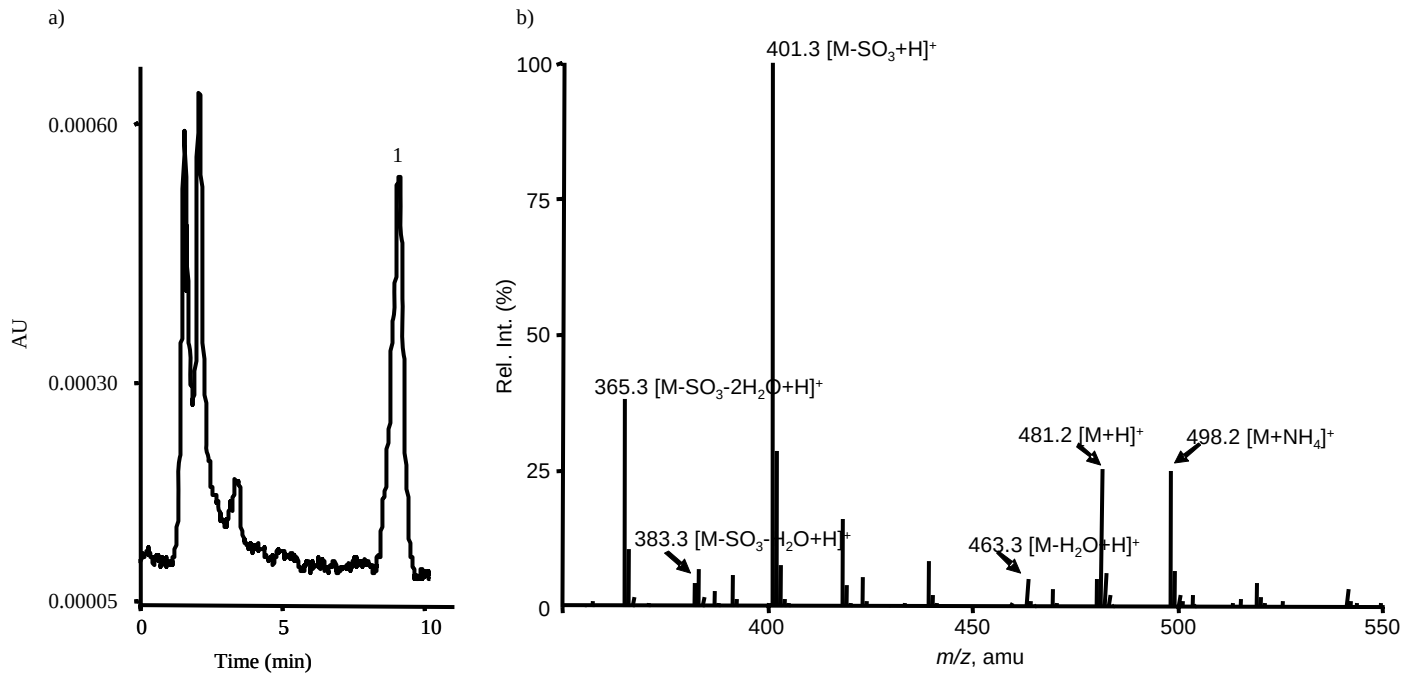

Fig.4. K. Shimada *et al*.

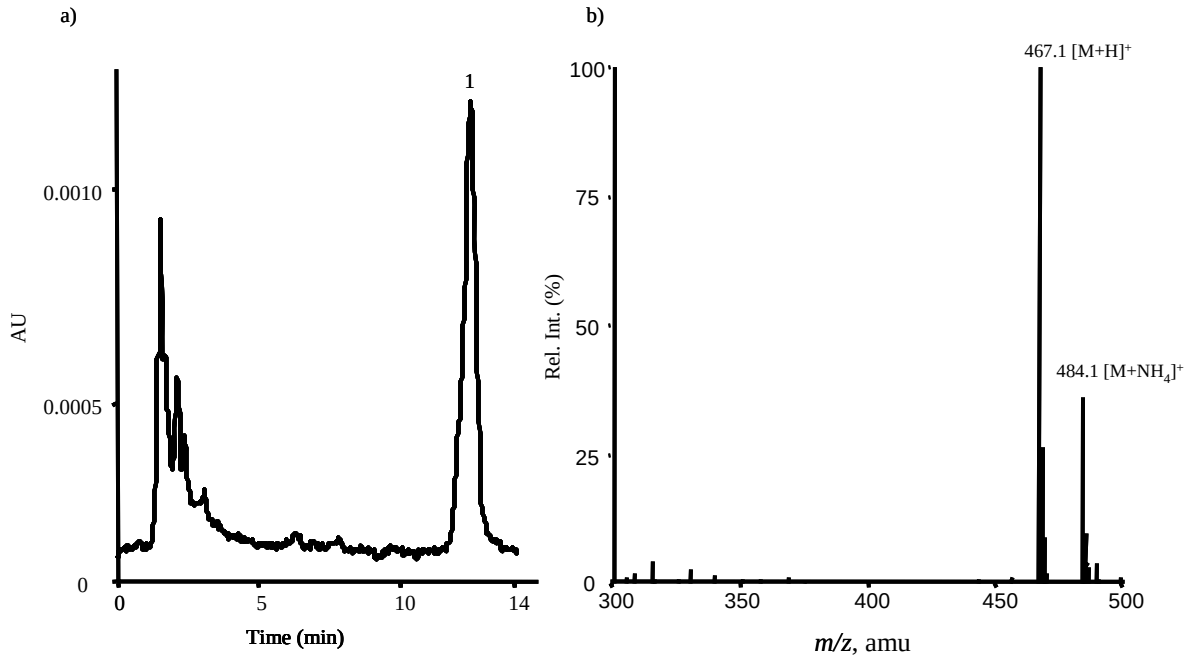

Fig. 5. K. Shimada *et al*.

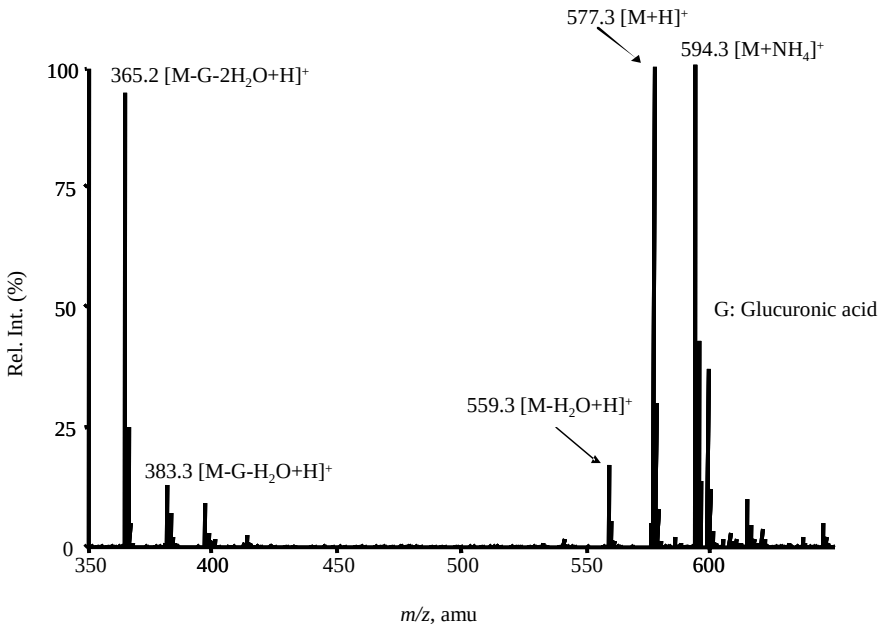

Fig. 6. K. Shimada *et al*.