Analyses of Biologically Active Steroids: Antitumor Active OSW-1 and Cardiotonic Marinobufotoxin, by Matrix-Assisted Laser Desorption/Ionization Quadrupole Ion Trap Time-of-Flight Tandem Mass Spectrometry

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Naturally occurring constituents of biological or pharmaceutical interest often exist in the form of glycosides or conjugates. Mass spectral investigations of these compounds require soft ionization techniques if information on molecular mass, sugar sequence, or conjugate content is desired. In this study, matrix-assisted laser desorption/ionization (MALDI) quadrupole ion trap (QIT) time-of-flight tandem mass spectrometry (TOF-MS^n) was used to identify both OSW-1, an acetylated cholestane diglycoside showing antitumor activity, and the cardiotonic steroid, bufotoxin. Each molecular-related ion was identified, and subsequent collision-induced dissociation experiments in which a molecular-related ion was selected as a precursor ion produced the characteristic product ions that are essential for structural elucidation. OSW-1 and its analogue with a modified side chain, thienyl OSW-1, were synthesized, and bufotoxins, *i.e.*, marinobufotoxin and its homologue, marinobufagin 3-pimeloylarginine ester, were isolated from toad venom. On MALDI-TOF-MS, sodium-adduct $[M+Na]^+$ ions were observed in the steroid glycosides, although protonated $[M+H]^+$ ions were relatively more abundant than sodium-adduct $[M+Na]^+$ ions in the bufotoxins. On the basis of tandem MS results, we propose key fragmentation pathways. The sugar moiety or side chain from the precursor ion was eliminated in OSW-1. However, characteristic product ions originating from the cleavage of the side chain with an ester formation were observed in the bufotoxins. Post-source decay (PSD) on MALDI-TOF-MS is also described when evaluating α-cyano-4-hydroxycinnamic acid or 2,5-dihydroxybenzoic acid as a matrix to obtain useful ions required for the identification of compound.

Key words matrix-assisted laser desorption/ionization time-of-flight MS^n; OSW-1; bufotoxin; post-source decay

Tandem mass spectrometry (MS^n) has been used for the identification and structural determination of steroids. Steroidal glycoalkaloids extracted from natural plants are identified using electrospray ionization (ESI) in ion trap (IT) MS,[1] and steroid glucuronides are analyzed using a triple-quadrupole (Q) instrument with a turbo ion-spray source.[2]

An acetylated cholestane diglycoside, OSW-1 (**1**), (3β,16β,17α-trihydroxycholest-5-en-22-one 16-*O*-{*O*-[2-*O*-(4-methoxybenzoyl)-β-D-xylopyranosyl]-(1→3)-2-*O*-acetyl-α-L-arabinopyranoside}), was isolated by Sashida and co-workers as the main constituent from the bulbs of *Ornithogalum saundersiae* (Liliaceae) and found to show potent cytotoxicity against a variety of malignant human tumor cells.[3] OSW-1 exhibits low toxicity against normal cells but inhibits the growth of malignant cells and is 10—100-fold more potent than clinically established anticancer agents such as mitomycin C, doxorubicin, camptothecin, and paclitaxel.[4] Fuchs and colleagues proposed that the active intermediate might be a 22-oxocarbenium ion, which could be generated from 22-carbonyl and 16α-hydroxy moieties.[5] Yu and colleagues have recently reported that both 22-methylene and 23-heteroatom (O, S, NH) analogues of OSW-1 were as potent as the parent natural products against the growth of tumor cells.[6] However, the precise mechanism by which OSW-1 exerts its effects remains unclear. A further study of the structure–activity relationships of OSW-1 analogues having heterocyclic rings, such as thiophene and thiazole, at the side chain was therefore designed. We have succeeded in the syntheses of OSW-1 (**1**) and one of its analogues, ThOSW-1, (20*S*,23*Z*,25*Z*)-3β,16β,17α-trihydroxy-23,26-epithiocholesta-5,23,25-trien-22-one 16-*O*-{*O*-[2-*O*-(4-methoxybenzoyl)-β-D-xylopyranosyl]-(1→3)-2-*O*-acetyl-α-arabinopyranoside} (**2**).[7,8] In a previous study, we investigated the optimum LC/MS conditions to obtain useful ions required to identify structures using ESI and atmospheric-pressure chemical-ionization (APCI)-QMS.[9]

Cardiotonic steroids are classified into two groups based on the type of unsaturated lactone ring substituted at the 17β-position of the steroidal skeleton, *i.e.*, cardenolides with a five-membered lactone ring and bufadienolides with a six-membered lactone ring, respectively. Toad steroids isolated from *Bufo* sp. have a six-membered lactone ring and are divided into two groups according to the form at the 3β-position of the steroid: bufogenin as an unconjugated steroid; and bufotoxin as a steroid conjugate. From ancient times, the skin secretions of native toads, known as *ch'an su* in China and as *senso* in Japan, have been employed as cardiac or diuretic agents, and the main cardiotonic constituent in toad venom has been elucidated to be bufotoxin.[10] Marinobufotoxin (**3**) with a suberoylarginine ester at the 3β-position is one of the main cardiotonic constituents. In addition to this, homologues with other dicarboxylic acids, *i.e.*, pimeric acid, succinic acid, or adipic acid, and analogues with other amino acids, *i.e.*, histidine or glutamine, are known to occur. In addition, other conjugate forms, *i.e.*, sulfates or glucuronides, are known to exist.[11] Forty types of bufotoxin and related

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compounds were investigated to determine their inhibitory activity against Na^+,K^+-adenosine triphosphatase, and their structure–activity relationships are discussed. The results showed that the inhibitory activities of bufotoxins are dependent upon the dicarboxylic acid and amino acid components in the side chain, and the activity of a bufotoxin was suggested to be superior to that of the corresponding bufogenin. Sulfates or glucuronides exhibited much less potency than the parent genins.[12)]

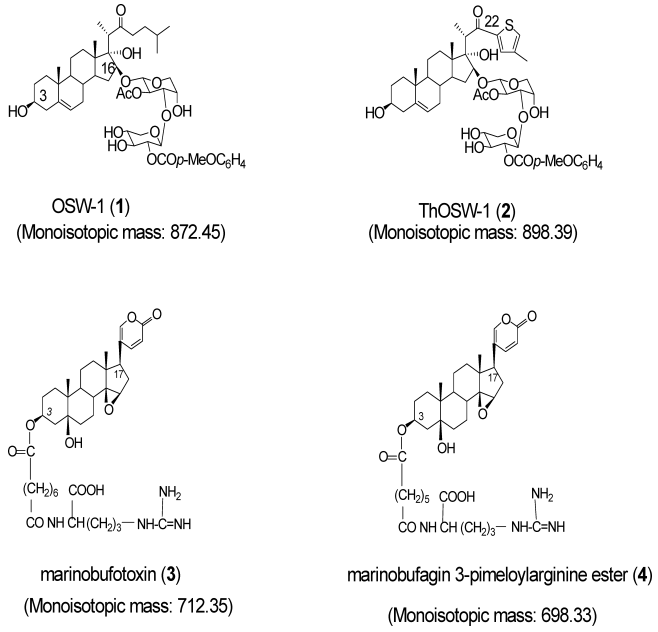

Chart 1. Structures of the Compounds Studied

Compound **3** and its homologue, marinobufagin 3-pimeloylarginine ester (**4**), were isolated from the skin of a tropical toad, *Bufo marinus* (L.) SCHNEIDER, and the structure of **4** was confirmed by the authors.[13)] In a previous study, MS^n utilizing an IT mass spectrometer with ESI and APCI modes was used for the analyses of cardiotonic steroids.[14)]

To the best of our knowledge, this is the first report on the detection of molecular-related ions and examination of the fragmentation pathways of structurally useful product ions using matrix-assisted laser desorption/ionization (MALDI)-quadrupole ion trap (QIT)-time-of-flight (TOF)-MS^n to identify biologically active steroids.

The post-source decay (PSD) technique using MALDI-TOF-MS was also investigated. This technique enables MS/MS-like analysis to obtain structural information,[15)] and the matrix used in measurement may influence the fragmentation pattern.[16)] In this study, PSD experiments on compounds **1** and **3** using two typical matrices, 2,5-dihydroxybenzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (CHCA), were also conducted, and ion species were compared with those obtained in tandem MS^n.

Experimental

Materials and Reagents OSW-1 (**1**) and its analogue ThOSW-1 (**2**) were synthesized in our laboratory. Marinobufotoxin (**3**) and its homologue, marinobufagin 3-pimeloylarginine ester (**4**), were isolated from a skin of the tropical toad *B. marinus* (L.) SCHNEIDER.

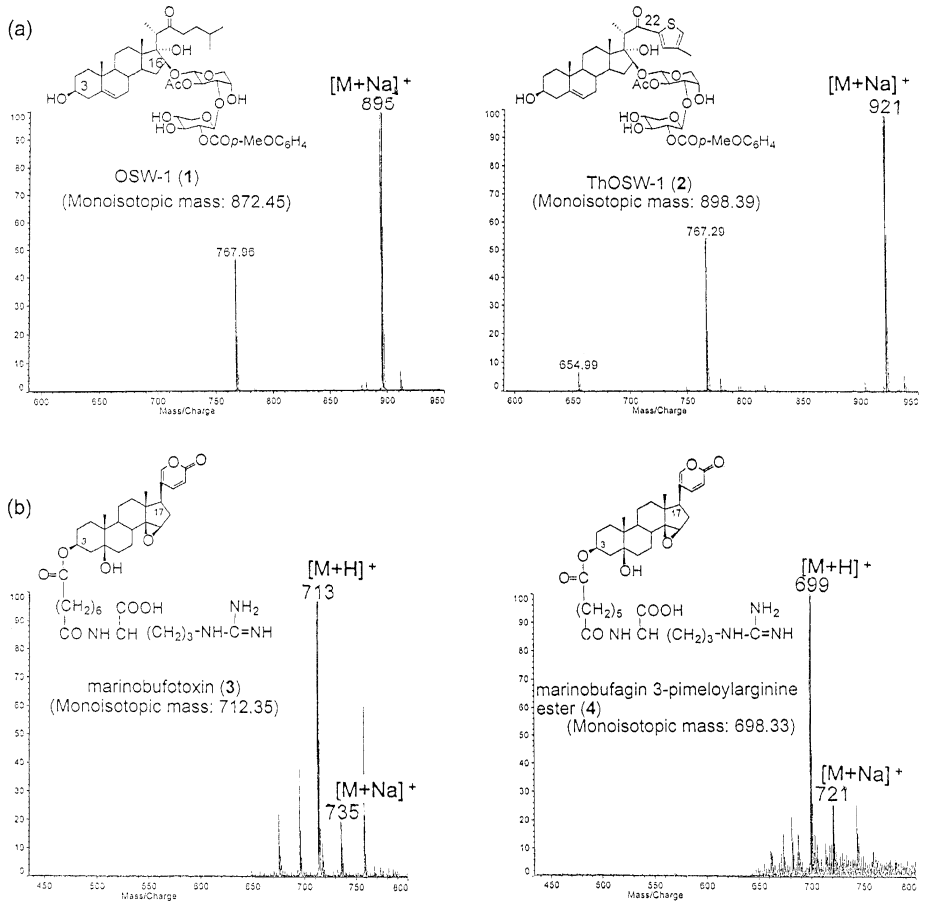

Fig. 1. Molecular-Related Ions Observed in MALDI-QIT-TOF-MS of (a) OSW-1 (**1**) and Its Analogue (**2**); (b) Marinobufotoxin (**3**) and Its Homologue (**4**)

Calibration standards, *i.e.*, angiotensin II (Ang II) and bradykinin 1-7 were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Acetonitrile (MeCN) of HPLC grade was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Trifluoroacetic acid (TFA) of HPLC grade was obtained from Wako Pure Chemical Industries (Osaka, Japan). DHB and CHCA matrices of MALDI-MS grade were purchased from Shimadzu GLC (Tokyo, Japan). All aqueous solutions were prepared using water filtered through a Milli-Q water system (Millipore, Bedford, MA, U.S.A.). Pipetman-P pipettes were obtained from Gilson (Middleton, WI, U.S.A.). The MALDI sample plate model 384 was equipped with AXIMA instruments.

Instruments and Methods Measurements were performed in positive-ion MALDI-MS. Tandem mass experiments with MALDI-QIT-TOF-MS^n (n=2, 3, or 4) were performed using argon gas as the collision gas on an AXIMA-QIT mass spectrometer (Shimadzu Corp., Kyoto, Japan) equipped with a nitrogen laser (337 nm) with the collision-induced dissociation (CID) control value set at 160, and PSD experiments using MALDI-TOF-MS were performed on an AXIMA-CFR^{PLUS} mass spectrometer (Shimadzu Corp.) with ion accelerating voltage of 20 kV.

Preparation of Sample Spots Ten milligrams of DHB or CHCA matrix was dissolved in 1 ml of 40% MeCN and 0.1% TFA. Matrix solution (0.5 μl) and 1% sample (0.5 μl) dissolved in methanol and water were deposited on a MALDI plate and left to dry at room temperature to prepare sample spots.

Calibration External calibrations were achieved using the standard reagents of CHCA (monoisotopic mass of $[M+H]^+$=190.1), Ang II (monoisotopic mass of $[M+H]^+$=1046.5), and bradykinin 1-7 (monoisotopic mass of $[M+H]^+$=757.4).

Results and Discussion

Species of Molecular-Related Ions In MALDI-QIT-TOF-MS, compounds **3** and **4** gave a mixture of protonated $[M+H]^+$ ions at *m/z* 713 and 699, and sodium-adduct $[M+Na]^+$ ions at *m/z* 735 and 721, while compounds **1** and **2** produced only a simple precursor ion peak as sodium-adduct $[M+Na]^+$ ions at *m/z* 895 and 921, as shown in Fig. 1. These results suggest that steroid conjugations involving suberoylarginine or pimeloylarginine ester have much higher affinity for protons than sodium cations. This may be responsible for the arginine residue contained in the ester moiety. The arginine guanidinium group would have greater thermodynamic stability with the addition of a proton, although the affinity of sodium cations for steroid glycosides is greater than that of protons.

Subsequently, tandem MS$^{2-4}$ experiments were performed to evaluate the structures of the characteristic ions produced.

MALDI-QIT-TOF-MS$^{2-4}$ Fragmentation. OSW-1 (1)

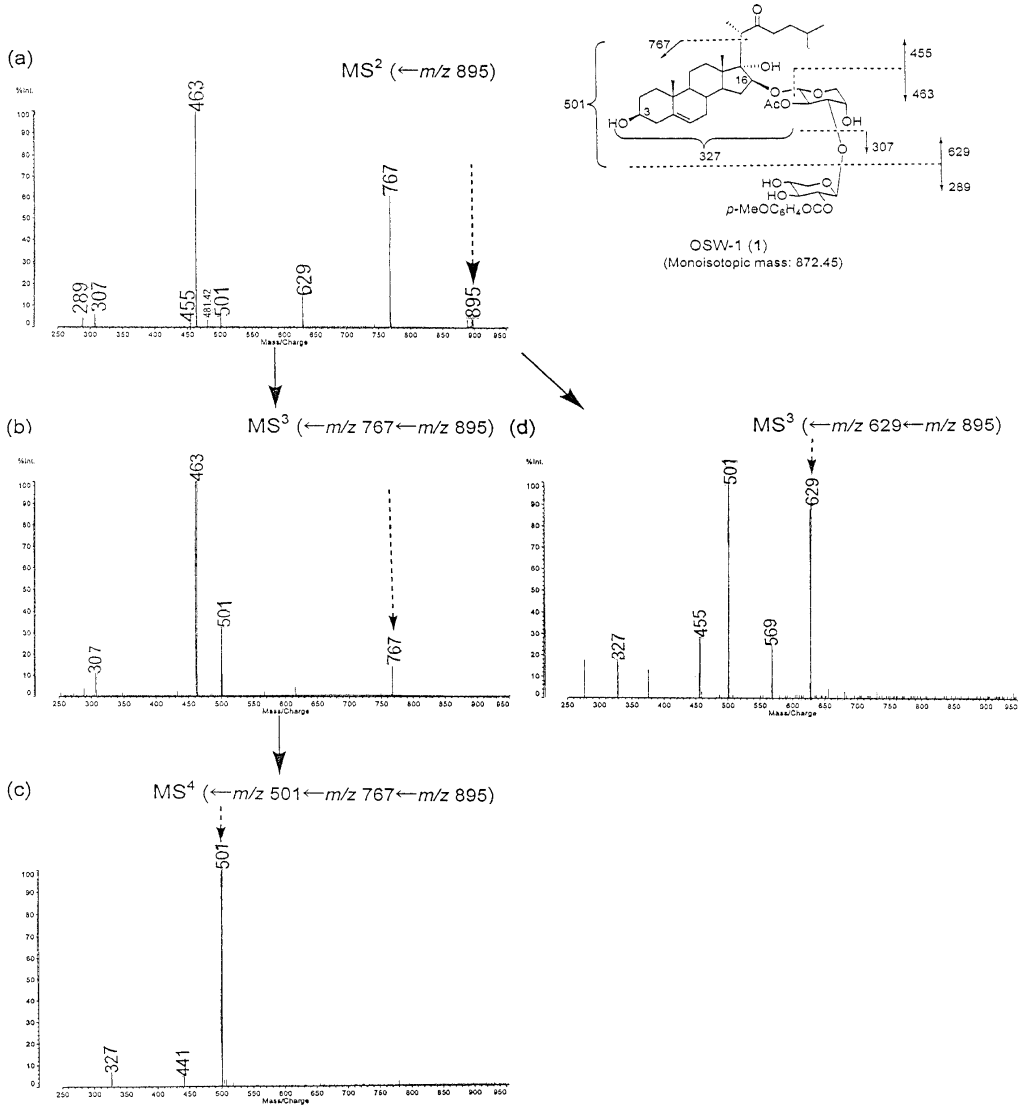

Fig. 2. MALDI-QIT-TOF-MS^n of OSW-1 (**1**) and Proposed Cleavage Parts for Characteristic Fragmentation Ions

(a) MS^2 spectrum selecting a precursor ion at *m/z* 895 $[M+Na]^+$. (b, d) MS^3 spectra selecting a precursor ion (b) at *m/z* 767 (← *m/z* 895); and (d) at *m/z* 629 (← *m/z* 895). (c) MS^4 spectrum selecting a precursor ion at *m/z* 501 (← *m/z* 767 ← *m/z* 895).

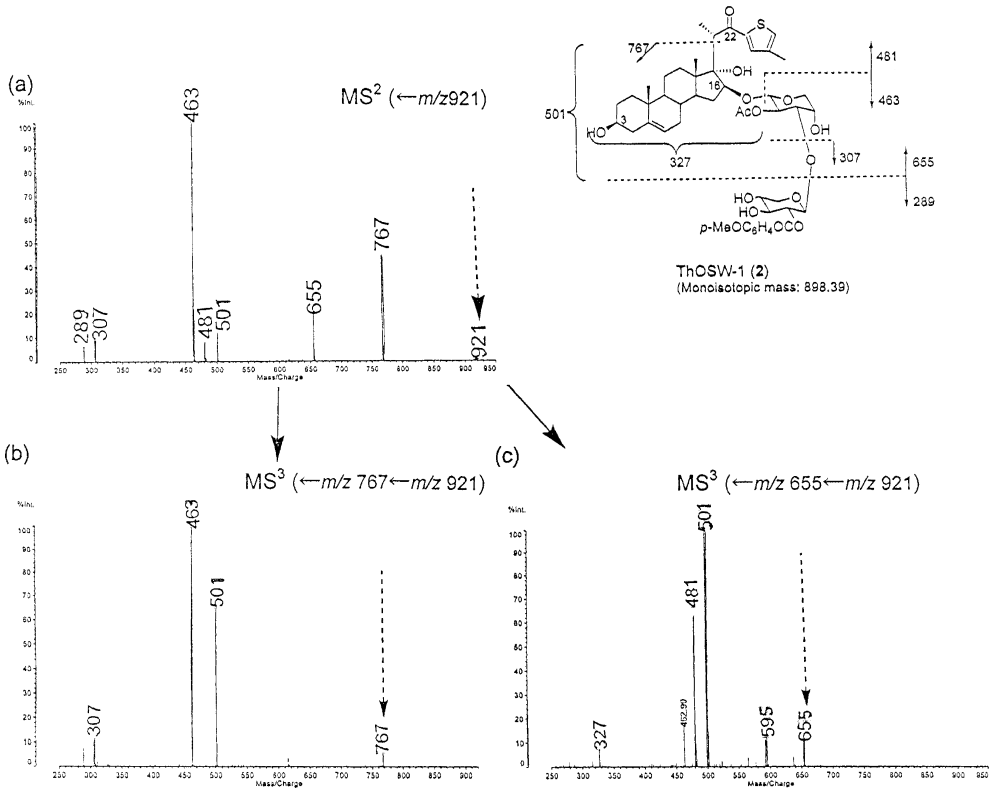

Fig. 3. MALDI-QIT-TOF-MS^n of ThOSW-1 (**2**) and Proposed Cleavage Parts for Characteristic Fragmentation Ions

(a) MS^2 spectrum selecting a precursor ion at *m*/*z* 921 $[M+Na]^+$. (b, c) MS^3 spectra selecting a precursor ion (b) at *m*/*z* 767 (← *m*/*z* 921); and (c) at *m*/*z* 655 (← *m*/*z* 921).

Table 1. Product Ions of OSW-1 (**1**) and ThOSW-1 (**2**) Obtained in MALDI-QIT-TOF-MS^n and MALDI-PSD

Precursor ion	MS² ⇒ Product ion	(*m*/*z*) MS³ ⇒ Product ion	MS⁴ ⇒ Product ion
895 [**1**+Na]$^+$	⇒ 767 (744+Na)	⇒ 501	⇒ 441
		463	327
		307	
		289	
	629 (606+Na)	⇒ 569	
		501	
		455	
		327	
	501 (478+Na)	⇒ 327	
	463 (diglycoside+Na)	⇒ 307	
		289	
		269	
	455 (aglycone+Na)		
	307 (sugar+Na)		
	289 (266+Na)		

Precursor ion	MS² ⇒ Product ion	(*m*/*z*) MS³ ⇒ Product ion
921 [**2**+Na]$^+$	⇒ 767 (744+Na)	⇒ 501
		463
		307
		289
	655 (632+Na)	⇒ 595
		501
		481
		327
	501 (478+Na)	⇒ 327
	463 (diglycoside+Na)	
	481 (aglycone+Na)	
	307 (sugar+Na)	
	289 (266+Na)	

	Matrix	
	CHCA [DHB]	
	PSD	
895 [**1**+Na]$^+$	⇒ 767	
	629	[629]
	501	
	463	[463]
	307	
	289	

and Its Analogue ThOSW-1 (2) The MS^2 spectrum of OSW-1 (**1**) when the ion at *m*/*z* 895 was selected as the precursor ion is shown in Fig. 2a, and the product ion is compared with that obtained in the MS^2 spectrum of its analogue (**2**), as shown in Fig. 3a, when the ion at *m*/*z* 921 was selected as the precursor ion. Compound **2** has a modified side chain, with a 4-methyl-2-thienyl group at position 22. Examining the mass spectra of these two compounds with different functional groups is interesting, and comparing the mass of the product ions of **1** with that of the product ions of **2** appears useful in confirming fragmentation pathways. If a difference of 26u between each *m*/*z* value of product ion obtained in each tandem MS^2 spectrum is observed, it means that the product ion includes position 22 in the side chain (isoamyl in **1** or 4-methyl-2-thienyl group in **2**).

The product ions can be divided into two groups, as indicated in Table 1. One group is the corresponding ions with 26 mass differences at *m*/*z* 629 of **1** or at *m*/*z* 655 of **2** and at *m*/*z* 455 of **1** or at *m*/*z* 481 of **2**, appearing to involve a 22-position side-chain moiety. The other group is the common product ions observed in both spectra at *m*/*z* 767, *m*/*z* 501, *m*/*z* 463, *m*/*z* 307, and *m*/*z* 289, appearing not to involve position 22.

First, our attention was focused on the product ion at *m*/*z* 767, which was also obtained in the MS spectra, as shown in Fig. 1a. Then the MS^3 experiment selecting the ion at *m*/*z* 767 (← *m*/*z* 895 $[\mathbf{1}+Na]^+$ or ← *m*/*z* 921 $[\mathbf{2}+Na]^+$) as a precursor ion was performed and product ions at *m*/*z* 501, *m*/*z* 463, *m*/*z* 307, and *m*/*z* 289 were obtained, as shown in Figs. 2b and 3b and Table 1. The product ion at *m*/*z* 463 was assumed to be derived from the diglycoside at the 16-position because the MS^3 experiment performed by selecting the ion at *m*/*z* 463 (← *m*/*z* 895 $[\mathbf{1}+Na]^+$) as the precursor ion gave product ions at *m*/*z* 307, *m*/*z* 289, and *m*/*z* 269, as indicated in Table 1 (MS spectrum is not shown), related to the *p*-methoxybenzoyl glycoside moiety. Thus the product ion at *m*/*z* 767 is assumed to be derived from the elimination of the 17-position side chain from the precursor ion. The fragmentation pathways and structures of product ions are proposed in Chart 2 and the cleavage parts are shown in Figs. 2 and 3.

Second, the product ion at *m*/*z* 629 (← *m*/*z* 895 $[\mathbf{1}+Na]^+$) or at *m*/*z* 655 (← *m*/*z* 921 $[\mathbf{2}+Na]^+$) with 26 mass differ-

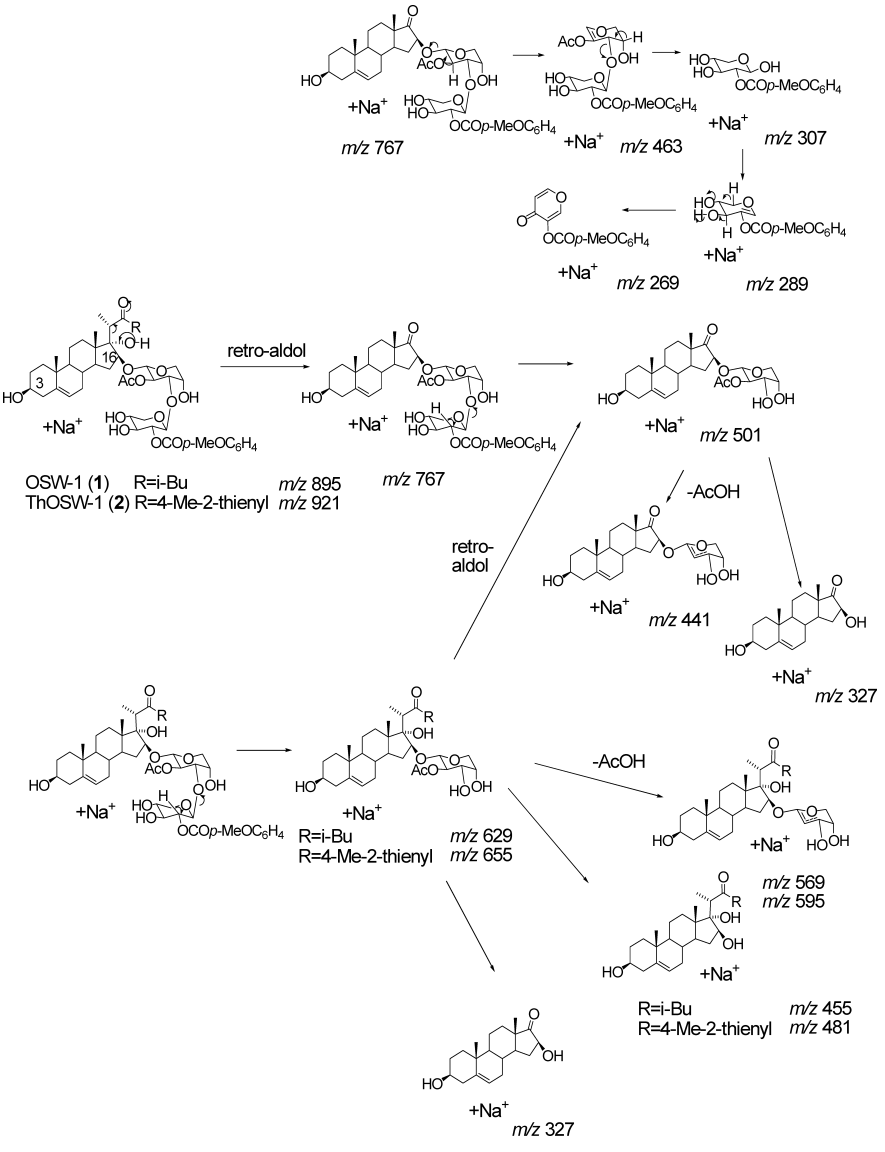

Chart 2. Fragmentation Pathways of OSW-1 (**1**) and Its Analogue (**2**) in MALDI-QIT-TOF-MS^n

Table 2. Product Ions of Marinobufotoxin (**3**) and Its Homologue (**4**) Obtained in MALDI-QIT-TOF-MS^n and MALDI-PSD

Precursor ion	MS^2 ⇒ Product ion	MS^3 ⇒ Product ion	(*m/z*)
713 [**3**+H]$^+$	⇒ 695 (713−H_2O)		
	331 (ester+H)	⇒ 313	
		296 (313−NH_3)	
		278 (296−H_2O)	
		175 (arginine+H)	
		158 (175−NH_3)	
	313 (331−H_2O)		
	278		

Precursor ion	MS^2 ⇒ Product ion	MS^3 ⇒ Product ion	(*m/z*)
699 [**4**+H]$^+$	⇒ 681 (699−H_2O)		
	317 (ester+H)	⇒ 299	
		282 (299−NH_3)	
		264 (282−H_2O)	
		175	
		158	
	299 (317−H_2O)	⇒ 282 (299−NH_3)	
		264 (282−H_2O)	
		175	
		158	
	264		

	Matrix	
	CHCA [DHB]	
	PSD	
713 [**3**+H]$^+$	⇒ 331	[331]
	278	[278]
	175	[175]
	158	[158]

ences, *i.e.*, involving a 22-position moiety, was investigated. The MS^3 experiment selecting each ion as a precursor ion gave both common product ions at *m/z* 501 and *m/z* 327, and 26u-gap product ions at *m/z* 569 of **1** or at *m/z* 595 of **2** and at *m/z* 455 of **1** or at *m/z* 481 of **2**, as shown in Figs. 2d and 3c and Table 1. In compound **1**, the ion at *m/z* 569 seems to be derived due to carboxylic acid elimination from the precursor ion at *m/z* 629, and the ion at *m/z* 455 is considered to correspond to aglycone. Thus the product ion at *m/z* 629 in **1** or at *m/z* 655 in **2** obtained in the MS^2 spectrum is assumed to originate due to the elimination of *p*-methoxybenzoyl glycoside moiety from the molecular-related ion as shown in Chart 2.

A query remains concerning the product ion at *m/z* 501, which is abundant in both the MS^2 and MS^3 spectra. In compound **1**, this product ion was obtained in both MS^3 experiments when not only a precursor ion having 26 mass differences at *m/z* 629 (← *m/z* 895 [**1**+Na]$^+$) but also a common precursor ion at *m/z* 767 (← *m/z* 895 [**1**+Na]$^+$) was selected as shown in Figs. 2b and d. To examine its structure, a subsequent MS^4 experiment selecting the ion at *m/z* 501 (← *m/z* 767 ← *m/z* 895 [**1**+Na]$^+$) as a precursor ion was performed, as shown in Fig. 2c. A product ions at *m/z* 441 and at *m/z* 327 were obtained, which are assumed to originate due to the elimination of carboxylic acid and a sugar moiety, respectively. Thus the product ion at *m/z* 501 is assumed to be derived from the elimination of both the 17-position side chain and *p*-methoxybenzoyl glycoside moiety from the molecular-related ion as shown in Chart 2.

Marinobufotoxin (3) and Its Homologue (4) The results of the MS^2 experiment when the ion at *m/z* 713 [**3**+H]$^+$ was selected as a precursor ion are shown in Fig. 4a, and the product ion is compared with that obtained in the MS^2 spectrum of its homologue (**4**) when the ion at *m/z* 699 [**4**+H]$^+$ was selected as the precursor ion in Fig. 4b. The only difference between compounds **3** and **4** is an acid species con-

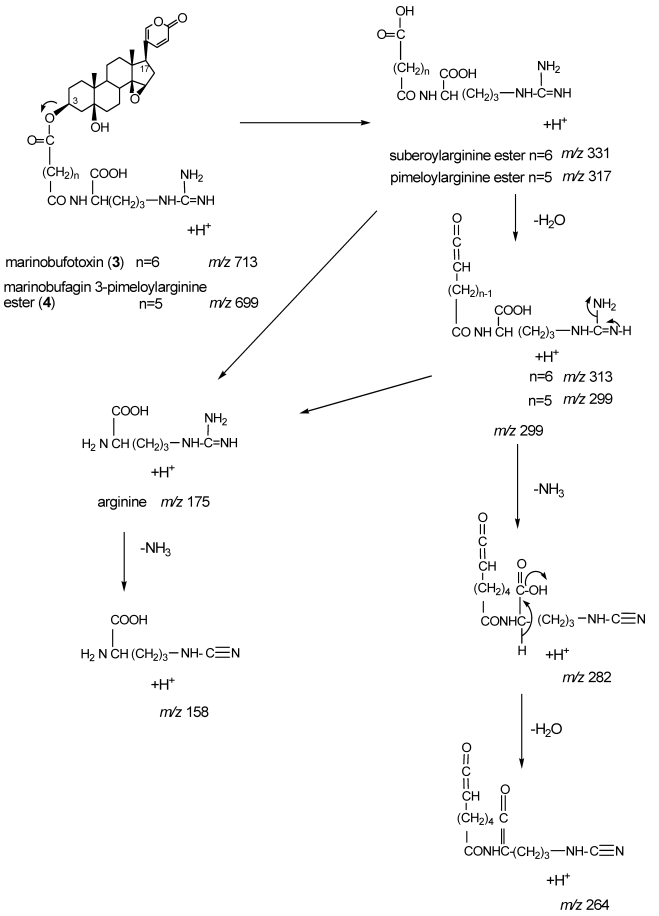

Chart 3. Fragmentation Pathways of Marinobufotoxin (**3**) and Its Homologue (**4**) in MALDI-QIT-TOF-MS^n

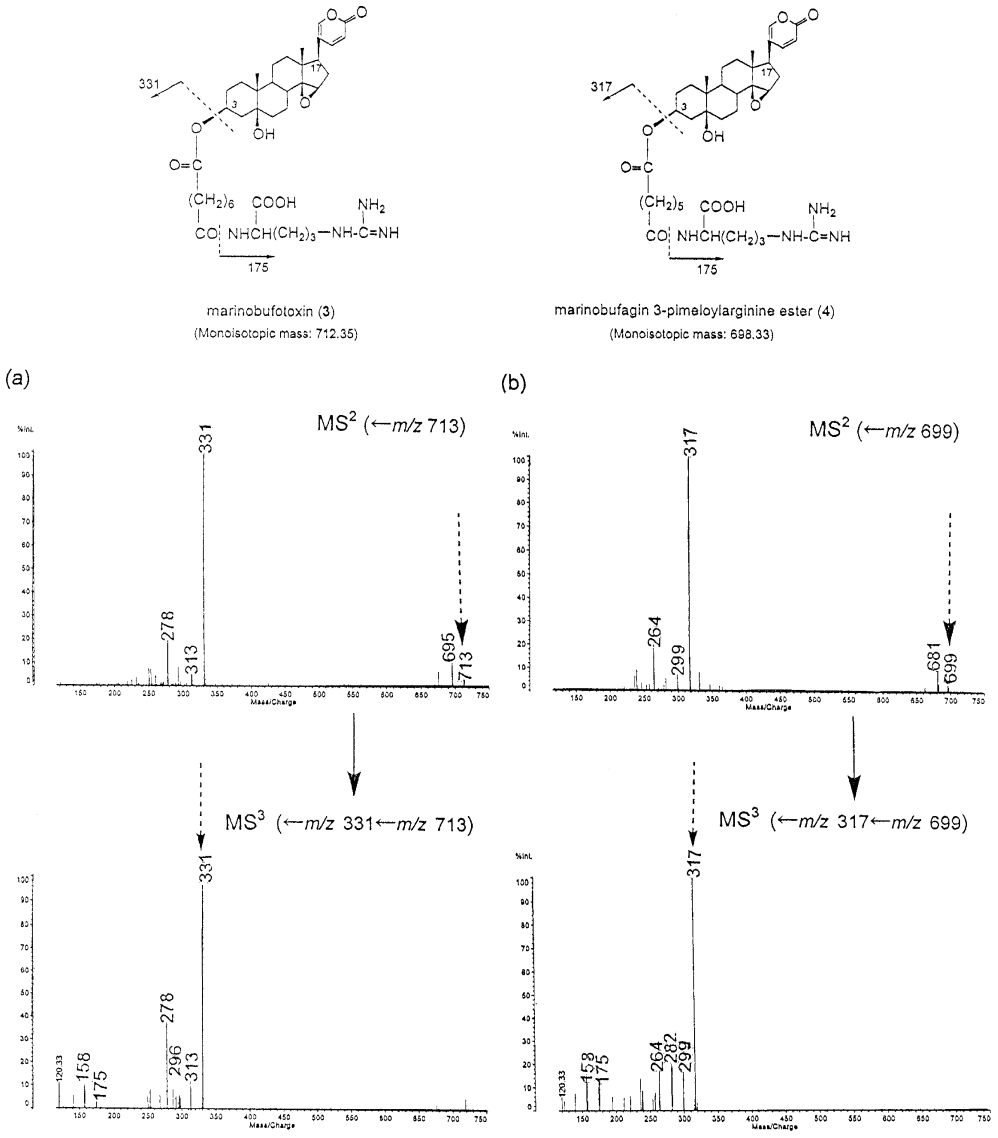

Fig. 4. MALDI-QIT-TOF-MS^n and Proposed Cleavage Parts for Characteristic Fragmentation Ions of (Left) Marinobufotoxin (**3**) and (Right) Its Homologue (**4**)

(a) MS^2 spectrum selecting a precursor ion at *m*/*z* 713 [**3**+H]$^+$; and MS^3 spectrum selecting a precursor ion at *m*/*z* 331 (← *m*/*z* 713). (b) MS^2 spectrum selecting a precursor ion at *m*/*z* 699 [**4**+Na]$^+$; and MS^3 spectrum selecting a precursor ion at *m*/*z* 317 (← *m*/*z* 699).

tained in the ester substituted at position 3 of the steroid, *i.e.*, suberoylarginine ester in **3** or pimeloylarginine ester in **4**, respectively. Thus comparing the mass of the product ion of **3** with that of the product ion of **4** appears useful in confirming cleavage portions. If a difference of 14u between each *m*/*z* value of product ion is observed, it means that the ion includes the acid portion contained in the ester.

As indicated in Table 2, the MS^2 experiment yielded product ions with 14 mass differences: a dehydrated ion at *m*/*z* 695 of **3** or at *m*/*z* 681 of **4**; at *m*/*z* 331 of **3** or at *m*/*z* 317 of **4**; at *m*/*z* 313 of **3** or at *m*/*z* 299 of **4**; and at *m*/*z* 278 of **3** or at *m*/*z* 264 of **4**, appealing to include the acid portion contained in the ester. The product ion at *m*/*z* 331 in **3** or at *m*/*z* 317 in **4** was the most abundant.

To confirm the cleavage portion that would yield the most abundant product ion, a subsequent MS^3 experiment was performed by selecting the ion at *m*/*z* 331 (← *m*/*z* 713 [**3**+H]$^+$) or at *m*/*z* 317 (← *m*/*z* 699 [**4**+H]$^+$) as the precursor ion. As shown in Table 2, the product ions at *m*/*z* 175 and *m*/*z* 158 were observed in both MS^3 spectra and considered to correspond to a common portion, *i.e.*, arginine and subsequent elimination of ammonia may have occurred.

To confirm the structure of the ion at *m*/*z* 313 of **3** or at *m*/*z* 299 of **4**, an MS^3 experiment selecting the ion at *m*/*z* 299 (← *m*/*z* 699 [**4**+H]$^+$) was performed as indicated in Table 2. (Mass spectrum is not shown.) In compound **4**, the product ion at *m*/*z* 282 seems to originate due to the elimination of ammonia from the precursor ion at *m*/*z* 299, and the ion at *m*/*z* 264 is assumed to be derived from subsequent dehydration. Thus the product ion at *m*/*z* 299 of **4** is assumed to originate due to dehydration from the pimeloylarginine ester ion at *m*/*z* 317. Based on the results of investigating product ions obtained in MS$^{2-3}$ experiments, fragmentation pathways, structures of product ions, and cleavage portions are proposed in Chart 3 and Fig. 4.

Fragmentation Pattern in PSD Experiments Using

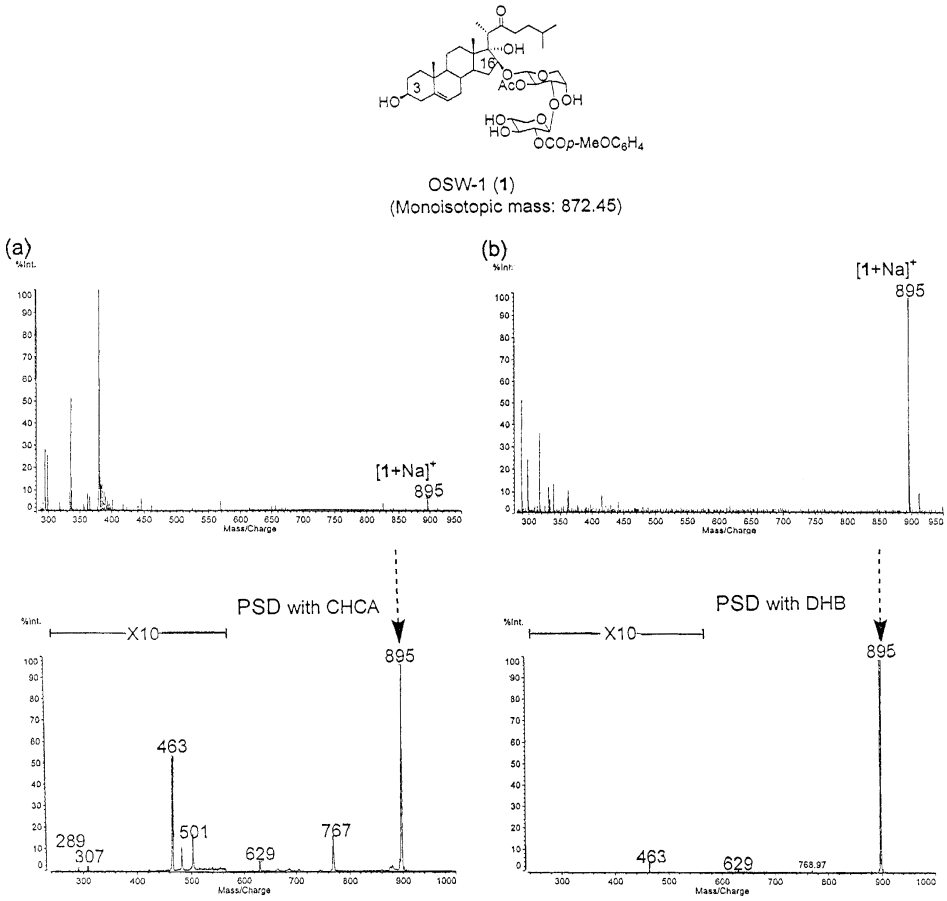

Fig. 5. Mass Spectra of OSW-1 (**1**) in (Upper) MALDI-TOF-MS and (Lower) PSD Experiment Selecting a Precursor Ion at *m/z* 895 $[\mathbf{1}+Na]^+$ Using (a) CHCA Matrix; and (b) DHB Matrix

MALDI-TOF-MS of OSW-1 (1) and Marinobufotoxin (3) Comparing DHB and CHCA Matrices Next, our attention focused on PSD experiments to determine the effects of the matrix on the yield of structurally important product ions. The results were compared with those obtained from the tandem MS^n experiments, as indicated in Tables 1 and 2 (lower). To investigate the effects of the matrix on the generation of ion peaks, we performed MALDI-TOF-MS and subsequent PSD experiments using CHCA or DHB as the matrix.

First, the MALDI-TOF-MS experiment was performed. As shown in Figs. 5 and 6 (upper panels), more fragmentation ions were observed when (a) the CHCA matrix was used compared with (b) the DHB matrix. As CHCA is known to be a "hot" matrix in MALDI and able to increase the internal energy of an ion, fragmentation is assumed to be facilitated during mass measurement. On the other hand, DHB facilitates the formation of molecular-related ions and is known to be a "cool" matrix.

Second, the PSD experiment when each molecular-related ion was selected as a precursor ion was performed. In compound **1**, as indicated in Fig. 5 (lower panel) and Table 1, the MALDI-PSD-MS spectrum using the CHCA matrix gave much more sensitive product ion peaks compared with that using the DHB matrix. In compound **3**, as indicated in Fig. 6 (lower panel) and Table 2, there were no differences in ion species obtained in MALDI-PSD-MS spectra using both the matrices, and the same product ion species were obtained as in the MS^n experiment.

The PSD experiments using the two typical matrices CHCA and DHB did not demonstrate different fragmentation patterns, and there were no differences between product ion species observed in the two spectra. The PSD spectrum measured in the presence of CHCA as a matrix yields more sensitive and abundant product ion peaks than those obtained with DHB.

Conclusions

The MALDI tandem mass spectra (MS^{2-4}) of biologically active steroids, *i.e.*, OSW-1 (**1**) and its analogue (**2**), marinobufotoxin (**3**) and its homologue (**4**), were obtained using MALDI-QIT-TOF-MS^n. Differences occurred in the molecular-related ion species observed in single stage MS. In the case of compounds **1** and **2** sodium-cation adduct $[M+Na]^+$ ions were observed, while in compounds **3** and **4** the protonated molecule $[M+H]^+$ ions were more abundant than $[M+Na]^+$ ions. Tandem mass spectra contain characteristic product ions. In compounds **1** and **2**, product ions derived not only from the sugar moiety but also from aglycone are observed. In compounds **3** and **4**, information on conjugation was observed, and product ions relating to ester contained in conjugation part are produced.

In PSD experiments on compounds **1** and **3**, the relative amount of structurally informative product ions was more abundant when the CHCA matrix was used compared with the DHB matrix.

The results of MALDI-TOF-MS^n experiments can thus

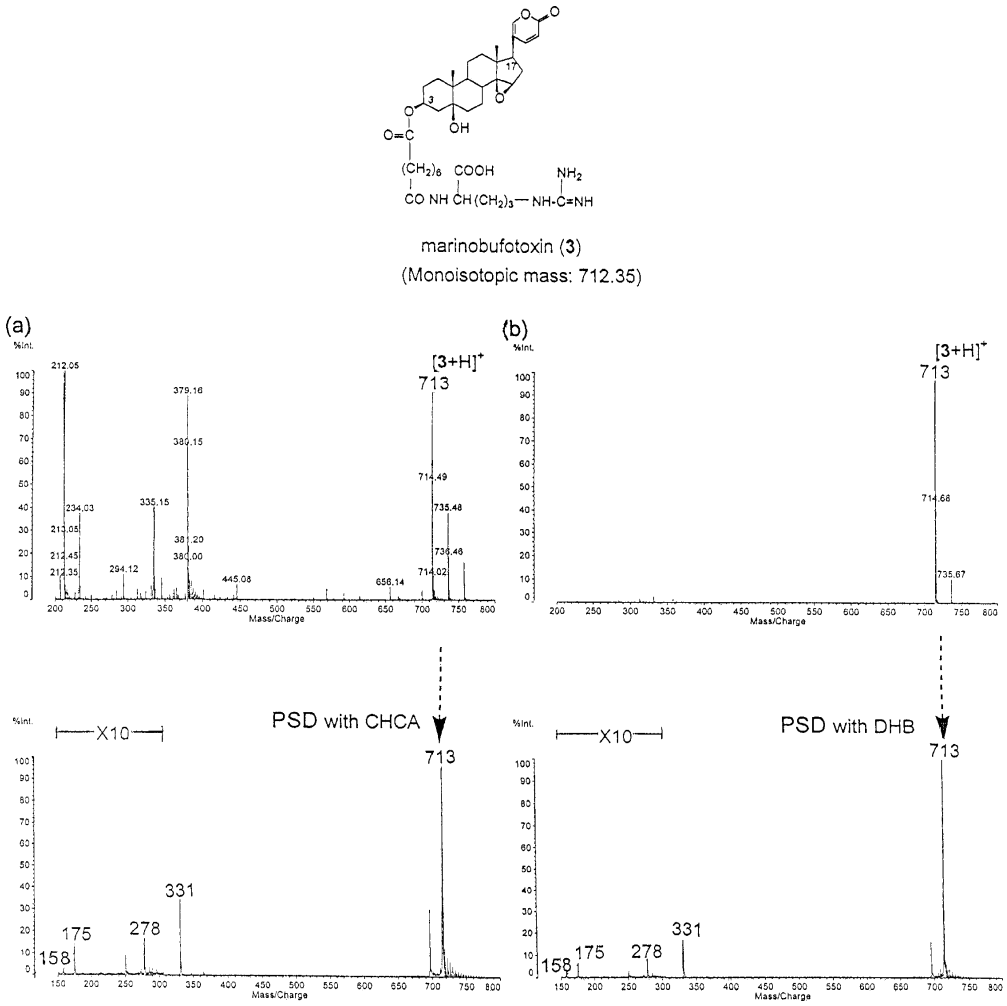

Fig. 6. Mass Spectra of Marinobufotoxin (**3**) in (Upper) MALDI-TOF-MS and (Lower) PSD Experiment Selecting a Precursor Ion at *m*/*z* 713 [**3**+H]$^+$ Using (a) CHCA Matrix; and (b) DHB Matrix

provide information on molecular mass, sugar sequence, or conjugated contents of these efficient steroids.

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