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Conformations of 3-carboxylic esters essential for neurotoxicity in veratrum alkaloids are loosely restricted and fluctuate

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Abstract—The lipid-soluble veratrum alkaloids, veratridine and cevadine, are plant neurotoxins that are agonists of voltage-gated sodium channel. Their conformations in a hydrophobic environment were analyzed by NMR spectroscopy in solution phase chloroform at low temperatures. The conformations around the 3-carboxylic esters which is essential for their neurotoxicity, was completely different from the previously reported X-ray crystallographic structure. The carbonyl oxygen atom (O28) of the carboxylic ester forms a weak intramolecular hydrogen bond with the OH proton at C4 (4-OH) that loosely restricts the conformation of the 3-veratroyl ester in veratridine and the 3-angeloyl ester in cevadine. Methylation at C4 hydroxyl group of veratridine had much reduced its neurotoxic activity relating to voltage-gated sodium channel. The results suggested that the loose conformational restrictions of the carboxylic esters are important for neurotoxicity of the veratrum alkaloids.

1. Introduction

Interactions between small molecules and target proteins have been studied to elucidate their biological functions, and in pharmaceutical chemistry these types of studies are applied to drug design and drug screening. Recently, attention has been focused on the relationships between structural fluctuations and protein functions. Fluctuations can also be important for protein–ligand binding. Biologically active organic molecules have partial flexibility in their structures. For example, the marine natural products ciguatoxins,[1] brevetoxins,[2] and maitotoxins,[3] include seven- to nine-membered rings, and the middle part of these molecules can fluctuate due to those rings. Such structural flexibility is thought to be essential for their toxic activity and for optimal binding to their target proteins.[4,5] Particularly in the case of lipid-soluble natural products binding specifically to target membrane proteins, these types of fluctuations are essential. Molecular motions generally become much slower in biomembranes than in solution, hence specific interactions between ligands and their receptors are statistically less frequent. Thus, it is an excellent strategy for the lipid-soluble neurotoxins to incorporate their own structural flexibility, even though they retain the preferable, but not fixed, conformations required for specific binding to the receptor proteins. These neurotoxins include such contradictory structural properties and thus elegantly solve this biological problem.

Veratridine **1** is one of the neurotoxic veratrum alkaloids isolated from *sabadilla* seed, whose primary targets are voltage-gated sodium channel.[6–8] It has a steroidal backbone 4α,9-epoxycevane substituted by 3,4-dimethoxybenzoate at C-3; in other words, it has a rigid backbone and a highly flexible and freely rotatable ester substitution (Fig. 1). Veratridine is known to bind to and to activate voltage-gated sodium channel, which makes the ion channel open and thereby depolarizes biomembranes.[6–8] A diverse set of lipid-soluble neurotoxins relating to sodium channel has been well studied, including tetrodotoxin, saxitoxin, brevetoxin, batrachotoxin, and veratridine.[9,10] Competitive binding studies with radiolabeled neurotoxin analogues have identified at least eight distinct neurotoxin binding sites, and those specific for veratrum alkaloids, as well as batrachotoxin, aconitin, and grayanotoxin,[9–14] were categorized as 'site-2'.[11–14]

Veratridine **1** and cevadine **2** have similar neurotoxic activities, but veracevine **3** is not toxic.[15] Previous research has shown that the functional groups at C-3,

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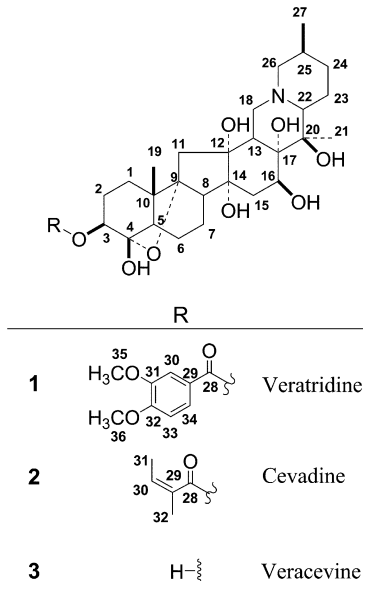


Figure 1. Structures of veratridine **1**, cevadine **2**, and veracevine **3**.

the aromatic and aliphatic carboxylic esters, are essential for the neurotoxicity of veratrum alkaloids.[15,16] These C-3 acyl groups seem to be highly flexible in the veratrum skeleton because the carboxylic esters have three free rotational axes. Considering the selectivity and toxic activity of veratrum alkaloids for targeted sodium channel, the conformations at the C-3 functional groups on the steroidal skeleton are expected to be restricted. However, the monomer structure indicated no conformational restriction of the 3-veratroyl group in the crystal structure previously reported.[17]

In this study, we investigated the conformational restriction and/or flexibility of the 3-carboxylic esters of veratrum alkaloids in chloroform as a representative hydrophobic environment, varying the temperature and using ^{1}H NMR to evaluate changes in the molecular dynamics of the system.

2. Results and discussion

2.1. Hydroxyl group proton signals and refined assignments

All signals in the ^{1}H and ^{13}C NMR spectra of **1**–**3** were re-assigned through DQF-COSY, HSQC, HMBC 2D-

Table 1. Revised Assignments of ^{1}H and ^{13}C NMR chemical shifts of veratridine **1**, cevadine **2** and veracevine **3**

Veratridine			Cevadine			Veracevine		
Position	δ_H	δ_C	Position	δ_H	δ_C	Position	δ_H	δ_C
1	1.62, 1.62	32.6	1	1.55, 1.55	32.6	1	1.49, 1.61	31.9
2[a]	1.73, 2.16	26.7	2[a]	1.53, 1.65	26.7	2	1.47, 1.58	27.7
3	5.10	75.7	3	4.98	75.1	3	3.76	73.5
4	—	105.0	4	—	105.1	4	—	106.4
5	2.07	46.1	5	2.00	46.0	5	2.20	44.1
6[a]	1.73, 1.95	18.9	6[a]	1.73, 1.92	18.8	6	—	18.9
7	1.73, 2.00	16.9	7	1.73, 2.00	16.9	7	1.78, 1.89	17.0
8	2.66	44.7	8	2.66	44.7	8	2.66	44.7
9	—	94.5	9	—	94.4	9	—	94.4
10	—	45.5	10	—	45.5	10	—	45.7
11	1.81, 2.18	42.0	11	1.80, 2.18	42.0	11	1.79, 2.18	42.0
12	—	81.7	12	—	81.7	12	—	81.7
13	2.10	36.9	13	2.10	36.9	13	2.10	36.9
14	—	80.3	14	—	80.3	14	—	80.3
15	1.75, 1.89	31.1	15	1.76, 1.90	31.0	15	1.75, 1.91	31.0
16	4.10	70.9	16	4.10	70.9	16	4.12	70.9
17	—	72.0	17	—	72.0	17	—	72.0
18	2.61	51.3	18	2.61	51.3	18	2.60	51.3
19	0.99	18.9	19	0.98	19.0	19	0.96	19.1
20	—	75.7	20	—	75.7	20	—	75.7
21	1.11	15.4	21	1.11	15.4	21	1.13	15.4
22	2.45	63.5	22	2.46	63.4	22	2.45	63.4
23[a]	1.40, 1.55	18.3	23[a]	1.40, 1.55	18.3	23	1.40, 1.55	18.3
24[a]	1.55, 1.55	29.0	24[a]	1.55, 1.55	29.0	24	1.55, 1.55	29.0
25	1.88	27.4	25	1.88	27.4	25	1.88	27.4
26	2.39, 2.66	61.2	26	2.39, 2.66	61.2	26	2.40, 2.65	61.2
27	1.06	17.0	27	1.06	17.0	27	1.07	17.1
28	—	167.1	28	—	168.9			
29	—	122.2	29	—	127.3			
30	7.48	112.4	30	6.11	139.7			
31	—	148.8	31	1.97	16.0			
32	—	153.5	32	1.88	20.6			
33	6.84	110.3						
34	7.61	123.8						
35	3.88	56.0						
36	3.90	56.0						

[a] The assignments labeled by asterisks were re-assigned to different signals from those in a previous report.[18]

NMR techniques, and a few assignments were revised as shown in Table 1. The mixture of veratrum alkaloids was measured directly in the previous report,[18] but purified samples were used in this study. The samples and solutions were prepared carefully as described in Section 4.

Hydroxyl group (OH) proton signals were observed in the ^{1}H NMR spectra of **1**–**3** in deuterated chloroform, in addition to the C–H proton signals (Fig. 2). Generally, OH and NH proton signals in deuterated chloroform can exchange easily and rapidly to contaminating deuterium in the solution, and therefore their signals disappear. When the OH and NH groups form hydrogen bonds, their signals can be detected. Thus, the observed OH signals indicate the formation of hydrogen bonds.

The OH proton signals showed temperature dependency; their chemical shifts migrated to the upfield, as seen in measurements at higher temperatures (Figs. 2 and 3). No migration of chemical shifts and no signal broadening were observed for the C–H proton signals under the same conditions. None of the proton signals of **1**–**3** depended on the concentrations (data not shown), indicating the sample molecules are monomeric. Thus, all the hydrogen bonds in these molecules **1**–**3** are intramolecular, rather than intermolecular.

2.2. β-Hydroxyl group (4-OH) neighboring the C-3 carboxylic ester

The four OH proton signals assigned to 12-OH, 14-OH, 16-OH, and 17-OH were observed commonly among **1**–

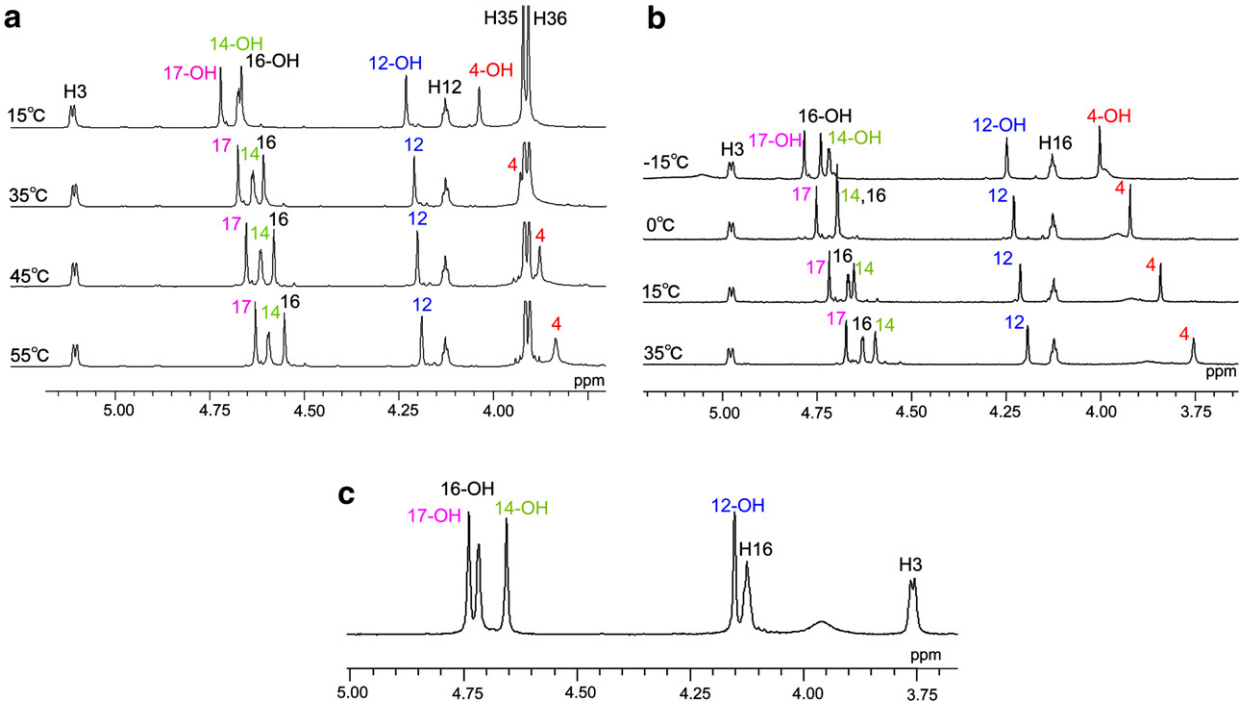


Figure 2. OH signal region spectra of veratridine **1** (a), cevadine **2** (b), and veracevine **3** (c). The spectrum of **3** was acquired at 35 °C.

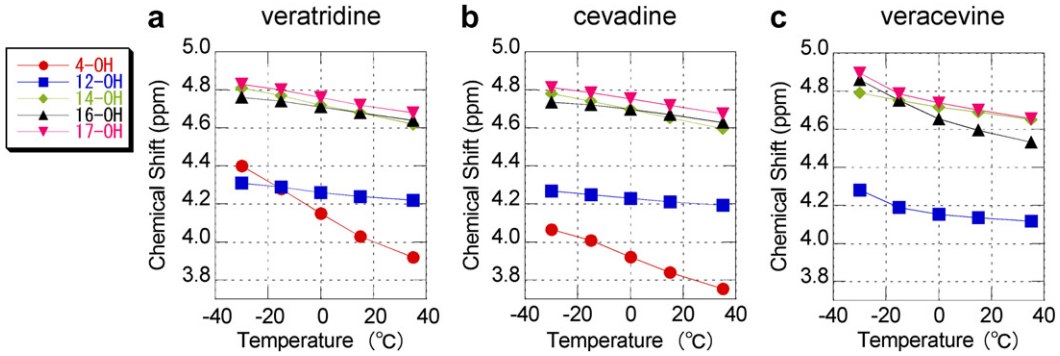


Figure 3. OH Signal migrations in the ^{1}H NMR spectra of veratridine (a), cevadine (b), and veracevine (c) at low temperatures. The chemical shifts are plotted for different temperatures at 35, 15, 0, −15, and −30 °C. The red circles are the C-4 hydroxy group proton signal (4-OH), the blue squares are the C-12 hydroxy group proton signal (12-OH), the light green diamonds are the C-14 OH proton signal (14-OH), the black triangles are the C-16 OH proton signal (16-OH), and the magenta reversed-triangles are the C-17 OH proton signal (17-OH).

3 (Figs. 2 and 3). The 4-OH proton signals of **1** and **2** were detected, in contrast, the 4-OH proton signal of **3** was not. The shape of the 4-OH proton signal changes most significantly. The 4-OH signal of **1** begun to broaden at 55 °C, that of **2** broadened at 35 °C as the measurement temperatures were elevated (Fig. 2). The chemical shift of 4-OH proton migrates further upfield than the other four OH proton signals at higher temperatures (Fig. 3). The OH proton signal migrations on the 12-OH, 14-OH, 16-OH, and 17-OH are gentle and of similar magnitude. These results suggest that the hydrogen bond of 4-OH is weaker and the proton exchange rate is faster than the others, and that the hydrogen bonding at 4-OH becomes strong and the exchange rate gets slower as the temperature decreases. Since the other four OH group signal shapes do not change, their hydrogen bonds must be quite stable. As described later, the hydrogen bond network in steroidal backbone included these hydroxyl groups at C12, C14, C17, and C16.

2.3. Hydrogen bond networks

The hydrogen bonding in the crystal structure of **1** was reported.[17] The hydroxyl groups at C-12, C-14, C-17, and C-20 form intramolecular hydrogen bonds both in liquid phase and crystal structure. The steroidal backbone conformation was stabilized by extensive intramolecular hydrogen bonds. The suggested hydrogen bond networks from the NMR data and that of the crystal structure are shown in Table 2. The hydrogen bond networks of 12-OH, 14-OH, 17-OH, and 20-OH were similar among the three veratrum alkaloids **1**–**3**. The difference between the liquid phase structure and the crystal structure is the hydrogen bond including 4-OH. The 4-OH in the liquid phase forms an intramolecular hydrogen bond, in contrast, that in the crystal structure forms an intermolecular one.

The 4-OH signal was observed in the spectra of **1** and **2**, but that was not in the spectrum of **3** (Figs. 2 and 3) as described above. The steroidal backbone is common among these three compounds (Fig. 1), but only **3** does not have the carboxylic ester. Therefore, the acceptor of the 4-OH proton is suggested to be the carbonyl oxygen.

The neighboring ester oxygen atom O3 could possibly accept a hydrogen bond with 4-OH by forming five-membered ring. If the 4-OH hydroxyl group formed a hydrogen bond to the neighboring O3 ester oxygen, they could bind each other in compound **3** without the 3-acyl substitution at the hydroxyl group. However, the 4-OH signal disappeared in the spectrum of **3** (Fig. 2), indicating that the oxygen atom of the neighboring hydroxyl group at C-3 (O3) is not an acceptor of the hydrogen atom in 4-OH. In addition, these vicinal hydroxyl groups (O3 and 4-OH) are not in the same plane, although they are both β-orientations, so it is difficult to form a hydrogen bond between them. Consequently, in veratridine and cevadine, the carbonyl oxygen atom O28 forms an intramolecular hydrogen bond with the 4-OH hydrogen atom. The structure of veratridine **1** was calculated with the Gaussian HF/3-21G(d) level of theory based on all intramolecular hydrogen bonds indicated by the NMR experiments as shown in Figure 4.

Table 2. Hydrogen bonding network

NMR	Crystal structure[b]
[a]O_{16}–H_{16}···O_{20}	O_{20}–H_{20}···O_{16}
O_{17}–H_{17}···O_{12}	O_{17}–H_{17}···O_{12}
O_{12}–H_{12}···O_{14}	O_{12}–H_{12}···O_{14}
O_{14}–H_{14}···O_{49}	O_{14}–H_{14}···O_{49}

[a] The difference between liquid phase and crystal structure.
[b] Ref. 17.

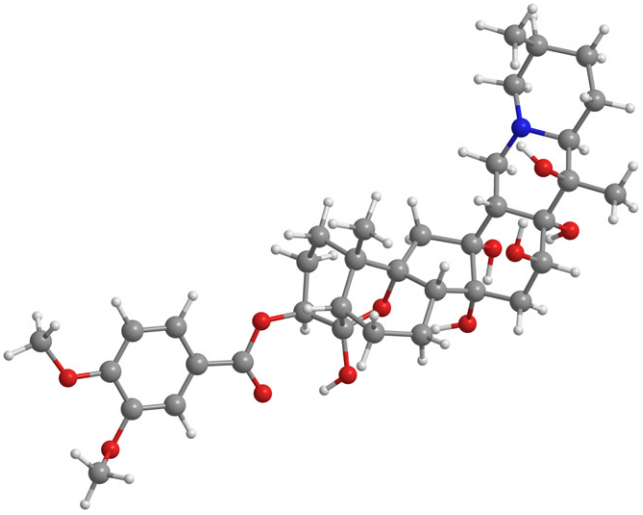


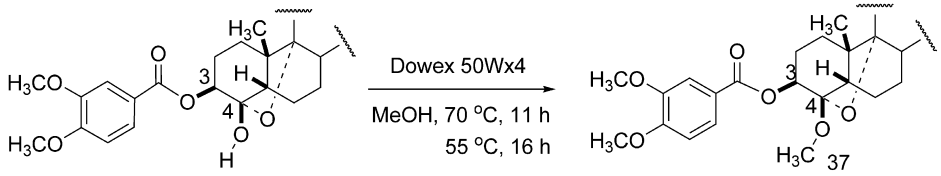
Figure 4. Structure of veratridine in solution phase calculated at the HF/3-21G(d) level of theory.

2.4. Hydrogen bond around 3-carboxylic esters and their toxic activity

Considering the necessity of 3-carboxylic ester for veratridine neurotoxicity, it is significant that the acceptor of the hydrogen bond of the 4-OH is the carbonyl oxygen atom O28 in the 3-veratroyl ester, rather than the ester oxygen atom (O3). If the hydrogen bonding acceptor was the O3 ester oxygen, there would be no factors restricting the rotation at the 3-veratroyl ester. The hydrogen bond formed between the 4-OH and the carbonyl oxygen atom O28 enables to restrict the conformation of the 3-veratroyl group to the steroidal backbone as shown in Figure 4. Moreover, this hydrogen bond is not strong, and the 3-veratroyl group conformation is loosely restricted rather than rigid, as described above. These structural features and properties are suggested to be important for neurotoxicity, that is, the structurally fixed steroidal backbone and the fluctuating 3-veratroyl group having a specific but loosely restricted conformation. This is an excellent strategy that allows the lipid-soluble neurotoxins to effectively target and specifically bind their target membrane proteins. Such strategies for binding membrane proteins are thought to be one of the common matters among the neurotoxins.

2.5. 4-Methoxy veratridine and its neurotoxicity

A previous structure-activity relationship study showed 4,16-diacetyl veratridine and 3α-epiveratridine had no



Scheme 1. Preparation of 4-methoxy veratridine. 4-OH of veratridine was methylated using cation exchange resin in methanol.

toxicity against the housefly.[16] The acetylation at the C-4 hydroxyl group in the former compound and the α-orientation of the 3-carboxylic ester in the latter compound prevent the formation of an intramolecular hydrogen bond between 4-OH and the carbonyl oxygen atom (4-OH::::O28). These results suggest that the 4-OH is important for veratridine neurotoxic activity and that, moreover, the conformational restriction of the 3-carboxylic esters in the veratrum alkaloids with an intramolecular hydrogen bond to 4-OH is important for their neurotoxicity. Here, we investigated the toxic activity of 4-methoxy veratridine to prove the importance of intramolecular hydrogen bonding for veratridine neurotoxicity, because that hydrogen bond cannot form in 4-methoxy veratridine. 4-methoxy veratridine was prepared from veratridine using a cation exchange resin and methanol (Scheme 1).[19]

To investigate toxic activity of 4-methoxy veratridine relating to voltage-gated sodium channel, a newly devised toxicity assay system was used.[20] A neuroblastoma cell expressing voltage-gated sodium channel (N18-RE-105 cell) was used in this assay system.[21] In general, the intracellular accumulation of sodium ion leads to cell apoptosis.[22] In the neuroblastoma cells, a channel opener such as veratridine can induce apoptosis in the presence of an inhibitor of Na^+/K^+ ATPase such as ouabain, because the intracellular Na^+ concentration is usually stabilized by the plasma membrane Na^+/K^+ ATPase (see the overview of the assay system in Supporting information 1).

Cell viability against 4-methoxy veratridine was much higher than that against veratridine at each concentration (Fig. 5), suggesting that the activity of 4-methoxy veratridine was effectively reduced by the methylation. Therefore, 4-OH is essential for veratridine neurotoxicity relating to voltage-gated sodium channel.

3. Conclusions

The neurotoxicity of veratrum alkaloids is well known to depend on the chemical structures of 3-carboxylic esters from the structure-activity relationship research. Nevertheless, their conformations have not been discussed because there was no useful structure information. In this study, hydrogen bond analyses of veratrum alkaloids in liquid phase using ^1H NMR spectroscopy indicate the intramolecular hydrogen bond around the 3-carboxylic ester, which cannot be observed in the X-ray crystal structure. The intramolecular hydrogen bond between 4-OH and 3-carbonyl oxygen atom restricts the conformations of 3-veratroyl ester in veratridine and 3-angeloyl ester in cevadine. It should be difficult that these hydrogen bonding restrictions and conformations were predicted computationally based on their crystal structures. The neurotoxicity relating to sodium channel was effectively reduced in 4-methoxy veratridine that does not have the hydrogen bond around 3-veratroyl ester. Therefore, the intramolecular hydrogen bond around 3-carboxylic ester is suggested to be essential for the neurotoxicity of veratrum alkaloids.

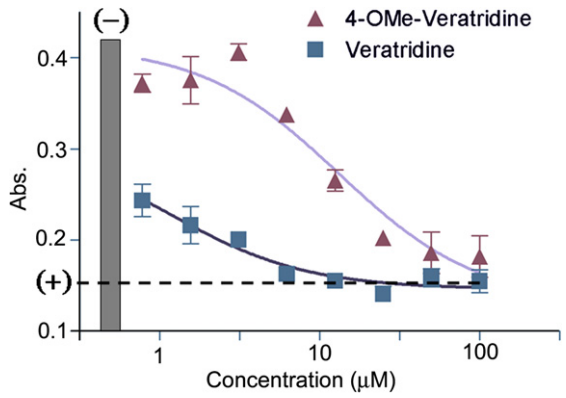


Figure 5. Selective activity for voltage-gated sodium channel in the neuroblastoma N18-RE-105 hybrid cell. The value of absorbance at 570 nm caused by formazan, reduced compound from 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, is correlated with cell viability. The cytotoxicity indicated the selective activation activity for sodium channel in this assay system. The gray bar labeled with (—) on the left side is the negative control in which only 1% (v/v, final concentration) ethanol solution was added to the cell. The dotted line drawn over 0.15 (abs 570 nm) is the positive control in which the 1.7 mM adriamysin (final concentration) was added. Curve approximation was performed using a Graph Pad Prism® sigmoidal dose–response program.

To investigate the structural common features of the agonists of sodium channel in their pharmacological activity, the structure superposition study was performed in the crystal structure of batrachotoxine and veratridine,[17] however, the superposed veratridine structure was limited in the steroidal backbone because, as one of the possibilities, the restriction of 3-carbonyl ester was not observed in the crystal structure of veratridine monomer. In these studies, the conformation study including the weak and labile restrictions or fluctuations becomes more important to understand the mechanisms how those pharmacologically important agonists and antagonists active to their receptors.

Interestingly, the intramolecular hydrogen bond of 3-carboxylic ester is weaker than those of steroidal back-

Table 3. Assignments of ^{1}H and ^{13}C NMR chemical shifts of 4-methoxy veratridine

Position	δ_H	δ_C
1	1.62, 1.62	32.6
2	1.73, 2.16	26.7
3	5.58	67.3
4	—	107.1
5	2.29	46.8
6	1.73, 1.95	19.2
7	1.73, 2.00	16.9
8	2.66	44.7
9	—	95.6
10	—	45.5
11	1.81, 2.18	42.0
12	—	81.7
13	2.10	36.9
14	—	80.3
15	1.75,1.89	30.9
16	4.14	70.9
17	—	72.0
18	2.61	51.3
19	0.99	19.2
20	—	75.7
21	1.11	15.4
22	2.46	63.4
23	1.40, 1.55	18.3
24	1.55, 1.55	29.0
25	1.88	27.4
26	2.39, 2.66	61.2
27	1.06	17.0
28	—	165.3
29	—	122.6
30	7.50	112.2
31	—	148.8
32	—	152.5
33	6.87	110.3
34	1.88	123.6
35	3.91	56.0
36	3.92	56.0
37	3.37	50.5

bone, that is, the conformations of 3-carboxylic ester are loosely restricted, not fixed in the molecules. 'his flexibility is thought to be essential to recognize the receptors in the ligand–receptor interactions. Recently, the structure fluctuations of proteins as the receptors were studied well, but those of the organic small molecules as the ligands were not so much. These concepts in the molecular fluctuations are excellent strategy to recognize the receptor and/or ligand to each other specifically and rapidly. These ideas supported by our study will be very useful and helpful for drug design, and understanding the small molecule interactions to their receptors in biomembrane.

4. Experimental

4.1. Samples

Veratridine (>90% purity at TLC) was purchased from Sigma–Aldrich, Japan, and was used without any further purification.

Cevadine was isolated from veratrine hydrochloride, a mixture of veratrum alkaloids (Sigma–Aldrich, Japan) by using reversed-phase HPLC (JASCO HPLC System, JASCO Corp., Hachioji, Japan). The effluents were detected by UV absorbance at 245 nm. The ^{1}H and ^{13}C NMR signal assignments are summarized in Table 1. The detected mass is $[M+H]^+$ 592.4 in MALDI-TOF-MS (AXIMA-CFRs MALDI TOF mass spectrometer, Shimadzu, Co., Kyoto, Japan).

Veracevine was prepared from veratridine by alkaline hydrolysis.[23] Veratridine (3.8 mg) in 100 μL of methanol was treated with 4 μL of 5 N aqueous NaOH and stirred at 0 °C for 24 h. The solution was diluted with 200 μL of ice water and extracted 10 times with 20 μL portions of chloroform. The extract was washed with 40 μL of water, evaporated, and applied to normal phase silica gel column chromatography, and then it was applied to reversed-phase HPLC. The effluent was monitored on an ISI RI-981 refractive index (LabSystem Instruments Ltd., Ome, Japan). The purified veracevine yield was 2.1 mg (80%). The ^{1}H and ^{13}C NMR signal assignments are summarized in Table 1. The detected mass was $[M+H]^+$ 510.2 in MALDI-TOFMS.

4.2. NMR and signal assignments

All NMR data were acquired on a JEOL ECA500 instrument (^{1}H, 500 MHz; JEOL Ltd., Tokyo, Japan) with Nanolac Probe and JEOL ECX400 instrument (^{13}C, 100 MHz; JEOL Ltd., Tokyo, Japan) with TH5 Probe in deuterium chloroform (Sigma, Japan). The chemical shifts were calibrated from the solvent signal $CHCl_3$ (δ_H 7.24, δ_C 77.0).

The ^{1}H NMR signals of **1**–**3** were assigned, and in particular the OH groups were assigned with HMBC. ^{1}H NMR signals depend on pH values for alkaloids, and we carefully prepared samples to maintain their reproducibility throughout our protocol, to avoid causing spectral changes by protonation of the amine in acid purification conditions; the sample dissolved in chloroform was washed in a 1-N sodium hydrate aqueous solution, evaporated, and freeze dried.

Two milligrams of purified cevadine and veracevine, and 10 mg of veratridine, were dissolved in 550 μL of $CDCl_3$, respectively. ^{1}H NMR spectra were recorded from −30 to 55 °C.

4.3. 4-Methoxy veratridine[19]

Veratridine (4.7 mg) in 2 mL of MeOH was stirred with 53.2 mg of cation-exchange resin Dowex 50Wx4 (Sigma–Aldrich, Japan) and heated to reflux for 11 h and subsequently at 55 °C for 16 h. The solution was filtered, washed with 1 N HCl/MeOH, 1:1, evaporated, and purified using reversed-phase HPLC (effluent monitored at UV 260 nm) to yield 654 μg (14%) of 4-methoxy veratridine. The ^{1}H and ^{13}C NMR signal assignments are summarized in Table 3. The 4-methoxy group was determined by an HMBC signal between C4 and H37. A high-resolution Mass (JEOL JMS-SX102 FAB mass spectrometer, JEOL Ltd., Japan) was found at $[M+H]^+$ 688.3667 for $C_{37}H_{54}NO_{11}$ (−3.0 mmu).

4.4. Cell culture

Neuronal mouse-rat hybrid cells N18-RE-105 (IFO50221, subdivided from JCRB Cell Bank, Japan) were cultured at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (Sigma–Aldrich, Japan) containing 1% HAT media supplement (the final reagents' concentrations are 100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine; Sigma–Aldrich, Japan), 5% penicillin/streptomycin (P/S, Sigma–Aldrich, Japan) and 5% fetal bovine serum (Sigma–Aldrich, Japan). Passage of cells was performed by pipetting. Experiments were carried out 2–3 days after seeding.

4.5. Voltage-gated sodium channel specific activity assay[16]

One hundred microliters of cell suspensions (100,000 cells/mL) in the medium was seeded in each well of a 96-well cell culture plate and incubated for 24 h. Cell culture medium (100 μL) containing 1 mM ouabain (final 500 μM, Sigma–Aldrich, Japan) and various concentration of veratridine or 4-methoxy veratridine (final 0.781, 1.56, 3.13, 6.25, 12.5, 25.0, 50.0, and 100 μM each) in ethanol (solvent max 1%), or an aqueous solution of adriamysin (final 1.7 mM, Sigma–Aldrich, Japan) as a positive control or ethanol (final 1%) as a negative control were added to each well and incubated for 48 h. 50 μL of aqueous 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/mL, Sigma–Aldrich, Japan) was added and incubated at 37 °C for 4 h. The culture supernatant was then decanted and 100 μL of dimethylsulfoxide was added as a solvent. The absorbance was measured at 570 nm subsequently using a Thermo Varioskan Flash micro plate reader (Thermo Fisher Scientific K.K., Japan).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.12.037.

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