JOURNAL OF NATURAL PRODUCTS

Article

pubs.acs.org/jnp

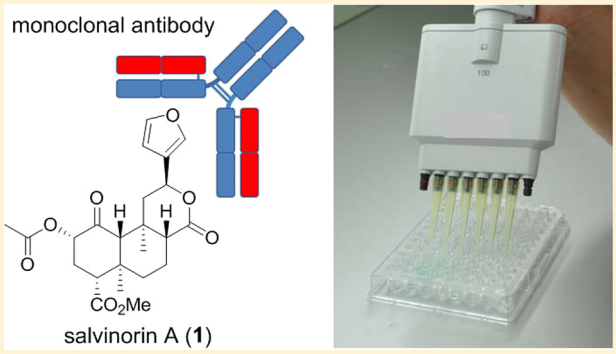
Development of an Enzyme Immunoassay Using a Monoclonal Antibody against the Psychoactive Diterpenoid Salvinorin A

Madan Kumar Paudel,[†] Osamu Shirota,[‡] Kaori Sasaki-Tabata,[†] Hiroyuki Tanaka,*[,†] Setsuko Sekita,[‡] and Satoshi Morimoto[†]

[†]Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

[‡]Laboratory of Pharmacognosy and Natural Products Chemistry, Kagawa School of Pharmaceutical Sciences, Tokushima Bunri University, Sanuki, Kagawa 769-2193, Japan

ABSTRACT: Salvinorin A (**1**), the main active constituent in *Salvia divinorum*, is a highly selective kappa-opioid receptor agonist with hallucinogenic effects, which is regulated in several countries. In the present study, a monoclonal antibody (mAb) against **1** was prepared, and an indirect competitive enzyme-linked immunosorbent assay (icELISA) system was developed for the detection of salvinorins. To raise mAbs against **1**, salvinorin B (**2**) hemisuccinate was synthesized and used to prepare the immunogen **2**–bovine serum albumin conjugate. This technique was used to prepare a hybridoma cell line, 3D5, which secreted a mAb that recognized **1**. The mAb was shown to have specificity for **1** and other salvinorins in cross-reactivity tests. The intra-assay calibration range by icELISA using the mAb against **1** was 0.0195–0.625 μg/mL. After validating the icELISA using intra- and interassays, a recovery experiment and analysis of several plants in the family Lamiaceae, including *S. divinorum*, confirmed that the analytical method based on ELISA is not only simple but also precise, accurate, sensitive, and sufficiently reliable. The results indicate that icELISA is a useful tool in the identification of *S. divinorum*.



Salvia divinorum Epling & Játiva-M. is a powerful psychoactive herb that belongs to the Lamiaceae (mint) family and is used in traditional spiritual and curative treatments by the indigenous Mazatec people of southern Mexico. Recently, this plant and its extracts have been used recreationally, which has led to social problems in the United States, Europe, Japan, and elsewhere. Thus, the sale of *S. divinorum* has been prohibited in several countries because of its psychoactive effects.

Salvinorin A (**1**) is a neoclerodane diterpene and an extremely potent and highly selective kappa-opioid receptor agonist.[1−4] It is the main active constituent isolated from the leaves of *S. divinorum*, while the structurally related salvinorins B (**2**), C (**3**), D (**4**), E (**5**), and F (**6**) are present in lower quantities.[5,6] At present, only **1** is known to be an effective kappa-opioid receptor agonist. It is capable of inhibiting excess intestinal motility (e.g., diarrhea), and its plant of origin is used in traditional healing practices for treating pain, inflammatory disorders, rheumatism, and headache.[7] In addition, it is an attractive substance for drug development because kappa-opioid receptors are potential targets for obesity, depression, anxiety, and alcoholism and can normalize cognitive and behavioral alterations.[8−14]

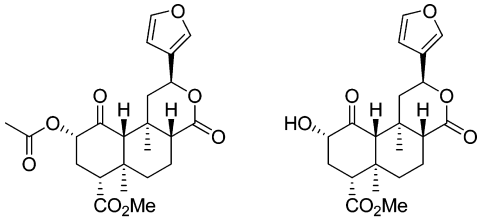
In humans, an inhaled dosage of 200–500 μg of **1** induces profound hallucinations that last up to 1 h, which include feelings of physical or mental displacement, exceptionally convincing illusions, and a loss of identity. Its potency is slightly inferior to that of lysergic acid diethylamide, the well-known synthetic hallucinogen.[15−17] Several recent studies have suggested the involvement of the endocannabinoid system in some of its effects.[18]

Analytical approaches for **1** include thin-layer chromatography and gas chromatography–mass spectrometry (GC-MS).[19−21] Additionally, high-performance liquid chromatography–mass spectrometry (HPLC-MS) was reported to be applicable for the quantitative analysis of plant materials and biological samples from consumers.[22,23] In contrast, immunochemical approaches have been used largely for the analyses of natural products, including drugs of abuse, such as morphine and cannabinoids.[24−34] The practicality and usefulness of the enzyme-linked immunosorbent assay (ELISA), immunochromatographic analysis, and eastern blotting have been validated in various experiments. To facilitate the development of an immunoassay for **1**, a polyclonal antibody against **1** has been prepared and made available by Randox Laboratories Ltd. (U.K.). However, the preparation of a monoclonal antibody (mAb) against **1** has not been reported previously.

Received: May 1, 2013

dx.doi.org/10.1021/np400358n | *J. Nat. Prod.* XXXX, XXX, XXX−XXX

Thus, the aim of the present study was to prepare a mAb against **1** and to develop an indirect competitive ELISA (icELISA) that could be used to identify *S. divinorum*.



RESULTS AND DISCUSSION

Synthesis of 2−Bovine Serum Albumin Conjugate (2−BSA) and Direct Determination of the Hapten−Carrier Protein Conjugates by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOFMS). Salvinorin A (**1**) lacks immunogenicity and does not possess ether a COOH or NH_2 group in its molecule. Thus, **1** had to be modified to prepare an appropriate hapten. This compound was converted to **2** (salvinorin B), which possesses an OH group and is able to be modified to a hemisuccinate after its reaction with succinic anhydride. Then, **2**-hemisuccinate was prepared successfully and conjugated with BSA as a carrier protein using the carbodiimide method (Figure 1). A conjugate with an adequate hapten number is essential for eliciting an immune response. Thus, the analysis of the **2**−BSA conjugate by MALDITOFMS, which is useful for the precise determination of the hapten number of a conjugate, was performed as previously reported.[35,36] The broad peak of **2**−BSA in the MALDITOFMS is shown in Figure 2. This revealed that there were **2**−BSA conjugates with different hapten numbers in the sample. On the basis of the peak around *m*/*z* 75 901 and the molecular weights of BSA and **2** (66 433 and 390, respectively), 24 molecules of **2** were conjugated to BSA in this particular conjugate. MALDITOFMS analysis indicated that the conjugates contained sufficient hapten numbers and could elicit adequate immunogenicity to raise antibodies against **1**. In addition, the broad peak of **2**−human serum albumin (**2**−HSA), which was used as a solid-phase antigen in ELISA, was also observed in the MALDITOFMS spectrum, and its hapten number was calculated as 25, similar to that of **2**−BSA.

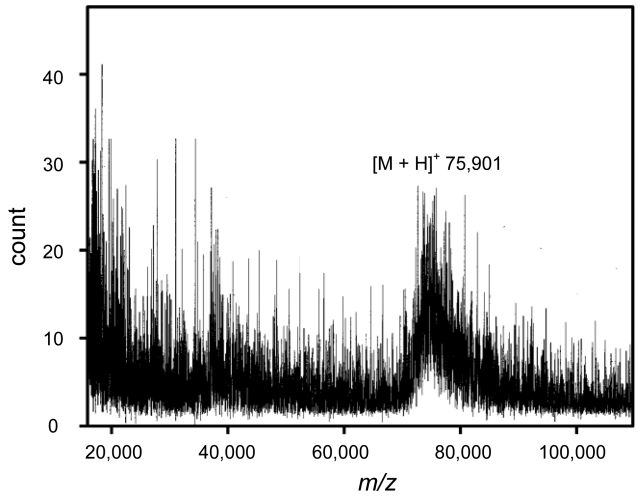


Figure 2. Direct determination of the hapten number of salvinorin B (**2**)−bovine serum albumin (**2**−BSA) conjugates by MALDITOFMS.

Production and Characteristics of mAbs against Salvinorin A (1). BALB/c mice were hyperimmunized with **2**−BSA to generate the cell clones used for this study, and splenocytes were obtained from these mice. These splenocytes were fused with SP2/0 myeloma cells using a previously published procedure.[37] After fusion, the cells were cultured in medium containing hypoxanthine, aminopterin, and thymidine to obtain hybridomas. Next, antibodies against **1** were screened using the supernatants from each well of the cell culture plates, by indirect ELISA and icELISA. Finally, a hybridoma cell that secreted a mAb against **1** was picked from a positive well and cloned. A hybridoma cell line that produced a mAb reactive to **1** (referred to as 3D5) was obtained and classified under the IgG2b category that possesses κ light chains.

Development of an Indirect Competitive Enzyme-Linked Immunosorbent Assay (icELISA) Using 3D5. To develop the

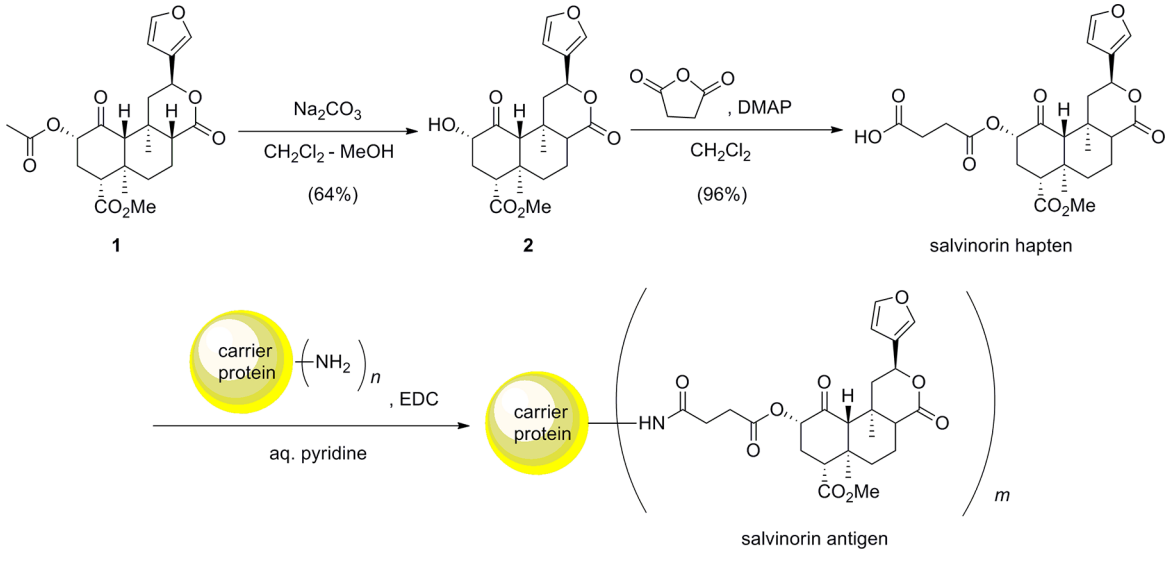


Figure 1. Synthetic scheme for salvinorin B (**2**)−carrier protein conjugates.

dx.doi.org/10.1021/np400358n | J. Nat. Prod. XXXX, XXX, XXX−XXX

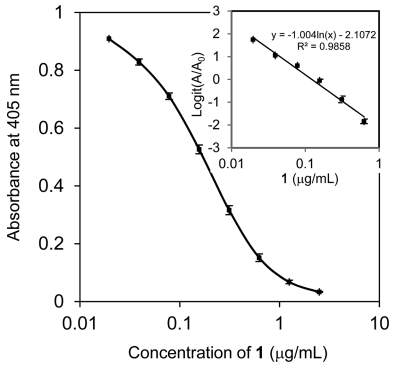


Figure 3. Standard and calibration curves for salvinorin A (**1**) based on icELISA using 3D5. The concentrations of 3D5 and the salvinorin B (**2**)−human serum albumin (**2**−HSA) coating conjugate were 333 ng/mL and 0.1 μg/mL, respectively.

icELISA, the optimal concentration of 3D5 was determined by indirect ELISA. Various concentrations of 3D5 were examined using a polystyrene microimmunoplate that was precoated with **2**−HSA. The level of antibody that bound to the **2**−HSA conjugate was calculated by direct comparison with the level of labeled peroxidase with the use of a secondary antibody. Color development was observed after adding 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) solution to the plate. Thus, the optimum concentration of 3D5 for icELISA was found to be 333 ng/mL, which yielded an absorbance of approximately 0.8−1.0 at 405 nm.

Under the conditions defined, the icELISA was used to characterize 3D5. During the initial antibody application step a 2-fold serial dilution of **1** was added to the microimmunoplate, which competed with the binding of 3D5 to **2**−HSA. Following treatment with a secondary antibody and addition of ABTS to the plate, the gradation of color development was distinguishable and depended on an increase in the concentration of **1** to form the standard curve, as shown in Figure 3. To assess the affinity of 3D5, an icELISA as described by Friguet et al. was used to estimate the dissociation constant (K_D) of the mAb against that of **1**, which was 5.1×10^{-7} M according to typical Scatchard plots.[38]

The specificity of the mAb was determined on the basis of its cross-reactivity (CR) with other compounds related to **1** in the icELISA, which was calculated using the equation developed by Weiler and Zenk.[39] Figure 4 shows the CRs of the mAb against **1** and several structurally related compounds and shows that it recognized **1** and other salvinorins. Compared with **1** (CR = 100%), the CRs for **2**−**6** were <0.084%, 26.2%, 9.79%, 9.33%, and 10.3%, respectively. In addition, four types of synthesized salvinorin derivatives with different side chains at the C-2 position were also examined for this characterization, as listed in Table 1. It was found that 3D5 could react with all of the additional compounds, except benzoyl-salvinorin (**14**). Considering their structural similarity to **2**-hemisuccinate, which was used as the immunogen, the specificity may be affected by the presence of the succinyl group at C-2 in the target molecule. On the basis of the low observed CRs of 3D5 against salvidivins, the furan ring at C-12 plays an essential role in this antigen and its interactions. In addition, the mAb may recognize the main characteristic structure of salvinorins precisely, because the CRs for C-8-epi-salvinorin A (**7**) and C-2-epi-salvinorin A (**9**) were reduced to 0.14% and 21.5%, respectively. As explained above,

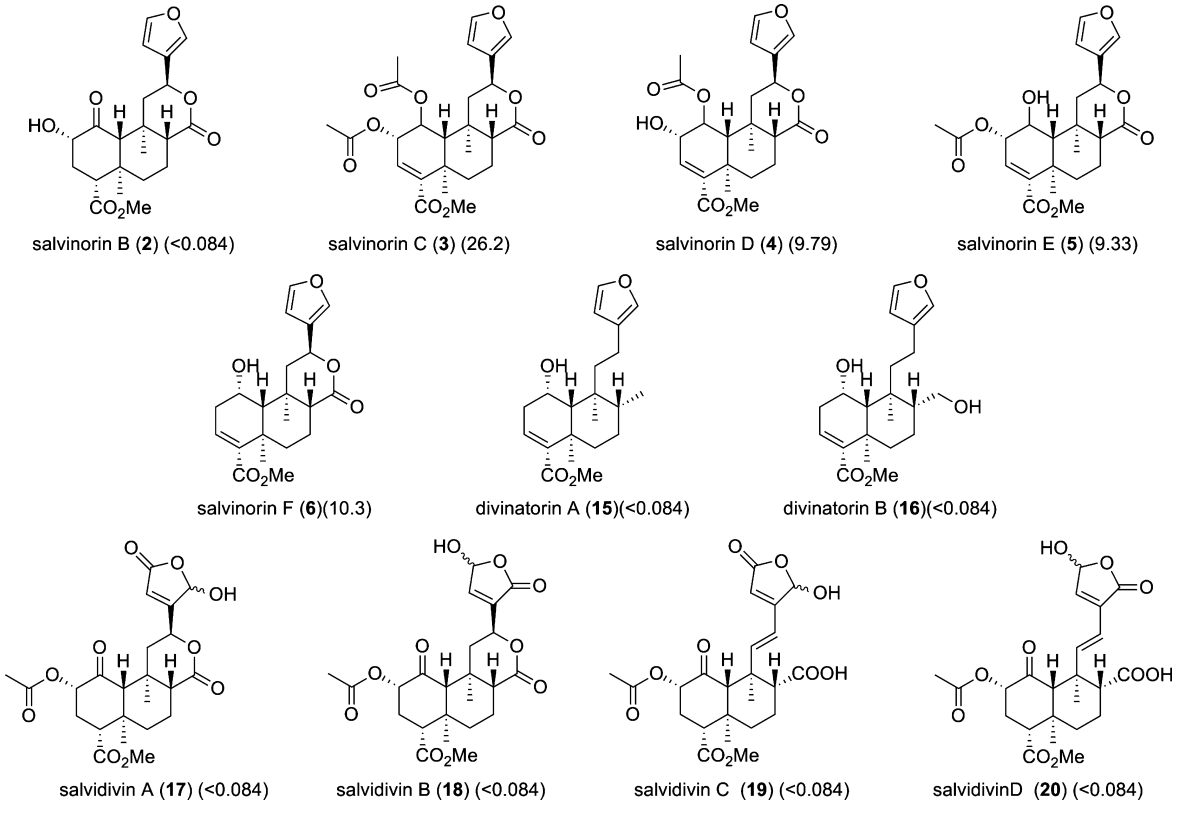


Figure 4. Structures of salvinorin A (**1**) derivatives for which the cross-reactivities (CRs) with 3D5 were tested. Each CR was calculated using the equation shown in parentheses.

dx.doi.org/10.1021/np400358n | J. Nat. Prod. XXXX, XXX, XXX−XXX

Table 1. Cross-Reactivities of 3D5 against 1 and Structurally Related Compounds[a]

compound	cross reactivity (%)
1	100
C-8-epi-salvinorin A (**7**)	0.14
C-8-epi-salvinorin B (**8**)	0.24
C-2-epi-salvinorin A (**9**)	21.5
C-2-epi-salvinorin B (**10**)	<0.084
propinoyl-salvinorin (**11**)	67.2
butyryl-salvinorin (**12**)	33.8
pivaloyl-salvinorin (**13**)	72.8
benzoyl-salvinorin (**14**)	<0.084

[a]Cross-reactivities are calculated by an equation described in the Experimental Section.

3D5 has the novel property of only reacting to salvinorins, which is a potent characteristic that can be used in the determination of salvinorins from *S. divinorum*.

Variation and Accuracy of icELISA Using 3D5. The intra- and interassay precision levels of icELISA using 3D5 were examined within the range 0.0195−2.5 μg/mL of **1**. The intra-assay precision was evaluated on the basis of the variability in the determination of **1** among wells (n = 6) on the same plate, whereas the interassay precision was obtained using different plates (n = 3). The results in Table 2 show that the maximum

Table 2. Intra- and Inter-assay Precision of the Analytical Method for Salvinorin A (1) Using icELISA[a]

1 (μg/mL)	intra-assay (%RSD,[b] n = 6)	interassay (%RSD, n = 3)
2.50	2.56	9.65
1.25	1.52	8.62
0.625	3.05	6.82
0.313	3.19	4.98
0.156	2.28	4.85
0.0781	1.64	4.98
0.0391	1.05	4.79
0.0195	0.88	2.78

[a]The values are means ± SD for three plates and six replicate wells for each concentration in one plate for three consecutive days. [b]RSD: relative standard deviation.

relative standard deviation (RSD) was 3.19% in the intra-assay. In contrast, the interassay RSD varied from 2.78% to 9.65%. The accuracy of icELISA was not high at concentrations of 1.25 and 2.5 μg/mL of **1**, as the interassay values at these concentrations were 8.62% and 9.65%, respectively. These data were sufficient to demonstrate the precision of the developed icELISA. The calibration curve to determine the concentration of **1** ranged from 0.0195 to 0.625 μg/mL, as shown in Figure 3, and the limit of detection was 0.0195 μg/mL.

A recovery experiment was conducted to confirm the reliability of the icELISA procedure developed. After spiking *S. divinorum* samples with **1** (10−40 μg/mg dry wt), sample extracts were prepared and the concentration of **1** in each sample was determined using the icELISA method. Table 3 shows that good recoveries of **1** were observed in this experiment, which ranged from 96.15% to 104.1% with an RSD of 1.05−2.26%. The recovery of **1** from each spiked sample was almost 100%, which demonstrated the high reliability of this system and confirmed that this technique is useful for the reliable determination of salvinorin derivatives from plant samples.

Analysis of *S. divinorum* and Other Plants in the Family Lamiaceae by icELISA Using 3D5. The mAb, 3D5, prepared in this study shows the novel characteristic of reacting with various salvinorins. Salvinorins can be isolated only from *S. divinorum*; therefore, icELISA using 3D5 serves as a useful differentiation method for menthol plant compounds. Several plants in the family Lamiaceae including *S. divinorum* were obtained, and their methanol extracts were tested using icELISA. Table 4 shows the results of the quantitative analysis of samples

Table 4. Determination of Salvinorins in *Salvia divinorum* and Other Plants in the Family of Lamiaceae by icELISA Using 3D5[a]

sample	salvinorin content (μg/mg dry wt)
S. divinorum 1	12.6 ± 0.6
S. divinorum 2	9.7 ± 0.67
S. divinorum 3	14.6 ± 0.4
S. divinorum 4	16.2 ± 0.9
S. divinorum 5	16.7 ± 0.5
Salvia farinacea	N.D.[b]
Salvia patens	N.D.
Salvia microphylla	N.D.
Ocimum basilicum	N.D.
Lavandula sp.	N.D.
Isodon japonicus	N.D.
Monarda citriodora	N.D.
Rosmarinus officinalis	N.D.

[a]Data are means ± SD from triplicate analysis of each sample. [b]N.D.: not detected.

where **1** was used as a standard preparation. As expected, salvinorins were detected only in *S. divinorum*. Quantitative data showed that the salvinorin content in *S. divinorum* ranged from 9.72 to 16.7 μg/mg dry wt, which differs from concentrations of **1** previously reported in this plant.[21,40] Probably this is likely due to the fact that the mAbs reacted with other salvinorins in the samples and the quantitative data reflected the approximate levels of total salvinorins.

Various immunochemical methods have been developed and used for the identification of morphine, cocaine, marijuana, and

Table 3. Recovery of Salvinorin A (1) from Spiked Samples Using icELISA

spiked level (μg/mg dry wt)	measured amount[a] (μg/mg dry wt) (%RSD[b])	expected amount (μg/mg dry wt)	recovery[c] (%)
0	12.7 ± 1.0 (1.36)	12.6	
10	23.0 ± 1.2 (1.06)	22.6	104.1
20	32.7 ± 4.4 (2.26)	32.6	100.4
40	51.1 ± 2.7 (1.05)	52.6	96.2

[a]All values are means ± SD from triplicate samples at each level. [b]RSD: relative standard deviation. [c]Recovery (%) = (measured amount − 12.69/added amount) × 100.

dx.doi.org/10.1021/np400358n | J. Nat. Prod. XXXX, XXX, XXX−XXX

other drugs.[24,25,41] These methods have novel features such as their cost-effective performance and the rapidity and simplicity with which they can be applied. In the present study, a mAb against salvinorin A (**1**), 3D5, was prepared successfully, and its characterization showed that it could recognize novel salvinorins. This mAb was then used to develop an icELISA to determine the concentrations of salvinorins, which are found only in *S. divinorum*. The icELISA procedure was validated using plant materials, including *S. divinorum,* which confirmed that this methodology is sufficiently sensitive, accurate, and reliable as a differentiation method. In addition, icELISA can analyze a large number of samples derived from complex and mixed materials in a short period of time without any requirement for a pretreatment procedure.

This is the first report of the production of a mAb against **1** and the development of an immunochemical method to analyze salvinorins and identify *S. divinorum*. Instrumental methods, such as HPLC-MS or GC-MS, have been used to analyze **1** and were evaluated in terms of their accuracy and reliability. However, these procedures must be performed by experienced laboratory staff, as they require a certain level of technical expertise to operate such complex instruments. In contrast, immunochemical approaches such as icELISA and immunochromatographic analysis are convenient to perform and do not require expensive equipment. Therefore, immunoassays can be used for the frequent screening of samples, and they could complement existing instrumental analytical methods for salvinorins to help assess *S. divinorum* abuse.

Various pharmacological and neurobiochemical studies regarding salvinorin A (**1**) have been conducted and reported.[42−49] Thus, **1** is widely recognized as a potential drug candidate. Immunoassays that use 3D5 to recognize this compound also have potential applications as analytical tools in the aforementioned research fields.

EXPERIMENTAL SECTION

General Experimental Procedures. Salvinorin A (**1**) and its related compounds, i.e., **2**−**6**, divinatorins A (**15**) and B (**16**), and salvidivins A (**17**), B (**18**), C (**19**), and D (**20**), were isolated from plant material.[50] The purities of these isolated compounds were checked by nuclear magnetic resonance spectometry and were >95%. Unnatural salvinorins, i.e., propinoyl-salvinorin (**11**), butyryl-salvinorin (**12**), pivaloyl-salvinorin (**13**), and benzoyl-salvinorin (**14**), were synthesized according to previously reported methods.[51,52] In brief, various types of carboxylic acids were esterified to the hydroxy group at C-2 of **2** using established methods. C-8-epi-Salvinorin B (**8**) was obtained by hydrolyzing **1** with lithium hydroxide. C-8-epi-Salvinorin A (**7**) was prepared by acetylating **8**. Finally, C-2-epi-salvinorin A (**9**) was prepared from **2** when subjected to Mitsunobu etherification conditions, and C-2-epi-salvinorin B (**10**) was obtained by hydrolyzing the resulting product. BSA and HSA were obtained from Sigma-Aldrich (Steinheim, Baden-Württemberg, Germany). Freund's complete and incomplete adjuvants were provided by Difco (Detroit, MI, USA). The peroxidase-labeled anti-mouse IgG was purchased from MP Biomedicals (Santa Ana, CA, USA). All other chemicals and organic solvents were standard commercial products and were of analytical reagent grade.

Plant Material. Dried *S. divinorum* leaves were purchased in June 2005 from Ethnogens.com (Lawrence, KS, USA) and were verified by one of the authors (O.S.). Voucher specimens were deposited at the Medicinal Herbarium, Kagawa School of Pharmaceutical Sciences, Tokushima Bunri University, as specimen #050601-001. Other Lamiaceae plants were cultivated in the medicinal plant station, Faculty of Pharmaceutical Sciences, Kyushu University. The leaves of other Lamiaceae plants were obtained in July 2011 in Fukuoka, Japan. The identities of plant materials were verified by one of the authors (H.T.), and voucher specimens [*Salvia farinacea* Benth., KYU-2011SF1; *Salvia patens* Cav., KYU-2011SP1; *Salvia microphylla* Kunth, KYU-2011SM1; *Ocimum basilicum* L., KYU-2011OB1; *Lavandula* sp., KYU-2011LA1; *Isoson japonicus* (Burm.) Hara, KYU-2011IJ1; *Monarda citriodora* Cerv. ex Lag., KYU-2011MC1; *Rosmarinus officinalis* L., KYU-2011RO1] were deposited in the Herbarium of the Faculty of Pharmaceutical Sciences, Kyushu University.

Sample Preparations. The leaf samples of *S. divinorum* and other Lamiaceae plants were each cut into small pieces, dried, and ground into a fine powder. Each sample (30 mg) was accurately weighed and extracted with 0.5 mL of methanol using an ultrasonic bath for 10 min. The extract was then centrifuged at 12 000 rpm for 10 min, and the supernatant was transferred to a microtube. The residue was extracted in the same way at least twice, and all the supernatants were mixed to prepare the sample solution. Next, the extracted solution was diluted to produce a 20% methanol solution, which was used for the immunoassays.

Synthesis of the Hapten Derivative. Salvinorin B (**2**) was obtained at a yield of 64% by hydrolyzing **1** (11.2 mg, 0.026 mmol) with sodium carbonate (3.3 mg, 0.032 mmol) in a dichloromethane−methanol (1:1) mixed solution (1.1 mL) at room temperature for 48 h. Next, the hapten, which had a carboxylic acid side chain at C-2 in **2**, was obtained at a yield of 96% by reacting **2** (15.1 mg, 0.039 mmol) with a succinic anhydride (35 mg, 0.35 mmol) in the presence of *N*,*N*-dimethyl-4-aminopyridine (catalytic amount) in dichloromethane (2 mL) at room temperature for 4 h.

Synthesis of Antigen Conjugates. The hapten was **2**-hemisuccinate, which was conjugated to BSA and HSA to produce the immunogen and the coating antigen, respectively. 1-Ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (6 mg, 0.031 mmol) and **2**-hemisuccinate (5 mg, 0.023 mmol) were added to a solution of 30% pyridine (0.5 mL). The reaction mixture was added dropwise to a H_2O solution (0.25 mL) that contained BSA (5 mg) or HSA (5 mg) before stirring at room temperature for 6 h. Subsequently, the mixture was dialyzed with five changes of H_2O at 4 °C for a period of 2 days and then lyophilized to yield 6.2 mg of **2**−BSA conjugate and 8.4 mg of **2**−HSA conjugate.

Animal Treatment. Male BALB/c mice (5 weeks of age) were purchased from KBT Oriental Co. (Saga, Japan). A standard diet (MF; Oriental Yeast Co., Tokyo, Japan) and water were provided ad libitum. The procedures and animal care were approved by the Committee on Ethics of Animal Experiments, Graduate School of Pharmaceutical Sciences, Kyushu University, Japan, and were conducted according to the Guidelines for Animal Experiments of the Graduate School of Pharmaceutical Sciences, Kyushu University (Animal Ethics Reference No. A22-113-0, Kyushu University, Japan).

Determination of the Hapten Number in the 2−Protein Conjugates by MALDITOFMS. The hapten numbers in the **2**−BSA and **2**−HSA conjugates were determined by MALDITOFMS. A small amount (1−10 pmol) of antigen conjugate was mixed with a 10^3-fold molar excess of sinapinic acid in an aqueous solution that contained 0.10% trifluoroacetic acid. The mixture was applied to a high-performance MALDITOFMS system (Autoflex III; Bruker Daltonics, Bremen, Germany). Data were analyzed using FlexAnalysis 3.0.92 (Bruker Daltonics, Bremen, Germany).

Immunization, Hybridization, and Production of the mAb. The **2**−BSA conjugate was dissolved in 8 M urea and diluted with phosphate-buffered saline (PBS) to prepare the immunogen solution. BALB/c male mice (5 weeks old) were injected intraperitoneally with 0.5 mL of **2**−BSA (100 μg/mL emulsified with an equal volume of Freund's complete adjuvant). In the second immunization, 50 μg of the conjugate in Freund's incomplete adjuvant was injected intraperitoneally 2 weeks after the initial injection. Subsequently, the mice were given boosters of immunogen (100 μg) without an adjuvant into the abdominal cavity in the third and final immunization. Mice were bled 4 days after each booster, and the titers of specific antibodies that recognized **2** in the sera were monitored by ELISA. On the third day after the final immunization (100 μg of protein), splenocytes were prepared from an isolated spleen and fused with an aminopterin-sensitive mouse myeloma cell line, SP2/0-Ag14, using the HVJ Envelop (Ishihara Sangyo Kaisha Ltd., Osaka, Japan).

dx.doi.org/10.1021/np400358n | J. Nat. Prod. XXXX, XXX, XXX−XXX

Hybridomas that produced mAbs reactive to **1** were detected by ELISA and cloned using the limiting dilution method. The established hybridoma clones were cultured in an enriched RPMI 1640-Dulbecco's-Ham's F12 (e-RDF) medium (Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan), which was supplemented with 10% fetal bovine serum. For the mAb, a serum-free medium, i.e., an e-RDF medium supplemented with 10 μg/mL insulin, 35 μg/mL transferrin, 20 μg/mL ethanolamine, and 25 nM selenium, was used to obtain a supernatant that contained the mAb against **1**.

Purification of mAbs. mAbs were purified using a Protein G FF column (0.46 cm × 11 cm; GE Healthcare, Uppsala, Sweden). The culture medium (500 mL) that contained the mAb (IgG type) was adjusted to pH 7 with 1 M Tris-HCl solution (pH 9) and applied to the column. The column was then washed with 10 mM phosphate buffer (pH 7). The IgG that adsorbed to the gel was eluted with 100 mM citrate buffer (pH 2.8) and was immediately neutralized with 1 M Tris-HCl (pH 9), dialyzed three times against H_2O, and lyophilized to yield 8.3 mg of mAb.

Indirect ELISA Using 2−HSA. A 96-well immunoplate (Maxisorb; Nalgene Nunc, Roskilde, Denmark) was coated with 100 μL of 0.1 μg/mL **2**−HSA conjugate in 50 mM carbonate buffer (pH 9.6) and incubated at 37 °C for 1 h. The plate was washed three times with 0.05% Tween 20-containing PBS (TPBS). Next, the plate was treated with 300 μL of PBS containing 5% skim milk (SPBS) for 1 h to reduce any nonspecific adsorption. The plate was washed three times with TPBS and reacted with 100 μL of test antibodies for 1 h. Next, the plate was washed again with TPBS three times, and mAbs were then combined with 100 μL of 1000-fold-diluted solution of peroxidase-labeled anti-mouse IgG (MP Biomedicals; Cappel Products, Santa Ana, CA, USA) for 1 h. After washing the plate three times with TPBS, 100 μL of the substrate solution, 0.1 M citrate buffer (pH 4.0) containing 0.003% H_2O_2, and 0.3 mg/mL 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Wako, Osaka, Japan) were added to each well, and the plates were incubated for 15 min. The absorbance was measured at 405 nm using a microplate reader (ImmunoMini, Nalgene Nunc). All the reactions were carried out at 37 °C.

icELISA Using mAb against 1. The **2**−HSA conjugate (100 μL, 0.1 μg/mL) was adsorbed onto the 96-well immunoplate, which was then treated with 300 μL of 5% SPBS for 1 h to reduce nonspecific adsorption. Salvinorins and plant sample extracts were dissolved with methanol and diluted with H_2O to yield 20% methanol solutions. Various concentrations of samples in 20% methanol (50 μL) were incubated with 50 μL of the mAb solution for 1 h. The plate was washed three times with TPBS, and the antibody was combined with 100 μL of a 1000-fold-diluted solution of peroxidase-labeled anti-mouse IgG for 1 h. After washing the plate three times with TPBS, 100 μL of the substrate solution was added to each well, and the plates were incubated for 15 min. The absorbance was measured at 405 nm using a microplate reader.

The CRs of the mAbs were evaluated with various compounds and calculated as follows using the method of Weiler and Zenk.[39]

$$\mathrm{CR}\ (\%) = \frac{\mathrm{IC_{50}\ for\ \mathbf{1}}}{\mathrm{IC_{50}\ for\ compound\ under\ investigation}} \times 100$$

Intra- and Interassay Variations of icELISA. Eight concentrations of **1** (2.50, 1.25, 0.625, 0.313, 0.156, 0.0781, 0.039, 1, and 0.0195 μg/mL in 20% methanol) were prepared and analyzed by a previously described icELISA using 3D5. Six replicate analyses of each solution were performed within one plate, and intra-assay variation was determined by RSDs of these data. The icELISA for the same eight concentrations of **1** was performed for three consecutive days, and interassay variation was determined by RSDs of data between the plates.

Recovery of 1 from Sample. Dried, powdered leaves of *S. divinorum* (30 mg) were spiked with various concentrations of **1**. The concentration of **1** in the unspiked sample was determined as 12.69 μg/mg dry wt by icELISA. Three concentrations of **1** (10, 20, and 40 μg/mg dry wt) were used to spike the powdered samples of *S. divinorum*. After drying the spiked samples, **1** was extracted using the same procedure described above, and the concentration of **1** in each sample was determined by icELISA. The recovery was calculated as follows on the basis of the concentration measured and the amount of **1** spiked in the sample.

$$\mathrm{Recovery}\ (\%) = \frac{\mathrm{Measured\ amount} - 12.69}{\mathrm{Added\ amount}} \times 100$$

AUTHOR INFORMATION

Corresponding Author

*E-mail: htanaka@phar.kyushu-u.ac.jp. Tel: +81(0)92-642-6582. Fax: +81(0)92-642-6582.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This study was supported in part by a Grant-in-Aid from the JSPS Asian CORE Program, the Ministry of Education, Culture, Sports, Science and Technology of Japan; a Health Labour Sciences Research Grant from the Ministry of Health, Labour and Welfare; and the research fund of Kyushu University Foundation.

REFERENCES

(1) Ortega, A.; Blount, J. F.; Manchand, P. S. *J. Chem. Soc., Perkin Trans. 1* **1982**, 2505−2508.
(2) Siebert, D. J. *J. Ethnopharmacol.* **1994**, *43*, 53−56.
(3) Roth, B. L.; Baner, K.; Westkaemper, R.; Siebert, D.; Rice, K. C.; Steinberg, S.; Ernsberger, P.; Rothman, R. B. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 11934−11939.
(4) Sheffler, D. J. *Trends Pharmacol. Sci.* **2003**, *24*, 107−109.
(5) Valdes, L. J.; Chang, H. M.; Visger, D. S.; Koreeda, M. *Org. Lett.* **2001**, *3*, 3935−3937.
(6) Munro, T. A.; Rizzacasa, M. A. *J. Nat. Prod.* **2003**, *66*, 703−705.
(7) Vortherms, T. A.; Roth, B. L. *Mol. Interventions* **2006**, *6*, 257−265.
(8) Scheerer, J. R.; Lawrence, J. F.; Wang, G. C.; Evans, D. A. *J. Am. Chem. Soc.* **2007**, *129*, 8968−8969.
(9) Yang, L.; Xu, W.; Chen, F.; Liu-Chen, L. Y.; Ma, Z. Z.; Lee, D. Y. W. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1301−1304.
(10) Hagiwara, H.; Suka, Y.; Nojima, T.; Hoshi, T.; Suzuki, T. *Tetrahedron* **2009**, *65*, 4820−4825.
(11) Yan, F.; Bikbulatov, R. V.; Mocanu, V.; Dicheva, N.; Parker, C. E.; Wetsel, W. C.; Mosier, P. D.; Westkaemper, R. B.; Allen, J. A.; Zjawiony, J. K.; Roth, B. L. *Biochemisty* **2009**, *48*, 6898−6908.
(12) Lee, D. Y. W.; Yang, L.; Xu, W.; Deng, G.; Guo, L.; Liu-Chen, L. Y. *Bioorg. Med. Chem. Lett.* **2009**, *20*, 5749−5752.
(13) Lozama, A.; Cunningham, C. W.; Caspers, M. J.; Douglas, J. T.; Dersch, C. M.; Rothman, R. B.; Prisinzano, T. E. *J. Nat. Prod.* **2011**, *74*, 718−726.
(14) Fichna, J.; Lewellyn, K.; Yan, F.; Roth, B. L.; Zjawiony, J. K. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 160−163.
(15) Chartoff, E. H.; Potter, D.; Damez-Werno, D.; Cohen, B. M.; Carlezon, W. A., Jr. *Neuropsychopharmacology* **2008**, *33*, 2676−2687.
(16) Braida, D.; Capurro, V.; Zani, A.; Rubino, T.; Viganò, D.; Parolaro, D.; Sala, M. *Br. J. Pharmacol.* **2009**, *157*, 844−853.
(17) McCurdy, C. R.; Sufka, K. J.; Smith, G. H.; Warnick, J. E.; Nieto, M. J. *Pharmacol., Biochem. Behav.* **2006**, *83*, 109−113.
(18) Capasso, R.; Borrelli, F.; Cascio, M. G.; Aviello, G.; Huben, K.; Zjawiony, J. K.; Marini, P.; Romano, B.; Di Marzo, V.; Capasso, F.; Izzo, A. A. *Br. J. Pharmacol.* **2008**, *155*, 681−689.
(19) Pichini, S.; Abanades, S.; Farre, M.; Pellegrini, M.; Marchei, E.; Pacifici, R.; de la Torre, R.; Zuccaro, P. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 1649−1656.
(20) Kennedy, J. H.; Wiseman, J. M. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 1305−1311.
(21) Jermain, J. D.; Evans, H. K. *J. Forensic Sci.* **2009**, *54*, 612−616.
(22) Schimidt, M. S.; Prisinzano, T. E.; Tidgewell, K.; Harding, W.; Kreek, M. J.; Murry, D. J. *J. Chromatogr. B* **2005**, *818*, 221−225.

(23) Tsujikawa, K.; Kuwayama, K.; Miyaguchi, H.; Kanamori, T.; Iwata, Y. T.; Yoshida, T.; Inoue, H. *Forensic Sci. Int.* **2008**, *180*, 105−109.
(24) Shoyama, Y.; Fukada, T.; Tanaka, T.; Kusai, A.; Nojima, K. *Biol. Pharm. Bull.* **1993**, *16*, 1051−1053.
(25) Tanaka, H.; Goto, Y.; Shoyama, Y. *J. Immunoassay* **1996**, *17*, 321−342.
(26) Yanagihara, H.; Sakata, R.; Minami, H.; Tanaka, H.; Shoyama, Y.; Murakami, H. *Anal. Chim. Acta* **1996**, *335*, 63−70.
(27) Shan, S. J.; Tanaka, H.; Shoyama, Y. *Anal. Chem.* **2001**, *73*, 5784−5790.
(28) Kim, J. S.; Tanaka, H.; Shoyama, Y. *Analyst* **2004**, *129*, 87−91.
(29) Phrompittayarat, W.; Putalun, W.; Tanaka, H.; Wittaya-Areekul, S.; Jetiyanon, K.; Ingkaninan, K. *Anal. Chim. Acta* **2007**, *584*, 1−6.
(30) Sakamoto, S.; Putalun, W.; Tsuchihashi, R.; Morimoto, S.; Kinjo, J.; Tanaka, H. *Anal. Chim. Acta* **2008**, *607*, 100−105.
(31) Kido, K.; Edakuni, K.; Morinaga, O.; Tanaka, H.; Shoyama, Y. *Anal. Chim. Acta* **2008**, *616*, 109−114.
(32) Juengwatanatrakul, T.; Sritularak, B.; Amornnopparattanakul, P.; Tassanawat, P.; Putalun, W.; Tanaka, H.; Morimoto, S. *Analyst* **2011**, *136*, 1013−1017.
(33) Kido, K.; Morinaga, O.; Shoyama, Y.; Tanaka, H. *Talanta* **2008**, *77*, 346−350.
(34) Paudel, M. K.; Putalun, W.; Sritularak, B.; Morinaga, O.; Shoyama, Y.; Tanaka, H.; Morimoto, S. *Anal. Chim. Acta* **2011**, *701*, 189−193.
(35) Xuan, L, J.; Tanaka, H.; Morimoto, S.; Shoyama, Y.; Akanuma, H.; Muraoka, K. *Spectrosc. Int. J.* **2001**, *14*, 85−92.
(36) Tanaka, H.; Xuan, L. J.; Morimoto, S.; Shoyama, Y.; Isobe, R.; Nojima, K. *Spectrosc. Int. J.* **2001**, *15*, 1−18.
(37) Sakata, R.; Shoyama, Y.; Murakami, H. *Cytotechnology* **1994**, *16*, 101−108.
(38) Friguet, B.; Chaffotte, A. F.; Djavadi-Ohaniance, L.; Goldberg, M. E. *J. Immunol. Methods* **1985**, *77*, 305−319.
(39) Weiler, E. W.; Zenk, M. H. *Phytochemistry* **1976**, *15*, 1537−1545.
(40) Gruber, J. W.; Siebert, D. J.; Der Marderosian, A. H.; Hock, R. S. *Phytochem. Anal.* **1999**, *10*, 22−25.
(41) Cone, E. J. *J. Forensic Sci.* **1989**, *34*, 991−995.
(42) Chavkin, C.; Sud, S.; Jin, W. Z.; Stewart, J.; Zjawiony, J. K.; Siebert, D. J.; Toth, B. A.; Hufeisen, S. J.; Roth, B. L. *J. Pharmcol. Exp. Ther.* **2004**, *308*, 1197−1203.
(43) Yan, F.; Mosier, P. D.; Westkaemper, R. B.; Stewart, J.; Zjawiony, J. K.; Vorthermas, T. A.; Sheffler, D. J.; Roth, B. L. *Biochemistry* **2005**, *44*, 8643−8651.
(44) Zhang, Y.; Butelman, E. R.; Schlussman, S. D.; Ho, A.; Kreek, M. J. *Psychopharmacology* **2009**, *179*, 551−558.
(45) Carlezon, W. A.; Beguin, C.; DiNieri, J. A.; Baumann, M. H.; Richards, M. R.; Todtenkopf, M. S.; Rothman, R. B.; Ma, Z. Z.; Lee, D. Y. W.; Cohen, B. M. *J. Pharmcol. Exp. Ther.* **2006**, *316*, 440−447.
(46) Capasso, R.; Borrelli, F.; Capasso, F.; Siebert, D. J.; Stewart, D. J.; Zjawiony, J. K. *Neurogastroent. Motil.* **2006**, *18*, 69−75.
(47) Vortherms, T. A.; Mosier, P. D.; Westkaemper, R. B.; Roth, B. L. *J. Biol. Chem.* **2007**, *282*, 3146−3156.
(48) Cunningham, C. W.; Rothman, R. B.; Prisinzano, T. E. *Pharmacol. Rev.* **2011**, *63*, 316−347.
(49) Su, D. S.; Riley, J.; Kiessling, W. J.; Armsead, W. M.; Liu, R. Y. *Anesthesiology* **2011**, *114*, 374−379.
(50) Shirota, O.; Nagamatsu, K.; Sekita, S. *J. Nat. Prod.* **2006**, *69*, 1782−1786.
(51) Beguin, C.; Richards, M. R.; Wang, Y.; Chen, Y.; Liu-Chen, L. Y.; Ma, Z.; Lee, D. Y. W.; Carlezon, W. A.; Cohen, B. M. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2761−2765.
(52) Beguin, C.; Richards, M. R.; Li, J.-G.; Wang, Y.; Xu, W.; Liu-Chen, L. Y.; Carlezon, W. A.; Cohen, B. M. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 4679−4685.