maintained at 37°C and continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2. The ileum was transmurally stimulated through platinum needle-ring electrodes with monophasic square wave pulses (0.2 Hz) of 0.1-ms duration by a stimulator (SEN-7203, Nihon Kohden, Tokyo, Japan). This transmural stimulation induces a twitch contraction via acetylcholine released from postganglionic cholinergic neurons [23]. Contractions were isotonically recorded with a displacement transducer (NEC, San-ei Instruments Ltd, Type 45347, Tokyo, Japan), a DC strain amplifier (San-ei 6M92, Tokyo, Japan) and a DC recorder (Hitachi, Mod 056, Tokyo, Japan). All concentration-response curves were constructed in a cumulative manner. The height of the twitch response to the transmural stimulation was measured before and after drug challenge. The twitch contraction percentage of the response after the addition of each compound was determined as described previously [5]. Agonist potency was expressed as pD_2, which is the negative logarithm of the concentration required to produce 50% of the maximum response to the drug (EC_{50}). Chemicals used for the pharmacological assay were: acetylcholine chloride (Dai-ichi Seiyaku Co., Ltd, Tokyo, Japan), and DAMGO {[D-Ala(2),*N*-Me-Phe(4),Gly(5)-ol]enkephalin}, naloxone hydrochloride and cyprodime (Sigma Chemical Co., St. Louis, Mo.). The solvent (dimethylsulfoxide 0.5%) did not affect the electrically stimulated or acetylcholine-induced contraction. The data are expressed as means ± standard error of the mean. Statistical analyses were performed by a one-way analysis of variance followed by a Bonferroni multiple comparison test. A p value < 0.05 was considered statistically significant.

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New Procedure to Mask the 2,3-π Bond of the Indole Nucleus and Its Application to the Preparation of Potent Opioid Receptor Agonists with a Corynanthe Skeleton

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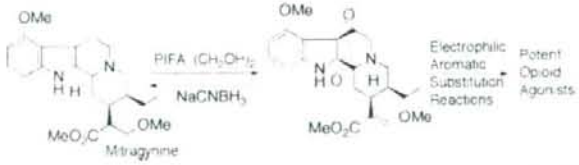
ABSTRACT



Treatment of indole alkaloids with hypervalent iodine in the presence of ethylene glycol provides 2,3-ethylene glycol bridged adducts that could be converted into the original indoles under mild reductive conditions. This procedure, which involves masking of the reactivity of the indole nucleus at the β-position, was utilized for the modification of the benzene ring of the indoline derivative and was applied to the preparation of potent opioid receptor agonists with the Corynanthe skeleton.

7-Hydroxy-7*H*-mitragynine (7-hydroxymitragynine, **1**)[1] is a minor constituent of a rubiaceous plant, *Mitragyna speciosa*,[2] that has long been used in Thailand for its opium-like effect. We have previously demonstrated in guinea pig ileum experiments that **1** inhibits electrically induced contraction through the opioid receptors, and its effect is approximately 13-fold more potent than that of morphine.[3] Further, **1** exhibits a potent antinociceptive effect in mouse tail-flick and hot-plate tests when administered subcutaneously or orally.[4] The antinociceptive effect of **1** is more potent than that of morphine in both tests and is induced mainly by

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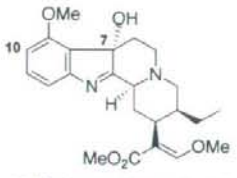
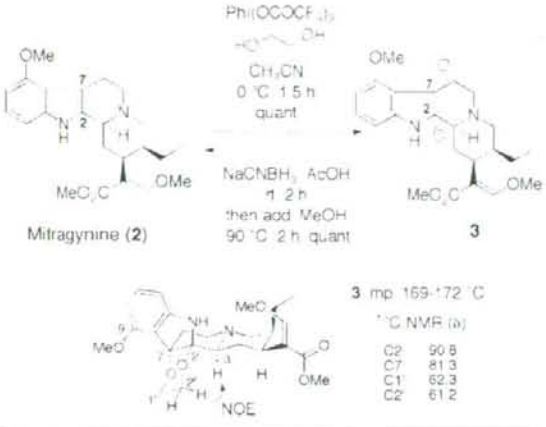
activating μ-opioid receptors. Development of tolerance to its antinociceptive effect and cross-tolerance to morphine antinociception were observed, indicating that **1** acts on μ-opioid receptors.[5] Furthermore, **1** induces constipation less potently than morphine at antinociceptive doses.[6] These interesting properties of **1**, which has a chemical structure different from that of morphine, have enabled us to pursue further investigations for the development of novel analgesics. To develop a more potent opioid receptor agonist based on **1**, we have initially planned the synthesis of compounds by modifying the benzene ring in **1** and the evaluation of their potency to opioid receptors. In the present study, we found a new method to protect the 2,3-π bond of indole alkaloids, which was applied to the preparation of derivatives having various substituents at the C-10 position in **1**. Among the synthetic derivatives, compound **11** showed the highest potency: 4-fold and 18-fold higher than that of **1** and morphine, respectively. In this communication, we report these chemical findings as well as the preliminary pharmacological results on the opioid agonistic effect of Corynanthe-type indole alkaloids.



7-Hydroxymitragynine (**1**)

Attempts at the direct introduction of electrophilic substituents on the benzene ring in **1** or in its parent compound, mitragynine (**2**),[7] were unsuccessful as expected. Then, we devised a method to protect the 2,3-π bond of indoles,[8] producing the aniline structure that should act as a reactive aromatic compound toward various electrophiles. When **2** was treated with 1 equiv of phenyliodine bis(trifluoroacetate) (PIFA)[9] in the presence of ethylene glycol (EG) in MeCN at 0 °C, a 2,3-ethylene glycol bridged indoline derivative (**3**) was obtained in quantitative yield. The structure of the adduct including the stereochemistry was determined from spectroscopic data, as shown in Scheme 1. Indoline **3** could be converted into starting indole **2** in almost quantitative yield upon reduction with $NaCNBH_3$ in AcOH at room temperature, followed by heating at 90 °C after addition of MeOH. Indoline **3** was put to practical use for the preparation of several benzene-substituted derivatives for the study of opioid receptor ligands, as described below.

Scheme 1



Using other indole alkaloids, we examined the generality of the newly developed method to mask the pyrrole moiety in the indole nucleus. Among the tested compounds, 2,3-dimethylindole, tetrahydrocarbazole, indoloquinolizidine, corynantheol, dihydrocorynantheol, and yohimbine, the corresponding EG adducts (**4**–**9**), were obtained in moderate yields. However, the best results (the yields are shown in Figure 1) were obtained when NH_4Cl was added to the reaction mixture (see Supporting Information).[10] In the case of reserpine, it was found that phenyliodine diacetate (PIDA) was a more suitable reagent than PIFA for the formation of the EG-bridged adduct (**10**), which was also useful as a starting material for the preparation of various kinds of A-ring-modified reserpine analogues.[11]

Using EG adduct **3** derived from mitragynine (**2**), various kinds of substituents were introduced onto the benzene ring, as shown in Scheme 2. Treatment of **3** with *N*-fluoro-2,6-dichloropyridinium triflate (FP-T800)[12] gave compound **11** fluorinated at the C-10 position in 53% yield. Exposure of **3** to NCS in AcOH afforded two chlorinated derivatives **12a** (10-Chloro) and **12b** (12-Chloro) in 88% and 11% yields, respectively. Using NBS in DMF, 10-bromo and 12-bromo derivatives (**13a** and **13b**) were obtained in 75% and 24% yields, respectively. To introduce the nitro group, a combination of CAN and concentrated H_2SO_4 in DCM[13] was used to give **14a** in 52% yield together with its 12-isomer (**14b**)

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(10) We found that treatment of 7-chloroindolenine derivatives, which were prepared by oxidation of indoles with *t*BuOCl, with ethylene glycol in the presence of TFA also afforded EG adducts, although the yields were inferior to those of the PIFA–EG-NH_4Cl method. The reaction mechanism for the formation of EG adducts with hypervalent iodines is still unclear.

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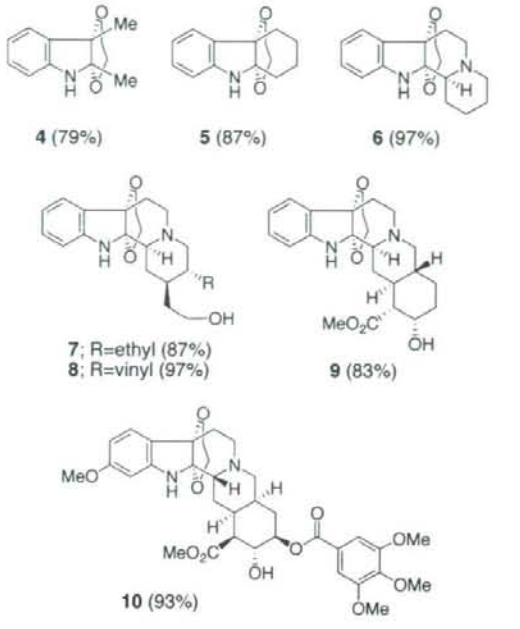
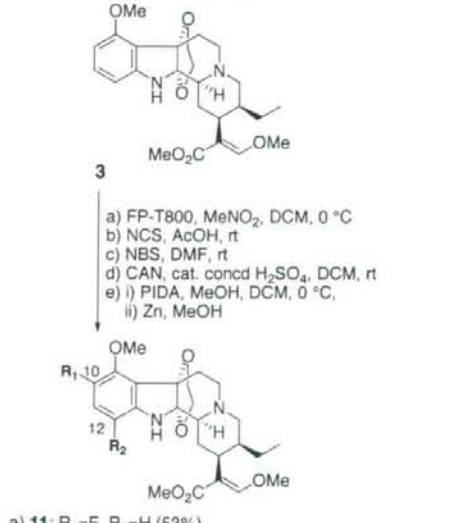


Figure 1. Ethylene glycol adducts of various indoles.

in 21% yield. 10-Methoxy derivative **15** was prepared in 64% yield by treatment of **3** with IBDA in MeOH, followed by the reduction of the resulting iminoquinone intermediate (see Supporting Information) with Zn in MeOH.[14]

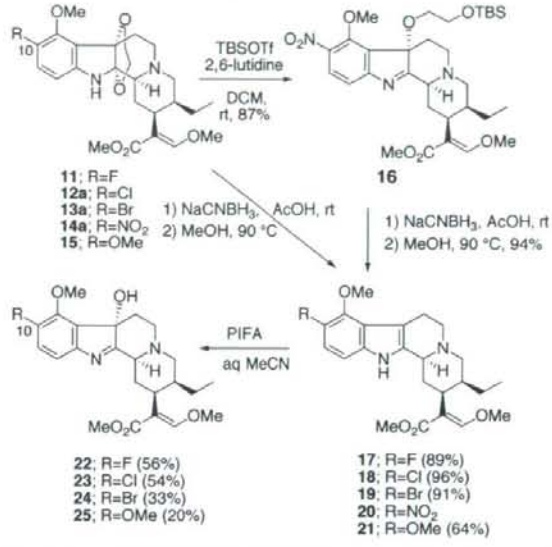
Scheme 2



a) **11**; R_1=F, R_2=H (53%)
b) **12a**; R_1=Cl, R_2=H (88%), **12b**; R_1=H, R_2=Cl (11%)
c) **13a**; R_1=Br, R_2=H (75%), **13b**; R_1=H, R_2=Br (24%)
d) **14a**; R_1=NO_2, R_2=H (52%), **14b**; R_1=H, R_2=NO_2 (21%)
e) **15**; R_1=OMe, R_2=H (64%, 2 steps)

The C10-substituted derivatives thus obtained as a major product of each electrophilic aromatic substitution reaction were converted into their indole derivatives in good yields by reduction with $NaCNBH_3$ in AcOH as described above (conversion from **3** into **2**). However, in the case of nitro derivative **14a**, a two-step procedure was needed; i.e., **14a** was treated with TBSOTf in the presence of 2,6-lutidine, and the resultant indolenine derivative **16** obtained in 87% yield was reduced with $NaCNBH_3$ to give the indole derivative **20** in 94% yield (Scheme 3). The thus obtained

Scheme 3



indole derivatives were, respectively, converted into 7-hydroxyindolenine derivatives (**22**−**25**) by oxidation with PIFA in aqueous MeCN.[15]

The series of C10-substituted mitragynine derivatives obtained by the above reactions was subjected to pharmacological evaluation. The opioid agonistic effect was evaluated in an experiment involving twitch contraction induced by electrical stimulation of guinea pig ileum. This experiment is generally used to study opioid analgesics. The results are shown in Table 1.

Among the EG-bridged derivatives (**3**, **11**, **12a**, **13a**, **14a**, and **15**) and the 7-hydroxyindolenine derivatives (**22**−**25**), C10-fluorinated derivatives (**11**, **22**) showed the highest potency. Derivatives having a chloro or bromo group at C10 showed lower potency than the corresponding fluorinated derivatives. These results suggest that the dimension or electronegativity of the functional group at the C10 position is important to elicit the opioid agonistic effect. None of the indole derivatives (**17**−**21**) showed any opioid agonistic

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Table 1. Opioid Effects of Mitragynine Derivatives on Twitch Contraction Induced by Electrical Stimulation in Guinea Pig Ileum[a]

compound	pD_2 value (−log M)	relative potency (%)	maximum inhibition (%)	inhibitory activity (%)
morphine	7.15 ± 0.05	100	87.2 ± 1.8	100
Ethylene Glycol Bridged Derivatives				
3	7.70 ± 0.10	354	35.0 ± 11.0	40
11	8.40 ± 0.02**	1778	83.4 ± 3.2	96
12a	7.61 ± 0.17	288	48.1 ± 9.3	55
14a	7.88 ± 0.18	537	65.0 ± 4.3	75
Mitragynine Derivatives				
2	6.50 ± 0.06**	22	72.0 ± 5.0	83
7-Hydroxyindolenine Derivatives				
1	7.78 ± 0.10	426	90.8 ± 3.4	104
22	7.87 ± 0.04	524	82.5 ± 1.8	95
23	7.53 ± 0.08	239	74.8 ± 3.0	86
24	7.45 ± 0.04**	199	61.7 ± 6.2	71

[a] Potency is expressed as a pD_2 value, which is the negative logarithm of the concentration required to produce 50% of the maximum response to each compound (EC_{50}). Relative potency is expressed as a percentage of the pD_2 value of each compound against that of morphine. Maximum inhibition (%), which is elicited by the compound when the response reaches a plateau, was calculated by regarding the twitch contraction as 100%. Relative inhibitory activity, which means intrinsic activity on opioid receptors, is expressed as a percentage of the maximum inhibition by each compound against that by morphine. Each value represents a mean ± the SEM of five or six animals. The asterisk (*) donates values that were significantly different from the morphine group by Student's *t*-test (**, $P < 0.01$). Compounds **13a**, **15**, **17**−**21**, and **25** did not show significant inhibition at 1 μM.

effect. Compound **22** showed potent agonistic effect, but its potency was nearly equal to that of 7-hydroxymitragynine (**1**). On the other hand, compound **11** showed the most potent opioid agonistic effect among the derivatives tested in the present study. Its potency was 18- and 4-fold higher than that of morphine and 7-hydroxymitragynine (**1**), respectively.

Next, we investigated the involvement of opioid receptor subtypes in the pharmacological effects of **11**. The μ-opioid receptor antagonist cyprodime (1 μM) and the κ-opioid receptor antagonist nor-binaltorphimine (30 nM) significantly reversed the inhibitory effect of **11** at 30 nM (data not shown), suggesting that **11** activates not only μ-opioid receptors but also κ-opioid receptors. Detailed results of the pharmacological and analgesic effects of **11** will be reported in due course.

In conclusion, we found a new method to mask the 2,3-π bond of indole alkaloids and to convert the protected compounds, i.e., 2,3-ethylene glycol adducts, back to the starting indoles. This procedure was utilized for the modification of the benzene ring of the indoline derivative and was applied to the preparation of potent opioid receptor agonists with the Corynanthe skeleton, one of which exhibited 18 times more potent opioid agonistic effect than morphine in in vitro experiments.

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Supporting Information Available: Experimental procedures and copies of ^1H and ^{13}C NMR spectral data for compounds **3**−**6**, **11**, **17**, and **22**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Involvement of μ-opioid receptors in antinociception and inhibition of gastrointestinal transit induced by 7-hydroxymitragynine, isolated from Thai herbal medicine *Mitragyna speciosa*

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Abstract

7-Hydroxymitragynine, a constituent of the Thai herbal medicine *Mitragyna speciosa*, has been found to have a potent opioid antinociceptive effect. In the present study, we investigated the mechanism of antinociception and the inhibitory effect on gastrointestinal transit of 7-hydroxymitragynine, and compared its effects with those of morphine. When administered subcutaneously to mice, 7-hydroxymitragynine produced antinociceptive effects about 5.7 and 4.4 times more potent than those of morphine in the tail-flick (ED_{50}=0.80 mg/kg) and hot-plate (ED_{50}=0.93 mg/kg) tests, respectively. The antinociceptive effect of 7-hydroxymitragynine was significantly blocked by the μ_1/μ_2-opioid receptor antagonist β-funaltrexamine hydrochloride (β-FNA) and the μ_1-opioid receptor-selective antagonist naloxonazine in both tests. Thus, 7-hydroxymitragynine acts predominantly on μ-opioid receptors, especially on μ_1-opioid receptors. Isolated tissue studies further supported its specificity for the μ-opioid receptors. Further, 7-hydroxymintragynine dose-dependently (ED_{50}=1.19 mg/kg, s.c.) and significantly inhibited gastrointestinal transit in mice, as morphine does. The inhibitory effect was significantly antagonized by β-FNA pretreatment, but slightly antagonized by naloxonazine. The ED_{50} value of 7-hydroxymitragynine on gastrointestinal transit was larger than its antinociceptive ED_{50} value. On the other hand, morphine significantly inhibits gastrointestinal transit at a much smaller dose than its antinociceptive dose. These results suggest that μ-opioid receptor mechanisms mediate the antinociceptive effect and inhibition of gastrointestinal transit. This compound induced more potent antinociceptive effects and was less constipating than morphine.

Keywords: 7-Hydroxymitragynine; Antinociception; Gastrointestinal transit; μ-Opioid receptor; Morphine

1. Introduction

7-Hydroxymitragynine (Fig. 1) is a minor constituent of *Mitragyna speciosa* (Ponglux et al., 1994). This herb has long been used in Thailand for its opium- (Burkill, 1935) and coca-like effects, and as a replacement for opium (Grewal, 1932; Suwanlert, 1975). We have studied the pharmacological activities of mitragynine, a major alkaloid of this herb (Watanabe et al., 1997; Matsumoto et al., 2005), and related alkaloids (Yamamoto et al., 1999; Takayama et al., 2002; Takayama, 2004; Matsumoto et al., 2006) and found that these compounds have opioid activities. Recently, we studied the opioid agonistic effects of the constituents of *M. speciosa* using in vitro assays. Among them, 7-hydroxymitragynine, which has a hydroxyl group at the C7 position of mitragynine, produced the most potent effect, which

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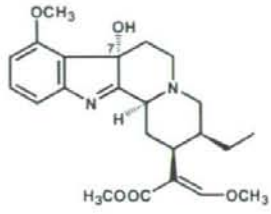

Fig. 1. Chemical structure of 7-hydroxymitragynine.

suggests that the opioid effect of *M. speciosa* is mostly based on the activity of 7-hydroxymitragynine (Horie et al., 2005). 7-Hydroxymitragynine induced potent antinociceptive effects in mouse tail-flick and hot-plate tests, and its effects were more potent than those of morphine when subcutaneously or orally administered (Matsumoto et al., 2004). Receptor-binding assays revealed that 7-hydroxymitragynine has a higher affinity for μ-opioid receptors than for the other opioid receptor types (Takayama et al., 2002; Matsumoto et al., 2004) (Fig. 1).

μ-Opioids represent the major class of strong analgesics, such as morphine, used clinically. Morphine plays an important role as a pain-relieving agent, but it has a number of adverse effects, e.g., respiratory depression, nausea, vomiting, constipation, tolerance, and dependence. Constipation can become a major problem during chronic opioid administration (Schug et al., 1992; McQuay, 1999; Portenoy, 1996), and relief from the adverse gastrointestinal effects markedly enhances the quality of life for patients. In the case of morphine, the dose required for its analgesic effect is much higher than that required for its constipating effect; thus, when morphine is used for analgesia, constipation is not a negligible issue (Megens et al., 1998).

Opioid receptors are widely distributed throughout the central and peripheral nervous system and play a fundamental role in pain and its adverse effects (Quock et al., 1999). In the present study, we studied the opioid receptor mechanisms of 7-hydroxymitragynine by using selective opioid antagonists in in vivo and in vitro assays to clarify the mechanism by which 7-hydroxymitragynine produces antinociceptive effects in mice. In addition, we investigated the inhibition of gastrointestinal transit to evaluate the constipating effect of 7-hydroxymitrgynine in comparison with morphine.

2. Materials and methods

2.1. Experimental animals

Male ddY-strain mice (Japan SLC, Hamamatsu, Japan) weighing 25–32 g and male albino guinea-pigs (Japan SLC) weighing 320–550 g were used. Animals were housed in a temperature-controlled room at 24 °C with lights on from 07:00–19:00 and had free access to food and water. All experiments were performed in compliance with the "Guiding Principles for the Care and Use of Laboratory Animals" approved by the Japanese Pharmacological Society and the guidelines approved by the Ethical Committee on Animal Care and Animal Experimentation of Josai International University (#12). The number of animals used was kept to the minimum necessary for a meaningful interpretation of the data, and animal discomfort was kept to the minimum.

2.2. Drugs

The drugs used in this study were morphine hydrochloride (Takeda Chemical Ind., Osaka, Japan), naloxone hydrochloride (MP Biomedicals, Irvine, CA), [D-Pen^2, D-Pen^5]-enkephalin (DPDPE; Bachem, Torrance, CA), naltrindole hydrochloride, cyprodime hydrobromide, nor-binaltorphimine dihydrochloride (norBNI), naloxonazine dihydrochloride, naloxone methiodide, [D-Ala^2, *N*-MePhe^4, Gly-ol^5]-enkephalin (DAMGO), (5α,7α,8β)-(+)-*N*-Methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide (U69593) (Sigma Chemical Co., St. Louis, MO, USA), and β-funaltrexamine hydrochloride (β-FNA; Tocris-Cookson, Bristol, UK). 7-Hydroxymitragynine was synthesized from mitragynine as described previously (Takayama et al., 2002). The purity (>99%) of these compounds was checked by high-performance liquid chromatography and ^{1}H-nuclear magnetic resonance (500 MHz) analysis (Takayama et al., 2002).

For the in vivo assays, 7-hydroxymitragynine was dissolved in phosphate-buffered saline (pH 5.5). The other drugs were dissolved in saline. 7-Hydroxymitragynine was administered subcutaneously (s.c.) using a volume of 0.1 ml/10 g body weight. The opiate antagonists, naloxone methiodide (3 mg/kg), naloxone (2 mg/kg), naltrindole (3 mg/kg), norBNI (20 mg/kg), naloxonazine (35 mg/kg), and β-FNA (40 mg/kg), were administered s.c. 15 min, 30 min, 30 min, 3 h, 24 h, and 24 h, respectively, before 7-hydroxymitragynine or morphine injection (s.c.). These protocols were described by Paul et al. (1989) and Jinsmaa et al. (2004). High doses of naloxone and naloxone methiodide were used to ensure maximum antagonism. For the in vitro assays, 7-hydroxymitragynine and cyprodime hydrobromide were first dissolved in 100% dimethylsulfoxide to yield a 5 mM solution, and then subsequently diluted with distilled water. The other drugs were dissolved in distilled water.

2.3. Antinociceptive activity

2.3.1. Tail-flick test

The method was adapted from that of D'Amour and Smith (1941). Mice respond to a focused heat stimulus by flicking or moving their tail from the path of the stimulus, thereby exposing a photocell located in the tail-flick analgesia meter (Ugo Basile Tail-flick Unit 7360, Ugo Basile, Comerio, Italy) immediately below the tail. The reaction time is automatically recorded. Prior to treatment with 7-hydroxymitragynine, morphine, vehicle, or saline, the nociceptive threshold was measured three times, and the mean of the reaction time was used as the pre-drug latency for each mouse. A cut-off time of 10 s was used to prevent tissue damage.

2.3.2. Hot-plate test

Animals were placed on an electrically heated plate at 55± 0.2 °C, and the latency period until the occurrence of nociceptive responses such as licking, shaking the legs, or jumping was measured. Prior to treatment with 7-hydroxymitragynine, morphine, vehicle, or saline, the nociceptive threshold was measured

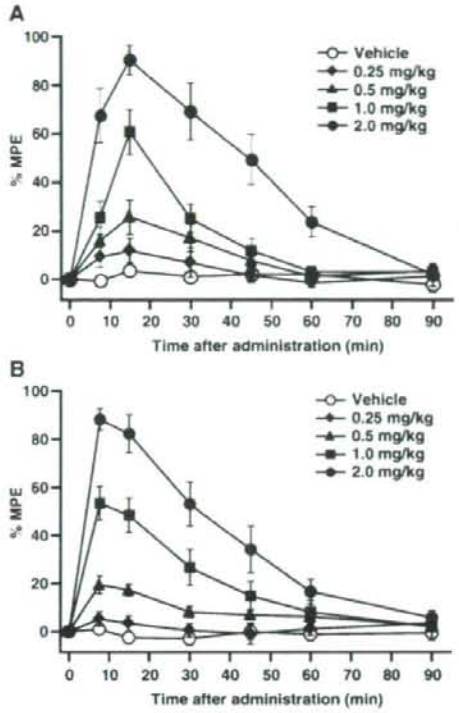

Fig. 2. Time course of the antinociceptive effects produced by s.c. administration of 7-hydroxymitragynine (0.25–2.0 mg/kg) in the tail-flick test (A) and hot-plate test (B) in mice. Each value represents mean ± S.E.M. of data obtained from seven or eight mice.

three times, and the mean reaction time was used as the pre-drug latency for each mouse. The cut-off time of 30 s was used to prevent tissue damage.

Antinociception in tail-flick and hot-plate tests was quantified using the percentage of maximum possible effect (% MPE) and calculated as: % MPE = [(test latency − pre-drug latency)/(cut-off time − pre-drug latency)] × 100.

2.4. Electrical stimulation of guinea-pig ileum

The guinea-pig ileum was dissected and placed in Krebs-Henseleit solution (mM): NaCl, 112.08; KCl, 5.90; $CaCl_2$, 1.97; $MgCl_2$, 1.18; NaH_2PO_4, 1.22; $NaHCO_3$, 25.00, and glucose, 11.49. The ileum was placed under 1 g tension in a 5 ml organ bath containing the nutrient solution. The bath was maintained at 37 °C and continuously bubbled with a mixture of 95% O_2 and 5% CO_2. Tissues were stimulated by a platinum needle-ring (the ring was placed 20 mm above the base of a 5 mm long needle) electrode. After equilibration, the ileum was transmurally stimulated (Cox and Weinstock, 1966) with monophasic pulses (0.2 Hz and 0.1 ms duration) by a stimulator (SEN-7203, Nihon Kohden, Tokyo, Japan). Contractions were isotonically recorded by using a displacement transducer (NEC Type 45347, San-ei Instruments Ltd., Tokyo, Japan). The effects of drug treatments on the twitch contractions evoked by transmural stimulation elicited through the ring electrodes were examined. The height of the twitch response to transmural stimulation was measured before and after the drug challenge. The responses were expressed as inhibition % of the twitch response to the transmural stimulation before the drug challenge.

2.5. Electrical stimulation of mouse vas deferens

The mouse vas deferens was dissected and placed in Krebs–Henseleit solution without $MgCl_2$. The tissues were placed under 0.2 g tension in a 5 ml organ bath containing the nutrient solution. The bath was maintained at 37 °C and continuously bubbled with a mixture of 95% O_2 and 5% CO_2. Tissues were stimulated by a platinum needle-ring (the ring was placed 20 mm above the base of a 5 mm long needle) electrode. After equilibration, the tissues were transmurally stimulated with a train of 10 pulses of 0.5 ms duration with 2 ms intervals by a stimulator (SEN-7203, Nihon Kohden, Tokyo, Japan) every 1 min. Contractions were isometrically recorded by using a displacement transducer (NEC Type 45347, San-ei Instruments Ltd., Tokyo, Japan). The effects of drug treatments on the twitch contractions evoked by

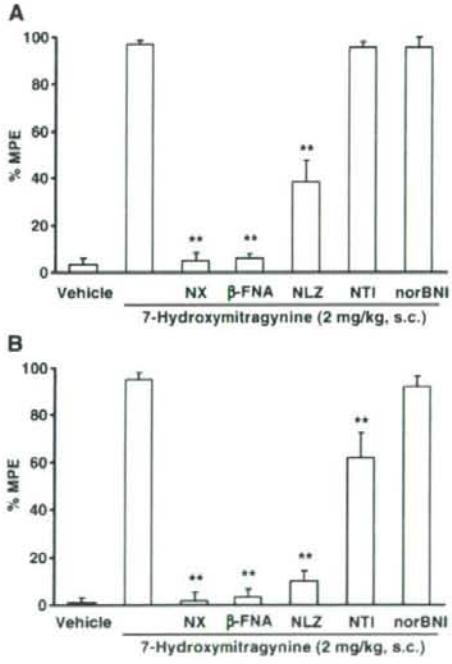

Fig. 3. Effects of opioid receptor antagonists on the antinociception by 7-hydroxymitragynine (2 mg/kg) after s.c. administration. The antinociceptive effect of 7-hydroxymitragynine was determined in the mice tail-flick test (A) and the hot-plate test (B) after s.c. administration of the following antagonists: naloxone (NX; 2 mg/kg), β-funaltrexamine (β-FNA; 40 mg/kg), naloxonazine (NLZ; 35 mg/kg), naltrindole (NTI; 3 mg/kg), and nor-binaltorphimine (norBNI; 20 mg/kg). Measurements were performed 15 and 7.5 min after s.c. administration of 7-hydroxymitragynine in the tail-flick and hot-plate tests, respectively. Each value represents mean ± S.E.M. of seven or eight mice. The asterisk (*) denotes values that were significantly different from 7-hydroxymitragynine-treated mice by Bonferroni test (**, $P<0.01$).

Table 1
Effect of cyprodime on twitch contraction inhibition by 7-hydroxymitragynine, DAMGO, and U69593 in guinea-pig ileum

Compound (concentration)	Contraction (%) inhibited by compound	Contraction (%) reversed by cyprodime	
		30 nM	1 μM
7-Hydroxymitragynine (100 nM)	19.5 ± 1.6	59.9 ± 5.0[a]	108.2 ± 9.2[a]
DAMGO (100 nM)	12.5 ± 3.8	41.7 ± 10.8	104.5 ± 9.2[a]
U69593 (1 μM)	11.7 ± 3.2	11.4 ± 2.6	15.7 ± 1.4

Each value represents the mean ± S.E.M. of five animals.
[a] $P < 0.01$ significantly different from the values before the addition of cyprodime (Bonferroni multiple comparison test).

transmural stimulation elicited through the ring electrodes were examined. The height of the twitch response to transmural stimulation was measured before and after the drug challenge. The responses were expressed as % inhibition of the twitch response to the transmural stimulation before the drug challenge.

2.6. Gastrointestinal transit

Mice were fasted, with water available ad libitum, for 18 h before the experiments. Fifteen minutes after s.c. injection of 7-hydroxymitragynine, morphine, vehicle, or saline, a charcoal meal (an aqueous suspension of 10% charcoal and 5% gum arabic) was orally administered at a volume of 0.25 ml. Thirty minutes after administration of the charcoal meal, the animal was sacrificed by cervical dislocation, and the small intestine from the pylorus to the cecum was carefully removed. Both the length of the small intestine from the pylorus to the cecum and the farthest distance to which the charcoal meal had traveled were measured. For each animal, the gastrointestinal transit (GIT) was calculated as the percentage of distance traveled by the charcoal meal relative to the total length of the small intestine. The inhibition of gastrointestinal transit (%) was calculated as: Inhibition of gastrointestinal transit (%) = [(saline or vehicle GIT − drug GIT) / (saline or vehicle GIT)] × 100.

2.7. Statistical analysis

The data are expressed as the mean ± S.E.M. Statistical analyses were performed with two-tailed Student's *t*-test for comparison of two groups, and by a one-way analysis of variance, followed by a Bonferroni multiple comparison test for comparison of more than two groups. A P value < 0.05 was considered statistically significant. ED_{50} values and 95% confidence limits were determined using the Litchfield–Wilcoxon method (Litchfield and Wilcoxon, 1949).

Table 2
Effect of nor-binaltrophimine (norBNI) on twitch contraction inhibited by 7-hydroxymitragynine, DAMGO, and U69593 in guinea-pig ileum

Compound (concentration)	Contraction (%) inhibited by compound	Contraction (%) reversed by norBNI	
		1 nM	30 nM
7-Hydroxymitragynine (100 nM)	15.6 ± 3.8	18.6 ± 3.7	29.2 ± 3.6
DAMGO (100 nM)	8.7 ± 2.9	9.4 ± 3.4	10.3 ± 3.2
U69593 (1 μM)	10.5 ± 5.3	13.8 ± 6.9	118.1 ± 8.5[a]

Each value represents the mean ± S.E.M. of five animals.
[a] $P < 0.01$ significantly different from the values before the addition of norBNI (Bonferroni multiple comparison test).

Table 3
Effect of naltrindole on twitch contraction inhibited by 7-hydroxymitragynine, DPDPE, DAMGO, and U69593 in mouse vas deferens

Compound (concentration)	Contraction (%) inhibited by compound	Contraction (%) reversed by naltrindole	
		3 nM	30 nM
7-Hydroxymitragynine (300 nM)	7.8 ± 1.5	8.4 ± 1.9	18.9 ± 2.9[a]
DPDPE (100 nM)	12.1 ± 3.2	42.5 ± 8.0[b]	83.8 ± 2.9[b]
DAMGO (300 nM)	11.9 ± 2.1	13.8 ± 3.3	19.4 ± 3.6
U69593 (1 μM)	19.1 ± 4.8	21.4 ± 5.2	24.0 ± 6.9

Each value represents the mean ± S.E.M. of five animals.
[a] $P < 0.05$.
[b] $P < 0.01$ significantly different from the values before the addition of naltrindole (Bonferroni multiple comparison test).

3. Results

3.1. Antinociceptive effect of 7-hydroxymitragynine in mice

7-Hydroxymitragynine (0.25–2 mg/kg, s.c.) induced dose-related antinociceptive responses in the tail-flick and hot-plate tests (Fig. 2). The effect peaked at 15 and 7.5 min after injection in the tail-flick and hot-plate tests, respectively. The ED_{50} values (95% confidence limits) for 7-hydroxymitragynine were 0.80 mg/kg (0.48–1.33) and 0.93 mg/kg (0.59–1.45) in the tail-flick and the hot-plate tests, respectively. The vehicle did not show any antinociceptive activity in either test.

Morphine (1.25–8 mg/kg, s.c.) produced a dose-related antinociceptive response with a peak effect at 30 min in both tests (data not shown). The ED_{50} values (95% confidence limits) for morphine were 4.57 mg/kg (3.12–6.69) and 4.08 mg/kg (2.75–6.06) in the tail-flick and hot-plate tests, respectively. Compared to morphine on a mg/kg (μmol/kg) basis, 7-hydroxymitragynine was 5.7 (6.3) and 4.4 (4.9) times more potent in the tail-flick and hot-plate tests, respectively (Fig. 4A, B, Table 5). Antinociception elicited by 7-hydroxymitragynine affected behavioral responses: 2 mg/kg of 7-hydroxymitragynine

Table 4
pD_2 values for inhibition of electrically stimulated contraction by 7-hydroxymitragynine, DAMGO, and U69593 in guinea-pig ileum, and pA_2 values of naloxone inhibition of 7-hydroxymitragynine, DAMGO, and U69593

	pD_2	pA_2	Slope
7-Hydroxymitragynine	7.78 ± 0.08	8.95 ± 0.30	0.91 ± 0.20
DAMGO	7.83 ± 0.07	8.77 ± 0.35	1.18 ± 0.18
U69593	9.01 ± 0.12	7.50 ± 0.36	1.19 ± 0.09

pD_2 values are the negative logarithm of the IC_{50} values. The pA_2 values are calculated from parallel shifts of the curves for the agonists. Data are expressed as the mean ± S.E.M. of five animals.

elicited an increase spontaneous locomotor activity and Straub tail, as did 8 mg/kg of morphine (data not shown).

In order to determine the opioid receptor type selectivity of 7-hydroxymitragynine antinociception, mice were pretreated with selective opioid receptor antagonists (Fig. 3). We chose a dose of 7-hydroxymitragynine that produces a response of 80–90% (Fig. 2) to detect the effects of the antagonist easily. In the tail-flick test, the antinociceptive effect of 7-hydroxymitragynine was significantly blocked by the non-selective opioid antagonist naloxone, the irreversible μ_1/μ_2-opioid receptor selective antagonist β-FNA, and the μ_1-opioid receptor selective antagonist naloxonazine. The selective δ-antagonist naltrindole and the selective κ-antagonist norBNI were ineffective against 7-hydroxymitragynine-mediated antinociception (Fig. 3A). In the hot-plate test, the effect of 7-hydroxymitragynine was significantly blocked by naloxone, β-FNA, and naloxonazine, and was partially (38%) blocked by naltrindole. The κ-opioid receptor antagonist norBNI was ineffective against 7-hydroxymitragynine-induced antinociception (Fig. 3B). When these opioid antagonists were administered subcutaneously alone at the doses used in the present study, they did not produce any changes in the tail-flick and hot-plate test results (data not shown).

3.2. Effects of 7-hydroxymitragynine on electrically induced contraction in guinea-pig ileum and mouse vas deferens

To investigate which opioid receptor subtypes are involved in the pharmacological effects of 7-hydroxymitragynine, in vitro biological activities were investigated in electrically stimulated guinea-pig ileum and mouse vas deferens assays using μ-, δ-, and κ-opioid selective agonists and antagonists (Tables 1–3). Isolated guinea-pig ileum was used to examine μ- and κ-opioid receptors and mouse vas deferens for δ-opioid receptors because it has been shown that predominantly μ- and κ-opioid receptors are expressed in the guinea-pig ileum (Chavkin and Goldstein, 1981), while δ-opioid receptors are expressed in mouse vas deferens (Hughes et al., 1975). Cyprodime (1 μM), a μ-opioid receptor antagonist, completely reversed the twitch contraction inhibition by 7-hydroxymitragynine and the μ-agonist DAMGO, but did not reverse the effect of the κ-opioid receptor agonist U69593 in guinea-pig ileum. In contrast, norBNI (30 nM), a κ-opioid receptor antagonist, completely reversed the inhibitory effect of U69593, but did not reverse the effect of 7-hydroxymitragynine and DAMGO in guinea-pig ileum. Naltrindole (30 nM), a δ-opioid receptor antagonist, completely reversed the inhibitory effect of the δ-agonist DPDPE and slightly reversed the effect of 7-hydroxymitragynine but not DAMGO and U69593 in the mouse vas deferens.

To further investigate the involvement of μ-opioid receptors in the effect of 7-hydroxmitragynine on twitch contraction induced by electrical stimulation in guinea-pig ileum, we compared the pA_2 values of naloxone in the concentration–response curves for DAMGO, U69593, and 7-hydroxmitragynine (Table 4). In the absence of naloxone, 7-hydroxmitragynine, DAMGO, and U69593 inhibited the ileal contraction. The concentration–response curves for 7-hydroxmitragynine, DAMGO, and U69593 were shifted to the right in the presence of naloxone (data not shown). The slope factors for 7-hydroxymitragynine, DAMGO, and U69593 were not significantly different from unity, suggesting competitive inhibition. The pA_2 values of naloxone were 8.95±0.30 for 7-hydroxymitragynine, 8.77±0.35 for DAMGO, and 7.50±0.36 for U69593.

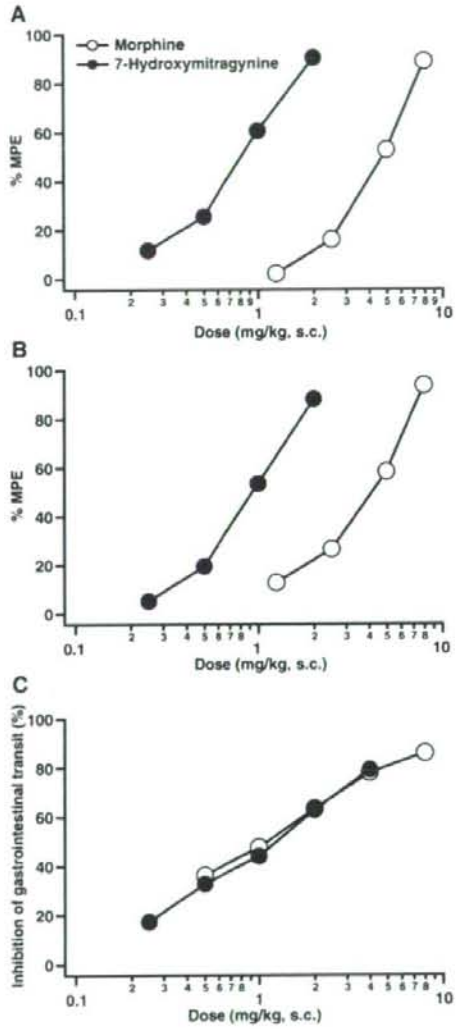

Fig. 4. Dose–response curves of antinociceptive effect and inhibitory effect on gastrointestinal transit of subcutaneous administration of morphine and 7-hydroxymitragynine in (A) tail-flick test, (B) hot-plate test, and (C) gastrointestinal transit. Antinociceptive effect of 7-hydroxymitragynine was assessed 15 and 7.5 min after the administration in the tail-flick and hot-plate tests, respectively. Antinociceptive effect of morphine was assessed 30 min after injection in the tail-flick and hot-plate tests. For the gastrointestinal transit study, 7-hydroxymitragynine and morphine were administered s.c. 15 min before oral administration of a charcoal meal. Gastrointestinal transit was measured at 30 min after administration of the charcoal meal.

Table 5
Antinociceptive, inhibitory effects on gastrointestinal transit (IGIT), and relative potency of 7-hydroxymitragynine compared with morphine in mice

Compound	Tail-flick (TF) ED_{50}	Hot-plate (HP) ED_{50}	IGIT ED_{50}	TF/IGIT	HP/IGIT
Morphine	4.57 (3.12–6.69)	4.08 (2.75–6.06)	1.07 (0.40–2.86)	4.27	3.81
7-Hydroxymitragynine	0.80 (0.48–1.33)	0.93 (0.59–1.45)	1.19 (0.54–2.63)	0.67	0.78
Relative potency	5.7	4.4	0.9		

ED_{50} represents the median effective dose (mg/kg) (95% confidence limits).
Relative potencies were calculated as morphine ED_{50}/7-hydroxymitragynine ED_{50} in each test.

3.3. Effect of 7-hydroxymitragynine on gastrointestinal transit

The effect of 7-hydroxymitragynine on the passage of a charcoal meal was examined. 7-Hydroxymintragynine (0.25–4 mg/kg, s.c.) and morphine (0.5–8 mg/kg, s.c.) dose-dependently and significantly inhibited gastrointestinal transit (Fig. 4C). The ED_{50} values (95% confidence limits) for 7-hydroxymitragynine and morphine were 1.19 mg/kg (0.54–2.63) and 1.07 mg/kg (0.40–2.86), respectively (Table 5).

The inhibitory effects of 7-hydroxymitragynine and morphine on gastrointestinal transit were similar, and both were significantly antagonized by pretreatment with the μ_1/μ_2-opioid receptor selective antagonist β-FNA (40 mg/kg). The μ_1-opioid receptor antagonist naloxonazine (35 mg/kg) slightly blocked the effects of 7-hydroxymitragynine and morphine. The peripheral opioid receptor antagonist naloxone methiodide slightly blocked the effect of 7-hydroxymitragynine and significantly blocked the effect of morphine (Fig. 5). No change in the gastrointestinal transit was observed when each antagonist was administered alone (data not shown).

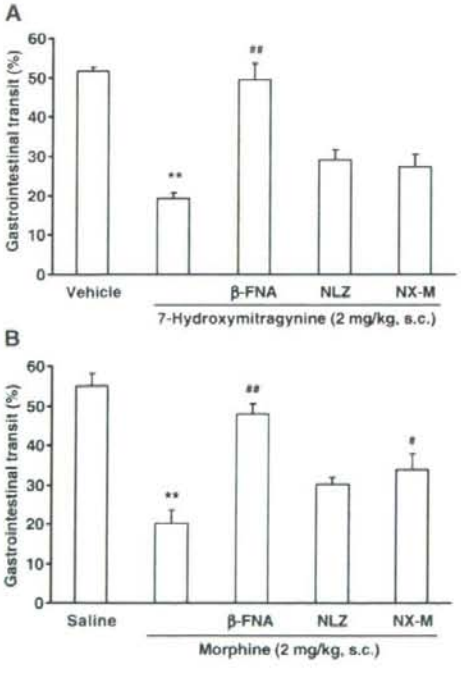

Fig. 5. Antagonism of the antitransit effect of a single dose (2 mg/kg, s.c.) of 7-hydroxymitragynine (A) and morphine (B) by β-funaltrexamine (β-FNA; 40 mg/kg), naloxonazine (NLZ; 35 mg/kg), and naloxone methiodide (NX-M; 3 mg/kg). Each value represents the mean ± S.E.M. of six or seven mice. The asterisk (*) denotes values that were significantly different from saline- or vehicle-treated mice by Student's *t*-test (**, $P<0.01$). # denotes values that were significantly different from mice treated with the agonist alone by Bonferroni test (*, $P<0.05$; **, $P<0.01$).

4. Discussion

We previously reported that 7-hydroxymitragynine is a more potent antinociceptive than morphine in mice when subcutaneously or orally administered (Matsumoto et al., 2004). The aim of this study was to investigate the mechanism of antinociception and the inhibitory effect on gastrointestinal transit of 7-hydroxymitragynine using selective opioid receptor antagonists, and to compare those effects with morphine. To evaluate the antinociceptive effect of the 7-hydroxymitragynine, acute thermal pain (tail-flick and hot-plate) tests were performed. The tail-flick test was used to study the possible involvement of spinal opioid receptors, whereas the hot-plate test was used to study the possible involvement of supraspinal receptors (Yaksh, 1999). 7-Hydroxymitragynine produced potent dose-dependent antinociceptive effects about 5.7 and 4.4 times more potent than morphine in the tail-flick and hot-plate tests, respectively. The higher potency and rapider effect of 7-hydroxymitragynine may be a result of its higher lipophilicity and its ease in penetrating the blood–brain barrier. Indeed, it has been shown that analgesics with high lipophilicity, such as fentanyl, rapidly penetrate the blood–brain barrier, and thus fentanyl produces more potent and rapid antinociception than morphine does (Narita et al., 2002).

Selective antagonists were employed in order to clarify the involvement of the opioid receptor subtypes in the antinociceptive effect of 7-hydroxymitragynine. μ-Opioid receptors are divided into two distinct subtypes that mediate antinociception at the spinal and supraspinal levels, the μ_1-opioid receptor being important for supraspinal antinociception, whereas the μ_2-opioid receptor is involved in spinal antinociception (Ling and Pasternak, 1983; Bodnar et al., 1988; Paul et al., 1989). To investigate the relative involvement of μ_1- and μ_2-opioid receptors in spinal and supraspinal antinociception of 7-hydroxymitragynine, the μ_1/μ_2-opioid receptor antagonist β-FNA and the μ_1-opioid antagonist naloxonazine were used (Sakurada et al., 1999). It was found that the antinociceptive effects of 7-hydroxymitragynine are mediated primarily through the μ-opioid

receptors because the μ_1 μ_2-opioid receptor antagonist β-FNA almost completely blocked the effect in the tail-flick and hot-plate tests. In addition, naloxonazine has been shown to preferentially block μ_1-opioid receptors rather than μ_2-opioid receptors (Sakurada et al., 1999). Naloxonazine significantly blocked the antinociceptive effect of 7-hydroxymitragynine in the tail-flick and hot-plate tests, suggesting that the antinociception induced by 7-hydroxymitragynine is highly involved with the μ_1-opioid receptors. However, it was also found that the effect of 7-hydroxymitragynine was partially blocked by the δ-selective antagonist naltrindole in the hot-plate test, suggesting partial involvement of the supraspinal δ-opioid receptors. In addition, Thongpradichote et al. (1998) revealed that mitragynine, which is a main constituent of *M. speciosa* and has structural similarities to 7-hydroxymitragynine, has an antinociceptive activity through the supraspinal μ- and δ-opioid receptors. At this moment, we can only speculate that the supraspinal δ-opioid receptors are involved in the antinociceptive effect of 7-hydroxymitragynine, and further studies are in progress to try to clarify the mechanism of its antinociception.

Studies of the isolated guinea-pig ileum and mouse vas deferens in electrical field stimulation preparations further established the opioid receptor specificity of the effect of 7-hydroxymitragynine. The μ-opioid selective antagonist cyprodime completely reversed the inhibitory effect of 7-hydroxymitragynine, confirming a mechanism of action through the μ-opioid receptors. The pA_2 values of the opioid antagonist naloxone against the inhibitory action of the μ opioid agonist DAMGO and κ opioid agonist U69593 represent the affinity of naloxone for μ- and κ-opioid receptors, respectively. The pA_2 value of naloxone against 7-hydroxymitragynine was very similar to that against DAMGO, but clearly different from that against U69593. These results support the hypothesis that 7-hydroxymitragynine inhibited the electrically stimulated ileum contraction through the μ-opioid receptors. Previous radioligand binding studies (Takayama et al., 2002; Matsumoto et al., 2004) also support the involvement of the μ-opioid receptors in the effect of 7-hydroxymitragynine. Taken together, the results obtained in vitro assay systems confirm that the antinociceptive effect of 7-hydroxymitragynine is mediated by μ-opioid receptors.

Opioids are well known to inhibit gastrointestinal transit. In the case of morphine, the dose required for its analgesic effect is much higher than required for its constipating effects. As previously reported, mitragynine has a minimal effect on gastric motility at its analgesic levels (Macko et al., 1972). We investigated the inhibition of gastrointestinal transit to evaluate the constipating effect of 7-hydroxymitragynine and its antinociceptive effect in comparison to morphine. 7-Hydroxymitragynine inhibited gastrointestinal transit in a dose-dependent manner, as morphine did. The ratios of ED_{50} values for the antinociceptive effect in the tail-flick or hot-plate test and inhibitory effect on gastrointestinal transit (IGIT) are shown in Table 5. The IGIT ED_{50} value of 7-hydroxymitragynine was larger than that of its antinociceptive ED_{50}. On the other hand, morphine significantly inhibited gastrointestinal transit at much smaller doses than its antinociceptive doses. The IGIT ED_{50} of morphine was about 4.3 and 3.8 times smaller than those of its tail-flick ED_{50} and hot-plate ED_{50} values, respectively. These results suggest that 7-hydroxymitragynine induces constipation 4.9–6.4 times less potently than morphine at antinociceptive doses.

It appears that among opiate receptors the μ-opioid receptors play a prominent role in morphine-induced constipation (Roy et al., 1998). We investigated the pharmacological effects of 7-hydroxymitragynine on gastrointestinal transit. The inhibitory effects of 7-hydroxymitragynine and morphine were markedly blocked by β-FNA, indicating that their effects are mediated by μ-opioid receptors. It is well known that the inhibitory effects on the gut of systemic morphine administration are mediated by opioid receptors located at central and peripheral sites (Goldberg et al., 1998; Shook et al., 1987). We investigated the effect of 7-hydroxymitragynine using centrally and peripherally acting antagonists. The inhibitory effects of 7-hydroxymitragynine and morphine were slightly blocked by the centrally acting μ_1-opioid antagonist naloxonazine. We also investigated the peripheral component using naloxone methiodide, which has restricted access to the central nervous system (Lewanowitsch and Irvine, 2002). Naloxone methiodide slightly blocked the effects of 7-hydroxymitragynine, although it moderately and significantly blocked the effects of morphine. These results suggest that 7-hydroxymitragynine inhibits gastrointestinal propulsive activity through central and peripheral action of the opioid receptors. These findings allow us to speculate that 7-hydroxymitragynine interacts less with the peripheral opioid receptors than morphine in the inhibition of gastrointestinal transit.

Opioid analgesia has been suggested to be mediated by μ_1-opioid receptors, whereas the μ_2-receptors seemed to be involved in the inhibition of gastrointestinal transit (Pasternak, 1993). The μ_1/μ_2-opioid receptor antagonist β-FNA significantly blocked the antinociceptive and anti-transit effect of 7-hydroxymitragynine. The μ_1-opioid antagonist naloxonazine significantly blocked the antinociceptive effect but slightly blocked the anti-transit effect. Taken together, it is suggested that antinociception and inhibition of gastrointestinal transit by 7-hydroxymitragynine are mediated predominantly by μ_1- and μ_2-opioid receptors, respectively.

7-Hydroxymitragynine appears to be a potent μ-opioid agonist with interesting pharmacological properties. The antinociceptive effect of 7-hydroxymitragynine was about 4.4–5.7 times more potent than that of morphine. Furthermore, 7-hydroxymitragynine is about 4.9–6.4 times less constipating than morphine at equi-antinociceptive doses. In conclusion, 7-hydroxymitragynine shows promising characteristics as an analgesic.

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NOTE

New heteroyohimbine-type oxindole alkaloid from the leaves of Thai *Mitragyna hirsuta*

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Abstract A new oxindole alkaloid, isomitraphyllinol (**1**), was isolated from the leaves of Thai *Mitragyna hirsuta*, together with five known oxindole alkaloids (**2**–**6**). The structure of the new compound was determined by spectroscopic analysis.

Keywords Mitragyna hirsuta · Rubiaceae · Alkaloid · Indole · Oxindole · Structure determination

Introduction

Our recent chemical and pharmacological studies on the *Mitragyna* alkaloids [1–4] have demonstrated that 7-hydroxymitragynine [5], a minor indole alkaloid from the leaves of *Mitragyna speciosa* (Rubiaceae), is a novel opioid agonist having a structure different [6] from that of morphine, and has potent analgesic activity when administered orally [6, 7]. These findings prompted us to further investigate the alkaloids of other *Mitragyna* plants [8]. We report, herein, the isolation and structure elucidation of one new heteroyohimbine-type oxindole alkaloid, isomitraphyllinol (**1**), from *Mitragyna hirsuta* Havil [9–11] collected in Thailand, together with five known alkaloids.

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Results and discussion

From the methanol (MeOH) extract of the leaves of *Mitragyna hirsuta* collected in Thailand one new heteroyohimbine-type oxindole alkaloid, isomitraphyllinol (**1**), was isolated, together with four heteroyohimbine-type oxindole alkaloids, i.e., mitraphylline (**2**) [12, 13], isomitraphylline (**3**) [12], isomitraphylline *N*-oxide (**4**) [14], and isopteropodine (**5**) [12], and one Corynanthe-type oxindole alkaloid, rhynchophylline (**6**) [13, 15] (Fig. 1). The structures of the known compounds were deduced from spectroscopic data and confirmed by comparison with authentic samples and reported data.

Alkaloid **1** was shown to have the molecular formula $C_{21}H_{26}N_2O_5$ from its high-resolution (HR)-FAB-MS (*m/z* 387.1928 $[MH]^+$), which indicated that **1** has an extra H_2O compared to isomitraphylline (**3**). The ultraviolet (UV) absorptions at 286.5, 252.0, and 207.5 nm revealed an oxindole nucleus. The ^{1}H-NMR spectrum of **1** showed signals indicating the presence of four aromatic protons of the A ring of the oxindole system at δ 7.37 (d, H-9), 7.05 (ddd, H-10), 7.21 (ddd, H-11), and 6.85 (d, H-12); an oxygenated methine proton at δ 4.20 (qd, H-19); a carboxymethyl group at δ 3.62 (3H, s); and a methyl group at δ 1.25 (3H, d, H_3-18); as well as a quartet signal assignable to H-14β at δ 0.76, which are very similar to those of **3**. Furthermore, a doublet signal of an acetal proton was observed at δ 5.05 (d, H-17), instead of the characteristic signal of a β-acrylic ester residue existing in **3**. The ^{13}C nuclear magnetic resonance (NMR) spectrum showed signals assignable to an acetal carbon at δ 90.9, a carbonyl carbon due to the oxindole nucleus at δ 180.7, and an ester carbonyl carbon at δ 172.1. Heteronuclear

multiple-bond correlation (HMBC) correlation between H-19 (δ 4.20) and the acetal carbon (δ 90.9) indicated that **1** possesses a hydroxyl group at C-17 position. The configurations at C-16 and C-17 positions were deduced from the coupling constants in the ^{1}H-NMR spectrum and nuclear Overhauser effect (NOE) experiments as follows (Fig. 2). The signal for H-14β appearing as a quartet with large coupling constants with H-3 and H-15 (J=11.8 Hz) indicated that H-14β, H-3, and H-15 are *trans–trans* diaxially oriented. The signal for H-17 appearing as a doublet with a coupling constant of 8.3 Hz with H-16 indicated that H-17 is *trans* diaxially related to H-16. NOE correlations of H-14β/H-16, H-14β/H-20, H-17/H-15, and H-17/H$_3$-18 suggested that 16-COOMe and 17-OH are α- and β-oriented, respectively. The similarity of its CD spectrum with that of isomitraphylline (**3**) and the upfield shift of H-14β caused by the anisotropic effect of the aromatic ring revealed that the absolute configuration at the C-7 spiro center is *S*. Therefore, the structure of the new alkaloid was deduced to be formula **1**. Compound **1** is a hydrate derivative of isomitraphylline (**3**) at the C-16-C-17 double bond.

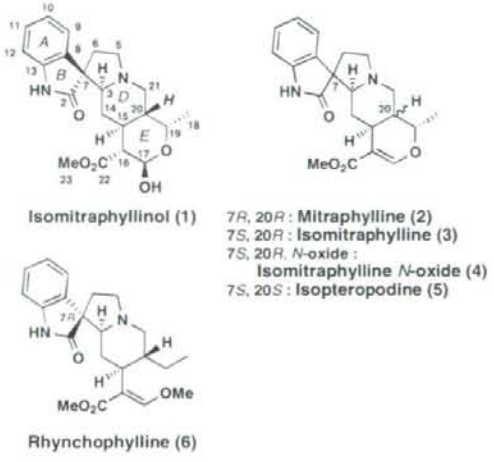

Fig. 1 Structures of isomitraphyllinol (**1**) and known alkaloids (**2–6**)

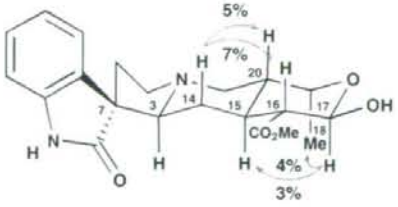

Fig. 2 Nuclear Overhauser effect (NOE) experiments in **1**

Experimental

General

^{1}H- and ^{13}C-NMR spectra were measured on a JEOL JNM A-500 at 500 (^{1}H) and 125.65 MHz (^{13}C), respectively. UV spectra were recorded on a JASCO V-560. For EI-MS, direct probe insertion was performed at 70 eV and the data were recorded on a JEOL GC mate. For HRFAB-MS, a JEOL JMS-HX110 was used. The CD spectra were measured with a JASCO J-720WI. For TLC, precoated silica gel 60 F_{254} plates (Merck, 0.25-mm thick) were used. Open-column chromatography was carried out over silica gel 60 (Merck, 70–230 mesh). For MPLC, C. I. G. prepacked column CPS-HS-221–05 (SiO_2, Kusano Kagakukikai) was used.

Plant material

The leaves of *Mitragyna hirsuta* Havil (1,159 g dry weight) were collected at Chulalongkorn University, Thailand, in February 2005 and identified by Dr. Sumphan Wongseripipatana, Chulalongkorn University, Thailand. A voucher specimen was deposited at the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

Extraction and isolation

The leaves of *M. hirsuta* (1,159 g dry weight) were extracted with MeOH (twice at room temperature and two times under reflux) to give a MeOH extract (325.5 g). The extract was partitioned between H_2O containing a small amount of MeOH and *n*-hexane to give the *n*-hexane extract (78.7 g). The aqueous layer was extracted with AcOEt and then 5% MeOH-$CHCl_3$ to give AcOEt extract (28.2 g) and MeOH-$CHCl_3$ extract (20.6 g), respectively. A portion of the MeOH-$CHCl_3$ extract (19.8 g) was separated by silica gel open-column chromatography with a $CHCl_3$/MeOH gradient to give 12 fractions: fr. A $CHCl_3$ (400 ml) 30 mg; fr. B $CHCl_3$ (600 ml) 26 mg; fr. C 3% MeOH/$CHCl_3$ (300 ml) 238 mg; fr. D 3% (300 ml) 1655 mg; fr. E 3% (900 ml) 856 mg; fr. F 5% (900 ml) 90 mg; fr. G 10% (200 ml) 100 mg; fr. H 10% (400 ml) 207 mg; fr. I 10% (800 ml) 214 mg; fr. J 20% (600 ml) 136 mg; fr. K 50% MeOH/$CHCl_3$ (800 ml) and MeOH (800 ml) 146 mg; and fr. L 10% H_2O-MeOH (800 ml) 54 mg. Fraction F (90 mg) was purified by MPLC (80% AcOEt-*n*-hexane and 5% MeOH-$CHCl_3$) to afford the new compound (**1**, 2.7 mg). Known alkaloids (**2–6**) were, respectively, isolated from the fractions of the

first-column chromatography, as follows: mitraphylline (**2**, 1646.6 mg) from fractions C–H; isomitraphylline (**3**, 246.1 mg) from fractions B–E; isomitraphylline *N*-oxide (**4**, 5.4 mg) from fraction J; isopteropodine (**5**, 0.1 mg) from fraction B; and rhynchophylline (**6**, 2.7 mg) from fraction F. The detailed spectroscopic data of isomitraphylline *N*-oxide (**4**) have not been reported elsewhere. Thus, we report them as follows.

Isomitraphyllinol (**1**)

Amorphous. ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 7.52 (1H, br s, NH), 2.45 (1H, dd, J=11.8, 2.6 Hz, H-3), 3.28 (1H, ddd, J=8.9, 8.9, 2.6 Hz, H-5), 2.50 (1H, ddd, J=8.9, 8.9, 8.9 Hz, H-5), 2.38 (1H, ddd, J=12.8, 8.9, 2.6 Hz, H-6), 2.02 (1H, ddd, J=12.8, 8.9, 8.9 Hz, H-6), 7.37 (1H, d, J=7.6 Hz, H-9), 7.05 (1H, ddd, J=7.6, 7.6, 1.1 Hz, H-10), 7.21 (1H, ddd, J=7.6, 7.6, 1.1 Hz, H-11), 6.85 (1H, d, J=7.6 Hz, H-12), 1.14 (1H, ddd, J=11.8, 2.6, 2.6 Hz, H-14α), 0.76 (1H, ddd, J=11.8, 11.8, 11.8 Hz, H-14β), 1.84 (3H, overlapped, H-15, H-20, and H-21), 1.98 (1H, dd, J=11.0, 8.3 Hz, H-16), 5.05 (1H, d, J=8.3 Hz, H-17), 1.25 (1H, d, J=7.0 Hz, H_3-18), 4.20 (1H, qd, J=7.0, 4.0 Hz, H-19), 3.04 (1H, br d, J=7.9 Hz, H-21), 3.62 (3H, s, H_3-23). ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 180.7 (C-2), 70.9 (C-3), 53.5 (C-5), 35.4 (C-6), 56.3 (C-7), 133.3 (C-8), 125.1 (C-9), 122.7 (C-10), 127.8 (C-11), 109.4 (C-12), 139.8 (C-13), 30.2 (C-14), 34.3 (C-15), 56.3 (C-16), 90.9 (C-17), 14.4 (C-18), 72.0 (C-19), 41.0 (C-20), 54.2 (C-21), 172.1 (C-22), 51.9 (C-23). UV λ_{max}(MeOH) nm (log ε): 286.5 (2.62), 252.0 (3.26), 207.5 (3.88). EI-MS m/z (%): 386 (M^+, 100), 368 (18), 241 (94), 223 (37). HRFAB-MS (NBA/PEG) m/z: 387.1928 (MH^+, calculated for $C_{21}H_{27}N_2O_5$: 387.1920). CD (c=0.260 mmol/l, MeOH, 24°C) $\Delta\varepsilon$ (nm) 0 (304), –2.6 (285), –1.7 (272), –6.0 (257), 0 (246), +10.3 (234), +5.1 (221).

Isomitraphylline *N*-oxide (**4**)

^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 8.86 (1H, br s, NH), 3.70 (1H, dd, J=12.2, 2.6 Hz, H-3), 3.88 (1H, dd, J=10.1, 7.7 Hz, H-5), 3.60 (1H, overlapped, H-5), 2.89 (1H, ddd, J=12.6, 12.6, 7.7 Hz, H-6), 2.47 (1H, dd, J=12.6, 7.3 Hz, H-6), 8.14 (1H, d, J=7.6 Hz, H-9), 7.05 (1H, ddd, J=7.6, 7.6, 1.2 Hz, H-10), 7.21 (1H, ddd, J=7.6, 7.6, 1.2 Hz, H-11), 6.85 (1H, d, J=7.6 Hz, H-12), 2.15 (1H, ddd, J=12.2, 2.6, 2.6 Hz, H-14), 1.75 (1H, ddd, J=12.2, 12.2, 12.2 Hz, H-14), 2.35 (1H, br dd, J=12.2, 12.2 Hz, H-15), 7.44 (1H, d, J=1.8 Hz, H-17), 1.14 (1H, d, J=6.5 Hz, H_3-18), 4.44 (1H, qd, J=6.5, 3.5 Hz, H-19), 3.21 (1H, dddd, J=12.2, 12.2, 3.5, 3.5 Hz, H-20), 3.64 (1H, dd J=10.7, 3.5 Hz, H-21), 3.05 (1H, dd, J=12.2, 10.7 Hz, H-21), 3.58 (3H, s, H_3-23). ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 181.2 (C-2), 80.4 (C-3), 68.6 (C-5), 36.2 (C-6), 54.9 (C-7), 130.8 (C-8), 128.6, and 128.5 (C-9 and C-11), 123.1 (C-10), 109.5 (C-12), 141.0 (C-13), 24.3 (C-14), 30.2 (C-15), 105.8 (C-16), 154.4 (C-17), 14.9 (C-18), 72.8 (C-19), 35.3 (C-20), 66.1 (C-21), 166.8 (C-22), 50.9 (C-23). CD (c=0.56 mmol/l, MeOH, 24°C) $\Delta\varepsilon$ (nm) 0 (307), –1.7 (286), –0.9 (272), –1.7 (264), 0 (254), +8.0 (232).

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—Articles—

幻覚性植物 *Salvia divinorum* 及び近縁植物の成分と基原種鑑別について

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Authentication and Ultra Performance Liquid Chromatography (UPLC)/MS Analysis of Magic Mint, *Salvia divinorum* and Its Related Plants

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Ultra performance liquid chromatography (UPLC)/mass spectrometry (MS) analysis was performed to investigate whether commercial *Salvia* cultivars available in the Japanese market contain salvinorin A (**1**), which is an hallucinogen present in magic mint (*Salvia divinorum*) prior to the regulation of *S. divinorum* by the Japanese Pharmaceutical Affairs Law. In addition, a previously reported method to authenticate *S. divinorum*, utilizing an amplification refractory mutation system (ARMS) was applied to the same samples to estimate the method's accuracy. As a result of the UPLC/MS analysis, it was clear that none of the tested cultivars possessed **1** while *S. divinorum* leaves and its processed products "concentrated salvia" contained **1** in the range from 0.19% to 0.58%. Furthermore, the ARMS method could clearly distinguish *S. divinorum* from the tested cultivars. In conclusion, the authentication method is considered to be useful for the practical regulation of *S. divinorum* due to its simplicity and accuracy.

Key words——*Salvia divinorum*; salvinorin A; amplification refractory mutation system (ARMS); 5S rRNA-non-transcribed spacer (NTS); ultra performance liquid chromatography (UPLC)/MS

INTRODUCTION

近年，麻薬・覚醒剤の代用として，様々な化学物質や植物が違法ドラッグ（いわゆる脱法ドラッグ）の名で流通している．特にマジックマッシュルームが流行し始めた2000年頃より，これらの違法ドラッグが原因と思われる死亡事故や殺人事件なども発生しており，社会問題の1つとなっている．また，違法ドラッグの乱用から真の麻薬・覚醒剤乱用に陥るといったゲートウェイドラッグとしての弊害も危惧されている．

厚生労働省では，深刻化する違法ドラッグ問題に対応するため，流通及び乱用実態を把握した上で，違法ドラッグ市場に存在する未規制薬物や植物の一部を，新たに麻薬及び麻薬原料植物に指定している．また，流通・乱用実態に即した迅速な規制を行うため，平成19年度より，薬事法内に，指定薬物制度を設け，幻覚性や中枢神経系への作用を有し，乱用される恐れのある物質の製造，輸入，販売の禁止，広告の制限を行っている.[1] 具体的には，tryptamine及びphenethylamine系の麻薬・覚醒剤類似化合物，亜硝酸エステル類など31化合物1植物が指定されている.[2]

植物として唯一指定された *Salvia divinorum* は，メキシコ原産のシソ科の多年草であり，本植物に含まれる主な幻覚成分は，salvinorin A (**1**) である (Fig. 1)．メキシコの原住民が伝統的に本植物の葉を噛んだり，抽出エキスを飲用するなどしており，その使用の歴史は長いと思われるが，広く世界的に

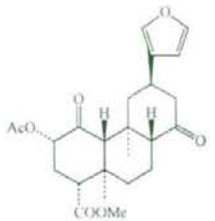

Fig. 1. The Structure of Salvinorin A

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認知されるのは，1962年に原植物が新種として報告され，[3,4] 1980年代に成分探索が行われた[5,6]後であり，比較的新しい幻覚性物質である．**1**は，他の多くの幻覚性物質と異なり，その構造中に窒素原子を含まない．また作用機序もκ-オピオイド受容体の賦活であると考えられており，[7,8] これまでとは全く違うタイプの幻覚剤である．

一方で，*Salvia*属植物は，地中海沿岸，中央及び東アジア地域，中南米を中心に900種以上が分布し，[9] その花は，非常に多様な色，形態を持つことから，園芸植物として多くの種（品種）が，市場に流通している．また，薬用セージ（*S. officinalis*）やタンジン（*S. miltiorrihiza*）などのように薬用に供されるものもある．

したがって，*S. divinorum*の指定薬物への移行に際しては，一般の園芸店など，身近に存在する園芸品種の*Salvia*属植物に**1**が存在しないことを確認するとともに，園芸品種と*S. divinorum*を区別する分析方法の確立が望まれる．

そこで本研究では，園芸植物として流通する*Salvia*属植物について，UPLC/MS分析を行い，**1**の有無を調査した．*Salvia*属植物は，*Heterosphace*節及び*Salviastrum*節に属する一部の例外を除き，ユーラシア及びアフリカ大陸分布種とアメリカ大陸分布種とで，分子系統的に異なることが示されており，[9] 前者の多くがabietane型のジテルペノイドを含有するのに対し，後者は，**1**と同じneoclerodane型のジテルペノイドを含有する．[10] そこで，調査対象としては，アメリカ大陸原産の*Salvia*属植物を基原とする品種を選んだ．

また，最近，Berteaらは，5S rRNA, non-transcribed spacer（NTS）塩基配列の違いを利用したamplification refractory mutation system（ARMS）法による*S. divinorum*の鑑定法を報告している．[11] しかし，鑑別の比較対象にヨーロッパ原産種1種のみを用いていることから，その精度には疑問が持たれる．そこで，*S. divinorum*により近い種であるアメリカ大陸産の*Salvia*属植物群を比較対象に用いて，文献の方法の精度及び有用性を検討した．

MATERIALS AND METHODS

1. Samples and Reagents　本研究に使用した園芸市場に流通する*Salvia*属植物及び違法ドラッグ市場に流通する*S. divinorum*製品をTable 1にまとめた．これらは，インターネット上の販売店及び国内の輸入雑貨店等より購入した．なお，表示の学名は，販売店による記載であり，植物分類学的な確認は行っていない．各試料は，乾燥後，1年間，室温にて保存したものを用いた．UPLC/MS分析に用いた**1**の標品は，徳島文理大香川薬学部の代田准教授が，*S. divinorum*より単離し，NMR, MSなどのスペクトルデータにより構造を確認したもの[12]を御恵与頂き，使用した．

Table 1. Commercial *Salvia* Cultivars and *Salvia divinorum* Products Used in This Study

Sample No.	Commercial name	Scientific name	Form
Sa-0	**Salvia divinorum**	***S. divinorum***	**seedling**
Sa-1	Prairie sage	*S. azurea*	seedling
Sa-2	Eyelish leaved sage	*S. blepharophylla*	seedling
Sa-3	Buchanan's sage	*S. blepharophylla*	seedling
Sa-4	Cacalia sage	*S. cacaliaefolia*	seedling
Sa-6	Salvia discolor	*S. discolor*	seedling
Sa-7	Pineapple sage	*S. elegans*	seedling
Sa-8	Honey Melon sage	*S. elegans*	seedling
Sa-9	Autumn sage	*S. greggii*	seedling
Sa-10	Anise scented sage	*S. guaranitica*	seedling
Sa-11	Rose leaf sage	*S. involucrata*	seedling
Sa-12	Mexican bush sage	*S. leucantha*	seedling
Sa-13	Mexican sage	*S. mexicana*	seedling
Sa-14	Patens	*S. patens*	seedling
Sa-15	Sinaloa sage	*S. sinaloensis*	seedling
Sa-16	Bog sage	*S. uliginosa*	seedling
Sa-17	Urica	*S. urica*	seedling
Sa-58	**Salvia X5**	***S. divinorum***	**unknown**
Sa-59	**Salvia X10**	***S. divinorum***	**unknown**
Sa-64	**Salvia·divinorum**	***S. divinorum***	**leaf**

Commercial *Salvia divinorum* products are indicated in bold letter.

2. UPLC/MS分析　試料溶液の調製は，次のように行った．細片化した試料20 mgに，MeOH 1 mlを加え，1時間，室温で振とう抽出したのち，遠心上清をMillex-HV（0.45 μm, Millipore）によりフィルターろ過し，その1 μlを分析に供した．

分析は，装置にWaters Acquity Ultra Performance LC system（Waters）及びWaters LCT Premier TOF mass spectrometer（Waters）を，カラムにAcquity UPLC BEH C_{18} column（100 mm×2.1 mm i.d., 1.7 μm particle size, Waters）を用いて行った．移動相は，H_2O（A）/acetonitrile（B）を20% B