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NOTE

The botanical origin of kratom (*Mitragyna speciosa*; Rubiaceae) available as abused drugs in the Japanese markets

Takuro Maruyama · Maiko Kawamura · Ruri Kikura-Hanajiri · Hiromitsu Takayama · Yukihiro Goda

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Abstract Kratom is the leaves of *Mitragyna speciosa* (Rubiaceae). Recently, kratom has been sold in street shops or on the Internet in Japan for the purpose of abuse due to its opium-like effects. In this study, we investigated the botanical origin of the commercial kratom products using the internal transcribed spacer (ITS) sequence analysis of rDNA in preparation for future regulation of this product. In addition, a previously reported method to authenticate the plant, utilizing polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was applied to the same products in order to estimate the method's accuracy and utility. The ITS sequence analysis of the commercial kratoms revealed that most of them were derived from *M. speciosa* or closely related plants, while the others were made from the same tribe plant as *M. speciosa*. The reported PCR-RFLP method could clearly distinguish kratoms from the other psychoactive plants available in the Japanese markets and also from related plants. The authentication method is considered to be useful for the practical regulation of the plant due to its wide range of application, high accuracy and simplicity.

Keywords Kratom · *Mitragyna speciosa* · rDNA internal transcribed spacer (ITS) · Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

Introduction

In recent years, many psychoactive substances and plants not controlled by Japanese laws are easily obtained through the Internet in Japan. Not only do these products induce health disorders in humans, but they can also function as 'gateway drugs' which catalyse the abuse of controlled narcotics and stimulants such as marijuana, opium and methamphetamine. Therefore, the Ministry of Health, Labour and Welfare, Japan, has regulated these psychoactive drugs as designated substances (Shitei-Yakubutsu) based on the Japanese Affairs Law since 2007[1]. Now, 39 chemical agents and a plant are controlled under this law[2].

The only plant material designated as "Shitei-Yakubutsu" is magic mint (*Salvia divinorum*; Labiatae). Therefore, other plants such as kratom (*Mitragyna speciosa*; Rubiaceae), sinicuichi (*Heimia salicifolia*; Lythraceae), blue lotus (*Nymphaea* spp.; Nymphaeaceae) and their processed products have often been found in the Japanese markets since the enforcement of the law.

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T. Maruyama · M. Kawamura · R. Kikura-Hanajiri · Y. Goda (✉)
National Institute of Health Sciences,
Division of Pharmacognosy,
Phytochemistry and Narcotics,
1-18-1 Kamiyoga, Setagaya-ku,
Tokyo 158-8501, Japan
e-mail: goda@nihs.go.jp

H. Takayama
Graduate School of Pharmaceutical Sciences,
Chiba University, 1-33 Yayoi-cho, Inage-ku,
Chiba 263-8522, Japan

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[1] The revision of the Pharmaceutical Affairs Law (Jun. 14, 2006), Law No. 69 (2006).

[2] Ordinance No. 14 (Feb. 28, 2007), Ministry of Health, Labour and Welfare, Japan. Ordinance No. 146 (Dec. 12, 2007), Ministry of Health, Labour and Welfare, Japan. Ordinance No. 172 (Dec. 17, 2008), Ministry of Health, Labour and Welfare, Japan.

Kratom is the leaves of *M. speciosa* endemic to tropical Southeast Asia. The leaves have been traditionally used for their opium-like effect and coca-like stimulant ability in Thailand and Malaysia, where the leaves are called 'Kratom' and 'Biak-Biak,' respectively [1, 2]. The leaves contain mitragynine (**1**) and its related indole alkaloids [1, 2], and 7-hydroxymitragynine (**2**) is reported as the active principle for their opium-like effect [2, 3].

During the course of our study of psychoactive plants [4–6], we investigated the botanical origin of the kratom obtained from the Japanese markets in preparation for its future regulation based on the internal transcribed spacer (ITS) sequence of the nuclear rDNA. In addition, the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method reported by Sukrong et al. [7] for the rapid identification of *M. speciosa* was applied to the commercial kratom products in order to estimate its accuracy and utility.

Materials and methods

Materials

Details of the referential plants and drugs which are recognised as kratom or related species in Thailand and Malaysia are shown in Table 1, together with their ITS genotypes. Among them, Mt-1 to Mt-3 were collected at Chulalongkorn University, Thailand, and were identified as *M. speciosa* (Mt-1 and Mt-2) and *M. hirsuta* (Mt-3) by Dr. Sumphan Wongseripipatana. Although Mt-1 and Mt-2 are not discriminated taxonomically, they have quite different leaves. So, we separately treated them as a big leaf for Mt-1 and a small leaf for Mt-2 based on their leaf size. Mt-4 to Mt-7 were kindly supplied by Ms. Latifah BT. HJ. Idris at the Pharmaceutical Services Division, Ministry of Health, Malaysia, through the Kanto-Shin'etsu Regional Narcotic Control Office, Japan. They were obtained as dried raw materials. Voucher specimens were deposited at the Division of Pharmacognosy, Phytochemistry and Narcotics, National Institute of Health Sciences, Japan. A map of the collection area is shown in Fig. S1, Supplementary Material. The phytochemical study of Mt-3 (*M. hirsuta*) has already been reported by Kitajima et al. [8].

Details of the commercial kratoms available in the Japanese markets are shown in Table 2, together with their ITS genotypes. They were purchased from shops on the Internet. They were in powder, dried leaves and gum product forms.

The leaves of magic mint (*S. divinorum*) and chacruna (putative *Psychotria viridis*; Rubiaceae) used for the negative controls in the PCR-RFLP assay were also purchased from shops on the Internet. Magic mint used in this study has already been identified as *S. divinorum* by genetic and phytochemical techniques [6].

rDNA, ITS sequence analysis

Ten mg of each sample was crushed using a mixer mill, MM-300 (Qiagen). The genomic DNA was extracted and purified from the powdered sample using a DNeasy Plant Mini Kit (Qiagen). The ITS region (small subunit rDNA-ITS1-5.8S rDNA-ITS2-large subunit rDNA) of the nuclear rDNA was two-steps (nested) PCR-amplified using the obtained genomic DNA as a template. PCR was separately performed with Gene TaqNT DNA polymerase (Nippon Gene) and KOD DNA Polymerase (Toyobo) using a DNA engine PTC-200 (Bio-Rad). The primer sequences are: ITS-S1 5′-GGA AGT AAA AGT CGT AAC AAG G-3′ and ITS-AS1 5′-TTT TCC TCC GCT TAT TGA TAT GC-3′ for the first-round PCR; and ITS-S2 5′-TCC GTA GGT GAA CCT GCG G-3′ and ITS-AS2 5′-GTA GTC CCG CCT GAC CTG-3′ for the second-round PCR. The amplicons were purified by Montage-PCR (Millipore) and were directly sequenced. The cycle sequencing reaction was performed using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). Subcloning of the amplicon by KOD DNA polymerase into the plasmid vectors was performed using a Zero Blunt PCR Cloning Kit (Invitrogen). The DNA sequences were aligned with a Clustal W program [9].

Table 1 Details of the referential plants and drugs used in this study

Sample no.	Scientific name or crude drug name	Locality	Form	Genotype
Mt-1[a]	*M. speciosa* (big leaf)	Bangkok, Thailand	Dried leaf	Type 1
Mt-2[a]	*M. speciosa* (small leaf)	Bangkok, Thailand	Dried leaf	Hybrid[b]
Mt-3	*M. hirsuta*	Bangkok, Thailand	Dried leaf (cutting)	Type 5
Mt-4	Kratom	Perlis, Malaysia	Dried leaf	Type 1
Mt-5	Kratom	Pahang, Malaysia	Dried leaf	Type 2
Mt-6	Kratom	Negeri Semblian, Malaysia	Dried leaf	Type 1
Mt-7	Kratom	Perlis, Malaysia	Dried leaf	Type 1

[a] Although they are not discriminated in taxonomically, they have quite different leaves

[b] Hybrid between *M. speciosa* and *M. diversifolia*/*M. hirsuta*

Table 2 Details of the commercial kratom products used in this study

Sample no.	Name	Form	Genotype
Mtk-1	Concd. kratom (50–60% pure)	Gum	No amplicon
Mtk-2	Kratom Crush Leaf KR-L	Dried leaves (cutting)	Type 3
Mtk-3	Kratom Gold KR-G	Powder	Type 4
Mtk-4	Kratom KR-1	Powder	Type 3
Mtk-5	Kratom	Dried leaves (cutting)	Type 4
Mtk-6	Kratom	Dried leaves	Type 3
Mtk-7	Mitragaina Extract Powder	Powder	Type 4
Mtk-8	Mitragaina	Dried leaves	Other[a]
Mtk-9	Mitragyna 40× resin	Gum	No amplicon
Mtk-10	Plant Sample Kratom	Dried leaves (cutting)	Type 3
Mtk-11	Kratom EX10	Dried leaves (cutting)	Other[a]
Mtk-12	Concd. Kratom KR-XV	Gum	No amplicon
Mtk-13	Kratom KR-1	Powder	Type 3

[a] BLAST search suggests a closely related plant within the same tribe, Naucleeae s.l. as *Mitragyna* (Table S1)

Table 3 Internal transcribed spacer (ITS) genotypes with nucleotide variable sites found in this study and the international nucleotide sequence database

Genotype	Aligned position															Accession no.
	ITS1						5.8S	ITS2								
	26	42	101	105	118	171	362	452	510	514	516	539	564	565	608	
Type 1	T	C	C	C	A	-	A	G	T	T	A	C	C	A	C	AB249645
Type 2	.	.	.	M	.	.	.	.	.	.	.	.	.	.	.	None
Type 3	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	None
Type 4	.	.	Y	M	.	.	.	.	.	.	.	M	.	.	.	None
Type 5	Y	G	T	.	-	G	C	A	-	C	C	.	T	G	A	None
M. hirsuta	.	G	T	.	-	G	C	A	-	C	C	.	T	G	A	AB249647
M. diversifolia																AB249646

A dot '.' and a hyphen '-' indicate the same nucleotide as *M. speciosa* type 1 sequence and a gap, respectively. M, A/C; Y, C/T

PCR-RFLP assay

The ITS1 DNA fragment was PCR-amplified with a new pair of primers, ITS-S1 and ITS1-AS1 (5′-TAT CCG TTG CCG AGA GTC-3′) using Nova Taq DNA polymerase Hot Start (Merck) and Ampdirect Plus (Shimadzu). The resulting PCR products were digested with a restriction enzyme, *Xma*I (New England BioLabs), at 37°C for 4 h. The digested fragments were analysed by 2.0% agarose gel electrophoresis and visualised by ethidium bromide staining.

Results and discussion

ITS sequence analysis

The genus *Mitragyna* consists of ten species around the world. A phylogenetic analysis of the *Mitragyna* plants has already been performed using nuclear rDNA, ITS and chloroplast DNA, *rbc*L and *trn*T-F sequences [7, 10], and it was shown that *M. speciosa* is well discriminated from other related plants based on the ITS sequence [7].

The ITS sequence of each genotype found in this study is summarised in Table 3, together with those on the international nucleotide sequence database (DDBJ, EMBL and GenBank; INSD), and the genotypes of all samples are shown in Tables 1 and 2. Among the referential plants and drugs (Mt-1 to Mt-7), the ITS sequence of Mt-1, 4, 6 and 7 were identical and the same as that of *M. speciosa* on the INSD (accession no.: AB249645; type 1 in Table 3). The ITS sequence of Mt-3 was also identical to those of *M. hirsuta* and *M. diversifolia* on the INSD (AB249646 and AB249647, respectively), except for one site with nucleotide additivity [11]. The sequence of Mt-2 seemed to be a mixture of the type 1 and *M. hirsuta/diversifolia* sequences on the INSD on direct sequencing. Therefore, the amplicon from Mt-2 was introduced into the plasmid vector and 12 clones were sequenced. As a result, six sequences each of

type 1 and *M. hirsuta/diversifolia* were detected, suggesting that Mt-2 was a hybrid between *M. speciosa* and *M. hirsuta/diversifolia*. This result was also supported by the contents of **1** and **2** in Mt-1 to Mt-3: 23.4 and 0.124 mg/g of **1** and **2** were present respectively in Mt-1, while 1.6 and 0.031 mg/g were present in Mt-2 and neither **1** nor **2** was detected in Mt-3 (Details will be reported in a future paper). Mt-5 has the same sequence as type 1 except for one site with nucleotide additivity [11]. These data indicate that most of the kratoms from Thailand and Malay peninsula have the type 1 sequence.

Among 13 commercial kratom products (Mtk-1 to -13), no amplicons were obtained from the gum samples (Mtk-1, -9 and -12), which appear to contain no organic tissue. The ITS sequences of Mtk-8 and -11 were identical, and the similarity survey for the sequence using the BLAST search program suggested that these products were made from the same tribe plant (Naucleeae *sensu lato*) [10] as *Mitragyna* (Table S1, Supplementary Material). The presence of these plants in the commercial kratoms can be dangerous for human health because of the various indole alkaloids with high pharmacological activities are reported from the Naucleeae s.l. plants [12]. Although the ITS sequences of the other samples (Mtk-2 to -7, -10 and -13) showed a great similarity to the type 1 sequence, all of them showed a one or three nucleotide(s) difference from the type 1 sequence (types 3 and 4 in Tables 2 and 3). Probably, a small number of nucleotide substitution(s) in types 3 and 4 sequences is (are) attributed to either the intraspecies nucleotide variation in *M. speciosa* or the closely related plants to *M. speciosa*. Unfortunately, the real truth remains inconclusive from the current study. A more detailed investigation including the wide sampling in other areas such as Sumatra, Borneo and the New Guinea islands might solve this question.

All of the commercial kratoms having types 3 or 4 sequences were found to contain the hallucinogens in the range from 12.1 to 21.4 mg/g of **1** and 0.114 to 0.393 mg/g of **2** in the LC/MS analysis (details will be reported in a future paper). Therefore, we subsequently examined the accuracy and utility of the reported authentication method of *M. speciosa* [7] in preparation for the future regulation of kratom, although the original species of the commercial kratoms remain unclear.

PCR-RFLP assay of the commercial kratom products

The PCR-RFLP method for the identification of *M. speciosa* established by Sukrong et al., is based on the nucleotide substitution at position 42 among the *Mitragyna* species [7]. Namely, *M. speciosa* has an *Xma*I site (CCCGGG) in the aligned position 42–47, while other species have the sequence GCCGGG in the same position. Although some different ITS genotypes from that in Sukrong et al.'s study [7] were found in the commercial kratoms, there are no nucleotide substitutions at position 42 among all of the ITS genotypes observed in this study. Therefore, it is expected that Sukrong et al.'s method can be utilised for the rapid identification of commercial kratom products. Hence, we applied the commercial kratoms to the method. For the negative controls in the assay, the leaves of chacruna and magic mint were selected because they are hallucinogenic plants normally used in the form of leaves, like the kratom products. The electropherogram of the assay is shown in Fig. 1. The image is also shown in colour in Fig. S2, Supplementary Material. Only the PCR products from the samples which have kratom genotypes were cleaved into two fragments by the *Xma*I digestion, while the original PCR products remained as a single band in the other samples (Fig. 1). The method could clearly distinguish kratoms from the other psychoactive plants (NC1 and NC2) and also from the same family (Mtk-8 and -11) and genus (Mt-3; *M. hirsuta*) plants as *M. speciosa* (Fig. 1). Simultaneously, our data indicate that the method is practical enough for application to processed products, except for gum samples.

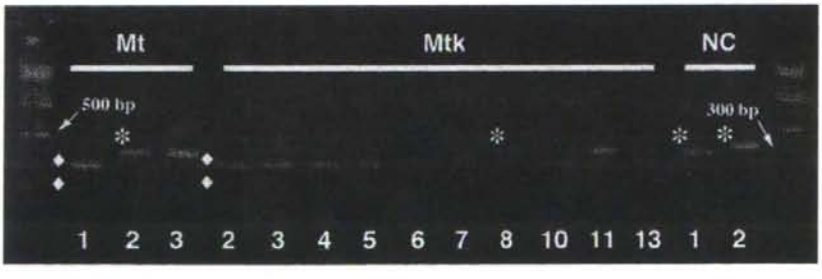

Fig. 1 Electropherogram in the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. NC: negative control. NC1, chacruna (putative *Psychotria viridis*); NC2, magic mint (*Salvia divinorum*). Details of Mt-1 to -3 and Mtk-2 to 13 are shown in Tables 1 and 2. An *asterisk* and a *diamond* indicate the original PCR product and the restriction fragments, respectively. The samples in which types 1 to 4 sequences were detected are shown as *underlined* numbers

In conclusion, the present study revealed that most of the commercial kratoms available in the Japanese markets are derived from *M. speciosa* or closely related plants and that the reported authentication method for *M. speciosa* utilizing PCR-RFLP has high practicality due to its wide range of application, high accuracy and simplicity.

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Identification of a Cannabinoid Analog as a New Type of Designer Drug in a Herbal Product

Nahoko UCHIYAMA, Ruri KIKURA-HANAJIRI, Nobuo KAWAHARA, Yuji HAISHIMA, and Yukihiro GODA*

National Institute of Health Sciences; 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan.

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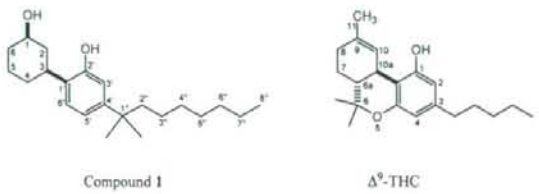
A new type of designer drug, a cannabinoid analog (1), was found in a herbal product distributed on the illegal drug market in Japan in expectation of its narcotic effect. The structure of 1 was identified by LC-MS, GC-MS, high-resolution MS, and NMR analyses. Compound 1 showed a molecular weight of 332, and accurate mass measurement exhibited its elemental composition to be $C_{22}H_{36}O_2$. Together, the mass and NMR spectrometric data revealed that 1 was (1*RS*,3*SR*)-3-[4-(1,1-dimethyloctyl)-2-hydroxyphenyl]cyclohexan-1-ol, which was first synthesized in 1979 by a group at Pfizer Inc. and reported as a potent cannabinoid analog possessing cannabinoid receptor binding activity and analgesic activity in the 1990s. This is the first report to identify a cannabinoid analog in an illegal drug.

Key words cannabinoid analog; designer drug; herbal product; (1*RS*,3*SR*)-3-[4-(1,1-dimethyloctyl)-2-hydroxyphenyl]cyclohexan-1-ol; drug abuse

Many types of chemicals are widely distributed and abused as psychotropic substances. In Japan every year this decade, a market survey of illegal drugs is performed by the Ministry of Health, Labour and Welfare.[1–9] Following the results of the survey, the compound identified and recognized as a designer drug[10–12] came to be strictly controlled by the Narcotics and Psychotropic Control Law or by the Pharmaceutical Affairs Law as designated substances (Shitei-Yakubutsu).[13–16] In 2008, seven new designer drugs were classified as narcotics or designated substances, and all of them are analogs of phenylethylamine or tryptamine.

Cannabis sativa L. (cannabis, hemp, marijuana, marihuana) is widely abused around the world because it contains psychoactive cannabinoids, such as Δ^9-tetrahydrocannabinol (Δ^9-THC), which contains no amine groups (Fig. 1). In the past few decades, a number of analogs of Δ^9-THC were synthesized, and their structure–activity relationships were studied.[17,18] In the 1980s, a group at Pfizer Inc. explored the development of analgesics using potent synthetic cannabinoids.[19–22] After the discovery of cannabinoid receptors, type 1 (CB_1, central type) and type 2 (CB_2, peripheral type), as well as the discovery of an endogenous cannabinoid, their physiological roles were elucidated; a number of cannabinoid analogs were then newly synthesized, and their pharmacological activity for the treatment of various diseases was studied.[23,24]



Compound 1 Δ^9-THC

Fig. 1. Structures of Compound 1 and Δ^9-Tetrahydrocannabinol (Δ^9-THC)

Recently, cannabis abuse seems to have spread in Japan. In this study, we identified a novel designer drug (**1**) possessing cannabinoid activity as an adulterant in a herbal product (Fig. 1). Compound **1** was first synthesized by Pfizer Inc. in 1979,[25] and reported as a cannabinoid analog in the 1990s.[26–30] Although many designer drugs having phenylethylamine, tryptamine, and piperazine structures have been found,[10–12] this is the first report to identify a non-nitrogenated compound, a phenylcyclohexane derivative having cannabinoid activity.

Experimental

Chemicals and Reagents HPLC-grade acetonitrile and all other chemicals (analytical grade) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Centrifugal filter devices (Ultrafree-MC, 0.45 μm filter unit) were from Millipore (Bedford, MA, U.S.A.).

Samples A product was purchased *via* the Internet (June 2008). The product was described as a herbal mixture and had the appearance of dried plants. The ingredients were listed as "Baybean," "Blue lotus," "Dwarf skullcap," "Indian warrior," "Lion's tail," "Maconha brava," "Marshmallow," "Pink lotus," "Red clover," "Rose," "Siberian motherwort," "Vanilla," and "Honey."

Preparation of Sample Solution A product (20 mg) was crushed into powder and extracted with 2 ml of methanol under ultrasonication for 10 min. After centrifugation (5 min at 3000 rpm), the solution was filtered through a centrifugal filter device.

Instrumentation Gas chromatography-mass spectrometry (GC-MS) in the electron impact (EI) mode at 70 eV of electron energy was used. Analysis was performed on a Hewlett-Packard 6890N GC with a 5975 mass selective detector using a capillary column (HP1-MS capillary, 30 m×0.25 mm i.d., 0.25 μm film thickness) and helium gas as a carrier. An initial column temperature of 80 °C was employed and the temperature was increased at a rate of 5 °C/min to 190 °C and at a second rate of 10 °C/min to 310 °C. Data were obtained in a full scan mode with a scan range of *m/z* 40–550. An ultra-performance liquid chromatography-electrospray ionization-mass spectrometer (UPLC-ESI-MS), consisting of a Waters ACQUITY UPLC system equipped with a Single Quadrupole Detector (SQD) mass detector and a photo diode array (PDA) (Waters, Milford, MA, U.S.A.), was also used. The sample solutions were separated using a Waters ACQUITY UPLC HSS T3 column (2.1×100 mm i.d., 1.8 μm; Waters) at 40 °C. The following gradient system was used with a mobile phase A (10 mM ammonium formate buffer, pH 3.5) and a mobile phase B (acetonitrile) delivered at 0.3 ml/min; A : B 50 : 50 (0 min)–20 : 80 (20–40 min). The injection volume was 5 μl. The wavelength of the PDA detector for screening was set from UV 190 to 400 nm, and chromatographic peaks were monitored at UV 254 and 280 nm. Mass analysis by the ESI was used in both a positive and a negative mode. Nitrogen gas was used for desolvation at a flow rate of 600 l/h at 350 °C. The capillary voltage was 3000 V, and the cone voltage was 30 V. MS data were recorded in the full scan mode (*m/z* 150–700). Preparative TLC was carried out using a silica gel plate (silica gel 60, 20×20 cm, 0.5 mm, Merck, Darmstadt, Germany).

Isolation of Compound 1 A product (3 g) was extracted with 100 ml of methanol by ultrasonication for 1 h. After the extraction was performed three times, the supernatant was evaporated to dryness. The extract was subjected to preparative silica gel TLC using $CHCl_3$–acetone (4/1) as developing solvent. A portion of the silica gel in the TLC plate was taken and eluted with $CHCl_3$–MeOH (1/1) to give a fraction 1. Repeated fractionation of fr. 1 by preparative silica gel TLC with $CHCl_3$–MeOH (20/1) gave compound **1** (15 mg) as an off-white solid.

Measurement of Accurate Mass The accurate mass of the target compound was measured by the LTQ OrbiTrap XL instrument (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) with the direct-infusion ESI positive and negative ion modes under the following conditions: solvent flow rate 5 μl/min, sheath gas flow rate 20 arb, auxiliary gas flow rate 10 arb, spray voltage 5 kV, capillary temperature 275 °C, capillary voltage 4 V, and tube

* To whom correspondence should be addressed. e-mail: goda@nihs.go.jp

lens 60 V. Tyrosine 1,3,6 standard was used as a mass calibrant of FT mass analyzer (resolution=100000), and tyrosine 3 standard was used as a lock mass ion (m/z 508.20783) during the measurement. Theoretical mass and delta value (mmu) were calculated by using the elemental composition tool of Xcalibur/Qual Browser software (Thermo Fisher Scientific Inc.). MS data were recorded in the full scan mode (m/z 100–1000).

NMR Analysis $CDCl_3$ (99.96%) and DMSO-d_6 (99.96%) were purchased from ISOTEC Inc., which is part of Sigma-Aldrich Inc. (St. Louis, MO, U.S.A.). The NMR spectra were obtained on ECA-600 and ECA-800 spectrometers (JEOL Ltd., Tokyo, Japan). Assignments were made *via* ^{1}H-NMR, ^{13}C-NMR, heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple-bond correlation (HMBC), double quantum filtered correlation spectroscopy (DQF-COSY), and rotating frame nuclear Overhauser effect (ROE) spectra.

Results and Discussion

In the sample solution of the product, an unknown main peak was detected by GC-EI-MS and by LC-ESI-MS analyses. The former found a peak at 47.9 min and showed four ion peaks at m/z (% relative intensity) 332 (16), 314 (14), 233 (80), and 215 (100). On the other hand, the latter detected a peak at 14.5 min and exhibited major ion peaks at m/z 333 $[M+H]^+$, 315 $[M+H-18]^+$ in the positive scan mode and at m/z 331 $[M-H]^-$ in the negative scan mode. The PDA-sliced UV spectrum of the peak exhibited maxima at 220 and 275 nm and minima at 212 and 249 nm. The accurate mass of **1** revealed $[M+H]^+$ at m/z 333.27918 in the positive scan mode and $[M-H]^-$ at 331.26442 in the negative scan mode, suggesting molecular formulae of $C_{22}H_{37}O_2$ and $C_{22}H_{33}O_2$, respectively. The errors between the observed mass and theoretical mass of $[M+H]^+$ and $[M-H]^-$ are +0.71 and −0.18 mmu, respectively. The ^{1}H- and ^{13}C-NMR spectra of **1** exhibited 36 protons and 22 carbons. These results suggested that **1** contained oxygen atoms but no nitrogen atoms.

The ^{1}H-NMR spectrum of **1** exhibited 36 non-exchangeable protons, including three methyl signals at δ 1.22 (6H, s) and 0.83 (3H, t, J=7.2 Hz), as well as ABX-type aromatic proton signals at δ 7.06 (1H, d, J=8.2 Hz), 6.84 (1H, dd, J=8.2, 2.0 Hz), and 6.67 (1H, d, J=2.0 Hz), as shown in Table 1. In addition, the ^{1}H-NMR spectrum also showed two methine proton signals at δ 2.86 (1H, tt, J=12.4, 3.1 Hz) and 3.76 (1H, tt, J=11.0, 4.1 Hz), and a characteristic signal assignable to hydroxy proton at δ 4.51 (1H, br d, J=4.6 Hz) and 9.01 (1H, br s). The ^{13}C-NMR spectrum of **1** showed 22 carbon signals, including three methyls, ten methylenes, two methines with one oxygenated carbon (δ 71.2) and one quaternary carbon, three aromatic carbons (δ 113.1, 118.5, 126.3), and three quaternary carbons (δ 128.7, 149.1, 152.3). The presence of three partial structures (1,3-substituted cyclohexyl group, 1,1-dimethyloctyl group, and 1,2,4-substituted phenyl) was suggested from its DQF-COSY, HMQC, and HMBC spectra (Table 1, Fig. 2). The connectivity of these groups was deduced from the HMBC spectrum (Table 1, Fig. 2). A methine proton at δ 2.86 (H-3) of the cyclohexyl group correlated to the phenyl carbons at δ 152.3 and 126.3 (C-2′, C-6′), and two aromatic protons, at δ 6.67 and 6.84 (H-3′, H-5′) of the phenyl group, showed correlations to the quaternary carbon at δ 37.3 (C-1″). In addition, the irradiation of the hydroxyl proton at δ 9.01 (2′-OH) resulted in ROE on the aromatic proton (H-3′), as shown in Fig. 3. The relative configuration between two methine protons at C-1 and C-3 established a *cis* configuration by the ROE

Table 1. NMR Data of Compound **1** in $CDCl_3$[a)]

No.	^{13}C	^{1}H	HMBC[b)]
1	71.2	3.76, 1H, tt, J=11.0, 4.1 Hz	2, 6[c)]
2	41.9	ax, 1.44, 1H, m, overlapped	3, 4, 1′
		eq, 2.16, 1H, br d, J=11.7 Hz	1, 3, 4
3	35.3	2.86, 1H, tt, J=12.4, 3.1 Hz	2, 4, 1′, 2′, 6′
4	31.7	ax, 1.30, 1H, m, overlapped	2, 3, 5, 1″[c)]
		eq, 1.82, 1H, d, J=13.1 Hz	2
5	24.5	ax, 1.44, 1H, m, overlapped	1, 4, 6
		eq, 1.87, 1H, dq, J=13.4, 3.4 Hz	1, 4, 6
6	35.5	ax, 1.27, 1H, m, overlapped	1, 2, 5
		eq, 2.05, 1H, br d, J=12.0 Hz	1, 2, 4
1′	128.7	—	—
2′	152.3	—	—
3′	113.1	6.67, 1H, d, J=2.0 Hz	1′, 2′, 4′, 5′, 1″
4′	149.1		
5′	118.5	6.84, 1H, dd, J=8.2, 2.0 Hz	1′, 2′, 3′, 6′, 1″
6′	126.3	7.06, 1H, d, J=8.2 Hz	3, 2′, 3′, 4′, 5′
1″	37.3	—	—
2″	44.6	1.51, 2H, m	4′, 1″, 3″, 4″, 1″-$(CH_3)_2$
3″	24.7	1.04, 2H, m	2″, 4″, 5″
4″	30.3	1.17, 2H, m, overlapped	2″, 3″, 5″
5″	29.2	1.17, 2H, m, overlapped	3″, 7″
6″	31.9	1.17, 2H, m, overlapped	5″, 7″
7″	22.6	1.24, 2H, m	6″, 8″
8″	14.1	0.83, 3H, t, J=7.2 Hz	6″, 7″
1″-$(CH_3)_2$	28.9	1.22, 6H, s	4′, 1

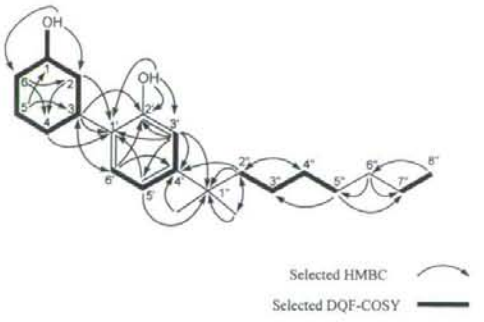


Fig. 2. Selected DQF-COSY and HMBC Correlations of Compound **1**

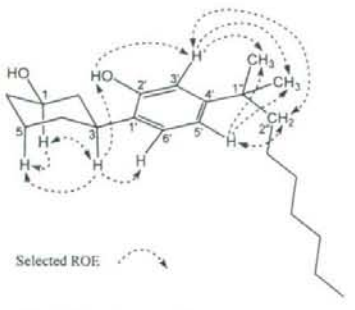


Fig. 3. Selected ROE Correlations of Compound **1**

correlations (Fig. 3). Therefore, the structure of **1** is finally elucidated as (1*RS*,3*SR*)-3-[4-(1,1-dimethyloctyl)-2-hydroxyphenyl]cyclohexan-1-ol.

The deduced structure has already been synthesized by Pfizer Inc. and reported as a cannabinoid analog.[25,26] Pharmaceutical studies showed that **1** has potent cannabinoid receptor binding activity *in vitro* and analgesic activity *in vivo* in mice.[27-30] Compton *et al.* reported that compound **1** was approximately 5-fold more potent than Δ^9-THC at the viewpoint of pharmacological activity.[28]

This is the first case in which **1** has been detected as a designer drug and an ingredient in a herbal product. Pfizer Inc. has also reported many analogs of **1** and has described their synthesis with pharmacological data.[19,22,31,32] Additionally, various cannabinoid analogs are synthesized one after another and their pharmacological activity studied for the development of new useful drugs for the treatment of a number of diseases.[23,24] This situation alerts us that these described cannabinoid analogs other than **1** may be found as designer drugs or adulterants in illegal products in the near future. To avoid health problems and abuse caused by new designer drugs, we have to continuously monitor such compounds during our surveillance.

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ORIGINAL ARTICLE

Identification of a cannabimimetic indole as a designer drug in a herbal product

Nahoko Uchiyama · Ruri Kikura-Hanajiri
Nobuo Kawahara · Yukihiro Goda

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Abstract A cannabimimetic indole has been identified as a new adulterant in a herbal product being sold illegally in Japan for its expected narcotic effect. Liquid chromatography-mass spectrometry and gas chromatography-mass spectrometry analyses indicated that the product contained two major compounds. One was identified as a cannabinoid analog (1*RS*,3*SR*)-3-[4-(1,1-dimethyloctyl)-2-hydroxyphenyl]cyclohexan-1-ol (**1**) by direct comparison with the authentic compound, which we reported previously. The other compound (**2**) showed a molecular weight of 341 daltons, and accurate mass spectral measurements showed its elemental composition to be $C_{24}H_{23}NO$. Both mass and nuclear magnetic resonance spectrometric data revealed that **2** was 1-pentyl-3-(1-naphthoyl)indole [or naphthalen-1-yl-(1-pentylindol-3-yl)methanone] being identical to JWH-018, which was synthesized by Wiley and coworkers in 1998. This compound was reported as a potent cannabinoid receptor agonist possessing a pharmacological cannabimimetic activity.

Keywords 1-Pentyl-3-(1-naphthoyl)indole · Naphthalen-1-yl-(1-pentylindol-3-yl)methanone · JWH-018 · Cannabimimetic indole · Designer drug · Herbal product

N. Uchiyama · R. Kikura-Hanajiri · N. Kawahara · Y. Goda (✉)
National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
e-mail: goda@nihs.go.jp

Introduction

Various psychotropic substances are being sold and distributed around the world via the Internet. Most recently, we found a synthetic cannabinoid analog (1*RS*,3*SR*)-3-[4-(1,1-dimethyloctyl)-2-hydroxyphenyl]cyclohexan-1-ol (**1**) [1], which contains no amino groups (Fig. 1), as an adulterant in a herbal product being commercially sold as an incense. This was the first report to identify a synthetic cannabinoid analog in a herbal product distributed on the illegal drug market for its expected narcotic effect. At almost the same time, we found another compound (**2**) that was also thought to be an adulterant in the same type of herbal products. This compound was finally found to be identical to JWH-018, a cannabimimetic aminoalkyl naphthoyl indole derivative; it had been first synthesized by Huffman and coworkers in 1998, and was reported as a potent cannabinoid receptor agonist possessing a cannabimimetic pharmacological activity in vivo [2–5]. Also, in January 2009, the Health Minister of Germany announced that **2** is an active component in a mislabeled mixture of herbs; **2** has been a controlled substance in Germany since 22 January 2009 [6]. However, no scientific report describing the isolation and identification of this compound from herbal products has been published. The present report deals with the details of its identification in a herbal product by various instrumental analyses.

Materials and methods

Materials and preparation

Acetonitrile (high-performance liquid chromatography grade) and all other chemicals (analytical grade) were

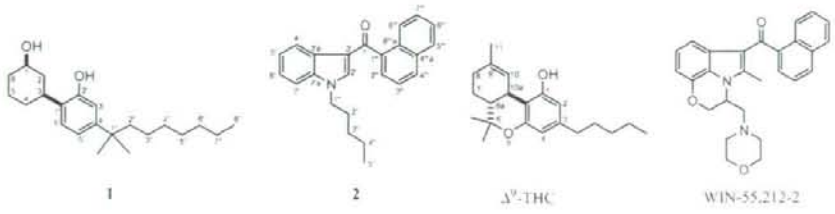

Fig. 1 Structures of detected compounds **1**, **2** and related compounds [Δ^9-tetrahydrocannabinol (Δ^9-THC) and WIN-55,212-2]

obtained from Wako (Osaka, Japan). A product, described as a herbal mixture and having the appearance of dried plants, was purchased via the Internet (December 2008). A 10-mg portion of the product was crushed into powder and extracted with 1 ml of methanol under ultrasonication for 10 min. After centrifugation for 5 min at 3000 rpm, the supernatant solution was passed through a centrifugal filter (Ultrafree-MC, 0.45 μm filter unit, Millipore, Bedford, MA, USA).

Instrumental analyses

Gas chromatography-mass spectrometry (GC-MS) was used in the electron impact (EI) mode at 70 eV of electron energy. The analysis was performed on a Hewlett-Packard 6890N GC with a 5975 mass-selective detector (Agilent, Palo Alto, CA, USA) using a capillary column (HP1-MS capillary, 30 m × 0.25 mm i.d., 0.25 μm film thickness, Agilent) and helium as carrier gas. An initial column temperature of 80°C was employed, and it was increased at a rate of 5°C/min to 190°C and then at a second rate of 10°C/min up to 310°C. The data were obtained in the full scan mode with a scan range of *m/z* 40–550. The analysis was performed under the same conditions as used in the analysis of designated drugs (Shitei-Yakubutsu) controlled by the Pharmaceutical Affairs Law of Japan [7].

The MS analysis was also made by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). The instrument consisted of an ACQUITY ultra-performance LC system connected with a single quadrupole mass detector and a photodiode array (PDA) detector (Waters, Milford, MA, USA). The sample solutions were separated using an ACQUITY UPLC HSS T3 column (2.1 mm i.d. × 100 mm, 1.8 μm; Waters) protected by a Van Guard column (2.1 mm i.d. × 5 mm, 1.8 μm; Waters) at 40°C. The following gradient system was used with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile) delivered at 0.3 ml/min; 50% A/50% B for 3 min, changing to 20% A/80% B over 2 min and held with the final composition over 5 min. The injection volume was 1 μl. The wavelength of the PDA detector for screening was set from 190 to 500 nm, and chromatographic peaks were monitored at 275 nm. Mass analysis by ESI was used in both positive and negative modes. Nitrogen gas was used for desolvation at a flow rate of 650 l/h at 350°C. The capillary and cone voltages were 3000 V and 30 V, respectively. MS data were recorded in the full scan mode (*m/z* 150–700).

The accurate mass spectrum of the target compound was measured using a direct analysis in real time (DART) ion source coupled to a time-of-flight (TOF) mass spectrometer (AccuTOF JMS-100LC, JEOL, Tokyo, Japan) operated in the positive ion mode. The measurements were made with the ion guide peak voltage set at 500 V, the reflectron voltage at 950 V, orifice 1 voltage at 15 V, orifice 2 voltage at 5 V, ring lens voltage at 5 V, and the orifice 1 temperature at 80°C. The mass range was 100–500 daltons. The DART ion source was used at a helium gas flow rate of 2.0 l/min, the gas heater temperature at 250°C, the discharge electrode needle setting at 3200 V, electrode 1 at 100 V, and electrode 2 at 250 V. Internal mass number calibration was achieved using PEG600, and diphenhydramine was used as an internal standard for each analysis.

For nuclear magnetic resonance (NMR) analysis, $CDCl_3$ (99.96%) was purchased from ISOTEC, a part of Sigma-Aldrich (St. Louis, MO, USA). The NMR spectra were obtained on ECA-600 and ECA-800 spectrometers (JEOL). Assignments were made via ^{1}H NMR, ^{13}C NMR, heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple-bond correlation (HMBC), double quantum filtered correlation spectroscopy (DQF-COSY), and rotating frame nuclear overhauser effect (ROE) spectra.

Isolation of compound 2

A 3-g portion of the herbal product was extracted with 100 ml of methanol by ultrasonication for 1 h. After the extraction was repeated three times, the combined supernatant was evaporated to dryness. The extract was loaded on a preparative silica gel thin layer chromatography (TLC) plate (Silica Gel 60, 20 × 20 cm, 2 mm, Merck, Darmdstadt, Germany) using hexane/acetone

(4:1) as developing solvent. A portion of the silica gel in the TLC plate was taken and eluted with CH_2Cl_2/methanol (2:1) to give fraction 1. Repeated fractionation of fraction 1 by preparative silica gel TLC with hexane/CH_2Cl_2 (1:20) gave compound **2** (15 mg) as an off-white solid.

Results and discussion

In the sample solution of the product, two major peaks were detected by LC-ESI-MS analysis (Fig. 2a–d). One peak, detected at 7.8 min, exhibited two ion peaks at *m/z* 333 $[M+H]^+$ and at 315 $[M+H-18]^+$ in the positive scan mode (Fig. 2e). A comparison with the mass spectrum of the authentic compound revealed that this peak was (1*RS*,3*SR*)-3-[4-(1,1-dimethyloctyl)-2-hydroxyphenyl]cyclohexan-1-ol (**1**) (Fig. 1), which was reported as an adulterant in a herbal product in our previous study [1]. Another unknown peak (**2**) detected at 7.5 min showed a major peak at *m/z* 342 $[M+H]^+$ (Fig. 2f). The PDA-sliced ultraviolet (UV) spectrum of the peak (**2**) exhibited maxima at 218, 247, and 314 nm and minima at 239 and 285 nm (Fig. 2h). These characteristics were completely different from those of **1** (UV λ_{max} 220, 275 nm; λ_{min} 212, 249 nm, Fig. 2g).

GC-EI-MS analysis showed two major peaks with a peak of α-tocopherol, which had been added as an antioxidant (Fig. 3a). One peak, detected at 47.9 min, showed a mass spectrum with four ion peaks at *m/z* (relative intensity) 332 (16), 314 (14), 233 (80), and 215 (100) as shown in Fig. 3b, which was identical to the mass spectrum of the authentic compound (**1**). An unknown peak (**2**), detected at 51.7 min, showed a mass spectrum with five ion peaks at *m/z* 341 (100), 324 (43), 284 (58), 214 (52), and 127 (32), as shown in Fig. 3c.

The accurate mass spectrum measured by TOF-MS showed a protonated molecular ion peak ($[M+H]^+$) at *m/z* 342.18579 in the positive mode, suggesting that the molecular formula of **2** was $C_{24}H_{24}NO$. The error between

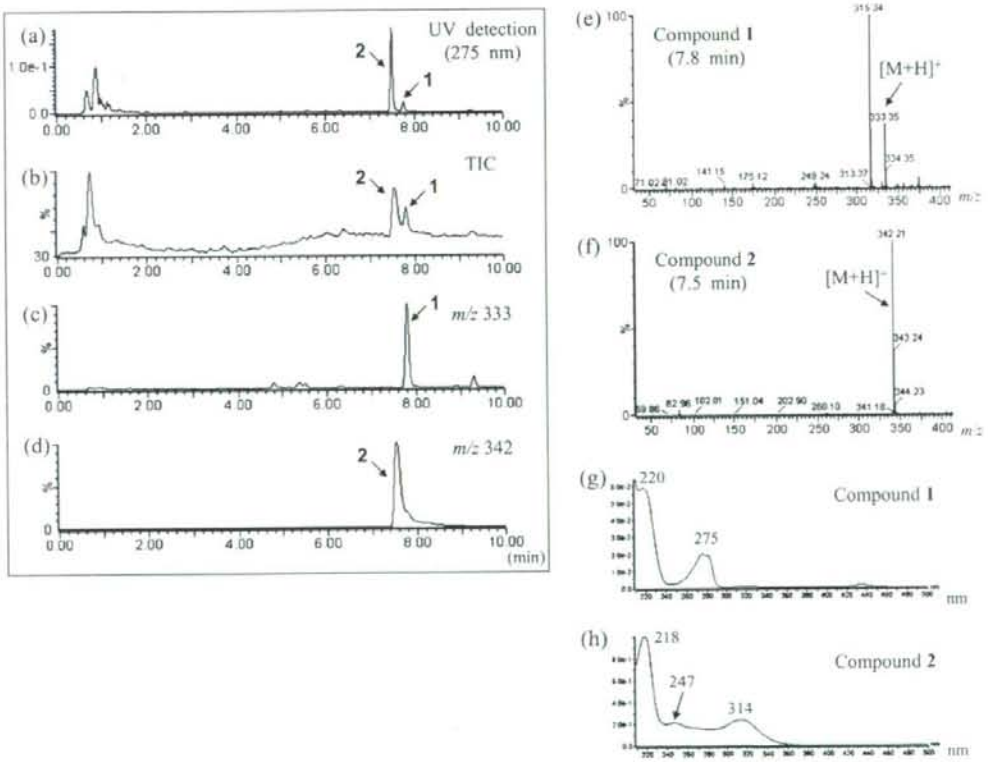

Fig. 2a–h Data from high-performance liquid chromatography with ultraviolet detection (**a**, **g**, **h**) and liquid chromatography-electrospray ionization-mass spectrometry (**b–f**) for the extract of the sample. Total ion chromatogram (**b**), mass chromatograms at *m/z* 333 (**1**) (**c**) and *m/z* 342 (**2**) (**d**), electrospray ionization mass spectra (**e**, **f**) and ultraviolet spectra (**g**, **h**) of each peak are shown

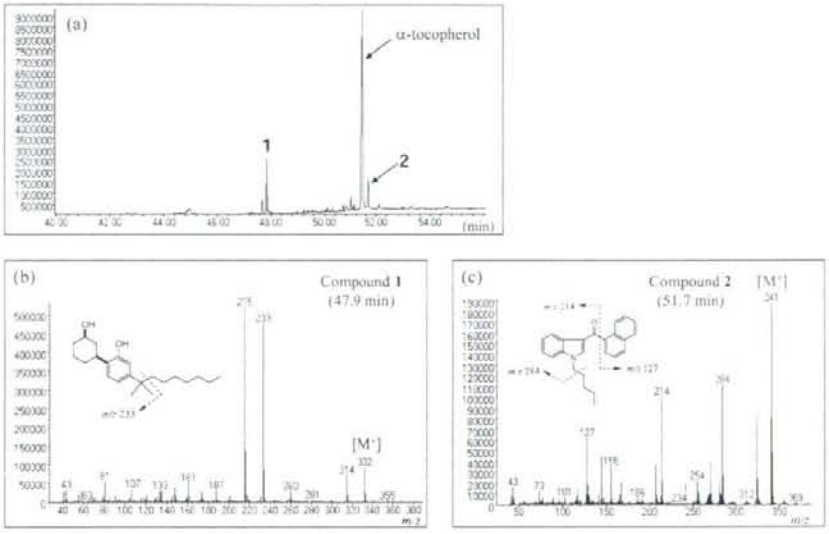

Fig. 3 Total ion chromatogram (**a**) and electron impact mass spectra of the peaks detected at 47.9 min (**1**) (**b**) and 51.7 min (**2**) (**c**) measured by gas chromatography-mass spectrometry

Table 1 Nuclear magnetic resonance data of compound **2**

No.	^{13}C	^{1}H	HMBC[a]
1	192.0	–	–
2'	137.9	7.33, 1H, s, overlapped	1, 3', 3'a, 7'a, 1"
3'	117.5	–	–
3'a	127.0	–	–
4'	122.9	8.47, 1H, m	3', 3'a, 6', 7'a
5'	122.8	7.35, 1H, m, overlapped	7'
6'	123.6	7.35, 1H, m, overlapped	7'a
7'	110.0	7.38, 1H, m, overlapped	3'a, 5', 7'a
7'a	137.0	–	–
1"	47.2	4.05, 2H, t, J = 7.4 Hz	2', 7'a, 2", 3"
2"	29.5	1.79, 2H, quint, J = 7.4 Hz	1", 3", 4"
3"	28.9	1.24, 2H, m, overlapped	1", 4", 5"
4"	22.2	1.28, 2H, m, overlapped	2", 3", 5"
5"	13.8	0.83, 3H, t, J = 7.0 Hz	3", 4"
1"'	139.1	–	–
2"'	125.8	7.64, 1H, dd, J = 7.1, 1.3 Hz	1, 3"', 4"', 8"'a
3"'	124.5	7.51, 1H, dd, J = 8.3, 7.1 Hz, overlapped	1"', 2"', 4"'a
4"'	129.9	7.95, 1H, brd, J = 8.3 Hz	2"', 4"'a, 5"', 8"'a
4"'a	133.7	–	–
5"'	128.1	7.90, 1H, brd, J = 8.3 Hz	4"', 7"', 8"'a
6"'	126.3	7.50, 1H, td, J = 6.9, 1.4 Hz, overlapped	4"'a, 7"', 8"'
7"'	126.7	7.45, 1H, ddd, J = 8.3, 6.9, 1.4 Hz	5"', 8"'a
8"'	126.0	8.17, 1H, brd, J = 8.3 Hz	1"', 4"'a, 6"', 8"'a
8"'a	130.8	–	–

Recorded in $CDCl_3$ at 600 and 800 MHz (^{1}H) and 150 and 200 MHz (^{13}C), respectively; data in δ ppm

[a]For heteronuclear multiple-bond correlation (HMBC), J = 8 Hz, the proton signal correlated with the indicated carbons

the mass number observed and theoretical mass number of $[M+H]^+$ was −0.10 amu.

The ^{1}H NMR spectrum of **2** showed 23 nonexchangeable protons, including a methyl signal at δ 0.83 (3H, t, J = 7.0 Hz), AB_2-type aromatic proton signals at δ 7.51 (1H, dd, J = 8.3, 7.1 Hz), 7.64 (1H, dd, J = 7.1, 1.3 Hz), and 7.95 (1H, brd, J = 8.3 Hz), and AA'BB'-type aromatic proton signals at δ 7.45 (1H, ddd, J = 8.3, 6.9, 1.4 Hz), 7.50 (1H, td, J = 6.9, 1.4 Hz), 7.90 (1H, brd, J = 8.3 Hz), and 8.17 (1H, brd, J = 8.3 Hz) as shown in Table 1. In addition, the ^{1}H NMR spectrum also showed three methylene proton signals, at δ 1.24 and 1.28 (each 2H, m) and at 1.79 (2H, quint, J = 7.4 Hz), as well as a characteristic methylene signal connected to a nitrogen atom at δ 4.05 (2H, t, J = 7.4 Hz). The ^{13}C NMR spectrum of **2** showed 24 carbon signals, suggesting the

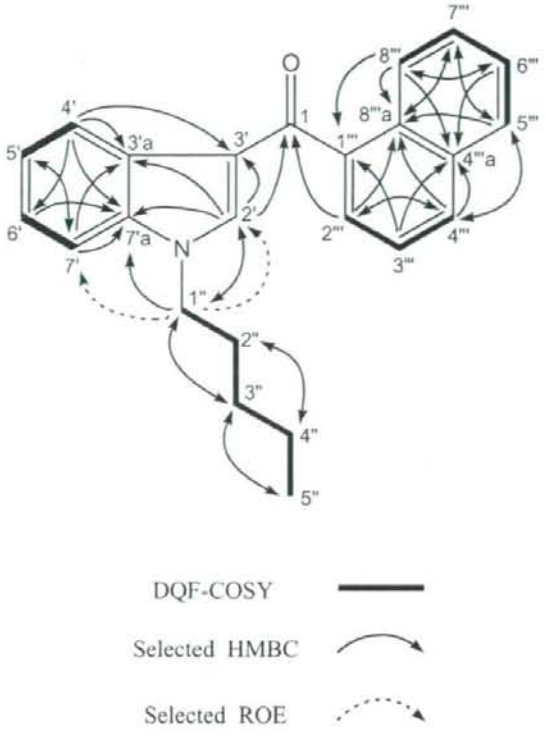

Fig. 4 Selected correlations for compound **2** by two-dimensional nuclear magnetic resonance spectroscopy techniques. *DQF-COSY*, Double quantum filtered correlation spectroscopy; *HMBC*, heteronuclear multiple-bond correlation spectroscopy; *ROE*, rotating frame nuclear overhauser effect spectroscopy

presence of a methyl, 4 methylenes with a nitrogenated carbon (δ 47.2), 12 aromatic carbons (δ 110.0, 122.8, 122.9, 123.6, 124.5, 125.8, 126.0, 126.3, 126.7, 128.1, 129.9, and 137.9), 6 aromatic quaternary carbons (δ 117.5, 127.0, 130.8, 133.7, 137.0, and 139.1), and a carbonyl carbon (δ 192.0). The presence of three partial structures (a 1,3-substituted indole group, a 1-substituted naphthalene group, and an *n*-pentyl group) was suggested from the DQF-COSY, HMQC, and HMBC spectra (Table 1, Fig. 4). The connectivity of these groups and the carbonyl group was deduced from the HMBC spectrum. An aromatic proton at δ 7.33 (H-2') of the indole group correlated to the carbonyl carbon at δ 192.0 (C-1), and the methylene carbon at δ 47.2 (C-1") of the *n*-pentyl group and an aromatic proton at δ 7.64 (H-2''') of the naphthalene group showed correlations to the carbonyl carbons at δ 192.0 (C-1). In addition, irradiation of the methylene protons at δ 4.05 (H-1") of the *n*-pentyl group resulted in ROE responses by the aromatic protons (H-2' and H-7') as shown in Fig. 4.

On the basis of the mass spectra (Figs. 2, 3) and NMR data (Table 1, Fig. 4), the structure of **2** was finally elucidated as 1-pentyl-3-(1-naphthoyl)indole [or naphthalen-1-yl-(1-pentylindol-3-yl)methanone]. The deduced compound had been already synthesized and named JWH-018 by Wiley et al. [2] in 1998. This compound is a potent cannabinoid receptor agonist possessing a pharmacological activity of a cannabinoid in vivo [2–5]. Wiley et al. [2] described that **2** showed a 4.5-fold more potent affinity for the CB_1 receptor ($K_i = 9 \pm 5$ nM) than did Δ^9-tetrahydrocannabinol (Δ^9-THC, Fig. 1), which is psychoactive and a major constituent of *Cannabis sativa* L. (cannabis, hemp, marijuana, marihuana) ($K_i = 41 \pm 2$ nM). Compound **2** produced potent cannabinoid effects of antinociception, hypomobility, hypothermia, and ring immobility in in vivo assays [2,3]. In the present study, we have identified compound **2** as a designer drug and an adulterant together with **1** in a herbal product.

The synthesis of many analogs of **1** and **2** together with pharmacological data has been already described [2–5,8–11]. In the past few decades, a number of analogs of Δ^9-THC have been synthesized based on the partially reduced dibenzopyran structure of THC, and their structure–activity relationships were studied [12,13]. In the 1980s, a group at Pfizer explored the development of analgesics using potent synthetic nontraditional cannabinoids, which lack the dibenzopyran structure present in the traditional cannabinoids but exhibit typical cannabinoid pharmacological effects [14–22]. On the other hand, D'Ambra et al. [23] reported in 1992 that aminoalkylindoles, such as WIN-55212-2, were bound to a cannabinoid brain receptor with high affinity (Fig. 1). A subsequent study by Huffman et al. [24] established that an aminoalkyl portion of the molecule, such as WIN-55212-2, could be replaced by an alkyl group to provide indole derivatives that have higher affinity for the brain receptor and exhibit typical cannabinoid pharmacological effects in vivo. These authors also described the structure–activity relationships of indole-derived, pyrrole-derived, and indene-derived cannabinoids [2,3,11]. After the discovery of cannabinoid receptors, CB_1 (central type) and CB_2 (peripheral type), as well as the discovery of endogenous cannabinoids, their physiological roles were elucidated to some extent [25]. A number of cannabinoid analogs, such as derivatives based on THC, indole, pyrrole, indene, and pyrazole, were then newly synthesized and their pharmacological activities applicable to the treatments of various diseases were studied [26,27]. This situation alerts us that these cannabinoid analogs other than **1** and **2** will be found as designer drugs or adulterants in illegal products as cannabis replacements in the near future. To avoid health problems and abuse caused by new designer

drugs, we must continuously monitor such compounds through surveillance.

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