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—Review—

コンピュータシミュレーションによる違法ドラッグの活性予測

栗原正明

Computational Study on Prediction of Bioactivity for Regulation of New Designer Drugs

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A method of prediction of biological activities of chemicals has been developed as a new drug-discovery technology. In recent years, a wide distribution of non-controlled psychotropic substances has become a serious problem in Japan. It takes a long time to evaluate their bioactivity *in vitro* and *in vivo*. Computer simulation could regulate new designer drugs in a short time. Prediction of biological activities of these drugs was performed by quantitative structure-activity relationship (QSAR) and pharmacophore-fingerprint method. A preliminary demonstration to predict the bioactivity of 4-methcathinone, a cathinone derivative that is widely distributed, by two methods is described herein.

Key words——designer drug; computer simulation; pharmacophore-fingerprint; Quantitative Structure-Activity Relationship (QSAR)

1. はじめに

違法ドラッグが大きな社会問題となっている．そこで，違法ドラッグを速やかに規制するためには，違法ドラッグの迅速な評価法が必要である．動物実験や生物学的試験には多くの時間が必要であり，迅速な規制のためにはコンピュータを用いたインシリコ評価法を開発することが必要である．化学物質の生物活性を予測する方法は創薬のために発展した技術である．筆者らもコンピュータシミュレーションを用いて受容体のリガンド，酵素の阻害剤等の創製を行ってきた．[1-4] 生物活性を予測する方法には大きく分けて2つの方法がある．1つは標的タンパク質の三次元構造が明らかな場合，もう1つは標的タンパク質が不明であるか判明しているが三次元構造が明らかでない場合である．前者の場合はドッキングスタディを行うことにより，活性を予測することが可能である．違法薬物であるシルデナフィル類似化合物の規制にはこの方法が有効であった．[5,6] それはシルデナフィルが作用する酵素（ヒトホスホジエステラーゼ5）が明らかであり，さらにX線構造解析も行われており3次元構造が解明されているからである．Figure 1にシクロペンチナフィルの構造とヒトホスホジエステラーゼ5との結合モデルを示した．

後者の場合はドッキングスタディを行うことができないので，リガンド側の情報のみで予測を行う．方法論としてはquantitative structure-activity relationship（QSAR；定量的構造活性相関）が一般的である．QSARとは化合物の構造と生物学的（薬学的あるいは毒性学的）な活性とを定量的に数学的な関係であらわしたものである．また，ファーマコフォアフィンガープリント法による構造類似性と生物活性から活性を予測する方法も検討した．QSAR法は活性を評価に入れた統計的モデルであるのに対して，ファーマコフォアフィンガープリント法は構造の類似性のみからモデルを作った予測法と言える．以下実際に行った1つの例として違法ドラッグとして流通した4-メチルメトカチノンの場合を述べる．4-メチルメトカチノンの構造はFig. 2に示した．

2. QSARによる違法ドラッグの活性予測

QSAR（定量的活性相関）法で4-メチルメトカチ

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本総説は，日本薬学会第132年会シンポジウムS09で発表したものを中心に記述したものである．

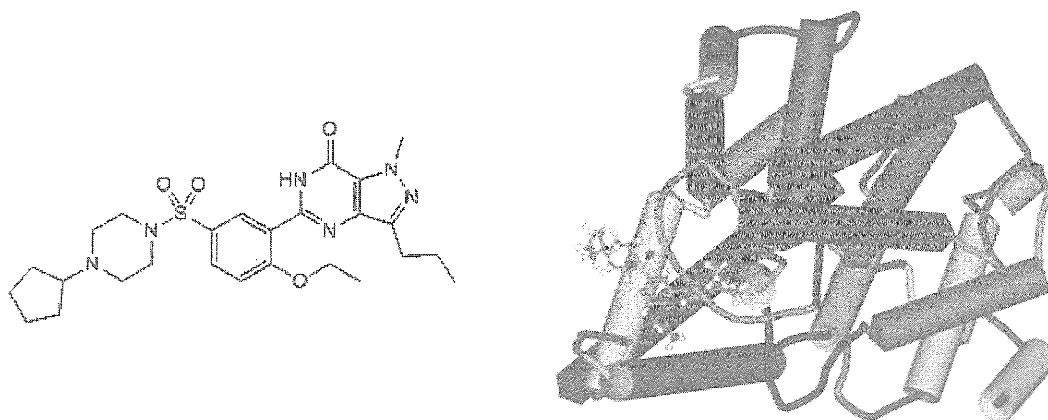

Fig. 1. Structure of Cyclopentynafil and PDE5

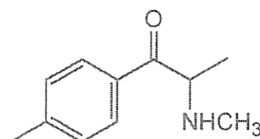

Fig. 2. Chemical Structure of 4-Methlmethcathinone

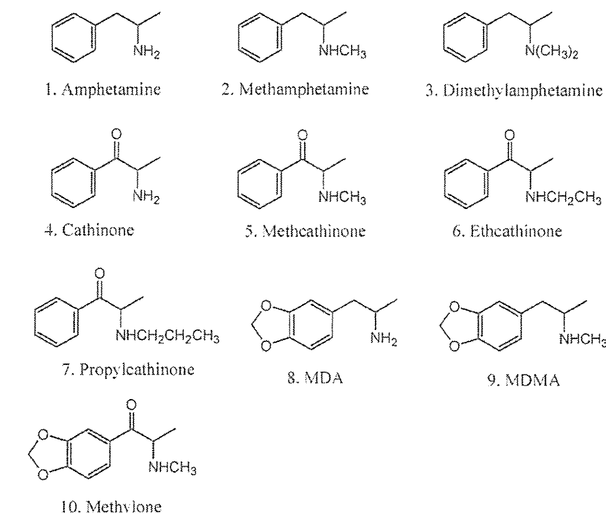

Fig. 3. Chemical Structures of Compounds **1–10**

ノンの活性予測を行った．QSAR のモデル構築には化学計算パッケージ MOE（CCG 社）を用いた．

活性が既知の化合物として Amphetamine **1**, Methamphetamine **2**, Dimethylamphetamine **3**, Catinone **4**, Methcatinone **5**, Ethcathinone **6**, Propylcathinone **7**, MDA **8**, MDMA **9**, Methylone **10** を用いた．**1–10** の化学構造を Fig. 3 に示した．活性値は（+）-アンフェタミン（1 mg/kg）で弁別したラットを用いて般化試験を行った際の構造類似化合物 **1–10** の実測活性値（ED_{50} 値）を採用した．[7] **1–10** の活性値は Table 1 に示した．

MOE に搭載されている AutoQuaSAR 法を使って妥当な QSAR モデル式を構築し，4-メチルメトカチノンの活性値を予測した．QSAR モデル式で用いた記述子は，MOE 上で動作する AutoQuaSAR プログラムによって，MOE に搭載されている 184 のすべての 2D 記述子から選択されたものである．QSAR 式は交差検定の R^2（相関係数の 2 乗）が最もよいものを用いた．QSAR の結果をグラフにしたものを Fig. 4（X 軸：活性値の予測，Y 軸：活性値）に示した．相関係数の 2 乗（R^2）=0.84, 交差検定の相関係数の 2 乗(XR^2)=0.92 であった．QSAR 式は Fig. 5 に示した．この QSAR 式で 4-メチルメトカチノンの活性予測をしたものを Fig. 6（X 軸：活性値の予測，Y 軸：活性値）に示した．

Table 1. Activities of Psychotropic Substances

No.	Psychotropic substances	Activities (ED_{50}, mg/kg)
1	Amphetamine	0.71
2	(±)-Methamphetamine	0.49
3	(+)-Dimethylamphetamine	2.92
4	(±)-Catinone	0.71
5	(±)-Methcatinone	0.37
6	Ethcathinone	0.77
7	Propylcathinone	2.03
8	MDA	2.29
9	MDMA	1.64
10	Methylone	2.36

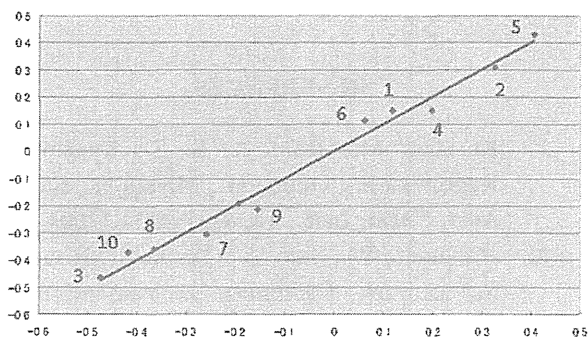

Fig. 4. Results of QSAR

$$
\begin{aligned}
pIC_{50} &= 1.40145 \\
&- 0.0365513 * PEOE_VSA\text{-}0 \\
&- 0.0848188 * PEOE_VSA\text{-}1 \\
&- 0.0168429 * SMR_VSA2 \\
&+ 0.0168429 * SlogP_VSA5
\end{aligned}
$$

PEOE: The Partial Equalization of Orbital Electronegativities method of calculating partial charges

SMR: Molecular refractivity

SlogP: Log of the octanol/water partition coefficient

Fig. 5. QSAR Equation

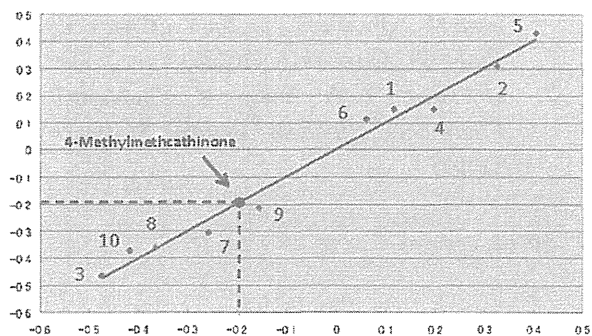

Fig. 6. Prediction of the Activity of 4-Methylmethcathinone

3. ファーマコフォアフィンガープリント法による違法ドラッグの活性予測

化学構造の類似性のみによる評価法として，ファーマコフォアフィンガープリント法による評価を行った．QSAR 同様化学計算パッケージ MOE（CCG 社）を用いた．2 点のファーマコファアのグラフ距離で分子の類似性を評価する TGD 法を用いた．活性が既知の化合物として Amphetamine **1**, Methamphetamine **2**, Dimethylamphetamine **3**, Catinone **4**, Methcatinone **5**, Ethcathinone **6**, Propyl-cathinone **7**, MDA **8**, MDMA **9**, Methylone **10** を用いた．活性の強い化合物 Methamphetamine **2**, Methcatinone **5** をテンプレートとして構造の類似性を算定した．類似性を Table 2 に示した．4-メチルメトカチノンの類似性を計算すると 0.813 となった．ファーマコフォアフィンガープリント法による化学構造の類似性と生物活性値との相関を Fig. 7（X 軸：構造の類似性，Y 軸：活性値）に示した．

麻薬，覚せい剤，指定薬物を含む構造類似化合物 10 化合物の活性（既知）と比較するために，QSAR（定量的活性相関）法及びファーマコフォアフィンガープリント法の 2 方法で評価した．4-メチルメトカチノンは，どちらの方法でもこれら規制化合物群と同程度の活性があることが予測された．このデータは 4-メチルメトカチノンを規制するための科学的データとして使われた．4-メチルメトカチノンのほかに QSAR 法及びファーマコフォアフィンガープリント法によりレギュレーションを行った化合物を Fig. 8 に示した．

Table 2. Similarity of Compounds **1**–**10**

Compounds	Similarity
Amphetamine **1**	0.844
Methamphetamine **2**	0.878
Dimethylamphetamine **3**	0.629
Catinone **4**	0.854
Methcatinone **5**	0.878
Ethcathinone **6**	0.860
Propyl-cathinone **7**	0.813
MDA **8**	0.698
MDMA **9**	0.699
Methylone **10**	0.671

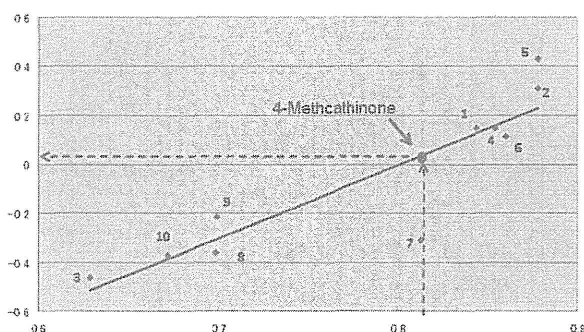

Fig. 7. Results of Pharmacophore-Fingerprint Method

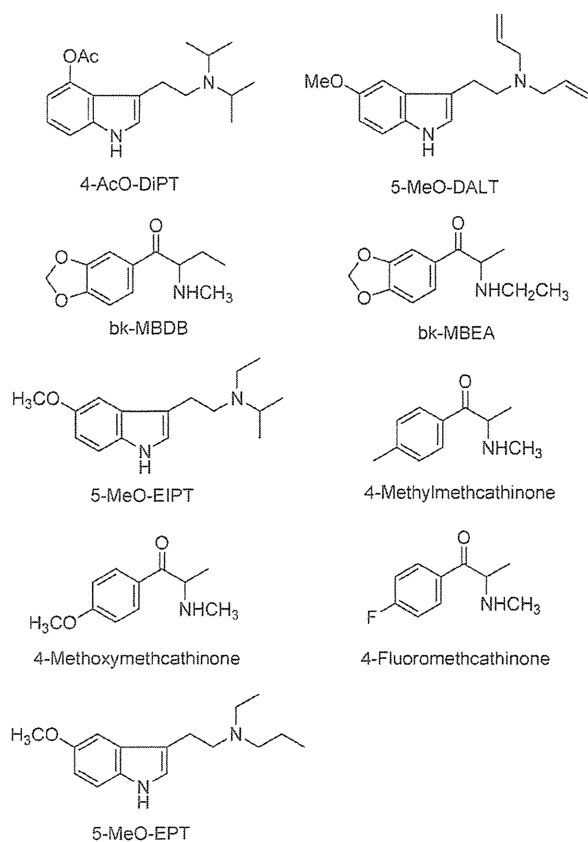

Fig. 8. Chemical Structures of Compounds Evaluation

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DNA sequence analyses of blended herbal products including synthetic cannabinoids as designer drugs☆

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ABSTRACT

In recent years, various herbal products adulterated with synthetic cannabinoids have been distributed worldwide via the Internet. These herbal products are mostly sold as incense, and advertised as not for human consumption. Although their labels indicate that they contain mixtures of several potentially psychoactive plants, and numerous studies have reported that they contain a variety of synthetic cannabinoids, their exact botanical contents are not always clear. In this study, we investigated the origins of botanical materials in 62 Spice-like herbal products distributed on the illegal drug market in Japan, by DNA sequence analyses and BLAST searches. The nucleotide sequences of four regions were analyzed to identify the origins of each plant species in the herbal mixtures. The sequences of "Damiana" (*Turnera diffusa*) and Lamiaceae herbs (*Mellissa, Mentha* and *Thymus*) were frequently detected in a number of products. However, the sequences of other plant species indicated on the packaging labels were not detected. In a few products, DNA fragments of potent psychotropic plants were found, including marijuana (*Cannabis sativa*), "Diviner's Sage" (*Salvia divinorum*) and "Kratom" (*Mitragyna speciosa*). Their active constituents were also confirmed using gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS), although these plant names were never indicated on the labels. Most plant species identified in the products were different from the plants indicated on the labels. The plant materials would be used mainly as diluents for the psychoactive synthetic compounds, because no reliable psychoactive effects have been reported for most of the identified plants, with the exception of the psychotropic plants named above.

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1. Introduction

In recent years, various herbal products (dried leaves, stems, petals and seeds mixtures) including the synthetic marijuana product "Spice," have been distributed around the world via the Internet [1–12]. In Japan, these products are readily available as herbal incense via the Internet and in "head shop". However, anonymous posters on various Internet web sites have reported experiencing cannabis-like effects after smoking these herbal products.

In 2009, our group and a group in Germany were the first to detect and identify the synthetic cannabinoids cannabicyclohexanol (CCH) and JWH-018 in these herbal products [5–7]. Since those reports, more than 30 synthetic cannabinoids have been detected as psychoactive ingredients in these herbal products in various countries around the world [1–12]. In addition, our group identified two new-type synthetic cannabinoids [12]. In Japan, 23 of these synthetic cannabinoids were controlled as "Designated Substances" under the Pharmaceutical Affairs Law as of July 2012 [13,14].

The labels of these herbal products indicate that these products contain several potentially psychoactive plants. In some instances, the ingredients are listed as common plant names, such as "Baybean," "Blue lotus," "Pink lotus," "Dwarf skullcap," "Indian warrior," "Lion's tail," "Maconha brava," "Marshmallow," "Red clover," "Rose," "Siberian motherwort," and "Vanilla". However, there is no precise information on their contents, and the actual plant species included in these herbal products have never been determined.

The identification of plant spices by morphology requires botanical knowledge and a great deal of experience. In addition, morphology and anatomy rarely confirm the source of the plant, particularly for degraded and fragmented materials, unless some content information already exists. Recently, a new genetic analysis technique in plants has been widely used for species

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identification and the determination of phylogenetic relationships. The advances in molecular genetics over the last few years have provided genetic markers involved in the conservation of plant genetic resources for easy and reliable identification of plant species [15,16]. The methodology of DNA barcoding is used not only in botany but also in forensic science and herbal medicine. Moreover, the use of short orthologous standard DNA sequences as a tool for species identification with a standardized protocol, known as DNA barcoding, has been proposed and initiated to facilitate biodiversity and taxonomic studies [17]. A variety of loci have been recently suggested as potential DNA barcodes in plants, in both the nuclear and chloroplast genomes (e.g., the internal transcribed spacer (ITS), external transcribed spacer (ETS), *trnH-psbA*, *rpl16* intron, *matK*, *rbcL*, and *trnL-trnF* intron [17–26]). In addition, The Plant Working Group of the Consortium for the Barcode of Life (CBOL) has recently proposed a two-locus combination of *matK* and *rbcL* as the standard plant barcode [23]. CBOL is fostering the development of international research alliances to build a barcode library for all eukaryotic organisms. The main purpose of DNA barcoding is to provide rapid and accurate identification of unidentified plant organisms whose DNA barcodes have already been registered in a sequence library (DNA database; BLAST or BOLD systems).

In this study, we investigated the plant species (genus or family) of botanical materials included in herbal products by sequencing three regions of the chloroplast genome (*trnL-trnF*, *matK*, and *rbcL*) and one of the nuclear genome (an ITS combination), and positive matches in two or more regions were considered sufficient for identification of a particular species. Moreover, we analyzed some cannabinoids in an herbal product in which Cannabis genome sequences were detected by using gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS).

2. Materials and methods

2.1. Plant materials

Sixty-two herbal products being sold in Japan were purchased via the Internet from 2008 to 2011. All products had different names and were contained in different packages. We reported that various synthetic cannabinoids were contained as psychoactive ingredients in all these herbal products (see Table 1) [5,6,8–12]. Diviner's Sage (*Salvia divinorum*) was from a laboratory collection [27].

2.2. Chemicals and reagents

Δ^9-Tetrahydrocannabinol (Δ^9-THC) was purchased from Cirilliant (Round Rock, TX). Authentic CCH was isolated from herbal products and identified in our previous studies [5,6]. Cannabidiol (CBD) and cannabinol (CBN) were purchased from Sigma–Aldrich. All reagents and chemicals used were of analytical reagent grade or high-performance liquid chromatography (HPLC) grade.

2.3. DNA extraction, amplification and sequencing

Each herbal product (dry minced leaves, stems, petals and seed mixtures) was separated to obtain a single fragment (tissue) and then the surface of the tissue was rinsed with 100% ethanol. Each separated sample (ca. 10 mg) was transferred into a 2.0 mL reaction tube, and crushed in an MM-300 mixer mill (Qiagen, Germany) under liquid nitrogen. Total genomic DNA was isolated from the different types of tissue (dry minced leaves, stems, petals and seeds) with a QIAGEN DNeasy plant mini kit (Qiagen, Germany) following the manufacturer's guidelines. Using different sets of primers, we amplified the three regions of the chloroplast DNA, i.e., the *trnL-trnF* (comprised of the *trnL* intron with its 3′exon, and the *trnL-trnF* spacer), *matK*, and *rbcL* regions and the single region of nuclear rDNA, i.e., the ITS region (comprised of the internal transcribed spacer 1 between 18S rDNA and 5.8S rDNA, and 5.8S rDNA, and internal transcribed spacer 2 between 5.8S rDNA and 26S rDNA) by a polymerase chain reaction (PCR) using the respective genomic DNA samples as a template (Fig. 1). Approximately 1–5 ng of template DNA was used in a 20 μL PCR reaction consisting of 10 μL 2× Ampdirect plus (Shimadzu, Japan), 0.5 μM of each primer, and 0.5 units of Ex Taq (5 U/μL, Takara, Japan). Amplification was performed using the following protocol: an initial cycle at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 90 s, and a final extension step at 72 °C for 10 min. The universal primers were designed from each of the sequences conserved between the plant genes [18,28]. Their sequences were as follows: *trn*-forward primer, 5′-CGAAATCGGTAGACGCTACG-3′; *trn*-reverse primer, 5′-ATTTGAACTGGTGACACGAG-3′; *matK*-forward primer, 5′-CGTACAGTACTTTTGTGTTTACGAG-3′; *matK*-reverse primer, 5′-ACCCAGTCCATCTGGAAATCTTGGTTC-3′; *rbcL*-forward primer, 5′-ATGTCACCACAAACAGAGACTAAAGC-3′; *rbcL*-reverse primer, 5′-GTAAAATCAAGTCCACCRCG-3′; ITS-forward primer, 5′-CCTTATCATTTAGAGGAAGGAG-3′; ITS-reverse primer, 5′-TCCTCCGCTTATTGATATGC-3′. PCR products were confirmed by separation on 1% agarose gels containing 0.3 μg/mL of ethidium bromide. The single PCR product was purified and concentrated using polyethylene glycol precipitation, and direct sequencing of purified PCR products was carried out with each of the forward and reverse PCR primer pairs with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems). In the case of mixed PCR products, the DNA fragments were subcloned into the pMD20-T vector of a Mighty TA-cloning Kit (Takara), and insert DNA was amplified using M13 primers by colony PCR, and then was sequenced by the method described above. For sequence similarity we used the nucleotide BLAST programme (http://blast.ncbi.nlm.nih.gov/) [29,30] to identify each sample sequence.

2.4. Identification of plant species

When two different regions among the four (*trnL-trnF*, ITS, *rbcL* and *matK*) showed very high similarity (more than 99%) to the sequences of the registered reference species in GenBank, we defined the sample plant as belonging to the reference species for purposes of this report.

2.5. Preparation of samples from herbal products for GC and LC–MS analyses

Ten mg of the product sample was crunched and extracted with 1 mL of methanol by ultrasonication for 10 min. After centrifugation (5 min at 3000 rpm), the solution was passed through a centrifugal filter (Ultrafree-MC, 0.45 mm filter unit; Millipore, Bedford, MA).

2.6. GC–MS conditions

MS analysis was performed by GC–MS in electron impact (EI) mode at 70 eV electron energy. The GC–MS 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. The initial column temperature was 80 °C, and was increased at a rate of 5 °C/min to 190 °C followed by 10 °C/min to 310 °C. Data were obtained in full scan mode with a scan range of m/z 40–550. The analysis was performed using the established method and under conditions described in our previous reports [13,14].

2.7. LC–MS conditions

LC–MS analysis was conducted on an ultra-performance liquid chromatography–electrospray ionization-mass spectrometer (UPLC–ESI-MS), consisting of an ACQUITY UPLC system, a single-stage quadrupole detector and a photo diode array (PDA) detector (Waters, Milford, MA). The sample solutions were separated using an Atlantis HSS T3 column (100 mm × 2.1 mm i.d., particle size 1.8 μm; Waters) protected by a Van Guard column (5 mm × 2.1 mm i.d., 1.8 μm; Waters) at 40 °C. The mobile phase consisted of 0.1% formic acid in water (solvent A) and acetonitrile/MeOH (1:1) containing 0.1% formic acid (solvent B). The LC flow programme was: initially 95% A, linearly changed to 80% A in 15 min, then changed to 20% A at 25 min, and held for 25 min [A:B 95:5–80:20 (15 min) – 20:80 (25–50 min)]. The flow rate of the mobile phase was 0.3 mL/min and the injection volume was 1 μL. The wavelength of the PDA detector was set from 190 to 500 nm.

3. Results and discussion

3.1. DNA analyses of herbal products

We attempted to investigate the origins of plant species in each unknown herbal minced mixture (herbal product) using the DNA barcoding method. The central concept in species identification is to match the sequence of the applied sample to a reference sequence through DNA sequence similarity searches. If the sequence data of the applied sample is completely matched with the data in the GenBank database by a BLAST search, we can obtain the information of the correct plant species. In addition, even if no sequence data for the appropriate species have been published in GenBank, we can obtain the information of the correct genus or family affiliation from the database.

The contents printed on the herbal product packages included the plants Baybean (*Canavalia rosea*), Indian warrior (*Pedicularis*

Table 1
List of botanical origins identified in herbal products using DNA analysis.

Sample no.	Damiana *Turnera diffusa*	Lamiaceae Mint herbs *Mellisa, Mentha, Thymus* sp.	Liquorice *Glycyrrhiza glabra*	Mullein *Verbascum thapsus*	Tea *Camellia sinensis*	Marshmallow *Althaea officinalis*	Astragalus *Astragalus membranaceus*	Red clover *Trifolium pratense*	Raspberry *Rubus idaeus*	Marigold *Calendula officinalis*
1		+								
2			+		+					
3			+		+					
4			+							
5		+	+							
6			+				+			
7										
8			+				+			
9							+			
10	+			+						
11		+								
12		+								
13	+			+			+			
14		+								
15		+								
16			+			+				
17			+							
18										
19										+
20	+									
21			+				+	+		
22				+						
23				+						
24				+						
25	+									
26	+			+				+		
27						+				
28										
29	+									
30	+				+					
31										+
32						+				
33						+				
34									+	
35					+					
36	+				+					
37						+				
38					+					
39	+			+						
40	+									
41									+	
42									+	
43		+								
44	+									
45		+								+
46								+		
47								+		
48		+								
49										
50	+									
51						+				+
52										
53	+				+					
54					+					
55	+									
56										
57		+								
58	+									
59	+									
60	+									
61		+								
62									+	

Sample no.	Others	Detected compounds[a]		
1		Cannabicyclohexanol	CP-474,97	α-tocopherol
2	*Morus alba*	Cannabicyclohexanol	α-tocopherol	
3		Cannabicyclohexanol		
4		Cannabicyclohexanol	JWH-018	α-tocopherol
5		Cannabicyclohexanol		
6		Cannabicyclohexanol	JWH-018	
7	*Urtica dioica*	Cannabicyclohexanol	JWH-018	
8		JWH-018		
9		Cannabicyclohexanol	JWH-018	

Table 1 (*Continued*)

Sample no.	Others		Detected compounds[a]					
10			Cannabicyclohexanol					
11	*Galium aparine*		Cannabicyclohexanol					
12			Cannabicyclohexanol	α-tocopherol				
13			Cannabicyclohexanol	α-tocopherol				
14			Cannabicyclohexanol	α-tocopherol				
15			Cannabicyclohexanol	JWH-018	α-tocopherol			
16			Cannabicyclohexanol	JWH-018	α-tocopherol			
17			Cannabicyclohexanol	JWH-018	α-tocopherol			
18	*Passiflora speciosa*		Oleamide	JWH-018	α-tocopherol			
19	*Nymphaea* sp.		Oleamide	JWH-018	α-tocopherol			
20			Oleamide	JWH-018	α-tocopherol			
21			JWH-018	α-tocopherol				
22			Oleamide	JWH-018				
23			Oleamide	JWH-018				
24			Oleamide	JWH-018				
25			Cannabicyclohexanol	JWH-073				
26	*Cannabis sativa*		Cannabicyclohexanol	THC				
27			JWH-018	α-tocopherol				
28	*Rosa* sp.		JWH-018	α-tocopherol				
29			JWH-073	α-tocopherol				
30			JWH-073					
31			JWH-018	JWH-073	α-tocopherol			
32			JWH-073	JWH-081				
33			JWH-073	JWH-250				
34			JWH-081	JWH-210	JWH-122	JWH-019		
35			JWH-250	JWH-015				
36			JWH-250					
37			JWH-250	JWH-081	JWH-073			
38			JWH-250	JWH-081	JWH-073	JWH-200		
39			Cannabicyclohexanol	JWH-018	α-tocopherol			
40			JWH-073					
41			JWH-251	JWH-081				
42			Cannabicyclohexanol					
43			Cannabicyclohexanol					
44			JWH-073	JWH-250	JWH-081			
45			JWH-250					
46			JWH-073					
47			JWH-210	JWH-019	AM-2201	JWH-203		
48	*Mitragyna speciosa*	*Salvia divinorum*	JWH-250	JWH-122	Mitragynine	Salvinorin A		
49	*Artemisia lactiflora*		JWH-073	JWH-250	JWH-081			
50			JWH-018	JWH-073	α-tocopherol			
51			JWH-122	JWH-210	JWH-019	AM-694	AM-2201	JWH-203
52	*Carthamus tinctorius*		JWH-203					
53			CB-13	APINACA				
54	*Centaurea cyanus*		JWH-210	JWH-019	AM-2201	JWH-203		
55	*Cannabis sativa*		RCS-4	CBD				
56	*Galium aparine*	*Gossypium barbadense*	APINACA	APICA				
57			APINACA	APICA				
58			JWH-019	JWH-203				
59	*Acleisanthes longiflora*	*Lippia* sp.	APINACA					
60	*Lippia* sp.		JWH-203					
61			APINACA	APICA				
62			APINACA	APICA				

[a] [5,6,8–12].

sp.), Lion's tail (*Leonotis leonurus*), Maconha brava (*Zornia* sp.) and Siberian motherwort (*Leonurus sibiricus*). The *trnL-trnF* and/or ITS sequence(s) data of these plants are present in the GenBank database. Moreover, the CBOL group suggested that *rbcL* and *matK* are good candidates for plant species identification [23]. Thus, we selected and analyzed the four sequence regions of *trnL-trnF*, ITS, *rbcL* and *matK* to identify plant species in the herbal products.

In this study, we used DNA sequence analyses to investigate botanical materials in 62 herbal products distributed in the illegal drug market in Japan. The plant species suggested by BLAST searches of the DNA fragments from each herbal product are shown in Table 1. In addition, all these herbal products were adulterated with synthetic cannabinoids. Identifications of these psychoactive compounds were described in detail previously [5,6,8–14]. As a result of the BLAST searches, in several cases, DNA fragments of two or more different plant species were detected from an herbal product (25/62) (Table 1). Damiana (*Turnrra diffusa*) DNA fragments were detected from the largest number of herbal products (18/62).

In Damiana, the obtained three sequences of ITS, *matK* and *rbcL* showed the highest homology to the published Damiana (*T. diffusa*) genome based on the BLAST search. However, the *trnL-trnF* sequence of Damiana is not registered in the GenBank database. Therefore, the obtained *trnL-trnF* sequence was most similar to *Turnera ulmifolia* (92% similarity) which is a species related to Damiana (Table 2). Moreover, in the case of Kratom (*Mitragyna speciosa*), two regions of the *trnL-trnF*and *matK* sequences were not registered in the GenBank database, and thus identification of Kratom was carried out by using the other two regions (ITS and *rbcL*).

In the case of the Lamiaceae (mint) family, the species sometimes was not clearly identified based on sequence research alone. That is, the BLAST searches sometimes gave the same score for more than one species. This is the reason that the genus name

Table 2
BLAST search results of *trnL-trnF*, ITS, *matK* and *rbcL* for the plant species for which corresponding sequences are published in GenBank.

Common name	Best similar sp. of *trnL-trnF*	Acc. No.	Similarity (%)	Best similar sp. of ITS	Acc. No.	Similarity (%)	Best similar sp. of *matK*	Acc. No.	Similarity (%)	Best similar sp. of *rbcL*	Acc. No.	Similarity (%)
Chamomile	*Matricaria matricarioides*	U82047	97%	*Matricaria recutita*	EU179212	97%	*Matricaria chamomilla*	JN894233	99%	*Matricaria chamomilla*	JN892268	99%
Common balm	*Melissa officinalis*	AJ505529	99%	*Melissa officinalis*	DQ667291	98%				*Melissa officinalis*	Z37414	99%
Damiana	*Turnera ulmifolia*	AY636110	92%	*Turnera diffusa*	AY973381	99%	*Turnera diffusa*	JQ588584	99%	*Turnera diffusa*	JQ593107	99%
Diviner's Sage	*Salvia divinorum*	HQ418964	99%	*Salvia divinorum*	DQ667249	99%	*Salvia divinorum*	In this study	100%	*Salvia divinorum*	L14407	99%
Kratom				*Mitragyna speciosa*	JF412827	99%	*Mitragyna rubrostipulata*	AY538390	98%	*Mitragyna speciosa*	AJ346988	99%
Liquorice	*Glycyrrhiza uralensis*	EF606870	93%	*Glycyrrhiza glabra*	AY065623	99%	*Glycyrrhiza glabra*	AB280742	100%			
Marigold	*Calendula officinalis*	HQ439868	99%	*Calendula officinalis*	AF422114	99%				*Calendula officinalis*	HM849835	99%
Marijuana/hemp	*Cannabis sativa*	AF501598	99%	*Cannabis sativa*	FJ572045	99%	*Cannabis sativa*	AF345317	99%	*Cannabis sativa*	JQ231002	99%
Marshmallow	*Althaea officinalis*	EF419727	100%	*Althaea officinalis*	EF679733	99%	*Althaea officinalis*	EU346765	99%	*Althaea officinalis*	JN891502	99%
Milkvetch	*Astragalus memoriosus*	AB485943	96%	*Astragalus membranaceus*	HQ891827	99%	*Astragalus membranaceus*	HM142236	100%			
Mugwort	*Artemisia argyi*	FJ710525	100%	*Artemisia argyi*	GQ396673	98%	*Artemisia argyi*	GQ436429	98%	*Artemisia argyi*	HM989725	99%
Mullein	*Verbascum speciosum*	AJ492271	99%	*Verbascum thapsus*	JQ801746	98%	*Verbascum thapsus*	AF052002	99%	*Verbascum thapsus*	L36452	99%
Passionflower	*Passiflora speciosa*	AY102402	99%	*Passiflora incarnata*	DQ344630	96%				*Passiflora incarnata*	HQ900864	99%
Raspberry				*Rubus idaeus*	AF055757	97%	*Rubus idaeus*	JN895011	99%	*Rubus idaeus*	HE574618	100%
Red Clover	*Trifolium pratense*	DQ311888	98%	*Trifolium pratense*	DQ312138	99%	*Trifolium pratense*	JN894446	99%			
Stickywilly	*Galium aparine*	GU357174	99%	*Galium aparine*	AF419186	96%	*Galium aparine*	HM850825	99%	*Galium aparine*	X81091	99%
Tea	*Camellia sinensis*	FR849963	99%	*Camellia sinensis*	FJ004863	97%	*Camellia sinensis*	AF380077	99%	*Camellia sinensis*	JN009632	99%
Water liliy	*Nymphaea gracilis*	AM422050	99%	*Nymphaea capensis*	AY707898	99%						
Zataria	*Zataria multiflora*	GU381626	99%	*Zataria multiflora*	GU381450	99%						

Blanks indicate that no investigation was run for this species.

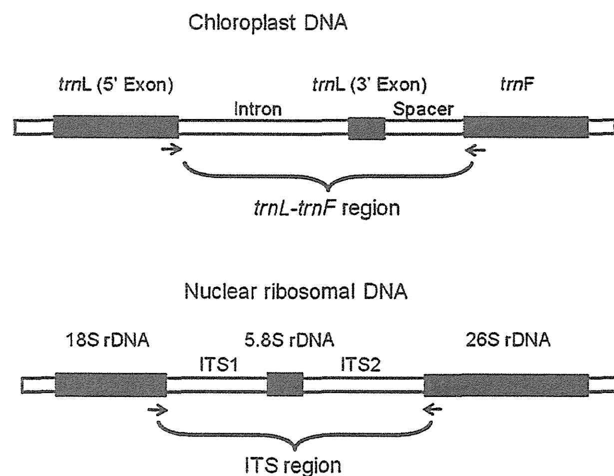

Fig. 1. Schematic representations of the *trnL-trnF* region in chloroplast DNA, and the ITS region in nuclear ribosomal DNA. The arrows indicate primer sites.

instead of the species name is given for the Lamiacea family members in Table 1.

Among the Lamiaceae plants, *S. divinorum* is a special case. Its *trnL-trnF*, ITS and *rbcL* sequences were present in GenBank, but the *matK* data was not registered. Thus, we obtained the *matK* sequence from genome DNA of *S. divinorum* [27]. In addition to the very high similarity of the other regions (*trnL-trnF*, ITS and *rbcL*), the *matK* sequence from sample No. 48 showed a 100% similarity with *S. divinorum* (in this study; Acc. No. AB734045).

In the case of two of the products (Nos. 26 and 55), the gene sequences, *trnL-trnF*, ITS, *matK* and *rbcL* amplified from a piece of plant tissue showed 99%, 99%, 99% and 100% similarities with *Cannabis sativa* (Acc. Nos. AF501598, FJ572045, AF345317 and JQ231002) in the BLAST search, respectively (Table 1; Fig. 2).

All of the examined products contained dry minced herbs and some of their packages indicated they contained herbal mixtures. The indicated ingredients were as follows: Baybean, Blue lotus, Dwarf skullcap, Indian warrior, Lion's tail, Maconha brava, Pink lotus, Siberian motherwort and Damiana. These plants seem to have been chosen because they are known or believed to have some psychoactive effects. For example, Lion's tail, Baybean, Siberian motherwort, and Maconha brava have been traditionally considered marijuana substitutes. These ingredients would be attractive to buyers who expect pharmacological effects from the product. However, the results of the sequence analyses were almost inconsistent with the plants indicated on the packages. Moreover, no reliable psychoactive effects have been reported for most of the identified plants. These facts suggested that the observed plant materials were used mainly as diluents for the psychoactive synthetic compounds. It is of interest that several plant species that are utilized for herbal tea in some countries, such as Damiana, Liquorice, Mullein and Marshmallow, were used as diluents for synthetic compounds in the present samples.

3.2. GC–MS and LC–MS analyses of herbal products

We also performed GC–MS and LC–MS analyses targeted especially to natural components of *Cannabis* plants. In the GC–MS total ion chromatogram of the methanol extract from product No. 26, three peaks at 43.84, 45.24, and 46.07 min were identified as CBD, Δ^9-THC and CBN, respectively, by comparing the mass spectra and retention times with those of authentic samples (Fig. 3), in addition to the peak of the synthetic cannabinoid, CCH, at 47.39 min. By the LC–MS analysis, the methanol extract of the

```
Cannabis sativa(FJ572045)    1:TTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAAACC-TGCAACAGCAGAACGACCCGTGAACACGT 69
ITS No. 26                   1:........................................-............................. 69
ITS No. 55                   1:........................................C............................. 70
HumlusLupulus(EF136401)      1:...................................T....-............................. 69

Cannabis sativa(FJ572045)   70:TTTAAACAGC-TTGGGCGGGCGAGAGGAGCTTGCTCCTTGGACCCGCCCGCACCTGCTGGGAGAAATCTC 138
ITS No. 26                  70:..........-........................................................... 138
ITS No. 55                  71:..........-........................................................... 139
HumlusLupulus(EF136401)     70:........A.C.....T.................................T........A........CT 139

Cannabis sativa(FJ572045)  139 GGCGGGCTAACGAACCCCGGCGCAATCTGCGCCAAGGAACAATAAAAGATTATCGCGTGGCTCGTGCGGT 208
ITS No. 26                 139 .....................T................................................ 208
ITS No. 55                 140 ...................................................................... 209
HumlusLupulus(EF136401)    140 ....................................................G.....TT.........A 209

Cannabis sativa(FJ572045)  209:GGCCCGGAGACGGTGTCCGCCAATCGAGATGCGTGTTTATCGAAATGTCTAAACGACTCTCGGCAACGGA 278
ITS No. 26                 209:...................................................................... 278
ITS No. 55                 210:...................................................................... 279
HumlusLupulus(EF136401)    210:.A.............CT....GC.....T........CT..A.T.......................... 279

Cannabis sativa(FJ572045)  279:TATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTG 348
ITS No. 26                 279:...................................................................... 348
ITS No. 55                 280:...................................................................... 349
HumlusLupulus(EF136401)    280:...................................................................... 349

Cannabis sativa(FJ572045)  349:AACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCACTAGGCCGAGGGCACGTCTGCCTGGGCGTCA 418
ITS No. 26                 349:...................................................................... 418
ITS No. 55                 350:...................................................................... 419
HumlusLupulus(EF136401)    350:...................................................................... 419

Cannabis sativa(FJ572045)  419:CACGCCGTTGCCCCCATGTGCACT-GCCAA-------AAGCGTG-----TTCAGGAGGGGCGGAGACTGG 475
ITS No. 26                 419:........................-.....-------.......-----..................... 475
ITS No. 55                 420:........................-.....-------.......-----..................... 476
HumlusLupulus(EF136401)    420:...A...........C.TGAAC..C.....TGCCTTA.T.G.A.AAGCA....A.T..........T... 489

Cannabis sativa(FJ572045)  476 CTTCCCATGAGCAT-TGCCTTGTGGTTGGCCTAAATTCGAGTCGTCGGCCGCAATCGCCTCGACATTCGG 544
ITS No. 26                 476 ..............-....................................................... 544
ITS No. 55                 477 ..............-....................................................... 545
HumlusLupulus(EF136401)    490 .C....G.....T.C..T..C......................A......T.G......G.......... 559

Cannabis sativa(FJ572045)  545 TGGTTTTCGATTATATCGGTGCCCCGTCGTGCGCGAATCCGTGACCGACTAGACCCGTACGACCCCAATG 614
ITS No. 26                 545 .....C.....................................................G.......... 614
ITS No. 55                 546 ...................................................................... 615
HumlusLupulus(EF136401)    560 .........................T..........T..T...G.T..G.......TA.G.......... 629

Cannabis sativa(FJ572045)  615:TGCTGCGAACGCAGTGCCTTCAACGCGACCCCCGTACAGGGGGGATCACCCGCTGAATTTAA 676
ITS No. 26                 615:................................A.GT.......................... 676
ITS No. 55                 616:................................A.GT.......................... 677
HumlusLupulus(EF136401)    630:C.....A..T......................A.GT..........T............... 691
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Fig. 2. Multiple alignments of the ITS region sequences from the herbal products Nos. 26 and 55, GenBank registered *Cannabis sativa* L. (Acc. No. FJ572045) and *Humulus luplus* L. (Acc. No. EF136401) as a related species of *Cannabis*. Identical sequences are indicated by (·) and gaps (–) are introduced to obtain maximum similarity. The upper box shows results for ITS1, and the lower box those for ITS2.

product also showed four peaks at 27.2 (CBD), 29.2 (CBN), 29.9 (CCH) and 30.5 (Δ^9-THC) min on a total ion chromatogram (Fig. 4). However, in the chromatograms of the No. 55 product by GC–MS and LC–MS, only CBD was detected as a natural cannabinoid (data not shown), although a *Cannabis* DNA fragment was identified from a plant tissue (Fig. 2).

It has been reported that the composition and contents of cannabinoids are highly variable among cannabis plants. Generally, the cannabis with a high-Δ^9-tetrahydrocannabinolic acid (THCA)/low-cannabidiolic acid (CBDA) chemotype is termed marijuana, whereas that with a low-THCA/high-CBDA chemotype is termed hemp [31]. Δ^9-THC and CBD are THCA and CBDA derivatives, respectively. Therefore, if the herbal diluents *C. sativa* of nos. 26 and 55 were marijuana (high-Δ^9-THC variety of *Cannabis*) and hemp (low-Δ^9-THC variety of *Cannabis*), respectively, this could explain the different results between the two products in the chemical analyses.

Salvinorin A of *S. divinorum* and mitragynine of *M. speciosa* are known as psychotropic constituents. In present study, both of these compounds were also found in the No. 48 product (data not shown), in which DNA fragments corresponding to both plants were detected (Table 1).

4. Conclusion

In this study, we identified plant species of botanical materials in Spice-like herbal products using the DNA barcoding method. We detected various plant species which were not indicated on the packaging labels of the herbal products. Most of these identified plants are not known to have psychoactive effects. Therefore, the plant materials would have been included mainly as diluents for the added synthetic cannabinoids. Also, it seemed that the content and constitution of the synthetic cannabinoids was unrelated to the plant species included in the products. On the other hand, in a few products, DNA fragments of some potent psychotropic plants were actually found along with their active natural constituents.

The Spice-like herbal products have become a serious social problem throughout the world. We must continuously monitor such herbal products by both chemical and molecular-biological techniques.

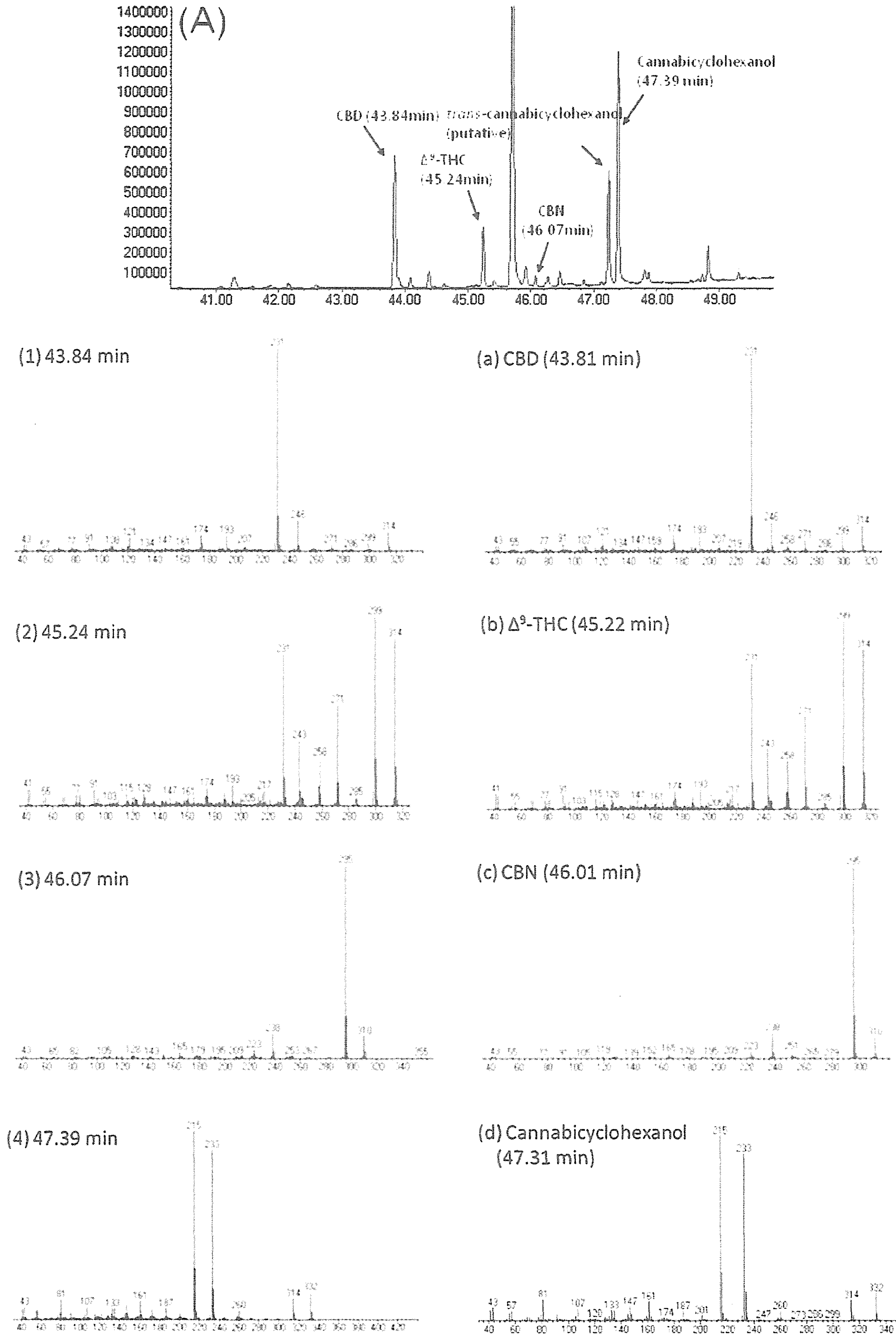

Fig. 3. GC–MS analysis of the methanol extract from the product No. 26. (A) Total ion chromatogram of the sample solution; (1)–(4) mass spectra of the peaks at 43.84, 45.24, 46.07 and 47.39 min, respectively; (a)–(d) mass spectra of authentic CBD, Δ^9-THC, CBN and CCH, respectively.

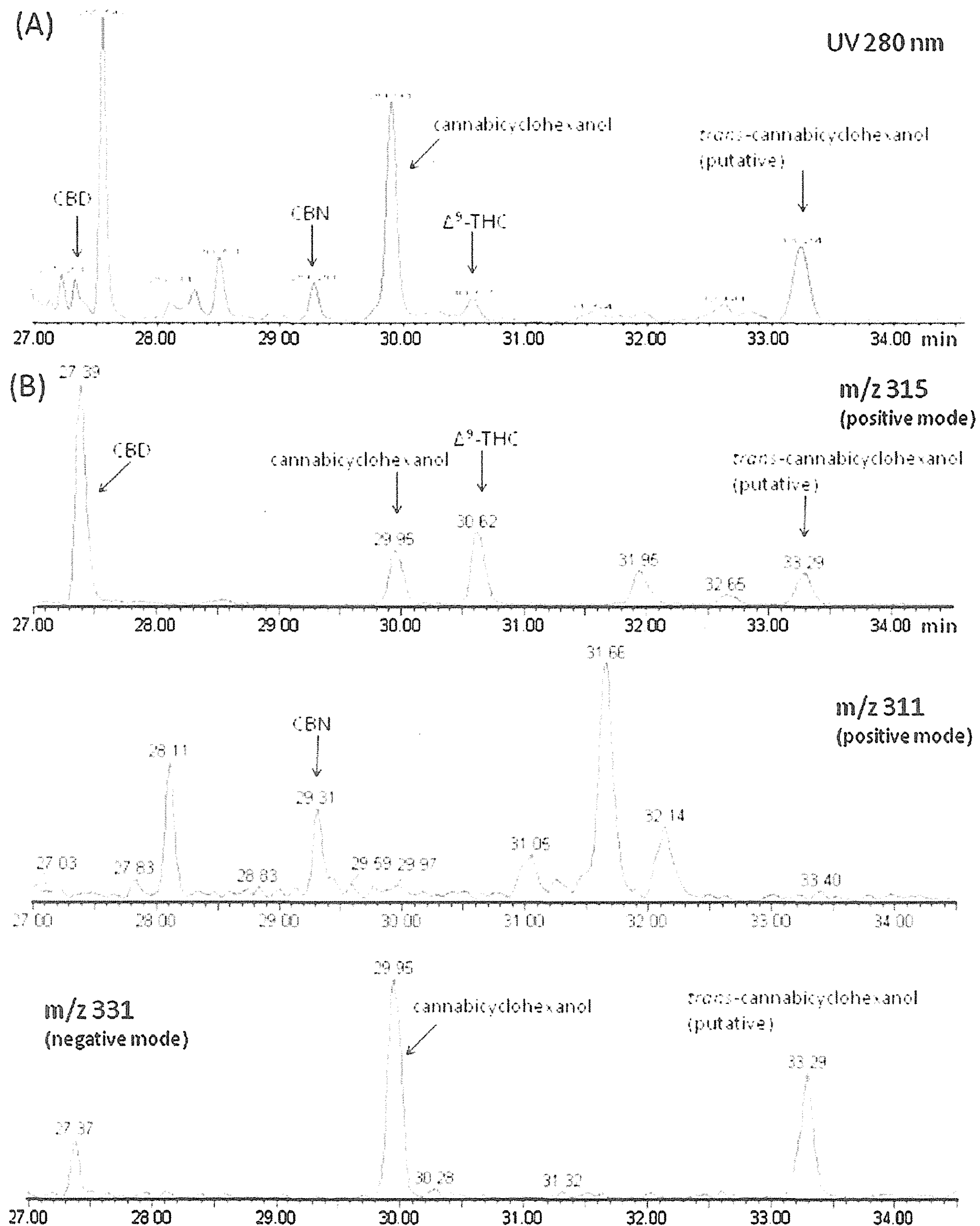

Fig. 4. UPLC–PDA–MS analysis of the methanol extract from the product No. 26. (A) UV chromatogram at 280 nm; (B) mass chromatograms at m/z 315, m/z 311 and m/z 331, respectively. The mass numbers of 315, 311 and 331 correspond to the $[M+H]^+$ ion of Δ^9-THC or CBD and the $[M+H-H_2O]^+$ ion of CCH, the $[M+H]^+$ ion of CBN, and the $[M-H]^-$ ion of CCH, respectively. The peaks at 27.2, 29.2, 29.9 and 30.5 min were identified as CBD, CBN, CCH and Δ^9-THC, respectively, by comparing their chromatographic and spectral data to those of the authentic compounds.

Acknowledgments

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SHORT COMMUNICATION

Identification and quantitation of JWH-213, a cannabimimetic indole, as a designer drug in a herbal product

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Abstract In our survey of designer drugs in the Japanese market, a cannabimimetic indole was identified as a new active compound in a herbal product. The structure of this compound was elucidated by liquid chromatography–photodiode array–mass spectrometry (LC–PDA–MS), gas chromatography–mass spectrometry (GC–MS), high-resolution MS, and nuclear magnetic resonance (NMR) analyses. The compound was finally identified as (4-ethyl-1-naphthalenyl)(2-methyl-1-pentyl-1*H*-indol-3-yl)methanone (JWH-213), an indole-based cannabinoid receptor ligand. To our knowledge, this is the first finding of JWH-213 as a designer drug in a herbal product. The quantitative LC–PDA analysis showed that the JWH-213 content in the product was 252 mg/pack.

Keywords (4-Ethyl-1-naphthalenyl)(2-methyl-1-pentyl-1*H*-indol-3-yl)methanone · JWH-213 · Designer drug · Synthetic cannabinoid · Drug of abuse

Introduction

In the past decade, numerous herbal and chemical products, which are often referred to as "legal drugs" or "legal highs," have become available via the Internet and street stores. These products are consumed for their euphoric and stimulant effects and generally contain designer drugs such as synthetic cannabinoids, tryptamine, phenetylamine, and cathinone derivatives [1–20]. To prevent their abuse, many countries have already undertaken legal measures to keep these compounds under control [13]. In Japan, many psychoactive substances are controlled as designated substances under the Pharmaceutical Affairs Law, carrying a penalty of imprisonment for not more than 5 years and/or a fine of not more than 5,000,000 yen. Nevertheless, a number of products including novel psychoactive substances continue to be sold. The chemical structures of substances contained in these products are very similar to those of the controlled substances [4–20].

In our recent survey of designer drugs obtained from the Japanese market, a synthetic cannabinoid was identified as a new active compound along with a synthetic cannabinoid, AM-2233, in a herbal product. This compound was isolated from a commercial sample and identified as (4-ethyl-1-naphthalenyl)(2-methyl-1-pentyl-1*H*-indol-3-yl)methanone (JWH-213). The structure–activity relationship of the substance at the cannabinoid CB_1 and CB_2 receptors was investigated by Huffman et al. [21] in 2005. In this study, we identified and quantified JWH-213 and AM-2233 in a herbal product using instrumental analysis. The structures of JWH-213 and AM-2233 are shown in Fig. 1.

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Materials and methods

Chemicals and reagents

Authentic AM-2233 and JWH-210 were purchased from Cayman Chemical Company (Ann Arbor, MI, USA) and authentic JWH-015 was purchased from Wako Pure Chemical (Osaka, Japan). $CDCl_3$ (99.96 %) was purchased

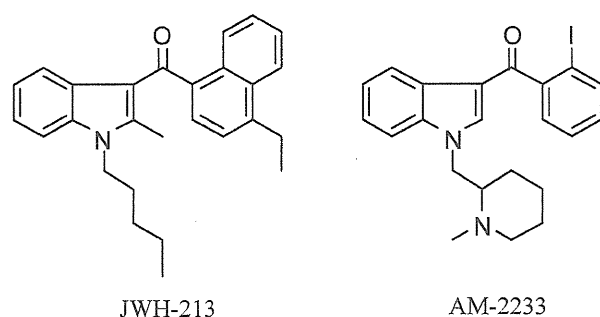

Fig. 1 Structures of cannabimimetic indoles detected in this study

from the ISOTEC division of Sigma-Aldrich (St. Louis, MO, USA) as a solvent for nuclear magnetic resonance (NMR) analysis. All other common chemicals and solvents were of analytical reagent grade or liquid chromatography (LC)–mass spectrometry (MS) grade.

Standard solution for qualitative analysis

The standard solution for qualitative analysis was prepared in methanol at 100 μg/ml.

Calibration curves and recovery rate

Calibration curves [peak area y versus concentration x (μg/ml)] using an external calibration method were constructed by LC–photodiode array (PDA) detection with peak areas at 312 and 319 nm for AM-2233 and the unknown compound, respectively. Each compound was diluted with methanol to prepare calibration solutions at 10, 25, 50, 100, and 250 μg/ml. To evaluate recovery rates, 50 mg of a sample (a herbal product described below) was accurately weighted and AM-2233 (5 mg) and the unknown compound (2 mg) were added to the sample. The sample solution was then prepared and analyzed by LC–PDA as detailed in the experimental section. The recovery rates were calculated according to the following formula; recovery rate (%) = (measured amount − original amount of the sample)/added amount × 100.

Sample preparation

In January 2012, a herbal product marketed in Japan was purchased via the Internet for analysis; the product contained 3 g of mixed dried plants. The herbal product was crushed into powder. A 50-mg portion of the powder was accurately weighed and extracted with 10 ml of methanol under ultrasonication for 30 min. After centrifugation (5 min, 3000 rpm), the supernatant solution was transferred to a 100-ml volumetric flask. The sediment was re-extracted using the same procedure described above, and the supernatant fractions were combined and diluted with methanol to 100 ml. After shaking the volumetric flask, the solution was filtered through a Millex LG hydrophilic PTFE filter (0.45-μm) (Merck Millipore, Darmstadt, Germany) to obtain the sample solution.

Analytical conditions

The sample solution was qualitatively and quantitatively analyzed by LC–PDA–MS with positive electrospray ionization. The instrument consisted of a Waters Alliance 2695 separation module, a ZQ mass spectrometer, and a 2996 PDA detector (Waters, Milford, MA, USA). The sample solution was separated by an XBridge C18 column (150 × 2.1 mm i.d., 3.5 μm, Waters). The mobile phases were 0.1 % formic acid aqueous solution (eluent A) and a mixture of acetonitrile and methanol (3:2, v/v) containing 0.1 % formic acid (eluent B). The gradient elution began at 50 % eluent A, decreasing linearly to 10 % eluent A over 30 min. The flow rate of the mobile phase was set at 0.3 ml/min, and the injection volume was 1 μl. The column temperature was maintained at 40 °C. The PDA detection wavelength was set from 190 to 400 nm, and max-plot chromatographic monitoring was performed (190–400 nm). The MS conditions were as follows: source temperature, 120 °C; desolvation temperature, 350 °C; capillary voltage, 3 kV; cone voltage, 30 V; and desolvation gas flow, 800 l/h. The mass range of the spectra was from m/z 100–700.

The sample solution was also analyzed by gas chromatography (GC)–MS in electron ionization mode at 70 eV. GC–MS was performed on an Agilent 6890N instrument equipped with a split/splitless injector operating in the splitless mode coupled to an Agilent 5975 mass-selective detector (Agilent, Palo Alto, CA, USA). Chromatographic separation was achieved on an HP1-MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, Agilent). The initial column temperature was 200 °C (held for 1 min); the temperature was increased at a rate of 5 °C/min to 310 °C (held for 7 min). The carrier gas was helium, and the flow rate was 1.1 ml/min. The injection volume was 1 μl. The injector and transfer line temperatures were held at 250 and 280 °C, respectively. The data were obtained in the full scan mode with a scan range of m/z 40–600.

The accurate mass spectrum of the unknown 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 measurement conditions were as follows: ion guide peak voltage, 500 V; reflectron voltage, 950 V; orifice 1 voltage, 15 V; orifice 2 voltage, 5 V; ring lens voltage, 5 V; orifice 1 temperature, 80 °C; mass range, m/z 100–1000. The conditions of the

DART ion source were as follows: helium gas flow rate, 2.0 l/min; gas heater temperature, 250 °C; discharge electrode needle voltage, 3200 V; voltages of electrodes 1 and 2, 100 and 250 V, respectively. Internal mass number calibration was achieved using PEG600. Diphenhydramine ($C_{17}H_{21}NO$) and verapamil ($C_{27}H_{38}N_2O_4$) were used as internal standards. The extract was directly exposed to the vicinity of the DART ion source.

NMR spectra were obtained on ECA-600 spectrometers (JEOL). Assignments were made using ^{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 the unknown compound

By ultrasonication for 30 min, 1 g of herbal product was extracted with 100 ml of methanol. The methanol solution was filtered and evaporated to dryness. The residue was dissolved in 4 ml of hexane and loaded onto a silica gel column (250 × 14 mm i.d.) packed with Silica Gel 60N (spherical and neutral; Kanto Chemical, Tokyo, Japan). The analytes were eluted from the column with a mixture of dichloromethane and hexane (9:1, v/v), and 10 ml of eluate was collected each time. Each eluate was evaporated to dryness and then dissolved in 10 ml of methanol. The solutions were analyzed by LC–PDA–MS according to the abovementioned method. All solutions containing only the unknown compound were pooled and evaporated to dryness. The unknown compound was obtained as a pale yellow gum (35 mg).

Results and discussion

Two main peaks were detected by LC–PDA–MS of the sample solution of the product (Fig. 2a). One peak detected at 4.8 min in the ultraviolet (UV) chromatogram was presumed to be AM-2233, which is a synthetic cannabinoid in herbal products [20], by comparison with the retention time (4.8 min) and the UV and mass spectra of the authentic compound. Another peak was detected at 25.2 min and exhibited maxima at 221 and 319 nm in the UV spectrum; the protonated molecule signal appeared at m/z 384 in the mass spectrum (Fig. 2b).

The total ion current chromatogram of the sample solution by GC–MS showed two intense peaks detected at 21.4 and 22.3 min (Fig. 3a); the peak at 21.4 min was deduced to be AM-2233 by comparing the retention time

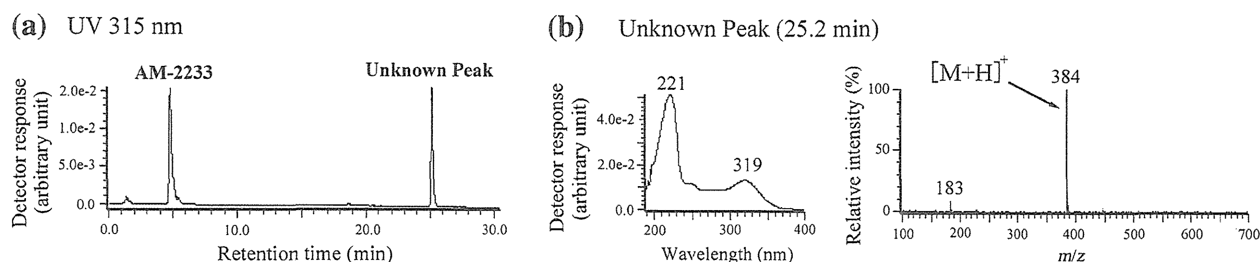

Fig. 2 Liquid chromatography (LC)–ultraviolet (UV) chromatogram of the sample solution at 315 nm (**a**), photodiode array (PDA) spectrum and electrospray ionization mass spectrum for the unknown peak appearing at 25.2 min (**b**)

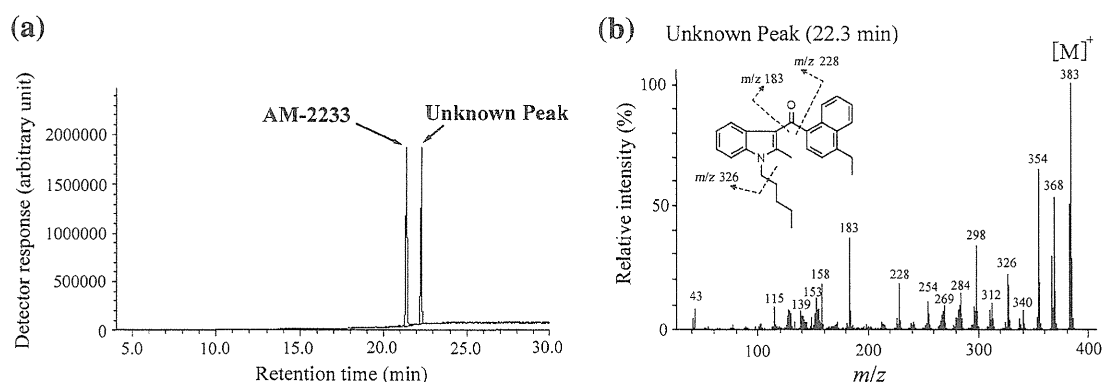

Fig. 3 Total ion current chromatogram (**a**) and electron ionization–mass spectrum at the peak of 22.3 min (**b**) obtained by gas chromatography–mass spectrometry of the sample solution

Table 1 Nuclear magnetic resonance (NMR) data for JWH-213 (unknown compound) and related compounds (JWH-210 and JWH-015) in $CDCl_3$

No.	JWH-210[a] ^{13}C	JWH-015[a] ^{13}C	JWH-213 (unknown compound)[a] ^{13}C	^{1}H	$HMBC^{b}$
1	192.3	193.4	193.7	–	–
2′	137.9	145.6	145.4	–	–
3′	117.6	114.9	115.0	–	–
3′a	127.0	127.0	127.1	–	–
4′	122.9	121.2	121.3	7.20, 1H, d, J = 7.9 Hz	3′, 6′, 7′a
5′	122.8	121.9	121.8	6.98, 1H, t, J = 7.9 Hz	3′a, 6′, 7′
6′	123.5	122.2	122.1	7.15, 1H, t, J = 7.9 Hz	4′, 7′, 7′a
7′	109.9	109.4	109.4	7.29, 1H, d, J = 7.9 Hz	3′a, 5′
7′a	137.0	136.1	136.0	–	–
1″	47.1	44.8	43.3	4.10, 2H, t, J = 7.6 Hz	2′, 7′a, 2″, 3″
2″	29.5	22.9	29.4	1.77, 2H, m	1″, 3″, 4″
3″	28.9	11.4	29.1	1.35, 2H, m, overlapped	4″, 5″
4″	22.2	–	22.4	1.35, 2H, m, overlapped	3″, 5″
5″	13.9	–	13.9	0.89, 3H, t, J = 6.9 Hz	3″, 4″
1‴	137.5	140.4	138.8	–	–
2‴	125.9	125.7	125.9	7.48, 1H, d, J = 7.3 Hz	1, 3‴, 4‴, 8‴a
3‴	123.5	125.1	124.0	7.34, 1H, d, J = 7.3 Hz	1‴, 2‴, 4‴a, E-1
4‴	142.5	129.9	142.6	–	–
4‴a	132.0	133.8	132.1	–	–
5‴	123.8	128.2	123.9	8.12, 1H, d, J = 7.6 Hz	4‴a, 7‴, 8‴a
6‴	126.1	126.2	126.0	7.51, 1H, t, J = 7.6 Hz	4‴a, 8‴
7‴	126.2	126.8	126.3	7.41, 1H, t, J = 7.6 Hz	5‴, 8‴a
8‴	126.8	125.6	126.4	8.15, 1H, d, J = 7.6 Hz	1‴, 4‴a, 6‴
8‴a	131.1	130.3	130.7	–	–
2′-Me	–	12.6	12.6	2.46, 3H, s	2′, 3′
E-1	26.2	–	26.2	3.17, 2H, q, J = 7.6 Hz	3‴, 4‴, 4‴a, E-2
E-2	14.9	–	15.0	1.41, 3H, t, J = 7.6 Hz	4‴, E-1

[a] Recorded in $CDCl_3$ at 600 MHz (^{1}H) and 150 MHz (^{13}C); data in δ ppm (J in Hz)

[b] J = 8 or 4 Hz; proton signal correlated with the indicated carbons

(21.4 min) and mass spectrum of the authentic compound. The peak at 22.3 min exhibited a mass spectrum with eight major ion signals at m/z (% relative intensity) 383 (100), 368 (54), 354 (65), 326 (22), 298 (32), 228 (16), 183 (32), and 158 (15) (Fig. 3b). LC–PDA–MS and GC–MS of the sample solution indicated that the herbal product contained two major compounds (AM-2233 and the unknown compound).

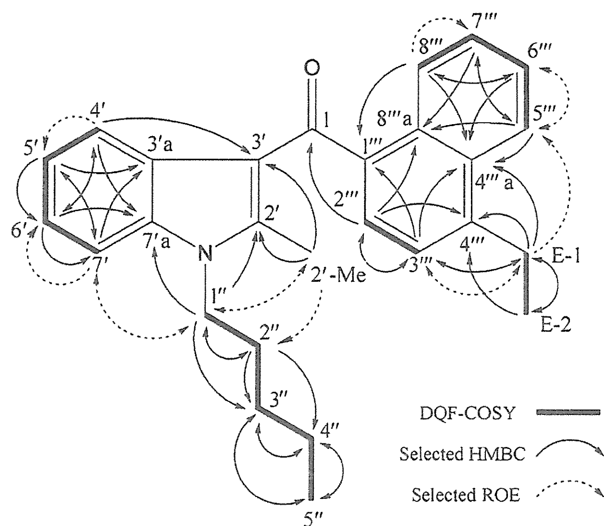

Fig. 4 Double quantum filtered correlation spectroscopy (DQF-COSY), selected heteronuclear multiple-bond correlation (HMBC), and selected rotating frame nuclear Overhauser effect (ROE) correlations of JWH-213 (unknown compound)

The isolated unknown compound was directly exposed to the DART ion source and the accurate mass spectrum was obtained. In the mass spectrum, the protonated molecule signal was shown at m/z 384.23228 (calculated 384.23274), corresponding to a molecular formula of $C_{27}H_{29}NO$.

As shown in Table 1, the ^{1}H NMR spectrum of the unknown compound exhibited signals for 29 protons including 3 methyl signals at δ 0.89 (3H, t, J = 6.9 Hz), δ 1.41 (3H, t, J = 7.6 Hz), and δ 2.46 (3H, s); 10 aromatic proton signals; 4 methylene proton signals at δ 1.35, δ 1.35 (each 2H, m, overlapped), δ 1.77 (2H, m), and δ 3.17 (2H, q, J = 7.6 Hz); and 1 methylene proton signal connected to the nitrogen atom at δ 4.10 (2H, t, J = 7.6 Hz). The ^{13}C NMR spectrum of the unknown compound exhibited 27 carbon signals including 3 methyl signals at δ 13.9, δ 15.0, and δ 12.6; 4 methylene signals at δ 22.4, δ 29.1, δ 29.4, and δ 26.2; 1 methylene connected to nitrogen at δ 43.3; 10 aromatic carbons; 8 aromatic quaternary carbons; and 1 carbonyl carbon (δ 193.7). Two-dimensional NMR analyses (HMQC, HMBC, DQF-COSY, and ROE) revealed that this compound has (4-ethylnaphthalen-1-yl)carbonyl and 2-methyl-1-pentyl-1*H*-indol moieties (Fig. 4). In fact, the ^{13}C NMR chemical shifts in the 2-methylindole moiety were very similar to those of JWH-015 (Fig. 5; Table 1). In addition, these ^{13}C NMR chemical shifts were concordant

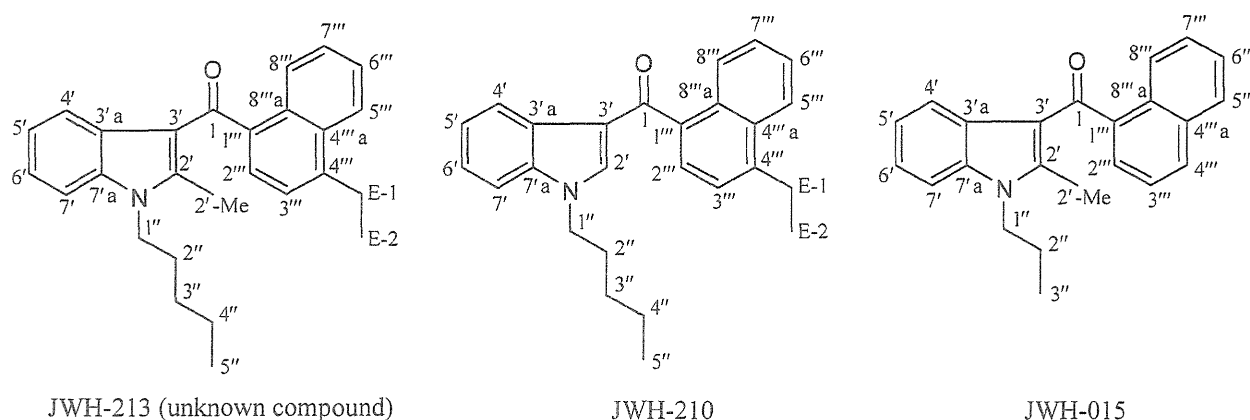

Fig. 5 Chemical structures of JWH-213 (unknown compound), JWH-210, and JWH-015

with those of JWH-210 except for the methylene carbon at δ 43.3 (C-1″) and the aromatic quaternary carbons at δ 145.4 (C-2′) and δ 115.0 (C-3′). The three different values of the ^{13}C NMR chemical shifts are probably due to the effect of the 2-methyl group of the indole (2′-Me), which is not present in JWH-210. The unknown compound was presumed to be (4-ethyl-1-naphthalenyl)(2-methyl-1-pentyl-1*H*-indol-3-yl)methanone on the basis of the accurate mass and NMR data. This deduced compound has been already synthesized and named JWH-213 by Huffman et al. [21]. Furthermore, the fragment ions corresponding to the ethylnaphthoyl and 2-methyl-1-pentyl-1*H*-indol-3-carbonyl fragments were observed at *m*/*z* 183 and 228, respectively, by GC–MS of the unknown compound (Fig. 3b). Therefore, the unknown compound was finally identified as JWH-213.

The AM-2233 and JWH-213 contents in the herbal product were determined by LC–PDA analysis. We used JWH-213 isolated from the herbal product as the reference standard to construct a calibration curve. The regression equations of these curves and their correlation coefficients (r^2) were calculated as follows: AM-2233, $y = 40.226x + 28.643$ ($r^2 = 1.000$); and JWH-213, $y = 72.870x + 143.318$ ($r^2 = 0.999$). Recovery rates of AM-2233 and JWH-213 were 99.2 ± 2.5 and 99.6 ± 1.0 % (mean ± standard deviation of three experiments), respectively. The AM-2233 and JWH-213 contents in the herbal product were 597 mg/pack (199 μg/mg) and 252 mg/pack (84 μg/mg), respectively.

Conclusions

To our knowledge, this is the first report on the detection of JWH-213 as a designer drug in a herbal product. JWH-213 exhibits potent affinity for cannabinoid CB_1 and CB_2 receptors (K_i values = 1.5 and 0.42 nM, respectively) [21]. In drug discrimination tests in rats, AM-2233 has been reported to substitute for Δ^9-tetrahydrocannabinol in the same way as JWH-018 [22]. In contrast, cannabimimetic activity of JWH-213 has not been reported. However, JWH-213 may have cannabimimetic activity because of its high affinity for CB_1 and CB_2 receptors, as seen in JWH-018 and AM-2233; their K_i values for CB_1/CB_2 receptors are 9.00/2.94 and 2.8/2.9 nM, respectively [23, 24].

Many cannabimimetic compounds such as JWH-018 and JWH-210 are regulated in Japan. However, it can be easily expected that new analogs will be distributed immediately after regulation is imposed. Therefore, continuous monitoring of such new compounds is crucial to revise regulatory measures.

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