Forensic Toxicol (2011) 29:25–37
DOI 10.1007/s11419-010-0100-3

ORIGINAL ARTICLE

Identification and quantitation of two cannabimimetic phenylacetylindoles JWH-251 and JWH-250, and four cannabimimetic naphthoylindoles JWH-081, JWH-015, JWH-200, and JWH-073 as designer drugs in illegal products

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Received: 14 June 2010 / Accepted: 9 August 2010 / Published online: 5 November 2010

Abstract Six cannabimimetic indoles have been identified as adulterants in herbal or chemical products being sold illegally in Japan, with four of the compounds being new as adulterants to our knowledge. The identifications were based on analyses using gas chromatography–mass spectrometry, liquid chromatography–mass spectrometry, high-resolution mass spectrometry, and nuclear magnetic resonance spectroscopy. The first two compounds were identified as phenylacetyl indoles JWH-251 (2-(2-methylphenyl)-1-(1-pentyl-1*H*-indol-3-yl)ethanone; **1**) and its demethyl-methoxylated analog JWH-250 (2-(2-methoxyphenyl)-1-(1-pentyl-1*H*-indol-3-yl)ethanone; **2**). Compound **2** was identical to that found as an adulterant in the UK and in Germany in 2009. The third compound was naphthoylindole JWH-081 (1-(4-methoxynaphthalenyl)-(1-pentyl-1*H*-indol-3-yl)methanone; **3**), and the fourth was JWH-073 (1-naphthalenyl(1-butyl-1*H*-indol-3-yl)methanone; **4**), which had been identified as an adulterant in our previous study. Two additional compounds were JWH-015 (1-naphthalenyl (2-methyl-1-propyl-1*H*-indol-3-yl)methanone; **5**) and JWH-200 (1-naphthalenyl(1-(2-(4-morpholinyl)ethyl)-1*H*-indol-3-yl)methanone; **6**). Compounds **1**–**4** and **6** were reported to be synthetic cannabinoids with selective affinity for cannabinoid CB_1 receptors, while compound **5** was reported to be a selective CB_2 receptor agonist causing immunosuppressive effects without psychotropic affects. One product contained both CB_1 and CB_2 receptor agonists in our collection. Quantitative analyses of the six cannabimimetic compounds in 20 products revealed that there was large variation in concentrations of the detected compounds among products; for herbal cutting products, the total amounts of these cannabinoids ranged from 26 to 100 mg.

Keywords JWH-251 · JWH-081 · JWH-015 · JWH-200 · Synthetic cannabinoid · Designer drug

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Introduction

Recently, a number of psychotropic herbal products have been marketed on the Internet under brand names such as "Spice" and "herbal blends" [1–5]. We have reported that two types of synthetic cannabinoids are present as psychoactive ingredients in herbal products [1, 2, 4]. German scientists have also found these compounds in some herbal products [6, 7]. The first drug group consists of cyclohexylphenols such as cannabicyclohexanol (CCH; (1*RS*, 3*SR*)-3-[2-hydroxy-4-(2-methylnonan-2-yl)phenyl]cyclohexan-1-ol) and CP 47497 ((1*RS*,3*SR*)-3-[2-hydroxy-4-(2-methyloctan-2-yl)phenyl]cyclohexan-1-ol); these compounds exert potent cannabimimetic actions [8–12]. The second group consists of naphthoylindoles, such as JWH-018 (1-naphthalenyl-(1-pentyl-1*H*-indol-3-yl)methanone) and JWH-073 (1-naphthalenyl-(1-butyl-1*H*-indol-3-yl)methanone, **4**). Although the chemical structures of JWH-018 and JWH-073 are greatly different from those of natural psychoactive cannabinoids such as Δ^9-tetrahydrocannabinol (Δ^9-THC), the former compounds have higher affinities for cannabinoid CB_1 receptors and activities that are comparable with or more potent than Δ^9-THC [13–16]. Since January 2009, JWH-018, CP 47497, and three homologs of CP 47497, including CCH, have been controlled in Germany [17], followed by Austria, France, Sweden, and other countries [18]. In Japan, CCH, CP 47497, and JWH-018

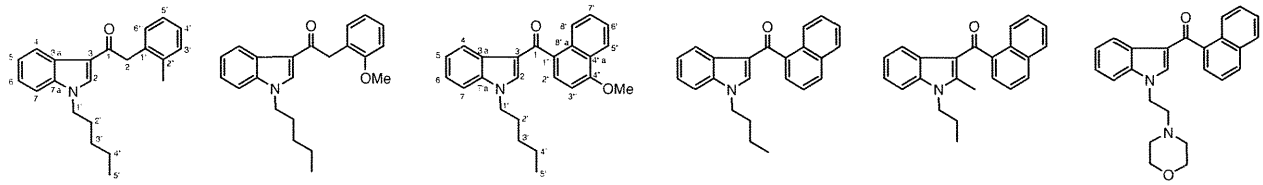

Fig. 1 Structures of cannabimimetic indoles detected in the present study

have been controlled as designated substances (Shitei-Yakubutsu) under the Pharmaceutical Affairs Law since November 2009. Despite such control measures, new synthetic cannabinoids are continuing to appear throughout the world. The classical cannabinoid HU-210 ((6a*R*,10a*R*)-3-(1,1-dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo[b,d]pyran-9-methanol), which is a potent cannabinoid receptor CB_1 agonist, was identified in herbal products in the USA and the UK [18]. In addition, two novel synthetic JWH cannabinoids, JWH-398 (4-chloro-1-naphthalenyl-(1-pentyl-1*H*-indol-3-yl)methanone) and JWH-250 (2-(2-methoxyphenyl)-1-(1-pentyl-1*H*-indol-3-yl) ethanone, **2**), were found in the UK and Germany in October 2009 [18]. Both compounds were reported as cannabinoid receptor agonists [19, 20]. However, no scientific reports with details of identification and isolation of these compounds from herbal or chemical products have been published. In the present study, we describe identification and quantitative analyses of six cannabimimetic aminoalkylindoles, including four new ones as designer drugs, in herbal or chemical products commercially available in Japan. The structures of the six compounds dealt with in this study are shown in Fig. 1.

Materials and methods

Chemicals and reagents

Betamethasone valerate (internal standard, IS) and JWH-015 (**5**) were purchased from Wako (Osaka, Japan); JWH-250 (**2**), JWH-073 (**4**), and JWH-200 (**6**) from Cayman Chemical Company (Ann Arbor, MI, USA). All other common chemicals and solvents were of analytical reagent grade or high-performance liquid chromatography (HPLC) grade.

Samples for analysis

Eighteen herbal products and two powder products being sold in Japan for their expected cannabis-like effects were purchased via the Internet from December 2009 to April 2010. All products had different names and were packaged differently. Sixteen of the herbal products were in the form of dried leaves and the remaining two were in the form of solids (resins). The labels on the packages indicated that the products contained 1–3 g of a mixture of plants. The two powder products were pale yellow powders, and the labels on the packages indicated that the products contained 200 and 250 mg, respectively. On the basis of the advertisements on the website, the single dosage amounts were estimated to be between 20 and 25 mg for powder products and between 200 and 300 mg for dried leaf products being finely cut.

Analytical conditions

The sample solutions were qualitatively and quantitatively analyzed by liquid chromatography-mass spectrometry (LC–MS) with positive electrospray ionization (ESI). The instrument consisted of an ACQUITY UPLC system, a mass detector, and a photodiode array (PDA) detector (Waters, Milford, MA, USA). The sample solutions were separated with an ACQUITY UPLC 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. Each analysis was carried out with a binary mobile phase consisting of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). An elution program (1) with a linear gradient was: 50% B (3-min hold) to 70% B (3–5 min), and 70% B with 7-min hold (5–12 min) at a flow rate of 0.3 ml/min. Another elution program (2) was also used for quantitation of **6** as follows: 30% B (3-min hold) to 70% B (3–5 min), and 70% B with 7-min hold (5–12 min). The injection volume was 1 μl for both programs. The wavelength of the PDA detector for screening was set from 190 to 500 nm.

The MS conditions for the LC–ESI–MS were: ionization, positive; desolvation gas, nitrogen at a flow rate of 650 l/h at 350°C; capillary and cone voltages, 3000 and 30 V, respectively; mass spectral range, *m/z* 50–500. For qualitative analysis of JWH-251 (**1**), **2**, JWH-081 (**3**), **4**, **5**, and **6**, the protonated molecular peaks ($[M + H]^+$) of the compounds and IS were monitored in the scan mode. The monitoring ions were: **1**, *m/z* 320; **2**, *m/z* 336; **3**, *m/z* 372;

4 and **5**, *m/z* 328; **6**, *m/z* 385; and betamethasone valerate (IS), *m/z* 477.

Gas chromatography-mass spectrometry (GC–MS) analysis was also performed in electron ionization (EI) mode at 70 eV of electron energy according to our previous report [21]. It 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; Hewlett-Packard, Palo Alto, CA, USA) with helium gas as a carrier at 0.7 ml/min. The conditions were: injector temperature, 200°C; injection, splitless mode for 1.0 min; oven temperature program, 80°C (1-min hold) and increase at a rate of 5°C/min to 190°C (15-min hold) followed by increase at 10°C/min up to 310°C (15-min hold); mass selective detector temperature, 280°C; scan range, *m/z* 40–550.

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 positive ion mode. The measurement conditions were: 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* 100–500. The conditions of the DART ion source were: 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, and diphenhydramine ($C_{17}H_{21}NO$) and verapamil ($C_{27}H_{38}N_2O_4$) were also used as ISs for each accurate mass analysis. The product itself or an extract was directly exposed to the vicinity of the DART ion source.

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

For isolation of compounds, a recycling preparative HPLC (Japan Analytical Industry, Tokyo, Japan) was used with a JAIGEL-GS310 column (500 mm × 20 mm i.d.; Japan Analytical Industry) and ultraviolet (UV) absorbance and refractive index (RI) detectors.

Isolation of compounds 1 and 3

A 3-g sample of a herbal product was extracted with 100 ml of chloroform by ultrasonication for 1 h. The extractions were repeated three times, the supernatant fractions were combined and evaporated to dryness. The extract was placed on a preparative silica gel thin-layer chromatography (TLC) plate (Silica Gel 60, 20 × 20 cm, 2 mm; Merck, Darmdstadt, Germany), which was then developed using hexane/ethyl acetate (4:1). A portion of the silica gel in the TLC plate was scraped and the target compound was eluted with chloroform/methanol (3:1) to get fractions 1 and 2. Each fraction was further purified by recycling preparative HPLC with chloroform/methanol (1:1) to give compound **1** (41 mg) as a pale yellow oil and compound **3** (111 mg) as a white solid, respectively.

Isolation of compound 2

A 60-mg sample of the pale yellow powder was dissolved in 2 ml of chloroform/methanol (1:1) and passed through a centrifugal filter (Ultrafree-MC, 0.45 μm filter unit; Millipore, Bedford, MA, USA). The solution was subjected to recycling preparative HPLC with chloroform/methanol (1:1) to obtain compound **2** (31 mg) as a white solid.

Standard solutions

For qualitative and quantitative analysis, standard solutions were prepared for each compound (**1**–**6**) at a concentration of 1.0 or 0.1 mg/ml in methanol.

Calibration curves

The concentrations of compounds **1**–**6** in the samples were calculated using the peak area ratios of **1** and **2** at 302 nm versus IS at 240 nm, and those of **3**–**6** at 314 nm versus IS at 240 nm, respectively. Compounds **1**–**6** were diluted with methanol to prepare calibration solutions containing 10, 25, 50, 100, 250, and 500 μg/ml (**1**–**4**) or 10, 25, 50, 100, and 250 μg/ml (**5** and **6**). The solutions also included IS (betamethasone valerate) at 100 μg/ml.

Precision and accuracy of the method

The precision and accuracy of the method were evaluated by analyzing triplicates of the standard solutions containing 10, 50, and 500 μg/ml (**1**–**4**) or 10, 50, and 250 μg/ml (**2**, **5** and **6**) for each compound. Accuracy, expressed as bias, was calculated as the percent difference between the amounts of each compound added and recovered.

Sample extraction procedure before instrumental analyses

For quantitative and qualitative analyses, the herbal product (5–10 mg) after being crushed into powder or the

powdery product (5 mg) was extracted with 1 ml of methanol including IS (100 μg/ml) under ultrasonication for 10 min. After centrifugation (5 min, 3,000 rpm), the supernatant solution was passed through a centrifugal filter (Ultrafree-MC, 0.45 μm filter unit; Millipore). If necessary, the solution was diluted with methanol to a suitable concentration before instrumental analyses.

Results and discussion

Identification of unknown peaks 1–3

Three unknown peaks **1**–**3** were observed in the total ion chromatogram (TIC) obtained by GC–MS for product No. 11 (Fig. 2a). Unknown peak **1** at 48.2 min showed a mass spectrum with four major ion signals at *m/z* (% relative intensity) 319 (5), 214 (100), 144 (17), and 105 (3) as shown in Fig. 2b. LC–MS analysis showed the corresponding three unknown peaks both in the PDA detection and TIC (Fig. 3a, e). Peak **1** at 9.0 min showed a major ion signal at *m/z* 320 $[M + H]^+$ and absorbance maxima at 246 and 303 nm in the UV spectrum (Fig. 3b, f). In the accurate mass spectrum obtained by DART–TOF–MS with direct exposure of the sample extract to the ion source, the major ion peak showed a protonated molecular ion signal ($[M + H]^+$) at *m/z* 320.19966 in the positive mode, suggesting that the molecular formula of **1** was $C_{22}H_{26}NO$. The error between the mass number observed and the theoretical mass number of $[M + H]^+$ was −1.78 mDa.

To elucidate the exact chemical structure of compound **1** by NMR analysis, we isolated **1** from the extract by TLC

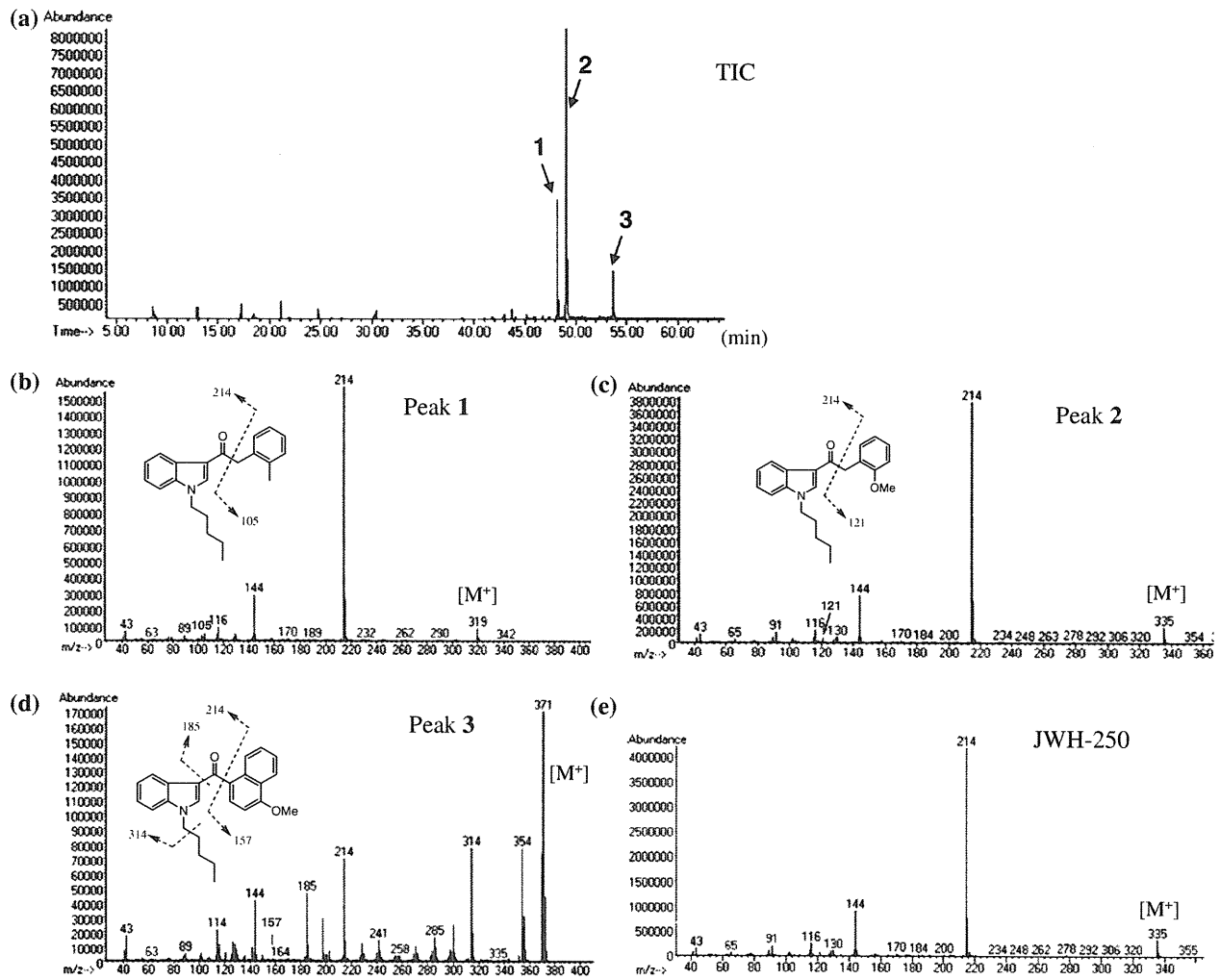

Fig. 2 Gas chromatography–mass spectrometry (GC–MS) analysis of product No. 11. Total ion chromatogram (TIC) (**a**), electron ionization (EI) mass spectra of the detected peaks at the retention times (RTs) 48.2 (**b**, **1**), 49.1 (**c**, **2**), and 53.7 min (**d**, **3**), and the EI mass spectrum of the standard of JWH-250 (RT 49.1 min, **e**)

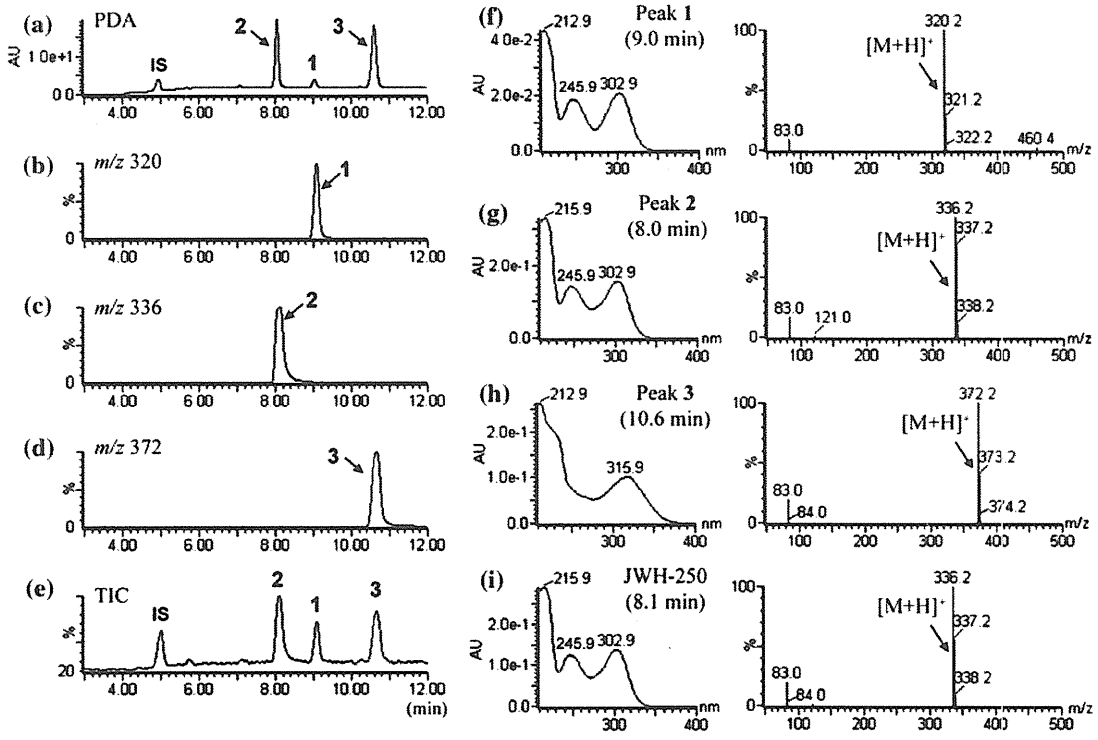

Fig. 3 Liquid chromatography (LC)-ultraviolet (UV) detection (**a**) and LC–MS analysis for the extract of product No. 11. Mass chromatograms obtained at *m/z* 320 (**b**, **1**), 336 (**c**, **2**), and 372 (**d**, **3**), and the TIC (**e**); UV spectra and electrospray ionization (ESI) mass spectra for each peak (**f**–**h**) and those of the standard of JWH-250 (**i**)

and preparative HPLC as described before. The ^{1}H NMR spectrum of **1** showed 25 nonexchangeable protons, including two methyl signals at δ 0.90 (3H, t, J = 7.2 Hz) and 2.33 (3H, s), and nine aromatic proton signals at δ 7.04, 7.16, 7.18, 7.19, 7.22, 8.41 (each 1H, m), 7.28 (1H, td, J = 6.9, 1.7 Hz), 7.30 (1H, td, J = 6.9, 1.7 Hz), and 7.74 (1H, s) as shown in Table 1. The spectrum also showed three methylene proton signals at δ 1.30, 1.35 (each 2H, m) and 1.87 (2H, q, J = 7.2 Hz), as well as two characteristic methylene signals connected to a nitrogen atom at δ 4.14 (2H, t, J = 7.2 Hz) and next to a carbonyl group at δ 4.20 (2H, s). The ^{13}C NMR spectrum of **1** showed 22 carbon signals, suggesting the presence of 2 methyls (δ 13.9 and 20.0), 5 methylenes with a nitrogenated carbon (δ 47.1) and a carbon adjacent to a carbonyl group (δ 44.9), 9 aromatic carbons (δ 109.8, 122.6, 122.8, 123.3, 126.0 126.9, 130.3, 130.3, and 134.4), 5 aromatic quaternary carbons (δ 116.2, 126.7, 134.6, 136.6, and 137.0), and 1 carbonyl carbon (δ 192.7). The analyses of DQF-COSY, HMQC, and HMBC spectra indicated that **1** has three partial structures (1,3-substituted indole group; 1,2-substituted phenyl group; and *n*-pentyl group), as shown in Table 1 and Fig. 4. The connectivity of these groups and the carbonyl methyl group was deduced from the HMBC spectrum. An aromatic proton at δ 7.74 (H-2′) of the indole group correlated to the carbonyl carbon at δ 192.7 (C-1) and the methylene carbon of the *n*-pentyl group at δ 47.1 (C-1″). A methylene proton next to a carbonyl group at δ 4.20 (H-2) showed correlations to the carbon at δ 116.2 (C-3′) of the indole group and the carbons of the phenyl group at δ 134.6 (C-2‴) and 130.3 (C-6‴). In addition, a methyl proton singlet resonance at δ 2.33 (Me-2‴) correlated to the carbons of the phenyl group at δ 137.0 (C-1‴) and 130.3 (C-3‴). Furthermore, the irradiation of the methylene proton at δ 4.14 (H-1″) of the *n*-pentyl group resulted in ROE correlations on the aromatic protons (H-2′ and H-7′) as shown in Fig. 4. The other methylene proton (H-2) also showed ROE correlations to the aromatic protons (H-2′ and H-6‴) and methyl proton (Me-2‴). On the basis of these mass and NMR spectral data (Figs. 2, 3, 4; Table 1), the structure of compound **1** was finally elucidated as 2-(2-methylphenyl)-1-(1-pentyl-1*H*-indol-3-yl)ethanone. The deduced compound had been synthesized as a potent cannabinoid receptor agonist and was named JWH-251 by Huffman et al. [20]. However, the present study is the first to detect compound **1** as a designer drug and an adulterant in illegal products. It should be noted that compound **1** is one of the cannabimimetic indoles with a phenylacetyl substituent, which are different from naphthoylindoles such as JWH-018 and JWH-073 (**4**) (Fig. 1).

Table 1 Nuclear magnetic resonance (NMR) data for compounds **1** and **3** in $CDCl_3$

No.	JWH-251 (**1**)[a]			Reference[b]	JWH-081 (**3**)[a]		
	^{13}C	^{1}H	HMBC[c]	^{13}C	^{13}C	^{1}H	HMBC[c]
1	192.7	–	–	191.7	191.8	–	–
2	44.9	4.20, 2H, s	1, 3′, 1‴, 2‴, 6‴	–	–	–	–
2′	134.4	7.74, 1H, s	1, 3′, 3′a, 7′a, 1″	137.4	137.4	7.41, 1H, s, overlapped	1, 3′, 3′a, 7′a, 1″
3′	116.2	–	–	117.6	117.7	–	–
3′a	126.7	–	–	127.1	127.2	–	–
4′	122.8	8.41, 1H, m	3′, 3′a, 5′, 6′, 7′a	122.7	122.9	8.46, 1H, m	3′, 3′a, 5′, 6′, 7′a
5′	122.6	7.28, 1H, td, J = 6.9, 1.7 Hz, overlapped	3′a, 4′, 6′, 7′	123.3	123.4	7.35, 1H, m, overlapped	3′a, 4′, 7′
6′	123.3	7.30, 1H, td, J = 6.9, 1.7 Hz, overlapped	4′, 7′, 7′a	122.5	122.6	7.34, 1H, m, overlapped	4′, 7′, 7′a
7′	109.8	7.04, 1H, m	3′a, 5′, 7′a	109.9	109.9	7.40, 1H, m	3′a, 5′, 6′
7′a	136.6	–	–	136.9	136.9	–	–
1″	47.1	4.14, 2H, t, J = 7.2 Hz	2′, 7′a, 2″, 3″	47.0	47.1	4.08, 2H, t, J = 7.3 Hz, overlapped	2′, 7′a, 2″, 3″
2″	29.5	1.87, 2H, q, J = 7.2 Hz	1″, 3″, 4″	29.4	29.5	1.82, 2H, q, J = 7.3 Hz	1″, 3″, 4″
3″	29.0	1.30, 2H, m. overlapped	1″, 2″, 4″, 5″	28.8	28.9	1.25, 2H, m, overlapped	1″, 2″, 4″
4″	22.2	1.35, 2H, m, overlapped	3″, 5″	22.1	22.2	1.31, 2H, m, overlapped	2″, 3″, 5″
5″	13.9	0.90, 3H, t, J = 7.2 Hz	3″, 4″	13.8	13.9	0.86, 3H, t, J = 7.0 Hz	3″, 4″
1‴	137.0	–	–	125.5/125.6	125.7	–	–
2‴	134.6	–	–	127.8	127.8	7.66, 1H, d, J = 7.9 Hz	1, 1‴, 3‴, 8‴a
3‴	130.3	7.19, 1H, m, overlapped	1‴, 4‴, Me	102.1	102.1	6.84, 1H, d, J = 7.9 Hz	1‴, 4‴, 4‴a
4‴	126.0	7.16, 1H, m, overlapped	2‴, 3‴, 6‴	156.9	157.0	–	–
4‴a	–	–	–	131.3	131.4	–	–
5‴	126.9	7.18, 1H, m, overlapped	1‴, 3‴, 6‴	122.0	122.0	8.34, 1H, m	4‴, 6‴, 8‴a
6‴	130.3	7.22, 1H, m, overlapped	2, 1‴, 2‴, 5‴	127.2	127.4	7.52, 1H, m, overlapped	5‴, 8‴
7‴	–	–	–	125.5/125.6	125.7	7.50, 1H, m, overlapped	5‴, 8‴, 8‴a
8‴	–	–	–	125.7	125.8	8.30, 1H, m	1‴, 4‴a, 7‴
8‴a	–	–	–	132.1	132.1	–	–
Me	20.0	2.33, 3H, s	1‴, 2‴, 3‴	–	–	–	–
OMe	–	–	–	55.6	55.7	4.08, 3H, s, overlapped	4‴

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

[b] Recorded in $CDCl_3$ at 300 MHz (^{1}H) and 75.5 MHz (^{13}C), respectively; data in δ ppm (J in Hz) for JWH-081 [22]

[c] J = 8 Hz; the proton signal correlated with the indicated carbons

An unknown peak **2** detected at 49.1 min in Fig. 2a showed a specific major GC–MS signal at *m/z* 214 (Fig. 2c), which was also observed in the mass spectrum of compound **1** (Fig. 2b), with a putative molecular ion signal at *m/z* 335. In the LC-PDA and LC–MS chromatograms as shown in Fig. 3a, e, the corresponding peak **2** at 8.0 min showed a protonated molecular ion signal at *m/z* 336 and a UV spectrum very similar to that of compound **1** (Fig. 3c, g). Using the isolated preparation of peak **2**, we measured its NMR spectra; the ^{1}H and ^{13}C NMR spectra of **2** showed one methoxy signal at δ_H 3.83 (3H, s) and δ_C 55.4 (data not shown). These data suggest that compound **2** has one methoxy group in place of a methyl group of compound **1**. Therefore, we purchased authentic JWH-250 and analyzed it to compare the data with those of unknown peak **2**. The GC–MS and LC–MS data (Figs. 2e, 3i) combined with the NMR results revealed that compound **2** was JWH-250, namely 2-(2-methoxyphenyl)-1-(1-pentyl-1*H*-indol-3-yl) ethanone. Compound **2** was also reported to be a potent cannabinoid receptor agonist, along with JWH-251 (**1**) [20]. In 2009, JWH-250 (**2**) was first detected as an adulterant in herbal products available on the UK and German markets [18].

The third unknown peak **3** detected at 53.7 min in Fig. 2a showed five major ion signals at *m/z* (% relative intensity) 371 (100), 314 (43), 214 (40), 185 (27), and 157 (10) by GC–MS analysis (Fig. 2d). In the LC-PDA analysis, the corresponding peak was detected at 10.6 min as

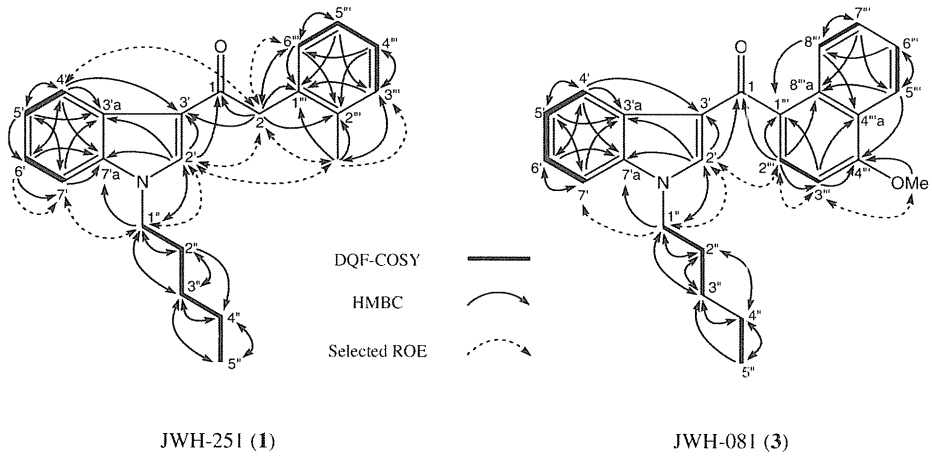

Fig. 4 Double quantum filtered correlation spectroscopy (*DQF-COSY*), heteronuclear multiple-bond correlation (*HMBC*) and selected rotating frame nuclear Overhauser effect (*ROE*) correlations of JWH-251 (**1**) and JWH-081 (**3**)

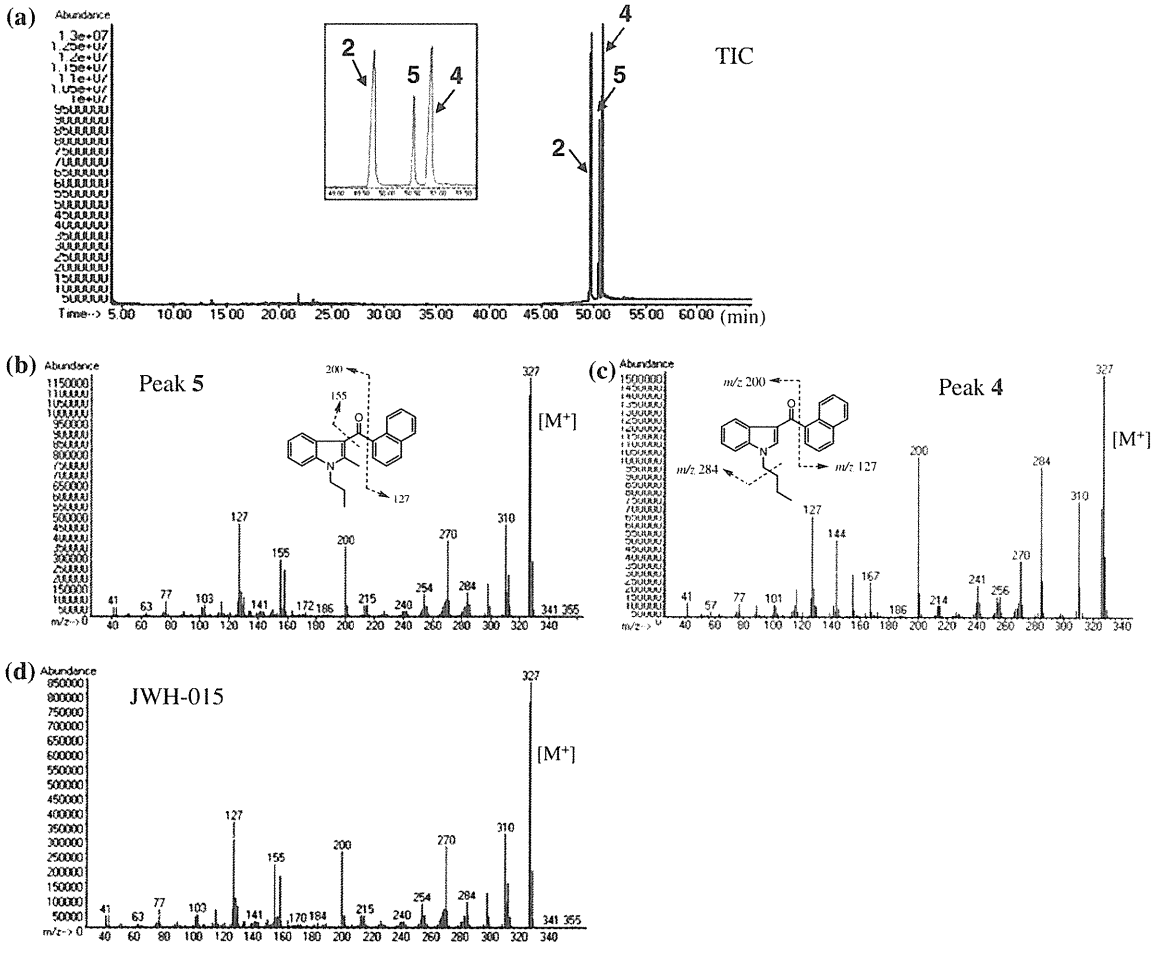

Fig. 5 GC-MS analysis for product No. 19. TIC (**a**), and EI mass spectra of peaks detected at 50.5 min (**b**, **5**), and 50.9 min (**c**, **4**) and of the peak of the standard of JWH-015 (**d**, RT 50.5 min)

shown in Fig. 3a; the peak showed a protonated ion signal at *m/z* 372 $[M + H]^+$ with an absorbance maximum at 316 nm in the UV spectrum, as shown in Fig. 3d, h. Although these characteristics were completely different from those of compounds **1** and **2** (Figs. 2b, c, 3f, g), they were similar to those of naphthoylindoles such as JWH-018

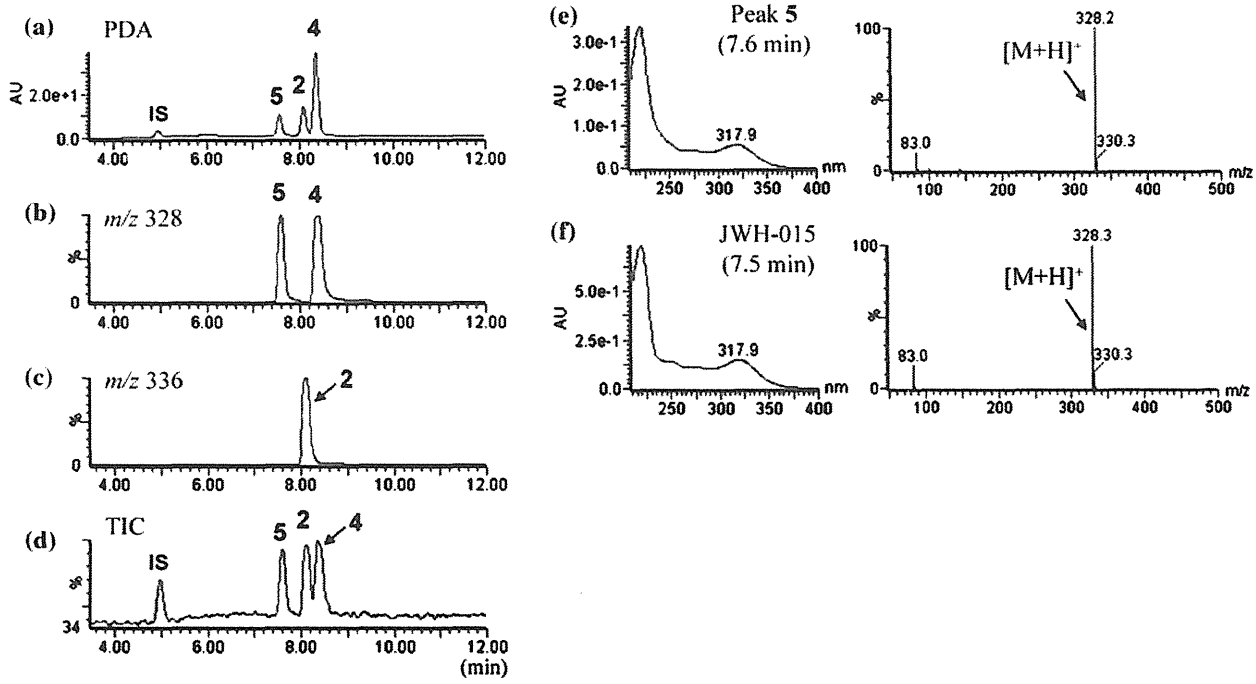

Fig. 6 LC-UV detection (**a**) and LC–MS analysis for the extract of product No. 19. Mass chromatograms at *m/z* 328 (**b**, **4** and **5**) and 336 (**c**, **2**), the TIC (**d**), and UV spectra and ESI mass spectra for peak **5** (**e**) and for the standard of JWH-015 (**f**)

and JWH-073 (**4**) (Fig. 5c) [2, 4]. The accurate mass spectrum measured by DART–TOF–MS in the positive mode showed the protonated ion peak at *m/z* 372.19494, suggesting that the molecular formula of compound **3** was $C_{25}H_{26}NO_2$. The error between the observed and theoretical mass numbers was −1.41 mDa. The ^{1}H NMR spectrum of compound **3** showed 25 nonexchangeable protons, including one methyl signal at δ 0.86 (3H, t, $J = 7.0$ Hz), one methoxy signal at δ 4.08 (3H, s), nine aromatic proton signals, AB-type aromatic proton signals, and one methylene proton signal connected to a nitrogen atom with three methylene proton signals as shown in Table 1. The ^{13}C NMR spectrum of compound **3** showed 25 carbon signals, containing 1 methyl signal, 1 methoxy signal (δ 55.7), 4 methylenes with a nitrogenated carbon (δ 47.1), 11 aromatic carbons, 7 aromatic quaternary carbons and a carbonyl carbon (δ 191.8). Following the two-dimensional NMR analyses shown in Table 1 and Fig. 4, compound **3** was identified as an aminoalkyl naphthoylindole. On the basis of the above mass and NMR data, the structure of compound **3** was deduced to be 1-(4-methoxynaphthalenyl)-(1-pentyl-1*H*-indol-3-yl)methanone. This compound has been reported as JWH-081 by Huffman et al. [22] and as a cannabinoid receptor agonist [14]. The ^{13}C NMR spectral data reported for JWH-081 [22] were identical to those of compound **3** (Table 1).

Identification of unknown peaks 4–6

An unknown peak **5** was detected with two other peaks **2** (JWH-250) and **4** (JWH-073) in the GC–MS and LC–MS chromatograms of product No. 19 (Figs. 5, 6). In the GC–MS chromatogram, the unknown peak **5** at 50.5 min in Fig. 5a showed five major fragment signals at *m/z* (% relative intensity) 310 (38), 270 (31), 200 (29), 155 (23), and 127 (38), with a putative molecular ion signal at *m/z* 327 (100) as shown in Fig. 5b. In the LC–MS chromatogram, the corresponding peak found at 7.6 min in Fig. 6a showed a protonated ion signal at *m/z* 328 $[M + H]^+$ and an absorbance maximum at 318 nm in the UV spectrum as shown in Fig. 6b, e. Although the putative molecular weight of compound **5** was the same as that of compound **4**, their characteristics differed (Figs. 5a–c, 6a, b, e). At the present time, several types of synthetic cannabinoids are available as commercial reagents. In order to rapidly identify compounds that are illegally added as designer drugs, we purchased a number of these compounds. Two of the purchased compounds were JWH-073 (1-naphthalenyl (1-butyl-1*H*-indol-3-yl)methanone) and JWH-015 (1-naphthalenyl(2-methyl-1-propyl-1*H*-indol-3-yl)methanone), both of which have a molecular weight of 327. Therefore, we measured their mass spectra by GC–MS and LC–MS. The compounds for peaks **4** and **5** were completely identical to

JWH-073 and JWH-015, respectively (Figs. 5, 6). It is of interest that compound **5** was reported as a selective CB_2 receptor ligand, but compounds **1–4** were CB_1 receptor agonists [14].

As shown in Figs. 7 and 8, an unknown peak **6** was detected together with peak **2** (JWH-250) in the GC–MS and LC–MS chromatograms of product No. 20. By GC–MS, the unknown peak **6** at 55.9 min in Fig. 7 showed five major EI-MS signals at *m/z* (% relative intensity) 384 (6), 207 (10), 155 (4), 127 (7), and 100 (100). In the LC–MS chromatogram, the corresponding peak appearing at 4.8 min in Fig. 8a showed a protonated ion signal at *m/z* 385 $[M + H]^+$ and an absorbance maximum at 312 nm in the UV spectrum as shown in Fig. 8b, e. As in the case of compounds **4** and **5**, both GC–MS and LC–MS spectra of the authentic (purchased) JWH-200, the molecular weight of which was 384, were measured (Figs. 7c, 8f); compound **6** was found to be identical to JWH-200 (1-naphthalenyl (1-(2-(4-morpholinyl)ethyl)-1*H*-indol-3-yl)methanone). Compound **6** has been reported as a synthetic cannabinoid possessing high affinity for CB_1 receptors and exhibiting Δ^9-THC-like pharmacological effects [16, 23].

Quantitation of the cannabimimetic compounds

As shown in Table 2, the calibration curves for LC-UV detection for solutions prepared by dilution of each standard solution using the gradient program 1 were linear over the concentration range 10–500 μg/ml (with calibration points at six different concentrations) (**1–4**) or 100–250 μg/ml (five concentrations) (**5**) with good correlation coefficients of $r^2 \geq 0.993$. Intraassay precision and accuracy were also

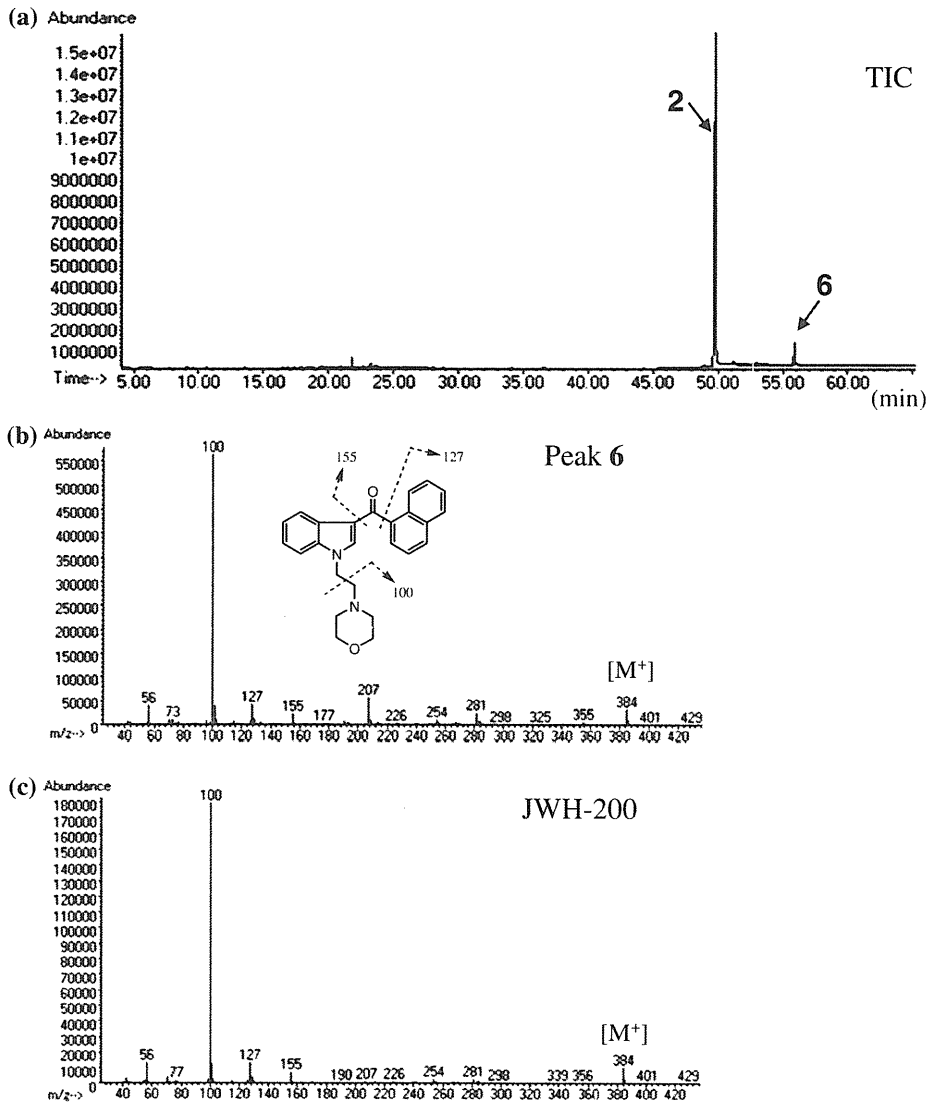

Fig. 7 GC-MS analysis for product No. 20. TIC (**a**), EI mass spectra of the peaks detected at 55.9 min (**b**, **6**) and of the peak of the standard of JWH-200 (RT 55.9 min, **c**)

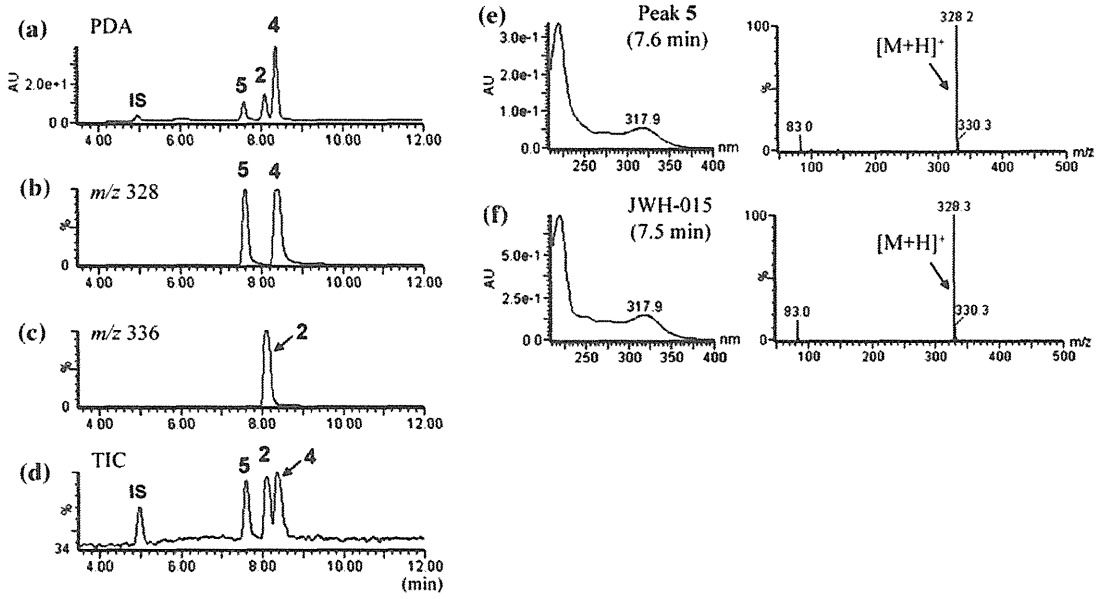

Fig. 8 LC-UV detection (**a**) and LC–MS analysis for the extract of product No. 20. Mass chromatograms at *m/z* 385 (**b**, **6** and **5**) and 336 (**c**, **2**), TIC (**d**), UV spectra and ESI mass spectra of peak **6** (**e**), and those of the standard of JWH-200 (**f**). The analysis was performed under the gradient program 2

Table 2 Linearity, precision, and accuracy obtained by the LC-UV analysis under gradient program 1 for cannabimimetic compounds **1–5**

Compound	Linear range (μg/ml)	Linearity	Concentration (μg/ml)	Precision (%)	Accuracy (%)
JWH-251 (**1**)	10–500	$y = 0.033x - 0.1838$	10	4.3	−1.8
		$r^2 = 0.9985$	50	0.6	0.4
			500	0.3	1.0
JWH-250 (**2**)	10–500	$y = 0.0122x - 0.0185$	10	4.9	−1.9
		$r^2 = 0.9993$	50	2.6	−2.6
			500	0.4	1.1
JWH-081 (**3**)	10–500	$y = 0.0147x - 0.1045$	10	1.3	−0.0
		$r^2 = 0.9934$	50	2.0	−1.3
			500	2.3	−0.2
JWH-073 (**4**)	10–500	$y = 0.0149x + 0.094$	10	1.5	−2.3
		$r^2 = 0.9931$	50	0.5	−0.4
			500	0.2	1.7
JWH-015 (**5**)	10–250	$y = 0.0152x + 0.0383$	10	0.1	0.2
		$r^2 = 0.9984$	50	0.1	0.2
			250	0.6	1.2

measured for the diluted standard solutions. The precision of the compounds ranged from 0.1% to 4.9%, and the accuracy ranged from −2.6% to 1.7% (Table 2). The calibration curves for compounds **2** and **6** obtained with the gradient program 2 were also linear over the concentration range 10–250 μg/ml (five concentrations) with good correlation coefficients of $r^2 \geq 0.992$ (Table 3). The precision of the two compounds ranged from 1.2% to 3.9%, and the accuracy ranged from −2.4% to 2.1%.

Eighteen herbal products and 2 powder products currently being sold in Japan for their expected cannabis-like effects were purchased via the Internet (Table 4). The concentrations of compounds **1–6** in the 20 products were measured using calibration curves (Tables 2, 3). The

Table 3 Linearity, precision, and accuracy obtained by LC-UV analysis under gradient program 2 for cannabimimetic compounds **2** and **6**

Compound	Linear range (μg/ml)	Linearity	Concentration (μg/ml)	Precision (%)	Accuracy (%)
JWH-250 (**2**)	10–250	$y = 0.0137x - 0.0837$	10	2.7	−1.5
		$r^2 = 0.9926$	50	1.7	2.1
			250	3.9	−0.1
JWH-200 (**6**)	10–250	$y = 0.013x - 0.0788$	10	3.3	−1.0
		$r^2 = 0.9921$	50	1.2	−2.4
			250	2.1	−0.3

Table 4 Concentrations of detected cannabimimetic compounds in the tested products

Product No.	Form	JWH-251 (**1**) (mg/g)	JWH-250 (**2**) (mg/g)	JWH-081 (**3**) (mg/g)	JWH-073 (**4**) (mg/g)	JWH-015 (**5**) (mg/g)	JWH-200 (**6**) (mg/g)
1	Dried leaf (cutting)	n.d.	n.d.	n.d.	37.3 ± 3.25	n.d.	n.d.
2	Solid (resin)	n.d.	n.d.	n.d.	91.5 ± 10.9	n.d.	n.d.
3	Solid (resin)	n.d.	n.d.	n.d.	107 ± 5.33	n.d.	n.d.
4	Dried leaf (cutting)	n.d.	n.d.	n.d.	79.8 ± 3.98	n.d.	n.d.
5	Dried leaf (cutting)	n.d.	25.9 ± 0.64	n.d.	n.d.	n.d.	n.d.
6	Dried leaf (cutting)	n.d.	51.5 ± 2.88	n.d.	n.d.	n.d.	n.d.
7	Dried leaf (cutting)	n.d.	16.9 ± 1.79	83.6 ± 0.94	n.d.	n.d.	n.d.
8	Powder	n.d.	321 ± 8.97	n.d.	n.d.	n.d.	n.d.
9	Powder	n.d.	340 ± 28.0	n.d.	n.d.	n.d.	n.d.
10	Dried leaf (cutting)	18.9 ± 4.64	n.d.	32.1 ± 7.49	n.d.	n.d.	n.d.
11	Dried leaf (cutting)	3.65 ± 0.81	41.7 ± 8.07	34.8 ± 7.18	n.d.	n.d.	n.d.
12	Dried leaf (cutting)	n.d.	30.5 ± 0.55	40.1 ± 1.77	29.6 ± 0.78	n.d.	n.d.
13	Dried leaf (cutting)	n.d.	n.d.	47.4 ± 7.51	n.d.	n.d.	n.d.
14	Dried leaf (cutting)	n.d.	n.d.	27.6 ± 4.08	26.7 ± 3.39	n.d.	n.d.
15	Dried leaf (cutting)	n.d.	n.d.	44.1 ± 6.72	n.d.	n.d.	n.d.
16	Dried leaf (cutting)	n.d.	n.d.	30.5 ± 7.76	24.7 ± 5.56	n.d.	n.d.
17	Dried leaf (cutting)	n.d.	n.d.	33.1 ± 3.10	27.6 ± 2.24	n.d.	n.d.
18	Dried leaf (cutting)	n.d.	n.d.	n.d.	88.3 ± 7.37	n.d.	n.d.
19	Dried leaf (cutting)	n.d.	23.6 ± 1.52	n.d.	45.2 ± 4.42	9.56 ± 0.55	n.d.
20	Dried leaf (cutting)	n.d.	41.7 ± 0.75	n.d.	n.d.	n.d.	21.7 ± 0.47

Data are given as the mean ± standard deviation, $n = 3$

n.d. not detected

results are summarized in Table 4. The concentrations of compounds **1**–**4** in these products were in the ranges of 3.65–18.9, 16.9–340, 27.6–83.6, and 24.7–107 mg/g, respectively. The concentrations of compounds **5** and **6** in the products were 9.56 and 21.7 mg/g, respectively (Table 4). These results indicate that there is great variation in the concentrations of illegal compounds added to these products. Two powder products (Nos. 8 and 9) and two resin products (Nos. 2 and 3) contained high contents of JWH-250 and JWH-073, respectively. In 9 out of 16 dried leaf (cutting) products, multiple drugs could be detected (Table 4); there are possibilities that a herbal material was adulterated with multiple drugs and that multiple herbal materials, each adulterated with a single or multiple adulterants, were combined together.

Conclusions

In the present study, we identified six synthetic cannabinoids in herbal or chemical products collected via the Internet from December 2009 to April 2010. Among the six compounds, four compounds (**1**, **3**, **5** and, **6**) are new as adulterants to our knowledge. Although "Spice" and other similar herbal products are known to have been sold on the Internet since as early as 2006 [18], the synthetic

cannabinoids were first reported as adulterants in these herbal products in early 2009 [1, 2, 6]. CCH, CP 47497, and JWH-018 were most frequently detected as adulterants in the products available in Japan from June 2008 to June 2009 [4]. However, new synthetic cannabinoids, especially indole derivatives belonging to the JWH series of cannabinoids, began to appear shortly thereafter. The compounds **1–6** detected in this study had been synthesized as cannabimimetic substances and were reported to have affinity actions on cannabinoid receptors. In addition, compounds **3–6** were reported to have in vivo pharmacological effects [13, 16, 23–25]. Compounds **1–4** showed affinity for CB_1 receptors three- to tenfold higher than for CB_2 receptors unlike JWH-018 [14, 20], whereas JWH-015 (**6**) showed affinity for the CB_2 receptor that was 24-fold that for the CB_1 receptor [14]. In the present study, it should be pointed out that product No. 19 contained not only CB_1 cannabinoid receptor agonists (compounds **2** and **4**) but also one CB_2 receptor agonist (compound **5**). This is the first case in which several synthetic cannabinoids possessing different types of activity were detected in a single product.

There is little information on pharmacology, toxicology, and safety of the cannabimimetic adulterants for humans, and there are possibilities of serious health damage for their abusers. For rapid identification of various new synthetic cannabinoids included as designer drugs in illegal products, we are collecting the GC–MS and LC–MS data of many cannabimimetic compounds to construct a database. In view of the worldwide trend to adulterating herbal or chemical products with designer drugs, an international system of cooperation to share the analytical information of such compounds is needed to prevent their worldwide spread.

Acknowledgments Part of this work was supported by a Health and Labor Sciences Research Grant from the Ministry of Health, Labour, and Welfare, Japan.

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Identification of a Novel Cannabimimetic Phenylacetylindole, Cannabipiperidiethanone, as a Designer Drug in a Herbal Product and Its Affinity for Cannabinoid CB_1 and CB_2 Receptors

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Received May 27, 2011; accepted June 30, 2011; published online July 4, 2011

A new cannabimimetic phenylacetylindole (cannabipiperidiethanone, 1) has been found as an adulterant in a herbal product which contains two other known synthetic cannabinoids, JWH-122 and JWH-081, and which is distributed illegally in Japan. The identification was based on analyses using GC-MS, LC-MS, high-resolution MS and NMR. Accurate mass spectrum measurement showed the protonated molecular ion peak of 1 at *m/z* 377.2233 $[M+H]^+$ and the molecular formula of 1 was $C_{24}H_{29}N_2O_2$. Both mass and NMR spectrometric data revealed that 1 was 2-(2-methoxyphenyl)-1-{1-[(1-methylpiperidin-2-yl)methyl]-1*H*-indol-3-yl}ethanone. Compound 1 has a mixed structure of known cannabimimetic compounds: JWH-250 and AM-2233. Namely, the moiety of phenylacetyl indole and *N*-methylpiperidin-2-yl-methyl correspond to the structure of JWH-250 and AM-2233, respectively. However, no synthetic, chemical or biological information about 1 has been reported. A binding assay of compound 1 to cannabinoid receptors revealed that 1 has affinity for the CB_1 and CB_2 (IC_{50}=591, 968 nM, respectively) receptors, and shows 2.3- and 9.4-fold lower affinities than those of JWH-250. This is the first report to identify cannabimimetic compound (1) as a designer drug and to show its binding affinity to cannabinoid receptors.

Key words synthetic cannabinoid; JWH-081; JWH-122; 2-(2-methoxyphenyl)-1-{1-[(1-methylpiperidin-2-yl)methyl]-1*H*-indol-3-yl}ethanone; JWH-250; designer drug

Numerous psychotropic products have been made readily available *via* the Internet. In Japan, various herbal products with brand names such as "Spice" and "herbal incense," hinting at cannabis-like effects, began to appear in 2008, following their advent in several European countries in 2006. In early 2009, we reported that these herbal products contained synthetic cannabinoids such as cannabicyclohexanol (CCH) and JWH-018 as psychoactive adulterants.[1,2] German groups have also found these compounds in some herbal products.[3] More than 20 synthetic cannabinoids have been detected as psychoactive ingredients in herbal products around the world since 2009,[4—10] and ten of those cannabinoids—CCH, CP-47,497, JWH-018, JWH-073, JWH-250, JWH-015, JWH-122 (**2**), JWH-081 (**3**), JWH-200 and JWH-251—were controlled as designated substances (Shitei-Yakubutsu) under the Pharmaceutical Affairs Law in Japan as of May 2011. Most of these compounds were synthesized as cannabimimetic substances having affinities to cannabinoid CB_1 and/or CB_2 receptors in the course of drug development.[11] However, some of these synthetic cannabinoids have been abused as psychoactive drugs in place of *Cannabis sativa* L. (cannabis, marijuana, hemp), which naturally contains psychoactive cannabinoids such as Δ^9-tetrahydrocannabinol (Δ^9-THC). During our successive survey of designer drugs distributed in Japan, we found a new compound (**1**) contained in a herbal product together with two known cannabimimetic substances, JWH-122 (**2**) and JWH-081 (**3**) (Fig. 1). In the present study, we describe the identification of the novel phenylacetylindole (**1**) and its affinity to cannabinoid CB_1 and CB_2 receptors.

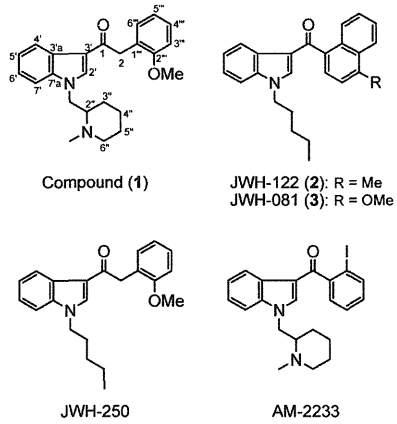

Fig. 1. Structures of the Detected Compounds (**1**—**3**) and Related Cannabimimetic Indoles

Experimental

Chemicals and Reagents JWH-122 (**2**), JWH-081 (**3**) and JWH-250 were purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). (*R*)-(+)-WIN-55,212-2 was purchased from Sigma (St. Louis, MO, U.S.A.). All other common chemicals and solvents were of analytical reagent grade or HPLC grade.

Sample for Analysis The analysis sample was purchased *via* the internet in January 2011 as a herbal product being sold in Japan. The product contained 2 g of mixed dried plants.

Preparation of Sample Solution For qualitative analyses, 10 mg of the herbal product was crushed into powder and extracted with 1 ml of MeOH under ultrasonication for 10 min. After centrifugation (5 min, 3000 rpm), the supernatant solution was passed through a centrifugal filter (Ultrafree-MC, 0.45 μm filter unit; Millipore). If necessary, the solution was diluted with MeOH to a suitable concentration before instrumental analyses.

Analytical Conditions The sample solution was analyzed by GC-MS (electron impact (EI)) and LC-MS (electrospray ionization (ESI)) analyses according to our previous report.[5] 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) in a positive mode.[12] The measurement conditions were as previously reported.[5]

For NMR analysis, pyridine-d_5 (99.96%) was purchased from the ISOTEC division of Sigma-Aldrich (St. Louis, MO, U.S.A.). The NMR spectra were obtained on ECA-600 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), one-dimensional total correlation spectroscopy (1D-TOCSY), and rotating frame nuclear Overhauser effect (ROE) spectra. For isolation of the compound, recycling preparative HPLC (Japan Analytical Industry, Tokyo, Japan) was used with a JAIGEL-GS310 column (500 mm×20 mm i.d.; Japan Analytical Industry) and monitored by UV absorbance and refractive index (RI) detectors.

Isolation of Compound 1 A 2-g sample of the herbal product was extracted with 150 ml of $CHCl_3$/MeOH (1 : 1) by ultrasonication for 1 h. The extractions were repeated three times, and the supernatant fractions were combined and evaporated to dryness. The extract was placed on a preparative TLC plate (Silica Gel 60, 20×20 cm, 2 mm; Merck, Darmdstadt, Ger-

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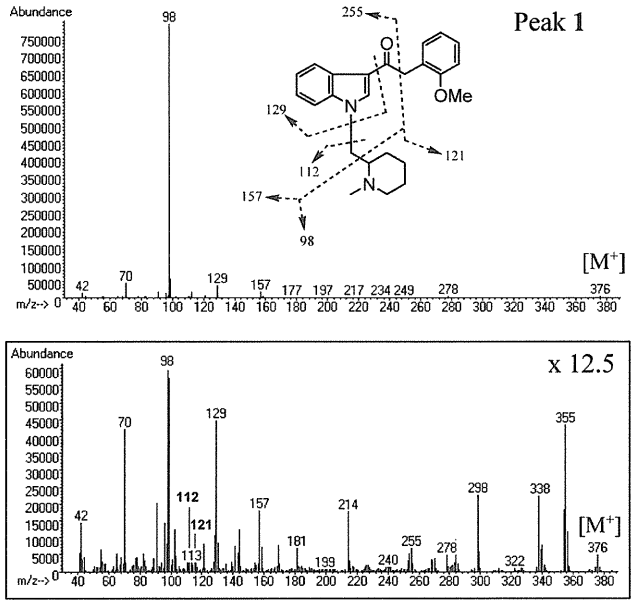

Fig. 2. GC-EI Mass Spectrum of the Detected Peak at 52.67 min (**1**)

many), which was then developed using $CHCl_3$/MeOH (20 : 1). A portion of the silica gel in the TLC plate that contained the target compound was detected by UV 254 nm and DART-TOF-MS, and scraped from the plate. The target compound was then eluted with $CHCl_3$/MeOH (1 : 1) to obtain fraction 1, and fraction 1 was further purified by recycling preparative HPLC with $CHCl_3$/MeOH (1 : 1) to obtain compound **1** (43 mg).

Compound **1**: A pale yellow oil; UV (MeOH) λ_{max} nm: 242, 300; ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz): see Table 1; EI-MS *m/z* (% relative intensity): 376 (0.6, $[M^+]$), 355 (5), 338 (3), 298 (3), 255 (0.8), 214 (2), 157 (2), 129 (6), 121 (1), 112 (2), 98 (100) and 70 (5), as shown in Fig. 2; DART-TOF-MS *m/z*: 377.2233 $[M+H]^+$ (Calcd for $C_{24}H_{29}N_2O_2$: 377.2229).

Binding Assay for Cannabinoid CB_1 and CB_2 Receptors The binding affinities of **1** and JWH-250 for the CB_1/CB_2 receptors were determined by the competition of agonist [^{3}H]-CP-55.940 (PerkinElmer Inc., MA, U.S.A.) binding to human recombinant cannabinoid CB_1/CB_2 receptors. To determine the IC_{50} values of the tested compounds, eight different concentrations of each compound in the range of 3 nM to 10 μM were investigated. (*R*)-(+)-WIN-55212-2, which is a cannabinoid receptor agonist, was used as a positive control.

Results and Discussion

Identification of Compound 1 An unknown peak **1** was detected along with two major peaks **2** (JWH-122) and **3** (JWH-081) in the GC-MS and LC-MS chromatograms of the herbal product (data not shown). The compounds for the peaks **2** and **3** were completely identical to JWH-122 and JWH-081, respectively, by direct comparison with the authentic samples.[5,9] The unknown peak **1** at 52.67 min in the GC-MS chromatogram showed a mass spectrum having 12 major ion peaks, as shown in Fig. 2. The LC-MS analysis determined that the peak **1** at 4.4 min showed a major ion peak at *m/z* 377 $[M+H]^+$ and absorbance maxima at 242 and 300 nm of the UV spectrum (data not shown). In the accurate mass spectrum obtained by DART-TOF-MS with direct exposure of the sample extract to the ion source, the major ion peak showed a protonated molecular ion peak ($[M+H]^+$) at *m/z* 377.2233 in the positive mode, suggesting that the molecular formula of **1** was $C_{24}H_{29}N_2O_2$.

The ^{1}H- and ^{13}C-NMR spectra of **1** exhibited 28 protons and 24 carbons as shown in Table 1. The NMR spectra of **1**

Table 1. NMR Data[a] of JWH-250 and Compound **1**

Position	JWH-250[b]	Compound **1**[c]	
	^{13}C	^{13}C	^{1}H
1	193.1	192.2	—
2	40.9	40.7	4.43, 1H, d, *J*=15.1 Hz, overlapped
			4.40, 1H, d, *J*=15.1 Hz, overlapped
2′	135.0	136.4	8.37, 1H, s
3′	116.1	116.1	—
3′a	126.8	126.6	—
4′	122.8	122.3	8.88, 1H, d-like, *J*=7.5 Hz
5′	123.1	123.0	7.36, 1H, m, overlapped
6′	122.4	122.0	7.38, 1H, m, overlapped
7′	109.7	110.3	7.55, 1H, d, *J*=6.8 Hz
7′a	136.6	137.3	—
N-CH_2	—	48.5	4.48, 1H, dd, *J*=14.1, 4.1 Hz
			4.00, 1H, dd, *J*=14.1, 8.3 Hz
1″	47.1	—	—
2″	29.5	62.3	2.31, 1H, m
3″	29.0	28.6	1.17, 1H, m
			1.10, 1H, m
4″	22.3	23.0	1.40, 1H, m, overlapped
			0.93, 1H, m
5″	13.9	25.0	1.38, 2H, m, overlapped
6″	—	56.2	2.74, 1H, d-like, *J*=11.3 Hz
			1.95, 1H, m
1‴	124.7	124.9	—
2‴	156.9	157.2	—
3‴	110.5	110.4	6.91, 1H, d, *J*=8.3 Hz
4‴	128.0	127.7	7.25, 1H, ddd, *J*=8.3, 7.6, 1.4 Hz
5‴	120.7	120.2	6.97, 1H, ddd, *J*=7.2, 7.6, 1.1 Hz
6‴	131.0	131.0	7.50, 1H, d-like, *J*=7.2 Hz
N-Me	—	42.7	2.34, 3H, s
OMe	55.4	54.7	3.65, 3H, s

a) Recorded at 600 MHz (^{1}H) and 150 MHz (^{13}C), respectively; data in δ ppm (*J* in Hz). *b*) Recorded in $CDCl_3$. *c*) Recorded in pyridine-d_5.

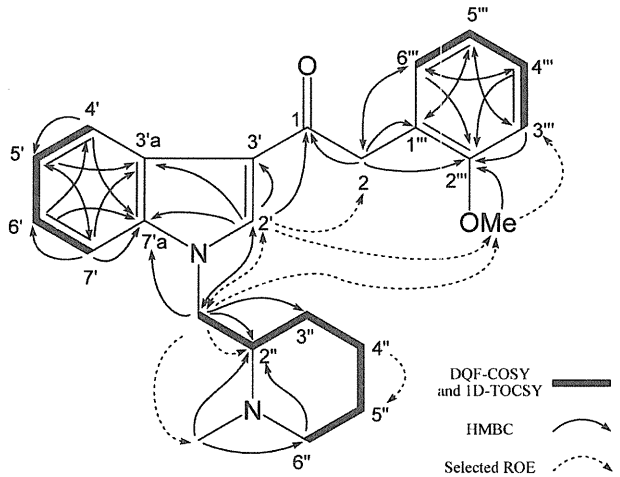

Fig. 3. DQF-COSY, 1D-TOCSY, HMBC and Selected ROE Correlations of **1**

(Table 1, Fig. 3) showed the presence of a methoxy group, a carbonyl carbon (C-1) with a methylene group which was adjacent to the carbonyl group (position-2), an indole group (positions-2′, 3′, 3′a, 4′, 5′, 6′, 7′, 7′a) and a phenyl group (positions-1‴ to 6‴). These spectra were very similar to those of the *o*-methoxy phenylacetyl indole, JWH-250 (Fig. 1, Table 1), except for the remaining data indicating a $C_7H_{14}N_1$

Table 2. Effect of Synthetic Cannabinoids on [^{3}H]-CP-55,940 Binding to Human Cannabinoid Receptors

Compound	IC_{50} (nM)		
	CB_1	CB_2	Ratio CB_1/CB_2
1	591	968	0.61
JWH-250	260	103	2.52
(*R*)-(+)-WIN-55,212-2[a]	45.6	13.8	3.30

a) Positive control, cannabinoid receptor agonist.

unit in place of the *n*-pentyl group. The HMBC and ROE spectra of **1** confirmed that the indole, methoxy, phenyl and acetyl groups were in the same arrangement as in JWH-250 (Figs. 1, 3). The ^{13}C-carbon, the HMQC, the DQF-COSY and the 1D-TOCSY spectra of the remaining unit suggested the existence of a 1,2,6-substiuted hexane moiety and one independent methyl group. The chemical shifts at the three carbons of C-2″, C-6″ and the independent methyl suggested that these carbons were connected to the nitrogen atom, and the HMBC correlations between the methylene protons (H-6″) and the methine carbon (C-2″) and between the *N*-methyl protons at δ_H 2.34 and the C-2″ and C-6″ carbons confirmed that the remaining unit of **1** was a *N*-methylpiperidin-2-ylmethyl group (Fig. 3). The connection of the remaining unit to the indole nitrogen was revealed by the HMBC correlations from the bridging methylene protons (*N*-C$\underline{H}_2$) to the two carbons (C-2′, C-7′a) and from the methine proton at the 2′-position to the bridging methylene carbon (*N*-$\underline{C}H_2$) (Fig. 3). The observed ROE correlations also supported the structure, as shown in Fig. 3. On the basis of these mass and NMR spectral data (Figs. 2, 3, Table 1), the structure of compound **1** was finally deduced as 2-(2-methoxyphenyl)-1-{1-[(1-methylpiperidin-2-yl)methyl]-1*H*-indol-3-yl}ethanone. This is the first report of this compound, and it was revealed that **1** has a mixed structure of known cannabimimetic compounds: JWH-250 and AM-2233 (Fig. 1). Considering its structure, compound **1** has been named cannabipiperidiethanone. By using chiral HPLC analysis, compound **1** has been revealed to exist as a racemic mixture (data not shown).

Binding Activity of Compound 1 to Cannabinoid CB_1 and CB_2 Receptors No chemical or biological information about compound **1** has yet been reported. However, **1** has a mixed structure of known cannabimimetic compounds, JWH-250 and AM-2233 (a racemic compound), and both compounds have been reported to possess affinity to cannabinoid CB_1 and CB_2 receptors (JWH-250: K_i=11, 33 nM, respectively; AM-2233: K_i=2.8, 2.9 nM, respectively).[13,14] Therefore, we thought that **1** might have some cannabinoid receptor-binding activity. Subsequently, the binding affinity of **1** to cannabinoid CB_1 and CB_2 receptors was determined in competition with agonist [^{3}H]-CP-55,940 binding, as shown in Table 2. As a result, **1** was shown to have affinity for the CB_1 and CB_2 receptors (IC_{50}=591, 968 nM, respectively), and to have 1.6-fold selectivity for the CB_1 receptor (Table 2). The affinities of **1** for the CB_1 and CB_2 receptors were 2.3- and 9.4-fold lower than those of JWH-250, and 13- and 70-fold lower than those of (*R*)-(+)-WIN-55,212-2, as shown in Table 2. Since the chiral resolution of AM-2233 has been reported and the (*R*)-(+)-enantiomer has very high affinities for the CB_1 and CB_2 receptors, 300- and 260-fold greater than those of the (*S*)-(−)-enantiomer,[14] it might be of additional interest to determine the affinities of each enantiomer of **1** from the view point of medicinal chemistry.

In this study, we first identified a novel cannabimimetic compound (**1**) in an illegal product and revealed its affinity for cannabinoid CB_1/CB_2 receptors. When certain synthetic cannabinoids became controlled substances under Japanese law, new analogs of the controlled substances replaced them as adulterants. Since the pharmacological and toxicological data for most of these cannabimimetic compounds have not been reported, there are serious health risks involved in their use. Therefore, we are continuously monitoring such compounds in illegal products to prevent their abuse.

Acknowledgments Part of this work was supported by a Health and Labor Sciences Research Grant from the Ministry of Health, Labour and Welfare of Japan.

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Forensic Science International 215 (2012) 179–183

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

Effects of synthetic cannabinoids on electroencephalogram power spectra in rats☆

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ARTICLE INFO

Article history:
Received 5 November 2010
Received in revised form 22 April 2011
Accepted 5 May 2011
Available online 2 June 2011

Keywords:
Electroencephalogram
Synthetic cannabinoids
Cannabicyclohexanol
CP-47,497
JWH-018
Locomotor activity

ABSTRACT

Several synthetic cannabinoids have recently been distributed as psychoactive adulterants in many herbal products on the illegal drug market around the world. However, there is little information on pharmacology and toxicology of such compounds. Although Δ^9-tetrahydrocannabinol (Δ^9-THC), a psychoactive cannabinoid of marijuana, was reported to affect electroencephalograms (EEG) of rats, the effects of synthetic cannabinoids are unknown. We examined the pharmacological activities of three synthetic cannabinoids; cannabicyclohexanol (CCH), CP-47,497 and JWH-018; by analyzing EEG power spectra and locomotor activity after intraperitoneal administration to rats and compared them with those of Δ^9-THC. The three compounds significantly increased the EEG power in the frequency range of 5.0–6.0 Hz for the first 3 h, while Δ^9-THC decreased the power spectra in the wide range of 7.0–20.0 Hz during the first hour. These results indicate that the effect of the three compounds on EEG is different from that of Δ^9-THC. Additionally, CCH, CP-47,497 and JWH-018 significantly decreased the locomotor activity for 11.5 h, 11 h and 4.5 h, respectively, after administration which was longer than that of Δ^9-THC (3.5 h). Furthermore, all three compounds significantly reduced the total amounts of locomotor activity during a 3-h, 6-h and 12-h period after injection, whereas no statistical difference was observed for the Δ^9-THC injection. Among the three compounds, CCH and CP-47,497 exerted a longer duration of the change in the EEG power spectra and suppression of the locomotor activity than JWH-018.

1. Introduction

Recently, many kinds of herbal products have been sold globally via the Internet under brand names such as "Spice" and "Herbal Incense" for its expected narcotic effects and as an alternative to cannabis (marijuana). In early 2009, the synthetic cannabinoids; cannabicylcohexanol (CCH, (1*RS*,3*SR*)-3-[2-hydroxy-4-(2-methylnonan-2-yl)phenyl]cyclohexan-1-ol), CP-47,497 ((1*RS*,3*SR*)-3-[2-hydroxy-4-(2-methyloctan-2-yl)phenyl]cyclohexan-1-ol) and JWH-018 (1-naphthalenyl(1-pentyl-1*H*-indol-3-yl)methanone), were first reported as psychoactive ingredients of herbal products (Fig. 1) [1–3] and as a consequence, have been controlled in Germany since January 2009 [4], followed by Austria, France, Sweden and other countries [5]. In Japan, CCH, CP-47,497 and JWH-018 have been controlled as designated substances (Shitei-Yakubutsu) under the Pharmaceutical Affairs Law since November 2009.

Despite the regulation by national governments, even more synthetic cannabinoids such as HU-210, JWH-398, JWH-073, JWH-250, JWH-251, JWH-081, JWH-015 and JWH-200 have recently been detected in many herbal products [5–8]. CCH and CP-47,497 are cyclohexylphenols and were originally developed by Pfizer during the 1970s and 1990s [9–13]. JWH-018 and other JWH compounds belonging to indole derivatives, such as naphthoylindoles and phenylacetylindoles, were mainly synthesized by Huffman and colleagues [14–16] since the 1990s. These compounds were reported as potent cannabinoid CB_1 and/or CB_2 receptor agonists [13,15,16] and in part possess in vivo pharmacological effects similar to that of Δ^9-tetrahydrocannabinol (Δ^9-THC, Fig. 1), a major psychoactive constituent of *Cannabis sativa* L. (marijuana) [9,12,14,17].

Since the end of 1970s, a number of cannabinoid analogs including THC derivatives and indole-, pyrrole-, indene-, and pyrazole-derivatives [18] were newly synthesized for the treatment of various diseases. However, there is little information on pharmacology, toxicology and safety of those compounds.

Various kinds of drugs including abused drugs affect electroencephalograms (EEG) in animals and humans [19]. Δ^9-THC reduced the power of local field potentials and EEG in various

☆ 48th Annual Meeting of the International Association of Forensic Toxicologists (TIAFT). Joint meeting with the society of Toxicological and Forensic Chemistry (GTFCh). August 29–September 2, 2010, Bonn, Germany, Guest edited by Thomas Kraemer, Hans. H. Maurer and Frank Musshoff.

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doi:10.1016/j.forsciint.2011.05.005

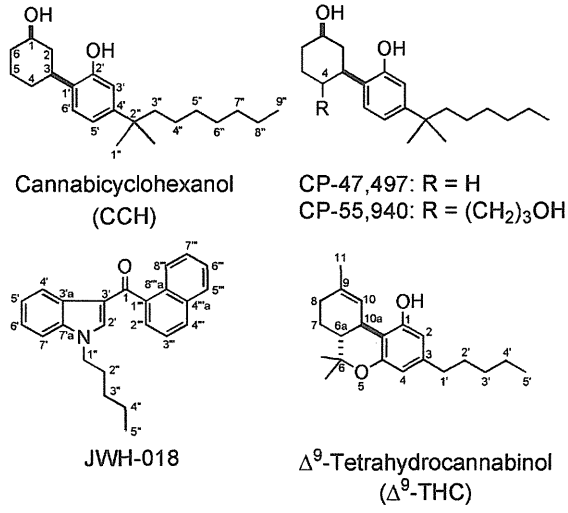

Fig. 1. Structures of synthetic cannabinoids and Δ^9-THC.

frequency bands such as theta (4–12 Hz) in the hippocampus and the neocortex in animals and humans and concomitantly, impair hippocampus-dependent memory tasks [20,21]. However, the psychotropic effects of the synthetic cannabinoids are unknown. In comparison with Δ^9-THC, we examined the pharmacological activities of the synthetic cannabinoids CCH, CP-47,497 and JWH-018 by analyzing EEG power spectra of rats after intraperitoneal administration.

2. Materials and methods

2.1. Chemicals

Cannabicyclohexanol (CCH) was synthesized in our laboratory according to the previously described method [22]. Then, NMR, GC–MS and LC–MS analyses were performed to confirm the identity and the purity of 99.9%. CP-47,497 and JWH-018 were purchased from Cayman Chemical (Ann Arbor, MI, USA) and Δ^9-THC was purchased from Cerilliant (TX, USA). CCH, CP-47,497, JWH-018 and Δ^9-THC were dissolved in a vehicle consisting of 5% ethanol, 5% emuphor (EL-620, a polyoxyethylated vegetable oil, GAF Corporation, Linden, NJ) and 90% saline (0.9% NaCl). This vehicle alone served as the control treatment. All compounds and vehicle were intraperitoneally (i.p.) administered.

2.2. Animals

Male Sprague–Dawley male rats, weighing 240–340 g (8 weeks old), were purchased from Japan SLC Inc. (Shizuoka, Japan) and housed at a constant temperature (24 ± 0.5 °C) with a relative humidity (60 ± 2%) on an automatically controlled 12:12 h light/dark cycle (light on at 08:00, illumination intensity ≈ 100 lx). Animals had ad libitum access to food and water. The experimental protocols were approved by the Animal Care Committee of Osaka Bioscience Institute.

2.3. EEG, electromyogram (EMG), locomotor activity recordings and signal analyses

Under pentobarbital anesthesia (50 mg/kg, i.p.), rats were implanted with EEG and EMG electrodes for polygraphic recording [23–26]. The implant consisted of three stainless steel screws (1 mm in diameter) that were inserted into the skull above the cerebral cortex (first screw: anteroposterior (AP), +2 mm; left–right (LR), −2 mm; second screw: AP, +0 mm; LR, 3–4 mm, third screw: AP, −2 mm; LR, −2 mm, AP from bregma, LR from lambda) according to the atlas of Paxinos and Watson [27] and served as EEG electrodes. The EEG electrodes and two stainless steel screws for anchorage were fixed to the skull with dental cement. Two stainless steel wires were placed into neck muscles as EMG electrodes. After 10 days of recovery, each animal was connected to an EEG/EMG recording cable in a sound-proof recording chamber and habituated for 3 days before polygraphic recording. The cortical EEG and EMG signals were amplified and filtered (EEG, 0.5–35 Hz; EMG, 16–128 Hz) and recorded using the analysis software SLEEPSIGN (Kissei Comtec, Nagano, Japan) [26]. Spectral analysis of EEG by fast Fourier transformation (FFT) was performed and the EEG power densities of each 0.5-Hz bin were averaged by calculating the percentage of each bin from the total power (0.5–35 Hz). The EEG power densities in frequency were then calculated as percentages of control values with the vehicle-treatment. The locomotion sensor was set on the animal cage to detect the movement of animals by infrared beams. The time spent in each state was scored and totaled for 0.5-h epochs of the observation period.

2.4. Pharmacological treatment

The EEG and EMG of each rat were recorded for 48 h. There is considerable individual variability on EEG pattern in animals. Therefore, we used the same rat continuously for evaluation of an effect of the drugs on EEG patterns. On day 1, rats were treated with vehicle (1 ml/kg body weight, i.p.) at 20:00 for served as control, followed by treatment with CCH, CP-47497, JWH-018 or Δ^9-THC (2.5 mg/kg, i.p.) at 20:00 on the second day. Four rats were assigned to each drug, and each rat received one drug only (preceded by one vehicle injection). Doses were chosen on the basis of previously reported behavioral actions of CP-47,497, JWH-018 and Δ^9-THC in rats and effect of Δ^9-THC on EEG in rats. CP-47,497 and Δ^9-THC exerted analgesic, motor depressant and anticonvulsant effects in the rats between 0.6–4.9 mg/kg and 7.1–94.1 mg/kg of ED_{50} values, respectively [9]. JWH-018 also had cannabimimetic in vivo potency at the ED_{50} value of 3.9 μmol/kg (1.3 mg/kg) [14]. In addition, Δ^9-THC affected EEG in the rats at a dose of 5 mg/kg i.p. [20]. We have also pre-tested to determine the injection doses of drugs in EEG experiments (data not shown). Three doses of the drugs (1, 2.5 and 5 mg/kg i.p.) were administered to the rats and we observed their behavioral action, such as immobility. We then decided on a dose of 2.5 mg/kg for the drugs for the first EEG experiments.

2.5. Statistical analysis

All results were expressed as means ± SEM ($n = 3$ or 4). Statistical analyses were performed by use of Student's t-test. In all cases, $p < 0.05$ was taken as the level of significance.

3. Results

3.1. Effects of CCH, CP-47,497 and JWH-018 administration on EEG spectra in rats

We examined the EEG spectra of rats after i.p. injection of CCH, CP-47,497, JWH-018 and Δ^9-THC at 20:00, the beginning of the active period of rats. Typical examples of EEG, EMG and locomotor activity of rats given vehicle or CCH at a dose of 2.5 mg/kg for 6 h after the injection are shown in Fig. 2A. The CCH injection remarkably enhanced the cortical EEG with large-amplitude, and reduced the EMG with low-amplitude and locomotor activity (Fig. 2A, lower panel) as compared with the vehicle injection

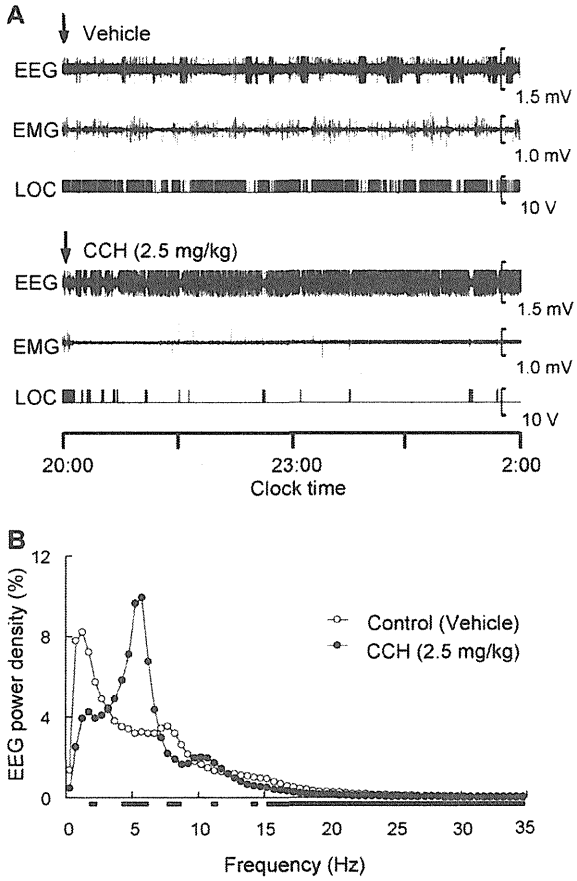

Fig. 2. Effect of CCH on EEG after administration to rats. (A) Typical examples of EEG, EMG and locomotor activity (LOC) after administration of vehicle (*Upper panel*) or CCH at a dose of 2.5 mg/kg (*Lower panel*). (B) EEG power density curve for 1.5–2.0 h after injection of CCH. The power of each 0.5-Hz bin was averaged by calculating the percentage of each bin from the total power in a whole range from 0.5 to 35 Hz. The horizontal bars indicate the frequency region with a statistical difference ($P < 0.05$) between vehicle and CCH (n = 4, each).

(Fig. 2A, upper panel). Fig. 2B showed the EEG power densities as FFT spectrum after injection. CCH significantly increased the cortical EEG with a peak of the EEG power density curve in a frequency range of 5.0–6.0 Hz for 1.5–2.0 h after administration as compared with the case of the vehicle injection.

We then calculated the EEG power densities in each frequency range as percentage of the control animals treated with vehicle to evaluate and compare the drug effects on the EEG of rats. Fig. 3A–D shows the effects of CCH, CP-47,497, JWH-018 and Δ^9-THC on EEG power densities in 0.5-h epochs during 3 h after the injection. CCH, CP-47,497 and JWH-018 strongly increased the EEG power in a frequency range of 5.0–6.0 Hz (theta) as compared with control animals (Fig. 3A–C). CCH increased EEG power in this frequency range up to 3.2-fold during 2.0–2.5 h (Fig. 3A) and more than 2-fold for over 6 h after administration (data not shown). CP-47,497 increased the EEG power up to 4.5-fold during 0.5–1.0 h (Fig. 3B) and more than 2-fold for over 6 h (data not shown). JWH-018 increased the EEG power up to 3.9-fold during 0.0–0.5 h after the i.p. injection and more than 2-fold during the first 3 h (Fig. 3C). CCH, CP-47,497 and JWH-018 injections reduced the EEG activity in a 12.0–35.0 Hz range immediately after administration, as compared to the control rats (Fig. 3A–C). In contrast, Δ^9-THC decreased the EEG power in a wide range of 7.0–20.0 Hz during 0.5–1.0 h after the drug injection and never increased 2-fold in the full range of 0–35.0 Hz (Fig. 3D). These results indicated that the

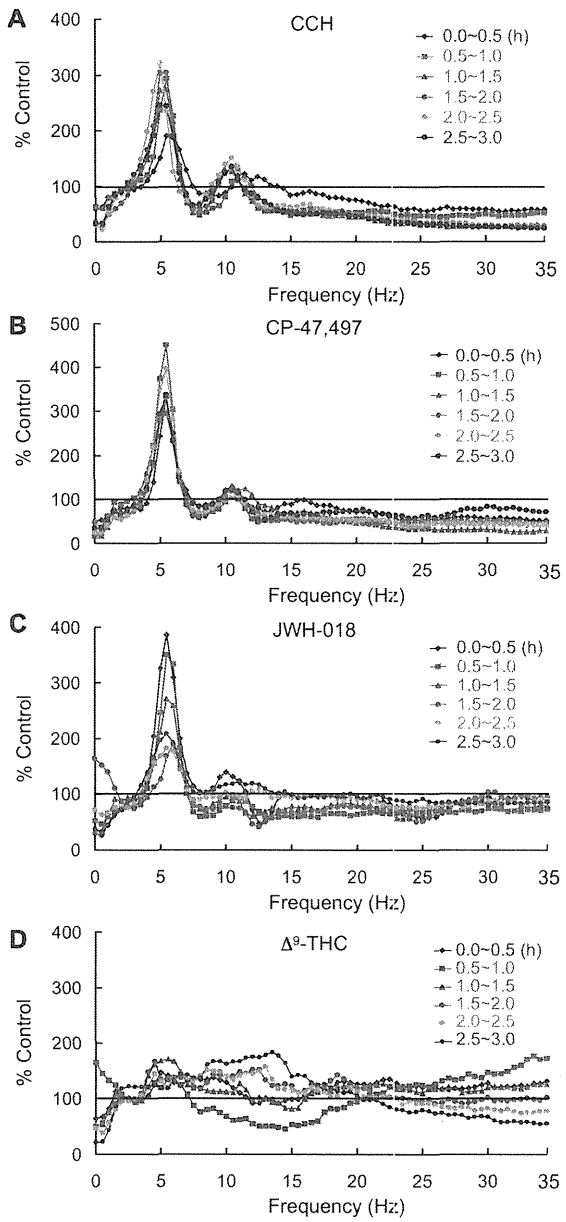

Fig. 3. Time courses of changes in EEG power density after i.p. injections of CCH (A), CP-47,497 (B), JWH-018 (C) and Δ^9-THC (D) at 2.5 mg/kg in rats. Each value is shown as percentage of the control value in animals treated with vehicle.

effect of the synthetic cannabinoids CCH, CP-47,497, JWH-018 on EEG is obviously different from that of Δ^9-THC.

3.2. Effects of CCH, CP-47,497 and JWH-018 administration on locomotor activity in rats

Fig. 4A–D shows time courses of the locomotor activity after i.p. administration of CCH, CP-47,497, JWH-018 or Δ^9-THC at a dose of 2.5 mg/kg in rats. CCH, CP-47,497, JWH-018 and Δ^9-THC significantly decreased the locomotor activity for 11.5 h (Fig. 4A), 11 h (Fig. 4B), 4.5 h (Fig. 4C) and 3.5 h (Fig. 4D), respectively, after i.p. administration. CCH and CP-47,497 suppressed the locomotor activity for approximately 3-times longer period than Δ^9-THC and JWH-018, slightly longer than Δ^9-THC.

Total amounts of locomotor activity during a 3-h, 6-h and 12-h period after injection were significantly decreased by CCH,

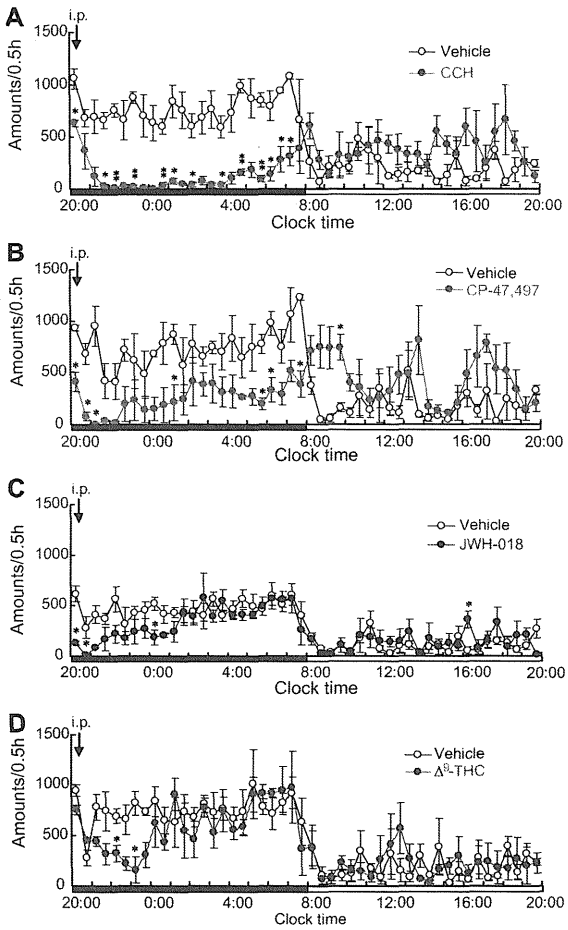

Fig. 4. Time courses of the locomotor activity after i.p. injections of CCH (A), CP-47,497 (B), JWH-018 (C) and Δ^9-THC (D) at 2.5 mg/kg in rats. Each value is shown as the locomotor count for 0.5 h. Values are means ± SEM ($n = 3$–4). $^*P < 0.05$; $^{**}P < 0.01$, significantly difference from the vehicle injection.

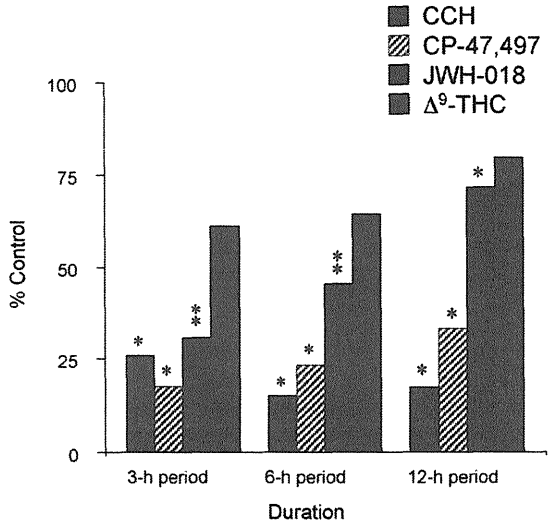

Fig. 5. Total amounts of locomotor activity during a 3-h, 6-h and 12-h period after injections of CCH, CP-47,497, JWH-018 and Δ^9-THC at 2.5 mg/kg in rats. Each value is shown as percentage of the control value in animals treated with vehicle. $^*P < 0.05$; $^{**}P < 0.01$, significantly difference from the vehicle injection.

CP-47,497 and JWH-018 (Fig. 5). CCH, CP-47,497, and JWH-018 decreased the total amounts of the locomotor activity during a 3-h, 6-h and 12-h period to 26%, 15% and 17%; 18%, 23% and 33%; and 31%, 45% and 72% of the control value, respectively. On the other hand, Δ^9-THC decreased the total amount of locomotor activity during 3-h, 6-h and 12-h period to 61%, 64% and 80%, respectively, although the difference was not statistically significant as compared with the control group. Thus, CCH, CP-47,497 and JWH-018 suppressed locomotor activity stronger and longer than Δ^9-THC, whereas the effects of CCH and CP-47,497 were more potent than that of JWH-018.

4. Discussion

In this study, we showed that the synthetic cannabinoids CCH, CP-47,497 and JWH-018 changed the cortical EEG power spectra and suppressed the locomotor activity of rats for longer duration and more significantly than Δ^9-THC. Previous studies reported that Δ^9-THC reduced the power of local field potentials and EEG in various frequency bands in both the hippocampus and the neocortex in animals and humans [20,21]. Robbe et al. [20] reported that Δ^9-THC and the potent cannabinoid CB_1 receptor agonist CP-55,940 (4-hydroxypropyl-CP-47,497, Fig. 1) decreased the EEG power spectra of the theta range (4–12 Hz) in the hippocampus of rats and that the effects were correlated with memory impairment in a hippocampus-dependent task. In this study, we confirmed that Δ^9-THC showed a similar effect on the cortical EEG power spectra in rats (Fig. 3D). However, CCH, CP-47,497 and JWH-018 exhibited much more remarkable changes in the EEG power than Δ^9-THC; the increase in a range of 5.0–6.0 Hz (theta) and the decrease in ranges of 8.0–9.0 Hz and 12.0–35.0 Hz (Fig. 3A–C). Furthermore CCH, CP-47,497 and JWH-018 exhibited a stronger and longer suppression effect on the locomotor activity than Δ^9-THC (Figs. 4A–C and 5).

Compton et al. [13,15] reported that CCH, CP-47,497 and JWH-018 had approximately 4–10-fold higher affinity for the cannabinoid CB_1 receptor with affinity constants (*Ki*) of 4.7, 9.5 and 9.5 nM, respectively, than Δ^9-THC (*Ki* = 40.7 nM). These synthetic cannabinoids were reported to induce cannabimimetic behavior [12,14], in which CCH and CP-47,497 showed pharmacological effects approximately 10- and 2-fold, respectively, more potent than Δ^9-THC but the effects of JWH-018 were similar in degree to that of Δ^9-THC. In this study, we found that the *Ki* values were well correlated to the suppressive effects of the three synthetic cannabinoids on the locomotor activity (Figs. 4 and 5).

The effects of the synthetic cannabinoids for a longer duration than that of Δ^9-THC are, at least in part, related to their metabolic pathway and rate. For instance, JWH-018 was detectable in human serum even 6 h after ingestion by smoking [28]. Sobolevsky et al. [29] reported monohydroxylation metabolites of JWH-018 bearing a hydroxyl group at the indole or the alkyl moiety and the dihydroxylation products carrying the hydroxyl groups at the indole and alkyl moieties or at the indole and naphthalene moieties. Although the metabolisms of CCH and CP-47,497 have not yet been reported, the oxidative metabolism of CP-55,940 (4-hydroxypropyl-CP-47,497) resulted in the formation of a number of alkyl side chain-hydroxylated metabolites without further oxidation [30]. An important route of metabolism of Δ^9-THC is allylic methyl hydroxylation at C-11, and 11-OH-Δ^9-THC is metabolized to an inactive carboxylic acid form [31]. However, CCH, CP-47,497 and CP-55,940 do not have an allylic methyl group at the position that corresponds to C-11 in Δ^9-THC (Fig. 1) so that the allylic methyl hydroxylation did not occur in these compounds. Namely, there are structural differences between CCH, CP-47,497 or CP-55,940 and Δ^9-THC suggesting that their metabolic fate is different from that of Δ^9-THC. The long-acting effects of CCH and