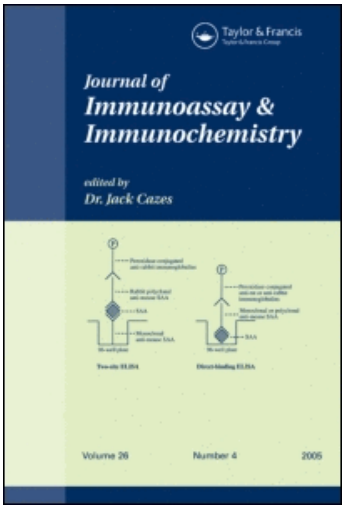

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Hiroyuki Tanaka[a]; Yuri Goto[a]; Yukihiro Shoyama[a]
[a] Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan

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MONOCLONAL ANTIBODY BASED ENZYME IMMUNOASSAY FOR MARIHUANA (CANNABINOID) COMPOUNDS+

Hiroyuki TANAKA, Yuri GOTO, Yukihiro SHOYAMA*

Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan

ABSTRACT

MAb against Δ^1-THCA was produced by fusing hybridoma with splenocytes immunized with Δ^1-THCA-BSA conjugate and hypoxanthine, aminopterine, thymidine-sensitive mouse myeloma cell line, P3-X63-Ag8-653. The cross-reaction of anti-Δ^1-THCA antibody against other cannabinoids was very wide, thus many cannabinoids and a spiro-compound were reactive suggesting that 2'-hydroxyl, 6'-hydroxyl or 6'-*O*-alkyl, 4'-alkylbenzene ring moiety is necessary for its reactivity. It became evident that this ELISA was able to be applied to the biotransformation experiments of cannabinoids in plant tissue culture system. The metabolites of Δ^6-THC such as two major metabolites, 7-oxo-Δ^6-THC and 7-hydroxyl-Δ^6-THC were also detectable by this ELISA.

(KEY WORDS: marihuana compound, THCA, monoclonal antibody, ELISA, non-specific MAb)

+ Part 25 in the series "Cannabis". For Part 24 see J.Am.Chem.Soc. 1995; 117:9766-7.

* Address for correspondence

Abbreviations: MAb, monoclonal antibody; Δ^1-THCA, Δ^1-tetrahydrocannabinolic acid; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; Δ^6-THC, Δ^6-tetrahydrocannabinol; CBN, cannabinol; MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; HSA, human serum albumin; PBS, phosphate buffer saline; GPBS, PBS containing 0.2% of gelatin; TPBS, PBS containing 0.05% of Tween 20; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; CBDA, cannabidiolic acid; MS, Murashige and Skoog; 2,4-D, 2,4-dichlorophenoxyacetic acid; CA1, CBDA-monoglucoside; CA2, hydroxyl-CBDA-monoglucoside; CBGA, cannabigerolic acid; CBNA, cannabinolic acid; CBLA, cannabicyclolic acid; CBGV, cannabigerovarin; CBGO, cannabigeorcin;

INTRODUCTION

Many metabolic studies on cannabinoids, especially pharmacologically active THC, have been carried out using animals, their organs and microsomal fractions (1-5) to find more pharmacologically active metabolites compared to the original Δ^1-THC (5). The THC receptor in the brain (6) and the endogenous ligands for the THC receptor in the brain (7) were recently found. Therefore, cannabinoids have attracted interest as sources of drugs.

In continuing studies on marihuana, we have examined the biotransformation of cannabinol (CBN) using plant tissue culture (8), the preparation and its characterization of a Δ^1-THCA-carrier protein conjugate by MALDI mass spectrometry (9), and an ELISA against naturally occurring biologically active compounds (10-12).

An immunological approach for assaying quantities of THC and its isomers in body fluids and cell preparation has been investigated

by many groups (13-16). Production of a broad-specificity MAb against cannabinoids has yet to be published. We present an immunoassay system for the detection of cannabinoids using MAb, its characteristics and application.

MATERIALS AND METHODS

Materials

BSA and HSA were provided by Pierce (Rockford; USA). All cannabinoids and spiro-compounds were isolated from *Cannabis* leaves in our laboratory (17-19), except 7-oxo-Δ^6-THC and 7-hydroxyl-Δ^6-THC that was generous gifts from Dr.A.Sawa, Meiji Seika Company, Yokohama, Japan. All other chemicals were standard commercial products of analytical grade.

Δ^1-THCA-BSA, Δ^1-THCA-β-alanine-BSA, Δ^1-THCA-HSA conjugates were synthesized as indicated previously (9).

Immunization and hybridization

Immunization and hybridization were investigated as previously described (12). Established hybridomas, 2A6, 3H11, 4A4, 4F5, 5D11, 6C6, 7C4, 7H12, 8A11 and 10F6 (Table 1) were cultured in eRDF medium supplemented with 10 μg/ml insulin, 35 μg/ml of transferrin, 20 μM ethanolamine and 25 nM selenium (20).

TABLE 1

List of monoclonal antibodies against Δ^1-THCA

MAb	IgG subclass	Light chain
2A6	2b	κ
3H11	1	κ
4A4	2b	κ
4F5	1	κ
5D11	1	κ
6C6	1	κ
7C4	1	κ
7H12	1	κ
8A11	2a	κ
10F6	1	κ

Purification of MAb and analysis by MALDI mass spectrometry

A MAb, 4A4 was purified using a CHROMATOP Protein A column (0.46 x 25 cm, NGK INSULATORS, LTD, Nagoya, Japan). The cultured medium (500 ml) containing the IgG was adjusted to pH 8 with Tris base and then subjected to the column. Absorbed IgG was eluted with 100 mM citrate buffer (pH 4). The eluted IgG was neutralized with 1 M Tris solution, then dialyzed against 100 vol. of PBS (pH 7.4) and finally lyophilized.

The purified MAb was analyzed by MALDI mass spectrometry as previously reported (12). A small amount (1-10 pmol) of the purified MAb was mixed with a thousand-fold molar excess of sinapinic acid in an aqueous solution containing 10% trifluoroacetic acid. The mixture was subjected to a JMS-IDI 1700 TOF mass monitor and irradiated with an N_2 laser (337 nm, 3 ns pulse). The

ions formed by each pulse were accelerated by a 30-kV potential into a 1.7-m evacuated tube.

Reactivity of MAbs to Δ^1-THCA-BSA

The reactivity of MAbs to Δ^1-THCA-BSA was determined by an ELISA. Δ^1-THCA-BSA conjugate (100 µl, 200 ng/ml) was adsorbed to the wells of a 96 well-immunoplate (NUNC. Roskilde, Denmark) then it was treated with 300 µl GPBS for 1 hr to reduce non-specific adsorption. The plate was washed three times with TPBS and reacted with 100 µl of testing MAb for 1 hr. The plate was washed three times with TPBS, and then MAb combined with 100 µl of a 1:1000 dilution peroxidase-labeled anti-mouse IgG (Organon Teknika Cappel Products, Westchester, USA) for 1 hr. After washing the plate three times with PBS, 100 µl of substrate solution, [0.1 M citrate buffer (pH 4.0) containing 0.003% H_2O_2, and 0.3 mg/ml ABTS (Wako, Tokyo, Japan)] was added to each well and incubated for 20 min. Absorbance was measured by a micro plate reader (MODEL 450 MICROPLATE READER BIO-RAD Laboratories) at 405 nm and 492 nm. All reactions were carried out at 37 °C.

Competitive ELISA

Δ^1-THCA-β-alanine-BSA (17 molecules of Δ^1-THCA per molecule of BSA) (9) (100 µl, 1 µg/ml) was adsorbed to the wells of a 96 well-immunoplate (NUNC. Roskilde, Denmark) then it was treated with 300 µl GPBS for 1 hr to reduce non-specific adsorption. 25 µl of various concentrations of Δ^1-THCA dissolved in 20% of MeOH solution was incubated with 25 µl (IgG: 1.34 µg) of a 1:40

dilution IgG for 45 min. The plate was washed three times with TPBS, and then the MAb was combined with 100 µl of a 1:1000 dilution of peroxidase-labeled anti-mouse IgG for 1 hr. After washing the plate three times with TPBS, 100 µl of substrate solution [0.1 M citrate buffer (pH 4) containing 0.003% H_2O_2, 0.3 mg/ml of ABTS] was added to each well and incubated for 15 min. The absorbance was measured by micro plate reader at 405 nm and 492 nm.

The cross-reactivities (CR%) of related cannabinoids were determined according to Weiler's equation (23):

$$CR\% = \frac{\mu g/ml \text{ of } \Delta^1\text{-THCA yielding } A/A_0=50\%}{\mu g/ml \text{ of compound under investigation yielding } A/A_0=50\%} \times 100$$

A is the absorbance in the presence of the test compound and A_0 is the absorbance in the absence of the test compound (20% MeOH soln.).

Biotransformation of CBDA, separation of biotransformed products by HPLC and detection by ELISA

An EtOH solution (2 ml) of cannabidiolic acid (CBDA)(40 mg) was sterilized by filtration using a Sterile Millex-HV Filter (0.45 µm Filter Unit, Millipore Products Division, Bedford, USA). It was then incubated with the tissue mass suspension of *Pinellia ternata* in MS medium (21) supplemented with 2,4-D (0.25 mg/l) on a shaker (60 rpm) at 25 °C under the dark as reported previously (8). Tissue masses were dried *in vacuo*, powdered (4.3 g) and extracted with MeOH. The extracts (1.96 g) were purified by HPLC using a Model

CCPM(Tosoh Co.Ltd) equipped with a Cosmosil 5C18-MS column and a variable wave length detector (268 nm). The mobile phase was a gradient acetonitrile-H_2O mixture (20 % to 100%) containing 5 mM phosphoric acid. The flow rate was 1.0 ml/min. The solvent of separated fractions 2, 6 and 8 was removed *in vacuo* to give CA 1, CA 2 and CBDA, respectively.

CA 1 and 2 were dissolved in MeOH, diluted with PBS (16 times), and then assayed using a competitive ELISA, respectively.

Ligand-binding assay

The ligand binding assay with the biotransformation products was performed by Prof. R. Mechoulam, Department of Natural Products, Hebrew University as described elsewhere (7).

RESULTS AND DISCUSSION

Production and characteristics of MAb against Δ^1-THCA and other cannabinoids

Ten hybridomas producing MAbs reactive to Δ^1-THCA were obtained, and classified as indicated in Table 1. They consisted of IgG1 (3H11, 4F5, 5D11, 6C6, 7C4, 7H12 and 10F6), IgG2a (8A11) and IgG2b (2A6 and 4A4) which had κ light chains. The reactivities of IgG type MAbs against Δ^1-THCA were investigated by performing a straight dilution curve resulting in different affinities as shown in Fig.1. Since 4A4 had a higher reactivity than the others, the original clone 4A4 secreted an anti-Δ^1-THCA antibody, and was purified by a

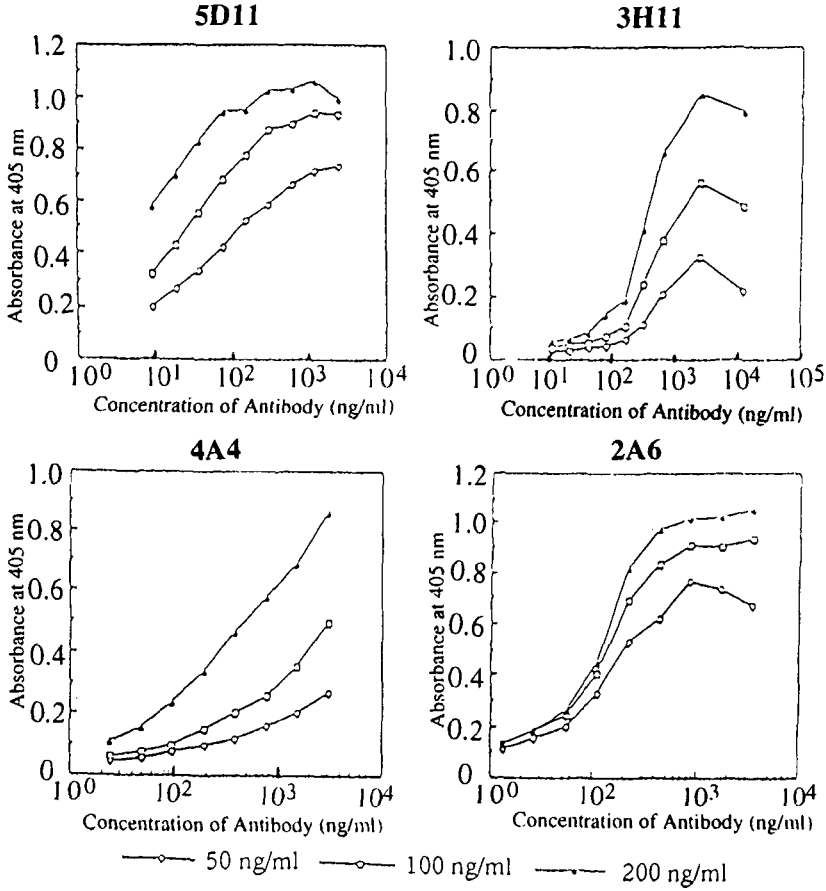


Fig.1 Reactivity of MAbs, 5D11, 3H11, 4A4 and 2A6 against Δ^1-THCA-β-alanine-BSA conjugate. To examine reactivity of MAbs, each IgG was added to each well of a 96 well-immunoplate coated with the Δ^1-THCA-BSA. Various concentrations of each IgG were added to each well of a 96 well-immunoplate coated with 200 ng/ml of Δ^1-THCA-BSA.

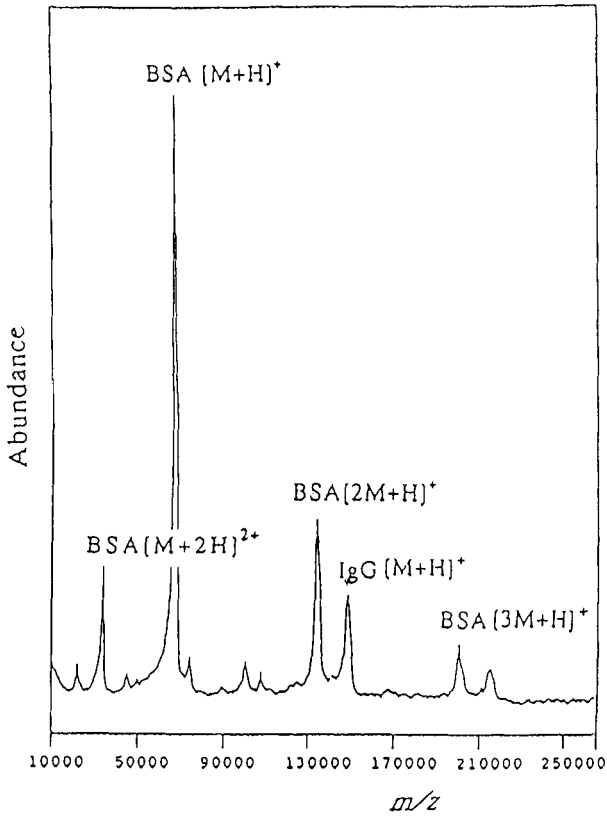


Fig.2 Analysis of purified IgG reactive to tetrahydrocannabinolic acid by co-matrix-assisted laser desorption/ionization mass spectrometry. $[M+H]^+$ and $[M+2H]^{2+}$ are single and double protonated molecule of BSA added as an internal standard and IgG, respectively.

Protein A affinity column chromatography. The purity of IgG was confirmed by the co-MALDI-mass spectrometry using BSA as an internal standard (Fig.2) as previously described (12). The molecular weight was 148,600 in good agreement with that of human IgG1 being determined as 146,000 (22). Furthermore, since only minor

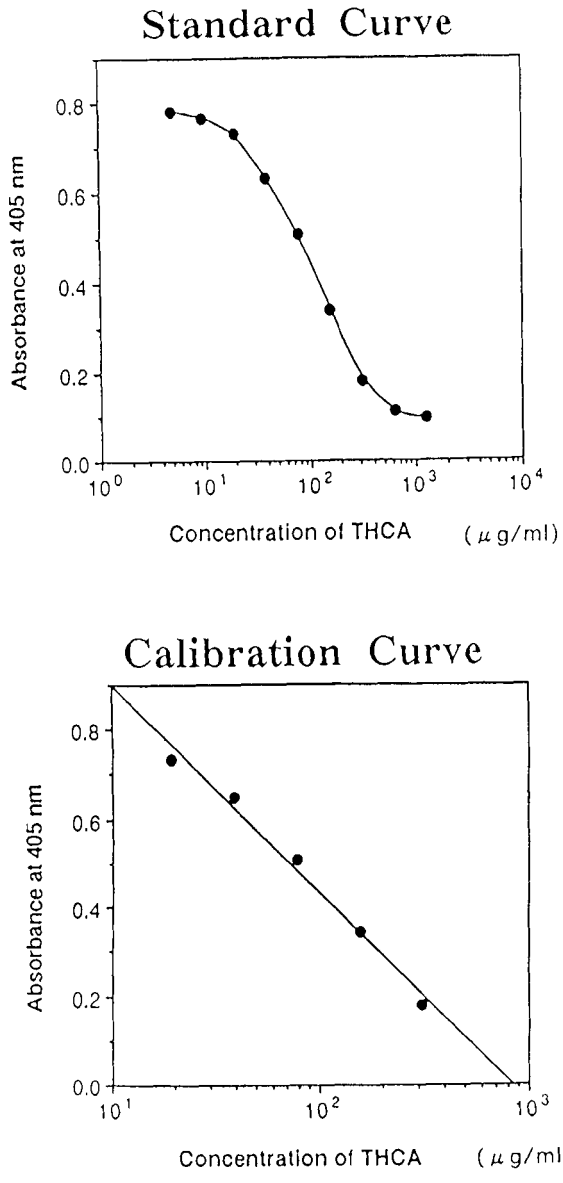


Fig.3 Calibration curve of Δ^1-THCA. Various concentrations of Δ^1-THCA were incubated with MAb in the pre-blocked plate, and incubated with Δ^1-THCA-BSA. The mixture was again incubated with peroxidase-labeled anti-mouse IgG. Absorbance was measured at 405 nm.

TABLE 2

Cross-reactions of monoclonal antibody against cannabinoids and related compounds

Compound	Cross-reactivity(%)
Δ^1-THCA	100
Δ^6-THCA	55
CBD	523
CBDA	93
CBDA diacetate	0
CBGA	1600
CBGV	391
CBGO	630
CBNA	174
CBLA	100
CBCA	138
Me-olivetolate	103
olivetol	17
cannabispirol	100

The cross-reactivities of cannabinoids and related compounds were determined according to Weiler's equation (Weiler et al. 1980; see MATERIALS AND METHODS section)

molecular peaks were observed except for those related to IgG, it became evident that the purification of IgG using protein A was good.

Competitive ELISA and cross-reactivity against cannabinoids and related compounds

The measuring range of the competitive ELISA extended from 10 µg/ml to 10^3 µg/ml of Δ^1-THCA (Fig. 3). The cross reactivities of related cannabinoids were determined according to Weiler's equation (23) and are summarized in Table 2.

The ELISA was not specific for Δ^1-THCA and showed cross-reactivities with various cannabinoids (Fig. 4), but did not react with other naturally occurring phenolics such as flavonoids and anthraquinones (Table 2). This wide cross-reactivity is the major advantage of the antibody reagent used in this ELISA.

Since CBGA, CBNA, CBDA and CBLA that the type of terpene moiety was different individually, and methylolivetolate which possessed no terpene moiety, were all reactive to MAb, the terpene moiety was not immunized. CBGV and CBGO had different cross-reactivities. Therefore, alkyl moiety was related to immunization. As we reported, marihuana contains spiro-compounds such as acetyl cannabispirol, cannabispirol, cannabispirone and cannabispirenone that have the same basic skeleton with cannabinoids (Fig.4) (19). One of them, cannabispirol, was assayed and resulted in almost the same reactivity as Δ^1-THCA (Table 2). From these results, the metabolites having 2'-hydroxyl, 6'-hydroxyl or 6'-*O*-alkyl, 4'-alkylbenzene ring moiety can be detected by this ELISA.

Many metabolites of cannabinoids have been found (1-5). These studies were concentrated almost entirely on pharmacological active THC. Nearly 70 metabolites of THC analogs have been found. However, only 7-oxo-Δ^6-THC and 7-hydroxyl-Δ^6-THC were determined as the major metabolites when Δ^6-THC was injected in animals (3). Since we have been having difficulties in obtaining such urine samples, we confirmed that two major metabolites, 7-oxo-Δ^6-THC and 7-hydroxyl-Δ^6-THC (Fig. 5) had clear reactivities (393 and 69% against Δ^1-THCA, respectively) for this ELISA. Thus, this newly established ELISA might be applied for the first screening for the urine samples of marihuana users.

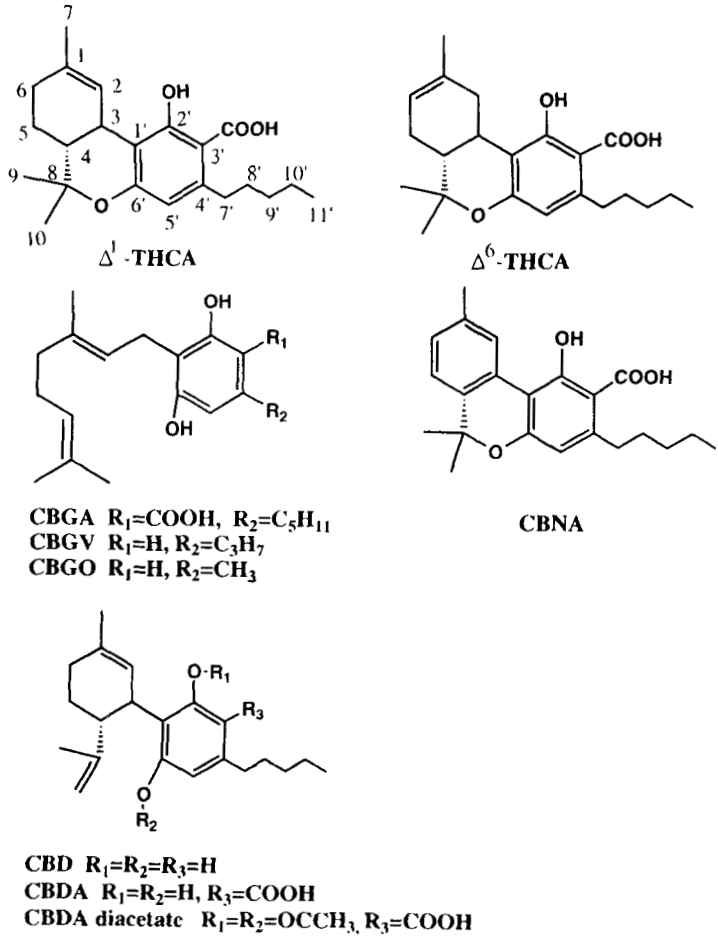
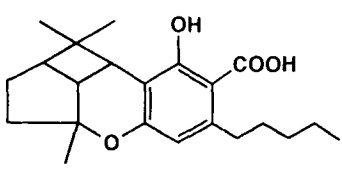
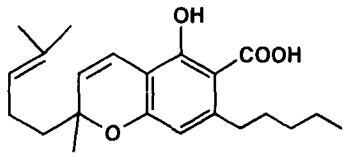


Fig.4 Structures of cannabinoids, cannabispirol, methyl olivetorate and olivetol.

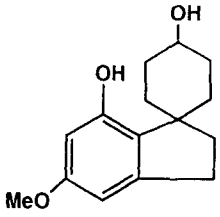
The marihuana samples and the fresh *Cannabis* leaf can be detected and distinguished from other plant species by this ELISA without any analytical instruments such as GC and HPLC (data not shown). Moreover, it is difficult to detect cannabinoids in old marihuana samples by usual methods such as GLC, HPLC and TLC



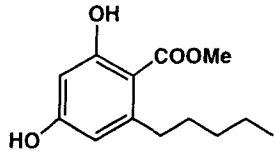
CBLA



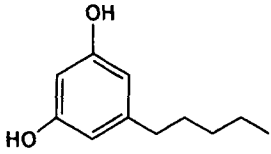
CBCA



cannabispirol



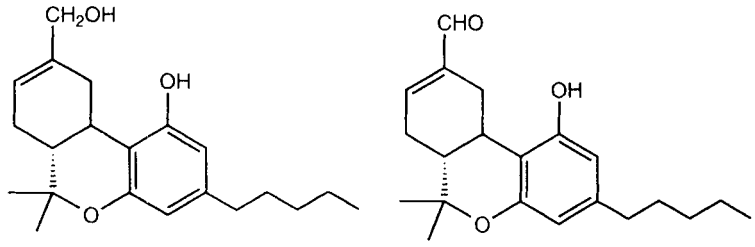
Me-olivetolate



olivetol

Fig. 4. Continued

because almost all cannabinoids are labile and decomposed by exposure to light (16), free radical oxidation (24), auto-oxidation (25), and/or high temperature (17) during storage. Spiro compounds are, however, stable compared to cannabinoids and were positive in the ELISA (Table 2). Thus, this newly established ELISA may be helpful for the analysis of old marihuana samples.



7-OH- Δ^6 -THC **7-oxo- Δ^6 -THC**

Fig.5 Structures of two major metabolites of Δ^6-THC by animals.

Application of ELISA for biotransformation experiments

In a previous paper, we reported the biotransformation of CBN into its glucoside and hydroxylated glucoside (8). Therefore, CBDA was incubated with *P. ternata* tissue segments for a novel application of this newly established ELISA. The biotransformation products of CBDA were analyzed by HPLC and several peaks detected (Fig.6). Individual peaks were assayed by ELISA and the reactivities of Fr.2, 5, 6, 7, 8 and 9 were 32, 27, 45, 65, 63 and 49% , respectively, compared to the control assay.

CA 1 and CA 2 contained in Fr. 2 and Fr. 6, respectively, were analyzed by FAB-MS and ^{13}C-NMR spectrometries. The negative FAB-MS spectrum of CA 1 showed a peak at (*m/z*): 519[M-H]$^-$, indicating a molecular weight of 520, which corresponds to an addition of a glucose moiety to CBDA. This was supported by a ^{13}C NMR spectrum of CA 1 (data not shown).

The negative FAB-MS spectrum of CA 2 indicated a peak at 535 that corresponds to an addition of a hydroxyl group to CA 1. The ^{13}C-NMR specrum of CA 2 (data not shown) was in good agreement with the result of FAB-MS analysis. From these results CA 1 and

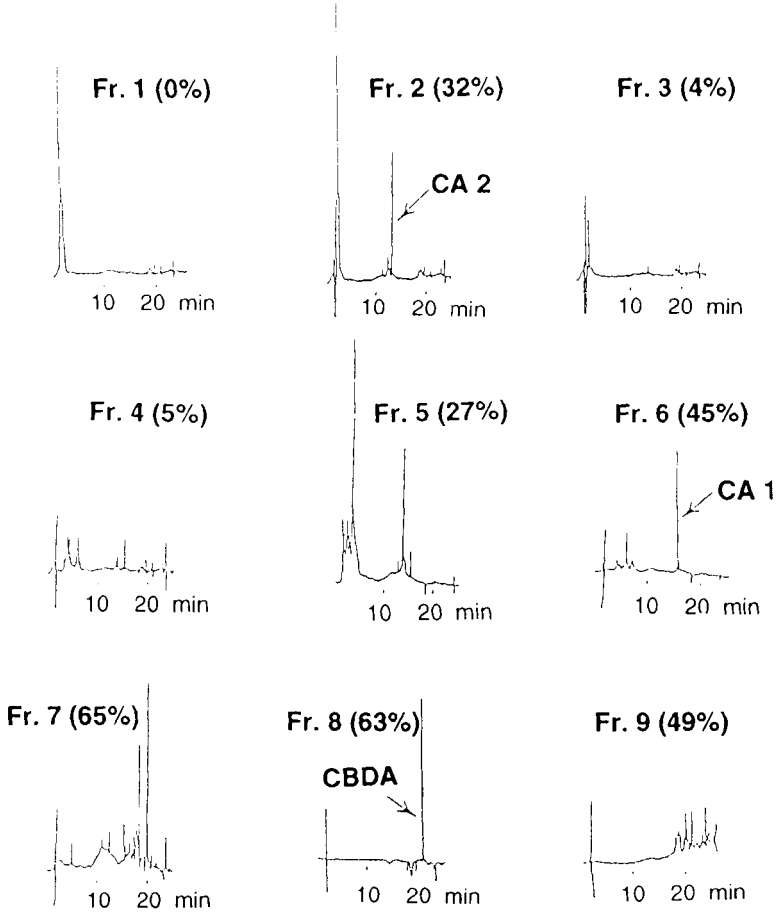
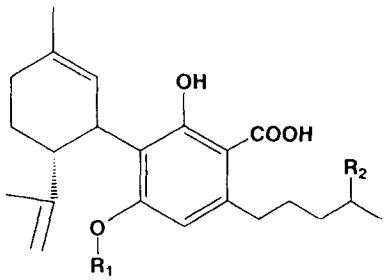


Fig.6 Separation of converted products of CBDA by HPLC. CBDA was incubated with the tissue mass suspension of *Pinellia ternata* in MS medium supplemented with 2,4-D on a shaker (60 rpm) at 25 °C under a 16 hr photoperiod. Tissue masses were dried, powdered and extracted with MeOH. The extractives were separated by HPLC. The HPLC was performed by a Cosmosil 5C18-MS column and a variable wave length detector (268 nm). The mobile phase was a gradient acetonitrile-H_2O mixture (20% to 100%) containing 5 mM phosphoric acid. The flow rate was 1.0 ml/min.



CA 1 R_1=Glc
CA 2 R_1=Glc, R_2=OH

Fig.7 Structures of cannabinoids converted by *Pinellia ternata* tissue mass

CA 2 were elucidated to be CBDA-monoglucoside and hydroxyl-CBDA-monoglucoside as indicated in Fig. 7, respectively.

Fr.8 was identified as CBDA by the comparison with the authentic sample by UV and FAB-MS spectra, although Fr.5 and Fr.7 were difficult to identify because they were present at very low concentrations.

In order to find new probes for the cannabinoid receptor, two biotransformed products were examined for their binding to CB1 (the cannabinoid receptor found mainly in the brain) according to the methods described by Devane et al. (7), but no significant binding was observed at concentrations lower than 20 μM, although THC showed binding constants of Ki = 46 ± 3 nM.

This is possibly the first report of an ELISA for the detection of marihuana compounds using monoclonal antibody and its application. Highly specific monoclonal antibody against Δ^1-THCA has been investigated. These results will be reported elsewhere.

ACKNOWLEDGMENT

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