



Cannabidiol metabolism revisited: tentative identification of novel decarbonylated metabolites of cannabidiol formed by human liver microsomes and recombinant cytochrome P450 3A4

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Received: 4 February 2019 / Accepted: 13 February 2019 / Published online: 1 March 2019
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Abstract

Purpose The purpose of the present study was to identify the structures of cannabidiol (CBD) metabolites during CO formation by human liver microsomes and human recombinant cytochrome P450 (CYP) enzymes.

Methods CBD was NADPH-dependently metabolized by human liver microsomes and human recombinant CYP enzymes. Less-polar metabolites were analyzed by gas chromatography–mass spectrometry monitoring, and their estimated molecular ions were m/z 286, 358 and 481 after non-derivatization, trimethylsilylation and pentafluorobenzyl oxime formation, respectively.

Results We tentatively identified novel decarbonylated metabolites of CBD as keto-enol tautomers. Among eight major recombinant human CYP enzymes, only CYP3A4 catalyzed the formation of decarbonylated metabolites.

Conclusions CBD was biotransformed to two decarbonylated metabolites, an enol-form (cyclopentadienol structure), and a keto-form (cyclopentenone structure) by human liver microsomes and CYP3A4.

Keywords Cannabidiol · Decarbonylation · Metabolism · Human liver microsomes · CYP3A4 · Cyclopentadienol

Introduction

Cannabidiol (CBD), one of the major phytocannabinoids in cannabis, has a variety of pharmacological effects, such as anticonvulsant, antiemetic, antioxidant, and anxiolytic effects, as well as barbiturate-induced sleep prolongation

[1–3], although this phytocannabinoid lacks psychoactivity, which is associated with its lower affinity for the CB₁ cannabinoid receptor [4]. For medical purposes, Sativex, a 1:1 mixture of Δ^9 -tetrahydrocannabinol and CBD as active ingredients, has been clinically used for the treatment of neuropathic pain and spasticity [5–7]. In addition, several studies have suggested that CBD has various medical benefits against a range of diseases, in particular cancer [8, 9] and drug-resistant epilepsy in children [10, 11].

CBD is metabolized in experimental animals and humans *in vitro*, as well as *in vivo* [12–15]. The primary metabolic reactions of CBD are allylic hydroxylations at the 6- and 7-positions that are catalyzed by cytochrome P450 (CYP) enzymes mainly expressed in the liver. Our previous study showed that CYP3A4 and CYP2C19 have major roles in the metabolism of CBD in human liver microsomes [16]. In earlier studies, we demonstrated that CBD generated CO during mouse liver microsomal metabolism [17, 18]. It was speculated that CO could be formed from CBD by CYP enzymes, in which the phenolic hydroxyl groups of CBD are a possible source for the CO formation, although a counterpart metabolite of CBD after CO formation had not

Shizuo Narimatsu: Deceased on 6 November 2017.

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been detected. In the field of forensic science, it is important to clarify the overall metabolism of CBD. The purpose of the present study was to tentatively identify the structures of CBD metabolites during CO generation by human liver microsomes and human recombinant CYP enzymes.

Materials and methods

Chemicals

CBD was isolated and purified from cannabis leaves by the method of Aramaki et al. [19]. The purity of CBD was confirmed to be more than 98% by gas chromatography (GC). NADP, NADPH, glucose 6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Oriental Yeast Co. (Tokyo, Japan); *N,O*-bis-(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane and pentafluorobenzylhydroxylamine (PFBHA) hydrochloride were from Wako Pure Chem. Co. (Osaka, Japan). Other chemicals and solvents used were of the highest quality commercially available.

Enzyme sources

Pooled human liver microsomes (HLMs) and microsomes from baculovirus-infected insect cells expressing human CYP enzymes (CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 with cytochrome b5) were purchased from BD Gentest (Woburn, MA, USA).

CBD metabolism

HLMs (20 µg protein) or human recombinant CYP enzymes (10 pmol) were used as enzyme sources. The incubation mixture consisted of 32 µM CBD, an enzyme source, an NADPH-generating system (0.5 mM NADP, 10 mM glucose 6-phosphate, 10 mM magnesium chloride, and 1 unit of glucose-6-phosphate dehydrogenase) or 2 mM NADPH, and 100 mM potassium phosphate buffer (pH 7.4) to make a final volume of 0.5 mL. The mixture was incubated at 37 °C for 30 min (or 60 min for recombinant CYP enzymes) and then extracted with 4 mL of *n*-hexane after addition of 0.5 mL 1 M monopotassium phosphate. Two additional incubations without enzyme sources or the NADPH-generating system were processed in the same way. A portion of the *n*-hexane extract was evaporated to dryness, and dissolved in 50 µL dry acetonitrile for direct gas chromatography–mass spectrometry (GC–MS) analysis. The remaining *n*-hexane extract was derivatized by trimethylsilylation as described previously [16, 20] or by pentafluorobenzyl oxime formation using the method of Kobayashi et al. [21].

Gas chromatography–mass spectrometry

GC–MS was conducted on a 7890B GC system (Agilent Technologies, Santa Clara, CA, USA), equipped with a DB5MS capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm; Agilent Technologies) and a JEOL JMS-Q1500 mass spectrometer (JEOL Ltd., Akishima, Japan). The GC–MS conditions were as follows: injection mode 1-µL sample injection and splitless; injection port temperature 250 °C; carrier gas He; flow rate 1 mL/min; column temperature 50 °C (1-min hold), 25 °C/min (5 min), 10 °C/min and 300 °C (8-min hold); ion source temperature 180 °C; interface temperature 240 °C; ionization conditions 70 eV in full scan mode (50–650 amu); emission current 100 µA. Estimated molecular ions of CBD metabolites were monitored at *m/z* 286 for non-derivatization, at *m/z* 358 for trimethylsilylation and at *m/z* 481 for PFBHA derivatization. Under these conditions, the retention times of the underivatized CBD and the trimethylsilyl (TMS) derivative of CBD were 13.33 and 11.44 min, respectively.

Results and discussion

The proposed metabolites of CBD could be tentatively identified as molecular ions of CBD decarbonylated metabolites. As shown in Fig. 1a, two peaks with retention times at 10.90 min (M-1) and 11.00 min (M-2) appeared in the extracted ion chromatogram (XIC) recorded by extracted ion monitoring of an estimated molecular ion at *m/z* 286. The retention times of M-1 and M-2 were earlier than that of the parent compound, CBD (13.33 min). M-1 (Fig. 1b) and M-2 (Fig. 1c) showed similar mass spectra, with a molecular ion at *m/z* 286 and typical fragment ions at *m/z* 203 and 134. The fragment ion at *m/z* 203 ($M^+ - 83$) is based on a similar fragmentation to that of CBD [22, 23], which is produced by retro Diels–Alder cleavage of the terpene ring and a methyl radical [23]. The base peak at *m/z* 134 is produced by the release of a CBD limonene moiety. These results probably indicate that CBD decarbonylation occurred in its olivetol moiety, as postulated by our earlier studies [17, 18], and that M-1 and M-2 seem to have similar structures, suggesting they are isomers of each other. When HLMs or the NADPH-generating system was omitted from the incubation mixture and processed in the same way, M-1 and M-2 were not observed on the XIC (data not shown).

Mass shifts and fragmentations by chemical derivatization can provide extremely useful information for the identification of unknown metabolites. After trimethylsilylation of the CBD reaction products, the peak at 10.90 min

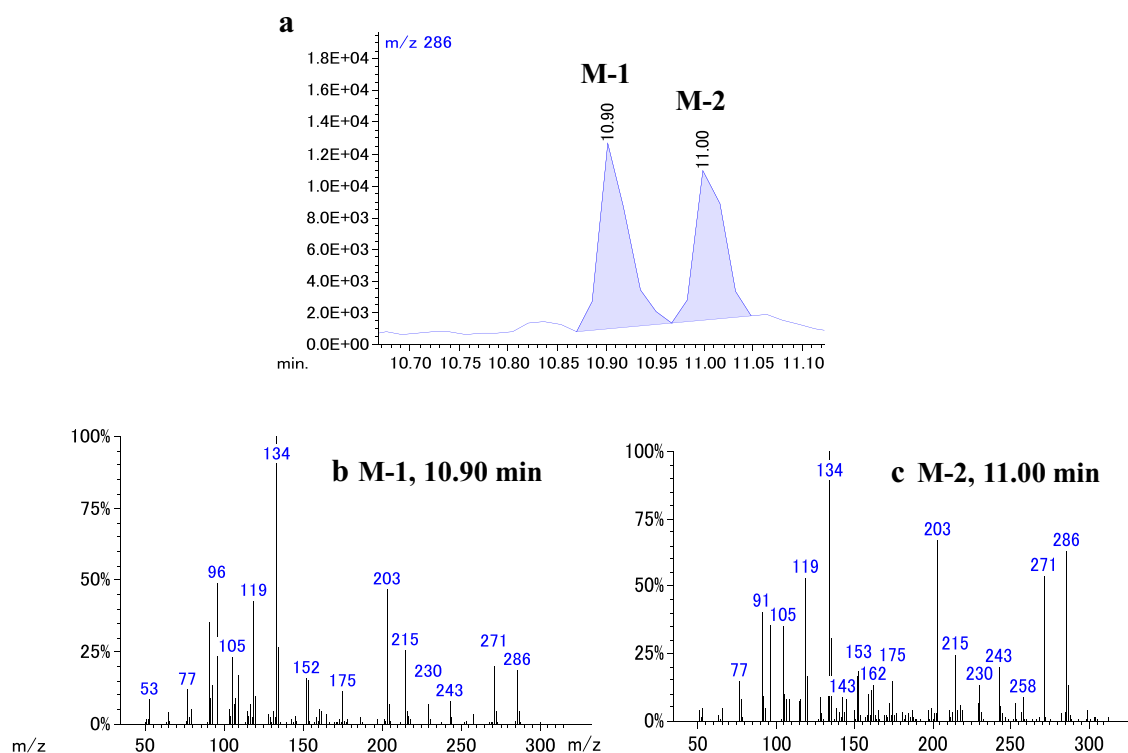


Fig. 1 Extracted ion chromatogram (XIC) and mass spectra of decarbonylated metabolites of cannabidiol (CBD) formed by human liver microsomes (HLMs) in the underivatized forms. **a** XIC at m/z 286

showing M-1 and M-2 peaks, **b, c** mass spectra for M-1 and M-2 peaks, respectively

disappeared (Fig. 2a), and then a new peak at a retention time of 10.16 min appeared on the XIC recorded by extracted ion monitoring of an estimated molecular ion of a TMS derivative at m/z 358 (Fig. 2b). The mass spectrum of the peak showed a molecular ion at m/z 358 and a base peak at m/z 290 (Fig. 2c), which was a similar fragmentation pattern to TMS derivatives of CBD and their monohydroxy metabolites reported previously [13, 23]. In contrast, M-2 was not derivatized by trimethylsilylation, and was present at the same retention time (Fig. 2a). These results indicate that the structure of M-1 has one hydroxyl group, and M-2 has no hydroxyl group, although both metabolites have the same molecular weight.

In the pyrolysis of phenol and dihydroxy benzene derivatives, CO is known to be a major final product, and cyclopentadiene, cyclopentadienol, and cyclopentenone were formed as intermediate products through fulvenone radical species [24, 25]. Considering these chemical findings and the data in Figs. 1 and 2, the possible structures of M-1 and M-2 are proposed to include keto-enol isoforms of cyclopentadienol and cyclopentenone-type structures. This possibility was further supported by an additional derivatization with PFBHA to form an oxime. PFBHA reacts with carbonyl functional groups, but not hydroxyl groups, to produce the corresponding oximes, which have been analyzed by

GC–MS [21, 26]. CBD metabolites formed by HLMs were processed with PFBHA, and analyzed by GC–MS using extracted ion monitoring of its estimated molecular ion of the M-2 oxime derivative at m/z 481. As shown in Fig. 3a, two main peaks appeared on the chromatogram at 14.23 and 14.87 min, both of which had a molecular ion at m/z 481 and a base peak at m/z 347, and a loss of the limonene moiety ($M^+ - 134$) (Fig. 3b, c). In PFBHA derivatization, it is well known that two *E/Z* oxime isomers are produced from a carbonyl compound, and are observed as double chromatographic peaks with the second peak generally much more prominent than the first peak [26].

In earlier studies, we suggested the possible role of CYP enzymes in CO formation during CBD metabolism by mouse liver microsomes [17, 18]. The CO formation was significantly inhibited by the addition of typical CYP inhibitors including SKF 525-A [17]. Among eight major recombinant human CYP enzymes (CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) examined in the present study, only CYP3A4 exhibited catalytic activity for the formation of decarbonylated metabolites of CBD. None of microsomes expressing other seven CYP enzymes and control baculosomes showed substantial formation of the decarbonylated metabolites. In GC–MS analysis of reaction products by CYP3A4, a similar

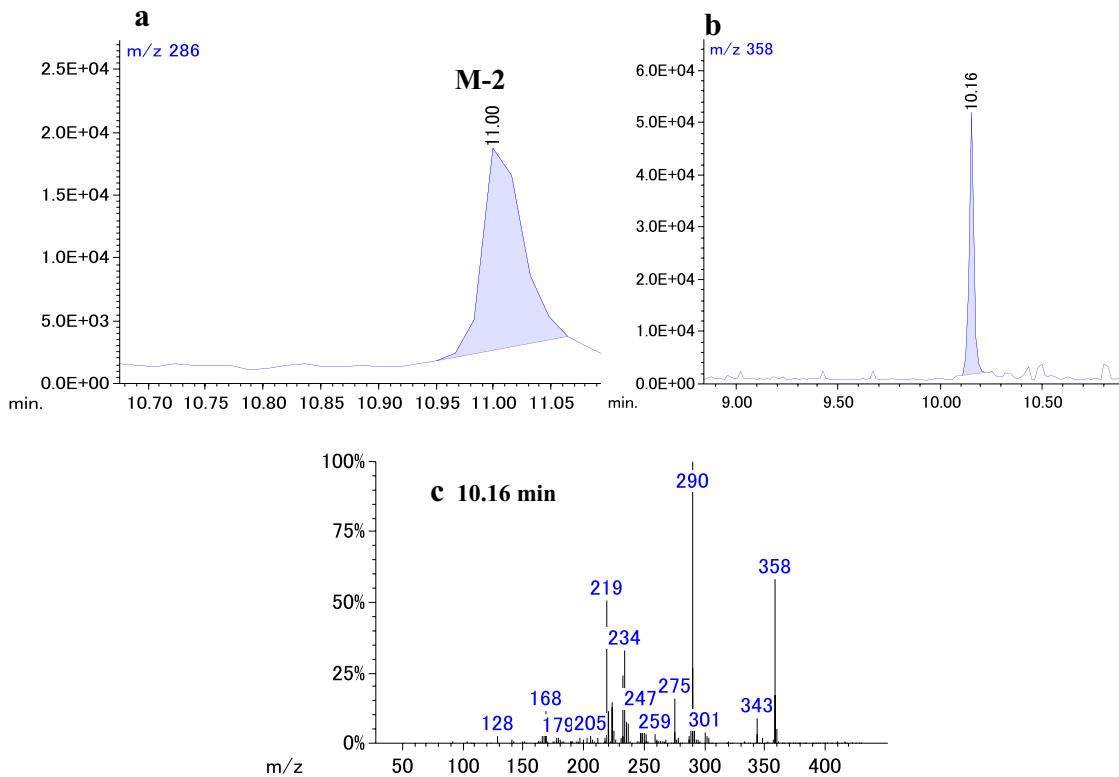


Fig. 2 XICs and the mass spectrum of the CBD metabolites after trimethylsilylation. **a** XIC without derivatization at m/z 286, **b** XIC after trimethylsilylation at m/z 358, **c** mass spectrum of the CBD metabolite after trimethylsilylation

mass chromatogram and mass spectra to those of M-1 and M-2 in Fig. 1 were observed as shown in Fig. 4. By trimethylsilylation and PFBHA oxime derivatization, the same mass shifts and fragmentations as those in Figs. 2 and 3 were also observed for two decarbonylated metabolites formed by human recombinant CYP3A4 (data not shown). These results strongly suggest that CYP3A4 is the main enzyme for the decarbonylation of CBD by HLMs.

Conclusions

In this study, we tentatively identified two less-polar metabolites of CBD formed with human liver microsomes and CYP3A4 as shown in Fig. 5. Both metabolites are formed from CBD by decarbonylation, and are present in keto-enol tautomers. Two metabolites, an enol-form and a keto-form, could be analyzed by GC–MS as a TMS derivative and PFBHA oximes, respectively (Fig. 5). This is the first report

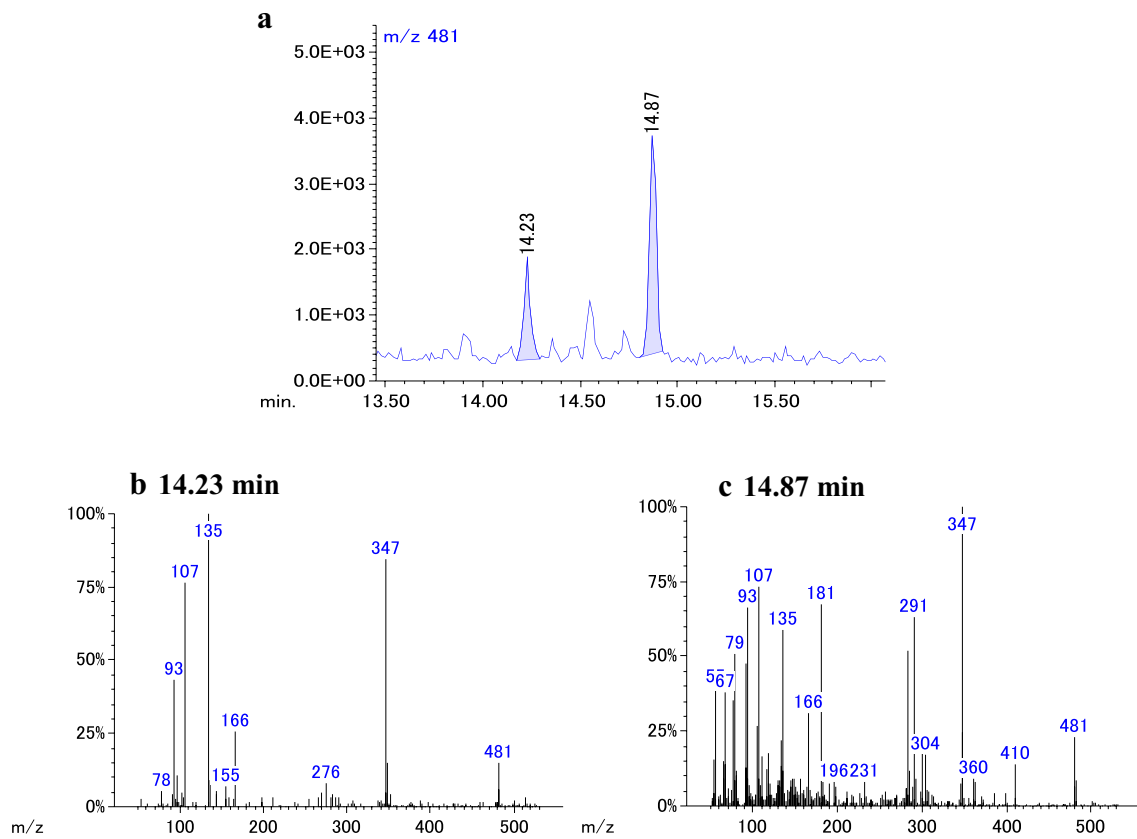


Fig. 3 XIC and the mass spectra of pentafluorobenzylhydroxylamine (PFBHA) derivatives of CBD metabolites formed by HLMs

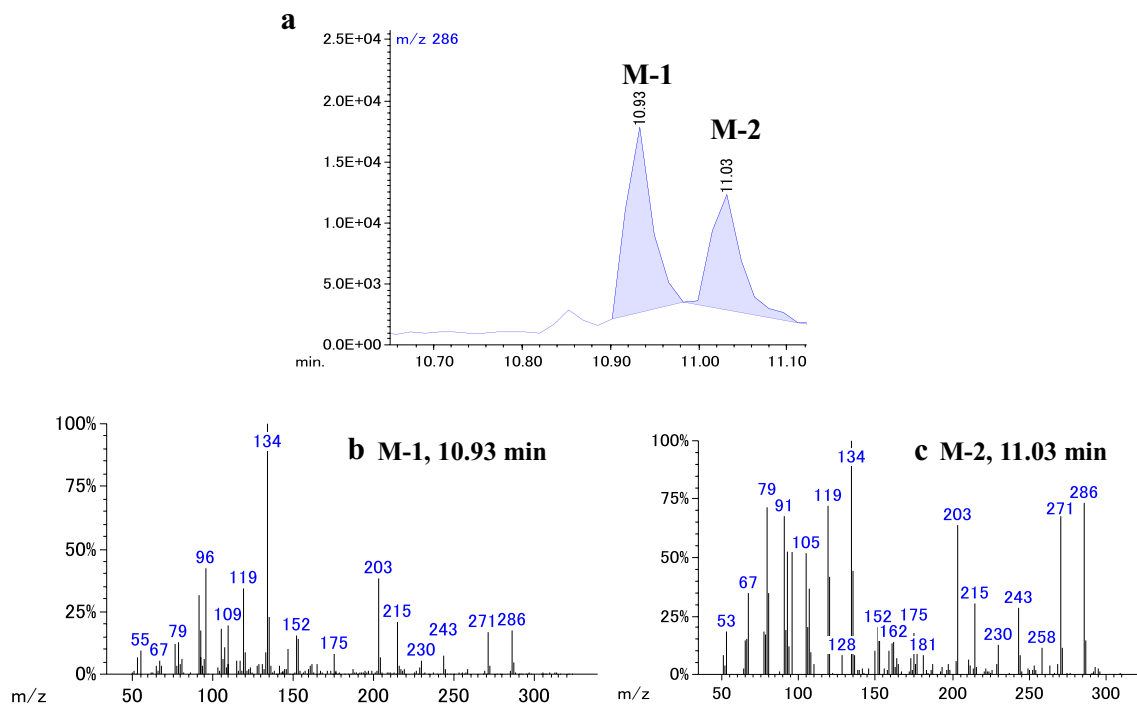
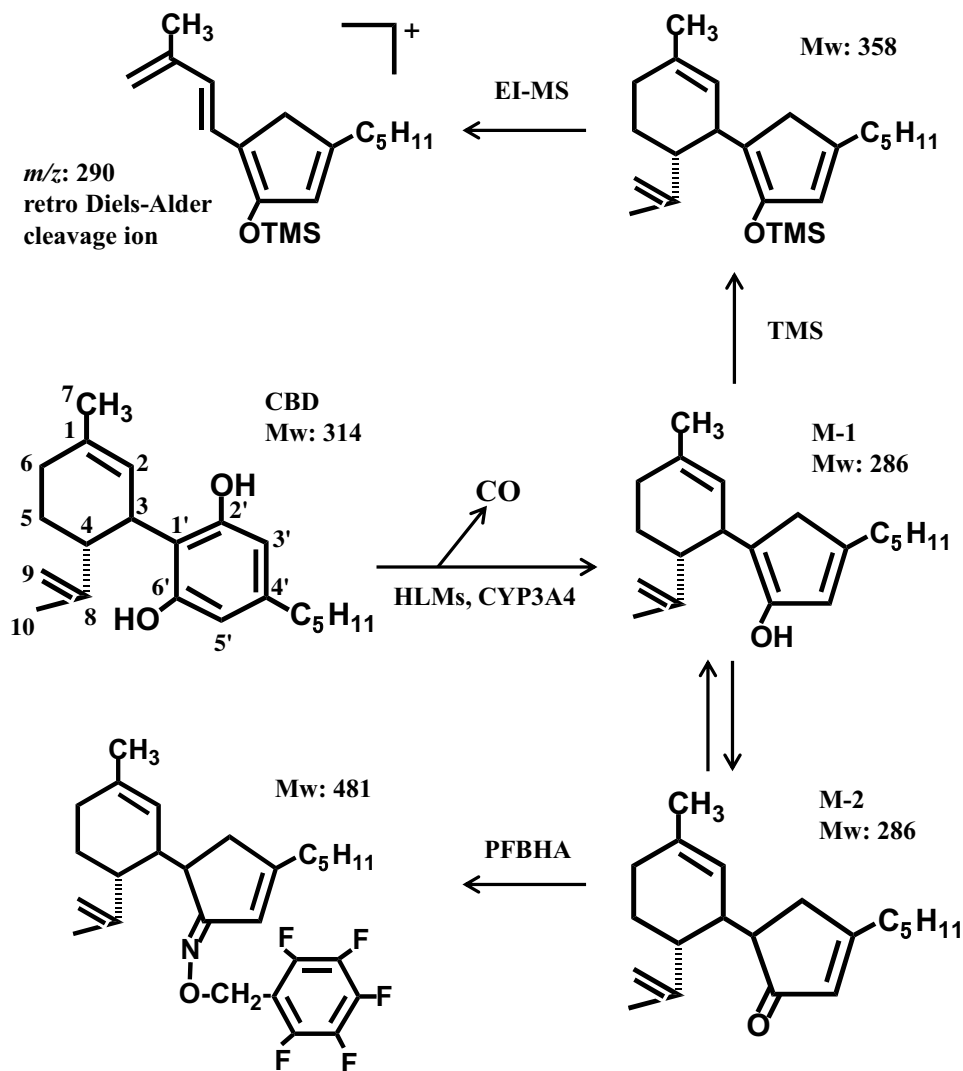


Fig. 4 XIC and mass spectra of decarbonylated metabolites of CBD formed by recombinant human cytochrome P450 3A4 (CYP3A4)

Fig. 5 Decarbonylation of CBD by HLMs and CYP3A4, and derivatizations of CBD metabolites. *EI-MS* electron ionization mass spectrometry, *TMS* trimethylsilyl



on the metabolic decarbonylation catalyzed by the human CYP enzyme, and further studies are needed to clarify the significance of this metabolic reaction of CBD *in vivo*, as well as the precise mechanism of the unique catalytic reaction by CYP3A4.

Acknowledgements This work was supported by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The authors thank Mr. Yuji Hasegawa for GC–MS measurements.

Compliance with ethical standards

Conflict of interest There are no financial or other relations that could lead to a conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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