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Highlights

- The fungus *Cunninghamella elegans* can metabolise JWH-018, JWH-073 and AM2201
- The majority of the metabolites was produced via phase I metabolism
- The fungal metabolites were found to be in good agreement with the human metabolites

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Biotransformation of synthetic cannabinoids JWH-018, JWH-073 and AM2201 by *Cunninghamella elegans*

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Running head: Metabolism of JWH-018, JWH-073 and AM2201

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Abstract

Being marketed as "legal" smoking blends or mixtures, synthetic cannabinoids are abused widely owing to its cannabis-like effect. Due to the rapid introduction of new generation analogues of synthetic cannabinoids to escape from legislative/judicial control, the investigation of the metabolic pathways of these substances is of particular importance for drug control, abstinence and forensic toxicology purposes. In this study, the *in vitro* metabolism of JWH-018, JWH-073 and AM2201 by the fungus *Cunninghamella elagans* has been investigated with the purpose of validating its potential as a complementary model for investigating synthetic cannabinoid metabolism. JWH-018, JWH-073 and AM2201 were incubated for 72 h with *C. elegans*. Detection of metabolites was based on liquid chromatography – tandem mass spectrometry and high resolution mass spectrometry analysis. *C. elegans* was found capable of producing the majority of the phase I metabolites observed in earlier *in vitro* and *in vivo* mammalian studies as a result of monohydroxylation, dihydroxylation, carboxylation, dehydrogenation, ketone formation, dihydrodiol formation, dihydrodiol formation with *N*-dealkylation and combinations thereof. *C. elegans* can thus be a useful and economic model for studying synthetic cannabinoid metabolism.

Keywords: Synthetic cannabinoids, metabolism, *Cunninghamella elegans*, JWH-018, JWH-073, AM2201

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Introduction

Abusive consumption of synthetic cannabinoids that are agonists at cannabinoid CB_1 receptors has been commonly reported since 2008 [1]. Usually sold as herbal blends or research chemicals in powder, synthetic cannabinoids mimic psychoactive effects of cannabis. However, unlike cannabis some synthetic cannabinoids are reported to be full agonists and thus create more serious public health issues [2]. When a synthetic cannabinoid is scheduled due to increased prevalence and health concerns, new molecules with similar or even stronger psychoactive effects are synthesized by slight structural modifications to bypass the laws [3, 4]. The lack of metabolism data of these new psychoactive molecules together with the lack of reference standards has made optimized detection in biological matrices, especially urine, difficult.

Due to rapid changes in product composition and continuous emergence of new compounds, identifying the unique fingerprint of drug metabolites is of vital importance for forensic-toxicological, clinical-toxicological and doping analysis. Several *in vivo* and *in vitro* models are being used to identify metabolites of synthetic cannabinoids. *In vivo* studies involving the researcher himself administering drug have been reported [5]. Despite the reliability of data obtained from such experiments, the adverse effects of these drugs are unknown and due to both health and ethical reasons it is difficult to perform *in vivo* human excretion studies to investigate the metabolism.

Other *in vivo* animal systems such as mouse, rat, and primates can be used as alternatives to human administration in metabolism studies. Unfortunately these models suffer from a number of limitations such as ethical constraints, cost of experimental models, time that must be spend on breeding animals etc. [6]. Moreover species differences in the excretory pathway may make the extrapolation from experimental animals to humans difficult [7]. Recently *in vivo* chimeric mouse models based upon the transplantation of primary human hepatocytes in uPA-SCID mice that suffer from a transgene-induced liver disease have been

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evaluated. This model proved to be an efficient alternative for human administration studies for the investigation of steroid metabolism and was recently used to reveal both phase I and phase II metabolism of synthetic cannabinoids, JWH-200 and JWH-122 [8-10]. Although the chimeric mouse is a promising model with respect to the array of metabolic pathways, the cost and complexity involved in the development of such a model with a high level of hepatocyte repopulation, low amount of urine that can be collected and the concentrated mouse urine matrix are some inherent limitations [10, 11].

In vitro platforms like perfused liver, hepatocytes or human liver microsomes are other valuable models for the elucidation of drug metabolism [6]. In particular, primary human hepatocytes give the closest metabolic profile of a drug to that of *in vivo* human and are hence considered as the 'gold standard' for predicting *in vivo* metabolic pathways of drugs [12]. Metabolic defects, restricted accessibility to suitable liver samples, unsuitability for quantitative estimations, inability of the cells to proliferate, quick degradation of P450 enzyme activities during culture and the requirement for specific culturing conditions are limitations of these *in vitro* models [10, 13].

The concept of using microorganisms, and in particular *Cunninghamella elegans*, as models of mammalian metabolism has been well documented [14-16]. It has been proved that *Cunninghamella* has CYP509A1 enzymes that are synonymous to that involved in xenobiotic detoxification in mammals [17] and can metabolize a wide variety of xenobiotics in a regio- and stereo-selective manner similar to mammalian enzyme systems [14, 15]. A recent review on *C. elegans* reports that the fungus shows similarities with mammalian metabolism for a wide variety of drugs, more than a hundred of them [15]. It is highly efficient in its production of metabolites, especially from antidepressants, antibiotics, steroids, alkaloids and related drugs [15]. The cultures of the fungus can be grown by transferring to new agar plates without complexity adding to advantages of the model [18].

The aim of the study was to elucidate the metabolite profile of (1-pentyl-1H-indol-3-

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yl)-1-naphthalenyl-methanone (JWH-018), (1-butyl-1H-indol-3-yl)-1-naphthalenyl-methanone (JWH-073) and [1-(5-fluoropentyl)-1H-indol-3-yl]-1-naphthalenyl-methanone (AM2201), three amino alkyl indoles with well-defined metabolic profiles, using the *C. elegans* model and to compare with previously reported *in vivo* and *in vitro* data to examine the potential of this model [19-23].

Materials and Methods

Chemicals

JWH-018 and JWH-073 were synthesized in-house following previously reported methods and characterized by mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy with no evidence of cross contamination [24, 25]. AM2201 (purity 99.4%) was obtained from the National Measurement Institute (North Ryde, NSW, Australia). Reference standards of JWH-018 metabolites namely JWH-018 *N*-(4-hydroxypentyl), JWH-018 *N*-(5-hydroxypentyl) and JWH-018 *N*-pentanoic acid and JWH-073 metabolites namely JWH-073 *N*-(3-hydroxybutyl), JWH-073 *N*-(4-hydroxybutyl) and JWH-073 *N*-butanoic acid were obtained from PM separations (Capalaba, QLD, Australia). Reagent grade dichloromethane and LC grade acetonitrile and methanol were obtained from Chemsupply (Gilman, SA, Australia). Potato dextrose agar, glucose, peptone, and yeast extract were purchased from Oxoid Australia (Adelaide, SA, Australia).

Microbial Culture and Biotransformation Conditions

Cultures of *C. elegans* ATCC 10028b (Cryosite Ltd, South Granville, NSW, Australia) were propagated on potato dextrose agar plates [26] at 27 °C for 5 days. The mycelia from five plates were then transferred to 20 mL of sterile physiological saline solution and homogenized for 5 min. Approximately 3 mL aliquots of the homogenate were used to inoculate 250 mL Erlenmeyer flasks containing 100 ml of growth media. The cultures were

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incubated for 48 h at 26 °C on an Infors HT Multitron rotary shaker (*In vitro* Technologies, Noble Park North, VIC, Australia) operating at 180 rpm. After 48 h, 10 mg of JWH-018, JWH-073 or AM2201 dissolved in 0.5 mL of methanol was added to the culture and incubated for further 72 h [18]. Control experiments consisted of cultures without cannabinoids and flasks containing only media and cannabinoid [27, 28].

Extraction, Isolation, and Identification of Metabolites

After 72 h of incubation, the contents of each flask, including the controls, were filtered through Buchner funnel into a separating funnel and extracted with three aliquots of dichloromethane (3 × 50 mL). The combined organic extracts were evaporated to dryness under vacuum at 40 °C using a Buchi rotary evaporator (*In vitro* Technologies, Noble Park North, VIC, Australia) and placed under high vacuum to remove traces of moisture. The residue was dissolved in acetonitrile to prepare 1 mg/mL stock solution and was filtered through 0.22 µM syringe filter before analysis. Cannabinoid parent drugs and metabolites were chromatographically separated using an Agilent Zorbax Eclipse XDB-C18 analytical column (150 × 4.6 mm, 5 µm). Mobile phases consisted of 0.1 % formic acid in water (mobile phase A) and acetonitrile (mobile phase B). The gradient used consisted of 30 % B (0 to 2 min), linear gradient from 30 % B to 50 % B (2 to 5 min), 50 % B to 90 % B (5 to 30 min, hold for 5 min) and 90 % B to 30 % B (35 to 40 min) run at 0.4 mL/min. MS data were acquired on an Agilent 6490 Triple Quadrupole mass spectrometer with an electrospray ionization source (ESI) source (positive ion mode), interfaced with an Agilent 1290 LC system. Samples prepared were injected in 2 µL volume to obtain full scan and product ion scan spectra. Product ion scan experiments were conducted on precursor ions that were presumed to be metabolites based on the comparison of full scan spectra of the samples and controls. A fragmentor voltage of 380 V with discrete collision energy of 10, 20, 30 and 40 eV (for product ion scan) was applied. The scanning mass range was set at *m/z* 100-1000 (scan

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time = 500 ms). The sheath gas temperature and flow were set to 250 °C and 11 L/min, respectively. The capillary and nozzle voltages were 3000 V and 1500 V, respectively.

High resolution quadrupole Time-of-Flight mass spectrometry (HRQToFMS) experiments were carried out on an Agilent 6510 Accurate Mass QToF Mass Spectrometer, equipped with ESI source operated in positive ion mode, in order to determine accurate masses of the metabolites. The LC system and conditions used were the same as above. The following operation parameters were used: injection volume 2µL (full scan) and 10 µL (product ion scan); capillary voltage 3500 V; nebulizer pressure 40 psi (275790 Pascal); drying gas 10.0 L/min; gas temperature 350 °C; fragmentor voltage 160 V; collision energy 10, 20 and 40 eV; skimmer voltage 60 V. HRQToFMS accurate mass spectra were recorded across the range from *m/z* 100 to *m/z* 1000. The mass axis was calibrated using the mixture provided by the manufacturer over the *m/z* 50-3200 range. A second orthogonal sprayer with a reference solution was used as a continuous calibration using the following reference masses: *m/z* 121.0509 and *m/z* 922.0098. The chromatographic conditions and column used were same as described above. The controls were subjected to the same analysis. Analysis of the chromatographic and mass spectrometric data was conducted using MassHunter Workstation Software Qualitative Analysis (version B.06.00, Agilent). Peaks present in the fungus sample, but not in the controls, were manually identified and their fragmentation patterns and accurate masses were examined to identify the metabolites. The signal-to-noise ratio of all the identified metabolites was greater than 5.

Results

Fig. 1 shows the product ion mass spectra of JWH-018, JWH-073 and AM2201 and their fragmentation patterns. These fragmentation patterns were used as a basis for determining the structures of fungal metabolites of each drug as follows. The presence of a product ion at *m/z*

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155 in a mass spectrum of a metabolite indicates that the naphthalene moiety of the metabolite has not been altered. If the ion at *m/z* 155 is absent, it suggests that the naphthalene moiety has undergone modification such as hydroxylation, dihydroxylation or dihydrodiol. The type of modification was deduced by the difference between the amu of the product ion of the parent drugs, i.e. *m/z* 155, and that of the product ion of the metabolite such as *m/z* 171 or *m/z* 189. A difference of 16 amu (*m/z* 171 – *m/z* 155) indicates an addition of an oxygen atom and hence the metabolite is considered to have undergone hydroxylation. Similarly, a difference of 34 amu (*m/z* 189 – *m/z* 155) suggests a formation of dihydrodiol. Product ions originating from other parts of metabolites, such as indole moiety or alkyl side chain, were analysed in the same way to deduce the whole structures of metabolites. The product ion mass spectra and their corresponding proposed fragmentation patterns of the most abundant metabolite for each drug (Ma15 for JWH-018, Mb13 for JWH-073 and Mc25 for AM2201) are shown in Fig. 1, illustrating the application of the above described approach in metabolite identification.

Overlaid extracted ion chromatograms of all the metabolites for each drug are shown in Fig. 2. The majority of phase I metabolites after incubation with *C. elegans* were oxidation products for all three drugs. In particular, hydroxylation was the most common transformation. Phase II metabolites due to glucosidation and sulfation were only detected for AM2201. The chromatograms of the control cultures without cannabinoids showed no metabolites or substrate present; those of the control flasks containing media and cannabinoid showed only the presence of the substrate. Metabolite identification was further supported by accurate mass data obtained from the high resolution quadrupole Time-of-Flight mass spectrometry analysis. These data were presented in Tables 1, 3, and 5 in Ref [29].

JWH-018

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Twenty one phase I metabolites (Ma1 – Ma21) were detected (Fig. 2A). Table 1 in Ref [29] lists all metabolites with suggested biotransformation, retention time, observed accurate mass, formula and major product ions. Table 2 in Ref [29] tabulates the key diagnostic product ions and their tentative structures used in elucidating the biotransformation pathways. Hydroxylation, dihydroxylation, carboxylation, dihydrodiol formation, dehydrogenation, ketone formation, combinations of some of these transformations, and dihydrodiol formation with *N*-dealkylation were observed. Phase II metabolites were not detected. Proposed metabolic pathway is given in Fig. 3. Hydroxylation and dihydroxylation were the most common transformations. The presence of the metabolites JWH-018 *N*-(4-hydroxypentyl) (Ma15), JWH-018 *N*-(5-hydroxypentyl) (Ma14) and JWH-018 *N*-pentanoic acid (Ma13) was confirmed by comparison with reference standards on retention time and product ion spectrum. The peak to the left of Ma13 in (a) has a *m/z* 372, but was found not to be a carboxylic acid metabolite based on the product ions. Mass errors of all metabolites in comparison with the calculated exact mass of proposed structures were all within 1.6 ppm. Comparison of metabolites obtained from *C. elegans* incubation in the present study with those obtained from human urine, human liver microsomes and rat urine samples in the literature is shown in Table 1. The majority of human urine metabolites reported in the literature were detected in the fungus sample including hydroxy and carboxy metabolites.

JWH-073

The metabolic transformation of JWH-073 was similar to that of JWH-018 except that there were fewer (seventeen, Mb1-Mb17) metabolites detected. Table 3 in Ref [29] lists all metabolites with suggested biotransformation, retention time, observed accurate mass, formula and diagnostic product ions. Table 4 in Ref [29] tabulates the key diagnostic product ions and their tentative structures used in elucidating the biotransformation pathways.

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Hydroxylation, dihydroxylation, carboxylation, dihydrodiol formation, dehydrogenation, ketone formation, combinations of some of these transformations, and dihydrodiol formation with *N*-dealkylation were observed. Phase II metabolites were not detected. Proposed metabolic pathway is given in Fig. 4. Hydroxylation was the most common transformation. The presence of the metabolites JWH-073 *N*-(3-hydroxybutyl) (Mb13), JWH-073 *N*-(4-hydroxybutyl) (Mb12) and JWH-073 *N*-butanoic acid (Mb11) was confirmed by comparison with reference standards on retention time and product ion spectrum. Mass errors of all metabolites in comparison with the calculated exact mass of proposed structures were all within 2.6 ppm. Comparison of metabolites obtained from *C. elegans* incubation in the present study with those obtained from human urine and human liver microsomes samples in the literature is shown in Table 2. Hydroxylation (human urine and human liver microsomes) and carboxylation (human urine) were reported in the literature and both were observed for fungus metabolites.

AM2201

The biotransformation of AM2201 was similar to the other two drugs, but produced extra metabolites including phase II metabolites. Forty eight phase I and II metabolites (Mc1-Mc48) were detected. Table 5 in Ref [29] lists all metabolites with suggested biotransformation, retention time, observed accurate mass, formula and diagnostic product ions. Table 6 in Ref [29] tabulates the key diagnostic product ions and their tentative structures used in elucidating the biotransformation pathways. Hydroxylation, dihydroxylation, trihydroxylation, oxidative defluorination, dihydrodiol formation, ketone formation, *N*-dealkylation, defluorination and demethylation were observed either alone or in combination. Glucosidation of hydroxy and dihydroxy metabolites and sulfation of dihydroxy metabolites were detected as Phase II transformation. Although the retention of the sulfate

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metabolites on the C18 column used appears unusually long, similar observations were reported previously when the sulfate conjugates had a longer retention than the unconjugated metabolites [28]. In contrast, glucuronidation was not observed. This lack of detection of glucuronides is less likely due to the extraction conditions as the similarly polar glucoside metabolites were successfully extracted and detected. Fig. 5 depicts the proposed metabolic pathways of AM2201 by *C. elegans*. Hydroxylation and dihydroxylation were the most common transformations. The presence of JWH-018 *N*-(5-hydroxypentyl) (Mc34) and JWH-018 *N*-pentanoic acid (Mc33) metabolites was confirmed by comparison with reference standards on retention time and product ion spectrum. Mass errors of all metabolites in comparison with the calculated exact mass of proposed structures were all within 1.4 ppm. Comparison of metabolites obtained from *C. elegans* incubation in the present study with those obtained from human urine, human postmortem heart blood, human liver microsomes and rat urine samples in the literature is shown in Table 3. The majority of human urine metabolites reported in the literature were detected in the fungus sample including JWH-018 *N*-(5-hydroxypentyl), JWH-018 *N*-pentanoic acid and AM2201 hydroxy metabolites.

AM2201 is a fluorinated analogue of JWH-018 at the terminal carbon of the pentyl side chain. Hence, in addition to the types of transformation observed for JWH-018 and JWH-073 metabolism, oxidative defluorination to form a terminal hydroxy or carboxy group was a common reaction.

Discussion

The metabolic transformation of JWH-018, JWH-073 and AM2201 by *C. elegans* was similar to one another as they are closely related structural analogues. The types of metabolic transformation detected were identical for JWH-018 and JWH-073, and they are carboxylation, dehydrogenation, dihydrodiol formation, dihydrodiol with hydroxylation,

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dihydrodiol with ketone formation, dihydrodiol with *N*-dealkylation, dihydroxylation, and hydroxylation, ketone formation, ketone formation with hydroxylation. AM2201 showed all the transformation except carboxylation, dehydrogenation, and ketone formation with hydroxylation. AM2201 underwent additional transformation including defluorination and oxidative defluorination, unique to compounds with a fluorine atom, as well as phase II metabolism including glucosidation and sulfation. Due to oxidative defluorination, some of the AM2201 metabolites are the same as JWH-018 metabolites: Mc4 and Ma2, Mc33 and Ma13, Mc34 and Ma14. Mc23 and Mc28 also matched well with Ma6 and Ma8, respectively, on both retention times and fragmentation patterns. Mc21, on the other hand, was a structural isomer of Ma7. The number of metabolites and metabolic transformation observed for AM2201 was higher than that for JWH-018 and JWH-073, likely because AM2201 has a fluorine atom enabling additional metabolic pathways.

Comparison of the fungus metabolites from the present study with human metabolites reported in the literature shows good agreement. Out of the twelve kinds of human urine metabolites reported in the literature for JWH-018, eight metabolite types were also detected among the *C. elegans* metabolites (Table 1), including the major human urine metabolites, namely *N*-(4-hydroxypentyl), *N*-pentanoic acid and *N*-(5-hydroxypentyl) metabolites. Minor human urine metabolites [30] such as demethylation with carboxylation (JWH-073 *N*-butanoic acid), dihydrodiol formation with dihydroxylation, *N*-dealkylation with hydroxylation and trihydroxylation metabolites were not observed using this model.

For JWH-073, all the reported human urine and human liver microsomes metabolites, i.e. hydroxylated and carboxylated metabolites, were observed in the *C. elegans* sample (Table 2). Compared to JWH-018 metabolites, very few have been reported as human urine or human liver microsomes metabolites. This may be because the detection of only the major metabolites were possible as the concentration of JWH-073 in the herbal products is usually low [31]. In any case, the fungus demonstrated the ability to produce the major human

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metabolites.

Among the ten human urine or human postmortem heart blood metabolites reported for AM2201, six metabolites were generated by the fungus (Table 3). Most importantly, these include the major human urine metabolites, JWH-018 *N*-(5-hydroxypentyl) and JWH-018 *N*-pentanoic acid metabolites [5], as well as JWH-018 *N*-(5-hydroxypentyl)-dihydrodiol, AM2201 hydroxy, AM2201 dihydroxy and AM2201 dihydrodiol metabolites. The four human urine metabolites that were not found in the fungus sample are *N*-dealkylation, JWH-018 *N*-(4-hydroxypentyl), JWH-073 *N*-butanoic acid and JWH-073 *N*-(4-hydroxybutyl) metabolites. The disagreement between the fungus and the human urine metabolites, however, may not be as significant. While JWH-018 *N*-(4-hydroxypentyl) metabolite was detected by Jang et al. [32], Hutter et al. [5] did not find its presence in authentic urine samples nor in self-administered urine samples. This may be such that JWH-018 *N*-(4-hydroxypentyl) metabolite detected by Jang et al. might come from unreported consumption of JWH-018 by the drug abusers. Also, despite the fact that JWH-073 metabolites were not observed in the present study, the fungus produced JWH-073 itself, suggesting the possibility that it can produce JWH-073 *N*-butanoic acid and JWH-073 *N*-(4-hydroxybutyl) metabolites if incubated longer. With regards to human postmortem heart blood metabolites, it is interesting to note that the reported metabolites are JWH-018 *N*-(5-hydroxypentyl), JWH-018 *N*-pentanoic acid and AM2201 *N*-(4-hydroxypentyl) metabolites and that the former two metabolites are the most abundant as with human urine metabolites [33]. Therefore, the fungus metabolites of AM2201 were consistent with not only human urine but also human postmortem heart blood metabolites.

Previously, the detection of JWH-018 itself as a metabolite of AM2201 has not been reported although JWH-018 metabolites, such as JWH-018 *N*-(5-hydroxypentyl) and JWH-018 *N*-pentanoic acid, derived from oxidative defluorination have been reported. If JWH-018 can be formed as a metabolite by humans, it would present difficulty in distinguishing

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AM2201 abusers from those who ingested both JWH-018 and AM2201. However, in a self-experiment where one of the authors ingested pure AM2201, the absence of JWH-018 *N*-(4-hydroxypentyl) metabolite, a major human metabolite of JWH-018, was reported in both serum and urine samples [5]. This suggests that oxidative defluorination is the only pathway to JWH-018 metabolites in humans and that if JWH-018 is formed at all, the concentration is likely to be so low that it has virtually no effects on production of its metabolites.

Unlike JWH-018 and JWH-073, phase II metabolites of AM2201 were also detected in the fungus sample. However, glucuronides, which were determined to be the major phase II human urine metabolites [34], were not observed. Instead, glucosides and sulfates were found. A previous study on drug metabolism by *C. elegans* showed that glucosides and sulfates were formed for the drugs whose main phase II human and equine metabolites are sulfates and glucuronides [28]. Although *C. elegans* has been reported to have the ability to produce glucuronides [35], its capacity to produce glucuronides may be limited and therefore the fungus is not suitable for producing the human phase II metabolites of synthetic cannabinoids, whose main phase II metabolites are reported to be glucuronides [34, 36]. The cause for more extensive metabolism observed for AM2201 is unknown.

In the present study, the incubation time was not optimised to obtain the best metabolic profiles that mimic human metabolism in terms of the quantity (measured by peak area) of the metabolites formed. Under the conditions reported here, however, JWH-018 *N*-(4-hydroxypentyl) metabolite (Ma15) and JWH-073 *N*-(3-hydroxybutyl) metabolite (Mb13) were found to be the most abundant metabolite for JWH-018 and JWH-073, respectively, consistent with human metabolism. On the other hand, carboxy metabolites (Ma13, Mb11), were present in a relatively lower amount in the fungal system. With AM2201, the most abundant metabolite was a dihydrodiol metabolite (Mc25), while the abundance of human metabolites JWH-018 *N*-(5-hydroxypentyl) (Mc34) and JWH-018 *N*-pentanoic acid (Mc33) was relatively low. Two limitations for using peak area as the quantity of the metabolites in

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these experiments should be noted: different metabolites may have significant differences in extraction recoveries resulting in a different ratio of metabolites observed by mass spectrometry from the actual ratio in the sample and mass spectrometric responses can be different for each metabolite due to different ionisation efficiencies.

Metabolism study is important not only for drug testing purposes by Police and hospital scientists but also for understanding pharmacology of the new designer drugs. However, currently the identification of metabolites of synthetic cannabinoids is largely relying on the analysis by mass spectrometry and thus the exact structures of metabolites are inconclusive without the use of reference standards. Analysis of metabolites by nuclear magnetic resonance spectroscopy after isolating them will provide more concrete structural elucidation, but this is usually limited by the low amount of metabolites obtained by the common models such as human hepatocytes, human liver microsomes or rats. With *C. elegans*, it is easy to scale up the incubation and hence obtain a large amount of metabolites [15], indicating the potential for isolation and purification of new synthetic cannabinoid metabolites for NMR analysis.

Conclusion

Cunninghamella elegans produced a large number of metabolites of JWH-018, JWH-073 and AM2201. Although the fungus cannot be reliable to produce phase II metabolites consistent with humans, it has demonstrated its ability to form the reported major human phase I metabolites of the investigated synthetic cannabinoids. Therefore, the fungus has the potential to be used as a complementary model to predict and characterise human metabolites of new synthetic cannabinoids.

Declaration:

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There is no conflict of interest to declare.

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Figure legends

Fig. 1. Product ion spectra (CE 20 eV) and the proposed fragmentation pathways for JWH-018, JWH-073, AM2201 and their most abundant metabolites (Ma15, Mb13 and Mc25).

Fig. 2. Overlaid extracted ion chromatograms of all the metabolites identified in the fungus sample of JWH-018 at *m/z* 306, 340, 356, 358, 372, 374, 376, 390, 392 (**a**); JWH-073 at *m/z* 306, 326, 342, 344, 358, 360, 362, 376, 378 (**b**); and AM2201 at *m/z* 306, 328, 342, 358, 372, 374, 376, 388, 392, 394, 408, 410, 426, 472, 538, 554 (**c**). Only the major metabolites are labelled for AM2201 (**c**).

Fig. 3. Proposed metabolic pathway of JWH-018 in *C. elegans*.

Fig. 4. Proposed metabolic pathway of JWH-073 in *C. elegans*.

Fig. 5. Proposed metabolic pathway of AM2201 in *C. elegans*. Parentheses indicate intermediate metabolites that were not detected in the study.

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Table 1

Metabolites of JWH-018 after *C. elegans* incubation in comparison with metabolites from other sources (human urine, human liver microsomes and rat urine) reported in literature, in alphabetical order. Square brackets in the metabolites column indicate the corresponding fungal metabolites in Fig. 3.

Metabolites	*CE*[*]	HU[*]	HLM[*]	RU[*]
Carboxylation [Ma13]	√	√ [20, 23, 30, 37-39]	√ [19]	
Dehydrogenation [Ma21]	√		√ [19]	
Demethylation + carboxylation (JWH-073 *N*-butanoic acid)		√ [30]		
Dihydrodiol formation [Ma12]	√	√ [37, 39]	√ [19]	
Dihydrodiol formation + dihydroxylation		√ [37]	√ [19]	
Dihydrodiol formation + hydroxylation [Ma2, Ma3]	√	√ [37, 39]	√ [19]	
Dihydrodiol formation + ketone formation [Ma4, Ma5]	√			
Dihydrodiol formation + *N*-dealkylation [Ma1]	√	√ [37]	√ [19]	
Dihydroxylation [Ma6 – Ma9, Ma11]	√	√ [20, 23, 37, 39]	√ [19]	
Hydroxylation [Ma14 – Ma16, Ma19, Ma20]	√	√ [20, 23, 30, 37-39]	√ [19, 23]	
Ketone formation [Ma17, Ma18]	√	√ [37]	√ [19]	
Ketone formation + hydroxylation [Ma10]	√	√ [37]	√ [19]	
N-dealkylation			√ [19]	√ [20]
N-dealkylation + hydroxylation		√ [20, 37, 39]	√ [19]	√ [20]
Trihydroxylation		√ [23, 37]	√ [19]	

[*] Abbreviations: *CE*, *Cunninghamella elegans*; HU, human urine; HLM, human liver microsomes; RU, rat urine.

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Table 2

Metabolites of JWH-073 after *C. elegans* incubation in comparison with metabolites from other sources (human urine and human liver microsomes) reported in literature, in alphabetical order. Square brackets in the metabolites column indicate the corresponding fungal metabolites in Fig. 4.

Metabolites	*CE*[*]	HU[*]	HLM[*]
Carboxylation [Mb11]	√	√ [38]	
Dehydrogenation [Mb17]	√		
Dihydrodiol formation [Mb8]	√		
Dihydrodiol formation + hydroxylation [Mb2, Mb3]	√		
Dihydrodiol formation + ketone formation [Mb4]	√		
Dihydrodiol formation + *N*-dealkylation [Mb1]	√		
Dihydroxylation [Mb5 – Mb7]	√		
Hydroxylation [Mb12, Mb13, Mb15, Mb16]	√	√ [20, 38]	√ [23]
Ketone formation [Mb14]	√		
Ketone formation + hydroxylation [Mb9, Mb10]	√		

[*] Abbreviations: *CE*, *Cunninghamella elegans*; HU, human urine; HLM, human liver microsomes.

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Table 3

Metabolites of AM2201 after *C. elegans* incubation in comparison with metabolites from other sources (human urine, human postmortem heart blood, human liver microsomes and rat urine) reported in literature, in alphabetical order. Square brackets in the metabolites column indicate the corresponding fungal metabolites in Fig. 5.

Metabolites	*CE*[*]	HU[*]	HPHB[*]	HLM[*]	RU[*]
Defluorination (JWH-018) [Mc48]	√				
Defluorination + Demethylation (JWH-073) [Mc47]	√				
Defluorination + hydroxylation (JWH-018 *N*-(4-hydroxypentyl))		√ [32]			
Dihydrodiol formation [Mc25, Mc30]	√	√ [34]		√ [34]	
Dihydrodiol formation + dihydroxylation [Mc9]	√				
Dihydrodiol formation + hydroxylation [Mc5, Mc6]	√				
Dihydrodiol formation + ketone formation [Mc10]	√				
Dihydrodiol formation + *N*-dealkylation [Mc1]	√				
Dihydroxylation [Mc2, Mc3, Mc7, Mc13, Mc16, Mc17, Mc19, Mc22, Mc24, Mc27, Mc29, Mc31, Mc32, Mc38, Mc42, Mc46]	√	√ [34]		√ [34]	
Hydroxylation [Mc12, Mc14, Mc35 – Mc37, Mc39 – Mc41, Mc44, Mc45]	√	√ [5, 32, 34, 40]	√ [33]	√ [33, 34]	√ [32]
Ketone formation [Mc43]	√				
N-dealkylation		√ [34]		√ [34]	
Oxidative defluorination (JWH-018 *N*-(5-hydroxypentyl)) [Mc34]	√	√ [5, 32, 34]	√ [33]	√ [33, 34]	√ [32]
Oxidative defluorination + dihydrodiol formation (JWH-018 dihydrodiol-hydroxy) [Mc4]	√	√ [34]		√ [34]	
Oxidative defluorination + hydroxylation (JWH-018 dihydroxy) [Mc15, Mc18, Mc21, Mc23, Mc28]	√				
Oxidative defluorination to carboxylic acid (JWH-018 *N*-pentanoic acid) [Mc33]	√	√ [5, 32, 34]	√ [33]	√ [33, 34]	√ [32]
Oxidative defluorination to carboxylic acid + decarboxylation + carboxylation (JWH-073 *N*-butanoic acid)		√ [5, 32]			√ [32]
Oxidative defluorination to carboxylic acid + decarboxylation + hydroxylation (JWH-073 *N*-(4-hydroxybutyl)		√ [5]			
Oxidative defluorination to carboxylic acid + hydroxylation [Mc20, Mc26]	√				
Trihydroxylation [Mc8, Mc11]	√				

[*] Abbreviations: *CE*, *Cunninghamella elegans*; HU, human urine; HLM, human liver microsomes; RU, rat urine.

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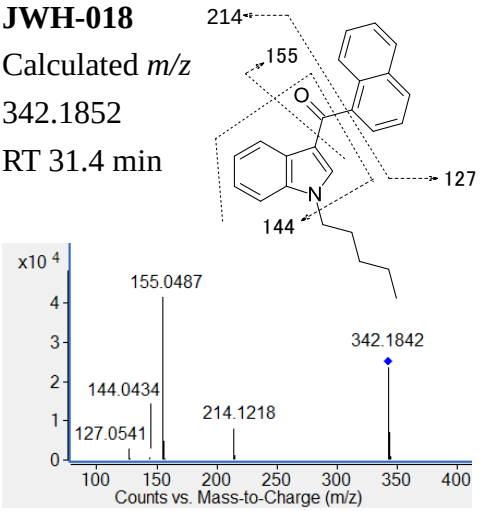
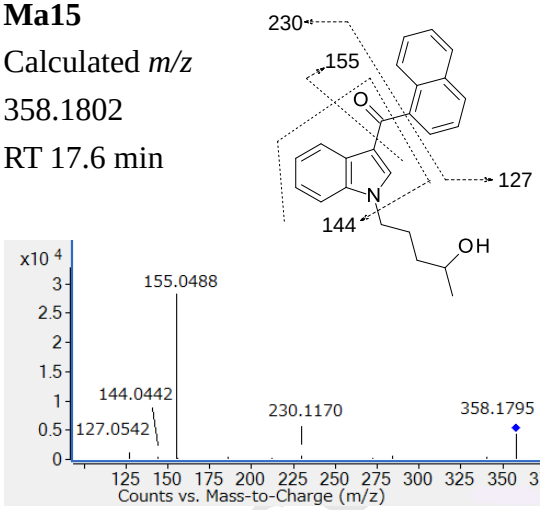
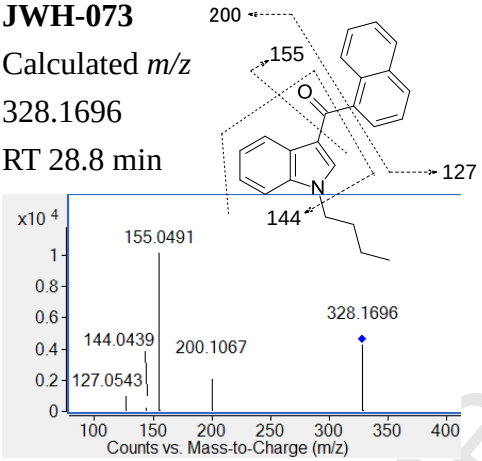
JWH-018
Calculated *m/z*
342.1852
RT 31.4 min



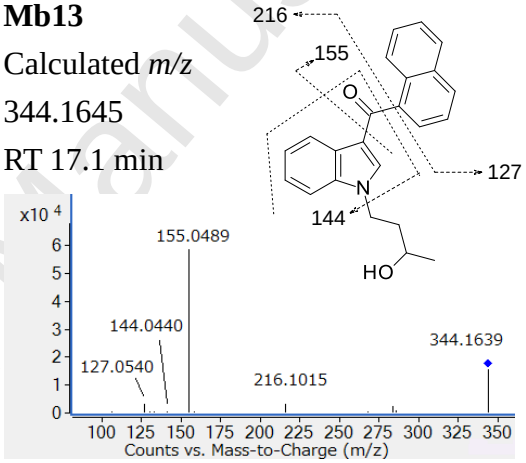
Ma15
Calculated *m/z*
358.1802
RT 17.6 min



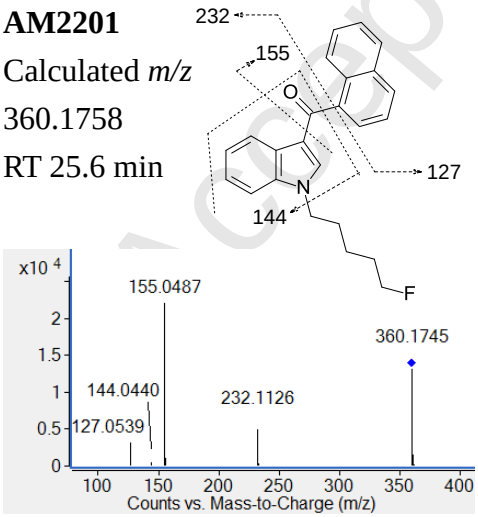
JWH-073
Calculated *m/z*
328.1696
RT 28.8 min



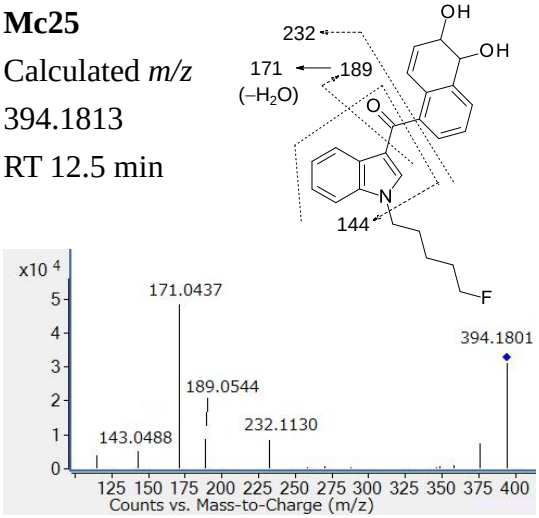
Mb13
Calculated *m/z*
344.1645
RT 17.1 min



AM2201
Calculated *m/z*
360.1758
RT 25.6 min



Mc25
Calculated *m/z*
394.1813
RT 12.5 min



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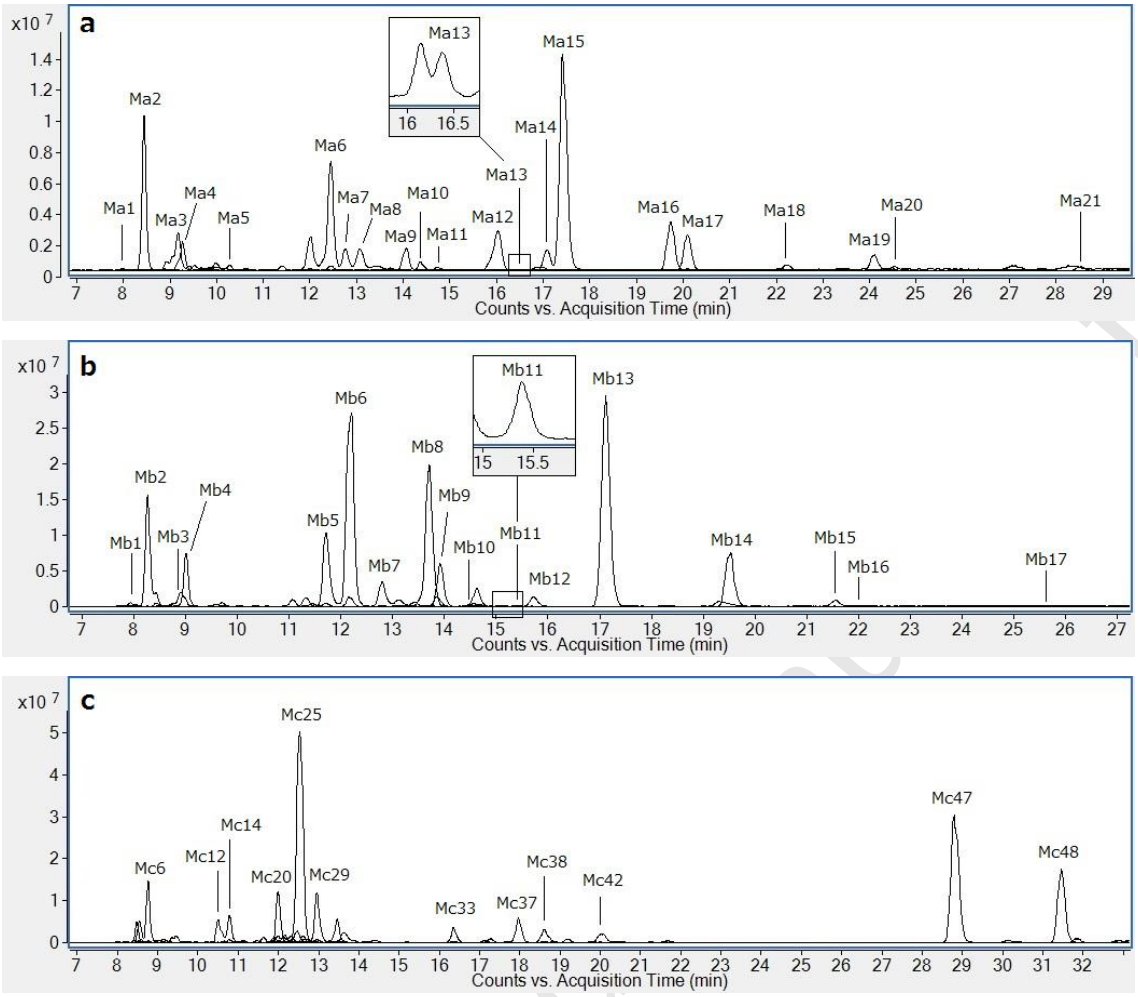

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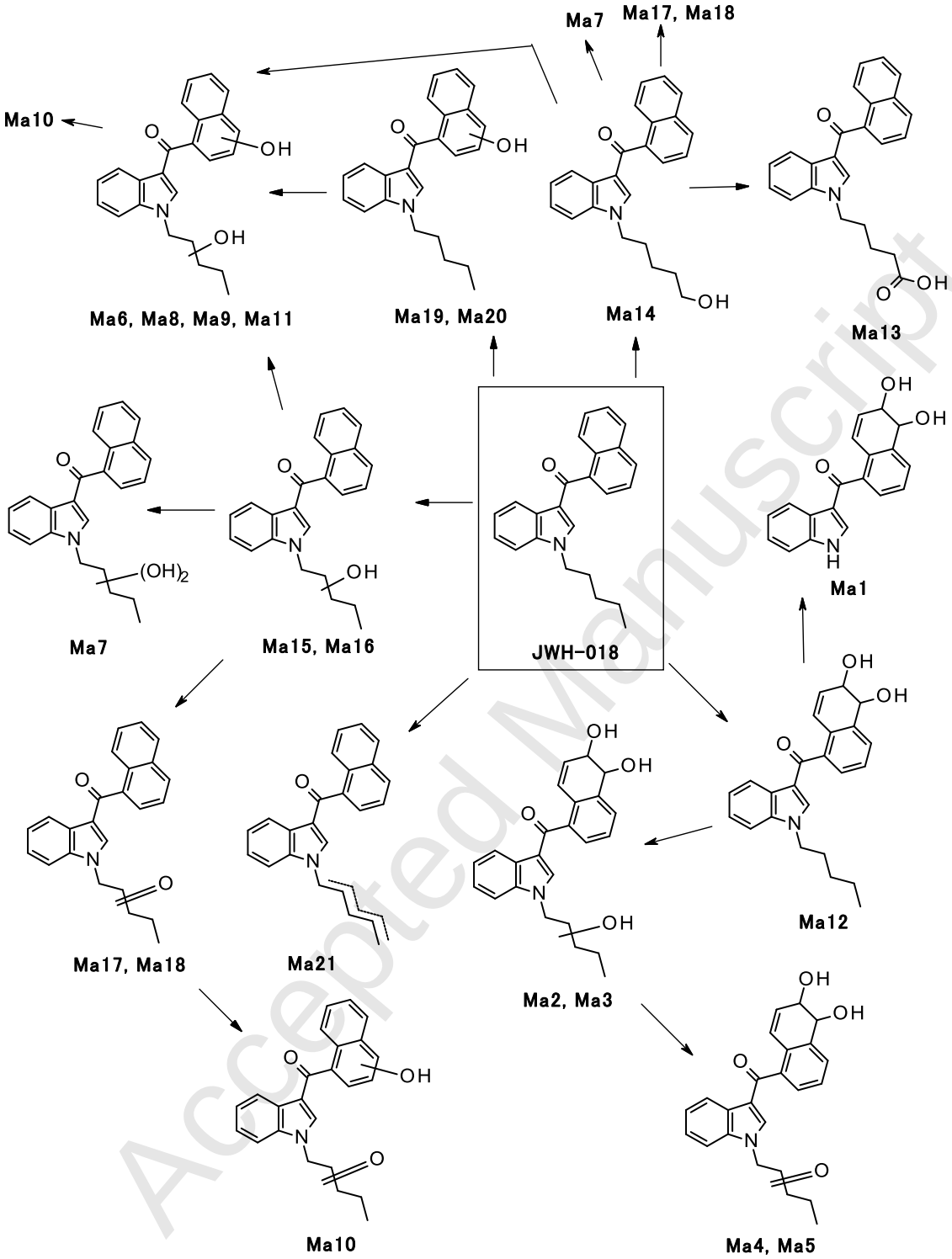

Fig. 3. Proposed metabolic pathway of JWH-018 in *C. elegans*.

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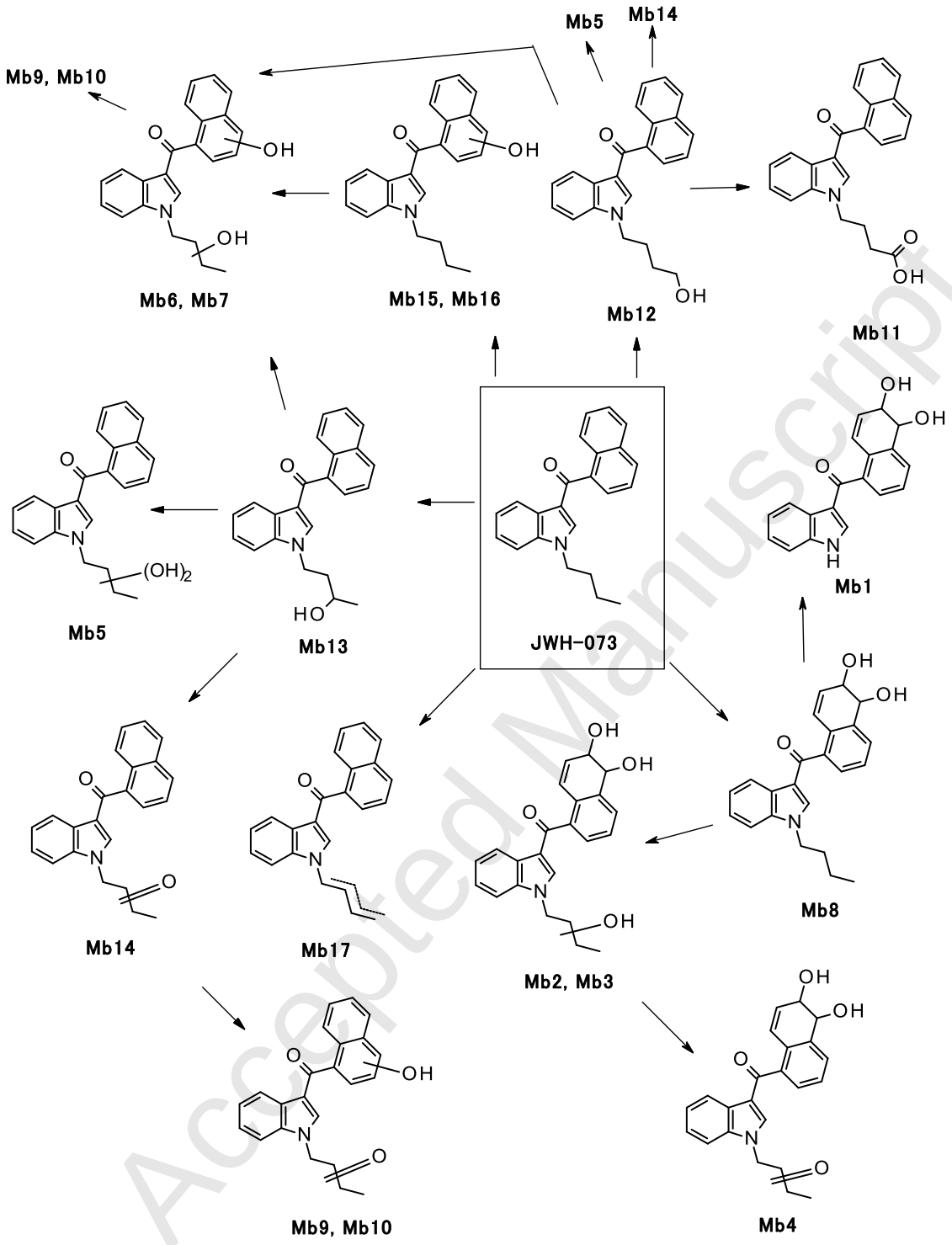

Fig. 4. Proposed metabolic pathway of JWH-073 in *C. elegans*.

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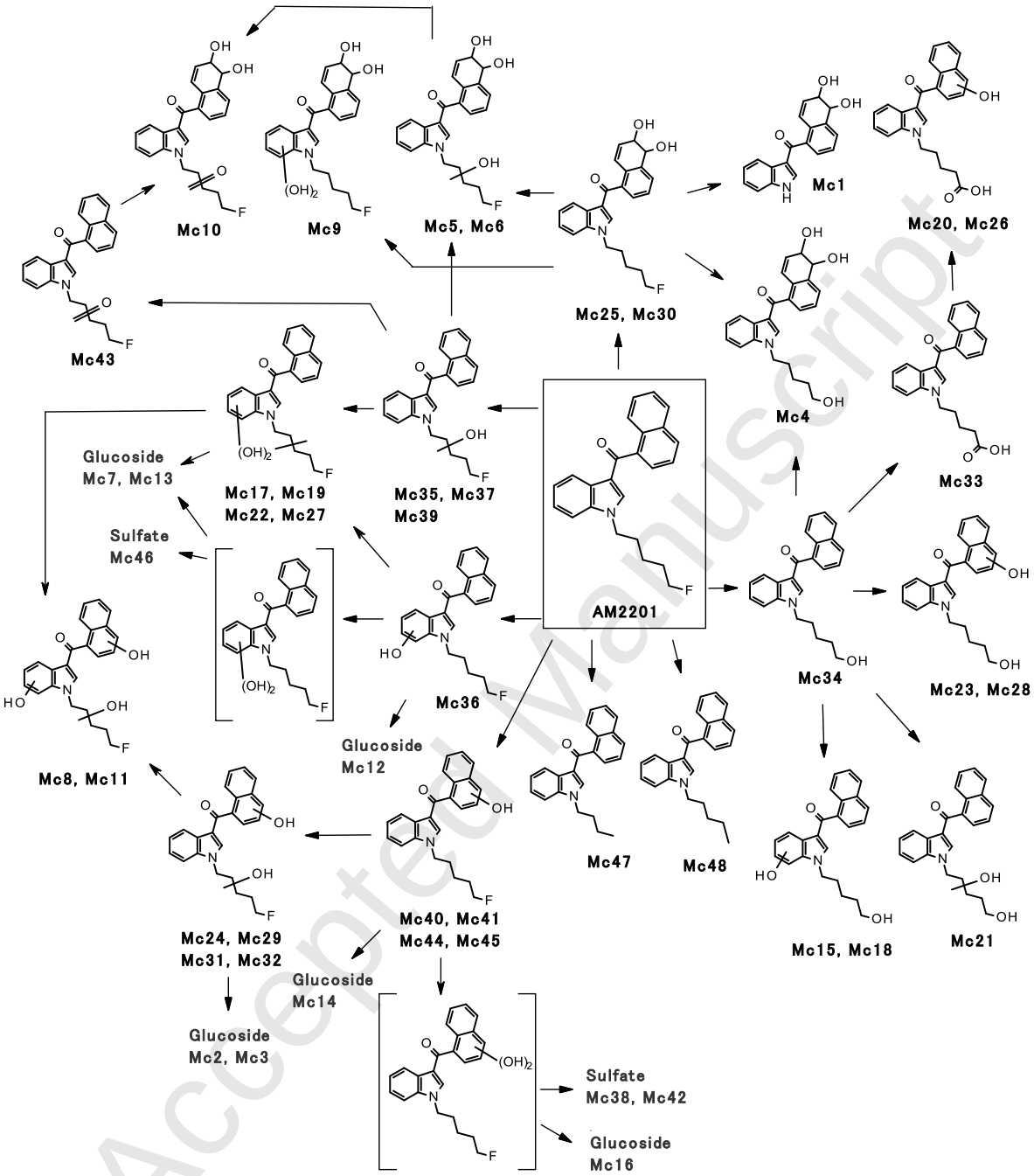

Fig. 5. Proposed metabolic pathway of AM2201 in *C. elegans*. Parentheses indicate intermediate metabolites that were not detected in the study.

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