Cannabinoids and zebrafish

Akhtar, M.T.

CHAPTER 3

Hydroxylation and further oxidation of Δ^9-tetrahydrocannabinol by alkane-degrading bacteria

Hassan Rashidi[‡], Muhammad Tayyab Akhtar[†], Frank van der Kooy[†], Robert Verpoorte[†], Wouter A. Duetz[‡]

[†] Natural Products Laboratory, Institute of Biology, Leiden University, The Netherlands.

[‡] Enzyscreen BV, Biopartner Centre, Wassenaarseweg 72, 2333 AL, Leiden, The Netherlands.

Abstract

The microbial biotransformation of Δ^9–tetrahydrocannabinol was investigated using a collection of 206 alkane-degrading strains. 15% of these strains, mainly Gram-positive strains from the genera *Rhodococcus, Mycobacterium, Gordonia*, and *Dietzia* yielded more polar derivatives. Eight derivatives were produced on a mg scale, isolated, purified, and their chemical structures were elucidated with the use of LC-MS, ^{1}H-NMR, and 2-D NMR (^{1}H-^{1}H COSY and HMBC). All eight biotransformation products possessed modified alkyl chains, with hydroxy, carboxy and ester functionalities. In a number of strains, β-oxidation of the initially formed C5 carboxylic acid led to the formation of a carboxylic acid lacking two methylene groups.

Introduction

Δ^9-Tetrahydrocannabinol (Δ^9-THC) is the decarboxylated product of the corresponding Δ^9-THC-acid, the major cannabinoid present in the cannabis plant (*Cannabis sativa* L., Cannabaceae). This compound is officially registered as a drug for the stimulation of appetite and antiemesis in patients under chemotherapy and HIV therapy regimes. Other biological activities ascribed to this compound include lowering of intraocular pressure in glaucoma, as analgesic for muscle relaxation, immunosuppression, sedation, bronchodilation, and neuroprotection (Grotenhermen 2003).

Δ^9-Tetrahydrocannabinol and many of its derivatives are highly lipophilic and poorly water-soluble with a logPo/w > 4.5 for Δ^9-THC (Thomas et al. 1990). Calculations of the n-octanol/water partition coefficient (Ko/w) of Δ^9-THC at neutral pH, vary between 6000 using the shake-flask method (Mechoulam 1982), and 9.44×106 by reverse-phase HPLC estimation (Thomas et al. 1990). The poor water solubility and high lipophilicity of cannabinoids cause their absorption across the lipid bi-layer membranes and fast elimination from blood

circulation. In terms of the 'Lipinsky rule of 5' (Lipinski 2000), the high lipophilicity of cannabinoids is hindering the further development of these compounds into large scale pharmaceutical products.

To generate more water-soluble analogues, one can either apply *de novo* chemical synthesis, or modify naturally occurring cannabinoids, e.g. by introducing hydroxy, carbonyl or carboxy groups. Chemical hydroxylation of compounds such as cannabinoids is difficult (Δ^9-THC is converted into Δ^8-THC under mild conditions), microbial biotransformation of cannabinoids is potentially a more fruitful option to achieve this goal.

So far, studies on biotransformation of Δ^9-THC were mainly focused on fungi, which lead to the formation of a number of mono- and dihydroxylated derivatives. Previous reports on the biotransformation of cannabinoids by various microorganisms are summarized in Table 1.

The aim of the present study was to test bacterial strains capable of transforming Δ^9-THC to new products (with potentially better pharmaceutical characteristics) at a higher yield and specificity than previously found for fungal strains. For this purpose, we have chosen to use a collection of alkane-degrading strains, since it was shown in previous studies (Duetz et al. 2001, Smits et al. 2002, Beilen et al. 2005) that alkane oxygenases often display a broad substrate range. Production of novel cannabinoid derivatives that might have interesting pharmacological activities was another objective of this project.

Materials and methods

Bacterial strains and growth conditions: A total of 206 alkane-degrading strains (mainly isolated from soil and surface water, see (Beilen et al. 2005) were screened. An optimized Evans mineral medium according to (Duetz et al. 2000) with four times more trace elements (E4T) supplemented with a carbon

Table 1. Previous biotransformation experiments conducted using various microorganims to transform cannabinoids

Cannabinoid(s)	Microorganism(s) used	No. of transformed Products	Reference
Δ^9-THC	Cunninghamella blakesleeana	6	(Binder 1976)
Δ^8-THC	*Pellicularia filamentosa*	4	(Vidic et al. 1976)
Δ^8-THC	*Streptomyces lavendulae*	4	(Vidic et al. 1976)
$\Delta^{6a, 10a}$-THC	400 cultures (Soil microorganisms)	various	(Abbott et al. 1977)
Nabilone	400 cultures (Soil microorganisms)	various	(Abbott et al. 1977)
$\Delta^{6a, 10a}$-THC	358 cultures containing bacteria, actinomycetes, and molds	3	(Fukuda et el. 1977)
Δ^9-THC, Δ^8-THC, CBD, CBN	*Syncephalastrum racemosum*, *Mycobacterium rhodochrous*	various	(Robertson et al. 1978)
Δ^9-THC	*Chaetomium globosum*	3	(Christie et al. 1978)
Δ^9-THC	51 fungal strains	8	(Binder and Miesenber 1978)
Nabilone	Microbes	various	(Archer et al. 1979)
Δ^9-THC	*Fusarium nivale*, *Gibberella fujikuroi* and *Thamnidium elegans*	8	(Binder and pop 1980)

sources was used throughout this study. All cultures were grown aerobically at 30 ^{o}C. To grow bacteria on agar-based media, pre-warmed Evans mineral medium (E4T) was mixed with a molten 4 % agar no.2 solution (1:1 v/v) and dispensed in either Petri dishes or microtiter plates. For growth on n-alkanes, Petri dishes containing E4T medium were incubated at 30 ^{o}C with n-alkanes

supplied through the vapor phase by placing an open Erlenmeyer flask with the mixture of alkanes in a sealed container holding the Petri dishes.

Miniaturized screening: The alkane degrading bacteria were screened using a microtiter based technology platform as described before (Duetz et al. 2000, Beilen et al. 2005). Briefly, multiple strains stored in one 96-well microtiter plate at -80°C were sampled simultaneously without thawing the bulk cultures by using a spring-loaded 96-pin replicator (Kühner, Basel, Switzerland) as previously described (Duetz et al. 2000) and were transferred to a regular sterile polystyrene microtiter plate (type 3072; Costar, Cambridge, Mass.). Each well (working volume, 350 µl) contained 180 µl of a solidified mineral medium agar (2% [wt/vol]) without a carbon source. The inoculated microtiter plate was placed in a desiccator together with a beaker of water and a 50-ml beaker containing 10 ml of a 1:1:1:1:1 (vol/vol/vol/vol/vol) mixture of hexane, octane, decane, dodecane, and hexadecane. The lid on top was kept 2 mm from the wells in order to allow for a sufficient supply of gaseous alkanes to the wells. After 7 days of growth at the ambient temperature (30°C), the cell mass that developed on the agar surface was harvested as follows. First, 110 µl of potassium phosphate buffer (50 mM, pH 7.0) was added to each well. Repeated lateral movement of the spring-loaded replicator in the wells resulted in suspension of a large part of the cell mass. The suspensions were subsequently transferred by using a 12-channel multipipette and wide-orifice tips to a microtiter plate with 0.5-ml conical wells (Maxi-plaque; Polylabo, Geneva, Switzerland). The microtiter plate was centrifuged for 15 min at 4,000 rpm in an Eppendorf type 5403 centrifuge. After disposal of the supernatant, the cells were resuspended in 100 µl of a buffer (50 mM potassium phosphate buffer [pH 7.0]) by repeated filling and emptying of wide-orifice pipette tips by using a 12-channel multipipette. Subsequently, 2 µL substrate (20 g/L Δ^9-THC in methanol) which was purified as previously described (Hazekamp et al. 2004),

was added to each well and the wells were closed by using a sandwich cover consisting of a pierced layer of soft silicone combined with a rigid polypropylene plate. The microtiter plate was incubated for 6h at 30 ^{o}C with orbital shaking at 300 rpm and an amplitude of 50 mm. To extract the metabolites, 50 µL of chloroform was added to each well after incubation. The microtiter plate's contents were transferred into 1.5 ml tubes and then centrifuged for 10 min at 12,000 ×g. The chloroform layer was transferred to another 1.5 ml tube and air-dried. The residue was re-dissolved in MeOH and used for analysis by HPLC and LC-MS.

Large scale Biotransformation: Three selected alkane degrading bacteria (ENZHR1, ENZHR3 and ENZHR5) were cultured in 500 ml flasks containing 100 ml E4T medium aerobically at 30 ^{o}C. A mixture of n-alkanes was supplied through the vapor phase using a small plastic container attached to the flask's cap, as described above. The flasks were incubated for 18-24h at 30 ^{o}C with orbital shaking at 300 rpm and an amplitude of 50 mm. Then 2 ml of substrate (20 g/L Δ^{9}-THC in methanol) was added to each flask and incubated for a further 24 hrs. Metabolites were produced on a 1-10 mg scale and were extracted as described above. In order to increase the extraction efficiency of acid derivatives, hydrochloric acid (HCl) was added to aqueous phase before extraction.

Purification of metabolites: All transformed metabolites were purified with a semi-preparative Agilent 1200 HPLC. Various ratios of aqueous methanol were employed to purify the different products. The column employed was a RP18 Phenomenex (250X10mm, 5µm). The yields of all metabolites were in the 1-10 mg range.

HPLC-APCI-MS Analysis: Analysis was performed by using a high-performance liquid chromatography (Agilent 1100 series, Hannover, Germany)

equipped with a diode array detector, and a mass detector in series. Solvent 1 (methanol containing 0.1% formic acid) and 2 (water containing 0.1% formic acid) were used at a 70:30 (v/v) ratio for 7 min isocratically. This was followed by a gradient to 100% solvent 1 over a period of 6 min. After 1 min the mobile phase was returned to the starting conditions and left to re-equilibrate for a further 3 min. (total run time of 17 min). A Macherey-Nagel (Duren, Germany) Nucleosil C_{18} column (70 mm length, 3µm internal diameter, 5µm particle size) equipped with an 8 mm-long pre-column of the same material was used to separate the components. The settings of the mass spectrometer (MS) were as follows: APCI mode; positive ionization; fragmentor voltage 100 and 240 V; gas temperature, 350 ^{o}C; vaporizer temperature, 400 ^{o}C; drying gas (N_2) flow rate, 4 liters min^{-1}; nebulizer pressure, 45 psig (lb/in); capillary voltage, 4000 V; corona current, 4.0 µA.

Determination of the maximal solubility of metabolites: 20 µL of 10 mM of each sample in methanol was dried in eppendorf tubes. 20 µL of 50 mM phosphate buffer pH 7.0 were added to each eppendorf, vortexed for 5 minutes and were left over night. After centrifugation for 10 minutes in 2000 g in room temperature, 5 µL of supernatant was diluted 20 times in methanol and was analysed by HPLC under the same condition as described above.

NMR: The purified compounds after evaporation of MeOH were re-dissolved in CD_3OD and ^{1}H-NMR (400 MHz) spectra were recorded by a Bruker model AV-400 NMR spectrometer with reference to residual solvent as standard.

Results

Screening. To identify appropriate bacterial strains for the bioconversion of cannabinoids to more polar derivatives, we screened 206 (largely unidentified) alkane-degrading strains grown with a mixture of alkanes as their sole source of carbon and energy. Strains selected for further study were identified by partial

16S RNA sequencing. Formation of more polar compounds was observed for 76 strains, of which 44 strains converted more than 50% of substrate after 6 hours incubation at a cell density of approximately 5 g dry wt / L. In seventy of the strains capable of converting Δ^9-THC into more polar derivatives, only compounds [**3]** and/or [**4]**, (Table 2) were formed in significant amounts. In only 6 of the strains other compounds were formed. In total eight different metabolites were identified (based on relative retention time, UV spectra, and mass spectra). The proposed structures (based on MS and NMR data; see further below) are given in (Table 2).

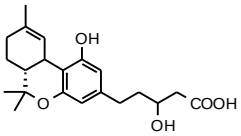
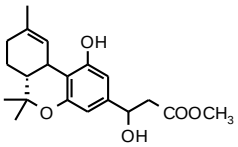
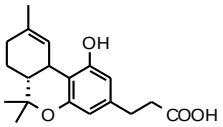
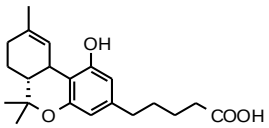
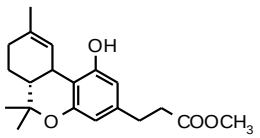
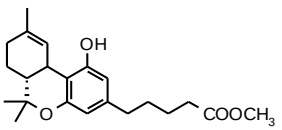
Metabolite **[1]** was the most polar derivative identified during the screening procedure and it was produced only by *Gordonia* sp ENZHR5 in relatively small quantities. Metabolite [**2]** was the second most polar derivative and was produced by only 4 of the screened strains, in a very low yield. The molecular mass of 347 [M+H], and the absence of any significant change in the UV spectra in comparison to Δ^9-THC suggested modification of the alkyl moiety. Metabolites [**3]** and [**4]** were the most abundant and were produced by all 76 strains, though in varying ratios. This ratio was observed to be not only strain-dependent, but was also influenced by the concentration of substrate, cell density and incubation time. In general, higher cell densities and longer incubation times resulted in increasing concentrations of compound [**3]**, suggesting compound [**3]** was a product of through-conversion of [**4]** to [**3]**. Metabolite [**5]** was produced by three strains, all of them also capable of production of metabolite [**2]**. A steady decrease in the concentration of metabolite [**5]** and an increase of metabolite [**2]** with a prolonged incubation time suggests that metabolite [**5]** is a precursor of metabolite [**2]**. Metabolite [**6]** was produced only by microorganisms capable of production of metabolites [**2]** and [**5]** in a time-dependent manner and a very low yield. Metabolites [**7]** and [**8]** were formed by only 1 of the screened strains.

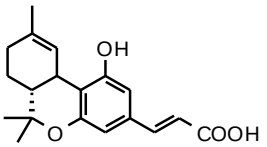
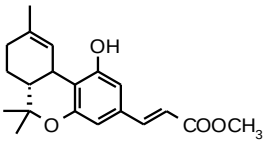
After preliminary analysis of the results, we focused on three strains that biotransformed Δ^9-THC at relatively high yields, and which covered all produced derivatives based on retention time of peaks and the molecular mass as determined by the LC-MS analysis. These strains were a *Dietzia* sp ENZHR1 yielding one major metabolite, a *Mycobacterium* sp ENZHR3 and a *Gordonia* sp ENZHR5, both producing a mixture of derivatives.

Structure elucidation. The chemical structures of metabolites [**1-8]** were further elucidated (Table 2) using ^{1}H-NMR, ^{1}H-^{1}H COSY and HMBC. The ^{1}H-NMR spectrum of all eight metabolites were in accordance with Δ^9-THC with regard to the signals due to two angular methyl groups [3H each, *s* at δ 1.41, C6 β methyl and δ 1.09, C6 α methyl], and three aromatic and olefinic protons (1H, *q*, *J*= 1.6 Hz, at δ 6.31, H-10; 1H, *d*, *J*=1.6 Hz at δ 6.14, H-4; 1H, *d*, *J*= 1.6 Hz at δ 6.06, H-2) (Choi et al. 2004). The changes were mostly limited to the signals representing protons in the alkyl side chain. The ^{1}H-NMR chemical shifts of the main signals of the metabolites and Δ^9-THC are shown in Table 3. On the basis of these proton NMR data, MS spectra and UV spectra, we propose compounds [**1-8]** to have the chemical structures as mentioned below, with the following argumentation:

3'-hydroxy-Δ^9-THC-5'-oic acid [1]: ^{1}H NMR (CD_3OD, 400MHz). The signals representing H-5' (tertiary methyl group at δ 0.87) disappeared and H-4' (2H, *m*, at δ 1.29) shifted up-field to δ 2.32 ppm. The signal representing H-3' (2H, *m*, at δ 1.29) could not be detected; instead a new single proton signal at δ 3.92 ppm was observed. The aromatic protons of H-2 and H-4 showed a slightly downfield shift from δ 6.06 and δ 6.14 to δ 6.09 and δ 6.16, respectively. The signal of H-2' (2H, *m* at δ 1.55) shifted to δ 1.70 (2H, *m*) and the signals representing the two H-1' (2H, *td* at δ 2.42 in (Δ^9-THC) divided into two signals with a slightly downfield shift to δ 2.45 and δ 2.57 with an intensity of one

Table 2: The proposed structures for the Δ^9-THC derivatives formed in the present study with their respective differences in physicochemical properties

Compound No	Chemical Name	rRT	UV_{max}	MW [b]	Proposed structure
	Δ^9-THC	1.000	210,280	314	NA
1	3'-Hydroxy- Δ^9-THC-5'-oic acid	1.000	210,280	314	
2	1'–hydroxy- Δ^9-THC-4',5'-bis, nor-3'-oic acid-methyl ester	0.225	210,280	346	
3	Δ^9-THC-4',5'-bis, nor-3'-oic acid	0.241	210,280	316	
4	Δ^9-THC-5'-oic acid	0.350	210,280	344	
5	Δ^9-THC-4',5'-bis, nor-3'-oic acid-methyl ester	0.375	210,280	330	
6	Δ^9-THC-5'-oic acid-methyl ester	0.540	210,280	358	

7	1',2'-dehydro- Δ ^{9}-THC-4',5'-bis, nor-3'-oic acid	0.350	232,300	314	
8	1',2'-dehydro- Δ ^{9}-THC-4',5'-bis, nor-3'-oic acid methyl ester	0.658	232,300	328	

proton each. APCI-MS *m/z* 361 $[M]^+$ (100), 343 (54), 325 (11). Based on the information provided above and ^{1}H-^{1}H cosy data (data not provided), we propose the structure as given in Table 2 for this compound.

1'-hydroxy-Δ^9-THC-4',5'-bis, nor-3'-oic acid-methyl ester [2]. ^{1}H NMR (CD_3OD, 400MHz). The signals representing H-5' (tertiary methyl group at δ 0.87), H-4' (2H, *m*, at δ 1.29) and H-3' (2H, *m*, at δ 1.29) was not observed. The aromatic protons of H-2 and H-4 showed a slightly downfield shift from δ 6.06 and δ 6.14 to δ 6.25 and δ 6.35 respectively. The signal of H-2' (2H, *m* at δ 1.55) shifted to δ 2.67 (1H, dd) and δ 2.61 (1H, dd); the signal representing H-1' (2H, *td* at δ 2.42 in Δ^9-THC) showed a strong downfield shift to δ 4.80 with an intensity of one proton. Presence of a new methyl ester signal at δ 3.66 was confirmed by HMBC which shows a long range coupling of a new signal representing a methyl ester group with carbonyl group around 173 ppm. APCI-MS *m/z* 347 $[M]^+$ (10), 329 (100). Based on this information we propose the structure as in Table 2 for this compound.

Δ^9-THC-5'-oic acid [4]. ^{1}H NMR (CD_3OD, 400MHz). The ^{1}H-NMR spectrum is in accordance with Δ^9-THC as explained above but the tertiary methyl group at δ 0.87 representing H-5' was not observed and signals representing H-3' and

4' (2H, *m*, at δ 1.29, H-3'; 2H, *m*, at δ 1.29, H-4') have shifted downfield to δ 1.59 and δ 2.29 respectively. A slightly downfield shift of the signal representing proton H-2' (2H, *m*) from δ 1.55 to δ 1.60 was observed but there were no significant change in the signal of H-1' (2H, *m*, at δ 2.42). APCI-MS *m/z* 345 $[M]^+$ (100), 327 (11). Based on the collected data, we propose that compound [**4]** is Δ^9-THC 5'-oic acid (Table 2).

Δ^9-THC-4',5'-bis, nor-3'-oic acid-methyl ester [5]. ^{1}H NMR (CD_3OD, 400MHz). The ^{1}H-NMR spectrum lacked the signals representing H-5' (tertiary methyl group at δ 0.87) H-3' (2H, *m*, at δ 1.29) and H-4' (2H, *m*, at δ 1.29). A significant downfield shift in the signal representing proton H-2' (2H, *t*) from δ 1.55 to δ 2.58 and slight downfield shift of H-1' (2H, *td*) from δ 2.42 to δ 2.74 and appearance of a new signal (3H, *s*) at δ 3.68 a typical signal of methyl ester group, were the main changes in the ^{1}H-NMR in comparison to Δ^9-THC. Long-range coupling of this new signal with a carbonyl group at 173 ppm in the HMBC spectra confirmed the presence of methyl ester substitution. APCI-MS *m/z* 331 $[M]^+$ (100), 299 (70). Based on the information provided above, we propose the structure as given in Table 2 for compound [**5]**.

Δ^9-THC-5'-oic acid-methyl ester [6]. ^{1}H NMR (CD_3OD, 400MHz). The ^{1}H-NMR spectrum is in accordance with Δ^9-THC as explained above and identical to compound [**4]** with only the presence of one additional signal (3H, *s*, at δ 3.63 typical for a O-methyl ester (confirmed by HMBC). APCI-MS *m/z* 359 $[M]^+$ (100), 327 (22). Based on the collected data, we propose that compound [**6]** is Δ^9-THC-5'-oic acid-methyl ester (Table 2).

1',2'-dehydro-Δ^9-THC-4',5'-bis, nor-3'-oic acid [7]. ^{1}H NMR (CD_3OD, 400MHz). Most of the ^{1}H-NMR spectrum is in accordance with Δ^9-THC as described above but the tertiary methyl group at δ 0.87 representing H-5' and signals representing H-3' and 4' (2H, *m*, at δ 1.29, H-3'; 2H, *m*, at δ 1.29, H-4';

Table 3. ^{1}H NMR chemical shifts (ppm) of the altered signals of new metabolites as compared to Δ^{9}-THC**.**

	Chemical shifts (ppm) for compound								
position	**Δ^{9}-THC**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
2	*6.06, d*	6.09, *d*	6.25, *d*	6.11, *d*	6.06, *d*	6.11, *d*	6.06, *d*	6.47, *d*	6.47, *d*
	(1.6)	(~1)	(1.5)	(1.6)	(1.2)	(~1)	(~1)	(~1)	(~1)
4	6.14, *d*	6.16, *d*	6.35, *d*	6.19, *d*	6.14, *d*	6.19, *d*	6.14, *d*	6.55, *d*	6.55, *d*
	(1.6)	(~1)	(1.5)	(1.6)	(1.2)	(~1)	(~1)	(~1)	(~1)
1'	2.42, *td*	2.45, *m*	4.8, *dd*	2.7, *td*	2.42, *td*	2.74, *td*	2.42, *td*	6.32, *d*	6.33, *d*
	(7.3, 1.6)		(8.8, 2.4)	(7.6, ~1)	(7.2, ~1)	(6.0, ~1)	(7.2, ~1)	(16)	(16)
1' (new signal)		2.57, *m*							
2'	1.55, *q*	1.7, *m*	2.67, *dd*	2.4, *td*	1.6, *m*	2.58, *td*	1.6, *m*	7.42, *d*	7.46, *d*
	(7.8)		(15.2, 8.8)	(7.6, ~1)		(6.0, ~1)		(16)	(16)
2' (new signal)			2.61, dd						
			(15.2, 2.4)						
3'	1.29, *m*	3.92, *bs*			1.59, *m*		1.59, *m*		
4'	1.29, *m*	2.32, *m*			2.29, *m*		2.32, *td*		
							(7.2, ~1)		
5'	0.9, *t* (7.0)								
New signal			3.66, *s*			3.68, *s*	3.63, *s*		3.76, *s*

respectively) disappeared. A significant downfield shift observed in the signals representing the aromatic protons of H-2 (1H, *d*) and H-4 (1H, *d*) from δ 6.06 and δ 6.14 to δ 6.47 and δ 6.55 respectively. Signals representing protons H-2' (2H, *t*, at δ 1.55) and H-1' (2H, *td*, at δ 2.42) changed to 1H doublets and shifted to 7.42 and 6.32 ppm, respectively. Significant changes in UV absorption of the compound suggested a further conjugation of a chromophore with the aromatic ring. A coupling constant of H-1 and H-2 (*J*=16 Hz) indicated a *trans* formation of the vicinal protons of a double bond between C-1' and C-2'. Fig. 1 shows the proposed structure for compound [**7]** with a *trans* configuration of the protons at C-1' and C-2'. APCI-MS *m/z* 315 $[M]^+$ (100), 297 (15).

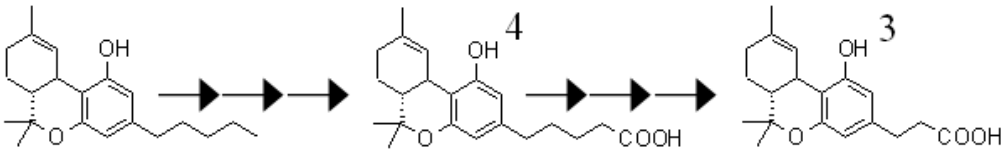


Figure 1: Biotransformation of Δ^9-THC by *Dietzia* sp ENZHR1

1',2'-dehydro-Δ^9-THC-4',5'-bis, nor-3'-oic acid-methyl ester [8]. ^{1}H NMR (CD_3OD, 400MHz). Most of the ^{1}H-NMR spectrum is in accordance with Δ^9-THC as described above and identical to compound **7** with only the presence of one additional signal (3H, *s*, at δ 3.68 typical for a O-methyl ester (confirmed by HMBC). APCI-MS *m/z* 329 $[M]^+$ (100), 297 (10). Table 2 shows the proposed structure for compound [**8]** with a *trans* configuration of protons at C-1' and C-2'. As shown in Fig. 1, production of new metabolites in *Dietzia* sp ENZHR1 was limited to [**3]** and [**4]** while the production of compound [**3]** yielded less than 5% of the total conversion based on their respective HPLC chromatogram areas. In *Mycobacterium* sp ENZHR3, compound [**3]** is one of the major

products (35-70% of total conversion based on the incubation condition) while compound [**5]** is the second most abundant metabolite. Compounds **[2], [6]** and **[8]** were found to be minor products. Formation of [**4]** is totally dependent on incubation conditions. Production of [**5]**, [**6]** and [**8]** in *Mycobacterium* ENZHR3 increased in a time-dependent manner accompanied by a decreasing amount of [**3]** and [**4]** (Fig. 2). *Gordonia* sp ENZHR5 also showed a multi-roduct pattern of production, although [**1]**, [**3]** and [**7]** were the most abundant metabolites formed (Fig. 3).

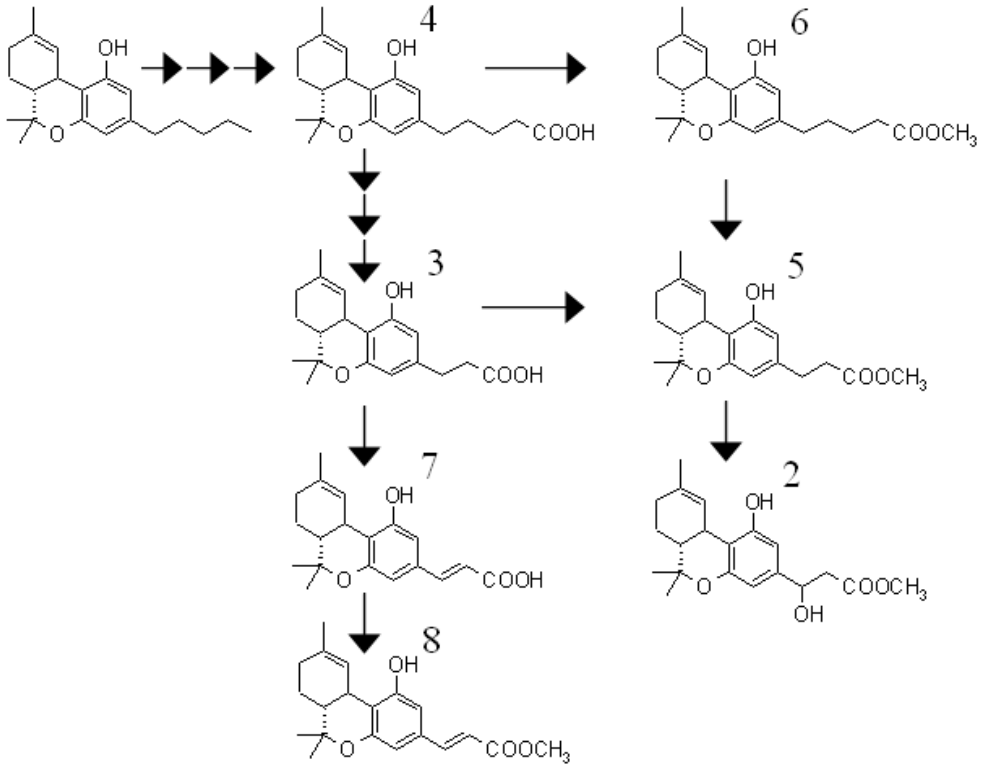


Figure 2: Production of new derivatives from Δ^9-THC by *Mycobacterium* sp ENZHR3

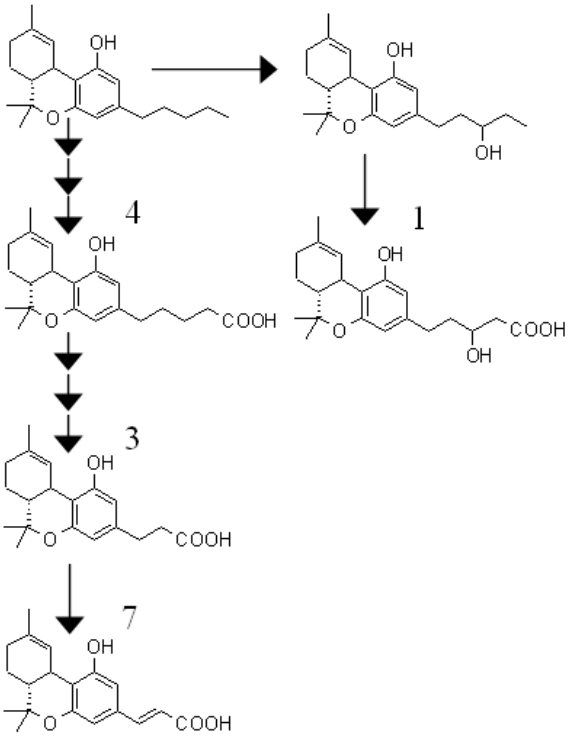


Figure 3: Metabolites by *Gordonia* sp ENZHR5 during biotransformation of Δ^9-THC

Solubility studies. The physicochemical properties of the derivatives were found to be significantly different from those of the parent compounds. For [3] and [4], the water-solubility at pH 7 are up to 8.5 and 4.5 mM respectively which is a dramatical increase in comparison to the solubility of Δ^9-THC and CBD (50 and 135 µM, respectively). Solubility of the methyl ester derivatives is also assumed to be improved based on their HPLC retention time in comparison to the parent compounds. The double bond between C-1' and C-2' has a negative effect on solubility; for example [7] has a 1.4 fold lower solubility compared to the saturated metabolite (6.2 mM for the dehydro metabolite).

Discussion

In this study, we established that a significant part (approx 37%) of a randomly-isolated collection of bacterial alkane degraders is capable of hydroxylation and further modification of the pentane moiety of tetrahydrocannabinol. A total of eight compounds were produced on a 1-10 mg scale. Their structures were elucidated using MS and UV spectrometry, ^{1}H-NMR and 2-D NMR (^{1}H-^{1}H COSY and HMBC).

The finding that Gram-positive alkane degraders from the CMN group (including Rhodococci and Mycobacteria) were overrepresented in the group of THC-converters possibly reflects their relatively good cell wall permeability for lipophilic compounds as a result of the absence of a LPS layer. The best producers were from the genera of Dietzia, Mycobacterium and Gordonia. A similar overrepresentation of Gram-positives was observed in similar screenings for the bioconversion of D- and L-limonene (van Beilen et al. 2005). A possibly broader substrate range of the (mainly P450) alkane monooxygenases in these strains may also have played a role in this finding.

The most frequently found metabolites were [**3]** and [**4].** Probably compound **[4]** was formed as a result of the action of an alkane monooxygenase, in combination with two aspecific dehydrogenases (possibly, but not necessarily alkanol dehydrogenases). The assumed initial product (Δ^9-THC-5'-OH) was not detected in significant amounts, probably reflecting the relatively high specific activities of the dehydrogenases responsible for the further oxidation of the alcohol. Compound **[3]** is also a carboxylic acid, but its alkane chain is two methylene groups shorter. In the light of the gradual conversion of compound **[4]** to compound **[3]** (as was observed for a number of strains) it seems reasonable to assume that compound **[3]** is formed as a result of β-oxidation of compound **[4]**.

Other metabolites were produced by only a few screened strains and normally in low yields, except compounds [**1]** and [**7]** that were produced in high yields by *Gordonia* sp ENZHR5. Further work is needed to assess which enzymes are responsible for these conversions.

Formation of Δ^9-THC derivatives with a C-3 side chain has been observed during an *in vivo* study by feeding mice with Δ^9-THC-5'-OH, Δ^9-THC-5'-oic acid and Δ^8-THC-5'-oic acid in another study (Harvey and Leuschner 1985).

The possible (pharmaceutical) application of the isolated derivatives is presently the subject of further study. The improved water solubility (8.5 and 4.5 mM for compounds [**3]** and [**4]**) would make them better pharmaceuticals in terms of the Lipinski rules. This positive effect might however be offset by changes in their affinities for the relevant receptors. Therefore, the isolated compounds are presently being tested with respect to their binding affinities for the CB1 and CB2 receptors.

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