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Hydroxylation and Further Oxidation of Δ^9-Tetrahydrocannabinol by Alkane-Degrading Bacteria▽

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The microbial biotransformation of Δ^9-tetrahydrocannabinol was investigated using a collection of 206 alkane-degrading strains. Fifteen percent 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, and purified, and their chemical structures were elucidated with the use of liquid chromatography-mass spectrometry, ^{1}H-nuclear magnetic resonance (^{1}H-NMR), and two-dimensional NMR (^{1}H-^{1}H correlation spectroscopy and heteronuclear multiple bond coherence). 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 C_5 carboxylic acid led to the formation of a carboxylic acid lacking two methylene groups.

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 human immunodeficiency virus therapy regimens. Other biological activities ascribed to this compound include lowering intraocular pressure in glaucoma, acting as an analgesic for muscle relaxation, immunosuppression, sedation, bronchodilation, and neuroprotection (11).

Δ^9-THC and many of its derivatives are highly lipophilic and poorly water soluble. Calculations of the *n*-octanol/water partition coefficient ($K_{o/w}$) of Δ^9-THC at neutral pH vary between 6,000, using the shake flask method (15), and 9.44×10^6, by reverse-phase high-performance liquid chromatography estimation (19). The poor water solubility and high lipophilicity of cannabinoids cause their absorption across the lipid bilayer membranes and fast elimination from blood circulation. In terms of the "Lipinsky rule of 5" (14), the high lipophilicity of cannabinoids hinders 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 (as, e.g., in reference 16) 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 easily converted into Δ^8-THC under mild conditions), and therefore 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 led 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 whether bacterial strains are capable of transforming Δ^9-THC into 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 (8, 18, 20) 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) (20) were screened. An optimized Evans mineral medium described in reference 9 with four times more trace elements (E4T) and supplemented with carbon sources was used throughout this study. All cultures were grown aerobically at 30°C. To grow bacteria on agar-based media, prewarmed Evans mineral medium (E4T) was mixed with a molten 4% agar no. 2 solution (1:1, vol/vol) and dispensed in either petri dishes or microtiter plates. For growth with *n*-alkanes, petri dishes containing E4T medium were incubated at 30°C with *n*-alkanes supplied through the vapor phase by placing an open Erlenmeyer flask with the mixture of *n*-alkanes in a sealed container holding the petri dishes.

Miniaturized screening. The alkane-degrading bacteria were screened using a microtiter-based technology platform (9, 20). Briefly, multiple strains stored in a 96-well microtiter plate at −80°C were sampled simultaneously without thawing the bulk cultures by using a spring-loaded 96-pin replicator (Enzyscreen, Leiden, The Netherlands) (9). The strains were transferred to a sterile polystyrene microtiter plate (type 3072; Costar, Cambridge, MA). 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. After 7 days of growth (at 30°C), the cell mass was harvested by addition of 110 μl of potassium phosphate buffer (50 mM, pH 7.0) to each well. Repeated lateral movement of the spring-loaded replicator in the wells resulted in suspension of the cell mass, after which the suspensions were transferred using a 12-channel multipipette and wide-orifice tips to a microtiter plate (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.

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TABLE 1. Previous biotransformation experiments conducted using various microorganisms to transform cannabinoids

Cannabinoid(s)[a]	Microorganism(s) used	No. of transformed products	Reference
Δ^9-THC	*Cunninghamella blakesleeana*	6	3
Δ^8-THC	*Pellicularia filamentosa*	4	21
Δ^8-THC	*Streptomyces lavendulae*	4	21
$\Delta^{6a,10a}$-THC	400 cultures (soil microorganisms)	Various	1
Nabilone	400 cultures (soil microorganisms)	Various	1
$\Delta^{6a,10a}$-THC	358 cultures containing bacteria, actinomycetes, and molds	3	10
Δ^9-THC, Δ^8-THC, CBD, CBN	*Syncephalastrum racemosum*, *Mycobacterium rhodochrous*	Various	17
Δ^9-THC	*Chaetomium globosum*	3	7
Δ^9-THC	51 fungal strains	8	4
Nabilone	Microbes	Various	2
Δ^9-THC	*Fusarium nivale*, *Gibberella fujikuroi*, and *Thamnidium elegans*	8	5

[a] CBD, cannabidiol; CBN, cannabinol.

After disposal of the supernatant, the cells were resuspended in 100 μl of buffer (50 mM potassium phosphate buffer [pH 7.0]). Subsequently, 2 μl substrate (20 g/liter Δ^9-THC in methanol), which was purified as previously described (13), was added to each well, after which 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 6 h at 30°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 a 1.5-ml tube and air dried. The residue was redissolved in methanol and used for analysis by high-performance liquid chromatography and liquid chromatography-mass spectrometry (LC-MS).

Large-scale biotransformation. Three selected alkane-degrading bacterial strains (*Dietzia* sp. strain ENZHR1, *Mycobacterium* sp. strain ENZHR3, and *Gordonia* sp. strain ENZHR5) were cultured in 500-ml flasks containing 100 ml E4T medium aerobically at 30°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 to 24 h at 30°C with orbital shaking at 300 rpm and an amplitude of 50 mm. Then 2 ml of substrate (20 g/liter Δ^9-THC in methanol) was added to each flask, and the flasks were incubated for a further 24 h. Metabolites were produced on a 1- to 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 the aqueous phase before extraction.

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

High-performance liquid chromatography–APCI-MS analysis. Analysis was performed with an HPLC (1100 series; Agilent, Hannover, Germany) equipped with a diode array detector and a mass detector in series. Solvents 1 (methanol containing 0.1% formic acid) and 2 (water containing 0.1% formic acid) were used at a 70:30 (vol/vol) 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 reequilibrate for a further 3 min (total run time of 17 min). A Macherey-Nagel (Duren, Germany) Nucleosil C_{18} column (70-mm length, 3-μm internal diameter, and 5-μm particle size) equipped with an 8-mm-long precolumn of the same material was used to separate the components. The settings of the mass spectrometer were as follows: atmospheric pressure chemical ionization (APCI) mode; positive ionization; fragmentor voltages, 100 and 240 V; gas temperature, 350°C; vaporizer temperature, 400°C; drying gas (N_2) flow rate, 4 liters min^{-1}; nebulizer pressure, 45 lb/in^2; capillary voltage, 4,000 V; corona current, 4.0 μA.

Determination of the maximal solubility of metabolites. Twenty microliters of a 10 mM solution of each purified metabolite in methanol was dried in Eppendorf tubes. Twenty microliters of phosphate buffer (50 mM, pH 7.0) was added to each Eppendorf tube, vortexed for 5 min, and left to stand overnight. After centrifugation for 10 min at 2,000 × *g* and room temperature, 5 μl of the supernatant was diluted 20-fold in methanol and was analyzed by high-performance liquid chromatography under the same conditions as described above.

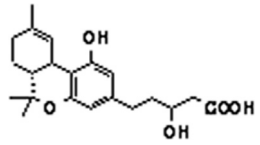
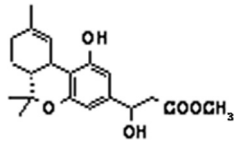
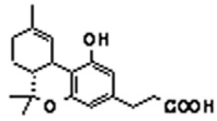
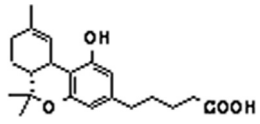
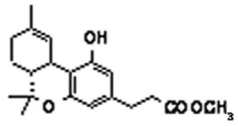
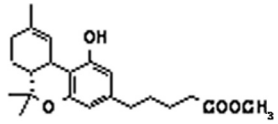
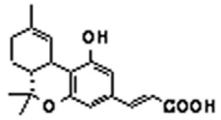
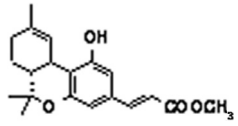
NMR. The purified compounds after evaporation of methanol were redissolved in perdeuterated methanol (CD_3OD), and ^{1}H-nuclear magnetic resonance (^{1}H-NMR; 400 MHz) spectra were recorded with a Bruker model AV-400 NMR spectrometer referenced to the residual solvent.

RESULTS AND DISCUSSION

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 h of incubation at a cell density of approximately 5 g dry weight/liter. In 70 of the strains, capable of converting Δ^9-THC into more-polar derivatives, only compounds 3 or 4 (or both) was formed in significant amounts (Table 2). In only six of the strains were other compounds 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) 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. strain ENZHR5 in relatively small quantities. Metabolite 2 was the second most polar derivative and was produced by only four of the screened strains, in a very low yield. The molecular weight 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 various ratios. The ratios were observed not only to be strain dependent but also to be 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 that compound 3 was a product of conversion from compound 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 suggest that metabolite 5 is a precursor of metabolite 2. Metabolite 6 was produced only by

TABLE 2. 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[a]	UV maxima	MW[b]	Proposed structure
NA[c]	Δ^9-THC	1.000	210, 280	314	NA
1	3′-Hydroxy-Δ^9-THC-5′-oic acid	0.220	210, 280	360	
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	

[a] rRT, relative retention time.
[b] MW, molecular weight.
[c] NA, not applicable.

microorganisms capable of production of metabolites 2 and 5 in a time-dependent manner and in a very low yield. Metabolites 7 and 8 were formed by only one 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 *Dietzia* sp. strain ENZHR1, yielding one major metabolite, *Mycobacterium* sp. strain ENZHR3, and *Gordonia* sp. strain ENZHR5, the last two producing a mixture of derivatives.

Structure elucidation. The chemical structures of metabolites 1 to 8 were further elucidated (Table 2) using ^{1}H-NMR, ^{1}H-^{1}H correlation spectroscopy (COSY), and heteronuclear multiple bond coherence (HMBC). The ^{1}H-NMR spectra 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, C-6 β methyl and δ 1.09, C-6 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) (6). 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 that compounds 1 to 8 have the chemical structures mentioned below, with the following argumentation.

3′-Hydroxy-Δ^9-THC-5′-oic acid (compound 1). ^{1}H-NMR (CD_3OD, 400 MHz) yielded the following results. The signals representing H-5′ (tertiary methyl group at δ 0.87) disap-

TABLE 3. ^{1}H-NMR chemical shifts of the altered signals of new metabolites compared to Δ^9-THC

Position	Shift (ppm) for compound:							
	Δ^9-THC	1	2	3	4	5	6	7	8
2	6.06, *d* (1.6)	6.09, *d* (~1)	6.25, *d* (1.5)	6.11, *d* (1.6)	6.06, *d* (1.2)	6.11, *d* (~1)	6.06, *d* (~1)	6.47, *d* (~1)	6.47, *d* (~1)
4	6.14, *d* (1.6)	6.16, *d* (~1)	6.35, *d* (1.5)	6.19, *d* (1.6)	6.14, *d* (1.2)	6.19, *d* (~1)	6.14, *d* (~1)	6.55, *d* (~1)	6.55, *d* (~1)
1′	2.42, *td* (7.3, 1.6)	2.45, *m*	4.8, *dd* (8.8, 2.4)	2.7, *td* (7.6, ~1)	2.42, *td* (7.2, ~1)	2.74, *td* (6.0, ~1)	2.42, *td* (7.2, ~1)	6.32, *d* (16)	6.33, *d* (16)
1′ (new signal)		2.57, *m*							
2′	1.55, *q* (7.8)	1.7, *m*	2.67, *dd* (15.2, 8.8)	2.4, *td* (7.6, ~1)	1.6, *m*	2.58, *td* (6.0, ~1)	1.6, *m*	7.42, *d* (16)	7.46, *d* (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*

peared, and H-4′ (2H, *m*, at δ 1.29) shifted upfield 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 proton each. APCI-MS yielded the following results: *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 (compound 2). ^{1}H-NMR (CD_3OD, 400 MHz) yielded the following results. 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) were 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. The 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 a carbonyl group around 173 ppm. APCI-MS yielded the following results: *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-4′,5′-bis, nor-3′-oic acid (compound 3). ^{1}H-NMR (CD_3OD, 400 MHz) yielded the following results. The ^{1}H-NMR spectrum is in accordance with Δ^9-THC except that there is a tertiary methyl group at δ 0.87 representing H-5′ and that signals representing H-3′ and H-4′ (2H, *m*, at δ 1.29, H-3′; 2H, *m*, at δ 1.29, H-4′, respectively) disappeared. A significant downfield shift in the signal representing proton H-2′ (2H, *td*) from δ 1.55 to δ 2.40 and slight downfield shift of H-1′ (2H, *td*) from δ 2.42 to δ 2.70 were observed in the ^{1}H-NMR spectrum of this compound. APCI-MS yielded the following results: *m*/*z* 317$[M]^+$ (100), 299 (18). Based on the LC-MS fragmentation and changes in signals in the ^{1}H-NMR spectra in comparison to Δ^9-THC, we propose the structure in Table 2 for compound 3.

Δ^9-THC-5′-oic acid (compound 4). ^{1}H NMR (CD_3OD, 400 MHz) yielded the following results. 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 H-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 was no significant change in the signal of H-1′ (2H, *m*, at δ 2.42). APCI-MS yielded the following results: *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 (compound 5). ^{1}H NMR (CD_3OD, 400 MHz) yielded the following results. 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

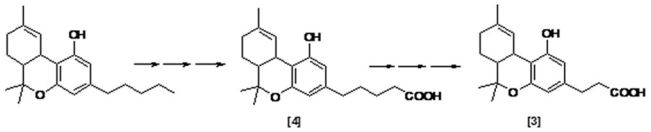


FIG. 1. Biotransformation of Δ^9-THC by *Dietzia* sp. strain ENZHR1.

signal representing proton H-2′ (2H, *t*) from δ 1.55 to δ 2.58, a slight downfield shift of H-1′ (2H, *td*) from δ 2.42 to δ 2.74, and the appearance of a new signal (3H, *s*) at δ 3.68, a typical signal of a methyl ester group, were the main changes in the ^{1}H-NMR spectrum in comparison to that of 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 yielded the following results: *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 (compound 6). ^{1}H-NMR (CD_3OD, 400 MHz) yielded the following results. The ^{1}H-NMR spectrum is in accordance with Δ^9-THC as explained above and identical to that for compound 4 except for the presence of one additional signal (3H, *s*, at δ 3.63 typical for a *O*-methyl ester; confirmed by HMBC). APCI-MS yielded the following results: *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 (compound 7). ^{1}H-NMR (CD_3OD, 400 MHz) yielded the following results. 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 H-4′ (2H, *m*, at δ 1.29, H-3′; 2H, *m*, at δ 1.29, H-4′, respectively) disappeared. A significant downfield shift was observed in the signals representing the aromatic protons 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′. Table 2 shows the proposed structure for compound 7 with a *trans* configuration of the protons at C-1′ and C-2′. APCI-MS yielded the following results: *m/z* 315 $[M]^+$ (100), 297 (15).

1′,2′-Dehydro-Δ^9-THC-4′,5′-bis, nor-3′-oic acid-methyl ester (compound 8). ^{1}H-NMR (CD_3OD, 400 MHz) yielded the following results: most of the ^{1}H-NMR spectrum is in accordance with Δ^9-THC as described above and identical to compound 7 except for the presence of one additional signal (3H, *s*, at δ 3.68 typical for a *O*-methyl ester (confirmed by HMBC). APCI-MS yielded the following results: *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. strain ENZHR1 was limited to compounds 3 and 4 while the production of compound 3 yielded less than 5% of the total conversion based on the compounds' respective high-performance liquid chromatography chromatogram areas. In *Myco-*

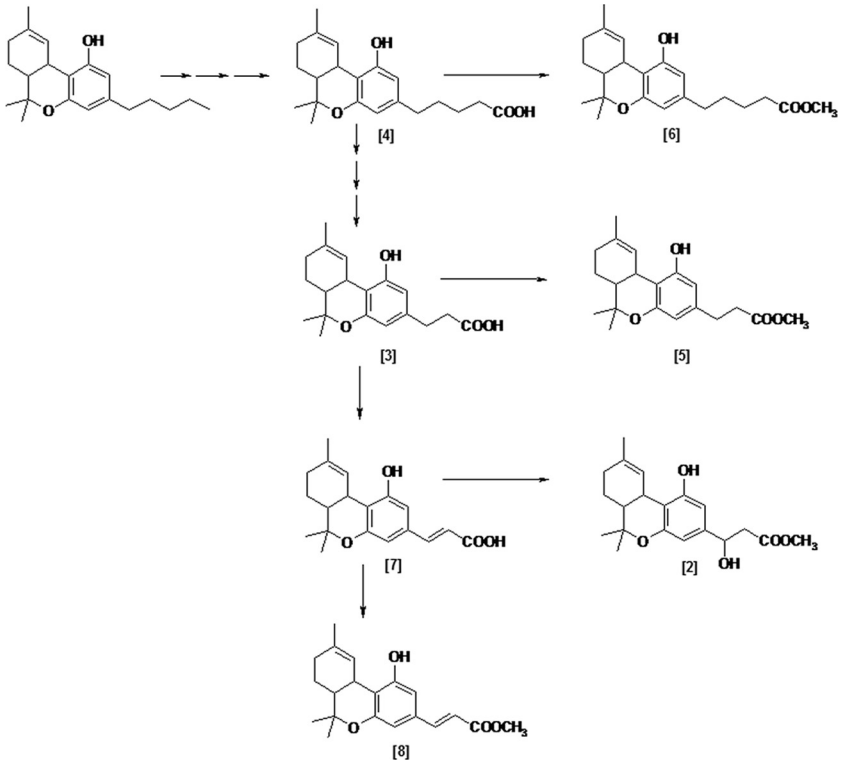


FIG. 2. Production of new derivatives from Δ^9-THC by *Mycobacterium* sp. strain ENZHR3.

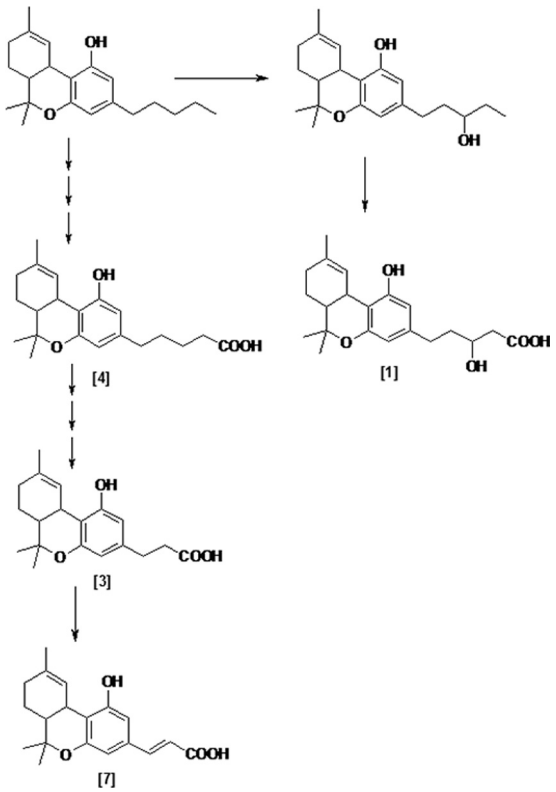


FIG. 3. Metabolites produced by *Gordonia* sp. strain ENZHR5 during biotransformation of Δ^9-THC.

bacterium sp. strain ENZHR3, compound 3 is one of the major products (35 to 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 compound 4 is totally dependent on incubation conditions. Production of compounds 5, 6, and 8 in *Mycobacterium* sp. strain ENZHR3 increased in a time-dependent manner, accompanied by decreasing amounts of compounds 3 and 4 (Fig. 2). *Gordonia* sp. strain ENZHR5 also showed a multiproduct pattern of production, although compounds 1, 3, and 7 were the most abundant metabolites formed (Fig. 3).

Solubility studies. The physicochemical properties of the derivatives were found to be significantly different from those of the parent compounds. For compounds 3 and 4, the water solubilities at pH 7 are up to 8.5 and 4.5 mM, respectively, which are dramatic increases in comparison to the solubilities of Δ^9-THC and cannabidiol (50 and 135 μM, respectively). Solubility of the methyl ester derivatives is also assumed to be improved based on their high-performance liquid chromatography retention times in comparison to the parent compounds. The double bond between C-1′ and C-2′ has a negative effect on solubility; for example, compound 7 has a 1.4-fold-lower solubility than the saturated metabolite (6.2 mM for the dehydro metabolite).

The most frequently found metabolites were compounds 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, which were produced in high yields by *Gordonia* sp. strain 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 (12). The possible (pharmaceutical) application of the isolated derivatives is presently the subject of further study. The improved water solubilities (8.5 and 4.5 mM for compounds 3 and 4, respectively) 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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