Microbiological transformation of cannabinoids

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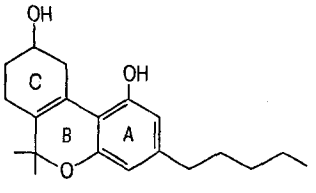
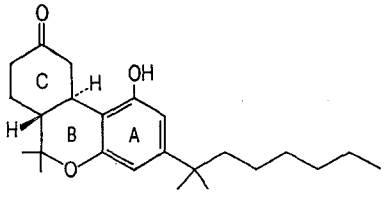
Summary. Microorganisms were screened for their ability to modify 2 synthetic cannabinoid substrates (**I** and **II**). Structure analyses revealed that microorganisms transformed the substrates by (a) primary oxidation of the side chain, β-oxidation of the side chain, ketone formation on the side chain or cyclohexene ring, (b) secondary hydroxylation on the side chain, (c) aromatization of the cyclohexene ring, and (d) tertiary hydroxylation at the b/c ring juncture.

The naturally-occurring cannabinoids, exemplified by Δ^9-THC the psychotomimetic principal of Cannabis sativa L., possess a number of interesting pharmacological activities. The potential clinical effectiveness of Δ^9-THC[1] in a number of disease states (e.g. anxiety, asthma, glaucoma, hypertension, etc.) would be hampered by the undesirable pharmacological side effects that THC possesses (e.g. euphoria, tachycardia, etc.). In attempts to separate the desirable activities from other actions or undesirable side effects, new cannabinoids have been made a) by de novo synthesis and b) by chemical modification of naturally-occurring cannabinoids[2]. Evolving from these efforts is a new group of synthetic cannabinoids which show potential as clinically useful agents[3–5]. Microorganisms may serve as important adjuncts to and extensions of synthetic chemical approaches by producing interesting new cannabinoids that would be difficult to prepare by other methods. To examine this potential, we chose the following compounds for biotransformation studies: $\Delta^{6a,10a}$-THC (**I**) and nabilone (**II**).



I



II

Compound **I** was available in large quantities and represents a basic THC typical of a series of synthetic compounds prepared by Adams and Todd in the 1940's and 1950's[2]. Nabilone is one of a new group of synthetic cannabinoids that have emerged from the synthetic efforts of the Lilly Research Laboratories. Its pharmacological profile in animals has been studied and it is currently undergoing clinical evaluation as a potential antianxiety agent[5].

The microorganisms used in this study were randomly selected soil isolates. Among the 400 cultures screened were bacteria, actinomycetes, and molds. Additionally, 22 alkane-metabolizing cultures were screened because they might metabolize the polymethylene side chain of **I** or **II**.

In the initial screen, microorganisms were grown in 10 ml of a nutrient medium in 50 ml Erlenmeyer flasks incubated at 25–30 °C on a rotary shaker. After 30 h incubation, 0.1 ml of ethanol containing 4 mg of either **I** or **II** was added to each culture. Control samples were prepared by sterilization (121 °C, 30 min) of a duplicate sample of each culture prior to addition of the substrate. Both sets of flasks were then incubated for an additional 96 h. Each flask (including controls) was extracted 3 times with ethyl acetate. The 3 extracts were combined, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure to give the crude product extracts. The extracts were examined by thin layer chromatography [silica gel 60 F254 plates developed in benzene: ethyl acetate (9:1) for conversion products of **I** and benzene: ethyl acetate (1:1) or benzene: ethyl acetate: hexane: methanol (1.5: 8:1:0.5) for conversion products of **II**; products were visualized by short wave UV light or by spraying the plates with Fast Blue B]. The microorganisms that appeared, by the above screening procedure, to produce cannabinoid metabolites were cultivated on a larger scale to obtain sufficient quantities of the products for structure determinations. The larger scale incubations were done in similar manner to the above except that 1–3 l of the appropriate culture medium and 300 mg to 1 g of substrate were employed. The crude ethyl acetate extracts were purified by column chromatography on Woelm Silica Gel (Act. I) or by high pressure liquid chromatography (HPLC) using Porasil A (60) as the packing. Final purification of the conversion products was generally achieved by preparative thin layer chromatography. Tables 1 and 2 show the structures of microbial transformation products of **I** and **II**. The structural assignments are based on physical chemistry data (high resolution mass spectra, nuclear magnetic resonnance (NMR), IR, UV, optical rotatory dispersion, and titration) and where available, on a comparison of physical chemistry data with authentic compounds from chemical syntheses. Thus structures **III**, **VIII** and **IX** were established by comparison with spectral data available from authentic samples. The remaining 9 structural assignments are strongly supported by analysis of physical chemistry data especially high resolution mass spectrometry and 100 mHz NMR. For instance, high resolution mass spectrometry yielded the molecular weights and empirical formulae for these products. Comparison of these empirical formulae with those of the starting substrates **I** and **II** clearly indicated that all of these microbial transformation products represented mono-oxygenated derivatives of the starting materials. Establishing the position of microbial oxygenation required in most cases a careful NMR-analysis making ample use of decoupling experiments and reference cannabinoids. Details of these structural assignments will be published elsewhere.

1 The dibenzopyran number system is used. See table 1 for positions and numbers.

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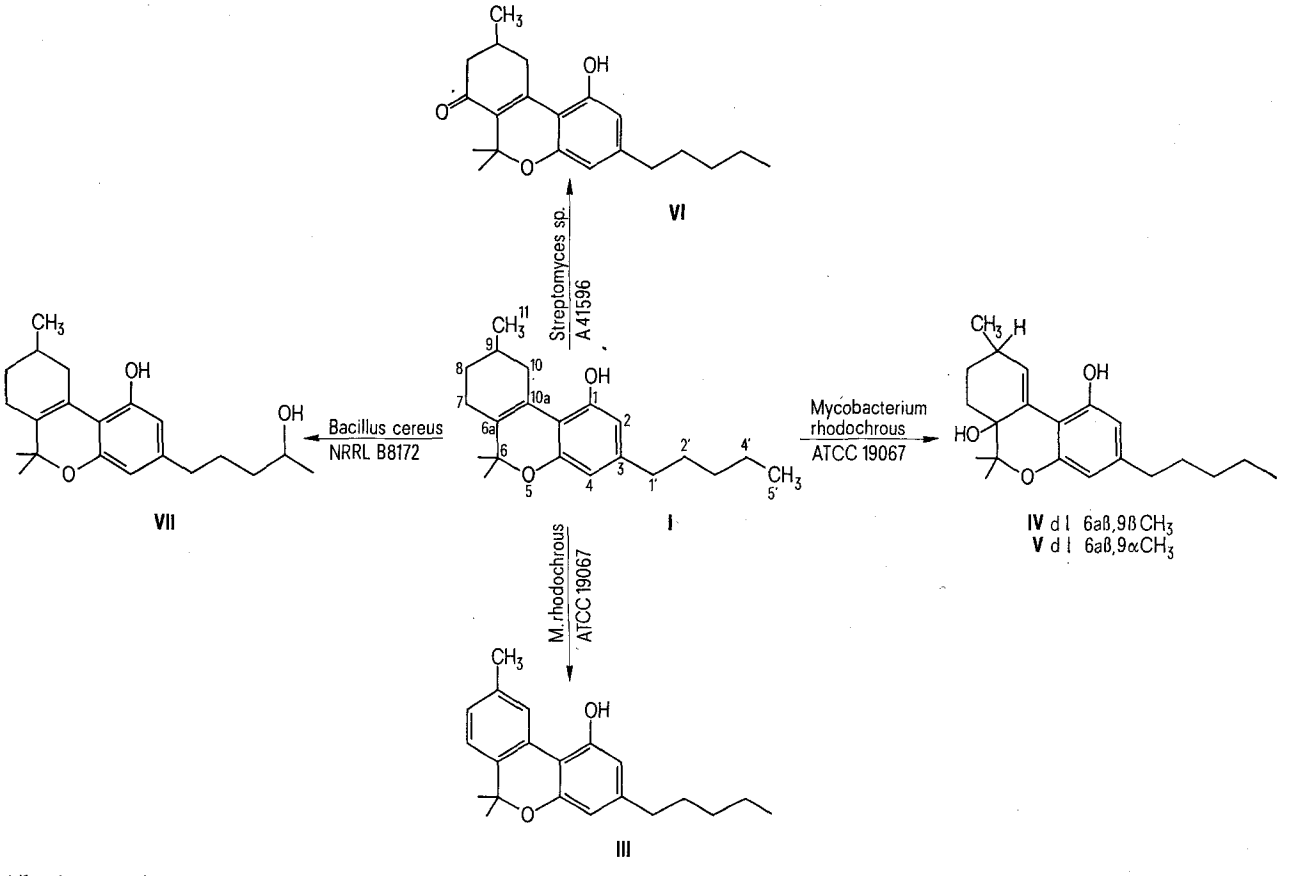
Although structures **III**, **VIII** and **IX** are available from chemical syntheses, all of the remaining microbial transformation products represent new and interesting cannabinoids which are not in most cases easily approachable by chemical syntheses. The amount of each product formed accounted for 1–5% of the amount of substrate (**I** or **II**) added to the culture. No attempts were made to enhance the production of any of the metabolites. However, a systematic study of the fermentation conditions could lead, as it has with other biotransformations, to higher product yields. The microbial reduction of **II** to a mixture of **VIII** and **IX** occured with several cultures in addition to those shown in table 2. With some of these microorganisms the yields of the reduction products of **II** were as high as 50%.

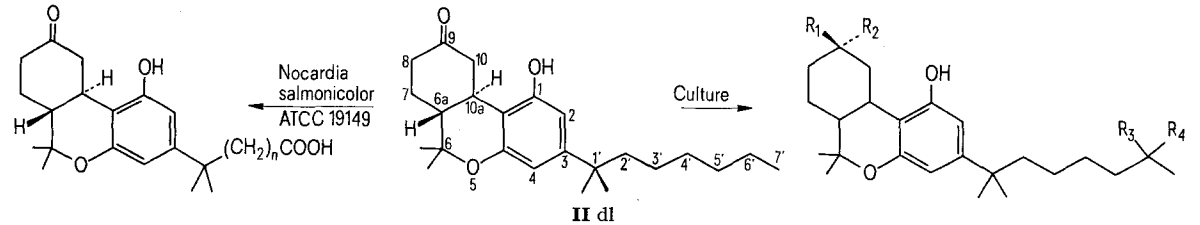
Since nabilone (**II**) is optically inactive, it exists as a 1:1 mixture of 6aR, 10aR and 6aS, 10aS optical isomers. Both products, **VIII** and **IX**, originate by stereospecific

Table 1. Microbial transformation products of $\Delta^{6a,10a}$-tetrahydrocannabinol (**I**)[1].



All microorganisms, except A41596, were cultivated in mineral salts medium[7] containing 3% glucose. Culture A41596 was grown in medium containing peptone and molasses (medium A from Boeck et al.[8]).

Table 2. Microbial transformation products of nabilone (**II**)[1].



Compound	n
XIV	3
XV	1

Compound	R_1 R^2	R_3 R^4	6a, 10a	Culture
VIII	H OH	H H	SS	B. cereus NRRL B8172
X	=O	=O	trans	NRRL B8172
XI	H OH	=O	trans	NRRL B8172
XII	=O	H OH	trans	NRRL B8172
XIII	H OH	H OH	trans	NRRL B8172
IX	H OH	H H	RR	Bacterium A24007

Culture A24007 was grown in trypticase soy broth (BBL). Culture ATCC 19149 was grown in a mineral salts medium containing 1–2% hexadecane and NRRL B8172 was grown in a similar medium that contained 3% glucose in place of hexadecane.

reduction of the 9-ketone of **II** the to 9S alcohols of **VIII** and **IX**. Compound **VIII** arizes from the stereospecific reduction of the 6aS, 10aS isomer of **II** and **IX** results from the stereospecific reduction of the 6aR, 10aR isomer of **II**.

Compounds **III**, **IV** and **V** occurred in control cultures albeit in amounts $< 10\%$ of that found in viable culture samples (the amounts of the transformation products were determined by scanning thin layer densitometry and by integration of gasliquid chromatograms). The small amounts of **III**, **IV** and **V** formed in the controls probably resulted from air oxidation of **I**.

The carboxylic acids, **XIV** and **XV**, were isolated from a hydrocarbon (alkane) oxidizing culture, Nocardia salmonicolor ATCC 19149. Notice that the alkyl side chain is either 2 or 4 carbon atoms shorter than the alkyl side chain of the starting substrate (**II**). These acids may arize from a β-oxidation mechanism similar to that reported for the microbial metabolism of alkanes[6]. By analogy to alkane metabolism, **II** may be converted by hydroxylation of the side chain terminal methyl group ($C_{7'}$) into an intermediate primary alcohol which is subsequently oxidized to a terminal carboxylic acid. Consistent with this mechanism, we have detected small amounts of other carboxylic acids and have made tentative structure assignments based on high resolution mass spectral data. Based on these assignments, the microbiological oxidation of **II** by culture 19149 appears to lead to 2 series of carboxylic acids: one containing a hydroxyl at $C_{9'}$ and carboxylic acid side chains of 5, 7, and 9 carbon atoms, respectively; the other series having a carbonyl at $C_{9'}$ and carboxylic acid side chains of 5, 7, and 9 carbon atoms, respectively.

The reactions described in this study may represent just a few of the many reactions that can be catalyzed using microorganisms. It is likely that microorganisms that modify **I** and **II** will catalyze similar reactions with other cannabinoid substrates. In addition, many other metabolites were detected in our screening program that have not yet been isolated or identified. Considering the reactions demonstrated and the potential that remains, we believe that microorganisms will play an important role in the development of new and possibly therapeutically useful cannabinoids.

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Isolation of 2-S-cysteinyldopa and 2,5-S,S-dicysteinyldopa from the urine of patients with melanoma[1]

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Summary. Urine from patients with melanoma metastases is shown to contain, in addition to the previously described 5-S-cysteinyldopa (**I**), 2 closely related metabolites which have been isolated and identified as 2-S-cysteinyldopa (**II**) and 2,5-S,S-dicysteinyldopa (**III**).

The urinary excretion of 5-S-cysteinyldopa (**I**) has been the object of extensive investigations showing its significance for the characterization of the metabolic activity of normal and pathological melanocytes[3,4]. In the pigment cell, this amino acid is formed by 1,6-addition of cysteine to dopaquinone and is subsequently converted to phaeomelanin pigments by oxidation, cyclisation, and coupling[5]. Normally only minute amounts of 5-S-cysteinyldopa are present in the urine, whereas a markedly increased level of excretion is observed in the urine of patients harbouring malignant melanoma[6].

In further scrutiny of melanoma urine for related metabolites of biochemical and/or clinical significance, we have isolated 2 additional catechol amino acids, 2-S-cysteinyldopa (**II**) and 2,5-S,S-dicysteinyldopa (**III**) (figure).

In a typical experiment, 850 ml of urine, collected for 24 h from a patient with melanoma metastasis, was adjusted to pH 1 with 6 M HCl and after filtration was passed through a column (1.8×10 cm) of Dowex 50 W X4, 200–400 mesh, H^+ form. After a prolonged washing with 0.5 M HCl (1000 ml), the column was eluted with 2 M HCl at a flow rate of 50 ml/h; 26 fractions of 40 ml each were collected and examined for the presence of catechol amino acids, both spectrophotometrically in the range 220–350 nm and chromatographically on precoated cellulose plates (Merck) with n-propanol-1 M HCl (3:2, v/v)[7], using as detecting reagents a ninhydrin solution and 3% ethanolic $FeCl_3$.

Fractions 1–8 forming the major elution peak were pooled and evaporated to dryness under reduced pressure to give a colourless residue which was taken up in water and rechromatographed on a Dowex 50 W column (1.8×10 cm), equilibrated with 2 M HCl. Elution with the same acid solvent gave a major peak fraction (57 mg) corresponding to 5-S-cysteinyldopa (**I**) and a faster moving band with a very similar UV spectrum. Further purification of this minor fraction on Whatman 3 MM paper with n-butanol-

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