Microbial Metabolism. Part 5.[1] Dihydrokawain

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Preparative scale fermentation of (6*S*)-dihydrokawain (1) with *Rhizopus arrhizus* (ATCC 11145) gave 3′-hydroxydihydrokawain (2) and (8*S*)-hydroxydihydrokawain (3). Structure elucidation of the metabolites was based on spectroscopic data. The C-8 absolute configuration of (3) was assessed *via* its Mosher's esters.

Key words dihydrokawain; microbial metabolism; kavalactone; (8*S*)-hydroxydihydrokawain

Piper methysticum FORST (Piperaceae) is a South Pacific plant used as an intoxicating beverage by the natives.[2] It is commonly referred to as kava-kava or kava. Investigations of kava preparations have demonstrated several biological activities.[3—6] Preparations are used in Europe and North America to manage mild anxiety disorders.[6] Chemical investigations of kava have led to the isolation and characterization of 19 styryl-α-pyrones (kavalactones) which are believed to be responsible for the pharmacological activities.[8] The most abundant of these lactones are desmethoxyyangonin, yangonin, dihydrokawain, kawain, dihydromethysticin and methysticin.[9]

Centuries of use of kava-kava by the South Pacific islanders showed no serious health risks. The standardized extracts of kava-kava prepared in Europe on the other hand had been shown to cause hepatotoxic effects.[10] This has been attributed to differences in the extraction procedures. Traditionally, the kava-extracts are prepared by macerating the roots of *P. methysticum* with water and coconut milk.[11] This procedure extracts kava lactones and glutathione in a 1 : 1 ratio.[12] In the standard extraction method the use of 60% aqueous ethanol or acetone results in a kava lactone concentration of over 30 times that found in the traditional extracts. It has been suggested that the resulting high concentration of lactones could saturate the detoxification pathways leading to hepatotoxicity. In addition, such extracts do not contain the tripeptide, glutathione, which plays a major role in the conversion of lactones into excretable products.[12] In another study which has demonstrated the formation of electrophilic quinoid metabolites when kava was incubated *in vitro* with hepatic microsomes, has led to the suggestion that the production of these quinoids might contribute towards hepatotoxicity in humans when metabolic pathways are altered.[13]

To evaluate the safety and efficacy of kava, knowledge of how its active constituents are metabolized is important. One such investigation[14] led to the detection of human urinary metabolites of the kava lactones following the consumption of kava prepared by the traditional method. Transformations observed were the reduction of the enolic 3,4-double bond and/or demethylation of the 4-methoxy group. In rat, however, in addition to metabolism occurring at the aromatic ring to form phenolic products, compounds resulting from the cleavage of the 5,6-dihydro-α-pyrone ring had been observed.[15] In both cases, unconverted kava lactones were detected in high concentrations.

The importance of using microbial models to generate mammalian metabolites of biologically active compounds is well documented.[16] The quantities of metabolites are usually sufficient to carry out complete structure elucidations and further pharmacological evaluations. The metabolites can furthermore be used as standards for detection in biological fluids. In the mammalian experiments cited above, the absence of such standards has led to the proposition of tentative structures to the metabolites, based on GC-MS data. Microbial transformations of the kava lactones, kawain (by *Cunninghamella elegans* ATCC 9245) and methysticin (by *Torulopsis petrophilum* ATTC 20225) resulted in the formation of the phenolic products, 4′-hydroxykawain and 3′-hydroxy-4′-methoxykawain, respectively.[17] The former may be identical to the metabolite detected in rat urine.[15]

As part of our program on microbial metabolism,[1] we now report the isolation and structure elucidation of 3′-hydroxydihydrokawain (**2**) and (8*S*)-hydroxydihydrokawain (**3**) formed when dihydrokawain (1) was metabolized by *Rhizopus arrhizus*.

Several of the 30 organisms screened showed the ability to transform dihydrokawain (**1**). Higher transformation efficiency led to the selection of *Rhizopus arrhizus* (ATCC 11145) for the preparative stage. The metabolites formed were 3′-hydroxydihydrokawain (**2**) (20 mg, 4% yield) and (8*S*)-hydroxydihydrokawain (**3**) (60 mg, 12% yield). These compounds are more polar than **1** and may contribute to the elimination of the parent compound from the human system.

High resolution electrospray ionization mass spectrometry (HR-ESI-MS) of **2** showed a molecular formula of $C_{14}H_{16}O_4$ indicating it to be a monoxygenated derivative of **1**. IR absorption due to hydroxyl was observed at 3310 cm^{-1}. The ^{1}H-NMR spectrum was similar to that of **1** except for reduction of the number of aromatic protons from five to four. The spectrum of **2** displayed the resonances of a 1,3-disubstituted phenyl ring indicating that *m*-hydroxylation of the aromatic ring in **1** had occurred. A singlet carbon signal at δ 154.6 corresponds to C-3′ in the ^{13}C-NMR spectrum. In the CD spectrum of **2**, a high amplitude positive Cotton effect at 249 nm permitted the assignment of the (6*S*)-configuration[18] indicating that the stereocenter did not undergo configurational change during microbial transformation. Thus, metabolite **2** was characterized as 3′-hydroxydihydrokawain.

The second metabolite (**3**) (60 mg, 12% yield) showed the same molecular formula ($C_{14}H_{16}O_4$) as that of **2**, indicating that it was also a monoxygenated product of **1**. This was supported by the presence of an –OH absorption band at 3402

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Table 1. ^{1}H and ^{13}C Data of Compounds **2** and **3**

Position	**2**		**3**	
	δ_H (J Hz)	δ_C	δ_H (J Hz)	δ_C
2	—	168.6	—	167.9
3	5.16, 1H, d, (1.5)	90.4	5.17, 1H, d, (1.5)	90.4
4	—	173.6	—	173.5
5	2.51, 1H, dd, (17.0, 4.0)	33.2	2.51, 1H, dd, (17.0, 4.0)	33.5
	2.32, 1H, dd, (17.0, 4.0)		2.37, 1H, dd, (17.0, 4.0)	
6	4.37, 1H, m	75.2	4.80, 1H, m	73.1
7	2.12, 1H, m, 1.91, 1H, m	36.2	2.10, 1H, m, 1.98, 1H, m	44.4
8	2.78, 1H, m, 2.73, 1H, m	31.0	5.13, 1H, dd, (10.0, 3.0)	69.5
1′	—	142.6	—	144.6
2′	6.74, 1H, br s	115.7	7.39, 1H, m	125.8
3′	—	154.6	7.35, 1H, m	128.8
4′	6.81, 1H, br d, (8.0)	115.8	7.29, 1H, m	127.8
5′	7.16, 1H, br t, (8.0)	129.9	7.35, 1H, m	128.7
6′	7.06, 1H, br d, (8.0)	129.7	7.39, 1H, m	125.8
4-OMe	3.75, s	56.4	3.75, s	56.3

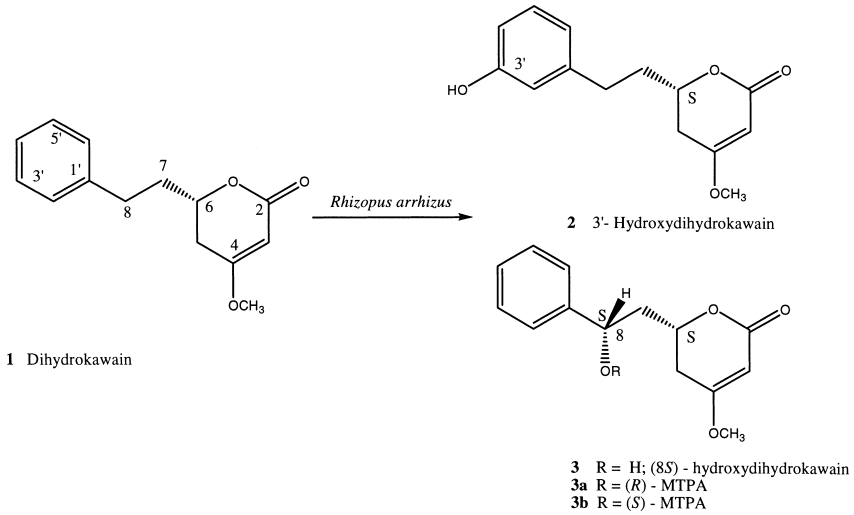

Fig. 1. Metabolites of Dihydrokawain (**1**) and the MTPA Esters **3a** and **3b** of (8*S*)-Hydroxydihydrocarwain (**3**)

cm^{-1} in the IR spectrum. The position of the hydroxyl group at C-8 in **3** was determined by comparing ^{1}H- and ^{13}C-NMR data of **1** and **3** which showed close similarities. The ^{1}H-NMR spectrum of **3** differed from that of **1** in the disappearance of multiplets at δ 2.84 (H-8) and δ 2.73 (H-8), the collapse of multiplets at δ 2.10 and δ 1.98 to double doublets and the appearance of H-8 as a double doublet at δ 5.13. The ^{13}C-NMR spectrum indicated the disappearance of the C-8 methylene carbon signal at δ 31.0 in **1** and the presence of an oxymethine signal at δ 69.5. In the CD spectrum of **3**, a high amplitude positive Cotton effect at 255 nm region permitted assignment of (6*S*)-configuration[18] indicating that, like **2**, the C-6 stereocenter did not undergo configurational change during microbial transformation. Thus, metabolite **3** is 8-hydroxydihydrokawain. The absolute configuration at C-8 was established *via* the Mosher's ester method[19–21] in conjunction with NMR analysis of derivatives **3a** and **3b**. Selective shielding of the H-5, H-6 and H-7 resonances in the (*S*)-MTPA ester **3b** compared to their chemical shifts in the (*R*)-MTPA ester **3a**, permitted assignment of *S*-configuration at C-8 (Fig. 2). Thus, the structure of metabolite **3** was established as (8*S*)-hydroxydihydrokawain. Compound **3** could be

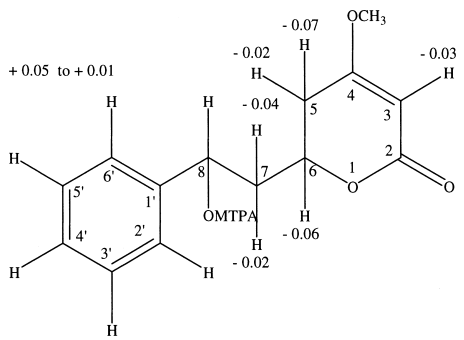

Fig. 2. $\Delta\delta^{SR}$ Values for MTPA Derivatives **3a** and **3b**

identical to that detected in the urine of rats fed with kavalactones.[15] The unidentified phenol in rat urine, however could not be compared with **2** since no data has been recorded for the former compound. Products formed *via* demethylation of the 4-methoxy group of the α-pyrone ring system observed in human urine[14] were also not detected in our experiments.

Thus, the microorganism *R. arrhizus* possesses the capac-

ity to convert dihydroxykawain (**1**) into 3′-hydroxydihydrokawain (**2**) and (8*S*)-hydroxydihydrokawain (**3**) in quantities sufficient to fully characterize the compounds and for future pharmacological studies.

Experimental

General Experimental Procedures IR spectra were run in $CHCl_3$ using an ATI Mattson Genesis series FTIR spectrophotometer. UV spectra were recorded on a Hewlett Packard 8452A diode array spectrometer. Optical rotations were measured with a Jasco DIP-370 digital polarimeter and CD measurements on a Jasco J-710 instrument in MeOH. ^{1}H- and ^{13}C-NMR were recorded in $CDCl_3$ on a Varian Unity Inova 600 spectrometer. HR-ESI-MS data were obtained using a Bruker GioApex 3.0.

Substrate Dihydrokawain was isolated from a methanol extract of the aerial parts of *Piper methysticum*. Its authenticity was established by physical methods including NMR and HPLC.

Organisms and Metabolism 31 Microorganisms from the collection of the National Center for Natural Products Research, University of Mississippi, were used to identify organisms capable of metabolizing dihydrokawain (**1**). A medium (referred to as medium-α)[17] consisting of dextrose, 20 g; NaCl, 5 g; K_2HPO_4, 5 g; bacto-peptone (Difco Labs, Detroit, MI), 5 g and yeast extract (Difco Labs), 5 g per liter of distilled water was used to carry out fermentations. Initial fermentations were conducted in 125 ml Erlenmeyer flasks containing 25 ml medium-α. The usual two-stage fermentation procedure was adopted.[22] **1** was added in dimethylformamide (0.5 mg/ml) to 24 h old stage II cultures. Incubation was at room temperature on a rotary shaker (New Brunswick Model G10-21) at 100 rpm for a period of 14 d. Sampling was carried out at seven-day intervals. Monitoring was by TLC using precoated Si gel 60 F_{254} plates (E. Merck) with EtOAc–hexane (3 : 2) as the solvent system. Spots were visualized by UV light (254 and 365 nm) and *p*-anisaldehyde as the spray reagent. Scale-up fermentations were performed under the same conditions with five 21 flasks, containing 500 ml of medium-α and 100 mg of substrate, each. Extractions of the culture filtrates and residues were carried out with EtOAc. The solvent was evaporated *in vacuo* at 40°C to obtain the residues. The isolation of metabolites was by column (Silica gel 230—400 mesh: E. Merck) and preparative thin layer (Silica gel 60 F_{254}) chromatography. Culture and substrate controls were run simultaneously with the above experiments.[17]

Microbial Metabolism of Dihydrokawain (1) by *R. arrhizus* The filtrate of the combined fermentation broth was exhaustively extracted with EtOAc. Evaporation of the solvent gave a light brown gummy mixture (500 mg). It was column chromatographed (Si gel 230—400 mesh: E. Merck, 30 g, column diameter: 20 mm.) using $CHCl_3$ gradually enriched with MeOH.

The fractions were combined and further purified by column and preparative layer chromatography (EtOAc–hexane, 3 : 2). 3′-hydroxydihydrokawain (**2**) was isolated as a white amorphous solid (20 mg, 4% yield). $[\alpha]_D^{26}$ +0.05° (c=0.74, MeOH). *Rf* 0.4; ^{1}H- and ^{13}C-NMR: see Table 1. UV λ_{max} (MeOH) nm (log ε): 206 (4.41), 226 (4.47), 276 (2.84). CD (MeOH) $[\theta]_{250}$=+52.7; IR ν_{max} ($CHCl_3$) cm^{-1}: 3310, 2925, 1677, 1619, 1516, 1456, 1397, 1227, 1041, 823. HR-ESI-MS $[M+Na]^+$: (m/z) 271.0958 (Calcd for $C_{14}H_{16}O_4$+Na: 271.09482).

(8*S*)-Hydroxydihydrokawain (**3**) was purified as a white amorphous solid (60 mg, 12% yield). $[\alpha]_D^{26}$ +0.05° (c=0.320, MeOH). *Rf* 0.3; ^{1}H- and ^{13}C-NMR: see Table 1. UV λ_{max} (MeOH) nm (log ε): 208 (4.21), 236 (4.09), 320 (3.68). CD (MeOH) $[\theta]_{256}$=+37.2. IR ν_{max} ($CHCl_3$) cm^{-1}: 3404, 2924, 1704, 1623, 1494, 1454, 1224, 1055, 825. HR-ESI-MS $[M+Na]^+$: (m/z) 271.0959 (Calcd for $C_{14}H_{16}O_4$+Na: 271.09482).

8-(*R*)-MTPA ester (**3a**) was prepared by adding (*S*)-(+)-α-methoxy-(trifluoromethyl)phenylacetyl chloride (MTPA chloride) (50mg) to **3** (5 mg) in dry pyridine (0.1 ml) under anhydrous conditions. The mixture was stirred at RT for 5 h. Completion of the reaction was determined by TLC monitoring. Chromatographic purification (EtOAc–hexane, 3 : 2) yielded the ester **3a**, as an amorphous white solid (8 mg). ^{1}H- and ^{13}C-NMR: see Table 2. HR-ESI-MS $[M+Na]^+$: (m/z) 487.1321 (Calcd for $C_{24}H_{23}F_3O_6$+Na: 487.1344).

Ester derivative (**3b**) was obtained as a white solid using (*R*)-(−)-α-methoxy-(trifluoromethyl)phenylacetyl chloride (MTPA chloride) under the same experimental conditions as above. ^{1}H- and ^{13}C-NMR: see Table 2. HR-ESI-MS $[M+Na]^+$: (m/z) 487.1314 (Calcd for $C_{24}H_{23}F_3O_6$+Na: 487.1344).

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