Microbial Transformation of Kawain and Methysticin

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Received June 5, 2000; accepted August 7, 2000

The styryl α-pyrones, *d*-kawain (1) and *d*-methysticin (2) are two of the major kavalactone constituents of the anxiolytic herb *Piper methysticum*, commonly known as kava. The use of fungal models to mimic the mammalian metabolism of 1 resulted in the production of 4′-hydroxykawain (1a) from the culture broth of *Cunninghamella elegans* (ATCC 9245), the same metabolite identified in rat urine. The fungus *Torulopsis petrophilum* (ATCC 20225) biotransformed 2 to 3′-hydroxy-4′-methoxykawain (2c) which is analogous, but not identical, to a known rat metabolite of methysticin.

Key words biotransformation; microbial transformation; *Piper methysticum*; kawain; methysticin; metabolism

Kava-kava, or kava, (*Piper methysticum* Forst., Piperaceae) is a South Pacific plant that has been used by indigenous peoples as an intoxicating beverage since ancient times.[1)] At present, various preparations of kava are marketed in Europe and North America to manage mild anxiety.[2)] Additional reports indicate that kava preparations may have analgesic,[3)] spasmolytic,[4)] neuroprotective[5)] and antimitotic activities.[6)] The main active constituents believed to be responsible for the pharmacological effects of *P. methysticum* are the styryl α-pyrones (kavalactones).[7)] The synthetic *dl*-form of one of the natural kavalactones, *d*-kawain (**1**), exhibits a synergistic sedative effect with kava extract and is marketed in Germany as a single entity for managing anxiety.[2)] *d*-Methysticin (**2**) is another major kavalactone that was reported to protect brain tissue against ischemic brain damage in a rat model.[5)] Various analytical techniques have been developed for the determination of **1**, **2**, and other kavalactones in kava products.[8)]

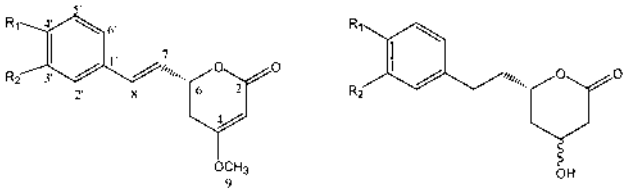
In addition to numerous pharmacological and mechanistic studies that aimed at corroborating the biological effects of *P. methysticum* extracts and isolates, two previous investigations have reported metabolites of **1** and **2** in rat and human urine.[9,10)] In both cases, however, unmetabolized kavalactones were detected in greater concentrations than any of their metabolites. The metabolites identified for **1** and for **2** were different for rats and humans. In rats, the metabolism of **1** and **2** occurred at the aromatic ring to produce the phenolic metabolites **1a** and **2a**, respectively. In humans, the major sites of metabolism were the styryl double bond (Δ^7) and the pyrone ring, resulting in the 3,4,7,8-tetrahydro-4-*O*-demethyl metabolites **1b** and **2b**, respectively (Chart 1).

In the present report, microbial models were utilized retrospectively in an attempt to generate the mammalian metabolites of **1** and **2**. Such metabolites can be used for further pharmacological evaluation alongside the already available kavalactones and also as analytical standards for detection and characterization of kava metabolites in biological fluids. Metabolite **1a** was identical to that detected in rat urine while metabolite **2c** was identical to a synthetic analog of **1**[11)] and analogous to the catechol rat metabolite **2b**.[9)] The analogy between **2b** and **2c** arises from the fact that both resulted from the hydrolysis of the methylenedioxy group of **2**, a well-documented metabolic reaction.[12)] It was not clear, however, whether **2c** resulted from a single step metabolic transformation, or *via* a multiple step pathway involving initial production of **2b** followed by *O*-methylation to **2c**. Although both metabolites **1a** and **2c** have been previously reported, no spectroscopic data were published for either compound. In this publication, we report the production of **1a** and **2c** *via* an alternative route and provide their full structure elucidation and spectroscopic data.

Twenty-one cultures were screened for their ability to biotransform **1** and **2** according to a standard two-stage procedure.[13)] TLC results showed that three fungi, *Cunninghamella blakeseana* (ATCC 8688a), *C. echinulata* (NRRL 3655) and *C. elegans* (ATCC 9245), produced the same metabolite **1a** from **1**. Of these, *C. elegans* was chosen for the preparative stage based on its relatively higher biotransformational efficiency as detected by TLC. For **2**, only one organism, *Torulopsis petrophilum* (ATCC 20225) produced a metabolite (**2c**).

Preparative scale fermentation of **1** by *C. elegans* provided metabolite **1a** in *ca*. 22% yield (based on 145 mg of biotransformed substrate). High resolution electrospray ionization mass spectrometric (HR-ESI-MS) analysis of **1a** provided a molecular formula of $C_{14}H_{14}O_4$ corresponding to a monoxygenated product of **1**. The IR spectrum showed a hydroxyl band at 3277 cm^{-1}. The ^{1}H-NMR spectrum of **1a** differed from that of **1** in the reduction of the total aromatic protons from **5** to **4** with the appearance of two doublets (AA′BB′ system, δ 6.82, 7.33) characteristic of a 1,4-disubstituted benzene and confirming the *p*-hydroxylation of the aromatic ring. Moreover, the ^{13}C-NMR spectrum of **1a** showed a singlet carbon signal at δ 158.0 corresponding to C-4′. Metabolite **1a** was thus characterized as 4′-hydroxykawain.[9)]

Preparative scale fermentation of **2** by *T. petrophilum*



1 R_1=R_2=H
1a R_1=OH, R_2=H
2 R_1, R_2=OCH_2O
2a R_1=R_2=OH
2c R_1=OCH_3, R_2=OH

1b R_1=R_2=H
2b R_1, R_2=OCH_2O

Chart 1. Structures and Major Metabolites of **1** and **2**

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Table 1. ^{1}H- and ^{13}C-NMR Data[a] of Compounds **1a**, **2** and **2c**

Position	**1a**[b]		**2**[c]		**2c**[b]	
	δ_C	δ_H (J Hz)	δ_C	δ_H (J Hz)	δ_C	δ_H (m, J Hz)
2	166.7 s	—	167.2 s	—	167.4 s	—
3	91.1 d	5.15 s	90.9 d	5.18 s	90.9 d	5.19 s
4	173.5 s	—	172.8 s	—	172.9 s	—
5	34.0 t	2.56 dd (17.1, 4.3)	33.8 t	2.51 dd (17.1, 4.4)	33.8 t	2.52 dd (17.1, 4.2)
		2.68 dd (18.0, 10.7)		2.64 ddd (17.1, 10.9, 0.8)		2.65 dd (17.1, 11.0)
6	77.0 d	5.03 m	76.4 d	5.01 m	76.6 d	5.02 m
7	124.3 d	6.20 dd (16.0, 6.7)	124.0 d	6.07 dd (15.8, 6.4)	124.1 d	6.10 dd (15.8, 6.4)
8	133.5 d	6.68 d (16.0)	133.4 d	6.62 d (15.8)	133.4 d	6.61 d (15.9)
9	56.7 q	3.80 s	56.5 q	3.75 s	56.5 q	3.75 s
10	—	—	101.6 t	5.95 s	56.4 q	3.88 s
1′	128.7 s	—	130.5 s	—	129.8 s	—
2′	129.0 d	7.33 d (8.5)	108.8 d	6.91 d (1.3)	112.5 d	7.00 d (1.7)
3′	116.4 d	6.82 d (8.5)	148.3 s	—	146.2 s	—
4′	158.0 s	—	148.5 s	—	147.4 s	—
5′	116.4 d	6.82 d (8.5)	106.2 d	6.75 d (8.0)	111.0 d	6.79 d (8.3)
6′	129.0 d	7.33 d (8.5)	122.2 d	6.81 dd (8.0, 1.3)	119.9 d	6.86 dd (8.3, 1.7
OH	—	8.64 s	—	—	—	5.85 s

Measured at 400 MHz (^{1}H) and 100 MHz (^{13}C). ^{13}C Multiplicities were determined by DEPT 135 experiments. *a*) Full NMR data of **1** and NMR data of **2** is included for comparison **2** have been previously reported.[15,16,17] *b*) In acetone-d_6. *c*) In $CDCl_3$.

yielded metabolite **2c** in 4% yield (based on 125 mg of biotransformed substrate). HR-ESI-MS analysis of **2c** provided a molecular formula of $C_{15}H_{16}O_5$ corresponding to a product of **2** with two more hydrogen atoms. The IR spectrum showed a strong hydroxyl band at 3364 cm^{-1}. The ^{1}H-NMR spectrum of **2c** resembled that of **2** in all aspects including the 3′,4′-disubstitution of the aromatic ring. However, the methylenedioxy singlet at δ 5.95 in **2** was replaced by another methoxyl singlet at δ 3.88 indicating the reductive cleavage of the methylenedioxy group. Long-range heteronuclear multiple bond correlation (HMBC) showed that the hydroxy and the methoxy groups were attached to the singlet carbons C-3 and C-4′ at δ 146.2 and 147.4, respectively. The exact position of the two substituents on the aromatic ring was determined by nuclear overhauser enhancement spectroscopy (NOESY) and HMBC experiments. The NOESY spectrum showed a correlation between the 4′-OMe protons (H-10) and H-5′, indicating that the methoxyl group was attached to C-4′. Also, the presence of an HMBC correlation between H-6′ and the singlet carbon at δ 147.4 (C-4′) and the absence of any correlation with the other singlet carbon at δ 146.2 (C-3′) further supported the NOESY data. Metabolite **2c** was thus determined as 3′-hydroxy-4′-methoxykawain.[11]

Experimental

General Experimental Procedure Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Optical rotations were measured using a Jasco DIP-370 digital polarimeter. UV spectra were obtained with a Hewlett Packard 8452A diode array spectrophotometer. The IR spectra were recorded with an ATI Mattson Genesis Series FTIR spectrophotometer. The ^{1}H- and ^{13}C-NMR spectra were obtained on a Bruker Avance DRX-400 FT spectrometer operating at 400 and 100 MHz, respectively. HR-ESI-MS analysis was conducted on a Bruker BioApex 3.0 mass spectrometer.

Chromatographic Conditions TLC: precoated Si 250F plates (Baker); developing system: hexane–EtOAc (3 : 7, v/v); visualization: UV light (254 nm), and *p*-anisaldehyde spray reagent. Column chromatography: Si gel 230—400 mesh (Merck).

Organisms and Metabolism Fungi were accessed from the culture collection housed in the National Center for Natural Products Research, University of Mississippi, and were originally obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, or from the National Center for Agricultural Utilization Research (NCAUR) (formerly Northern Regional Research Laboratories (NRRL)), Peoria, Illinois. Stock cultures were maintained on agar slants of media recommended by ATCC and were stored at 4 °C. All screening and scale-up fermentations were run in a complex culture medium (medium α) of the following composition: 5 g yeast extract (Difco Labs, Detroit, MI), 5 g bacto-peptone (Difco Labs), 5 g NaCl, 5 g K_2HPO_4, 20 g dextrose, and distilled H_2O to 1 l. For screening, cultures were grown in 25 ml of medium-α held in 125-ml Erlenmeyer flasks and equipped with stainless steel caps. Compounds **1** and **2** were separately added to 1-day-old stage II culture media as a 10% acetone solution (0.2 mg of substrate/ml of culture medium). Cultures were incubated at room temperature on a rotary shaker (New Brunswick Model G10-21) at 150 rpm for a maximum period of 14 d with sampling and TLC monitoring at three-day intervals. The production of **1a** and **2c** started at day 6 and 12, respectively. Preparative scale fermentations followed the same general procedure with the difference that, for each organism, 2 ml of 10% solutions of **1** and **2** in acetone were equally divided on 4 1-l Erlenmeyer flasks, each containing 250 ml of stage II culture of the respective organism (50 mg/flask). Incubation periods for the preparative scale fermentations were 9 and 14 d for **1** and **2**, respectively. Work-up followed a routine procedure[14] that comprised exhaustive EtOAc extraction of both culture filtrates and residues as determined by TLC. The combined biomass and filtrate extracts were concentrated *in vacuo* at 40 °C to yield residues that were subsequently subjected to column chromatography for metabolite isolation and purification. Appropriate substrate and culture controls were simultaneously run alongside each preparative scale fermentation.

Substrates Pure **1** and **2** were isolated from crude *P. methysticum* extract (Botanicals International, Long Beach, CA) and their identities were confirmed by comparing their chromatographic and spectroscopic characteristics with literature values.

Biotransformation of 1 with *C. elegans* At the end of the fermentation period, the media were filtered. Both the filtrate and biomass were exhaustively extracted with EtOAc to yield an orange residue (*ca*. 350 mg). The residue was chromatographed on a Si gel column using gradient elution (EtOAc in hexane, 10→100%). Unchanged **1** was recovered in earlier fractions (55 mg), then a white residue was obtained from later fractions. The residue was rechromatographed with the same solvent system and crystallized from hexane/EtOAc to yield metabolite **1a** (0.033 g, 22 %).

Biotransformation of 2 with *T. petrophilum* Fermentation media were filtered at the end of the 14-d fermentation period. Both the filtrate and biomass were exhaustively extracted with EtOAc to yield a brownish residue (*ca.* 300 mg), which was chromatographed on a silica gel column using the same gradient as before. Unchanged **2** was recovered in earlier fractions (75 mg). Late eluting semi-pure fractions were concentrated and purified by

preparative TLC to yield metabolite **2c** (0.005 g, 4%).

Metabolite **1a**, 4′-Hydroxykawain [6-(4′-Hydroxystyryl)-4-methoxy-5,6-dihydropyran-2-one]: Colorless needles; mp 165—166 °C; *Rf* 0.70; $[\alpha]_D^{25}$ +35.1° (*c*=0.02, MeOH); UV (MeOH) λ_{max} (log ε) 264 (3.85) nm; IR ($CHCl_3$) ν_{max} 3277, 3028, 2924, 2858, 1688, 1618, 1516 cm^{-1}; ^1H- and ^{13}C-NMR: see Table 1; HR-ESI-MS *m*/*z* $[M+Na]^+$: 269.0788 (Calcd for $C_{14}H_{14}O_4Na$: 269.0789).

Metabolite **2c**, 3′-Hydroxy-4′-methoxykawain [6-(3′-Hydroxy-4′-methoxystyryl)-4-methoxy-5,6-dihydropyran-2-one]: White residue; mp 139—140 °C; *Rf* 0.60; $[\alpha]_D^{25}$ +73.5° (*c*=0.02, MeOH); UV (MeOH) λ_{max} (log ε) 236 (4.05), 264 (4.01) nm; IR ($CHCl_3$) ν_{max} 3364, 3011, 2934, 2851, 1692, 1621, 1511 cm^{-1}; ^1H- and ^{13}C-NMR: see Table 1; HR-ESI-MS *m*/*z* $[M+H]^+$: 277.1092 (Calcd for $C_{15}H_{17}O_5$: 277.1076).

Acknowledgments We are grateful to Ms. Rangavalli B. Manyam for assistance with the fermentation and isolation procedures. Ms. Julie R. Mikell is acknowledged for providing the kavalactone substrates. This work was supported, in part, by the United States Department of Agriculture, Agricultural Research Service Specific Cooperative Agreement No. 58-6408-7-012.

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