Microbial Transformation of Kawain and Methysticin

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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) spasmylic,4) neuroprotective5) 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.8) Various analytical techniques have been developed for the determination of 1, 2, and other kavalactones in kava products.9)

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 (Δ1) 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.11) 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.11) TLC results showed that three fungi, Cunning-hamella 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 C14H14O4 corresponding to a monooxygenated product of 1. The IR spectrum showed a hydroxyl band at 3277 cm⁻¹. The 1H-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 13C-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

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yielded metabolite 2c in 4% yield (based on 125 mg of bio-transformed substrate). HR-EEl-MS analysis of 2c provided a molecular formula of C_{15}H_{16}O_{5} corresponding to a product of two with 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 \(\delta 5.95\) in 2 was replaced by another methoxy singlet at \(\delta 3.88\) 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 carbon at \(\delta 146.2\) and 147.4, respectively. The \(^1\)H-NMR spectrum showed a correlation between the 4\(^{-}\)-OMe protons (H-10) and the singlet carbon at \(\delta 147.4\) (C-4') and the absence of any correlation with the other singlet carbon at \(\delta 146.2\) (C-3') further supported the NOESY data. Metabolite 2c was thus determined as 3'-hydroxy-4'‐methoxykawain.\(^{13}\)

**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-EEl-MS analysis was conducted on a Bruker BioApex 3.0 mass spectrometer.

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

**Organisms and Metabolism** Fungi were accessed from the 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 \(m\)) of the following composition: 5 g yeast extract (Difco Labs, Detroit, MI), 5 g bacto-peptone (Difco Labs), 5 g NaCl, 5 g K\(_2\)HPO\(_4\), 20 g dextrose, and distilled H\(_2\)O to 1 l. 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 was determined as follows: 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 41-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 \(P.\) \textit{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 \textit{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 redissolved in EtOAc and crystallized from hexane/EtOAc to yield metabolite 1a (0.033 g, 22 %).

**Biotransformation of 2 with \textit{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; [α]D25 135.1° (c = 0.02, MeOH); UV (MeOH) λmax (log ε) 264 (3.85) nm; IR (CHCl3) νmax 3277, 3028, 2858, 1688, 1618, 1516 cm⁻¹; 1H- and 13C-NMR: see Table 1; HR-ESI-MS m/z [M+Na]⁺: 269.0788 (Calcd for C14H14O4Na: 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; [α]D25 73.5° (c = 0.02, MeOH); UV (MeOH) λmax (log ε) 236 (4.05), 264 (4.01) nm; IR (CHCl3) νmax 3364, 3011, 2934, 2851, 1692, 1621, 1511 cm⁻¹; 1H- and 13C-NMR: see Table 1; HR-ESI-MS m/z [M+H]⁺: 277.1092 (Calcd for C15H17O5: 277.1076).

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References