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Piperidine alkaloids from *Piper methysticum*

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Abstract

Pipermethystine (**1**), 3 α ,4 α -epoxy-5 β -pipermethystine (**2**) and awaine (**3**) were isolated from the aerial parts of kava (*Piper methysticum* G. Forster, Piperaceae) and identified by HRMS and NMR spectroscopic analysis. **1** was concentrated in the stem peelings and leaves. **2** and **3** are new alkaloids with **2** found only in cv. Isa among the 11 cultivars examined, and **3** occurred primarily in young leaves of all cultivars. The stem peelings have been used in recent years as a source of kavalactones in kava dietary supplement industry. Quantitative aspects of these piperidine alkaloids in *P. methysticum* and their potential activities on human physiology are discussed.

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1. Introduction

The root of the kava shrub (*Piper methysticum* G. Forster, Piperaceae) is the source of perhaps the most important traditional beverage for many South Pacific Island peoples. This relaxing drink is prepared for ceremonial and recreational purposes (Lebot et al., 1997). During the past decade, kava has become a popular remedy in Europe (Dentali, 1997) and North America (Blumenthal, 1999) due to its anxiolytic properties (Bilia et al., 2002). Tablets, capsules and tinctures prepared from the lipophilic extracts have been widely available as non-prescriptive botanical dietary supplements. As a result, kava became an important economic crop throughout the South Pacific (Davis and Brown, 1999). Since 1998, however, several European countries reported cases of liver damage allegedly due to medicinal usage of kava (Stoller, 2000). The products were subsequently banned in Germany (BfArM, 2002) and several other countries around the world.

Extensive work has been done on the chemistry and physiological effects of kavalactones, also known as

styryl α -pyrones, the major lipophilic natural products in roots. They have been found to be responsible for the anxiolytic effect (Schulz et al., 1998), and kava with high kavalactone content is generally considered to be of high quality. One cepharadione (Jaggy and Achenbach, 1992) and two pyrrolidine alkaloids (Achenbach and Karl, 1970) have also been isolated and identified from kava roots as minor components. Their physiological activity is unclear.

Since the major plant parts in traditional use of kava have been the rootstock and roots, far less research has been done on the chemistry of the aerial parts. In recent years, however, stem peelings are included as a raw material in kava commerce (Bennett, 2002 personal communication; NIH, 1998) due to the high demand by the pharmaceutical industry. Also, leaves and branches have been used in folk medicine (Cambie and Ash, 1994; WHO, 1998) primarily for topical applications and kava leaf tea has appeared in health food stores in recent years in Hawaii.

Smith (1979, 1983) discovered the alkaloid pipermethystine (**1**; Fig. 1) in leaves and stems of kava. Other research on aerial parts of kava includes kavalactone content (Siméoni and Lebot, 2002; Lebot et al., 1999) and receptor binding (Dinh et al., 2001). Interestingly, compared to root extracts, the leaf extracts showed

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stronger interactions with several CNS receptors in vitro (Dinh et al., 2001).

This study reports the presence of alkaloids in relatively high concentrations in the aerial parts of kava plants. Two new piperidine alkaloids, 3 α ,4 α -epoxy-5 β -pipermethystine (**2**) and awaine (**3**) are isolated and identified in the stem peelings and unopened young leaves, respectively. Distribution and possible toxicological implications of these and the known alkaloid pipermethystine (**1**) are discussed.

2. Results and discussion

2.1. Pipermethystine (**1**)

2.1.1. Stems

Alkaloid **1** has been reported as a minor compound in the stems based on its relative GC peak area, and it was absent from roots (Smith, 1983). In the present study, mature basal stem segments (0–20 cm above the rootstock) were cut and the green to dark purple peelings, which contained primarily epidermis and cortex tissues, were separated by knife from the woody inner part. **1** was found to be concentrated in these stem peelings, as determined by GC using a standard curve, with an average content of 0.23% (wt/dry wt) (Table 1). Isolated as a colorless oil, its identity was confirmed by comparison to the existing data (Smith, 1979). Three known kavalactones, 7,8-dihydrokavain, 7,8-dihydromethysticin and 5,6,7,8-tetrahydroangonin were also found as major volatile components in the peelings. In the peeled stems, however, only negligible amounts of alkaloids were detected, but the kavalactone profile was similar to that of the roots (data not shown).

Stem peelings as a commercial product consist of crude shavings of the outer stem, often including part of

the woody tissue. In Fiji, this product is explicitly referred to as *civi-civi* (Bennett, 2002, personal communication), but in other regions basal stem peelings are sold as part of the ‘peelings of the rootstock’. These products served as popular raw material for the pharmaceutical industry (Secretariat of the Pacific Community, 2001). In commercial *civi-civi* (cultivar unknown), only traces of **1** were present together with the major kavalactones commonly found in roots (data not shown). However, concentrations of **1** can be as high as 0.85% in the freshly prepared peelings as shown in Table 1. The discrepancy may be attributed to cultivar differences, the instability of the alkaloid, and greater thickness, resulting in a higher proportion of woody tissue in the commercial peelings.

2.1.2. Leaves

Alkaloid **1** has previously been isolated and identified as a major constituent (0.17% yield) in the dried leaves (Smith, 1979). In the present report, **1** was determined in unopened and the adjacent opened leaves in all 11 cultivars examined (Table 1). Concentrations ranged from 1.02 to 2.43% (wt/dry wt) in the former, and from 0.32 to 2.29% in the latter, respectively.

2.2. 3 α ,4 α -Epoxy-5 β -pipermethystine (**2**)

The novel epoxy alkaloid **2** was found only in *P. methysticum* cv. Isa, and was not detectable in the other 10 cultivars (Table 1). HRMS data corresponded to the molecular formula C₁₆H₁₇NO₅. The EIMS fragmentation pattern indicated a structural similarity to compound **1**. The presence of a 172 fragment in the MS spectrum of **2**, which corresponded to the 156 fragment in the MS spectrum of **1**, suggested the structure of **1** with an epoxide ring. ¹H and ¹³C NMR spectroscopic data (Table 2) showed epoxidation at 3,4 position, δ

Table 1
Distribution of pipermethystine (**1**), 3 α ,4 α -epoxy-5 β -pipermethystine (**2**), and awaine (**3**) in aerial *Piper methysticum* (% wt/dry wt)

Cultivar	Origin	Stem peelings ^a			Leaf blade, unopened			Leaf blade, opened		
		1	2	3	1	2	3	1	2	3
1. Apu	Hawaii	0.12	– ^b	–	1.57	–	1.45	1.70	–	0.03
2. Hiwa	Hawaii	0.39	–	–	1.40	–	0.88	2.29	–	–
3. Isa (PNG)	Papua New Guinea	0.85	0.93	–	2.43	0.36	2.67	0.84	0.26	–
4. Iwi	Papua New Guinea	0.38	–	–	1.14	–	1.51	0.32	–	–
5. Kumakua	Hawaii	0.09	–	–	1.03	–	0.73	1.04	–	–
6. Mahakea	Hawaii	0.13	–	–	1.57	–	0.16	1.60	–	–
7. Moi	Hawaii	0.25	–	–	1.61	–	1.17	2.19	–	0.05
8. Nene	Hawaii	t ^c	–	–	1.02	–	0.82	0.44	–	–
9. Nene ele ele	Hawaii	0.11	–	–	1.18	–	1.57	0.34	–	0.09
10. Rahmedel	Pohnpei	0.06	–	–	2.08	–	0.57	1.26	–	–
11. SIG	Hawaii	0.11	–	–	2.17	–	1.58	1.36	–	0.02

^a Obtained from basal stem (0–20 cm above ground).

^b –Not detectable.

^c t, traces (<0.02%).

52.5 (C-3) and δ 53.4 (C-4) of pipermethystine in comparison to the existing data (Arrayás et al., 2001). The relative stereochemistry at C-3, C-4 and C-5 were determined by comparing ^1H – ^1H coupling constants. The *syn* relationship for H-3 and H-4 was established by a 4 Hz coupling constant observed between the two protons. A four bond coupling constant ($J=1.4$ Hz) observed between H-4 and H-6 β placed the two protons at equatorial positions in the six membered ring. Finally, small coupling constants observed for H-5 and H-6 $\alpha\beta$ indicated that H-5 was in the equatorial position. **2** is therefore 3 α ,4 α -epoxy-5 β -pipermethystine, or 2-piperidinone, 1-(1-oxo-3-phenylpropyl)-3 α ,4 α -(epoxy)-5 β -(acetyloxy).

The concentration of **2** in the dried peelings was 0.93%. The ratio between **1** and **2** was ca. 1:1 (wt/wt) in the basal stem peelings of 'Isa'. Lower concentrations of **2** were found in unopened (0.36%) and adjacent opened (0.26%) leaves (Table 1).

The cultivar Isa, also known as 'PNG' in Hawaii, appears to be unique in containing this epoxy alkaloid. 'Isa' has been introduced to Hawaii from Papua New Guinea, where it has been used only occasionally for drinking purposes since it causes prolonged nausea (Lebot and Lévesque, 1989). However, as a source of pharmaceuticals, 'Isa' has recently gained popularity. In Hawaii, it is the only cultivar currently known to be less affected by kava dieback, a devastating viral disease (Nelson, 2000).

Two 7,8-epoxidized kavalactones have previously been isolated from kava roots and recently been linked to hepatotoxicity of kava (Ono, 2002). Unlike the alkaloidal epoxides in 'Isa' stem peelings, however, concentrations of these two lactonic epoxides in roots were very low.

2.3. Awaine (**3**)

Kava is called 'awa in Hawaiian, hence the name awaine. This new alkaloid was obtained as a colorless oil with the molecular formula $\text{C}_{14}\text{H}_{17}\text{NO}_2$, based on HRMS. Initially, the ^1H and ^{13}C NMR spectral data (Table 2) indicated that **3** was a mixture of two compounds, however, closer examination of the data revealed the existence of two conformers, which were present in a 3:2 ratio.

^1H – ^1H gCOSY and ^1H – ^{13}C gHSQC spectra indicated that **3** consisted of two fragments, a 1-oxo-3-phenylpropyl portion, which was also present in **1** and **2**, and a 4-hydroxypiperidine moiety. Sequential COSY correlations from H-2 to H-6 followed by a gHMBC correlation from H-2 to C-6 completed the structure of the piperidine unit. Although there was no direct NMR spectroscopic evidence to connect the two fragments in **3**, the amide bond at C-7 was established by the presence of conformers in the ^1H and ^{13}C NMR spectra. A mixture of conformers has been shown to be a result of the *cis/trans* isomerization of tertiary amides in peptides (Kofron et al., 1992; Nogle et al., 2002). Thus confirming structure **3** for awaine, or Δ^2 -piperidine, 1-(1-oxo-3-phenylpropyl)-4-(hydroxy). Oddly enough, the optical rotation for a sample of **3** was negligible indicating that the compound was racemic.

3 Was found in all 11 kava cultivars examined. Awaine occurred primarily in the unopened leaves at a concentration range from 0.16 to 2.67% (Table 1). When leaves of different developmental stages from the same branch were compared, the concentration reduced sharply; only about half remained in the partially

Table 2
 ^1H NMR and ^{13}C NMR spectroscopic data (acetone- d_6) for 3 α ,4 α -epoxy-5 β -pipermethystine (**2**) and awaine (**3**)

C#	3 α ,4 α -epoxy-5 β -pipermethystine			Awaine			
	δ_{C}	δ_{H} (J in Hz)	C#	Major conformer		Minor conformer	
				δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)
2	169.2	—	2	127.3	6.83, <i>d</i> , 8.2	125.7	7.17, <i>d</i> , 8.2
3	52.5	3.67, <i>d</i> , 4.0	3	110.6	4.97, <i>dd</i> , 8.2, 4.0	111.2	5.08, <i>dd</i> , 8.6, 4.5
4	53.4	3.88, <i>ddd</i> , 4.0, 3.1, 1.4	4	61.4	4.15, <i>m</i>	61.1	4.15, <i>m</i>
5	66.8	5.64, <i>ddd</i> , 3.1, 2.6, 2.4	5	32.0	1.78, <i>m</i>	32.0	1.78, <i>m</i>
6	41.8	4.45, <i>ddd</i> , 14.9, 2.4, 1.4 3.33, <i>dd</i> , 14.9, 2.6	6	36.9	3.93, <i>dt</i> , 13.0, 5.1 3.40, <i>ddd</i> , 13.0, 8.7, 5.6	40.0	3.75, <i>dt</i> , 12.4, 4.6 3.55, <i>ddd</i> , 12.4, 7.8, 6.0
7	175.0	—	7	170.1	—	170.1	—
8	41.7	3.24, <i>ddd</i> , 17.5, 9.1, 6.6 3.17, <i>ddd</i> , 17.5, 8.6, 6.6	8	35.3	2.76, <i>t</i> , 7.3	35.7	2.76, <i>t</i> , 7.3
9	31.3	2.91, <i>m</i>	9	31.5	2.91, <i>t</i> , 7.3	31.7	2.91, <i>t</i> , 7.3
10	142.1	—	10	142.4	—	142.5	—
11,15	129.3	7.26, <i>m</i>	11,15	129.3	7.26, <i>m</i>	129.3	7.26, <i>m</i>
12,14	129.1	7.26, <i>m</i>	12,14	129.1	7.26, <i>m</i>	129.1	7.26, <i>m</i>
13	126.8	7.17, <i>m</i>	13	126.7	7.17, <i>m</i>	126.7	7.17, <i>m</i>
$\text{CH}_3\text{--}\overset{\text{O}}{\parallel}\text{C}$	170.3	—	4-OH	—	3.81, <i>d</i> , 5.4	—	3.79, <i>d</i> , 5.5
$\text{CH}_3\text{--}\overset{\text{O}}{\parallel}\text{C}$	20.6	2.02, <i>s</i>					

opened (data not shown), and minor amounts or none were found in the opened leaves (Table 1).

The chemical diversity of the three alkaloids (Fig. 1), formally due to dehydrogenation, hydroxylation and epoxidation on the same basic skeleton, may be achieved by closely related enzymes, as it has been shown for enzymes capable of these reaction types in other pathways (for a review see Shanklin and Cahoon, 1998). Perhaps a 2,3 dehydrogenation as in awaine (**3**) precedes the oxygenation at C-2 of compounds **1** and **2**. The fate of **3** during early leaf development would therefore be an interesting subject for future work.

The effects of these piperidine alkaloids on human physiology are unknown and their possible toxicity on the liver remains to be investigated. Several pyridone alkaloids with structures similar to **1** have been shown to be cytotoxic (Duh and Wu, 1990; Duh et al., 1990). Furthermore, **1** decomposes on standing at room temperature due to hydrolysis of the amide, to give 3-phenylpropionic acid and the dihydropyridone **4** (Smith, 1979). Compounds **1** and **4** exhibit structural features of 2,5-dihydropyridine, which has been shown to affect DNA integrity in vitro due to its ability to redox cycle (Kim and Novak, 1990, 1991).

Compound **2** is the first alkaloidal epoxide isolated from the kava plant. Its high concentration in the 'Isa' peelings suggests the ease of enzymatic epoxidation of **1**, resulting in a relatively stable product. Epoxidation of certain natural or xenobiotic compounds in liver has been well established as one of the mechanisms pertinent to hepatotoxicity (Zimmerman, 1999).

None of the three piperidine alkaloids reported here were detectable in the commercial root powders from Fiji, Tonga, or Hawaii in our routine analyses using HPLC and GC. The Pacific peoples prepare the time-proven kava drink using primarily the underground parts of certain preferred kava cultivars, and the use of alkaloid-rich stem peelings is avoided in general. In contrast, the raw material for extraction in the pharmaceutical industry may include stem peelings (Dentali, 1997; Secretariat of the Pacific Community, 2001). Although we have no direct evidence on the presence of piperidine alkaloids in kava dietary supplements, the quantitative data (Table 1) suggest that products using kava peelings contain the piperidine alkaloids and their decomposition products. We therefore are compelled to advise caution on using the above-ground plant parts for human consumption.

3. Experimental

3.1. General

All CC was performed on silica gel (40 μm). Solvents used were HPLC grade. NMR for ^1H (500 MHz) and

^{13}C (125 MHz) were performed on a Varian Unity INOVA 500 spectrometer. HRMS–EIMS was obtained with a VG 70SE spectrometer. GC–FID Hewlett-Packard 5890II equipped a 7673 Auto Sampler, a 3396A Integrator and DB-5MS glass capillary column of 30 m, 0.25 i.d., 0.25 μm film thickness (J & W Scientific). The conditions were: injector, 200 $^\circ\text{C}$; detector, 300 $^\circ\text{C}$; oven, initial temp. 100 $^\circ\text{C}$ for 1 min, then increased at 5 $^\circ\text{C}/\text{min}$ to 300 $^\circ\text{C}$ and held for 2 min. For GC–MS analysis, a HP 5890 Gas Chromatograph interfaced with an HP-5989A Mass Spectrometer was used under EI mode at 70 eV.

3.2. Plant material

A voucher specimen of *Piper methysticum* cv. Isa (PNG) has been deposited at the herbarium of the Bishop Museum, Dragull s.n. (BISH 695258-61).

3.2.1. Plant material for preparing standards

Compounds **1** and **2** from peelings—fresh aerial parts of *Piper methysticum* cv. Isa were collected at the Magoon Greenhouse Facility (University of Hawaii at Manoa, Hawaii). Selected healthy mature 2-year-old plants were cut at the base of the stem and the fresh stem peelings were obtained by using a sharp knife with minimum inclusion of the inner woody tissue. The peelings were air-dried at 22 $^\circ\text{C}$ in the dark for 1 day, followed by oven-drying overnight at 40 $^\circ\text{C}$, then milled to pass a 2 mm sieve.

Compound **3** from leaves—unopened and partially opened young leaves excluding petioles were collected from the same location and cultivar as above. Drying conditions were also the same as above. Immediately after oven-drying, the leaves were ground into a fine powder with mortar and pestle.

3.2.2. Plant material for quantitation

One mature kava stem from each of the 11 kava cultivars (Table 1) was collected from local nurseries on the islands of Oahu and Hawaii, and the Waimea Arboretum, Oahu. The basal stem segments (from the base to 20 cm above the rootstock) were peeled within 3 h using a sharp knife. Two young leaves per plant (without petioles, 1 opened and 1 unopened) adjacent to each other on the same branch were used. All samples were air-dried at 22 $^\circ\text{C}$ for 1 day, then oven-dried at 40 $^\circ\text{C}$ overnight.

3.3. Extraction and isolation

3.3.1. Compounds **1** and **2**

The milled stem peelings (100 g), obtained from the mature, ca. 1.5 m long stems, were extracted with a total of 700 ml EtOAc by cold percolation at 22 $^\circ\text{C}$ for 3 days. The solvent was evaporated in vacuo at 26 $^\circ\text{C}$ to

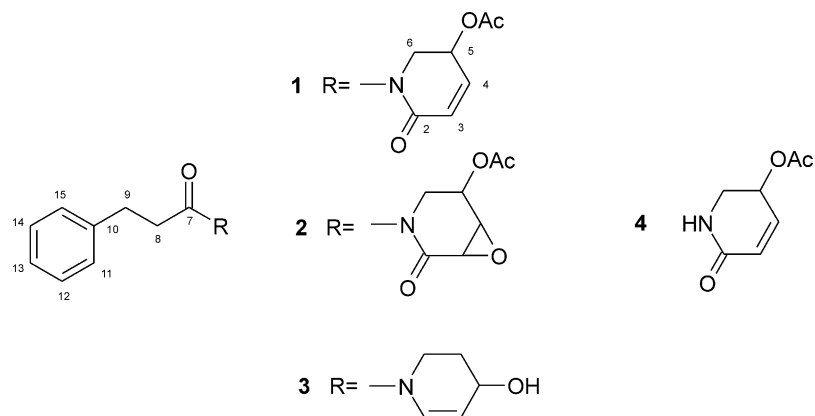


Fig. 1. Structures of pipermethystine (1), 3 α ,4 α -epoxy-5 β -pipermethystine (2), awaine (3), and the dihydropyridone (4).

give a grayish-green residue (13 g). For CC a gradient of EtOAc/hexane was used with increasing proportions of EtOAc (10, 20, 33, 40, 50, 75, 100%) to give crude **1** and **2**. Further purification was achieved by repeated isocratic flash chromatography using silica gel with EtOAc/hexane (1:1) as eluant. Yields of GC-pure compound **1** and **2** were ca. 0.5 g each.

3.3.2. Compound 3

Young leaves (10 g) gave a dark green extract residue (1.6 g) using the same extraction, isolation and purification procedures as in Section 3.3.1, except for flash chromatography; here the solvent used was EtOAc. Yield of GC-pure **3** was ca. 120 mg.

3.4. Identification

3.4.1. Pipermethystine (1)

Colorless oil. $[\alpha]_D^{23} -176.4^\circ$ (Me₂CO; *c* 0.49). Identification of **1** was based on the comparison of the UV and HRMS data with the reported values (Smith, 1979).

3.4.2. 3 α ,4 α -Epoxy-5 β -pipermethystine (2)

Colorless crystals (needles) from hexane, mp 59 °C; $[\alpha]_D^{22} -98.8^\circ$ (Me₂CO; *c* 0.5); ¹H NMR and ¹³C NMR: Table 2; HREIMS: *m/z* 303.1117 (C₁₆H₁₇NO₅ requires *m/z* 303.1106); EIMS (64 eV) *m/z* (rel. int.): 303 [M]⁺ (5), 172 (24), 132 (14), 105 (38), 104 (100), 91 (61).

3.4.3. Awaine (3)

Colorless oil; ¹H NMR and ¹³C NMR: Table 2; HREIMS: *m/z* 231.1294 (C₁₄H₁₇NO₂ requires *m/z* 231.1259). EIMS (64 eV) *m/z* (rel. int.): 231 [M]⁺ (2), 213 [M-H₂O]⁺ (12), 122 (19), 105 (26), 91 (61), 81 (46), 80 (100).

3.5. GC Quantitation

Dried, individual leaf blades, 1 g of peelings, and 1 g of peeled stem slices were ground separately by mortar

and pestle into fine powders. Each (50 mg) were extracted in a screw-capped culture tube with 5 ml EtOAc by sonication in an unheated water bath for 15 min. After centrifugation, part of the supernatant was transferred into a sample vial and subjected to GC. Peak identity was assured by GC-MS. Compounds were quantified using standard curves, established with each authentic kava alkaloid. The injection volume was 1 μ l.

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