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TOXIC TETRANORTRITERPENES OF THE FRUIT OF *MELIA AZEDARACH*

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Key Word Index—*Melia azedarach* L. var. *australasica*; Meliaceae; white cedar tree; tetranortriterpenes; meliatoxins A_1, A_2, B_1, B_2; toxicity; pathology.

Abstract—Four new tetranortriterpenes, meliatoxins A_1, A_2, B_1 and B_2 have been isolated and identified as toxic constituents of the fruit of *Melia azedarach* L. var. *australasica*. Toxicity and pathological results confirm that the meliatoxins are responsible for most but not all of the symptoms resulting from the ingestion of whole fruit.

INTRODUCTION

Melia azedarach L. is a small attractive deciduous tree often grown for shade or ornament on roadsides, parks and other open situations. It can be also found in the tropical rain forests of Queensland and New Guinea where it can grow to a height of 45 m [1]. The plant is reported to be a native of Persia, China and India [2], and is naturalized in a number of countries including Africa, Australia and the Americas. It has been used medicinally in many of these places for the treatment of a variety of human disorders [3]. On the Island of Maritius and in China, extracts of the bark are used as an antihelminthic. In Algeria the plant is used as a tonic and antipyretic, and in South Africa for the treatment of leprosy, eczema and the relief of asthmatic attacks.

There are early records of the plant being poisonous to man [1–3]. The leaves, bark and flowers have been shown to be toxic, but the great majority of cases occur from ingestion of fruits. Children are reported to have died after eating six–eight ripe fruits and are said to have developed malignant ulcers after forcing fruit into the nose. Death may not occur until some days after eating the fruit [1], and is preceded by symptoms of nausea, vomiting, diarrhoea, severe thirst, cold sweats, grinding of teeth, sleepiness and convulsions.

Numerous records shown that *Melia azedarach* L. is toxic to livestock. Pigs are most commonly affected, but poisoning of cattle, sheep, goats and poultry has been reported [3]. The symptoms are of two types [4]: the first produces nausea, vomiting, constipation or scouring often with blood; the second produces more acute nervous symptoms of excitement or depression, weakened heart action, dyspnea and death. On autopsy acute cases show evidence of gastro-intestinal irritation only, but in animals that survive for several days, histological examination shows fatty degeneration and hyperaemia of the liver and kidneys.

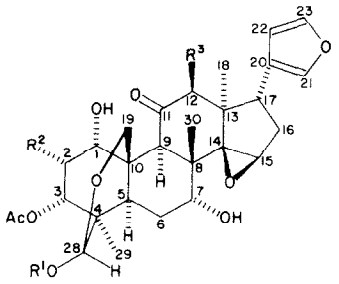
There has been little agreement about the nature of the toxic principle. Morrison and Grant [5] in feeding tests with guinea pigs found that the toxin was in the resinous portion of the fruit, and that the resin contained an 'alkaloidal substance' of unknown structure. Steyn and Rindl [6] reported that the toxic principles are not alkaloids, toxalbumins or glycosides, but are probably bitter principles. According to Carratola [7] the fruit contains an alkaloid azaridine, a resin, tannin and meliotannic and benzoic acids. It appears that the toxin is the same in different forms of the species that occur in Australia, Africa, Asia or America [1].

Experimental feeding of the fruit to pigs and sheep has established its toxicity to be close to 0.5% of the animal's body weight [4], but there are some cases of feeding experiments where large amounts of the fruit failed to produce symptoms. It has been suggested that toxicity may vary with location or stage of growth, and may be entirely absent in some trees. In Argentina farmers regard the fruit as harmless, because children and livestock have access to them and no cases of poisoning are recorded [2]. Children in California are said to swallow the fruit with impunity, but they are considered toxic in the West Indies and Guam. This observation was supported by Morrison and Grant who found that fruit from trees growing at Grafton, N.S.W., were non-toxic, but that fruit collected from trees in two other areas of N.S.W. was toxic [5].

This article records the isolation and characterization of four new tetranortriterpenes of the limonoid class, meliatoxins A_1, A_2, B_1 and B_2 from the fruit of *Melia azedarach* L. var. *australasica*. Evidence is presented to show that these compounds are responsible for the acute nervous symptoms and death in pigs. In addition an attempt has been made to relate some clinical and histopathological findings to poisoning by these toxins. A full article describing the pathology of these findings will be published in due course.

RESULTS AND DISCUSSION

The four compounds in the toxic extract from *M. azedarach*, meliatoxins A_1, A_2, B_1 and B_2 were very sensitive to traces of acid and decomposed on attempted TLC purification on silica. It was, therefore, necessary to use MPLC and HPLC separation techniques to achieve a reasonable recovery of purified compounds. The structures of meliatoxins A_1 **(1)**, A_2 **(2)**, B_1 **(5)** and B_2 **(6)** were

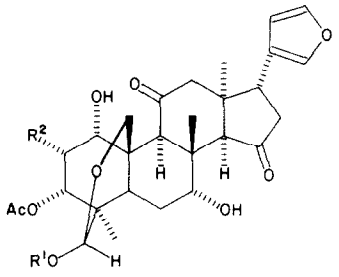


1 R^1 = $MeCH_2CHMeCO$, R^2 = AcO, R^3 = H

2 R^1 = Me_2CHCO, R^2 = AcO, R^3 = H

3 R^1 = $MeCH_2CHMeCO$, R^2 = AcO, R^3 = OH

4 R^1 = H, R^2 = H, R^3 = H



5 R^1 = $MeCH_2CHMeCO$, R^2 = AcO

6 R^1 = Me_2CHCO, R^2 = AcO

7 R^1 = H, R^2 = H

elucidated mainly by comparison of their high field ^{1}H NMR spectra with those published for other related limonoids, in particular, trichilin A (**3**) [8], amoorastatin (**4**) [9] and amoorastatone (**7**) [10].

Meliatoxin A_1 differs from trichilin A, an insect antifeedant extracted from *Trichilia roka* [8], only in the lack of the hydroxyl substituent at C-12. The ^{1}H NMR spectra of the two compounds are very similar although the reported assignments for H-5 and H-17 in trichilin A need to be reversed [Nakanishi, K., personal communication]. Nakanishi has also briefly reported the isolation from the same plant of trichilin D, which he has proposed to have structure **1** [8]. There is insufficient published data available on trichilin D for us to confirm its identity with meliatoxin A_1 at this time. Amoorastatin (**4**), a cytotoxic limonoid isolated from *Aphanamixis grandifolia* B1 [9], has the same B, C and D ring substituents and relative stereochemistry as meliatoxin A. This is reflected in the similarity of the chemical shifts and coupling constants for the protons on these rings in **1** and **4**. The structure of amoorastatin has been confirmed by X-ray crystallography [9]. Meliatoxin A_2 (**2**) is identical to A_1 except that the ester moiety at C-28 is 2-methylpropionoyl in place of the 2-methylbutanoyl group in **1**.

Meliatoxin B_1 (**5**) and B_2 (**6**) are isomeric with A_1 and A_2 respectively, the only structural difference being that the epoxide ring in **1** and **2** is replaced by a five-membered ring ketone at C-15. The evidence for their structure is based on a comparison of the ^{1}H NMR spectra of **5** and **6** with amoorastatone (**7**), the epoxide ring-opened isomer of amoorastatin **4** [10].

Toxicology and pathology

The yield of meliatoxins in the original sample of fruit was *ca* 0.5% of its wet wt. That similar fruit from three other areas contained no meliatoxins supports the conclusions of previous workers that certain areas or individual trees are not toxic [5]. The fourth sample of fruit analysed was collected when it was well past maturity, and it is possible that loss of toxin may have occurred due to drying or some other process. Because of the initial difficulty in separating the meliatoxin components in large quantities they were tested together in the following study, since previous work had shown that the A and B fractions were of equal toxicity to mice.

The toxins were tested using purified ethanolic solutions; the approximate LD_{50} for orally dosed pigs was found to be 6.4 mg/kg, and intraperitoneally injected mice 16 mg/kg. At all stages during the isolation, testing with mice gave comparable results to pigs. The first clinical signs in the pigs that died were observed from 2–4 hr after drenching, and were marked by severe and rapid muscular contractions. The animals then collapsed and frequently the contractions were replaced by spasmodic quivering of the muscles similar to shivering. The heart rate was rapid (180 beats/min) and the pupils were dilated. By 8 hr the animals were comatose with weak heart beat (100 beats/min) and subnormal body temperature (less than 35°) and this was followed by death at *ca* 30 hr.

The gross pathological findings in pigs that died were unremarkable but the microscopic lesions in the intestine were considered characteristic. These lesions commenced in the glandular stomach and extended throughout the length of the intestines. The basic change was necrosis of cells in the interglandular area of the laminar propria, particularly in the superficial half. Lymphoid tissue of lymph nodes, splenic white pulp and gastrointestinal tract had many necrotic cells with pink cytoplasm and fragmented pyknotic nuclei. Moderate numbers of renal tubules contained proteinaceous casts.

With pigs receiving sublethal doses of whole fresh fruit, the major clinical sign was severe scouring (frequently with blood and fragments of the intestinal mucosa) which commences *ca* 20 hr after dosing, resolved over the next 2–3 days. These symptoms were never observed in mice or pigs dosed at lethal or sublethal levels with the meliatoxins. It appears that the meliatoxins are, therefore, responsible only for the acute nervous symptoms and rapid death in pigs. Work is in progress on the isolation from the fruit of *M. azedarach* L. of the compound responsible for scouring.

EXPERIMENTAL

^{1}H NMR: 200 MHz, TMS as int. standard. EIMS: 70 eV, direct probe, source temp. 200–250°. TLC: Si gel G (0.25 mm layer) $CHCl_3$–*n*-BuOH (96:4) solvent, sprayed with 18N H_2SO_4 followed by heating (90°) for 30 min.

Bioassay of the toxin. Throughout this work white mice (20–30 g) were used as test animals to detect and assay toxicity. To assay the toxin in various fractions, dried aliquots were dissolved in purified EtOH and 10–50 μl doses injected intraperitoneally. Pigs, 5–7 months-old (10–30 kg body wt) were used to test the

isolated toxin, which was administered orally in *ca* 10 ml purified EtOH.

Source of fruit. Melia azedarach L. var. *australasica* (A. Juss.) C. DC. fruits were collected from Miriam Vale in central Queensland on 21 March 1979. A voucher specimen (BRI-243355) was deposited in the Queensland Herbarium. The fruits were commencing to turn yellow when picked, and after collection were stored at −5°.

Extraction and isolation of meliatoxins A_1, A_2, B_1 and B_2. Frozen fruit (100 g) was extracted × 3 in a laboratory blender with EtOH and the extract concd under red. pres. to a thick syrup. H_2O (200 ml) was added and the mixture of solid and soln extracted × 4 with Et_2O. The Et_2O extract was concd under red. pres. to dryness, and the residue redissolved in toluene (200 ml). The soln was added to a silicic acid column (30 g) packed with toluene. Elution with this solvent (100 ml) was followed by a stepwise elution with 100 ml vol. of the following solvents: $CHCl_3$–toluene (1:1); $CHCl_3$–toluene (4:1); $CHCl_3$; $CHCl_3$–*n*-BuOH (99:1); $CHCl_3$–*n*-BuOH (98:2); $CHCl_3$–*n*-BuOH (96:4). Fractions (10 ml) were monitored using TLC and bioassay with mice. The toxin was recovered by concn of the fractions under red. pres.

The dried toxic residue (525 mg) from above was dissolved in MeOH–H_2O–$ClCH_2CH_2Cl$–HOAc (85:9:5:1) (5 ml) and added to a column of micronized XAD-2 resin (50 g) [11]. Elution with this solvent mixture was carried out at a flow rate adjusted to *ca* 1 ml/min. Fractions (2 ml) were monitored using TLC and bioassay with mice. Those containing the toxin were combined and concd to give a white amorphous solid (430 mg) which was shown by TLC to be a mixture of at least two compounds, A and B (R_fs 0.43 and 0.57). Attempted TLC separation of them led to extensive decomposition of the sample.

Passage through a larger column (100 g) using a slower flow rate gave a partial separation of the two toxic fractions. Bioassay using mice showed they were of equal toxicity. Medium pressure liquid chromatography on Merck Lichroprep Si 60 (40–63 μm) using CH_2Cl_2–EtOAc (2:3) separated the toxic extract from CC (180 mg) into two fractions, meliatoxins A (38 mg) and B (83 mg). Both fractions A and B were further separated isocratically on a Waters μBondapak C_{18} column (7.3 × 300 mm, 10 μm) using a Waters 6000A HPLC system with RI detector (MeCN–H_2O (3:2), 3 ml/min) to give pure meliatoxins A_1 (18 mg) and A_2 (8 mg), B_1 (38 mg) and B_2 (25 mg).

Meliatoxin A_1 **1**. White powder, mp 148–154° d. Found: $[M]^+$ 658.29718. Calc. for $C_{35}H_{46}O_{12}$: 658.29898. UV λ_{max}^{MeOH} nm: 210 (ε 6560). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3610, 3460, 3030, 1745, 1710, 1430, 1375, 1250, 1050, 910, 870. CD (MeOH): 222 nm (Δε + 1), 294 nm (Δε − 5.0). ^1H NMR ($CDCl_3$): δ 0.85 (3H, *s*, H-29), 0.94 (3H, *t*, *J* = 7 Hz, H-4′), 1.09 (3H, *s*, H-30), 1.18 (3H, *d*, *J* = 7 Hz, Me-2′), 1.24 (3H, *s*, H-18), 1.55 and 1.72 (2H, *m*, H-3′), 1.78 (1H, *m*, H-6α), 1.88 (1 H, *dd*, *J* = 14, 11 Hz, H-16α), ~ 2.0 (1 H, *m*, H-6β), 2.02 (3H, *s*, MeCOO), 2.14 (3H, *s*, MeCOO), 2.26 (1 H, *dd*, *J* = 14, 6 Hz, H-16β), 2.46 (2H, *s*, H-12), 2.51 (1 H, *m*, H-2′), 2.75 (1H, *dd*, *J* = 14, 4 Hz, H-5), 2.76 (1H, *dd*, *J* = 11, 6 Hz, H-17), 3.69 (1H, *t*, *J* = 3 Hz, H-7), 3.71 (1H, *s*, H-15), 4.24 (1H, *d*, *J* = 4.4 Hz, H-1), 4.40 and 4.50 (2H, AB*q*, *J* = 13 Hz, H-19), 4.61 (1H, *s*, H-9), 5.52 (1H, *d*, *J* = 4.4 Hz, H-3), 5.78 (1H, *s*, H-28), 5.91 (1H, *t*, *J* = 4.4 Hz, H-2), 6.14 (1H, *s*, H-22), 7.14 (1H, *s*, H-21), 7.38 (1H, *s*, H-23). EIMS *m/z* (% rel. int.): 658 $[M]^+$ (2), 640 $[M-H_2O]^+$ (3), 598 $[M-MeCOOH]^+$ (5), 580 $[M-H_2O-MeCOOH]^+$ (2), 557 (18), 556 $[M-C_4H_9COOH]^+$ (15), 539 (10), 538 $[M-2\times MeCOOH]^+$ (10), 496 $[M-MeCOOH-C_4H_9COOH]^+$ (32), 454 (29), 436 (18), 426 (13), 394 (16), 377 (16), 327 (61), 163 (38), 121 (18), 107 (56), 95 (37), 85 (33), 74 (25), 57 (100).

Meliatoxin A_2 **2**. White powder, mp 155–160° d. Found: $[M]^+$ 644.28351. Calc. for $C_{34}H_{44}O_{12}$: 644.28325. UV and IR spectra identical to meliatoxin A_1. CD(MeOH): 222 nm (Δε + 1), 294 nm (Δε − 5.1). ^1H NMR ($CDCl_3$): identical to A_1 except for ester group at C-28, δ 1.21 (3H, *d*, *J* = 7 Hz, Me-2′), 1.22 (3H, *d*, *J* = 7 Hz, Me-2′), 2.69 (1H, *m*, H-2′). EIMS *m/z* (% rel. int.): 644 $[M]^+$ (4), 626 $[M-H_2O]^+$ (7), 584 $[M-MeCOOH]^+$ (3), 557 (25), 556 $[M-C_3H_7COOH]^+$ (19), 538 $[M-H_2O-C_3H_7COOH]^+$ (18), 496 $[M-MeCOOH-C_3H_7COOH]^+$ (76), 454 (36), 436 $[M-2\times MeCOOH-C_3H_7COOH]^+$ (22), 426 (19), 327 (100), 163 (59), 121 (26), 107 (74), 95 (49), 71 (61), 57 (21).

Meliatoxin B_1 **5**. White powder, mp 140–150° d. Found: $[M-H_2O]^+$ 640.2897. Calc. for $C_{35}H_{44}O_{11}$: 640.2883. UV λ_{max}^{MeOH} nm: 207 (ε 8500). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600, 3480, 3040, 1745, 1710, 1462, 1375, 1250, 1050, 905, 875. CD(MeOH): 224 nm (Δε + 5), 293 nm (Δε − 4.7). ^1H NMR ($CDCl_3$): δ 0.86 (3H, *s*, H-29), 0.94 (3H, *t*, *J* = 8 Hz, H-4′), 0.99 (3H, *s*, H-30), 1.05 (3H, *s*, H-18), 1.19 (3H, *d*, *J* = 7 Hz, Me-2′), 1.53 and 1.72 (2H, *m*, H-3′), 1.83 (1H, *dt*, *J* = 15, 3.5 Hz, H-6α), 2.04 (3H, *s*, MeCOO), 2.14 (3H, *s*, MeCOO), 2.14 (1H, *m*, H-6β), 2.31 and 2.68 (2H, AB*q*, *J* = 17 Hz, H-12), 2.50 (1H, *m*, H-2′), 2.59 (2H, *d*, *J* = 10 Hz, H-16), 2.66 (1H, *m*, H-5), 3.16 (1H, *s*, H-14), 3.34 (1H, *t*, *J* = 10 Hz, H-17), 3.48 (1H, *s*, H-9), 3.97 (1H, *t*, *J* = 3 Hz, H-7), 4.27 (1H, *d*, *J* = 4.4 Hz, H-1), 4.27 and 4.66 (2H, AB*q*, *J* = 13 Hz, H-19), 5.57 (1H, *d*, *J* = 4.4 Hz, H-3), 5.78 (1H, *s*, H-28), 5.90 (1H, *t*, *J* = 4.4 Hz, H-2), 6.31 (1H, *s*, H-22), 7.29 (1H, *s*, H-21), 7.42 (1H, *s*, H-23). EIMS *m/z* (% rel. int.) 640 $[M-H_2O]^+$ (0.5), 598 $[M-MeCOOH]^+$ (1), 557 (31), 556 $[M-C_4H_9COOH]^+$ (35), 539 (17), 496 $[M-MeCOOH-C_4H_9COOH]^+$ (66), 468 (19), 454 (29), 436 $[M-2\times MeCOOH-C_4H_9COOH]^+$ (26), 426 (22), 327 (100), 234 (83), 177 (30), 163 (44), 149 (32), 136 (19), 95 (22), 57 (35), 43 (30).

Meliatoxin B_2 **6**. White powder, mp 155–162° d. Found: $[M-H_2O]^+$ 626.26978. Calc. for $C_{34}H_{42}O_{11}$: 626.27276. UV and IR spectra identical to meliatoxin B_1. CD(MeOH): 225 nm (Δε + 6), 295 nm (Δε − 4.8). ^1H NMR ($CDCl_3$): identical to B_1 except for ester group at C-28, δ 1.21 (3H, *d*, *J* = 7 Hz, Me-2′), 1.22 (3H, *d*, *J* = 7 Hz, Me-2′). EIMS *m/z* (% rel. int.): 626 $[M-H_2O]^+$ (0.5), 557 (6), 538 $[M-H_2O-C_3H_7COOH]^+$ (1.5), 525 (5), 496 $[M-MeCOOH-C_3H_7COOH]^+$ (11), 468 (6), 454 (13), 436 $[M-2\times MeCOOH-C_3H_7COOH]^+$ (8), 426 (6), 408 (7), 377 (15), 327 (40), 163 (49), 121 (13), 95 (27), 71 (22), 43 (100).

Analysis of M. azedarach *L. fruit samples from other areas.* Fruit from four areas in SE. Queensland was analysed for the presence of the meliatoxins using the preceding method of extraction and isolation. Three of these samples were gathered when fruit was at the same stage of growth as the original, but the fourth was at a later stage of growth. Of these only the fourth was shown to contain a low concn of meliatoxins.

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