PRODUCTION OF CYTOTOXIC CANTHIN-6-ONE ALKALOIDS BY *AILANTHUS ALTISSIMA* PLANT CELL CULTURES

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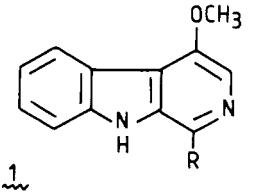
ABSTRACT.—*Ailanthus altissima* (Mill.) Swingle was established as callus- and cell-suspension cultures. Canthin-6-one and 1-methoxycanthin-6-one were isolated by a combination of preparative tlc and preparative hplc. The two alkaloids were identified by their uv, ms, and ^{1}H-nmr spectra. The combined yield of the two alkaloids was 1.38% of dry weight from callus and 1.27% of dry weight from cell suspensions. The cytotoxicities of canthin-6-one, 1-methoxycanthin-6-one, 5-methoxycanthin-6-one, and canthin-6-one-3-*N*-oxide to guinea pig ear keratinocytes have been compared, and the IC_{50} values range from 1.11 to 5.76 μg/ml. There is no significant difference in activity among these four cytotoxic alkaloids.

A number of species from the Simaroubaceae have been used in traditional medicine for treating tumors (1) and amoebic dysentery (2), and also as anthelmintics (3). Chemical studies have shown that most species contain degraded triterpenes, which are known collectively as quassinoids (2), and that indole alkaloids are common constituents of the family. A number of quassinoids have been shown to have marked anticancer activity and one of them, bruceantin, is currently undergoing phase II clinical trials (4). The indole alkaloids are either of the simple β-carboline type or canthin-6-ones (5). It has been demonstrated that some canthin-6-ones can act as antimicrobial agents (6). The potential of the family to produce biologically active compounds has prompted us to investigate their production by plant cell cultures.

The deciduous "tree of heaven," *Ailanthus altissima* (Mill.) Swingle, has yielded a series of alkaloids, quassinoids, flavonoids, and quinones (5,7). The following β-carboline alkaloids have been isolated from the root bark: 1-acetyl-4-methoxy-β-carboline (**1**, R=$COCH_3$) (8), 1-carboxymethyl-4-methoxy-β-carboline (**1**, R=$COOCH_3$) (7), 1-(2′-hydroxyethyl)-4-methoxy-β-carboline (**1**, R=CH_2CH_2OH) (8), and 1-(1′,2′-dihydroxyethyl)-4-methoxy-β-carboline (**1**, R=$CH[OH]CH_2OH$) (8,9). Canthin-6-one (**2**, R=H), its 3-*N*-oxide, and 1-methoxycanthin-6-one (**2**, R=OCH_3) have been obtained from both root bark and wood (7-10). 1-Methoxycanthin-6-one-3-*N*-oxide is a constituent of root bark (8), and 1-hydroxycanthin-6-one (**2**, R=OH) has been isolated from wood (11) of *A. giraldii* Dode, a synonym of *A. altissima* (Mill.) Swingle (5,12). The roots of this plant have also been reported to contain 4-methoxy-1-methyl-3-[3,3-dimethylallyl]-quinol-2-one (**3**) (13).



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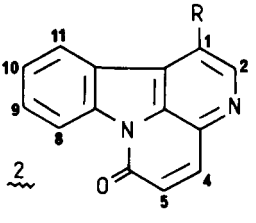

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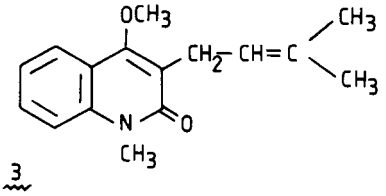

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To date, some 11 quassinoids have been isolated from various parts of *A. altissima*, and they include ailanthone (14,15), amarolide, amarolide-11-acetate (16,17), chaparrinone (14), chaparrolide (18), glaucarubinone (19), $\Delta^{13(18)}$-dehydroglaucarubinone (20), $\Delta^{13(18)}$-dehydroglaucarubolone (21,22), shinjudilactone (23), shinjulactone B (24), and shinjulactone C (25). Antileukemic and cytotoxic activities have been reported for ailanthone, chaparrinone, and glaucarubinone (4). Ailanthone is reported to have antiamoebic activity (26). *A. altissima* also contains quercetin 3-glucoside (27) and 2,6-dimethoxy-*p*-benzoquinone (28).

RESULTS AND DISCUSSION

The callus and cell suspensions of *A. altissima* consisted of fast-growing, homogeneous, creamy white cells. Thin layer chromatographic (tlc) and high performance liquid chromatographic (hplc) analyses of extracts obtained from callus and suspension cultures indicated the presence of canthin-6-one (**2**, R=H) and 1-methoxycanthin-6-one (**2**, R=OCH_3). The former alkaloid was co-chromatographed with an authentic sample, and confirmation of identity of both alkaloids was obtained from their uv, ms, and ^{1}H-nmr spectra. The chemical shifts obtained for the ^{1}H-nmr spectra of both alkaloids are listed in the Experimental section and show differences with published data (10), but are nevertheless consistent with the identifications. Based on comparison with the ^{1}H-nmr spectrum of the *N*-oxide, the assignment of the signals for C-4 and C-5 protons are reversed from previous literature assignments (8), inasmuch as the signal for the C-4 proton in the spectrum of the *N*-oxide appears downfield by 0.34 ppm, indicating the deshielding effect at this position.

In the whole plant, canthin-6-one has been found to be the major alkaloid of the root bark and wood. 1-Methoxycanthin-6-one proved to be the major alkaloid present in callus culture, whereas there were similar quantities of both alkaloids in cells of the suspension cultures (table 1). The alkaloids were obtained only from the cells of suspension cultures and were not detected in the media. Canthin-6-one alkaloids previously have not been reported from plant cell cultures, and this is the first report of such cultures from a species of the Simaroubaceae. No evidence was obtained for the presence of quassinoids in either callus or suspension cultures.

TABLE 1. Yield of alkaloids from plant cell culture of *Ailanthus altissima*.

Culture	Dry wt. of cells (g)	% yield of alkaloid	
		Canthin-6-one	1-Methoxycanthin-6-one
Callus	0.162	0.09	1.29
Cell suspension . .	0.963	0.72	0.55

Plant cell cultures are potentially a viable source of secondary plant products (29,30). However, only a few cultures have been shown to synthesize secondary metabolites in concentrations comparable to those of their parent plants (31). In particular, the yields of alkaloids by plant cell cultures generally have been low. Notable exceptions, however, are yields of ajmalicine (1.02% dry weight of cells) from *Catharanthus roseus* cultures (32) and jatrorrhizine (10% dry weight of cells) from *Berberis* cultures (33). These high yields have been achieved by employing cell selection procedures. In the present investigation, the yield of canthin-6-one alkaloids from *A. altissima* callus was 1.38% and from cell suspensions, 1.27% of dry weight. These

yields are considerably higher than those values given in the literature for the whole plant material, inasmuch as the root bark reportedly contains 0.01% and the wood 0.001% of total alkaloid (8,10). It is possible that, by utilizing cell selection techniques and optimizing medium and environmental conditions, the alkaloid yield can be increased even further.

It has been reported that canthin-6-one, 1-methoxy-canthin-6-one, and 5-methoxycanthin-6-one are not sufficiently active in the KB test system to meet the required criteria of an active compound (34). However, the antibacterial activity of extracts of *Zanthoxylum elephantiasis* Macf. has been attributed to canthin-6-one, which showed significant activity against *Staphylococcus aureus, Klebsiella pneumoniae, Mycobacterium smegmatis,* and *Candida albicans* (6). This broad-based activity is apparently specific to canthin-6-one, because 5-methoxycanthin-6-one, 4-methylthio-canthin-6-one, 4,5-dimethoxycanthin-6-one, and benz[4,5]-canthin-6-one were devoid of meaningful activity. In the present investigation, the cytotoxic activities of canthin-6-one, 1-methoxycanthin-6-one, 5-methoxycanthin-6-one, and canthin-6-one-3-*N*-oxide to guinea pig ear keratinocytes (GPK) (35) have been compared, and all four compounds were shown to have marked cytotoxic activities (table 2). The inhibitory effects on DNA synthesis in GPK epithelial cells are of the same order of magnitude for the four compounds; statistically, there is no significant difference between the IC_{50} values obtained.

TABLE 2. Cytotoxicity (GPK epithelial cells) of some canthin-6-one alkaloids.

Alkaloid	IC_{50} (μg/ml)
Canthin-6-one	1.11
1-Methoxycanthin-6-one . . .	5.76
5-Methoxycanthin-6-one . . .	5.44
Canthin-6-one-3-*N*-oxide . .	4.21

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The uv spectra were determined on a Perkin Elmer 402 uv-visible spectrophotometer, and ms were obtained using a ZAB-1F (VG-Micromass Ltd.) mass spectrometer. ^{1}H-nmr spectra were determined either on a Bruker WM-250 or WM-400 spectrometer using $CDCl_3$ solutions containing TMS as internal standard. Silica gel GF-254 (Merck) tlc plates, and ethylacetate was the mobile phase. An Altex Isocratic Liquid Chromatograph (Model 330) equipped with variable Hitachi uv spectrophotometer (Model 100-10) was used to obtain hplc separations. Columns of Lichrospher Si 100, 5 μm (15 cm x 4.5 mm, internal diameter) were eluted with hexane-ethylacetate (75:25), and alkaloids were detected at 280 nm.

PLANT MATERIAL.—*A. altissima* (Mill.) Swingle seeds were obtained from the Royal Botanic Gardens, Wakehurst Place. The seeds were surface sterilized in sodium hypochlorite (2%) containing Triton-X-100 for 12 minutes and then germinated on wetted filter paper in petri dishes. Aseptic cotyledon seedlings were transferred to solid (1%) agar Murashige and Skoog's medium (36) containing 2,4-dichlorophenoxyacetic acid (1 mg/ml), kinetin (0.1 mg/liter), polyvinylpyrrolidone (1%), and sucrose (5%). The creamy, undifferentiated callus produced was maintained at 25° under continuous illumination and subcultured every four weeks onto fresh medium. Suspension cultures were initiated by transferring third subcultures of callus to 250-ml conical flasks containing 50 ml of liquid medium and were shaken at 100 rpm on an orbital shaker. Suspensions were maintained at 25° under continuous illumination and subcultured every two weeks. Fourth subcultures of suspension cultures were harvested and extracted.

EXTRACTION AND ISOLATION PROCEDURES.—The suspension cells were removed from the media, and the cells were dried at 40° for 12 h. Methanolic extracts of suspension cells and of callus were fractionated using a silica gel "Sep-Pak" cartridge (Waters Associates). Hexane, chloroform, chloroform-methanol (1:1), and finally methanol were used in sequence for elution. Alkaloids were detected on tlc plates by their uv fluorescence and by spraying with Dragendorff's reagent. Quassinoids were not detected in any of the fractions obtained. The alkaloids were isolated by preparative tlc and further purified on an analytical hplc column (table 1).

ALKALOID IDENTIFICATION.—*Canthin-6-one* (**2**, R=H), uv λ max (MeOH) 250, 259, 267, 300, 346, 360, 378 nm, identical with literature values (9); ^{1}H-nmr (250 Mhz), δ 7.00 (1H, d, J=10 Hz, C-5 H), 7.55 (1H, m, J=8 Hz, C-10 H), 7.73 (1H, m, J=8 Hz, C-9 H), 8.00 (1H, d, J=5 Hz, C-1H), 8.05 (1H, d, J=10 Hz, C-4 H), 8.14 (1H, d, J=8 Hz, C-11 H), 8.70 (1H, d, J=8 Hz, C-8 H), 8.85 (1H, d, J=5 Hz, C-2 H) (9); ms, *m/z* (%) 220 ($M^{+\cdot}$; 100), 192 (52), 166 (5), 165 (7), 164 (7), 139 (7), 114 (12), 96 (10) (9); identical tlc Rf value (0.43) and hplc Rt value (6.25 min) with authentic canthin-6-one.

1-Methoxycanthin-6-one (**2**, R=OCH_3), uv λ max (MeOH) 259, 270, 280, 346, 360, 378 (9); ^{1}H-nmr (250 MHz), δ 4.28 (3H, s, C-1, OCH_3), 6.87 (1H, d, J=10 Hz, C-5 H), 7.54 (1H, m, J=8 Hz, C-10 H), 768 (1H, m, J=8 Hz, C-9 H), 7.99 (1 H, d, J=10 Hz, C-4 H), 8.25 (1H, J=10 Hz, C-11 H), 8.52 (1 H, s, C-2 H), 8.70 (1H, d, J=10 Hz, C-8 H) (9); ms, *m/z* (%), 250 ($M^{+\cdot}$; 100), 235 (9), 222 (9), 220 (8), 208 (6), 207 (37), 192 (5), 180 (12), 179 (11), 152 (11) (9); tlc Rf value (0.33) and hplc Rt value (9.25 min).

PREPARATION OF CANTHIN-6-ONE-3-*N*-OXIDE.—Canthin-6-one (25 mg) was dissolved in chloroform (1.5 ml) and methanol (2 drops) and cooled in ice; *m*-chloroperbenzoic acid (17.5 mg), dissolved in chloroform (0.5 ml), was slowly added to the stirred alkaloid solution. The mixture was allowed to reach room temperature, and stirring continued for 18 h. The organic solution was washed with 5% sodium bicarbonate solution and water, and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure and the *N*-oxide purified by preparative tlc using chloroform as mobile phase. Canthin-6-one-*3*-*N*-oxide (3 mg) was obtained as an amorphous solid.

Canthin-6-one-3-N-oxide, uv λ max (MeOH) 246, 278.5, 366 nm; ^{1}H-nmr (400 MHz), δ 6.93 (1H, d, J=10 Hz, C-5 H), 7.53 (1H, t, J=8 Hz, C-10 H), 7.65 (1H, t, J=8 Hz, C-9 H), 7.83 (1H, d, J=6.5 Hz, C-1 H), 7.99 (1H, d, J=10 Hz, C-11 H), 8.35 (1H, d, J=6.5 Hz, C-2 H), 8.39 (1H, d, J=10 Hz, C-4 H), 8.66 (1H, d, J=10 Hz, C-8 H); ms, *m/z* (%) 236 ($M^{+\cdot}$; 48), 222 (4), 220 (100), 208 (8), 207 (4), 192 (72), 191 (12), 179 (8), 166 (12), 165 (16), 164 (20), 153 (12), 151 (4), 139 (16).

CYTOTOXICITY TEST USING GPK EPITHELIAL CELLS (35).—Cells for experiments were derived from guinea pig ear epidermis (GPK cells). Cultures were routinely screened by autoradiography to exclude mycoplasma contamination. The culture medium was composed of Eagle's Minimum Essential Medium (Flow Laboratories) (modified with Earle's salts and 20 mM Hepes buffer) and with 10% fetal bovine supplementation (Gibco) (virus and mycoplasma screened). The medium contains penicillin (10 Ku%) and streptomycin (10 mg%). Cells were seeded in multiwell trays (Falcon) at an initial density of 5 x 10^4 cells/ml and incubated at 1 ml/well of medium for two days at 37° in an incubator. The medium was then decanted and replaced by fresh medium warmed to 37° and containing the reagents to be tested, each being initially dissolved in 20 μl sterile DMSO. The cultures were returned to the incubator for 3 h when 50 μl/well of [^{3}H]-thymidine (Amersham International Ltd.) was added to give a final radioisotope concentration of 10 μ Ci/ml. Incubation at 37° was then continued for 30 min, after which time the medium was poured off and the cells washed twice with cold phosphate buffered saline (PBS, Oxoid, Dulbecco A) and twice with cold 5% trichloracetic acid. The second wash was left on the cells for 5 min; then the cells were washed twice with PBS and dried in a stream of warm air. The cell sheets were then digested overnight at 37° in 1 N sodium hydroxide solution (250 μl/well), and the radioactivity of each sample culture was determined from a 200-μl aliquot placed in BBOT (BDH Ltd.) scintillation fluid and counted in a Packard Tri-Carb Liquid Scintillation counter. The IC_{50} values obtained are recorded in table 2.

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