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OCCURRENCE OF INDOLE ALKALOIDS IN *AILANTHUS ALTISSIMA* CELL CULTURES

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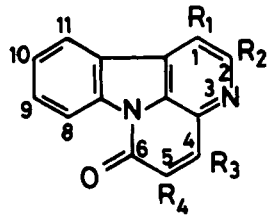
ABSTRACT.—Production of alkaloids with both canthin-6-one and β-carboline structure by *Ailanthus altissima* cell cultures is reported. Besides canthin-6-one (**1**), 1-methoxycanthin-6-one (**2**), canthin-6-one-3-oxide (**7**), 1-methoxycanthin-6-one-3-oxide (**8**), 1-hydroxycanthin-6-one (**3**), 5-hydroxycanthin-6-one (**6**), β-carboline-1-propionic-acid (**11**), and 4-methoxy-β-carboline-1-carboxylic acid methylester (**10**), three new alkaloids are described. These are 2-hydroxycanthin-6-one (**4**), 4-hydroxycanthin-6-one (**5**), and 4,5-dihydrocanthin-6-one (**9**).

Ailanthus altissima Swingle (Simaroubaceae) is known to be a producer of indole alkaloids and has been extensively studied (1-4). These alkaloids belong to the class of either the simple β-carboline or the more complex canthin-6-one derivatives. The literature has recently reported (5) cell cultures of *A. altissima* that produced canthin-6-one (**1**) and 1-methoxycanthin-6-one (**2**), which are the two most abundant alkaloids in the intact plant.

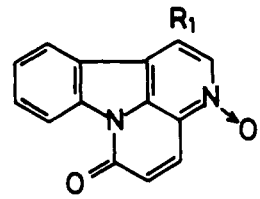
We investigated the production of some minor alkaloids whose presence had been suspected during a previous study undertaken in order to clarify the different steps of their biosynthesis (6). Eleven alkaloids were identified and isolated in satisfactory yields from a batch of 50 liters of cell suspension cultures of *A. altissima*. Besides the main alkaloids **1** and **2**, cell cultures produced their corresponding 3-oxides, **7** and **8**, respectively, as well as β-carboline-1-propionic acid (**11**), 4-methoxy-β-carboline-1-carboxylic acid methylester (**10**), 5-hydroxycanthin-6-one (**6**) previously found in *A. altissima* (1,2,4), and 1-hydroxycanthin-6-one (**3**) found in *Ailanthus giraldii* (7). Furthermore, the following three alkaloids never before found in nature, 2-hydroxycanthin-6-one (**4**), 4-hydroxycanthin-6-one (**5**), and 4,5-dihydrocanthin-6-one (**9**), have been extracted. In spite of the predominant alkaloid canthin-6-one (**1**) produced in the amount of 300 μg/ml of cell suspension, each of the new compounds **4, 5,** and **9** represent 0.3% of the total alkaloids, and their production is 2 μg/ml or less.

RESULTS AND DISCUSSION

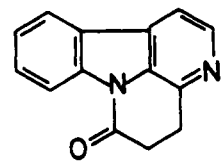
In order to obtain a good amount of crude alkaloids and to identify all the alkaloids present in it, a batch of about 50 liters of cell suspension culture was prepared by pooling 900 Erlenmeyer flask cultures. Cells only were extracted as reported in the Experimental section, the medium being discarded because of its negligible content in alkaloids and high concentration of nutrients. The toluene extract of the cells was loaded on a silica gel column in toluene and eluted with increasing amounts of EtOAc. By this method pure **1** and **2** were obtained, while alkaloids **2, 7, 8,** and **10** required further purification. Compound **9** submitted to eims showed a molecular ion at *m/z* 222, corresponding to a dihydrocanthin-6-one. The fragmentation pattern was similar to that



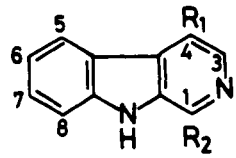
	R_1	R_2	R_3	R_4
1	H	H	H	H
2	OCH_3	H	H	H
3	OH	H	H	H
4	H	OH	H	H
5	H	H	OH	H
6	H	H	H	OH



7 R_1=H
8 R_1=OCH_3



9



10 R_1=OCH_3, R_2=$COOCH_3$
11 R_1=H, R_2=CH_2-CH_2-COOH

of canthin-6-one, the only difference being the presence of $[M-H]^+$ and $[M-H-CO]^+$ ions of high intensity. ^1H nmr established that while the indole ring and positions 1 and 2 remained unchanged, the reduction had taken place at carbons 4 and 5 giving rise to two multiplets at δ 3.49 and δ 3.22 each corresponding to a methylene group (see Table 1). The two alkaloids **4** and **5** obtained as a mixture from the *n*-BuOH extract (see Experimental section) were separated and purified by preparative and analytical tlc.

Their mass spectra gave the same molecular weight (*m/z* 236), which corresponds to an oxygen atom more than that of canthin-6-one. The fragmentation patterns of both compounds were similar, both recalling that of canthin-6-one, showing consecutive losses of CO and HCN from the molecular ion. In compound **4** the possible sites of hydroxylation were position 1 and 2, since ^1H nmr showed that the indole nucleus and positions 4 and 5 were untouched (see Table 1). Comparison of acetylated **4** with an authentic sample of 1-acetoxycanthin-6-one showed that hydroxylation had taken place in position 2, even though chemical shift values were not in agreement with the expected substitution effects (8). Possibly the nearby nitrogen influenced the position in space of the acetyl substituent thus causing unexpected anisotropic effects on H-1 (see Table 1).

Alkaloid **5** was identified based on the acetylated derivative, which showed the indole ring and positions 1 and 2 unchanged; the chemical shift value of H-5 at δ 7.64 was in good agreement with known substituent effects (8) (H-5 in **5**: δ 7.60).

Although the two alkaloids **4** and **5** have never been described in nature, the reported presence of 4-methoxycanthin-6-one in *Charpentiera obovata* (9) suggests that production of **5** is likely. It is noteworthy that in cell cultures of *A. altissima* canthin-6-one is oxidized only in positions 1,2,3,4, and 5, i.e., in all the possible positions of

TABLE 1. ^{1}H-nmr Chemical Shift Values [coupling constants (Hz) in parentheses]

Compound	Solvent	Proton							
		H-1	H-2	H-4	H-5	H-8	H-9	H-10	H-11
1	$CDCl_3$	7.96 (5.0)	8.82 (5.0)	6.98 (9.8)	8.02 (9.8)	8.67 (8.1, 1.0, 1.0)	7.70 (8.1, 7.7, 1.0)	7.52 (7.7, 7.7, 1.0)	8.11 (7.7, 1.0, 1.0)
3	DMSO-d_6	8.71	—	6.86 (9.8)	8.11 (9.8)	8.54 (8.1, 1.0)	7.75 (8.1, 7.5, 1.0)	7.59 (7.5, 7.5, 1.0)	8.43 (7.5, 1.0)
4[a]	$CDCl_3$	8.56	—	6.90 (9.8)	7.98 (9.8)	8.68 (8.2, 1.0)	7.70 (8.2 7.5, 1.0)	7.55 (7.5, 7.5, 1.0)	8.17 (7.5, 1.0)
5	DMSO-d_6	8.17 (5.2)	8.78 (5.2)	—	7.60	8.55 (8.0, 1.0)	7.78 (8.0, 1.2)	7.60 (8.0, 1.2)	8.37 (8.0, 1.0)
5[a]	$CDCl_3$	7.90 (5.1)	8.80 (5.1)	—	7.64	8.68 (8.0, 1.0	7.71 nd[b]	7.54 nd	8.11 (7.7, 1.0)
9	$CDCl_3$	7.72 (5.5)	8.52 (5.5)	3.49 (7.9)[c]	3.22 (7.9)[c]	8.53 (8.3, 1.0)	7.67 nd	7.48 nd	8.05 (7.7, 1.0)

[a]Product acetylated.
[b]Values not determined.
[c]Apparent coupling constant.

rings C and D. In accordance with the literature, no oxidation occurs in ring A; oxidations in this region of the molecule of **1** occur in plants different from *A. altissima*, such as *Odyendyea gabonensis, Simaba multiflora*, and *Amaroria soulameoides* in which, respectively, 8-hydroxyoanthin-6-one (10), 10-hydroxycanthin-6-one (11), and 11-hydroxycanthin-6-one (12) have been found. All these plants belong to the family Simaroubaceae.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined using a Kofler hot stage and are uncorrected. Unless otherwise stated, uv spectra were obtained in MeOH solution. ^{1}H-nmr spectra were recorded at 200 MHz on a XL-200 Varian spectrometer in $CDCl_3$ or DMSO-d_6 solutions. Eims of all compounds were recorded on a Varian mat 311 A mass spectrometer at an electron energy of 70 eV. The temperature of the direct inlet probe was 60°-180°, and the source temperature was 250°. Tlc analyses were carried out on silica gel plates G (Merck) with the following solvent systems: (a) *n*-hexane-Me_2CO (60:40); (b) CH_2Cl_2-iPrOH (92:8); (c) CH_2Cl_2-MeOH (90:10); (d) CH_2Cl_2-MeOH (80:20); (e) CH_2Cl_2-MeOH-HOAc-H_2O (80:20:7:3).

PLANT MATERIAL.—Callus cultures were obtained from leaves, stems, and shoots of *A. altissima* and transferred to a solid Murashige and Skoog's (MS) medium (13) containing 1 mg/liter of 2,4-dichlorophenoxyacetic acid (2,4-D). These cultures produce virtually the same mixture of alkaloids determined by tlc or hplc.

Submerged cutures were obtained by transferring the callus into 300-ml Erlenmeyer flasks with 60 ml of MS liquid medium containing 2,4-D (1 mg/liter) and stirring on a rotary shaker (100 rpm) at 28° in the dark. Cultures were harvested at 25 days when they produced an average of 400 mg/liter of alkaloids, 90% of which corresponded to **1**.

ALKALOID EXTRACTION.—More than 50 liters of cell suspension cultures of *A. altissima* were processed. The cultures were centrifuged in the cold at 3,000×*g* and the supernatant discarded. The cells were

suspended in 80% EtOH, homogenized with an Ultra-turrax apparatus (Janke & Kukel), and centrifuged. The sediment was extracted twice in the same way, and each extraction was checked spectrophotometrically for canthin-6-one derivatives. The pooled extracts were reduced to one fifth of their initial volume under reduced pressure. The aqueous solution was brought to pH 9 with NH_3 and extracted three times with an equal volume of toluene. The combined toluene extracts were dried over Na_2SO_4 and evaporated under reduced pressure. The resulting aqueous layer was brought to pH 4 with HCl and extracted repeatedly with *n*-BuOH saturated with H_2O. The extracts were pooled, neutralized, and evaporated under reduced pressure.

CHROMATOGRAPHY.—The alkaloid mixture extracted in toluene was chromatographed on a silica gel Merck (230-400 mesh) (1.2 kg) column in toluene. More than 100 one-liter fractions were collected, and each was checked spectrophotometrically and by tlc.

The column washed with toluene (fractions 1-18) afforded an oil corresponding mainly to the triglycerides present in the cells. Elution started with toluene and 10% EtOAc) fractions 19-45) and gave 26.6 g of canthin-6-one (**1**). Increasing EtAOc to 20% (fractions 53-70), a mixture of 1-methoxycanthin-6-one (**2**), and 4-methoxy-β-carboline-1-carboxylic acid methylester (**10**) was obtained. Further elution with 30% of EtOAc (fractions 71-80) gave 92 mg of pure 4,5-dihydrocanthin-6-one (**9**). Following elution with toluene and 10% MeOH (fractions 81-90), a mixture of canthin-6-one-3-oxide (**7**) and 1-methoxycanthin-6-onbe-3-oxide (**8**) was obtained (358 mg expressed as **7** by uv absorbance). The two alkaloids were separated by tlc in systems a and b.

The column was then eluted with toluene and 50% MeOH (fractions 91-100) and finally with pure MeOH (fractions 101-110). In the last fractions only traces of 1-hydroxycanthin-6-one were present. In order to separate the mixture of **2** and **10** obtained from fractions 52-70, a reverse phase column chromatography was performed on a Lichroprep RP-18 (Merck) column, particle size 25-40 μm in H_2O, and one-liter fractions were collected. Elution started with 20% MeOH in H_2O (fractions 1-4), continued with 50% MeOH (fractions 5-8), and concluded with MeOH (fractions 9-12). Fractions 1-4 yielded 2.5 g of pure **2** and fractions 5-8, 0.153 g of pure **10**.

The *n*-BuOH extract was evaporated and the brownish syrupy residue resuspended in H_2O. Because of the large amount of impurities, only one half of it was processed. A reverse phase column chromatography was performed on a column Lichroprep RP-18, particle size 25-40 μm (g 150); 300-ml fractions were collected, analyzed by tlc, and pooled according to their chromatographic behavior. After washing with H_2O (fractions 1-5), elution was performed with H_2O and 10% MeOH (fractions 6-17), 25% MeOH (fractions 18-29), 40% MeOH (fractions 30-38), and with pure MeOH (fractions 39-50). Fractions 21-24 showed the presence of compound **11** which was purified by subsequent analytical tlc in systems c and d (mg 370). Fractions 25-34 were pooled because of the predominance of products **4** and **5**. These two alkaloids were then separated and purified by preparative and analytical tlc in systems d and e (53 and 45 mg, respectively). Fractions 35-42 showed the presence of alkaloids **3** and **6**, which were isolated and purified by tlc in the same way as **4** and **5**.

IDENTIFICATION OF ALKALOIDS.—The identities of canthin-6-one (**1**) and 1-methoxycanthin-6-one (**2**) were confirmed by analytical data in comparison with authentic samples and are in agreement with the literature (1). 1-Hydroxycanthin-6-one (**3**) was identified by its uv, eims, and ^{1}H-nmr spectra, all data being in agreement with the literature (7).

2-Hydroxycanthin-6-one (**4**) was obtained as a colorless amorphous solid from MeOH: uv λ max (MeOH) nm (log ε) 256 (3.43), 266 (3.55), 274 (3.56), 315 (3.41), 342 (3.50), 358 (3.73), 375 (3.70); uv λ max (MeOH+HCl) nm (log ε) 256 (3.45), 270 (3.54), 342 (3.52), 358 (3.68), 375 (3.70); uv λ max (MeOH+NaOH) nm (log ε) 256 (3.55), 266 (3.61), 274 (3.63), 315 (3.45), 342 (3.52), 358 (3.73); eims *m/z* (rel. int.) 236 (M^+, 100), 208 (61), 181 (12), 153 (33), 152 (11), 126 (15), 73 (18), 60 (34), 57 (20), 43 (21). ^{1}H-nmr data are listed in Table 1.

4-Hydroxycanthin-6-one (**5**) was obtained as a yellow amorphous solid from MeOH; uv λ max (MeOH) nm (log ε) 238 (3.49), 244 (sh. 3.44), 260 (3.18), 294 (3.06), 338 (3.13), 352 (3.36), 370 (3.35); uv λ max (MeOH+HCl) nm (log ε) 244 (3.45), 200 (2.90), 308 (3.23), 320 (3.22), 345 (3.16), 364 (3.40), 382 (3.44); uv λ max (MeOH+NaOH) nm (log ε) 338 (3.30), 352 (3.40), 370 (3.37); eims *m/z* (rel. int.) 236 (M^+, 100), 180 (45), 179 (30), 153 (15), 73 (33), 60 (35), 57 (48), 45 (62). ^{1}H-nmr data are listed in Table 1.

Acetyl derivatives of **4** and **5** were obtained with Ac_2O/pyridine and then purified by tlc in solvent systems b and c. 5-Hydroxycanthin-6-one (**6**) was identified by its uv, eims, and ^{1}H-nmr spectra, all data being in agreement with the literature (1,4,14).

Canthin-6-one-3-oxide (**7**) and 1-methoxycantin-6-one-3-oxide (**8**), were crystallized from MeOH and identified by eims (M^+ 236 and M^+ 266, respectively) and ^{1}H nmr in agreement with published values (1,3). Comparison with 3-oxides obtained by oxidation of **1** and **2** as reported in the literature (1,3) confirmed their identities.

4,5-Dihydrocanthin-6-one (**9**) was obtained as pale yellow needles (CH_2Cl_2/petroleum ether) mp 128°; uv λ max (CH_2Cl_2) nm (log ε) 260 (4.35), 270 (sh. 4.25), 282 (4.25), 300 (sh. 3.78), 314 (3.99), 326 (4.06); eims *m/z* (rel. int.) 222 (M^+, 57), 221 (22), 194 (70), 193 (100), 192 (15), 168 (21), 140 (30), 139 (16), 113 (18), 63 (21). ^{1}H-nmr data are listed in Table 1. The product was identical with an authentic sample obtained by reduction of **1** with Zn dust (3,15).

4-Methoxy-β-carboline-1-carboxylic acid methylester (**10**) was identified by its uv, eims, and ^{1}H-nmr spectra, all data being in agreement with the literature (1,2).

β-Carboline-1-propionic acid (**11**) was identical with an authentic sample obtained by oxidation of **1** (4), and all its data (uv, eims, and ^{1}H nmr) were in agreement with the literature (16).

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