Biotransformation of Chanoclavine by *Euphorbia calyptrata* Cell Culture

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Structures of oxepino[5,4,3-*c*,*d*]indole derivatives (**2**, **3**) and 3,4-disubstituted indoles (**4**, **5**) resulting from biotransformation of chanoclavine by *Euphorbia calyptrata* cell culture have been elucidated by NMR and mass spectroscopy.

Ergot alkaloids and their derivatives exhibit various physiological effects widely used in medicine.[1−5] The majority of newly developed drugs of this group are semisynthetic compounds.[6] Some simple representatives of this class (e.g., agroclavine or chanoclavine) are available in large quantities from submerged cultivation of certain *Claviceps* strains.[2,7] One possible way that their functionalization may lead to new prospective drugs is through biotransformation.[9] So far, biooxidation of agroclavine, targeted to its hydroxy derivatives, has been extensively studied.[10] Glycosidation of chanoclavine (**1**) by *Claviceps fusiformis*[11] and several glycosidases[12,13] was reported. To the best of our knowledge, no oxidative biotransformation of this compound was ever tried. This paper presents new results of biotransformation of **1** by plant cell cultures.

Results and Discussion

Suspension plant cell cultures of *Euphorbia calyptrata* var. *involucrata* Bett. (Euphorbiaceae),[14] *Atropa belladona* L. (Solanaceae), *Armoracia rusticana* Gaertn., Mey. L. Schreb. (Brassicaceae), and *Solanum aviculare* Forst. (Solanaceae)[15] were tested for biotransformation of **1**. Even though all tested cultures produced a similar spectrum of biotransformation products from **1**, *E. calyptrata* gave highest yields, and therefore it was chosen for preparatory purposes. In biotransformation of **1** with *E. calyptrata* cells, the first new Ehrlich-positive compounds appeared during 4−12 hours of cultivation, and **1** quickly disappeared. At least four new substances were detected on TLC in 70-h cultures: one (**4**) gave the usual blue color with Ehrlich reagent, one (**5**) produced an azure spot typical for aldehyde-containing ergot alkaloids,[8] and two compounds (**2**, **3**) gave initially green spots that later turned to pink.

EISM of compound **2** exhibited a $M^{\bullet+}$ ion at *m*/*z* 259 ($C_{15}H_{17}NO_3$) and an ion at *m*/*z* 228 ($M^+ - CH_2OH$). The difference in elemental composition between **1** and **2** ($-CH_3N$, O_2) suggested a loss of an *N*-methyl group and the introduction of two oxygen atoms. Indeed, the *N*-methyl signal was absent in the ^{1}H-NMR spectrum of **2**, which still contained signals of a disubstituted indole nucleus, one CH_2CH_2O- moiety, one CH_2O-, two OCH, and one $C-CH_3$ group. Fifteen protons are attached to carbons, that leaves two hydrogen atoms bonded to heteroatoms. One of them accounts for the indole N−H; the second, for the primary hydroxy group inferred from MS. Therefore, the two remaining oxygen atoms are of the ether type. Long-range couplings showed that the indole substituents are the CH_2CH_2O group and the isolated oxymethine (s, 5.750 ppm), assigned to H-4 and H-10, respectively. To account for the chemical shifts of H-10, C-5, and C-10, an oxygen bridge between these atoms is suggested. Other inter-proton long-range couplings observed among CH_2OH, CH_3, and the second oxymethine singlet (3.816 ppm) indicated presence of an 8,9-epoxide. Finally, a long-range coupling was also observed between H-10 and the terminal methyl, which suggested structure **2**. Small or negligible $J_{9,10}$ values indicate that the corresponding dihedral angle is close to 90°. Some fragmentations found in EIMS [loss of CH_3CO from $M^{\bullet+}$, loss of CH═O (228 − 198)], could be rationalized by rearrangements and migrations observed in the mass spectra of many substituted oxiranes.[16]

As indicated by its ^{1}H-NMR spectrum, compound **3** contains a disubstituted indole ring, in addition to CH_2-CH_2O-, $CH(O-)CH(O-)$, CH_2OH, and CH_3 groups.

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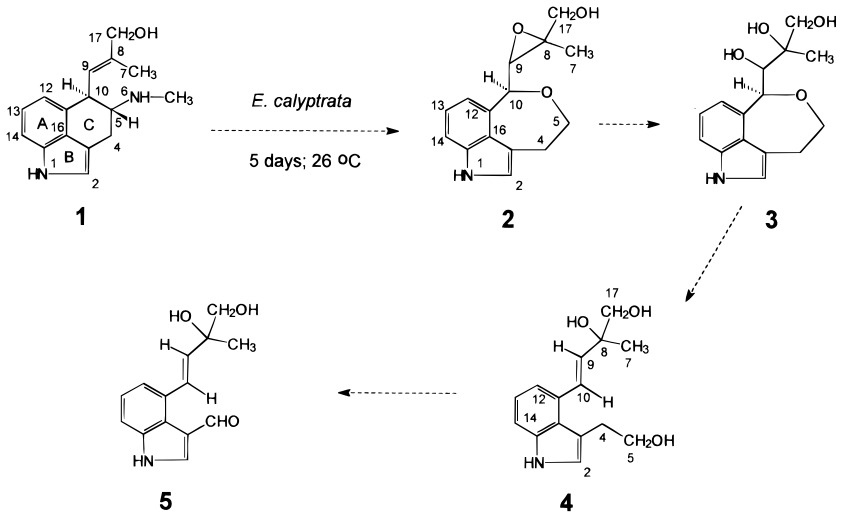
Scheme 1



Observed long-range couplings (H-2 to both H-4s, H-13 to H-10, H-9 to H-7) and chemical shift considerations for C-5 and C-10 led to structure **3**. The molecular ion was not observed in EIMS, CIMS, or FABMS, probably because of thermal lability of this compound. Main fragment ions in EIMS are due to the loss of two H_2O (*m*/*z* 259 and 241), CH_2OH (*m*/*z* 210), and CH═O (*m*/*z* 180).

The molecular ion of compound **4** *m*/*z* 261 ($C_{15}H_{19}NO_3$) readily undergoes dehydration, giving the base peak *m*/*z* 243. This ion either stepwise loses CH_2OH, C_2H_2, and CH_2═O, giving ions *m*/*z* 212, 184, and 154, or eliminates CH_3 and then CH_2═O with formation of ions at *m*/*z* 228 and *m*/*z* 198. Comparison of the molecular formula with the number of carbon-attached protons indicates four hydrogens bonded to heteroatoms. Subtraction of an indole N−H left three OH groups. The ^{1}H-NMR spectrum indicated that **4** contained a disubstituted indole moiety, one transdisubstituted double bond (J = 15.9 Hz), one CH_2CH_2OH side chain, and CH_2OH, CH_3, and OH groups. The latter three groups are likely located on the same carbon (C-8, 74.81 ppm) because of a long-range coupling between CH_2OH and CH_3 protons. The couplings of this type observed between H-2 and both C-4 protons and between one olefinic proton and H-12 and H-13 led to structure **4**.

The last isolated compound, **5**, contained only 14 carbon atoms, one aliphatic methyl, one OCH_2, seven sp^2-type CH (including one CH═O), one aliphatic C−O, and four sp^2-hybridized quaternary carbons. The ^{1}H-NMR spectrum suggested a disubstituted indole, one trans-disubstituted double bond (J = 16.2 Hz), a tertiary methyl, and an isolated CH_2OH. The presence of a tertiary OH group was also assumed in order to satisfy all observed facts. Long-range couplings (H-2 to CH═O, H-10 to H-12 and H-13, H-9 to C═CH_3, C-CH_3 to CH_2-OH) led to the formula **5**. No molecular ion was observed in EIMS. The ions with higher mass (*m*/*z* 227, 214, and 198) correspond to M − H_2O, M − CH_2OH, and 227 − CH═O, and so support the proposed structure.

Reactions of the ergoline system with peroxidases are well documented.[9,17] Various 8-hydroxy, 10-hydroxy, and epoxy compounds are known, and N-6 demethylation has been observed.[9,17−20] Another reactive site in this skeleton is position 2. Substitution at position 2 leads to 2,3-dihydro-2-oxo- or 2,3-dihydro-2-oxo-3-hydroxyderivatives.[21,22] Oxidative B-ring opening was reported in later fermentation stages of *Claviceps purpurea*.[23] A 9,10-epoxide ring, present in the metabolite **2**, was previously found in epoxyagroclavine-I.[24] The oxepino[5,4,3-*c*,*d*]indole system, in **2** and **3**, has an aza analogue in aurantioclavine[25] and clavicipitic acids I and II.[26] Two 3,4-disubstituted indoles—*N*-methyl-4-dimethylallyltryptophan[27] (an important biosynthetic precursor of ergot alkaloids) and 4-(4-hydroxy-3-methylbut-2-enyl)tryptophan—are well known metabolites of *C. purpurea* and biosynthetic precursors of ergot alkaloids.[28−30] Analogues of **4** and **5** were described as intermediates in the syntheses of 6,7-secoagroclavine.[31,32] Therefore, the observed oxidative biotransformation of chanoclavine (Scheme 1) mimicks the synthetic process in the reverse order. In fact, our results show that the described process is an oxidative biodegradation. It is probably not general, because chanoclavine, as a typical secoclavine, contains a secondary amino group instead of a tertiary one present in the majority of ergot alkaloids.

Experimental Section

General Experimental Procedures. Ergoline numbering (Scheme 1) was adopted throughout this paper to facilitate comparison with the parent compound. ^{1}H- and ^{13}C-NMR spectra were measured on a Varian VXR-400 spectrometer (399.95 and 100.58 MHz, respectively) in CD_3OD at 25 °C. Residual solvent signal (δ_H 3.33, δ_C 49.3) served as an internal reference. Chemical shifts are given in the δ scale; J values are given in Hz, digital resolution was 0.0002 and 0.006 ppm, respectively. Carbon signal multiplicity was determined by an APT (Attached Proton Test) experiment. Manufacturer's software was used for 2D NMR (COSY, ROESY, HOM2DJ). EIMS were recorded on Finnigan MAT 90 (Finnigan MAT, Bremen, Germany) double-focusing instrument (electron energy 70 eV, electron current 0.5 mA, source temperature 250 °C, accelerating voltage 5 kV, direct inlet). Accurate mass determinations (peak matching with perfluorokerosene) were performed on a JEOL JMS-D100 (JEOL Ltd. Tokyo, Japan) double-focusing instrument (electron energy 80 eV, electron current 0.3 mA, source temperature 150 °C, accelerating

voltage 3 kV). UV spectra were measured on a Pye Unicam 8740 UV/vis spectrophotometer in MeOH.

HPLC analyses were performed under the following conditions: SP 8800 ternary gradient pump, SP 8880 autosampler, Spectra Focus scanning UV/vis detector, column 150 × 3.3 mm packed with Separon SGX C18, 7 μm (Tessek, Prague, Czech Republic). Gradient-elution separation was used; 4−90% MeOH-H_2O (+ 0.04% NH_4OH), gradient time 60 min, flow rate 0.5 mL/min, scan 210−360 nm. Preparative HPLC was performed on the Spectra Physics modular preparative HPLC system (San Jose, CA) comprising of SP8810Ti pump, Rheodyne injection port with a 100-μL sample loop, Spectra 100 variable wavelength UV/vis detector set at 285 nm, and ChromJet SP 4400 integrator. The column (250 × 8 mm) was packed with 7-μm Separon SGX C18 (Tessek, Prague, Czech Republic) and eluted with MeOH-H_2O-NH_4OH (40:60:0.04) at a flow rate of 2 mL/min.

Plant Cell Cultures and Feeding Experiments. The cultures of *S. aviculare*, *A. belladonna*, and *A. rusticana* are deposited at the Institute of Organic Chemistry and Biochemistry, Czech Academy of Science, Prague, Czech Republic (maintained on a Gamborg medium),[33] *Duboisia myoporoides* culture was a kind gift of Prof. A. Jacquin-Dubreuil, Université de Picardie Jules Verne, Amiens, France; and the cultures of *E. calyptrata*[14] are deposited in the culture collection of the Department of Pharmaceutical Sciences, University of Bologna, Bologna, Italy. Media and cultivation conditions were reported in detail elsewhere.[14,15,33] Chanoclavine (**1**) was kindly provided by Galena Pharmaceuticals Co., Opava, Czech Republic. Portions (0.5 mL) of MeOH solution of **1** (20 mg/mL) were fed to suspension plant cell cultures (80 mL) in 300-mL conical flasks cultivated on rotary shaker at room temperature and exposed to normal day light.

Sampling and Chromatography. Samples for analysis (cells + medium) were taken immediately after addition of **1** and then at 4-, 10-, 24-, 48-, and 72-h intervals. Samples (10 mL) were treated with an equal amount of MeOH, homogenized by Ultra-Turrax, and centrifuged. The pellet was re-extracted with Me_2CO (10 mL). Both extracts were pooled and evaporated to less than one-tenth of the original volume in order to remove all organic solvents. The aqueous phase was diluted with H_2O to ca. 5 mL and loaded onto an SM-2 prepacked column (Bio-Rad, USA). The column was washed with H_2O (20 mL) and eluted with EtOH (20 mL). The eluate was evaporated to 2 mL and analyzed by TLC in solvent system C. The spots were inspected under UV light and visualized by Ehrlich reagent.[8] The following solvent systems were used for the TLC analyses (Si gel 60, F_{254}, Merck): A = CH_2Cl_2−MeOH (8:2), B = CH_2Cl_2−MeOH (93:7, twice developed), C = CH_2Cl_2−MeOH (9:1).

Isolation and Charaterization of 2−5. Compound **1** (90 mg, dissolved in 3 mL MeOH) was evenly dispensed into six 300-mL flasks each containing 100 mL of 5-day old *E. calyptrata* submerged culture in modified Gamborg medium.[10] After a further 2 days of cultivation, another portion of **1** (90 mg) dissolved in MeOH was dispensed into the flasks and cultivated another 3 days.

The cultures were pooled, an additional equal amount of MeOH was added, and the suspension was homogenized by Ultra-Turrax (3 × 1 min). After centrifugation the pellet was re-extracted with Me_2CO (150 mL), and the extracts were pooled. This solution was reduced to one-tenth by evaporation, diluted with H_2O to 500 mL, and slowly loaded onto a column filled with XAD-2 nonionic porous resin (C. Erba, Italy) (600 g) in H_2O. The resin was washed extensively with H_2O (2 L) and then eluted with MeOH (600 mL), EtOH (150 mL), and Me_2CO (150 mL). Combined solutions were evaporated to a syrup, and most of the plant-cell products were separated by flash chromatography [SiO_2, Merck 60, 43−60 μm, CH_2Cl_2-MeOH (8:2 + 0.1% NH_4OH)]. The fractions containing substances with higher R_f values (**2** + **3**) (40 mg) and the fractions containing **4** + **5** (27 mg) were separated by medium-pressure chromatography using a prepacked column Lobar size A (Si gel) (Merck, Germany). Elution with CH_2Cl_2-MeOH (95:5) containing 0.1% NH_4OH, flow rate 2 mL/min, linear gradient (5−10% MeOH), gradient time 80 min, afforded **2** (18 mg) and **3** (9 mg). The second pre-separated fraction afforded pure **4** (3 mg) and **5** (6 mg).

Alkaloid 2: colorless amorphous solid; UV (MeOH) λ max [nm] 218, 250 (br), 282.5; EIMS *m/z* 259 ($M^{\bullet+}$; 83, $C_{15}H_{17}NO_3$, calcd 259.1208, measured 259.1213), 228 (28; $C_{14}H_{14}NO_2$, calcd 228.1024, measured 228.1011), 216 (71; $C_{13}H_{14}NO_2$, calcd 216.1024, measured 216.1020), 198 (50; $C_{13}H_{12}NO$, calcd 198.0919, measured 198.0934), 170 (46), 168 (39), 158 (82), 156 (69), 154 (100; $C_{11}H_8N$, calcd 154.0657, measured 154.0659), 144 (77), 143 (66), 130 (83), 129 (51), 127 (45), 115 (48), 77 (30), 43 (63); ^{1}H NMR (CD_3OD, 399.95 MHz) δ 7.137 (1H, dd, J = 8.1, 1.0 Hz, H-14), 7.159 (1H, dd, J = 0.9, 0.9 Hz, H-2), 7.106 (1H, dd, J = 8.1, 7.4 Hz, H-13), 6.932 (1H, ddd, J = 7.4, 1.0, 0.7 Hz, H-12), 5.750 (1H, s, H-10), 3.972 (1H, d, J = 10.1 Hz, H-17d), 3.960 (1H, d, J = 10.1 Hz, H-17u), 3.896 (1H, m, H-5d), 3.846 (1H, m, H-5u), 3.816 (1H, s, H-9), 3.181 (2H, m, H-4), 1.607 (3H, s, H-7); ^{13}C NMR (CD_3OD, 100.58 MHz) δ 125.40 (d, C-2), 122.37 (d, C-13), 117.22 (d, C-14), 79.04 (d, C-10), 71.10 (t, C-5), 67.18 (d, C-9), 64.46 (t, C-17), 32.27 (t, C-4), 14.10 (q, C-7).

Alkaloid 3: yellowish amorphous solid; UV (MeOH) λ max [nm] 250 (br), 293 (br); ^{1}H NMR (CD_3OD, 399.95 MHz) δ 7.348 (1H, dd, J = 7.7, 1.5 Hz, H-14), 7.140 (1H, dd, J = 7.7, 1.5 Hz, H-12), 7.152 (1H, dd, J = 1.0, 1.0 Hz, H-2), 7.118 (1H, dd, J = 7.7, 7.7 Hz, H-13), 5.131 (1H, s, H-10), 3.875 (2H, m, H-5), 3.697 (1H, d, J = 7.5 Hz, H-9), 3.627 (1H, d, J = 12.4 Hz, H-17d), 3.537 (1H, d, J = 12.4 Hz, H-17u), 3.216 (2H, m, H-4), 1.165 (3H, s, H-7); ^{13}C NMR (CD_3OD, 100.58 MHz) δ 125.37 (d, C-2), 122.32 (d, C-13), 118.51 (d, C-12), 113.05 (d, C-14), 71.63 (d, C-10), 66.81 (t, C-5), 65.52 (d, C-9), 64.66 (t, C-17), 31.86 (t, C-4), 15.04 (s, C-7).

Alkaloid 4: white amorphous solid; UV (MeOH) λ max [nm] 224, 243 (sh), 300 (br), EIMS *m/z* 261 ($M^{\bullet+}$, 6; $C_{15}H_{19}NO_3$, calcd 261.1365, measured 261.1375), 243 (100), 212 (43), 210 (15), 198 (43), 197 (18), 196 (13), 194 (17), 184 (49), 182 (26), 181 (16), 180 (17), 172 (11), 170 (33), 169 (21), 168 (40), 167 (43), 155 (26), 154 (62), 144 (17), 130 (34), 115 (17); ^{1}H NMR (CD_3OD, 399.95 MHz) δ 7.423 (1H, ddd, J = 15.9, 0.8, 0.5 Hz, H-10), 7.240 (1H, dd, J = 8.0, 1.7 Hz, H-14), 7.123 (1H, ddd, J = 7.3, 1.7, 0.8 Hz, H-12), 7.080 (1H, t, J = 0.7 Hz, H-2),

7.037 (1H, ddd, J = 8.0, 7.3, 0.5 Hz, H-13), 6.247 (1H, dq, J = 15.9, 0.7 Hz, H-9), 3.828 (2H, t, J = 7.4 Hz, H-5), 3.574 (1H, d, J = 10.8 Hz, H-17d), 3.557 (1H, d, J = 10.8 Hz, H-17u), 3.158 (2H, td, J = 7.4, 0.7 Hz, H-4), 1.430 (3H, s, H-7); ^{13}C NMR (CD_3OD, 100.58 MHz) δ 139.22 (s, C-15), 136.30 (d, C-10), 132.46 (s, C-11), 129.26 (d, C-9), 126.40 (s, C-16), 125.11 (d, C-2), 122.64 (d, C-13), 117.83 (d, C-12), 113.27 (s, C-3), 111.88 (d, C-14), 74.81 (s, C-8), 71.27 (t, C-5), 64.70 (t, C-17), 32.41 (t, C-4), 25.02 (q, C-7).

Alkaloid 5: brownish amorphous solid; UV (MeOH) λ max [nm] 226 (sh), 283 (br), EIMS m/z 277 ([M−H_2O]$^{\bullet+}$; 42; $C_{14}H_{13}NO_2$, calcd 227.0946, measured 227.0960), 215 (20), 214 (27), 199 (11), 198 (39), 184 (15), 183 (14), 182 (12), 181 (17), 180 (15), 172 (100), 170 (64), 168 (30), 167 (25), 158 (17), 156 (22), 155 (22), 154 (74), 144 (17), 130 (25), 115 (37); ^{1}H NMR (CD_3OD, 399.95 MHz) δ 9.872 (1H, s, H-4), 8.163 (1H, s, H-2), 8.093 (1H, dd, J = 16.2, 0.5 Hz, H-10), 7.480 (1H, ddd, J = 7.5, 1.1, 0.5 Hz, H-12), 7.387 (1H, dd, J = 8.0, 1.1 Hz, H-14), 7.267 (1H, ddd, J = 8.0, 7.5, 0.5 Hz, H-13), 6.323 (1H, d, J = 16.2 Hz, H-9), 3.610 (1H, d, J = 11.0, H-17d), 3.588 (1H, d, J = 11.0 Hz, H-17u), 1.486 (3H, s, H-7); ^{13}C NMR (CD_3OD, 100.58 MHz) δ 186.75 (d, C-4), 142.54 (d, C-10), 140.54 (s, C-3), 135.85 (d, C-9), 133.59 (s, C-11), 131.85 (d, C-13), 125.46 (d, C-2), 124.32 (s, C-15), 121.67 (s, C-16), 120.72 (d, C-12), 112.61 (d, C-14), 74.91 (s, C-8), 71.25 (t, C-17), 24.66 (q, C-7).

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