Dimerization of Lysergene by *Euphorbia calyptrata* Cell Cultures

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A new spiro-oxa dimer of lysergene (**3**) has been isolated as a product of the biotransformation of lysergene (**1**) by *Euphorbia calyptrata* suspension cell culture. Its structure has been determined by spectral methods.

Lysergene (**1**) is the only ergot alkaloid known to date containing an exomethylene group. Owing to conjugation with the $\Delta^{9,10}$ double bond, this molecule is rather susceptible to oxidation or addition. The compound can be prepared easily from elymoclavine, making **1** a common starting material for various semisynthetic ergot alkaloids. Attempts to derivatize or functionalize **1** often produce complex mixtures. Therefore, we turned to biotransformations that operate under mild conditions and are often regioselective or enantioselective.[1]

The biotransformation of lysergene (**1**) has not been investigated, except for the study of its incorporation into other ergot alkaloids (lysergol, isolysergol, penniclavine, and isopenniclavine) by *Claviceps purpurea*.[2,3] Plant cell cultures have proven to be very suitable for the transformation of ergot alkaloids.[1,4] Most of the reactions observed are oxidative in nature. Therefore, cultures of *Euphorbia calyptrata* Coss & Dur (Euphorbiaceae), exhibiting a high oxidative capacity, were tested to study the biotransformation of lysergene (**1**).

The biotransformation of **1** was achieved by suspension cultures of *E. calyptrata* var. *involucrata*[5] after 70 h of cultivation. New Ehrlich's reagent-positive spots appeared after 4−12 h of cultivation. After 70 h, at least four new substances were detected by TLC; however, only one of these prevailed, and **1** disappeared.

The new (prevailing) compound **3** gave a blue spot with Ehrlich's reagent in contrast to lysergene, which exhibited a blue-gray color. It was slightly more polar than the parent compound, and it did not fluoresce in long-wave UV light, whereas a strong blue fluorescence is typical for **1**. The shift of the UV maximum from 343 to 314 nm indicated decreased conjugation in the molecule. The remaining double bond must still be conjugated with an aromatic moiety because $\Delta^{8,9}$-ergolene derivatives have a lower UV maximum, typically at 282 nm.

The molecular ion at *m*/*z* 488 of **3** corresponded to a molecular formula of $C_{32}H_{32}N_4O$. Sixteen signals observed in the ^{13}C-NMR spectrum (one CH_3, three CH_2, six CH, and six quaternary carbons) indicated a symmetrical dimer. ^{1}H−^{1}H COSY NMR revealed spin systems typical for $\Delta^{9,10}$-ergolenes with a breaking point (quaternary carbon) at C-8. The only hint of the presence of a larger compound was the signal of an isolated aliphatic methylene at 2.08−2.21 ppm. Its 14 lines were incompatible with a single CH_2 group but were consistent with the AA′BB′ pattern expected for a $-CH_2CH_2-$ moiety. The chemical shift of the sp^3-hybridized quaternary carbon (79.16 ppm) suggested its participation in the formation of an oxygen bridge formation, which also accounted for the only oxygen atom present in the molecule. The second point of linkage was formed by the above-mentioned ethylene bridge. The deduced structure might be formally derived from a putative intermediate, 8,17-epoxylysergene (**2**), produced possibly through the action of a peroxidase. Addition of this compound to **1** (probably by a free-radical mechanism) could then lead to dimer **3** (Scheme 1). The observed symmetry in the NMR spectra of **3** is consistent with an 8*R*,8′*R* or 8*S*,8′*S* configuration only.

The observed mass spectrum was interpreted in terms of structure **3**. The ion at *m*/*z* 445 is a product of a retro-Diels−Alder cleavage by the loss of $CH_2{=}NCH_3$. The abundant ions *m*/*z* 233−237 correspond to a lysergene moiety with different degrees of unsaturation. Due to the high temperature of the direct-insertion probe necessary for evaporation (ca. 300 °C), partial decomposition of the sample cannot be excluded. Therefore, some of the ions observed (e.g., in the low mass region) might correspond to pyrolytic products. The most abundant ions *m*/*z* 471 and 235 observed in collision−activated dissociation fast-atom bombardment mass spectrometry (CAD-FABMS) of the $[M + H]^{\bullet+}$ ion *m*/*z* 489 can be attributed to consecutive losses of H_2O and lysergene (236 amu).

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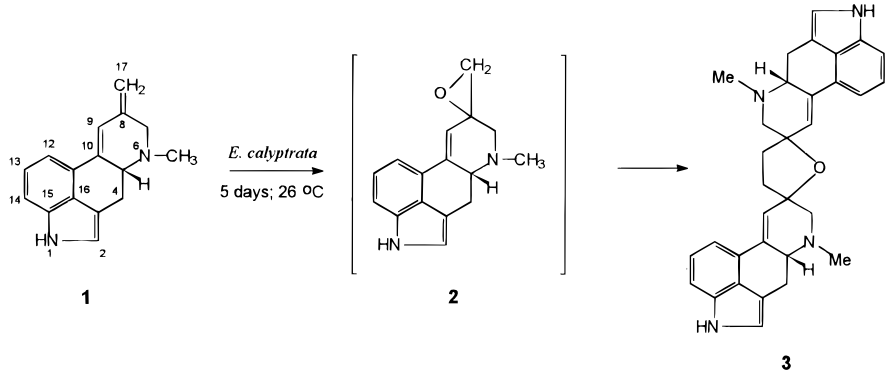
Scheme 1. Biotransformation of Lysergene (**1**) by *E. calyptrata*



1 **2** **3**

The existence of dimeric ergot alkaloid derivatives is not without precedence. 2,2-Dimeric products of ergot alkaloids have been obtained from the reaction of ergolines with boron trifluoride ethyl etherate in HOAc.[6] The 1,1-bridge resulting from presence of CH_2Cl_2 was formed from terguride in alkaline conditions under phase-transfer catalysis.[12] A 1,1-dimer of agroclavine I and a mixed dimer of agroclavine I and epoxyagroclavine I were found in the culture of *Penicillium sizovae*.[7] The same organism produces another dimeric ergot alkaloid, namely, 1,1′-bi(6,8-dimethyl-8,9-epoxy-5a,10-ergoline).[8] However, compound **3** is the first example of an ergot alkaloid dimer at position C-17. Analogous compounds might be expected as the products of the modification of lysergene (**1**).

Experimental Section

General Experimental Procedures. ^{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, with J values presented in Hz, and the digital resolution being 0.0002 and 0.006 ppm, respectively. ^{13}C-NMR signal multiplicity was determined by an APT (attached proton test) experiment. Manufacturer's software was used for 2D NMR (COSY, ROESY, HOM2DJ). The alkaloid numbering is given in Scheme 1, and the letters *a* and *e* refer to axial and equatorial protons in diastereotopic methylene groups, respectively, and the letters *d* and *u* mean the downfield and upfield resonating protons, respectively. EIMS spectra were recorded on a 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, sample was introduced via a direct insertion probe). FABMS measurements were performed using a standard saddle-field FAB gun (Ion Tech Ltd., Teddington, U.K.), operating at 2 mA current and 6 kV energy with Xe as bombardment gas. *m*-Nitrobenzyl alcohol was used as the matrix. MS/MS experiments were performed using a collisional cell located in the first field-free region with He as a collision gas. Pressure was adjusted for 50% attenuation of the primary ion beam. Products of collision-activated decomposition were analyzed by fragment ion linked scan (B/E constant). Accurate mass determinations 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). Samples were introduced *via* a direct insertion probe. A conventional peak-matching method was used with perfluorokerosene as internal standard. The instrument was tuned to a resolution of 10 000 (50% valley). UV spectra were recorded in MeOH on a Pye Unicam 8740 UV/vis spectrophotometer.

Preparation of 1. Elymoclavine (kindly donated by Galena Pharmaceutical Works, Opava, Czech Republic) (3 g) was dissolved in dry pyridine (90 mL). The solution under N_2 was cooled to 0 °C, and *p*-toluenesulfonyl chloride (9 g) was added in portions with stirring. After 1.5 h 6-methyl-8-(pyridiniomethyl)-$\Delta^{8,9}$-ergolene *p*-tosylate precipitated. The precipitate was separated by filtration, washed with Me_2CO, and dried (yield 95%). The quaternary salt obtained was suspended in EtOH (46 mL) and mixed with 2 M NaOH (32 mL). The stirred mixture was heated under reflux for 1 h and then cooled and allowed to crystallize at 0 °C overnight. Dark-brown needles of **1** were filtered off, washed with ice-cold EtOH and Me_2CO, and dried (yield 74%). The EIMS of the preparation was identical to that published previously,[9] and the NMR data are in qualitative agreement with previously published data of **1** in DMSO.[10]

Lysergene (1): UV (MeOH) λ max (log ϵ) 248 (4.22), 266 (sh, 4.07), 343 (4.01) nm; ^{1}H NMR ($CDCl_3$) δ 7.26 (1H, dd, J = 6.8, 1.8 Hz, H-12), 7.24 (1H, dd, J = 8.0, 1.8 Hz, H-14), 7.20 (1H, dd, J = 8.0, 6.8 Hz, H-13), 6.98 (1H, m, H-9), 6.94 (1H, dd, J = 2.0, 1.9 Hz, H-2), 5.07 (1H, m, H-17*d*), 4.96 (1H, m, H-17*u*), 3.52 (1H, ddd, J = 14.6, 5.9, 0.5 Hz, H-4*e*), 3.51 (1H, d, J = 13.2 Hz, H-7*a*), 3.62 (1H, dddd, J = 11.4, 5.9, 2.0, 0.8 Hz, H-5), 3.22 (1H, ddd, J = 13.2, 1.9, 1.8 Hz, H-7*e*), 2.77 (1H, ddd, J = 14.6, 11.4, 1.9 Hz, H-4*a*), 2.59 (3H, s, N-Me); ^{13}C NMR ($CDCl_3$) δ 140.6 (s), 136.4 (s), 134.0 (s), 128.0 (s), 126.5 (s, C-16), 123.4 (d, C-13), 121.8 (d, C-9), 118.4 (d, C-2), 112.6 (d, C-12), 111.1 (s, C-3), 111.0 (t, C-17), 109.8 (d, C-14), 62.5 (d, C-5), 58.8 (t, C-7), 43.0 (q, N-Me), 27.1 (t, C-4).

Feeding Experiments. Compound **1** was dissolved in MeOH (20 mg/mL), and 0.5 mL was fed to 80 mL of a suspension culture of *E. calyptrata* (9 g dry wt/L) in a 300-mL Erlenmeyer flask. The medium used and cultivation conditions have been reported elsewhere.[5] The cell suspensions were cultivated on a rotary shaker at room temperature and exposed to normal daylight. Samples of the culture (cells and medium) were taken immediately after addition of **1** (0 h) and during cultivation (4, 10, 24, 48, and 72 h).

Assay of the Samples. The analytical samples (10 mL) were extracted as in the preparatory experiment (see below) and loaded onto SM-2 prepacked columns (Bio-Rad, Hercules, CA). The columns were washed with H_2O (20 mL) and eluted with EtOH (20 mL). The eluates were evaporated to 2 mL and analyzed by TLC (SiO_2 gel 60, F_{254}, Merck; mobile phase CH_2Cl_2−MeOH, 9:1). The spots were inspected under UV light and visualized by Ehrlich's reagent.[11]

Isolation and Characterization of 3. For preparative purposes, **1** (90 mg), dissolved in MeOH, was fed as described above into 15 flasks containing the culture of *E. calyptrata*. After 2 days of cultivation on a rotary shaker, another portion of **1** (90 mg), dissolved in MeOH, was dispensed into the flasks and cultivated for another 3 days. The cultures were pooled, treated with an equal amount of MeOH, homogenized with a Ultra-Turrax homogenizer, and centrifuged. The pellet was reextracted with Me_2CO (100 mL). Both extracts were pooled and evaporated to less than 1/10 of the original volume in order to remove all organic solvent. The aqueous phase was diluted with H_2O to about 500 mL and slowly loaded onto a column filled with XAD-2 nonionic porous resin (Carlo Erba, Italy) (600 g) in H_2O. The resin was washed extensively with H_2O (2 L) and then eluted with MeOH (600 mL). Eluent was evaporated to a syrup, and most of the plant-cell products were removed by flash chromatography (SiO_2, Merck 60, 43−60 μm, CH_2Cl_2−MeOH, 8:2 + 0.1% NH_4OH). The alkaloid-containing fractions were separated by medium-pressure chromatography using a prepacked Lobar column, size A (Si gel 60, Merck). Elution with mobile phase CH_2Cl_2−MeOH, 95:5 + 0.1% NH_4OH, flow rate 2 mL/min, linear gradient (5−10% MeOH), gradient time 80 min, affording **3** (9 mg, yield 5%).

Compound 3: UV (MeOH) λ max (log ϵ) 227 (4.32), 243 (4.31), 314 (3.91) nm; ^{1}H NMR ($CDCl_3$) δ 8.02 (2H, d, J = 1.8 Hz, 2 × N-H), 7.20 (2H, m, 2 × H-14), 7.15 (4H, m, 2 × H-12, H-13), 6.31 (2 H, dd, J = 2.1, 1.0 Hz, 2 × H-9), 3.38 (2H, dd, J = 14.6, 5.5 Hz, 2 × H-4*e*), 3.14 (2H, ddd, J = 11.4, 5.5, 2.1 Hz, 2 × H-5), 3.03 (2H, dd, J = 11.5, 1.0 Hz, 2 × H-7*e*), 2.79 (2 H, ddd, J = 14.6, 11.4, 1.8 Hz, 2 × H-4*a*), 2.61 (2 H, d, J = 11.5 Hz, 2× H-7*a*), 2.53 (6H, s, 2 × N-Me), 2.08−2.21 (4H, AA′BB′, 2 × H-17); ^{13}C NMR ($CDCl_3$) δ (each signal represents 2C) 136.5 (s, C-10), 133.9 (s, C-11), 128.5 (s, C-15), 126.6 (s, C-16), 125.3 (d, C-9), 123.0 (d, C-13), 118.3 (d, C-2), 112.2 (d, C-12), 111.3 (s, C-3), 109.6 (d, C-14), 80.0 (s, C-8), 64.1 (t, C-7), 62.4 (d, C-5), 43.5 (q, N-Me), 36.0 (t, C-17), 25.9 (t, C-4); EIMS m/z 488.2573 ($M^{\bullet+}$; $C_{32}H_{32}N_4O$, calcd 488.2576, 38), 445 (24), 250 (34), 249 (63), 248 (47), 247 (38), 238 (51), 237 (98), 236 (48), 235 (93), 234 (89), 233 (63), 223 (33), 222 (34), 221 (65), 220 (55), 219 (74), 212 (34), 207 (31), 198 (41), 197 (49), 183 (43), 169 (63), 168 (89), 167 (58), 154 (100).

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