Journal of Natural Products
Vol. 47. No. 3 pp. 433-438. May-Jun 1984

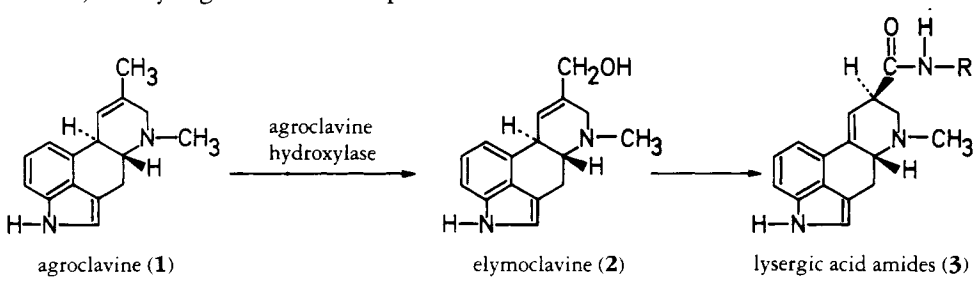
MICROBIOLOGICAL C-17-OXIDATION OF CLAVINE ALKALOIDS, I. SUBSTRATE SPECIFICITY OF AGROCLAVINE HYDROXYLASE OF *CLAVICEPS FUSIFORMIS*

REINHOLD SIEBEN, URSULA PHILIPPI, and ECKART EICH*

Fachbereich Pharmazie der Universität Mainz, D-6500 Mainz, Federal Republic of Germany

ABSTRACT.—Microbiological transformation studies have been conducted with agroclavine derivatives to obtain the corresponding derivatives of elymoclavine. This should be the first step of a directed biosynthesis leading to lysergic acid amides, with corresponding substitutions of the ergoline ring system. *Claviceps fusiformis* strain SD 58 was able to transform 1-alkyl-, 1-benzyl-, 1-hydroxymethyl-, 2-halo-, 2,3-dihydro-, and 6-ethyl-6-nor-agroclavine to the corresponding elymoclavine-derivatives in submerged cultures. It was shown that the substrate specificity of the agroclavine hydroxylase is high with respect to the 8,9-double bond and to the tertiary state of N-6, whereas the specificity is low for variations in the pyrrole partial structure (N-1, C-2, C-3).

Ergot alkaloids modified at the ergoline ring system for instance, methysergide and bromocriptine, are increasingly used in therapy. Compounds such as these are obtained by partial synthetic transformation of natural lysergic acid derivatives (1). The yields of these reactions are not very satisfactory because of the lability of the starting material. Agroclavine (**1**) and elymoclavine (**2**), biogenetic precursors of lysergic acid amides (**3**) (Figure 1), are more stable than **3** derivatives, so that they ought to endure, for instance, 1-alkylation or 2-halogenation with higher yields than do lysergic acid derivatives. This is true especially for **1**, because **2** shows side reactions (e.g., O-alkylation when alkylated at N-1) that are impossible for **1**. If such substituted **1** could be transformed microbiologically to the corresponding **2** and afterwards to the corresponding **3** in good yields, this would be of practical interest as an alternative way to prepare, for instance, methysergide or bromocriptine.

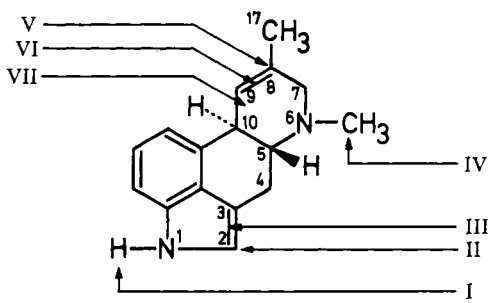


agroclavine (**1**) elymoclavine (**2**) lysergic acid amides (**3**)

FIGURE 1. Formation of lysergic acid amides from agroclavine in *Claviceps*.

The oxidation of **1** to **2** is catalyzed by agroclavine hydroxylase characterized for *Claviceps purpurea* (2) and *Claviceps fusiformis* strain SD 58 by Anderson and co-workers (3). We wish to report the realization of the first step of such a directed biosynthesis (substituted **1** to substituted **2**) on a small scale by means of *C. fusiformis* strain SD 58 well known as a producer of **2** and other clavine alkaloids in submerged culture (4). The significance of this study is increased by the fact that such substituted clavines show considerable antibiotic and strong cytostatic activity, as we reported recently (5-7). A modified sucrose-asparagine medium according to Brack *et al.* (8) was employed. The preparation of the different substrates from **1** was already published by our team (9-12), except five which were synthesized according to other authors (13-15). The partial synthesis of the corresponding derivatives of **2** is communicated in the present paper. The alkaloid (**2**) was alkylated at the indole nitrogen by reaction with potassium and alkyl

iodide in NH_3 as already described earlier for 1-methyl-**2** (**11a**) (9). The compound **11h**, 1-benzyl-**2**, was prepared from **2** in a similar manner by means of benzyl bromide. The 2-halo-**2** derivatives (**12a** and **12b**) were synthesized by reaction of **2** with N-bromo- and N-iodo-succinimide, respectively. Compound **14a** (6-nor-**2**) could be prepared by means of the cyanogen bromide method; 6-ethyl-6-nor-**2** (**14b**), by alkylation with ethyl bromide. The substrates (Figure 2) were added as a solution in aqueous tartaric acid to a final concentration of 100 μg/ml in the culture medium. Samples were withdrawn every 2 days and analyzed by tlc. An incubation period of 3 days before the addition of the ergolines was necessary for a sufficient growth of the fungus; later addition resulted in lower yields because of the concurrence between the added ergoline and the natural substrate **1** synthesized, in the meantime, in the submerged culture.



				Corresponding derivatives of elymoclavine (**2**)
I	alkylation:	1-methyl-**1** (**4a**), 1-ethyl-**1** (**4b**), 1-propyl-**1** (**4c**), 1-allyl-**1** (**4d**), 1-butyl-**1** (**4e**), 1-isobutyl-**1** (**4f**), 1-octyl-**1** (**4g**)	→	**11a-11g**
	benzylation:	1-benzyl-**1** (**4h**)	→	**11h**
	hydroxymethylation:	1-hydroxymethyl-**1** (**4i**)	→	**11i**
II	halogenation:	2-bromo-**1** (**5a**), 2-iodo-**1** (**5b**)	→	**12a, 12b**
III	hydrogenation of the 2,3-double bond:	2,3-dihydro-**1** (**6**)	→	**13**
IV	1) demethylation:	6-nor-**1** (**7a**)	⇸	**14a**
	2) ethylation:	6-ethyl-6-nor-**1** (**7b**)	→	**14b**
V	hydroxylation and shift $\Delta^{8,9} \rightarrow \Delta^{9,10}$:	setoclavine (**8**)	⇸	**15** (penniclavine)
VI	hydrogenation of the 8,9-double bond:	festuclavine (**9a**), 2-bromo-festuclavine (**9b**)	⇸	**16a** (dihydrolysergol-I), **16b** (2-bromo-dihydro-lysergol-I)
VII	isomerization ($\Delta^{8,9} \rightarrow \Delta^{9,10}$):	lysergine (**10a**), isolysergine (**10b**)	⇸	**17a** (lysergol), **17b** (isolysergol)

FIGURE 2. Agroclavine (**1**) derivatives accepted and not accepted as substrates of *Claviceps fusiformis* agroclavine hydroxylase

The 1-substituted alkyl- and benzyl-agroclavines were converted to the corresponding elymoclavines. Even traces of the substrates were not at all detectable after a certain period specific for the individual substances: the transformation rate was dependent on

the polarity of the substances (Figure 3). There were no further metabolic transformations except one which relates to the 1-octyl derivatives: besides 1-octyl-**2** (**11g**), another 1-substituted elymoclavine derivative was detected by tlc (typical dark violet color of 1-substituted ergolines with van Urk's reagent). Compound **11a** (1-methyl-**2**) and the 1-ethyl- (**11b**), 1-propyl- (**11c**), and 1-allyl- (**11d**) homologs were isolated from the medium. Their identity was proved by comparing their ir spectra and tlc behavior (cochromatography) with those of the corresponding synthesized compounds. Their identity was confirmed by mixed mps. In addition, two elymoclavine derivatives isolated from the culture medium (**11b, 11d**) were subjected to an elementary analysis. 1-Butyl-**2** (**11e**) and the 1-isobutyl- (**11f**), 1-octyl- (**11g**), and 1-benzyl- (**11h**) homologs were extracted from the medium by means of CH_2Cl_2 after alkalinization. These crude extracts were compared and cochromatographed with the corresponding synthesized compounds by tlc in five different solvents. In the case of an incubation for a longer time (>30 days), all 1-substituted elymoclavines were increasingly transformed to compounds that show a blue fluorescence at 365 nm and grey-green colored spots on tlc with van Urk's reagent, attributes which are characteristic for 8-hydroxy-ergol-9-enes. The transformation rate of 1-hydroxy-methyl-**1** (**4i**) was much lower compared with the 1-alkylated derivatives. Nevertheless, the substrate was converted without any residual material. 2-Halo-agroclavines (**5a, 5b**) and 2,3-dihydro-**1** (**6**) were also transformed to the corresponding elymoclavines (**12a, 12b, 13**) by agroclavine hydroxylase of strain SD 58. In these cases, the oxidation at C-17 showed a transformation percentage of only 50-60; still, 40-50% of the substrates were detectable in the medium even after 30 days.

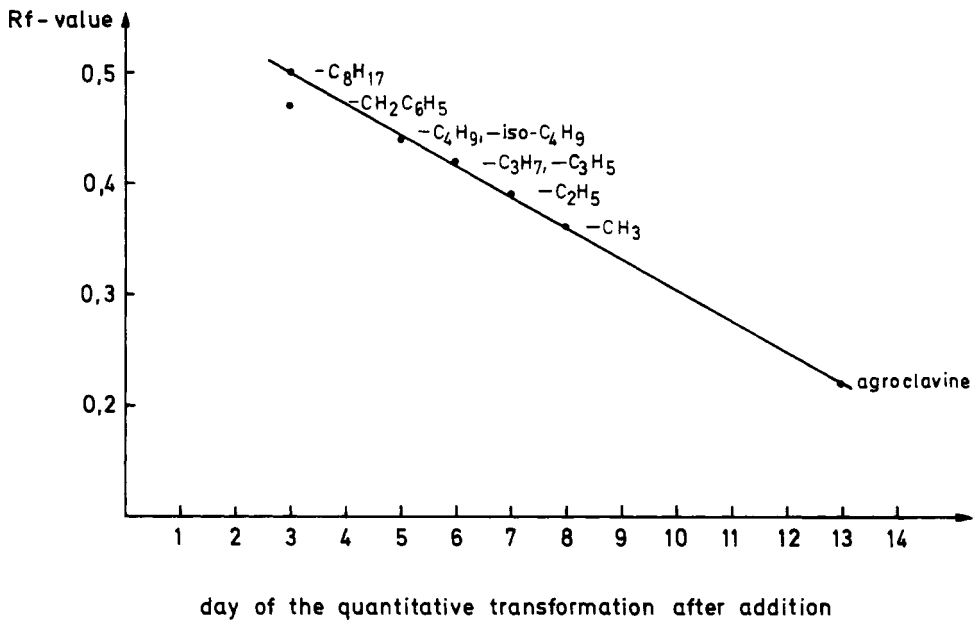


FIGURE 3. Relation between the polarity of 1-substituted agroclavines expressed by the Rf-values ($CHCl_3$-EtOH, 80:20) and the rate of transformation to the corresponding elymoclavines by *Claviceps fusiformis*, strain SD 58.

6-Nor-**1** (**7a**) cannot serve as a substrate, whereas the 6-ethyl homolog of **1** (**7b**) was converted to 6-ethyl-6-nor-**2** (**14b**) without detectable substrate remaining in the medium. Finally, neither the 8,9-dihydro-**1** derivatives (festuclavine (**9a**), 2-bromo-festuclavine (**9b**)), the 5R, 8R- and 5R, 8S-Δ^9 isomers of **1** (lysergine (**10a**), isolysergine (**10b**)), nor the 8 α-hydroxy derivative of **10a**, setoclavine (**8**), were oxidized at C-17.

These results show that the substrate specificity of the strain SD 58 agroclavine hydroxylase is high as to the 8,9-double bond: no compound lacking this structural feature (by hydrogenation or $\Delta^{9,10}$-isomerization) was oxidized at C-17. It can be assumed that the allylic double bond is necessary because it activates the hydrogen at C-17. The basic N-6 must be tertiary (methyl, ethyl): 6-nor-agroclavine is not accepted. The substrate specificity of the enzyme is clearly lower for the pyrrole partial structure: variations of the molecule at N-1, C-2, and C-3, respectively, led to the analogous elymoclavines. Compound **4h** was transformed quantitatively within 3 days, whereas 13 days were needed for the same amount of added **1** (Figure 3). This may be due to the better penetration of the substituted substances into the fungus cell as a consequence of more lipophilic attributes. Alternatively, it may be assumed that the affinity to the agroclavine hydroxylase and/or the V_{max}-value is larger. Perhaps all these points are significant. In-vitro studies with the isolated enzyme could give conclusive information concerning this. Consequently, the first step for a directed biosynthesis of 1-, 2-, or 6-substituted lysergic acids or their amides became realizable with the *Claviceps fusiformis*, strain SD 58, in saprophytic submerged culture. The yields were very acceptable (65-75%) for the N-1-substituted and 6-ethyl-6-nor derivatives and satisfactory (30-35%) for the 2-halo- and 2,3-dihydro derivatives.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined with a Tottoli apparatus and are uncorrected. Ir spectra were recorded on a Beckman IR 33 with KBr disks. Tlc was performed on 0.25-mm-thick layers of silica gel GF_{254} (Merck) on glass plates. Five mg of the pure compounds or of the crude bases was dissolved in 20 ml CH_2Cl_2-MeOH (70:30); 10 µl of this solution were applied to the tlc by means of a precision piston pipette. Tlc systems used included: solvent A, $CHCl_3$-EtOH (80:20); solvent B, $CHCl_3$-$(Et)_2NH$ (90:10); solvent C, $CHCl_3$-$(Me)_2CO$-EtOH (60:40:40); solvent D, $CHCl_3$-MeOH (70:30); solvent E, $CHCl_3$-$(Me)_2CO$-$(Me)_2NCHO$-NH_4OH (92:23:20:0.5). Visualization of developed tlc plates (fluorescence quenching: ergol-8-enes; fluorescence: ergol-9-enes) was accomplished by spraying with van Urk's reagent (0.2 g 4-dimethylaminobenzaldehyde dissolved in 100 ml 35% HCl with an addition of 3 drops of aqueous $FeCl_3$ solution (10%)).

SYNTHESIS OF THE SUBSTRATES (DERIVATIVES OF **1**).—The synthesis of compounds **4a** (9), **4b-4i, 5b, 7a, 7b** (10), compound **6** (11), and compound **9a** (12) is already described elsewhere. Compounds **5a** and **9b** were prepared according to Yamatodani (13), **8** according to Hofmann *et al.* (14), **10a** and **10b** according to Schreier (15).

SYNTHESIS OF 1-ALKYL-ELYMOCLAVINES (**11b-11h**).—Potassium (0.46 g = 11.74 mmol) was dissolved at −50° in 700 ml dried NH_3. The blue solution was stirred until it became colorless. After addition of 2.14 g (8.4 mmol) **2** (dried in vacuo), it was stirred again at −45° for about 30 min until almost all alkaloid was dissolved. Finally, 11.74 mmol of the alkyl iodide (for **11h**: benzyl bromide) dissolved in 3 ml absolute Et_2O was added, stirring further for 30 min. After evaporation of the NH_3, the residue was dissolved in aqueous $NaHCO_3$ solution and extracted with CH_2Cl_2. The concentrated CH_2Cl_2 solution containing the crude base was chromatographed by means of a column with 40 g silica gel (Woelm, Aktivitätsstufe 1). Elution was started with CH_2Cl_2 and continued with mixtures of CH_2Cl_2 and low but rising amounts (0.5%, 1%, 2% etc.) of MeOH. Those fractions containing the converted substrate were pooled and evaporated to dryness. The residue was dissolved in a suitable solvent for recrystallization (see individual substances). Unsubstituted **2** (20-35%) was recycled.

SYNTHESIZED 1-ETHYL-ELYMOCLAVINE (**11b**).—Compound **11b** gave the following data: mp 159-161° (dec.) [isopropyl ether]; ir 3400, 3060, 2830, 2800, 1610, 1460 cm^{-1}; Calcd for $C_{18}H_{22}N_2O$: C, 76.6; H, 7.85; N, 9.9. Found: C, 76.3; H, 7.83; N, 9.9. Rf-value (solvent A) 0.15, color with van Urk's reagent: dark violet.

SYNTHESIZED 1-PROPYL-ELYMOCLAVINE (**11c**).—Compound **11c**: mp 124-126 (dec.) [petroleum ether]; ir 3400, 3070, 2870, 2830, 1620, 1460 cm^{-1}; Calcd for $C_{19}H_{24}N_2O$: C, 77.0; H, 8.18; N, 9.5. Found: C, 76.7; H, 8.38; N, 9.4. Rf (A) 0.17, van Urk's reagent: dark violet.

SYNTHESIZED 1-ALLYL-ELYMOCLAVINE (**11d**).—Compound **11d**: mp 169-171° (dec.) [petroleum ether]; ir 3400, 3060, 2830, 2800, 1610, 1460 cm^{-1}; Calcd for $C_{19}H_{22}N_2O$: C, 77.5; H, 7.53; N, 9.5. Found: C, 77.2; H, 7.47; N, 9.5. Rf (A) 0.17, van Urk's reagent: dark violet.

SYNTHESIZED 1-BUTYL-(**11e**)- , 1-ISOBUTYL-(**11f**)- , AND 1-OCTYL-ELYMOCLAVINE (**11g**).—Compounds **11e-11g** were synthesized for tlc comparison with the corresponding metabolites of authentic **4e-4g** from the cultures. Further characterizations of these bases were not carried out.

SYNTHESIZED 1-BENZYL-ELYMOCLAVINE (**11h**).—Compound **11h**-picrate: mp 86-89° (dec.) [H_2O]; ir 3440, 3060, 2920, 2880, 2680, 1650, 1630, 1585, 1555, 1470, 1450, 1380, 1335, 1280 cm^{-1}; Calcd for $C_{23}H_{24}N_2O \cdot C_6H_3N_3O_7$: C, 60.7; H, 4.74; N, 12.2. Found: C, 60.4; H, 4.78; N, 11.8. Rf (A) free base 0.24, van Urk's reagent: dark violet.

SYNTHESIS OF 2-HALO-ELYMOCLAVINES (**12a, 12b**).—The solution of 1.0 g **2** in 40 ml absolute dioxane was mixed with the solution of 0.9 g *N*-bromosuccinimide (or 1.2 g *N*-iodosuccinimide) in 40 ml absolute dioxane and heated at 65° on the H_2O bath for 30 min. Afterwards, the solvent was distilled off in vacuo and the residue was dissolved in 0.1 N H_2SO_4. After addition of NH_4OH, it was extracted with Et_2O several times. The Et_2O extracts were pooled and evaporated to dryness. The residue was dissolved in a minimum amount of CH_2Cl_2 and purified with a silica gel column as described above. Unsubstituted **2** was recycled.

SYNTHESIZED 2-BROMO-ELYMOCLAVINE (**12a**).—Compound **12a** (yield: 53%): mp 207-209° (dec.) [C_6H_6]; ir 3400, 3060, 2830, 1610, 1440 cm^{-1}; Calcd for $C_{16}H_{17}N_2OBr$: C, 57.7; H, 5.14; N, 8.4; Br, 24.0. Found: C, 57.5; H, 5.29; N, 8.5; Br, 24.0. Rf (A) 0.22, Rf (B) 0.23, van Urk's reagent: yellow.

SYNTHESIZED 2-IODO-ELYMOCLAVINE (**12b**).—Compound **12b** (yield: 54%): mp not determinable (sublimation/dec.) [C_6H_6]; ir 3400, 3060, 2830, 2800, 1610, 1440 cm^{-1}; Calcd. for $C_{16}H_{17}N_2OI$: C, 50.5; H, 4.51; N, 7.3; I, 33.4. Found: C, 50.2; H, 4.43; N, 7.0; I, 33.3. Rf (A) 0.23, Rf (B) 0.24, van Urk's reagent: blue (I is split).

SYNTHESIS OF 6-ETHYL-6-NOR-ELYMOCLAVINE (**14b**).—6-Nor-**2** (**14a**) (3.6 mmol) dissolved in 8 ml *N,N*-dimethylformamide was stirred together with 0.8 g K_2CO_3 and 0.41 g (3.8 mmol) ethyl bromide at 25° for 5 h. Afterwards, the mixture was filtered. The filtrate was diluted with H_2O and extracted with CH_2Cl_2. The CH_2Cl_2 extract was evaporated under reduced pressure to dryness. The residue was dissolved with minimal amounts of $CHCl_3$ and chromatographed on silica gel (Woelm), eluted with $CHCl_3$ and rising additions of MeOH up to 80:20. Those fractions containing only **14b** were pooled and evaporated under reduced pressure to dryness. Recrystallization with isopropyl ether. mp 183-185° (dec); ir 3430, 2800, 1610, 1460 cm^{-1}; Calcd for $C_{17}H_{20}N_2O$: C, 76.1; H, 7.51; N, 10.4. Found: C, 76.0; H, 7.44; N, 10.2. Rf (A) 0.14, Rf (B) 0.30, van Urk's reagent: blue.

SYNTHESIS OF FURTHER ELYMOCLAVINE DERIVATIVES.—The synthesis of compounds **11a** (9), **13** (16), **16a** (12), and **17a** (17) is already described elsewhere. Compound **14a** was prepared according to Fehr (18); **17b** according to Schreier (15). Compound **15** had been isolated from submerged cultures of *Claviceps* strain 47a.

FERMENTATION PROCEDURE.—*Claviceps fusiformis*, strain SD 58, was maintained on a sucrose-peptone-agar medium. The strain is deposited at the Institute für Biochemie der Pflanzen, Akademie der Wissenschaften der DDR, Halle (Salle). Surface mycelia of 22-day-old agar slant cultures were suspended with sterile distilled H_2O (10 ml/tube). Wide-necked Erlenmeyer flasks (300 ml) containing 100 ml of the following media were inoculated with 5 ml of the mycelium suspension: sucrose 100.0 g, asparagine 10.0 g, yeast extract 0.1 g, $Ca(NO_3)_2$ 1.0 g, $MgSO_4 \times 7\ H_2O$ 0.25 g, KH_2PO_4 0.25 g, KCl 0.12 g, $FeSO_4 \times 7\ H_2O$ 0.83 mg, $ZnSO_4 \times 7\ H_2O$ 0.34 mg, distilled water to 1000.0 ml. Incubations were conducted on rotary shakers operating at 24° and 120 rpm (excentricity 30 mm). The substrates were added to the three-day-old cultures as a solution in 0.5% aqueous tartaric acid to a final concentration of 100 μg/ml of culture medium. The solutions were sterilized in an autoclave at 120° for 20 min. Labile substances (**4i, 8**) were injected into the medium by means of a sterile filter. Samples were withdrawn every 2 days and analysed by tlc. In the case of the faster reactions (**4g, 4h**), samples were withdrawn every day. Controls consisted of cultures grown without substrate and of incubations containing medium and substrate without fungus. For preparative purposes, 0.2-1.0 g of alkaloid base as hydrogen tartrate were dissolved in 100-500 ml 0.5% aqueous tartaric acid and distributed equally to 20-100 Erlenmeyer flasks containing SD 58 cultures as described above. The cultures were harvested after different periods depending on the chemical structure of the added substrate: 1-benzyl- , 1-octyl-: 3 days after addition of the substrate; 1-butyl-, 1-isobutyl-: 5 days; 1-allyl-, 1-propyl-: 6 days; 1-ethyl-: 7 days; 1-methyl-: 8 days; 1-hydroxymethyl-: 23 days; 2-bromo-, 2-iodo-, 2,3-dihydro-: 25 days after addition.

ISOLATION OF THE ELYMOCLAVINE DERIVATIVES.—The culture filtrates including the washings of the mycelia with 3% aqueous tartaric acid were alkalized by $NaHCO_3$ to pH 8 and extracted repeatedly with CH_2Cl_2. The concentrated extract was dissolved again in a minimum amount of CH_2Cl_2 applied to

silica gel columns, and eluted as described above. The yields were 30-75% depending on the chemical structure and the added amount of the substrate.

IDENTIFICATION OF THE ELYMOCLAVINE DERIVATIVES FORMED IN SUBMERGED CULTURES OF *CLAVICEPS FUSIFORMIS*.—Compounds **11a, 11b, 11c, 11d, 11i, 12a,** and **12b** were isolated from the culture filtrate as described above and recrystallized like the corresponding synthesized compounds. All these compounds were identical by mmp, tlc (solvent A, **12a** and **12b** also solvent B), and ir spectra with the synthesized compounds described above. In addition, compounds **11b** and **11d** isolated from the culture filtrate were subjected to elementary analysis.

Compounds **11h, 13,** and **14b** isolated as crude bases from the culture filtrate were also compared with authentic synthesized and pure substances. In these cases, cochromatography (tlc) in five solvents (A-E) resulted in identical spots and van Urk reaction.

Compounds **11e, 11f,** and **11g** were isolated as crude bases from the culture filtrate and compared with the corresponding synthesized crude bases by the same cochromatography procedure in five solvents (A-E) resulting in tlc identity (Rf and color of van Urk reaction). In all cases, the synthesized and the isolated compound formed only one spot after development.

ACKNOWLEDGMENTS

We thank Prof. Dr. D. Gröger, Institut für Biochemie der Pflanzen, Akademie der Wissenschaften der DDR, Halle (Saale), for his *Claviceps fusiformis* strain SD 58 and Dr. K. Mayer, Mainz, for 2-bromodihydrolysergol-I (**16b**).

LITERATURE CITED

1. F. Troxler and A. Hofmann, *Helv. Chim. Acta*, **40**, 1721 (1957).
2. J.C. Hsu and J.A. Anderson, *Biochim. Biophys. Acta*, **230**, 518 (1971).
3. I.S. Kim, S.U. Kim, and J.A. Anderson, *Phytochemistry*, **20**, 2311 (1981).
4. D. Gröger, *Arch. Pharm.* (Weinheim), **292**, 389 (1959).
5. D. Eichberg and E. Eich, *Planta Med.*, **45**, 146 (1982).
6. G. Schwarz and E. Eich, *Planta Med.*, **47**, 212 (1983).
7. E. Eich, D. Eichberg, and W.E.G. Müller, *Biochem. Pharmacol.*, **33**, 523 (1984).
8. A. Brack, R. Brunner, and H. Kobel, *Arch. Pharm.* (Weinheim), **295**, 510 (1962).
9. E. Eich, *Arch. Pharm.* (Weinheim), **316**, 781 (1983).
10. E. Eich, R. Sieben, and C. Becker, *Arch. Pharm.* (Weinheim), (in press).
11. R. Sieben, Dissertation, Universität Mainz (1976).
12. K. Mayer and E.Eich, *Pharmazie*, (in press).
13. S. Yamatodani, *Ann. Rep. Takeda Res. Lab.*, **19**, 15 (1960).
14. A. Hofmann, R. Brunner, H. Kobel, and A. Brack, *Helv. Chim. Acta*, **40**, 1358 (1957).
15. E. Schreier, *Helv. Chim. Acta*, **41**, 1984 (1958).
16. U. Philippi, Dissertation, Universität Mainz (1978).
17. E. Eich, *Pharmazie*, **30**, 516 (1975).
18. T. Fehr, Dissertation, ETH Zürich (1967).

Received 14 February 1983