Journal of Natural Products
Vol. 52, No. 5, pp. 1003-1007, Sep-Oct 1989

NEW ALKALOIDS OF *CLAVICEPS PASPALI*

M. FLIEGER,* R. LINHARTOVÁ, P. SEDMERA, J. ZIMA, P. SAJDL,

Institute of Microbiology, Czechoslovak Academy of Sciences, Vídeňská 1083, 142 20 Prague 4, Czechoslovakia

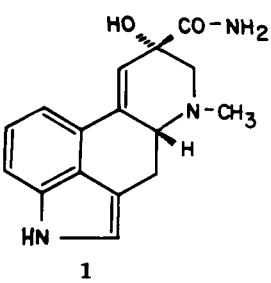
J. STUCHLÍK, and L. CVAK

Galena, Opava-Komárov, Czechoslovakia

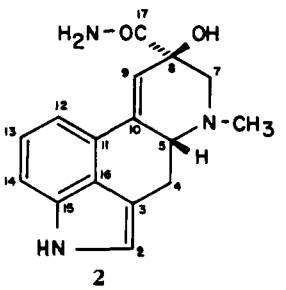
ABSTRACT.—Two new alkaloids, 8-hydroxyergine [**1**] and 8-hydroxyerginine [**2**], are produced during the post-production phase of submerged cultivation of the strain *Claviceps paspali* MG-6. Their structures were determined on the basis of uv, ms, and 2D-nmr data. A mechanism of formation of these compounds is proposed. The C-8 configuration of 8-hydroxyergotamine was solved by comparison with nmr spectra of **1** and **2**.

Submerged fermentation of various strains of *Claviceps paspali* Stevens et Hall is a long-studied subject (1–6). Only a few authors (1,2,6) have mentioned a decline in the alkaloid content in the later production phase. Our results, published earlier (6), induced us to study the effect of prolonged submerged fermentation on the extracellular alkaloid content. It was found that both the alkaloid concentration and the composition of the alkaloid mixture undergo dramatic changes.

The present paper deals with the isolation and structure determination of the main transformation products. A possible biosynthetic route to their formation is proposed.



1



2

EXPERIMENTAL

STRAIN.—The strain *C. paspali* MG-6 was isolated from the grass *Paspalum dilatatum* in the vicinity of Rome by Prof. H. Rochelmayer, Institute of Pharmacy, University of Mainz. Cultivation conditions and total alkaloid and dry wt determination are described elsewhere (6).

ALKALOID SEPARATION.—Alkaloids were separated by adsorption on bentonite (7). A crude alkaloid mixture (50–70 mg) was loaded on tlc preparative plates, 20 × 20 cm, layer thickness 1 mm (Kieselgel 60 F_{254}, Merck), and eluted with $CHCl_3$-iPrOH-NH_3 (4:1:0.01) mixture. The R_f values of **1** and **2** are 0.50 and 0.91, respectively.

CHROMATOGRAPHY.—Prepurified alkaloids were loaded on a Separon SGX C-18 column (Tessek, Czechoslovakia), particle size 7 μm, 25 × 0.8 cm i.d. The mobile phase for elution consisted of two different mixtures of MeOH and NH_3 in H_2O: mixture A is MeOH-H_2O-NH_3 (90:10:0.036), and mixture B is MeOH-H_2O-NH_3 (20:80:0.036). Prior to analysis the column was equilibrated for 10 min with 4% A in B. Subsequently, the concentration was increased in a linear manner up to 54% A in B within 20 min; this concentration was then maintained for another 10 min. The flow rate was 2 ml/min, column temperature ambient, detection uv at 310 nm. Capacity factors of the alkaloids under study were **1** 4.0., ergine 8.6., **2** 8.9., and erginine 10.8. A Separon SGX C-18 column (15 × 0.33 cm i.d., particle size 7 μm, Tessek) was used for checking purity under the same chromatographic conditions at the flow rate 0.8 ml/min.

GENERAL SPECTRAL PROCEDURES.—The uv spectra were recorded as published earlier (7). ^{1}H- and

^{13}C-nmr spectra were measured on a Varian VXR-400 at 400 and 100 Mz, respectively, in CD_3OD at 25°. Chemical shifts are given in the δ scale. Eims were taken on a Finigan MAT 90 instrument by the DI-ei technique; the temperature of the inlet system was 250°, probe temperature rose from 25° to 200° at a rate of 1°/sec, ion current 1.0 mA, electron energy 70 eV, accelerating voltage 5.0 kV. The eims are summarized in Table 1.

TABLE 1. Electron Impact Mass Spectra of Compounds **2** and **1**.

m/z	Elemental composition	Relative intensity	
		2	**1**
283	$C_{16}H_{17}N_3O_2$	100	61
266	$C_{16}H_{16}N_3O$	14	27
265	$C_{16}H_{15}N_3O$	29	37
248	$C_{16}H_{12}N_2O$	35	59
240	$C_{14}H_{12}N_2O_2$	93	86
223	$C_{14}H_9NO_2$	31	25
221	$C_{15}H_{13}N_2$	42	42
206	$C_{14}H_{10}N_2$	12	19
195	$C_{13}H_9NO$	60	36
194	$C_{13}H_8NO$	61	32
181	$C_{13}H_{11}N$	50	26
180	$C_{13}H_{10}N$	20	26
167	$C_{12}H_9N$	94	83
154	$C_{11}H_8N$	96	100

RESULTS AND DISCUSSION

Strain *C. Paspali* MG-6, similar to the strain *C. paspali* FA (3,10), produced at the beginning of the production phase (4-6 days) mainly lysergic acid α-hydroxyethylamide. Under conditions of submerged fermentation this compound undergoes isomerization and degradation reactions, forming isomers, epimers, and ergine. Maximum production is reached at the end of the exponential growth phase (12–14 days) (Figure 1), and the formation of other transformation products starts. After 28 days of fermentation these products **1** and **2** formed about 80% of total extracellular

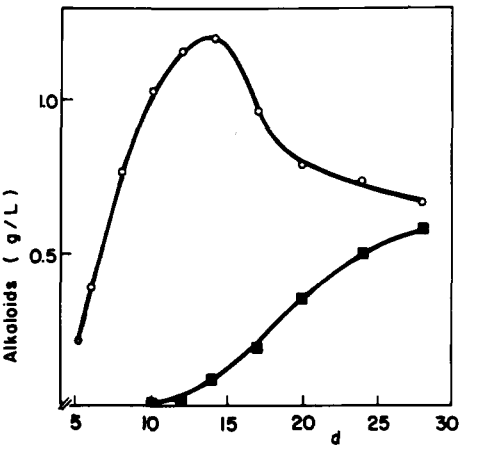


FIGURE 1. Formation of **1** and **2** (■) during submerged fermentation of *Claviceps paspali* MG-6. ○=Total alkaloids.

alklaloids. Substances **1** and **2** were separated from the culture broth by tlc and hplc techniques.

STRUCTURE DETERMINATION.—Both compounds exhibit a molecular ion *m/z* 283 ($C_{16}H_{17}N_3O_2$). Their eims (Table 1) contain the same prominent fragments differing in relative intensity only. ^{13}C-nmr spectra (Table 2) reveal an identical distribution

TABLE 2. Carbon Chemical Shifts (100 MHz, CD_3OD, 25°).

Carbon	Compound		
	2	**1**	8-Hydroxy-ergotamine
C-2	120.53	120.60	120.66
C-3	110.55	110.28	110.52
C-4	28.27	27.39	27.15
C-5	64.11	64.44	64.20
C-7	63.06	63.48	62.71
C-8	71.87	73.72	73.84
C-9	124.30	121.47	121.00
C-10	139.13	140.38	139.91
C-11	128.04	128.08	128.13
C-12	113.15	113.39	113.43
C-13	123.96	123.96	123.93
C-14	111.72	111.87	111.95
C-15	135.95	135.98	136.02
C-16	127.99	127.91	128.13
C-17	179.59	179.65	177.92
N-Me	43.39	43.92	43.75

of carbons: one carbonyl, five sp^2 methines, five quarternary sp^2 carbons, two aliphatic methylenes, one aliphatic methine, one quarternary sp^3 carbon (C-O type), and one *N*-methyl. Thirteen hydrogens are attached to carbon; that leaves four hydrogens bonded to heteroatoms. Among them are the $CONH_2$ group and N_1-H so that the remaining hydrogen atom is a part of an OH group. The latter-mentioned group has to be a tertiary one, because of the presence of one sp^3 C-O carbon in ^{13}C-nmr spectra. Two structures are consistent with these results: 8-OH (double bond in position C-9–C-10) and 10-OH (double bond in position C-8–C-9). According to ^{1}H-nmr and COSY experiments, the spin-spin coupling topology of both compounds is the same. The chemical shifts (Table 3) are slightly different but the corresponding ^{1}H-^{1}H coupling constants (Table 4) are very similar. The magnitude of $J_{(5,9)} = 2.2$ Hz in both cases allows us to reject the 10-OH alternative because coupling of this size is impossible in this arrangement. Therefore, compounds **1** and **2** are isomeric 8-hydroxyergines. This conclusion is in agreement with the uv spectra of these substances.

The magnetic nonequivalence of C-7 protons is large in **2** (0.454 ppm) and small in **1** (0.060 ppm). The signal of H-7 is strongly shifted upfield in **2**. Such behavior can be rationalized by a long-range shielding effect of the amide carbonyl group that has to be in the C-8 position. Thus, **2** is 8-hydroxyerginine and **1** is 8-hydroxyergine.

Using the data on both these isomers, the old problem of hydroxyl group configuration in 8-hydroxyergotamine (9) could be tackled. The small magnetic nonequivalence of C-7 protons (0.029 ppm) (Table 3) in this compound places it to the "normal" series. Comparison of ^{13}C-nmr spectra (without the carbonyl resonance) (Table 2) shows 12 carbons within the 1 ppm tolerance limit in **2** and 15 in **1**. The corresponding similar-

TABLE 3. Proton Chemical Shifts (400 MHz, CD_3OD, 25°, TMS).

Proton	Compound		
	2	**1**	8-Hydroxy-ergotamine
H-2	6.974	6.971	6.984
H-4a	2.649	2.746	2.749
H-4b	3.606	3.577	3.569
H-5	3.150	3.263	3.265
H-7a	3.080	2.966	2.936
H-7b	2.626	3.026	2.965
H-9	6.268	6.378	6.358
H-12	7.118	7.147	7.193
H-13	7.090	7.083	7.107
H-14	7.219	7.204	7.231
N-Me	2.614	2.606	2.590

ity factors S (9) are 0.8585 for **2** and 0.9123 for **1**. That definitely places the hydroxyl group of 8-hydroxyergotamine in the 8-alpha position.

It has been proved that oxidation of agroclavine to setoclavine and isosetoclavine is catalyzed by peroxidase (11,12). Additional evidence for the involvement of peroxidase in the biological oxidation of another clavine alkaloid, elymoclavine, was the formation of penniclavine and isopenniclavine (13). Peroxidase is common in fungi and plants, and Johansson (14) has demonstrated its presence in ergot. From all the above-mentioned results we suppose that the formation of **1** and **2** and even 8-hydroxyergotamine is the result of peroxidase action. Peroxidase of the strain *C. paspali* MG-6 is synthesized de novo at the beginning of the stationary phase (unpublished data). A possible mechanism of formation of **1** and **2** is given in Figure 2. On the basis of chromatographic data we suppose that **1** and **2** are mainly formed by the reaction of peroxidase with ergine, but some small part of lysergic acid α-hydroxyethylamide is probably transformed to 8-hydroxylysergic acid α-hydroxyethylamide prior to decomposition.

TABLE 4. Coupling Constants (Hz).

Pairs	Compound		
	2	**1**	8-Hydroxy-ergotamine
2,4a	1.6	1.8	1.8
2,4b	> 0	> 0	> 0
4a,4b	−14.6	−14.5	−14.5
4a,5	11.5	11.5	11.8
4b,5	5.9	5.9	5.9
4b,9	0.8	0.6	> 0
5,9	2.2	2.2	2.1
7a,7b	−11.3	−11.8	−11.7
7a,9	1.5	1.3	1.0
12,13	7.0	7.4	7.4
12,14	1.7	0.8	0.7
13,14	7.2	7.9	7.9

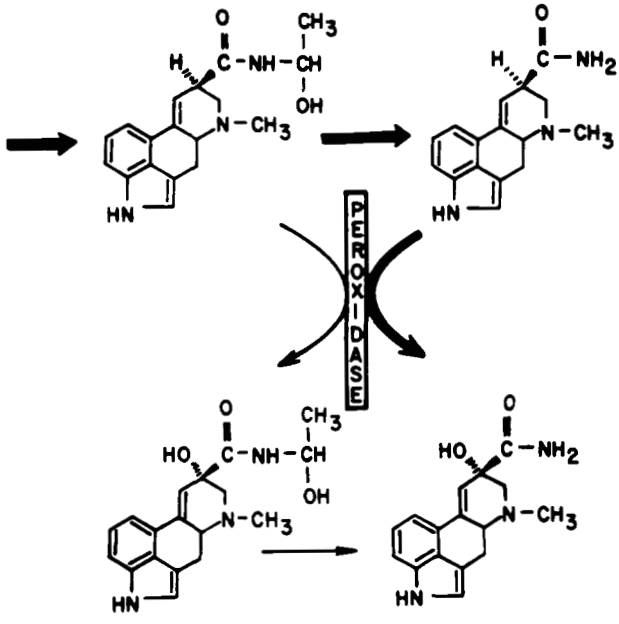


FIGURE 2. Possible mechanism of formation of 8-hydroxyergine [**1**].

LITERATURE CITED

1. L.R. Pacifici, W.J. Kelleher, and A.E. Schwarting, *Lloydia*, **25**, 37 (1962).
2. N.Y. Mary, W.J. Kelleher, and A.E. Schwarting, *Lloydia*, **28**, 218 (1965).
3. A. Řičicová, M. Flieger, and Z. Řeháček, *Folia Microbiol. (Prague)*, **27**, 433 (1982).
4. E. Pertot, J. Čadež, S. Miličič, and H. Sočič, *Appl. Microbiol. Biotechnol.*, **20**, 29 (1984).
5. H. Sočič, V. Gaberc-Porekar, E. Pertot, A. Puc, and S. Miličič, *J. Basic Microbiol.*, **26**, 533 (1986).
6. V. Rylko, R. Linhartová, P. Sajdl, and Z. Řeháček, *Folia Microbiol. (Prague)*, **33**, 425 (1988).
7. M. Flieger, N.F. Zelenkova, P. Sedmera, V. Křen, J. Novák, V. Rylko, P. Sajdl, and Z. Řeháček, *J. Nat. Prod.*, **52**, 506 (1989).
8. A. Krajíček, B. Trtík, J. Spáčil, P. Sedmera, J. Vokoun, and Z. Řeháček, *Collect. Czech. Chem. Commun.*, **44**, 2255 (1979).
9. W. Bremser, M. Klier, and E. Meyer, *Org. Magn. Reson.*, **7**, 97 (1975).
10. M. Flieger, P. Sedmera, J. Vokoun, A. Řičicová, and Z. Řeháček, *J. Chromatogr.*, **236**, 453 (1982).
11. E.H. Taylor, K.J. Goldner, S.F. Pong, and H.R. Shough, *Lloydia*, **29**, 239 (1966).
12. E.H. Taylor and H.R. Shough, *Lloydia*, **30**, 197 (1967).
13. W.-N. Chan Lin, E. Ramstad, and E.H. Taylor, *Lloydia*, **30**, 202 (1967).
14. M. Johansson, *Physiol. Plant.*, **17**, 547 (1964).

Received 8 March 1989