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NEW ALKALOIDS FROM A SAPROPHYTIC CULTURE OF *CLAVICEPS PURPUREA*

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ABSTRACT.—Stationary cultivation of *Claviceps purpurea* D-3-18 on a liquid medium enriched with D,L-isoleucine yielded sclerotial mycelium containing, apart from α- and β-ergokryptine (**1,2**) and ergocornine, three new alkaloids: a 5′-epimer of β-ergokryptine (**3**), β-ergokryptam (**6**), and a lactam alkaloid from a new group of ergopeptam alkaloids containing L-isoleucine as the first amino acid, i.e., N[N(d-lysergyl)-L-isoleucyl]-L-isoleucyl-D-proline lactam (**5**). For this substance the name β,β-ergoannam is proposed.

Many new ergot alkaloids of the ergopeptine and ergopeptam groups have been isolated in the past decade (1-3). The new series of ergopeptines containing α-aminobutyric acid as the second amino acid of the peptide moiety (4) has seemed to exhaust the possibilities of mutual exchange of non-polar amino acids in the peptide part of ergopeptines.

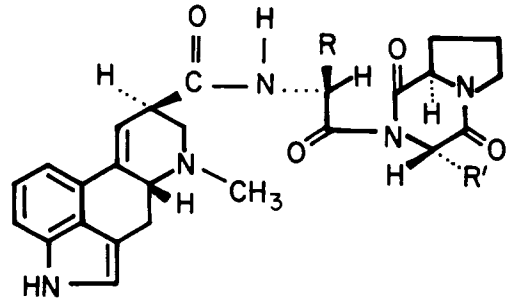
The group of ergot alkaloids can be extended either by preparing their semisynthetic analogues or by adding altered amino acids to the culture media. It was shown that one of the amino acids of the peptide chain of the ergot alkaloids can be replaced by some of its analogues added to the producing culture. *Claviceps purpurea* (Fr) Tul strain MUT 168/2, which produced ergosine in submerged culture, incorporated L-thiazolidine-4-carboxylic acid, i.e., an analogue of the common amino acid of the ergopeptines, L-proline (5). A submerged culture of *C. purpurea* S-40 Phe-, producing ergocristine utilized an analogue of one amino acid of the ergocristine peptide chain to form the corresponding ergocristine analogue (6). In the case of ergotoxine alkaloids, the addition of L-valine, L-leucine, or L-isoleucine, respectively, to the nutrient medium significantly affected the proportions of the corresponding alkaloids ergocornine, α-ergokryptine, and β-ergokryptine (7). All these data led to the conclusions that neither of the two enzyme activities responsible for the peptide bond formation and the "cyclol synthetase" is strictly specific, and the biosynthesis of the peptide moiety of the ergot alkaloids is controlled by the relative concentration of these amino acids in the internal pool (5-7). Two natural types of the ergot peptide alkaloids were isolated, i.e., ergopeptams (Table 1) and ergopeptines. So far, only ergopeptams of the ergotoxam group have been isolated (3,8,9). They formed only a negligible part of the total alkaloid production.

C. purpurea strain D-3-18 produces saprophytically a mixture of ergocornine, α-ergokryptine (**1**), and β-ergokryptine (**2**). To increase the production of β-ergokryptine (**2**), D,L-isoleucine at the concentration 0.3% was added to culture medium. Apart from a four-fold increased production of **2**, the mycelium was found to contain three new alkaloids that formed 40% of total alkaloids.

EXPERIMENTAL

STRAIN.—*C. purpurea* strain D-3-18 was cultivated in a polyethylene bag (10) in a stationary manner with dimensions 1200×2500 mm filled with a nutrient medium of the following composition: sucrose, 33%; corn steep liquor, 0.4%; KH_2PO_4, 0.045%; $MgSO_4 \cdot 7\ H_2O$, 0.11%; $Ca(NO_3)_2 \cdot 4\ H_2O$, 0.16%; triammonium citrate, 0,839%; D,L-isoleucine, 0.3%; tap H_2O. Prior to filling into the bag, the medium was inoculated with vegetative inoculum (0.5%). After a 21-day cultivation at 24°, the culture formed a

TABLE 1. General Formula and Groups of Ergopeptam Alkaloids



R′	Ergotamams R=CH_3	Ergoxams R=C_2H_5	Ergotoxams R=$CH(CH_3)_2$	β-Ergoannams R=$CH(CH_3)CH_2CH_3$
$CH_2C_6H_5$	Ergotamam	Ergostam	**Ergocristam**[a]	—
$CH_2CH(CH_3)_2$	α-Ergosam	α-Ergoptam	**α-Ergokryptam**	α,β-Ergoannam
$CH(CH_3)CH_2CH_3$	β-Ergosam	β-Ergoptam	**β-Ergokryptam**	**β,β-Ergoannam**
$CH(CH_3)_2$	Ergovalam	Ergonam	**Ergocornam**	—
CH_2CH_3	Ergobam	Ergobutam	**Ergobutyram**	—

[a]Names printed in bold letters are those of known compounds.

continuous layer of biomass. One bag yielded 4 kg dry mycelium with the total content of alkaloids, 0.55%.

ANALYTICAL PROCEDURES.—Hplc, ms, ^{1}H-nmr, and ^{13}C-nmr as previously described (11).

ALKALOID EXTRACTION AND PURIFICATION.—The mycelium was wetted and then extracted with Et_2O-EtOH (9:1). From the extract, alkaloids were extracted into 1% tartaric acid solution. The alkaloids were precipitated by adjusting the pH to 7.5 and were collected by filtration and dried. The crude alkaloid mixture was defatted with petroleum ether; the resulting amount of alkaloids (20 g) was chromatographed on a six-fold volume of silica gel and eluted with $CHCl_3$. The resulting alkaloids were purified by hplc on a semipreparative column: MicroPak NH_2 (50×0.8 cm), particle size 10 μm; mobile phase, Et_2O-EtOH (22:3); flow rate, 220 ml/h; uv detection at 240 nm. A fifty-fold repetition of the semipreparative chromatographic cycle yielded 20-25 mg pure alkaloids. Purity checking and determination of capacity factors (Table 2) were performed on an analytical column: Perkin-Elmer NH_2 (25×0.46 cm); particle size 10 μm; mobile phase, hexane-Et_2O-EtOH (41:41:8); flow rate 0.6 ml/min; detection uv, 240 nm.

TABLE 2. Capacity Factors of the Investigated Alkaloids

Compound	Capacity factors, k'
α-ergokryptine (**1**)	3.92
β-ergokryptine (**2**)	3.55
ergocornine	4.27
ergocristine	5.27
5′-epi-β-ergokryptine (**3**)	3.77
β,β-ergoannam (**5**)	3.62
β-ergokryptam (**6**)	4.38

DEGRADATION PROCEDURES-ACID HYDROLYSIS.—The sample (5 mg) was dissolved in HCl and heated in a closed flask on a boiling water bath for 8 h. The contents of the flasks were then evaporated to dryness, dissolved in aqueous MeOH (50%), filtered through activated charcoal (Carboraffin), and again evaporated to dryness. The residue was dissolved in 0.25 ml MeOH and 2 μl of the resulting solution was applied to a silica gel layer and developed in the system, phenol-H_2O (3:1). Detection was done with ninhydrin. Individual alkaloids were found to have the following amino acid composition: **3**—proline, isoleucine; **5**—proline, isoleucine; **6**—proline, valine, isoleucine.

AMMONOLYSIS.—The sample (2 mg) was dissolved in an Me_2CO-concentrated NH_3 (9:1) mixture and incubated for 30 min at room temperature. The products were analyzed by tlc on silica gel previously alkalinized with NH_3 fumes, in the solvent system, $CHCl_3$-toluene-Me_2CO-absolute EtOH (5:3:2:1). Alkaloid **3** was not degraded; alkaloid **5** yielded the degradation product lysergylisoleucine amide; alkaloid **6** yielded lysergylvaline amide.

RESULTS AND DISCUSSION

The above extraction and chromatographic methods aided us in isolating new alkaloids **3, 5,** and **6,** each in an amount of 20 mg, from the sclerotial mycelium of the strain *C. purpurea* D-3-18. The results of the ammonolytic degradation of these alkaloids indicate that **3** has a cyclol structure and that **5** and **6** are lactams. Lactam and cyclol alkaloids can also be reliably distinguished by spectroscopic methods. In the region of 164-176 ppm of the ^{13}C-nmr spectrum, lactam alkaloids have four singlets, while cyclol alkaloids have three. In the region of 85-105 ppm, lactam alkaloids have no singlet and cyclols have two singlets. In the region of 58-65 ppm, lactams exhibit four doublets, whereas cyclols exhibit three. Protons of the HN groups yield in ^1H-nmr spectra of lactams a singlet and doublet [owing to the vicinal interaction with the H(2′) proton] while cyclols yield two singlets. In eims, lactam alkaloids, in contrast to their cyclol counterparts, yield molecular ions of sufficient intensity. The lysergic acid residue of lactam alkaloids gives rise to the *m/z* 221 ion, and in cyclol alkaloids, to the *m/z* 267 ion. Differences are also found in the fragmentation of the peptide moiety of the molecule whose detailed interpretation makes it possible to derive the size of the residues on carbons C(2′) and C(5′) (12).

On the basis of the results of ammonolysis, nmr, and ms, alkaloid **3** (Figure 1) was classified among the cyclols. Its mass spectrum (Table 3) is practically the same as the spectra of **1** and **2**. According to the ^1H-nmr spectrum (Table 4), the substance differs from **1** and resembles **2**. The signal of H(5′) is a doublet (J=3.1 Hz); the methyl region features doublets of three secondary methyl groups and a triplet of one primary methyl group. Comparison of cmr spectra (Table 5) points to the identical ergine part of the molecule and the first amino acid valine. Significant differences in chemical shifs occur in the second amino acid portion. They are 0.7, 0.4, −1.6, −0.1, and 1.4 ppm for car-

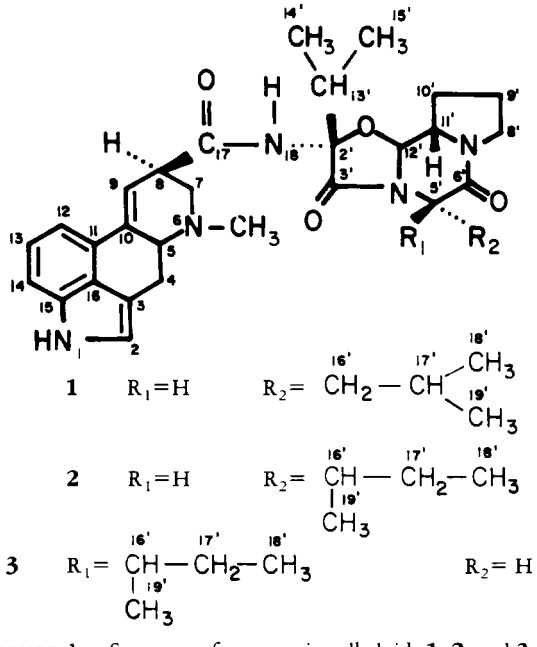


FIGURE 1. Structure of ergopeptine alkaloids **1, 2,** and **3**.

TABLE 3. Mass Spectra Data of Alkaloids **1-6**

Ion	*m/z*	composition	relative intensity (%)					
			1	**2**	**3**	**4**	**5**	**6**
M	573	$C_{33}H_{43}N_5O_4$	—	—	—	—	4	—
	559	$C_{32}H_{41}N_5O_4$	—	—	—	1	—	1
l	363	$C_{22}H_{25}N_3O_2$	—	—	—	—	7	—
	349	$C_{21}H_{23}N_3O_2$	—	—	—	4	—	2
m	335	$C_{21}H_{25}N_3O$	—	—	—	—	12	—
	321	$C_{20}H_{23}N_3O$	—	—	—	6	—	4
c	308	$C_{16}H_{24}N_2O_4$	3	2	1	—	—	—
b	267	$C_{16}H_{17}N_3O$	22	47	15	—	—	—
n	221	$C_{15}H_{13}N_2$	—	—	—	15	21	9
d	210	$C_{11}H_{18}N_2O_2$	14	8	8	—	—	—
e	209	$C_{11}H_{17}N_2O_2$	33	22	22	—	—	—
f	154	$C_7H_{10}N_2O_2$	53	90	67	79	91	100
h	125	$C_6H_9N_2O$	8	17	12	25	22	26
k	86	$C_5H_{12}N$	6	10	8	19	12	13
j	71	C_4H_7O	61	44	47	—	—	—
i	70	C_4H_8N	100	100	100	100	100	87

bons C(5′) and C(16′)-C(19′). Since D,L-isoleucine was added to the fermentation medium, it is highly probable that **3** differs from **2** only in containing D-isoleucine instead of L-isoleucine.

Alkaloid **5** (Figure 2) has the molecular formula $C_{33}H_{43}N_5O_4$. The above spectroscopic features and ammonolysis placed it among the lactam alkaloids. The differences in the elemental composition of fragments *m* and *n* (12) (Table 3) indicate that the residue on C(2′) has four carbons. The composition of fragment *k* points to the same size of the substituent on C(5′). The finding that the signal of proton H(2′) (Table 4) has,

TABLE 4. Comparison of Selected ^{1}H-nmr Data of Compounds **1-6**[a]

	1	**2**	**3**	**4**	**5**	**6**
N(1)-H	8.37 s	8.21 s	8.47 s	8.00 s	8.00 s	8.30 s
H(2)	6.94 s	6.92 s	6.94 s	6.92 s	6.92 s	6.92 s
H-(9)	6.37 dd	6.35 dd	6.36 dd	6.47 d	6.46 dd	6.46 dd
	(6.4,1.5)	(6.1,1.4)	(5.5,1.3)	(4.9)	(4.4,1.5)	(4.3,1.2)
N(6)-CH_3	2.63 s	2.63 s	2.65 s	2.69 s	2.67 s	2.67 s
N(18)-H	9.86 s	9.88 s	9.84 s	8.16 d	8.26 d	8.22 d
				(8.6)	(9.8)	(9.2)
H(2′)				5.75 dd	5.94 dd	5.83 dd
				(8.6,2.5)	(9.8,2.4)	(9.2,3.1)
H(5′)	4.52 t	4.49 d	4.53 d	5.08 t	4.94 d	4.96 d
	(6.4)	(2.5)	(3.1)	(6.1)	(9.3)	(9.2)
H(11′)					4.36 t	4.36 t
					(7.6)	(7.8)
C(2′)-side chain methyls	1.03 d	1.11 d	1.03 d	1.18 d	0.70 d	0.75 d
	(6.1)	(6.1)	(6.1)	(7.3)	(6.8)	(6.7)
	0.91 d	0.87 d	0.89 d	0.73 d	0.90 d	0.94 d
	(6.1)	(6.1)	(6.1)	(7.3)	(7.3)	(6.7)
C(5′)-side chain methyls	1.02 d	1.04 d	1.14 d	0.97 d	1.06 d	0.98 d
	(7.3)	(6.8)	(6.8)	(6.1)	(6.8)	(6.7)
	1.02 d	0.94 t	0.96 t	0.97 d	0.92 t	0.93 t
	(7.3)	(7.3)	(7.3)	(7.3)	(7.3)	(7.3)

[a]59.797 MHz, $CDCl_3$, 25°, δ-scale, coupling constants in Hz given in parentheses.

TABLE 5. ^{13}C-nmr Chemical Shifts of Compounds **1-6**[a]

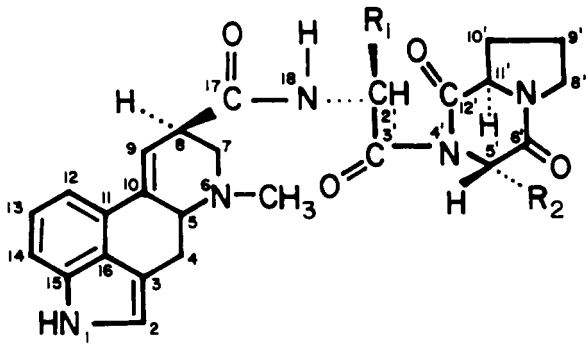
Carbon	1	2	3	4	5	6
Ergolene part						
2	119.2	119.1	119.2	119.8	118.8	118.9
3	110.6	110.7	110.5	111.0	111.1	110.9
4	26.5	26.6	26.7	29.1	29.8	29.6
5	64.5	64.0	64.1	62.5	63.3	63.2
7	48.1	48.0	48.2	50.8	50.4	50.6
8	40.9	40.9	40.9	41.8	41.7	41.7
9	118.8	119.2	118.9	118.8	119.8	119.2
10	139.2	139.2	139.0	137.6	137.9	137.8
11	129.6	129.7	129.6	129.4	129.7	129.4
12	111.9	112.0	111.9	111.9	111.8	111.8
13	123.3	123.3	123.2	123.5	123.3	123.2
14	110.1	110.1	110.2	109.8	109.8	109.9
15	133.9	133.8	133.9	133.9	133.9	133.9
16	126.3	126.3	126.3	126.4	126.3	126.3
17	176.3	176.3	176.1	174.5	174.7	174.4
N-CH_3	44.3	44.3	44.3	44.3	44.4	44.3
Peptide part						
2′	89.7	89.5	89.5	58.2	56.5	58.1
3′	165.8	164.6	165.0	173.7	173.6	173.6
5′	53.3	59.7	60.4	59.0	60.0	60.0
6′	166.2	166.6	167.1	165.6	165.6	165.6
8′	46.0	45.9	46.0	45.5	45.5	45.5
9′	21.6	21.3	21.7	22.9	22.9	22.9
10′	22.2	22.2	22.1	23.3	23.1	23.3
11′	59.3	59.2	59.3	59.5	60.3	60.4
12′	103.4	103.6	103.7	169.9	170.0	170.0
13′	34.3	34.3	34.3	30.3	36.8	30.3
14′	15.3[b]	15.3[b]	15.3[b]	16.0[b]	27.4	16.1[b]
15′	16.9[b]	17.0[b]	16.9[b]	20.1[b]	11.7	20.1[b]
16′	43.5	39.4	39.8	40.3	13.6	37.2
17′	25.1	27.9	26.3	25.1	37.3	25.7
18′	22.1[c]	12.6	12.5	21.6[c]	25.7	10.9
19′	22.6[c]	15.2	16.6	23.2[c]	10.9	15.5
20′					15.5	

[a]15.036 MHz, $CDCl_3$, 25°, δ-scale.

[b,c]Chemical shifts denoted by the same letter can be interchanged.

apart from the coupling with HN proton, only one other interaction implies that the neighbouring group is a methine. The same situation obtains with the doublet of the H(5′) proton. Proton spectrum (Table 4) requires the presence of two secondary and two primary methyl groups. All these facts and the results of amino acid analysis are consistent solely with substitution of C(2′) and C(5′) by secondary butyl groups.

Analogous analysis revealed that alkaloid **6** (Figure 2) with the molecular formula $C_{32}H_{41}N_5O_4$ is also a lactam. The mass spectrum (Table 3) indicates that the substituent on C(2′) contains three carbon atoms and the substituent on C(5′), four. Signals H(2′) and H(5′) are a doublet of a doublet and a doublet (Table 4). This fact, along with the quality of methyl groups (three secondary and one primary methyl) and the multiplicity of appropriate ^{13}C-nmr signals in an off-resonance experiment, permits us to deduce that the substituent on C(2′) is isopropyl and on C(5′), secondary butyl. The similarity of chemical shifts of carbon C(13′)-C(15′) in **4** and **6** indicates that the valine residue has, most probably, the L-configuration. Comparison of chemical shifts of car-



4 R_1= $-CH(CH_3)(CH_3)$ R_2= $CH_2-CH(CH_3)(CH_3)$

6 R_1= $-CH(CH_3)(CH_3)$ R_2= $CH(CH_3)-CH_2-CH_3$

5 R_1= $CH(CH_3)-CH_2-CH_3$ R_2= $CH(CH_3)-CH_2-CH_3$

FIGURE 2. Structure of ergopeptam alkaloids **4**, **5**, and **6**.

bons C(16′)-C(19′) in **5** and **6** reveals that the configuration of the isoleucine part of the molecule in the two new alkaloids is identical. Alkaloid **6** is thus β-ergokryptam.

The results of cultivations of production strains of *C. purpurea* in nutrient media with some amino acids or their derivatives imply that these medium components may be incorporated into alkaloids. Highly significant is the heretofore unreported finding of a direct formation of a C(5′) epimer documented in 5′-epi-β-ergokryptine (**3**). Whether this is a direct incorporation of a D-amino acid into the peptide part of the molecule or an isomerization reaction of some of the intermediates is a subject of further study.

The new lactam alkaloid, β,β-ergoannam (**5**), contains isoleucine as the first amino acid of the peptide moiety. Lactam alkaloids of the ergotoxam group form a minor component in a mixture with ergotoxin alkaloids; however, no cyclol counterpart to β,β-ergoannam has been found. This fact is interpreted as follows: because of the bulk of the carbon chain bound to C(2′), the lysergyl tripeptide probably cannot be bound to the binding site of "cyclol synthetase" despite its low specifity. This further implies that the probability of formation of lactam alkaloids decreases with the bulk of the carbon chain of the amino acid bound in position one, in the sequence *sec*-butyl≥isobutyl>isopropyl>ethyl>methyl. This may also partially explain why lactam alkaloids of the ergoxam and ergotamam group have not yet been isolated.

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