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Free and bonded homoisoleucine in sclerotia of the parasitic fungus *Claviceps purpurea*

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Summary. Homoisoleucine, an unusual amino acid recently discovered in the structure of the ergopeptine alkaloid ergogaline, was determined in the parasitic fungus *Claviceps purpurea* Fr. (Tul.). growing on rye *Secale cereale* (L.) and in its host plant. Free homoisoleucine was detected by gas chromatography-mass spectrometry (GC-MS) in the amino acid pool of sclerotia of all fungal strains examined. Since homoisoleucine was not detected in rye, it seems that the amino acid is synthetized by the fungus. Furthermore, the ratio of leucine/homoisoleucine in the free amino acid pool of sclerotia is in good agreement with the ratio of the corresponding alkaloids α-ergokryptine/ergogaline estimated by high performance liquid chromatography (HPLC). Thus, homoisoleucine is incorporated into the ergopeptines randomly with the similar specificity as leucine.

Keywords: Amino acids – Homoisoleucine – Ergot alkaloids – *Claviceps purpurea*

Introduction

Homoisoleucine ((2S,4S)-2-amino-4-methylhexanoic acid, homo-Ile) is an unusual amino acid which origin is attributed to the non-specificity of enzymes involved in leucine biosynthesis (Jakubowski and Goldman, 1992), Fig. 1. Till date, homo-Ile was found in a free form in the bacterium *Serratia marcescens* (Kisumi et al., 1976) and in seeds of the plant *Aesculus californica* (California buckeye) (Boyle and Fowden, 1971; Fowden and Mazelis, 1977; Fowden and Smith, 1968). It was also described as a constituent of an antibiotic peptide isolated from *Streptomyces diastaticus* (Shoji et al., 1970; Shoji and Sakazaki, 1970). Recently, we revealed homo-Ile in the structure of the ergot alkaloid

isolated from the parasitic fungus *Claviceps purpurea* (Cvak et al., 1994; Szántay et al., 1994), Fig. 2.

Biological importance of the homo-Ile biosynthesis still remains obscure. It was proposed that in seeds of *Aesculus californica* homo-Ile serves as a nitrogen-storage material which is degraded during the subsequent germination of the seeds making the pool of nitrogen readily available (Fowden and Smith, 1968). In bacteria, biosynthesis of the uncoded amino acids is considered to be important for the production of antibiotics (Jakubowski and Goldman, 1992; Kisumi et al., 1976). It is not clear, however, if these metabolites represent a real specific target, or a route how to get rid of useless side products of a primary metabolism. Since homo-Ile was found to be a leucine antagonist in *Escherichia coli* and *Leuconostoc dextranicum* (Edelson et al., 1959), an intriguing question has to be explained, whether homo-Ile might be a product of a nonspecific defence mechanism of the infected plant,

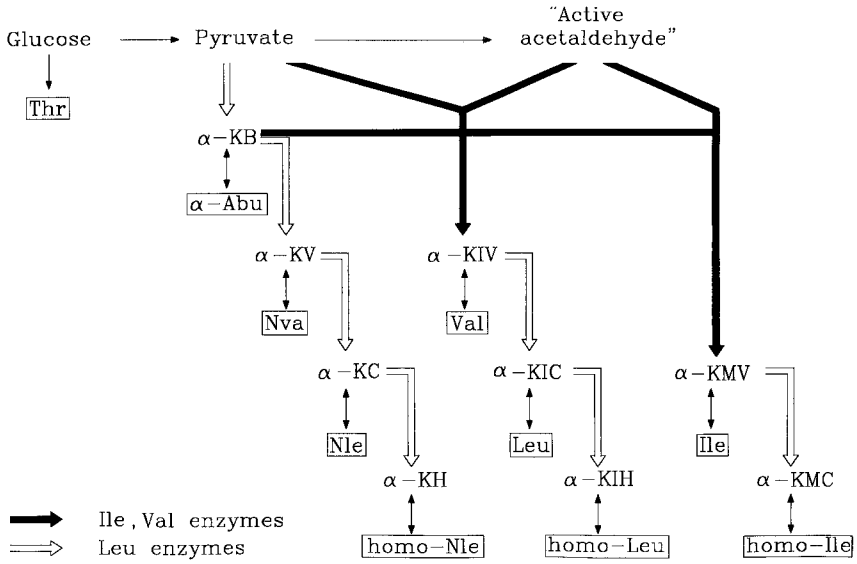

Fig. 1. Amino acids biosynthetic pathways, *KB* ketobutyric acid, *KV* ketovaleric acid, *KIV* ketoisovaleric acid, *KC* ketocaproic acid, *KIC* ketoisocaproic acid, *KMV* keto-3-methylvaleric acid, *KH* ketoheptanoic acid, *KIH* ketoisoheptanoic acid, *KMC* keto-4-methylcaproic acid

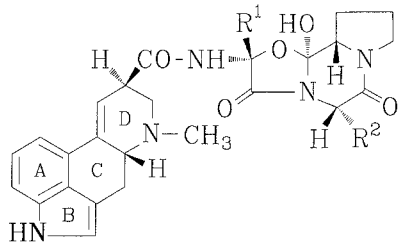

Fig. 2. Structure of ergopeptine alkaloids: R^1 methyl, R^2 benzyl – ergotamine; R^1 isopropyl, R^2 isopropyl – ergocornine; R^1 isobutyl, R^2 isopropyl – α-ergokryptine; R^1 isopropyl, R^2 benzyl – ergocristine; R^1 isopropyl, R^2 2-methyl-butyl – ergogaline

or is synthetized by the fungal pathogen. This work was conducted to obtain more detailed information about the occurence and the possible source of homo-Ile in ergot.

Materials and methods

Fungal and plant material

Four strains of *Claviceps purpurea* denoted as Gal 012 (CCM 8178), Gal 130 (CCM 8058), Gal 310 (CCM 8057), and Gal 401 (CCM 8177), were cultivated in field on the sterile rye sort Hyclaro in the year 1993 at the Northern Moravia. Fungal sclerotia were finely grounded prior to use. As a reference material, the residual grains of rye were collected; most of them neighboured directly with the ergot horns. The same sort of rye was also cultivated in the laboratory box under the simulated natural conditions. The whole plants were collected after 45 days and the fresh material was used for the amino acid determination.

Chemicals

Ergogaline monohydrate was isolated from the fungus *Claviceps purpurea* as described recently (Cvak et al., 1994). Homo-Ile standard was used as an acid ergogaline hydrolysate. For this purpose, 1 mg of ergogaline was hydrolysed in 2 ml of 6 M HCl at 105°C for 48 h. Leucine, cycloleucine, N-(tert-butyldimethylsily)-N-methyltrifluoroacetamide (MTBSTFA), dichloro methane, dimethylformamide, isooctane (all solvents puriss, dried over the molecular sieve), and 6 M HCl were purchased from Fluka (Buchs, Switzerland). Water and methanol were redestilled in glass before use.

Instrumentation

HPLC analyses were performed on a Lachrom HPLC chromatograph (Merck, Darmstadt, Germany) equipped with a Rheodyne valve, 10 μl loop and a UV detector set at 313 nm. GC-MS analyses were carried out on a Hewlett-Packard 5890 gas chromatograph equipped with a split/splitless injector, and linked to a Profile double focusing mass spectrometer (Kratos, Manchester, Great Britain). The compounds were separated on a 30 m $\times$ 0.25 mm I.D. DB-1 fused silica capillary column (J & W Scientific, Folsom, USA). The column temperature was held at 100°C for 3 min, then programmed at 10°C/min to 260°C. The column was directly coupled to the ion source via transfer line maintained at 250°C. Injector temperature was 250°C. Helium was used as a carrier gas at a velocity 25 cm/s; the split flow was 25 ml/min. Mass spectrometer operating conditions were: 70 eV electron energy, ion source temperature 200°C, ion current 100 μA. EI mass spectra were recorded by scanning mass range 50–500 at 0.6 sec/decade and resolution 600. For the trace analysis of homo-Ile, leucine and cycloleucine (internal standard) the respective ion m/z 214.200, 200.183 and 198.168 were monitored with 0.5 s cycle time and 113 ms dwell time on each particular mass at resolution 3000; i.e. by selected ion monitoring technique (GC-MS-SIM). High resolution mass spectral data were obtained from the prepared TBDMS-leucine derivative by peak matching technique (resolution 7500, static, static, 10% valley) with perfluorokerosene as reference in the ion source.

Determination of ergot alkaloids

1 g of grounded ergot sclerotia was suspended in 10 ml of methanol. Alkaloids were extracted at ultrasound bath 5 min, then the suspension was allowed to stand for additional 30 min. 10 μl aliquots were applied on a reversed-phase column (Hypersil ODS,

5μm, 250 × 4.6, mm I.D., from Shandon, U.K.). Isocratic elution was carried out with the acetonitrile/water/triethylamine 340/660/20 v/v/v mixture, flow 1.5 ml/min. Due to the partial epimerization of ergot alkaloids at the C-8 atom (the formation of -inines), the content of particular alkaloid was calculated as a sum of both epimers.

Identification of homoisoleucine in ergogaline hydrolysate

100μl (0.1645μmol) of the ergogaline hydrolysate was evaporated to dryness under a stream of nitrogen on a water bath kept at 40°C. Traces of water were removed with 2 × 200μl dichloromethane. MTBSTFA (40μl) and dimethylformamide (70μl) were added to the residue and the vial was tightly capped. The mixture was agitated on a vortex mixer for a min and heated at 80°C for 30 min. The TBDMS products were then extracted with 2 × 75μl of isooctane and 0.8μl aliquot was injected into the gas chromatograph for the GC-MS analysis. Split valve was opened after 0.75 min.

Quantitative GC-MS analysis of homoisoleucine and leucine

The ergogaline hydrolysate, containing approximatly molar aliquots of homo-Ile at 119.5μg/ml (1.645μmol), and the leucine stock solution (1 mg/ml) in 0.1 M HCl were employed for the construction of calibration plots. The stock solutions were serially diluted with 0.1 M HCl, containing each 10, 1, 0.1 and 0.01μg/ml of the corresponding amino acid. Initial 4-point calibration was made with 1μg/ml cycloleucine internal standard over the range of interest for each of the target compounds. One point recalibration was made regularly with the amino acid standard mixture at the 1μg/ml level after every six sample analyses.

Homo-Ile and leucine were determined in the grounded ergot sclerotia and various plant parts; both infected and noninfected by the fungal pathogen. The total amino acid content was obtained for the 10 mg of each sample (dry weight) hydrolysed with 1 ml of 6 M HCl (48 h, 105°C). Free amino acids were extracted from 1 g of the grounded material with 10 ml of 50% aqueous ethanol for two hours. After centrifugation 100–200μl extract aliquots were transferred into the 2 ml derivatization vials (Supelco, Gland, Switzerland), and cycloleucine (0.1μg) was added as internal standard. After evaporation of solvents the samples were derivatized and subjected to the GC-MS-SIM analysis as described above.

Results

A slightly modified method, which we developed recently for the GC-MS analysis of protein and nonprotein amino acids and other constituents as their TBDMS derivatives (Šimek et al., 1994) was applied for the analysis of samples in the present work. The EI mass spectrum of the TBDMS-homo-Ile is very similar to the homologous amino acids showing intensive diagnostic ions $(M-57)^+$, $(M-85)^+$ and $(M-159)^+$ at relatively high masses in all cases, Fig. 3a–c. Elemental composition of the three diagnostic ions was verified by high resolution mass spetrometry in the case of the TBDMS-Leu, confirming the losses of C_4H_9, C_4H_9, CO, and $-CO_2$ TBDMS radicals from the molecular ion. The high resolution data are summarized in Table 1.

The target compounds, homoisoleucine and leucine were identified in samples, if present, by monitoring of their characteristic fragment masses in the predetermined retention time window ±6 s at the enhanced resolution. For the trace analysis, the monitoring of the characteristic $(M-159)^+$ ions was

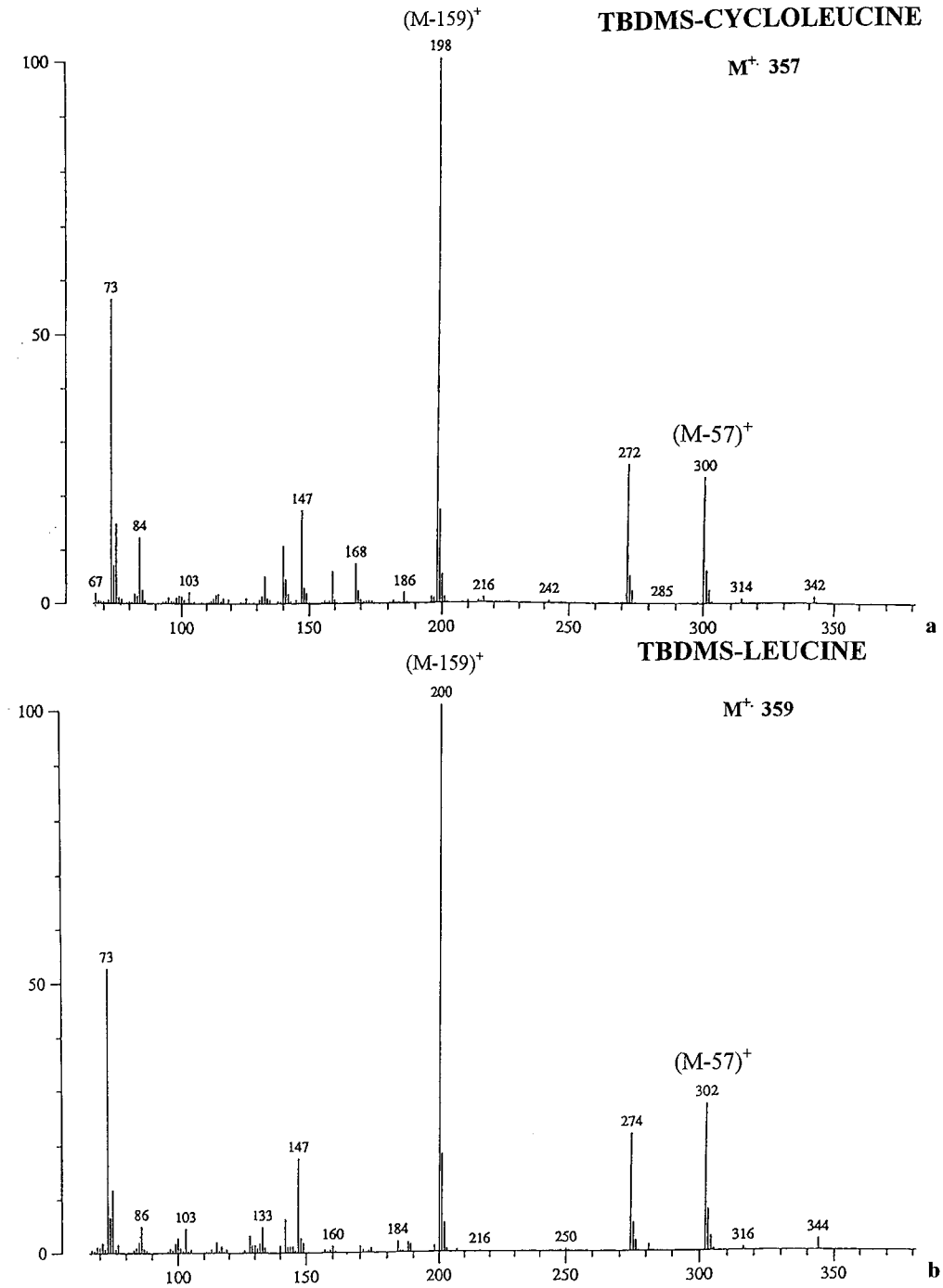

Fig. 3. EI mass spectra of the TBDMS amino acids, **a** cycloleucine (used as internal standard); **b** leucine; **c** (see page 14) homoisoleucine

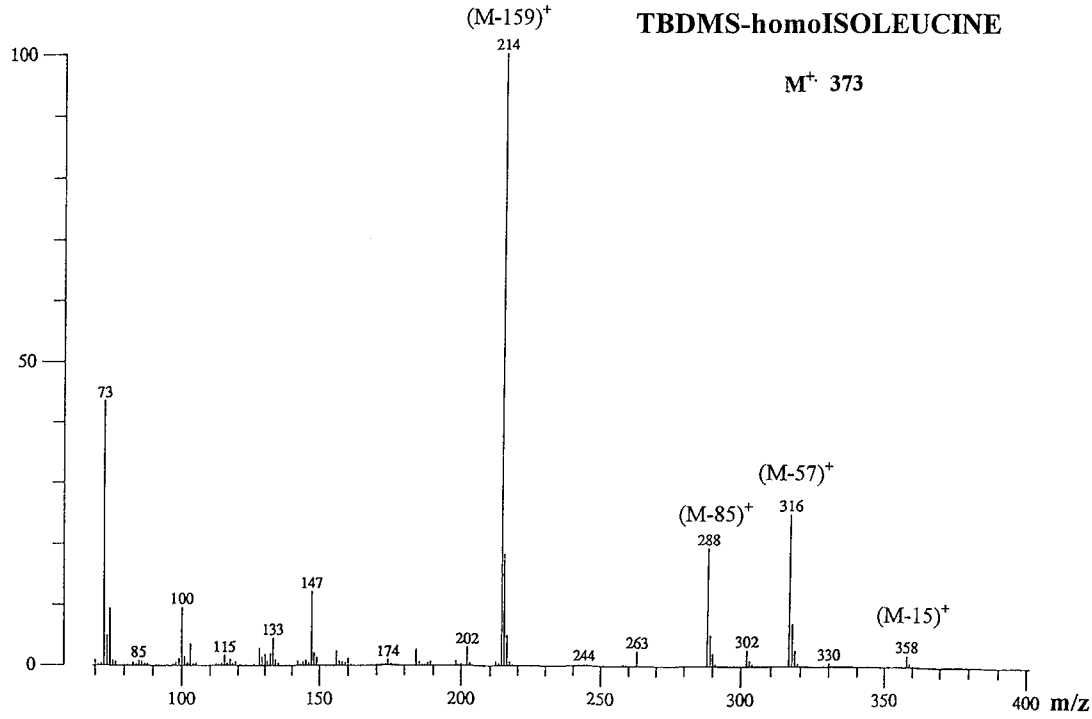

Fig. 3c

Table 1. Elemental composition of diagnostic ions from the EI high resolution mass spectrum of the leucine TBDMS derivative (n = 3)

Ion	Mass calc.	Mass obs.	Composition	I [%]
M^+	359.2676	359.2667	$C_{18}H_{41}N_2OSi_2$	0.1
$(M-57)^+$	302.1934	302.1927	$C_{14}H_{32}N_2OSi_2$	25.7
$(M-85)^+$	274.2023	274.2029	$C_{13}H_{32}N_2OSi_2$	21.1
$(M-159)^+$	200.1835	200.1840	$C_{11}H_{26}NSi$	100.0

I relative intensity of the ions in the EI mass spectrum.

found to be the most convenient. Under these conditions, selectivity and also the precision and accuracy of the peak areas were better than those with integral m/z values thus allowing quantitative determination of homo-Ile over more than three orders of concentration. Retention times of the target amino acids, ions selected for the GC-MS-SIM, the relative response factors (RRF) calculated for each concentration (n = 3) and parameters of the calibration lines are presented in Table 2.

At each concentration level the relative standard deviation was better than 10% Detection limits were estimated 1.4ng/ml, 1.2ng/ml and 2.4ng/ml for homo-Ile, leucine and cycloleucine standards, respectively. Because only traces of homo-Ile were present in the samples, its detection limit was also affected by the sample matrix. The performance of the method was therefore

Table 2. Retention time, the monitored ions and relative response factors (RRF, n = 3) of leucine, homoisoleucine, and cycloleucine used for the GC-MS SIM calibration

Amino acid	Leucine	Homoisoleucine	Cycloleucine
RT [min]	13.85	14.90	15.30
Ion m/z	200.184	214.200	198.168
[μg/ml]	RRF	RRF	I.S.
10	12.031	10.861	10.000
1	1.134	1.068	1.000
0.1	0.124	0.095	0.100
0.01	0.014	0.011	0.010

Coefficients of calibration lines: Homo-Ile, Y = Bx + A, B = 10.8710, A = −0.01009, r = 0.999; Leucine, Y = Bx + A, B = 12.0463, A = −0.02011, r = 0.999.

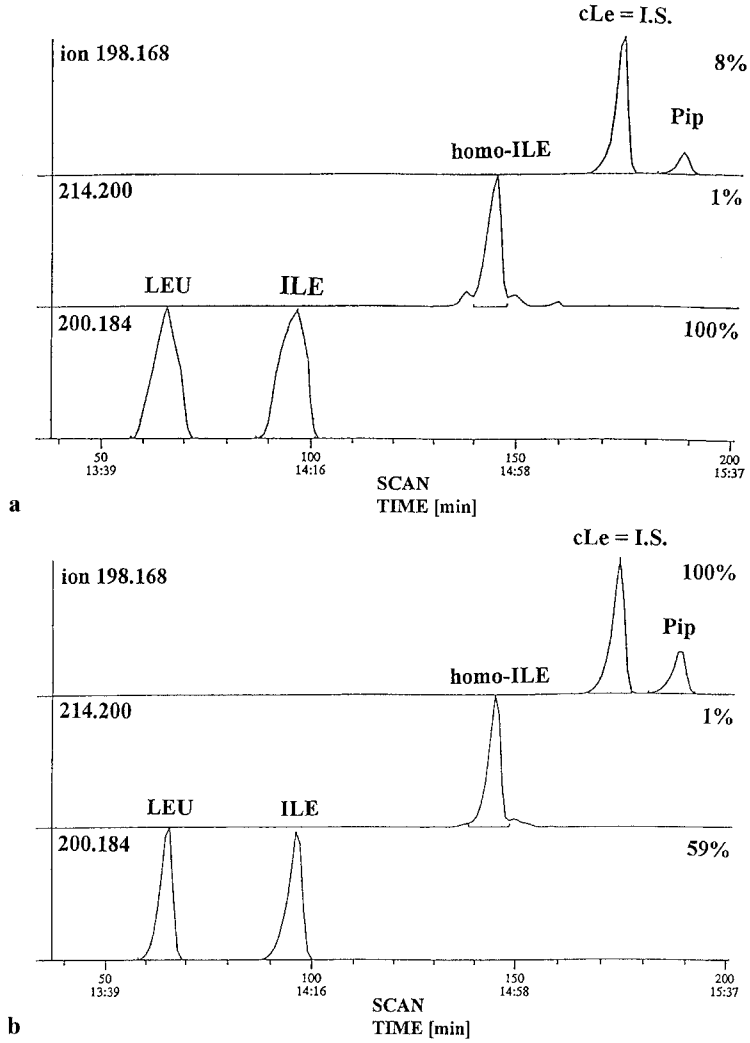

Fig. 4. Typical GC-MS-SIM traces for the ions characteristic of cycloleucine (*cLe* int. std., 198.168) and pipecolic acid (Pip, 198.168), homo-Ile (214.200), leucine and isoleucine (Ile, 200.184) at the ergot sclerotia of the strain Gal 012; **a** acid hydrolysate, **b** ethanolic extract of free amino acids

Table 3. Absolute amounts of homo-Ile, Leu, and ergopeptine alkaloids in rye grains and ergot sclerotia

Sample	Homoleucine μg/g		Leucine μg/g		Ergogaline μg/g	Other alkaloids* μg/g	homo-Ile/Leu ratio [%]		
	hydrolyzate	free AA	hydrolyzate	free AA			hydrolyzate	free acid	Gal/α-Kry
rye grains	n.d.	n.d.	3800	37	n.d.	n.d.	–	–	–
Gal 012	12.2	0.13	1030	5.1	22	α-Kry 2800 Cri 116	1.18	2.47	0.79
Gal 130	n.d.	0.32	2460	5.0	6	α-Kry 780 Cri 5700	–	6.45	0.77
Gal 310	8.8	0.30	470	3.2	32	α-Kry 2000 Cor 3200	1.85	9.37	1.60
Gal 401	n.d.	0.33	1280	6.5	n.d.	α-Kry n.d. Cri 39 Tam 3900	–	5.11	–

**n.d.* not detected, *α-Kry* α-ergokryptine, *Cri* ergocristine, *Cor* ergocornine, *Tam* ergotamine (see Fig. 2).

checked by regular analysis of blank samples and sample extracts spiked with a known amount of homo-Ile (1 μg/ml) prior to derivatization. With respect to the background, the determination limits for homo-Ile were estimated 2.4 ng/ml and 10 ng/ml for the ethanolic extracts and acid hydrolysates, respectively. Typical ion chromatograms representing the GC-MS-SIM amino acid analysis of the ethanolic extract containing free amino acids and the acid hydrolyzate of ergot sclerotia are documented in Fig. 4a and 4b, respectively. The summarized results are in Table 3 and for convenience are expressed in μg/g.

Discussion

Free homo-Ile and leucine pool was determined in ethanolic extracts of both the infected and noninfected plants and in sclerotia of the parasitic fungus. Leucine was chosen as a reference amino acid in biological material with respect to its similar chemical properties and the possible homo-Ile/leucine competition in the incorporation into ergopeptine alkaloids. As a free amino acid, homo-Ile was found in sclerotia of all *Claviceps* strains examined, however, was not detected in any part of rye. In Table 3, the analytical data for individual ergot strains are summarized together with an axample of rye grain analysis. The results demonstrating the absence of homo-Ile in any other part of either infected or noninfected plants are omited for clarity. In order to obtain the total balance of homo-Ile in the biological material, all samples were also hydrolyzed with 6M hydrochloric acid. Homo-Ile was detected at ppm level in all α-ergokryptine-producing ergot strains, but again here, not in any plant material. As expected, the relative abundance of homo-Ile in hydrolyzates was lower compared with the free amino acid pool, since its incorporation into ordinary proteins can hardly be expected.

Parasitsm by *Claviceps purpurea* is described as a competition for photosynthates between the fungus and rye seeds (Bacon and Luttrell, 1987). It seems likely that any defense mechanism against the fungus is not known. Thus the absence of even traces of homo-Ile in the plant favors its production by the fungus and the hypothesis, that the plant might produce homo-Ile as a nonspecific defence mechanism against the parasite can be abanded. Since homo-Ile was found in ppm concentrations only, its importance as a possible nitrogen storage material can also be neglected. It is interesting to note, that the presence of homo-Ile in ergot indicates, that at least some part of amino acids in the fungus is synthetised *de novo*. Pointing this fact, the homo-Ile/Leu ratio reflects relatively poor specifity of enzymes involved in the synthesis of amino acids in the fungus. It seems likely that the loss of enzyme specifity might be just caused by the adaptation of fungus to the parasitism.

For the estimation of specifity of the homo-Ile incorporation into ergopeptines expressed as the ratio of total homo-Ile content and its part incorporated into ergogaline (homo-Ile/Leu vs. ergogaline/α-ergokryptine) we also determined individual alkaloids in ergot sclerotia. Ergogaline was found in all α-ergokryptine producing strains, but not in the strain incorporating phenylalanine at the same position (i.e., ergotamine, ergocristine, see

Fig. 2). Ergogaline was not detected in sclerotia where the homo-Ile/Leu ratio was below 0.01.

Ergot alkaloids are synthetized by a multienzyme system extraribosomally (Maier et al., 1987). This process is less specific than the ordinary peptide translation and, consequently, a variety of structurally related amino acids can be incorporated (Cvak et al., 1996). Since the alkaloid production depends primarily on the relative concentration of free amino acids in cells (Beacco et al., 1978; Bianchi et al., 1982; Crespi-Perellino et al., 1992; Cvak et al., 1996; Flieger et al., 1984; Keller et al., 1980), the comparison of the homo-Ile/Leu ratio in the amino acid pool, Table 3, with that in the corresponding alkaloids, i.e. the ergogaline/α-ergokryptine ratio, indicates that the production of ergogaline in ergot is not a targeted process and homoisoleucine is incorporated into ergopeptines with slightly lower specificity than leucine.

Selective ion monitoring provided in addition two interesting results. For ion m/z 214, several related congeners were detected (Fig. 3a) in both the acid hydrolyzate and free amino acid pool of ergot sclerotia, which might be assigned to some of homo-Ile isomers (i.e., homo-Nle, homo-Leu, or homo-allo-Ile). Similarly as with homo-Ile, these amino acids originate as a side product in the amino acid biosynthesis, Fig. 1 (Jakubowski and Goldman, 1992). Due to the poor specifity of enzymes involved in the biosynthesis of ergot alkaloids, ergopeptines containing these unusual amino acids might be expected. It should be noted, that the occurence of homo-Leu-containing diketopiperazine in ergot was reported already in 1976 by Ohmono and Abe (Ohmono and Abe, 1976). They suggested also the names ergohexine (Fig. 2, R^1 = methyl) and ergoheptine (R^1 = *iso*propyl) for the corresponding predicted ergopeptine alkaloids (R^2 = *iso*amyl). The higher proline homologue – pipecolic acid (Pip) was detected by SIM at m/z 198 (Fig. 3c). Since misincorporation of Pip instead of Pro is well known to occur in similar multienzyme systems (Pais et al., 1981), theoretically also ergopeptines containing Pip could exist. Pipecolic acid, however, have not been detected in hydrolyzates of crude ergot alkaloid mixtures yet.

References

Bacon CW, Luttrell ES (1981) Competition between ergots of *Claviceps purpurea* and rye seeds for photosynthates. Phytopathology 72: 1332–1336

Beacco E, Bianchi ML, Minghetti A, Spalla C (1978) Directed biosynthesis of analogues of ergot peptide alkaloids with *Claviceps purpurea*. Experientia 34: 1291–1293

Bianchi ML, Crespi Perellino N, Gioia B, Minghetti A (1982) Production by *Claviceps purpurea* of two new peptide ergot alkaloids belonging to a new series containing α-aminobutyric acid. J Nat Prod 45: 191–196

Boyle JE, Fowden L (1971) Biosynthesis of 2-amino-4-methylhex-4-enoic acid in *Aesculus californica*: the route from isoleucine and the role of aminotransferases. Phytochemistry 10: 2671–2678

Crespi-Perellino N, Malyszko J, Ballabio M, Gioia B, Minghetti A (1992) Directed biosynthesis of unnatural ergot peptide alkaloids. J Nat Prod 55: 424–427

Cvak L, Jegorov A, Sedmera P, Havlíček V, Ondráček J, Hušák M, Pakhomova S, Kratochvíl B, Granzin J (1994) Ergogaline, a new ergot alkaloid produced by

Claviceps purpurea: isolation, identification, crystal structure and molecular conformation. J Chem Soc Perkin Trans 2: 1861–1865

Cvak L, Minář J, Pakhomova S, Ondráček J, Kratochvíl B, Sedmera P, Havlíček V, Jegorov A (1996) Ergoladinine, a new ergot alkaloid. Phytochemistry 42: 231–233

Edelson J, Skinner CG, Ravel JM, Shive W (1959) Structural specificity of some amino acid antagonists. J Am Chem Soc 81: 5150–5153

Flieger M, Sedmera P, Vokoun J, Řeháček Z, Stuchlík J, Malinka Z, Cvak L, Harazim P (1984) New alkaloids from a saprophytic culture of *Claviceps purpurea*. J Nat Prod 47: 970–976

Floss HG, Tcheng-Lin M, Kobel H, Stadler P (1974) On the biosynthesis of peptide ergot alkaloids. Experientia 30: 1369–1370

Fowden L, Mazelis M (1971) Biosynthesis of 2-amino-4-methylhex-4-enoic acid in *Aesculus californica*: the precursor role of isoleucine. Phytochemistry 10: 359–365

Fowden L, Smith A (1968) Newly characterized amino acids from *Aesculus californica*. Phytochemistry 7: 809–819

Jakubowski H, Goldman E (1992) Editing of errors in selection of amino acids for protein synthesis. Microbiol Rev 56: 412–429

Keller U, Zocher R, Kleinkauf H (1980) Biosynthesis of ergotamine in protoplasts of *Claviceps purpurea*. J Gen Microbiol 118: 485–494

Kisumi M, Sugiura M, Chibata I (1976) Biosynthesis of norvaline, norleucine, and homoleucine in *Serratia marcescens*. J Biochem 80: 333–339

Maier W, Erge D, Schumann B, Gröger D (1981) Incorporation of L-[U-^{14}C]leucine into ergosine by cell-free extracts of *Claviceps purpurea* (Fr.) Tul Biochem Biophys Res Commun 99: 155–162

Ohomono S, Abe M (1976) Production of alkaloids and related substances by fungi. Part XIII. A new prolyldiketopiperazine produced by ergot fungi. Nippon Nogei Kagaku Kaishi 50: 543, [Chem Abst (1977) 86, 190303]

Pais M, Das BC, Ferron P (1981) Depsipeptides from *Metarhizium anisopliae* Phytochemistry 20: 715–723

Shoji J, Sakazaki R (1970) A new antibiotic complex S-520. IV. Isolation of three new amino acids from the hydrolysate. J Antibiot 23: 519–521

Shoji J, Kozuki S, Mayama M, Shimaoka N (1970) A new peptide antibiotic complex S-520. I. Isolation and characterization. J Antibiot 23: 429–431

Szántay C Jr, Bihari M, Brlik J, Csehi A, Kassai A, Aranyi A (1994) Structural elucidation of two novel ergot alkaloid impurities in α-ergokryptine and bromokryptine. Acta Pharm Hung 64: 105–108

Šimek P, Heydová A, Jegorov A (1994) High resolution capillary gas chromatography and gas chromatography – mass spectrometry of protein and non-protein amino acids, amino alcohols, and hydroxycarboxylic acids as their tert-butyldimethylsilyl derivatives. HRC – J High Res Chromatogr 17: 145–152

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