Liquid chromatography–tandem mass spectrometry characterization of ergocristam degradation products

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ABSTRACT

The UPLC method with diode array UV detection was developed for qualitative determination of ergocristine and ergocristam including degradation products. The mechanism of the ergocristam disruptive reaction was described based on MS/MS characterization of ammonolytic product, *N*-(D-lysergyl)-L-valinamide (A1) and two methanolytic products, methyl ester of *N*-(D-lysergyl)-L-valine (M2), and *N*-[*N*-(D-lysergyl)-L-valyl]-L-phenylalanyl-D-prolyl methyl ester (M1). The influence of extraction conditions on epimerization and degradation of ergocristine and ergocristam was tested and conditions for reproducible decomposition of ergocristam were found. The presented method could potentially be applied for ergot alkaloids determination in sclerotia, fermentation broth, mycelium, and possibly contaminated food products, *i.e.* corn, flour, bread, *etc.*, and feeding stuffs containing ungrounded cereals.

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1. Introduction

In general, there are two possible methods of ergot alkaloid extraction from different solid matrices. The first one is an extraction of water soluble salts (tartarates, phosphates, acetates or others) [1–5] with water, water/alcohol, and water/acetonitrile mixtures. The obtained crude extracts are generally alkalized with NH$_4$OH (pH 9) to remove the co-extracted compounds and free alkaloid bases are re-extracted into organic solvents. The second one is the extraction of alkaloid bases from alkalized (pH 9) matrices with organic solvents [6]. Purification of these crude alkaloid bases is frequently performed *via* re-extraction to acidic solutions (*e.g.* 4% tartaric acid) followed by alkalization and re-extraction into organic solvent [7]. Very common use of methanol and ammonia for isolation and/or purification of ergot alkaloid extracts could be a source of problems when ergopeptams (non cyclol ergot alkaloids) are present in original mixture.

Ergopeptams were isolated from different *Claviceps purpurea* strains producing ergot alkaloids namely of ergotoxine group [1,2,6,8]. Structures of these compounds were assigned by ^{1}H and/or ^{13}C NMR and mass spectrometry with electron impact [8,9] and fast atom bombardment ionization [10]. A role of ergopeptams in biogenesis of the peptide part of ergot alkaloids has been studied by Floss and co-workers [11–13] who described an effect of competitive reaction bringing about a reversal of configuration of optically active carbon of L-proline to the D-configuration. The resulting compound cannot enter the biochemical reaction which leads to the formation of a non cyclol structure.

Ergopeptams were found to be rather unstable in organic solvents containing bases (pyridine, ammonia, or triethylamine) [14–16]. When mixture of ergocristine and ergocristam (for structure see Fig. 1) was chromatographed on TLC in dichlormethane–diisopropylether–methanol–benzene–ammonia elution phase, more spots have been found than expected. Four of them corresponded to ergocristine, ergocristinine, ergocristam and ergocristinam the other two were determined as *N*-(D-lysergyl)-L-valine methyl ester and *N*-(D-lysergyl)-L-valinamide. These compounds have been previously isolated from ergot [17] and described as artifacts originated from ergopeptams [18]. Due to the predominance of α-ergokryptine/ergocristine producing *C. purpurea* strains in nature where production of ergocristam could be expected the simultaneous determination of both alkaloids is necessary namely in samples of newly isolated wild type strains and subsequently in samples of possibly contaminated food products and feeding stuffs which could represent a risk for public and animal health related to their presence.

The aim of this study was to examine the influence of extraction conditions on α-ergokryptine, ergocristine, and ergocristam epimerization and degradation. The structures of degradation products have been extensively investigated by different mass spectrometry techniques to prepare a base for future off- and/or on-line

(A)

(B)
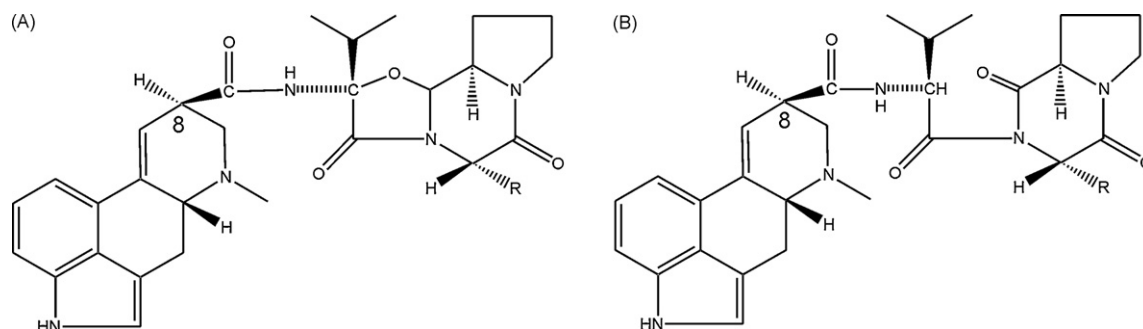
Fig. 1. Genaral formulas of ergotoxines (A) and ergotoxams (B).

A	B	R
Ergocornin	Ergocornam	$CH(CH_3)_2$
α-Ergokryptine	α-Ergokryptam	$CH_2CH(CH_3)_2$
Ergocristine	Ergocristam	$CH_2C_6H_5$

LC–MS/MS method allowing determination of trace amounts of ergopeptams in different matrices. Further, the UPLC procedure allowing simultaneous determination of α-ergokryptine, ergocristine, ergocristam and all degradation products including C(8) epimers was developed. This method was used for the analysis of ergot alkaloids in sclerotia of wild strain of *C. grohii* [19].

2. Experimental

2.1. Chemicals

Ergocristine, ergocristinine, α-ergokryptine, α-ergokryptinine, ergocristam, and ergocristinam were isolated in our laboratory [2]. Methanol, ethanol and acetone were purchased from Merck (Darmstadt, Germany) and ammonium hydroxide A.C.S. reagent (24% aqueous solution of NH_4OH), acetic acid, MRFA peptide, human angiotensin I, deuterated methanol, and α-cyano-4-hydroxy-

cinnamic acid from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Acetonitrile (J.T.Baker, Deventer, the Netherlands) used in UPLC was of HPLC grade.

2.2. Extraction procedure

Sclerotia of the strain *C. grohii* were extracted with solution of methanol–water–NH_4OH (80:20:0.1, v/v/v) for 2 h at laboratory temperature.

2.3. UPLC method

An Acquity UPLC system (Waters, Milford, Massachusetts), equipped with 2996 PDA detector operating at 225 and 310 nm was used for analysis. Data were processed with Empower 2 software (Waters). Samples were analyzed on Waters BEH C18 column (50 mm × 2.1 mm I.D, particle size 1.7 μm), column temperature,
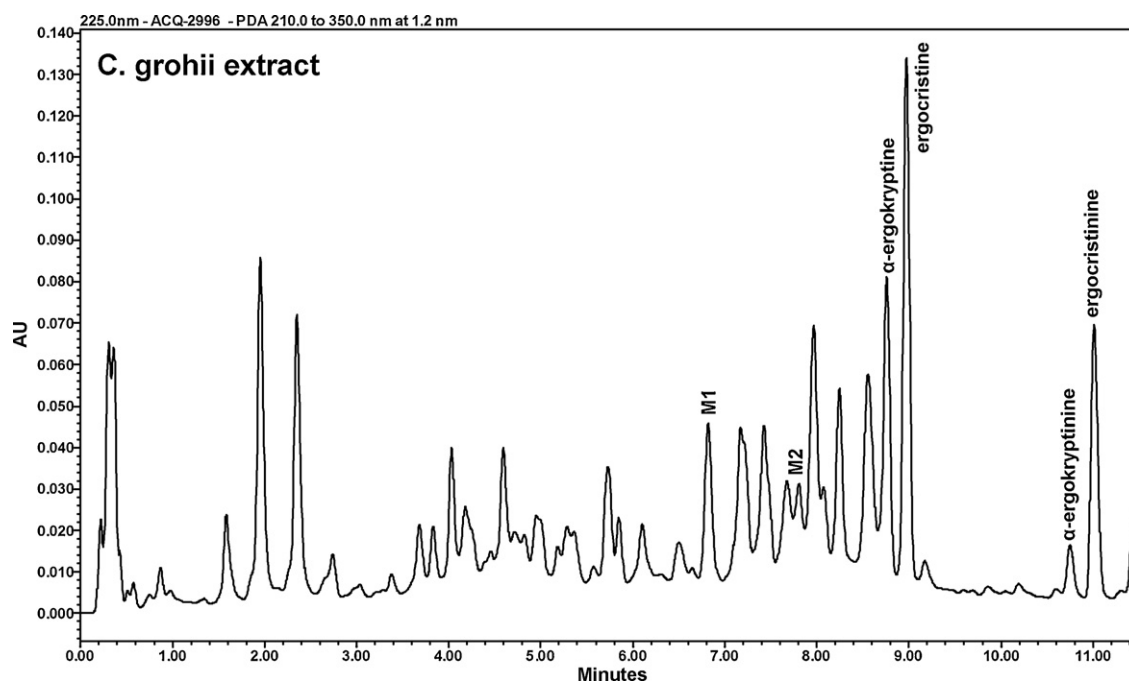
Fig. 2. UPLC analysis of *C. grohii* crude alkaloid mixture extracted from sclerotia with solution of methanol–water–NH_4OH (80:20:0.1; v/v/v) for 2 h at laboratory temperature. Analytes retention time: M1 (6.8 min), α-ergokryptine (8.8 min), α-ergokryptinine (10.7 min), ergocristine (8.9 min) and ergocristinine (11.0 min). UPLC conditions: Waters BEH C18 column (2.1 mm × 50 mm I.D, particle size 1.7 μm); mobile phase: solvent A, water, and solvent B, acetonitrile; both containing NH_4OH 0.04%, flow rate, 0.4 ml min^{-1}; column temperature, 35 °C; injection volume, 5 μl; gradient elution, (5–61% B) in 12 min, washing step (95% B) 1 min, equilibration step (5% B) 1.0 min. UV detection at 225 nm.

35 °C; data sample rate, 20 pts s^{-1}; filter constant, 0.5; injection volume, 5 μl; analysis time, 12 min; flow rate, 0.4 ml min^{-1}. Mobile phases consisted of water (A) and acetonitrile (B), both containing NH$_4$OH 0.04%. Gradient elution started at 5% B (0 min), increasing linearly to 61% B within 12 min. Each analysis was followed with column washing step (95% B, 1 min) and equilibration step (1 min).

Three fractions corresponding to one product of ammonolysis (A1), and two products of methanolysis (M1 and M2) were collected for MS/MS structure determination and characterization of fragmentation pattern. Fractions were evaporated to dryness under reduced pressure.

2.4. Method calibration

Calibration curves over the range from 0.78 to 250.00 μg ml^{-1} for ergocristine and ergocristam, respectively, were determined. Calibration standards were prepared by serial dilution of stock solution (1 mg ml^{-1} of standard in methanol). The representative regression equation for ergocristam was $y = 3.00 \times 10^4 x + 1.22 \times 10^4$. The representative regression equation for ergocristine was $y = 2.78 \times 10^4 x - 5.25 \times 10^4$. Determination coefficient (r^2) was in both cases 0.999.

2.5. Lower limit of quantification (LLOQ)

LLOQ for UPLC method was determined as the lowest concentrations of ergocristine and ergocristam quantified with precision and accuracy lower then 20%. Six replicates of QC (quality control) of both alkaloids at concentration 0.78 μg ml^{-1} were measured (concentrations at which the signal-to-noise ratio was found to be larger than 10). The LLOQ of ergocristam was 0.78 μg ml^{-1} with RSD of 4.9% and accuracy of 102.7% ($n = 6$). The LLOQ of ergocristin was 0.78 μg ml^{-1} with RSD of 2.6% and accuracy of 101.5% ($n = 6$).

2.6. Solution stability testing

The stability of α-ergokryptine, ergocristine, and ergocristam solutions (0.1 mg ml^{-1}) was performed in methanol containing either 0 (control), 0.1, 1.0 or 10.0% of NH$_4$OH, respectively. The mixtures were stirred and left to react for 2 and 10 h at room temperature. After that the solutions were evaporated to dryness under reduced pressure and reconstituted in 50 μl of methanol before UPLC and MS analysis. All experiments were done in triplicates for determination of relative standard deviations (RSDs). The stability of the same sample set was tested under acidic conditions in solutions containing 20% acetic acid in methanol (v/v).

2.7. Mass spectrometry analysis

Mass spectra were measured on a matrix-assisted laser desorption/ionisation reflectron time-of-flight (MALDI-TOF) mass spectrometer BIFLEX (Bruker-Franzen, Bremen, Germany) equipped with a nitrogen laser (337 nm) and gridless delayed extraction ion source. Ion acceleration voltage was 19 kV and the reflectron voltage was set to 20 kV. Spectra were calibrated externally using the monoisotopic [M+H]$^+$ ion of matrix peak at m/z 379.1, peak of MRFA peptide at m/z 524.3, and human angiotensin I peak at m/z 1269.7. A 10 mg ml^{-1} solution of α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile with 0.3% acetic acid was used as a MALDI matrix. A 1 μl of matrix solution was mixed with a 1 μl of the sample and 1 μl of this premix was loaded on the target and allowed to dry at ambient temperature. The MALDI-TOF and PSD (post source decay) spectra were collected in a reflectron mode.
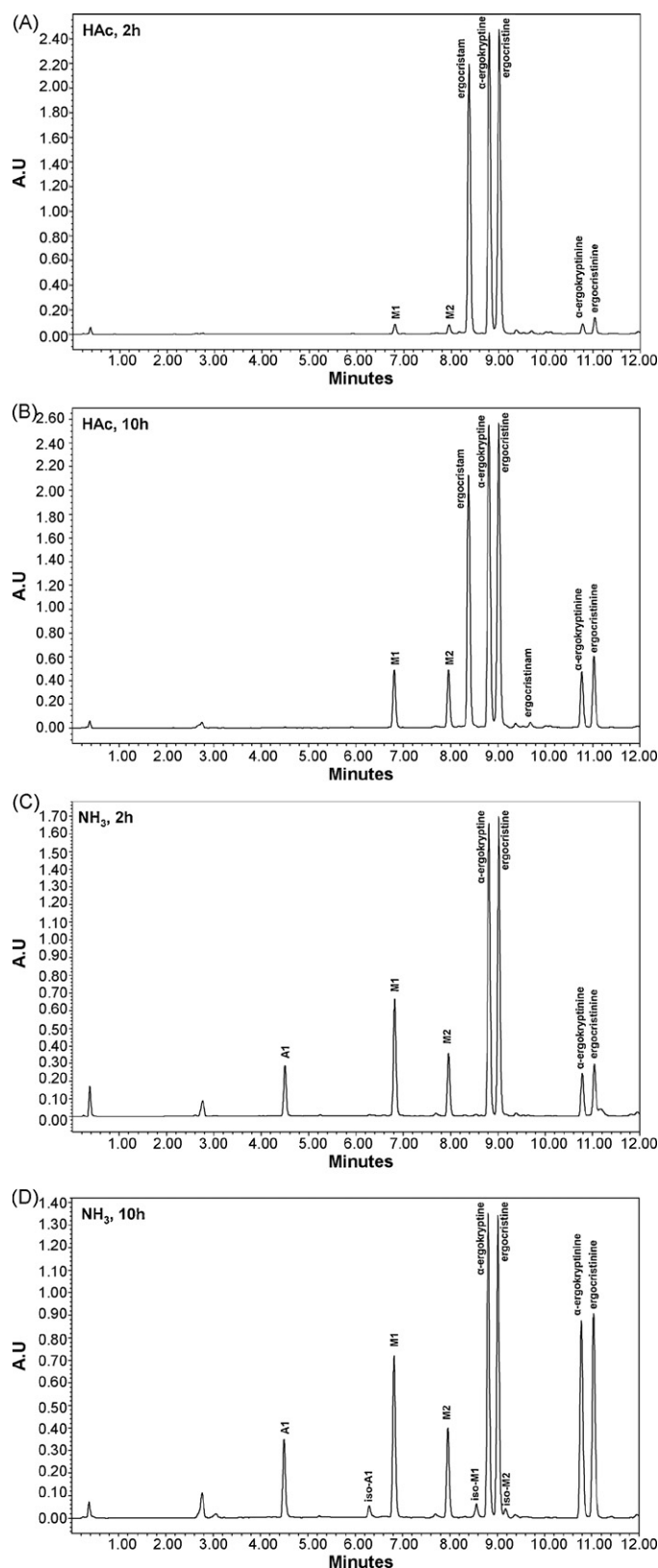

Fig. 3. UPLC analysis of ergocristam degradation mixture after 2 (A) and 10 (B) hours of incubation in the mixture acetic acid–methanol (20:80; v/v) and after 2 (C) and 10 (D) hours in the mixture NH$_4$OH–methanol (10:90; v/v). Analytes retention time: A1 (4.5 min), M1 (6.8 min), M2 (8.0 min), ergocristam (8.4 min), α-ergokryptine (8.8), ergocristine (8.9 min), α-ergokryptinine (10.7 min), ergocristinine (11.0 min). For chromatographic conditions see Fig. 2.

MS/MS experiments were performed on LCQ^{DECA} ion trap (IT) mass spectrometer (ThermoQuest, San Jose, CA) equipped with a static nanoelectrospray ion source (nanoESI). Spray voltage was held at 1.2 kV, tube lens voltage was 30 V. The heated capillary was kept at 175 °C with a voltage 10 V. Positive-ion full scan were acquired over m/z range 150–2000. Collisions were done from the most intense ion in full MS scan. Collision energy was kept at 25–40 units range with the activation time 30 ms for collision-induced dissociation (CID) experiment. All obtained data were interpreted manually with respect to described fragmentation rules.

High resolution MS data of samples were acquired on a APEX-Q FTMS instrument equipped with a 9.4 T superconducting magnet and a Combi ESI/MALDI ion source (Bruker Daltonics, Billerica MA) in the positive ion mode with 1 M data points and 128 time-domain transients was obtained in non mass-selective (rf-only) mode in m/z range 150–2000. The instrument was externally calibrated using fragments of angiotensin I, which results in typical mass accuracy below 0.5 ppm.

Each fraction from UPLC was dissolved in 50% of methanol with 0.5% acetic acid (v/v) and sonicated for 5 min prior to mass spectrometry analysis.

2.8. Degradation of ergocristam in deuterated methanol

For degradation reaction, 2 μg of ergocristam standard were dissolved in 20 μl of deuterated methanol (CD$_3$OD) containing 10% NH$_4$OH. After 2 h incubation at room temperature the mixture was loaded on UPLC and appropriate fractions were collected. After SPEED-VAC to dryness the fractions were reconstituted in 10 μl of 50% acetonitrile, containing 0.5% acetic acid (v/v) by sonication in water bath. The 1 μl was directly loaded on MALDI target and MALDI-TOF MS was performed with α-cyano-4-hydroxy-cinnamic acid matrix in positive and reflectron mode. As a positive control a standard linear peptide (human angiotensin I) and as a negative control ergocristine were used. The PSD fragmentation of observed signals was performed to corroborate their structure.

2.9. Esterification protocol for mass spectrometry analysis

For esterification, a reconstituted UPLC fraction was dried on SPEED-VAC (Savant, USA) and reconstituted in a 4:21 (v:v) acetylchloride–absolute ethanol mixture in water-bath sonicator. After 3 h of incubation at room temperature the mixture was dried and reconstituted with 20 μl of 50% MeCN, 0.3% acetic acid and 1 μl was directly loaded on MALDI target. As a positive control a standard linear peptide of human angiotensin I and as a negative control an ergocristine were used.

2.10. Carboxypeptidase degradation

For carboxypeptidase degradation, a reconstituted UPLC fraction was dried on SPEED-VAC (Savant, USA) and reconstituted in water in a water-bath sonicator. After addition of carboxypeptidase Y (100 ng, Roche Diagnostics GmbH, USA), incubation at 37 °C was held and 1 μl of reaction mixture was premixed with matrix to stop reaction and directly loaded on MALDI target in time dependence. As a positive control a standard of linear peptide human angiotensin I (20 pmol/μl) and as a negative control an ergocristine UPLC fraction were used.

3. Results

UPLC analysis of ergot alkaloid content in extract (80% methanol with 0.1% NH$_4$OH, 2 h) of wild strain of *C. grohii* revealed some unknown alkaloids (M1, M2; Fig. 2) with typical UV spectra of ergopeptines (λ_{max} 223, 240 and 310 nm). Isolated fractions were analyzed by MS and two protonated molecules at m/z 382.1 (M2) and 626.2 (M1) were detected. Both compounds were previously described as products of ergocristam degradation. In contrast to M2 no structural characterization of M1 was available in the literature [8]. Since no data were available also on the influence of extraction conditions on degradation of ergocristam this process was studied in details.
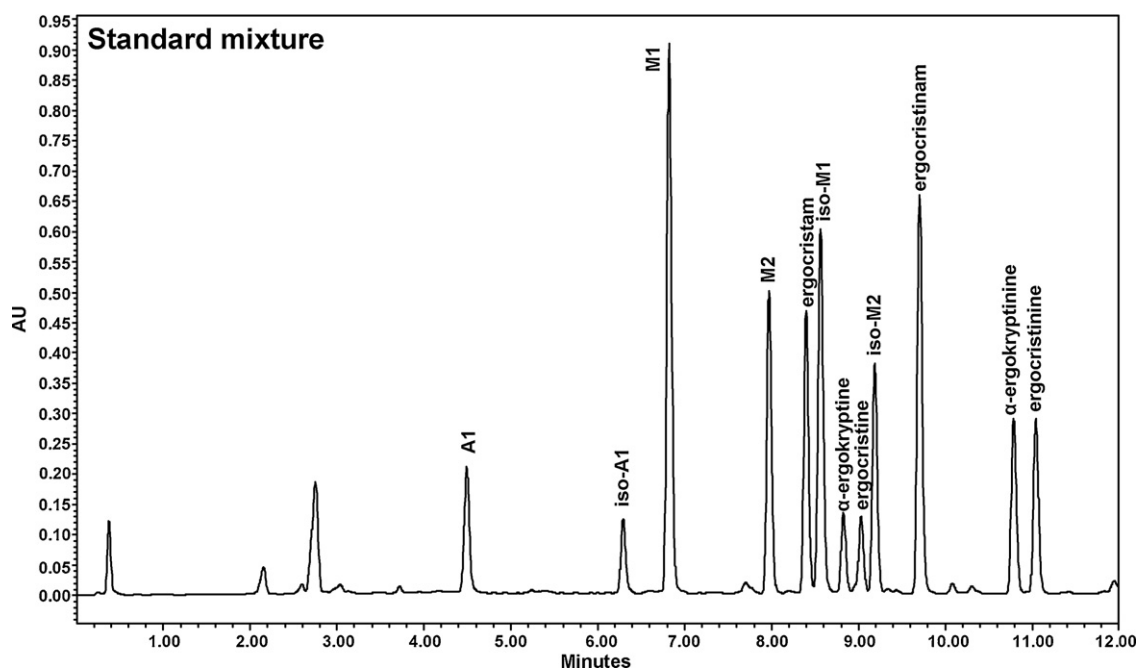

Fig. 4. UPLC analysis of the standard mixture of the α-ergokryptine, ergocristine, and ergocristam C(8)-epimerization and degradation products. Analytes retention time: A1 (4.5 min), iso-A1 (6.3 min), M1 (6.8 min), M2 (8.0 min), ergocristam (8.4 min), iso-M1 (8.6 min), α-ergokryptine (8.8 min), ergocristine (8.9 min), iso-M2 (9.2), ergocristinam (9.7 min), α-ergokryptinine (10.7 min), ergocristinine (11.0 min). For chromatographic conditions see Fig. 2.

3.1. Influence of extraction condition on epimerization and degradation of α-ergokryptine, ergocristine, and ergocristam

Stability of ergot alkaloid mixtures containing α-ergokryptine, ergocristine, and ergocristam in acidic (20% HAc, Fig. 3A, B) and alkaline (10% NH$_4$OH, Fig. 3C, D) methanolic solutions frequently used for alkaloid extraction was tested. Under acidic conditions ergocristine and α-ergokryptine form only C(8) epimers, i.e. ergocristinine and α-ergokryptinine. The production of ergocristinam is negligible but both products of methanolysis i.e. M1 and M2 were observed (Fig. 3A, B). When alkaline conditions are used the ergocristine and α-ergokryptine undergo again only C(8) epimerization but with the reaction rate about 4× higher. Much more complex is the degradation of ergocristam which undergoes simultaneous ammonolysis and methanolysis products A1, M1, and M2, see Fig. 3C and C(8) epimerization of all products thus forming iso-M1, iso-M2, iso-A1 (Fig. 3D).

To study the degradation of ergocristam, its standard solution in methanol was mixed with NH$_4$OH in different concentrations, i.e. 0.0 (control), 0.1, 1.0 and 10.0%, respectively, and incubated for 2 h at laboratory temperature. It was found that degradation is strongly dependent on NH$_4$OH concentration. No standard compound was found in reaction mixture containing 10% of NH$_4$OH and only three degradation products were determined (A1, M1, and M2, see Fig. 3C). Prolongation of reaction time (10 h) led to the formation of C(8) epimers of degradation product (i.e. iso-A1, iso-M1, and iso-M2, Fig. 3D). In reaction mixtures containing 0.1 and 1.0% of NH$_4$OH, methanolytic products (M1 and M2) form the major part and only trace amount of ergocristam was found. Without addition of NH$_4$OH no degradation was observed. During further detailed study we found that degradation of ergocristam in methanol solutions containing 10% of NH$_4$OH proceeds within 1 min at laboratory temperature and formation of degradation products A1:M1:M2 is in constant ratio (23:55:22). Therefore, con-
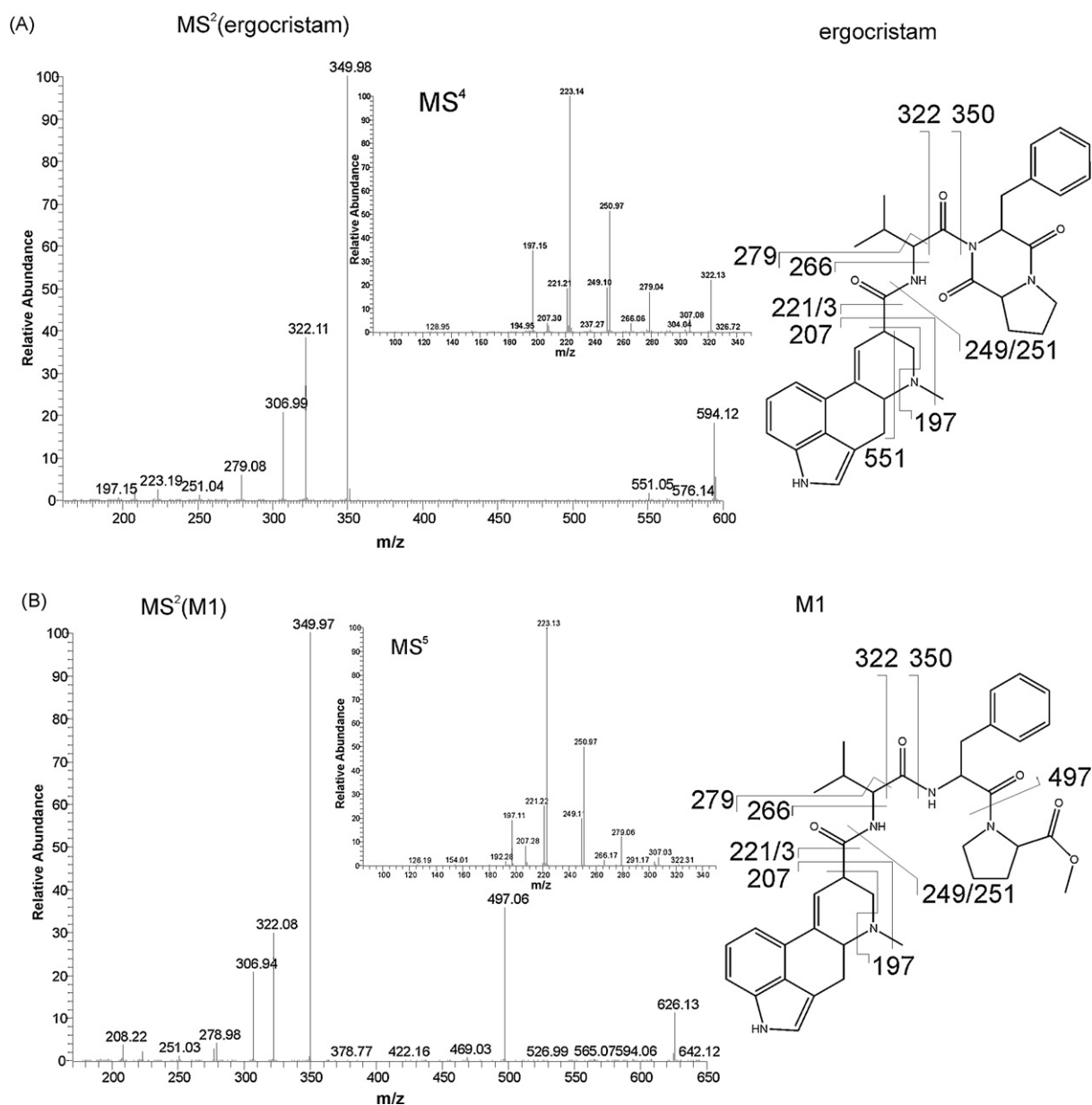

Fig. 5. The MS^2 and MS^4 (594-350-322) spectra of ergocristam (m/z 594.1 of [M+H]$^+$ ion) and the MS^2 and MS^5 (626-496-350-322) spectra of ergocristam degradation product M1 (m/z 626.2 of [M+H]$^+$ ion) with proposed fragmentations.

ditions of simultaneous ammonolysis and methanolysis could be used for quantification of original lactam alkaloid content. All experiments were performed simultaneously with ergocristine and α-ergokryptine where no degradation except formation of ergocristinine and α-ergokryptinine was observed. As an example of reaction complexity a UPLC chromatogram of all standard compounds including their C(8) epimers is given in Fig. 4.

3.2. Mass spectrometry structure determination

Compounds A1, M1 and M2 were prepared using UPLC and the three protonated molecules at m/z 367.1 (A1), 382.1 (M2) and 626.2 (M1) were detected. In the first step of structure determination of these degradation products, both MS techniques (MALDI-TOF, nanoESI-IT) were used to characterize ergocristam standard. A protonated molecule of ergocristam at m/z 594.1 was acquired and

its fragmentation was studied by detailed CID and/or PSD analysis. The detected fragments in PSD spectrum (data not shown) corresponded with previously published EI data [9]. The resulted CID mass spectra of ergocristam with fragments description are shown in Fig. 5A. The structures of the A1 and M2 degradation products of ergocristam previously described [8,16,20,21] were confirmed by collection of PSD or collision spectra. The suggested fragments of corresponding mass peaks from acquired CID fragmentation analysis are presented in Fig. 6. Comparison of CID spectra of ergocristam shown in Fig. 5A with fragmentation of both described products A1 and M2 (Fig. 6), respectively, easily indicate their structure; product A1 as D-lysergyl-L-valinamide and product M2 as methyl ester of D-lysergyl-L-valine. This finding corresponds with described results in literature [1].

The MS analysis of M1 product revealed on both MALDI-TOF MS and nanoESI-IT MS (LCQ^{DECA}) an abundant protonized ion at m/z
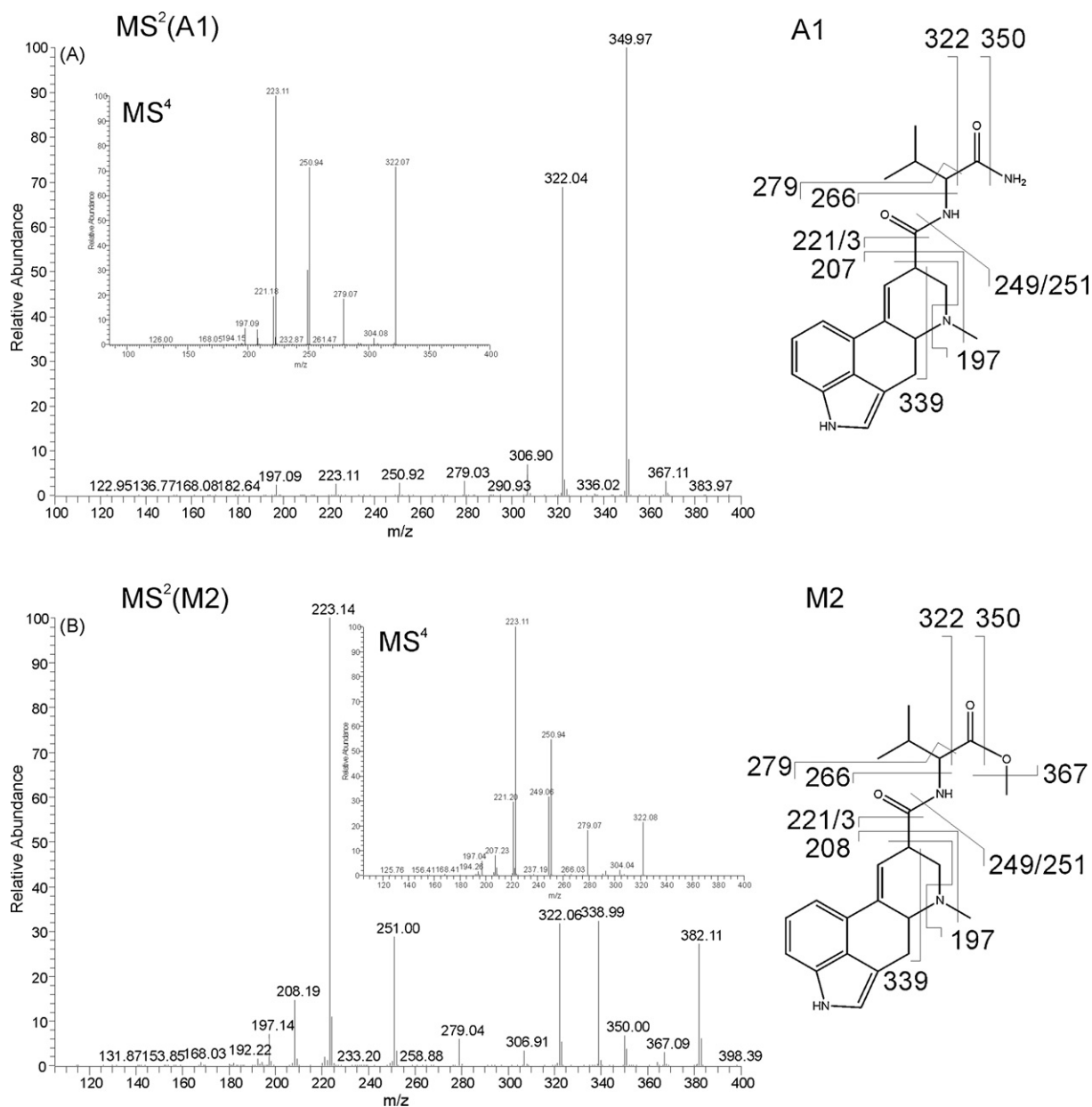

Fig. 6. The MS^2 and MS^4 (367/382-350-322) spectra of ergocristam degradation products A1 and M2 (m/z 367.1 (A1) and 382.1 (M2) of [M+H]$^+$ ions) with proposed fragmentation.
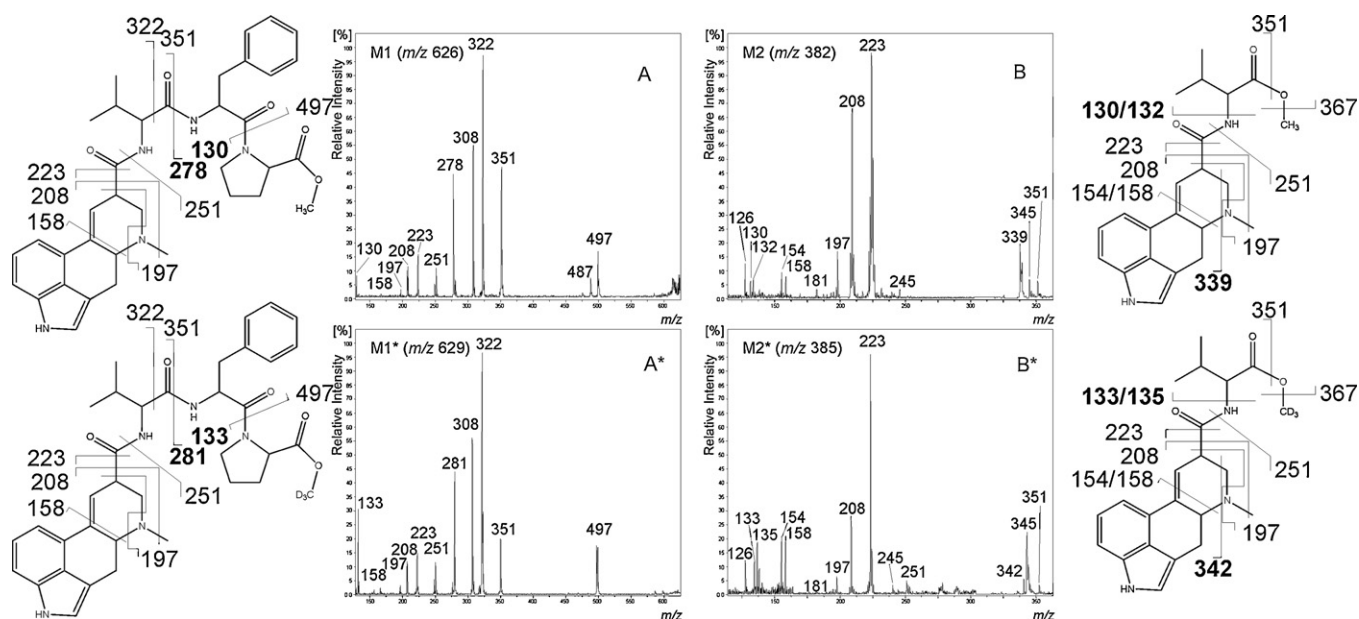
Fig. 7. The PSD spectra of ergocristam degradation products M1 (A) and M2 (B) prepared in methanol (m/z 626.1 and 382.1 of [M+H]⁺ ions) or in deuterated methanol (marked with *) (m/z 629.1 and 385.1 of [M+H]⁺ ions) with proposed fragmentations (bold means the characteristic fragment corroborated a methyl ester moiety at carboxy group).
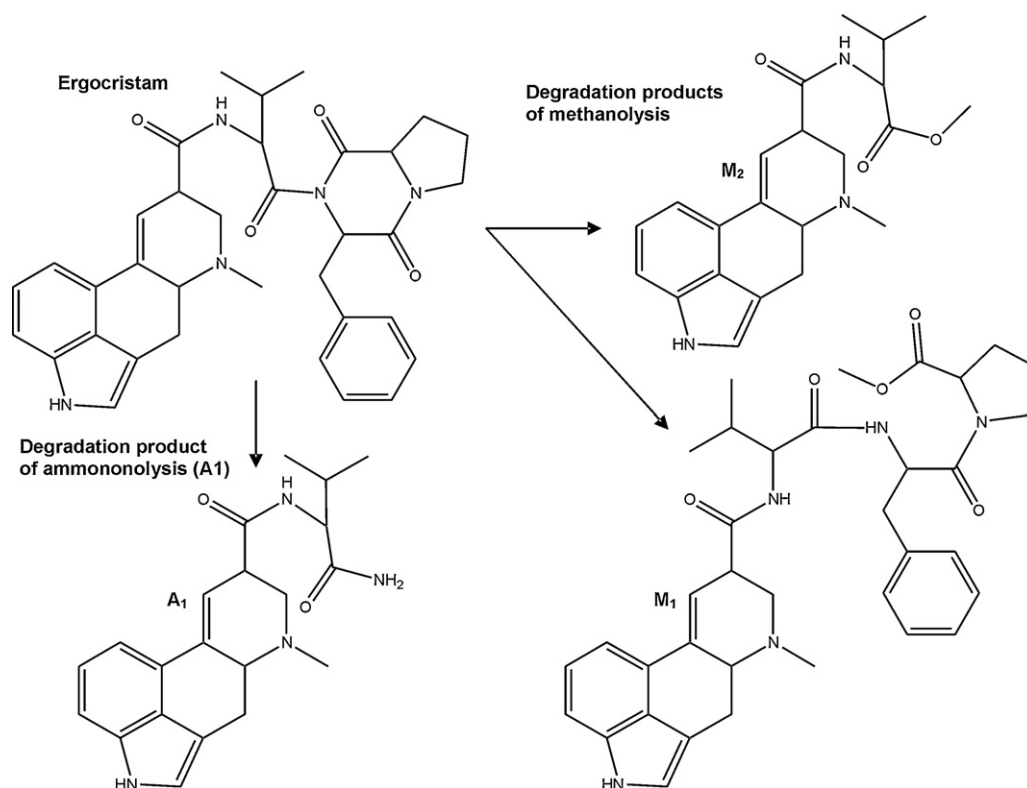

Fig. 8. A general scheme of ergocristam ammonolysis and methanolysis and characteristics of analyzed compounds.

Mark	Name	Formula	[M+H⁺]	MS/MS fragmentation (see in Fig.)
Ergocristam	N-[N-(D-lysergyl)-L-valyl]-cyclo(L-phenylalanyl-D-proline)	C35H39N5O4	594.1	Fig. 5A
M1	N-[N-(D-lysergyl)-L-valyl]-L-phenylalanyl-D-proline methyl ester	C36H43N5O5	626.2	Fig. 5B
M2	N-(D-lysergyl)-L-valine methyl ester	C22H27N3O3	382.1	Fig. 6B
A1	N-(D-lysergyl)-L-valinamid	C21H26N4O2	367.1	Fig. 6A

626.2 as previously described without any structure characterization [8]. The high resolution MS data on ESI-FT-ICR MS contained base peak at m/z 626.33422. Based on this result, the molecular formula $C_{36}H_{43}N_5O_5$ was proposed with theoretical mass 626.33367 and mass error tolerance 0.9 ppm. The final molecular structure of the analyzed substance was determined based on detailed CID fragmentation of $[M+H]^+$ ion which was found to offer good yield of fragment ions on ion trap LCQ^{DECA} (Fig. 5B). The structure of an opened lactam ring with methylated carboxyl group of ergocristam was disclosed. This proposed structure was in good agreement with Pellegrin rule determining the total number of rings and double bonds base on obtained molecular formula [22]. Both M1 degradation product of ergocristam standard and degradation product in *C. grohii* crude extract revealed the same MALDI-TOF and nanoESI-IT molecular ion, high resolution MS data, CID fragmentation, and PSD MS spectra.

Moreover, to corroborate the methyl ester moiety at carboxyl group, the re-esterification experiment and carboxypeptidase degradation of molecule M1 were performed. Both experiments revealed the methyl ester moiety at carboxyl group of opened lactam ring. The MALDI-TOF analysis revealed that carboxypeptidase did not degrade this compound due to no free carboxyl group in molecule, however, the simultaneous time dependent degradation of linear peptide with free carboxyl group (human angiotensin I) was observed (data not shown). Similarly, the esterification/re-esterification procedure with absolute ethanol resulted in mass shift of MALDI-TOF detected ion from m/z 626.2 to m/z 640.3 that corresponded to re-esterification of presented methyl ester group in molecule M1. Simultaneously the esterification of angiotensin I standard was successfully performed to verify used conditions (data not shown).

Based on all findings, the degradation procedure in 10% NH_4OH solution of deuterated methanol was performed. In correspondence with the proposed structure of opened lactam ring with methyl ester moiety at carboxyl group of ergocristam (product M1), the mass peak at m/z 626.2 has to be moved to m/z 629.3. The resulted MALDI-TOF analysis detected this shift and also the PSD analysis (Fig. 7A) confirmed our hypothesis. Moreover, the peak at m/z 382.1 (product M2) was similarly displaced by m/z 385.1 with PSD fragmentation spectrum (Fig. 7B) to verify its structure as methyl ester of D-lysergyl-L-valine. Simultaneously the esterification of angiotensin I standard was successfully performed and no transformation was observed for ergocristin used as a negative control (data not shown).

To summarize all results (tandem mass spectra, high resolution MS, carboxypeptidase degradation, re-esterification experiment and methanolysis in deuterated methanol) the structures of all ergocristam degradation products (A1, M1 and M2) are schematized in Fig. 8.

4. Discussion

An alkaloid extract of *C. grohii* (80% methanol with 0.1% NH_4OH) analyzed by UPLC method revealed besides of ergopeptines (α-ergokryptine, ergocristine) and ergopeptinines (α-ergokryptinine, ergocristinine) one alkaloid (M1) previously described as ergocristam degradation product [8]. The detailed MS/MS characterization of the M1 structure provided an idea to use this product as a diagnostic molecule for qualitative determination of original ergopeptam. The CID MS/MS spectrum offered abundant diagnostic daughter fragment at m/z 497.1 (Fig. 5B). Its difference (129 mass units) from molecular ion at m/z 626.2 clearly demonstrated the methyl ester moiety of proline residue on the end of linearized molecule of ergocristam and should be same for all ergopeptams. MS^3 spectra of the ion at m/z 497.1 and also MS^2 of ergocristam (see Fig. 5A) revealed the base peak at m/z 349.9 corresponding to loss of phenylalanine residue (147 mass units) and fitted to b-ion of peptide bond cleavage between the valine and the second variable amino-acid in the molecule of ergopeptam. In general the mass unit difference from mentioned diagnostics daughter fragment should determine residue of the second variable amino-acid (AA2) in original ergopeptam structure. Proposed diagnostic pattern of ergopeptams degradation product M1 is: M1 (molecular ion), M1-129 (lost of proline methyl ester, diagnostic daughter fragment), m/z 350 (350 = M1-129-(AA2)).

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