

MICROBIAL TRANSFORMATION OF LYCORINE BY
TALAROMYCES PINOPHILUS ISOLATED FROM THE
RHIZOSPHERE OF *LEUCOJUM AESTIVUM* L.

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Abstract

In the present study we report on the isolation of 12 strains of filamentous fungi from the rhizosphere of *Leucojum aestivum* L. (Amaryllidaceae). They were screened for ability to transform the Amaryllidaceae alkaloid lycorine. Gas chromatography-mass spectrometry was applied to analyze the samples. The *Talaromyces pinophilus* S120 strain was found to transform 19% of lycorine into its 2-oxo-derivative. To the best of our knowledge, this is the first report on transformation of lycorine by a filamentous fungus.

Key words: lycorine, *Leucojum aestivum* L., *Talaromyces pinophilus*, microbial transformation, 2-oxolycorine

Introduction. Lycorine (Fig. 1) is one of the most distributed Amaryllidaceae alkaloids. Since its discovery in 1877, lycorine and its derivatives have been drawing interest in the medicinal field due to their divergent chemical structures and strong biological effects [1]. Derivatives with a wide area of activities and prepared by different means were reported. There are some semi-synthetic

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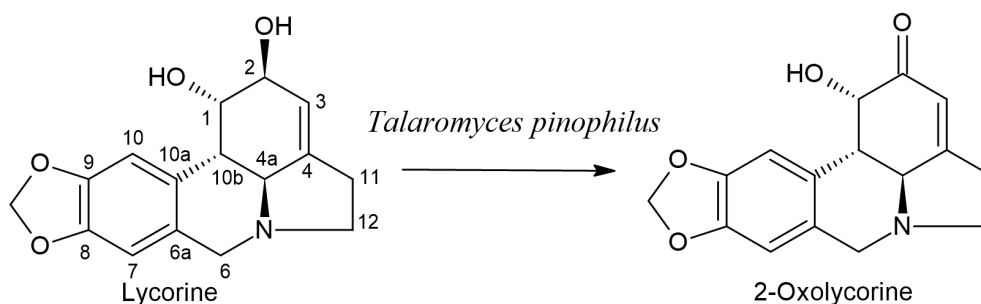


Fig. 1. Biotransformation of lycorine into 2-oxolycorine by *Talaromyces pinophilus* S120

derivatives of lycorine revealing antitumour [2] and cytochrome P450 3A4 inhibiting properties [3]. Acylated and etherified derivatives of lycorine as potential dual inhibitors of hBChE and hAChE were also reported [4].

Derivatives of lycorine were obtained via in vivo and in vitro incubations of lycorine with plasma from beagle dogs and with rat liver microsomes, respectively [5]. There are also two lycorine derivatives with antibiotic activity obtained via microbial transformation carried out by a *Pseudomonas* strain [6]. However, despite the interest in fungi as an ideal model for drug biotransformation [7] they still have not been reported for lycorine biotransformation.

Here, we present first results from the investigation of lycorine biotransformation performed by filamentous fungi isolated from the rhizosphere of *Leucojum aestivum* L., a well-known producer of lycorine.

Material and methods. Chemicals. All chemicals used in the study were of analytical grade. Lycorine was obtained from the aerial parts of the *Leucojum aestivum* L. as described by BERKOV et al. [8].

Isolation of fungal strains. The filamentous fungi were isolated from soil samples collected from the rhizosphere of *Leucojum aestivum* L. populations growing near Archar, Vidin region in Bulgaria according to the methods of soil serial dilution and soil sprinkle [9]. The isolated single colonies were transferred to new Petri dishes with fresh potato dextrose agar. The isolated fungal strains are deposited to the Mycological collection of the Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences and maintained at 4 °C.

Fungal strain identification and cultivation. The isolated fungi were identified up to species level by molecular biological methods and studied by ITS-based molecular techniques. The isolated gene DNA samples were amplified with universal primers ITS1 and ITS4 as described in our previous study [10]. The nucleotide sequences were determined using the sequencing service of Macrogen Inc. (Amsterdam, the Netherlands). The obtained sequences were checked for ambiguity, compared and analyzed through the global databases: GenBank and BLAST program, part of the resources of the National Center for Biotechnology

Information (NCBI, USA <http://www.ncbi.nlm.nih.Gov/>) and MYCOBANK Database (<http://www.mycobank.org/>). The sequences obtained in this study were deposited in the NCBI GenBank database under accession numbers from MW142512 to MW142523.

For the isolation of DNA as well as for carrying out the microbial transformation reaction fungal strains were cultured in 500 mL conical flasks containing 74 mL of potato-dextrose medium pH 5.1 and 6 mL of a standardized spore suspension. For the spore suspension fungal strains were freshly sub-cultured on sterile potato-dextrose agar and incubated at 28 °C for 7 days. The resultant spores were washed into sterile solution of Triton X-100 and adjusted to a concentration of 2×10^8 spores mL⁻¹. Flasks were incubated at 28 °C on an orbital shaker for about 60 h. Mycelium was harvested by filtration.

Microbial transformation reaction, sampling, and sample preparation. Stationary phase mycelium was harvested by filtration and washed twice with 0.05 M phosphate buffer. The transformation reaction was performed by 100 mg (wet weight) mycelium and 10 mg lycorine in 100 mL flasks containing 10 mL 0.05 M phosphate buffer pH 6.2 on an orbital water bath shaker at 28 °C within a period of 210 h.

Samples of 1 mL from the liquid medium were taken periodically, basified by a drop of 10% ammonia, and extracted with 1 mL of chloroform after addition of 50 µg of codeine as internal standard. The organic solvent was evaporated and the residue was dissolved in chloroform for gas chromatography-mass spectrometry (GC-MS) analysis. After the analysis, the solvent was evaporated and the sample dissolved in 50 µL of pyridine and 50 µL *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) purchased from Sigma-Aldrich (St. Louis, MO, USA). After heating at 80 °C for 2 h and adding of 200 µL of chloroform, the derivatized sample was analyzed again by GC-MS.

GC-MS analysis. The GC-MS spectra were recorded on a Thermo Scientific Focus GC coupled with Thermo Scientific DSQ mass detector operating in EI mode at 70 eV. A DB-5MS column (30 m × 0.25 mm × 0.25 µm) was used. The temperature programme was: 100–180 °C at 15 °C × min⁻¹, 180–300 °C at 5 °C × min⁻¹ and 10 min hold at 300 °C. The injector temperature was 250 °C. The flow rate of carrier gas (Helium) was 0.8 mL min⁻¹. The split ratio was 1:10. One microlitre of the solution was injected.

The GC-MS spectra of the compounds in the samples were deconvoluted by AMDIS 2.64 software (NIST, National Institute of Standardization and Technology, Gaithersburg, MD) before their comparison with those of the standard compound lycorine. The rate of lycorine transformation was determined as a percentage of its biotransformation product found in the samples. The latter is expressed as a percentage of the total ion current (TIC) of all alkaloids detected in the sample.

Results. Identification of the soil filamentous fungi. Altogether twelve fungal strains were isolated from the soil samples collected from the rhizosphere of *Leucojum aestivum* L. They were identified at genus level using classical taxonomy as described by BARSEGHYAN and WASSER [11] and found to belong to the genera *Penicillium*, *Aspergillus* and *Talaromyces* being the predominant one. The isolated filamentous fungi were identified to the species level and were given respective accession numbers of ITS sequences in NCBI GenBank database (Table 1).

T a b l e 1

List of the isolated fungal strains and accession numbers of ITS sequences in GenBank database

Strains	Strain number	Similarity, %	GenBank accession number
<i>Talaromyces pinophilus</i>	S 119	99	MW142512
<i>Talaromyces pinophilus</i>	S 120	99	MW142513
<i>Talaromyces pinophilus</i>	S 121	99	MW142514
<i>Talaromyces pinophilus</i>	S 122	98	MW142515
<i>Talaromyces pinophilus</i>	S 123	99	MW142516
<i>Penicillium chrysogenum</i>	S 124	99	MW142517
<i>Penicillium chrysogenum</i>	S 125	99	MW142518
<i>Aspergillus fumigatus</i>	S 126	99	MW142519
<i>Talaromyces pinophilus</i>	S 127	99	MW142520
<i>Talaromyces pinophilus</i>	S 128	99	MW142521
<i>Talaromyces muroii</i>	S 129	99	MW142522
<i>Aspergillus fumigatus</i>	S 130	99	MW142523

Microbial transformation of lycorine. All isolated filamentous fungi were found to transform lycorine in its 2-oxo-derivative as follows: eleven strains transform between 2 and 3% of lycorine, only the strain *Talaromyces pinophilus* S120 (Genbank MW142513) was found to transform 19% of lycorine within the frame of 168 h (Fig. 1). There were neither degradation products of lycorine nor of its derivative detected within the tested period.

Discussion and conclusions. Microbial transformation reactions of alkaloids are of interest as they have a potential for providing new compounds or intermediates for the synthesis of new or improved drugs [13,14]. That is why search for microorganisms capable to transform alkaloids is of primary importance. Traditionally, for carrying out microbial transformation processes are used representative of genera known for their large biochemical potential. Recently, CARMONA-VIGLIANCO et al. [15] screened 11 fungal strains from different collections belonging to genera *Aspergillus*, *Rhizopus*, *Cunninghamella* and *Fusarium* for biotransformation of the analogues of the Amaryllidaceae alkaloid norbelladine.

In our study we attempted screening of filamentous fungi capable of transformation of lycorine among those isolated from the rhizosphere of *L. aestivum* L., a well-known producer of lycorine. The rationale behind our approach was that the rhizospheric soils are among the most complex ecosystems on Earth and their microbiome utilizes nutrients and other compounds released by plants [16] thus being responsible for the biogeochemical cycling and organic substances turnover [17]. Indeed, the only investigation on lycorine biotransformation was carried out by *Pseudomonas* sp. strain ITM 311 isolated from the rhizosphere of *Sternbergia lutea* Ker Gawl (a wild *Amaryllidaceae* plant) collected during the wintering period near Bari, Italy) and it resulted in identification of some lycorine derivatives [6].

The product obtained within the transformation of lycorine by the strain *Talaromyces pinophilus* S120 was identified as 2-oxolycorine (Fig. 1). This compound was co-eluted with lycorine (Fig. 2A) and its MS spectrum was extracted and deconvoluted (Fig. 2B) with AMDIS 2.64 (NIST) software. The compound showed a molecular ion peak $[M]^+$ at m/z 285, a $[M-H]^+$ ion peak with higher intensity than the $[M]^+$, a base ion peak at m/z 266, intensive ion peaks at m/z 226 and 227, and ion peaks at the low mass region identical with those of lycorine (at m/z 65, 77, 91, 119, 135, and 147). These data strongly suggested a lycorine-like biotransformation product with 2 protons less than lycorine. There are two logical possibilities for lycorine to lose two protons, from C11 and C12 like in 11,12-didehydroanhydrolycorine [8] and oxidation of one of its hydroxyl groups. Derivatization of the sample with BSTFA resulted in better separation of the compounds in the mixture (Fig. 2C) and revealed one hydroxyl group (the $[M]^+$ of the TMS-derivative is shifted with 72 amu at m/z 357, Fig. 2D) suggesting that the other hydroxyl group of the biotransformation product is oxidized to a keto group. The hydroxyl group was assigned to C1 due to the loss of 19 amu ($[M]^+ - H - H_2O$), following the fragmentation rules for lycorine-type alkaloids as described recently by Berkov et al. [8]. The GC-MS fragmentation of 2-oxolycorine is presented in Fig. 3. Its stereochemistry was tentatively assigned as in the parent compound lycorine. The presence of the electron-withdrawing keto-group at C2 (in contrast to electron-donating hydroxyl group in lycorine) explained the easier loss of the adjacent hydroxyl group leading to the base ion. Also, the conjugated $C=C-C=O$ system led to easier loss of a H from C4a and more intensive $[M-H]^+$ than $[M]^+$ peak ion. The observed MS spectrum was congruent with those reported for 2-oxolycorine in the literature [12].

It is worth mentioning that all twelve fungal strains isolated from the rhizosphere of *L. aestivum* transformed lycorine into its 2-oxo-derivative. Among them the strain *Talaromyces pinophilus* S120 transformed 19% of lycorine into 2-oxolycorine without neither accumulation of side products nor destruction of the lycorine itself. What makes important this compound is that being designed via oxidation of the hydroxyl group at C2 position of lycorine, 2-oxo-derivative was

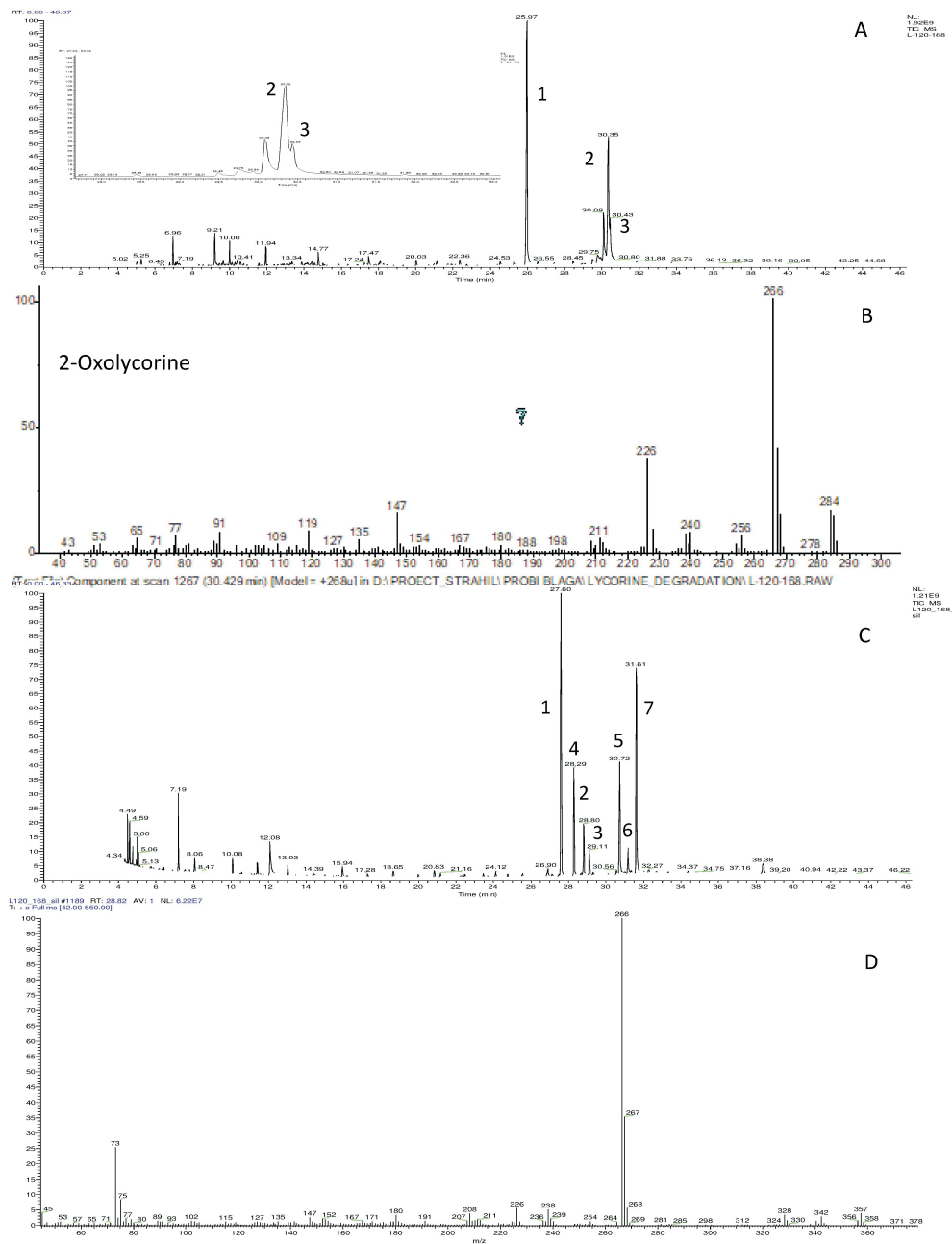


Fig. 2. GC-MS analysis of a sample from microbial biotransformation of lycorine. **A**: GC-MS chromatogram of underivatized sample (1-codeine as internal standard; 2-lycorine; 3-2-oxolycorine); **B**: MS spectrum of 2-oxolycorine; **C**: GC-MS chromatogram of TMS-derivatized sample (1-codeine-TMS as internal standard; 2-lycorine-TMS; 3-2-oxolycorine-TMS; 4-7-non-alkaloid fungal metabolites-glycerols), **D**: MS spectrum of 2-oxolycorine-TMS

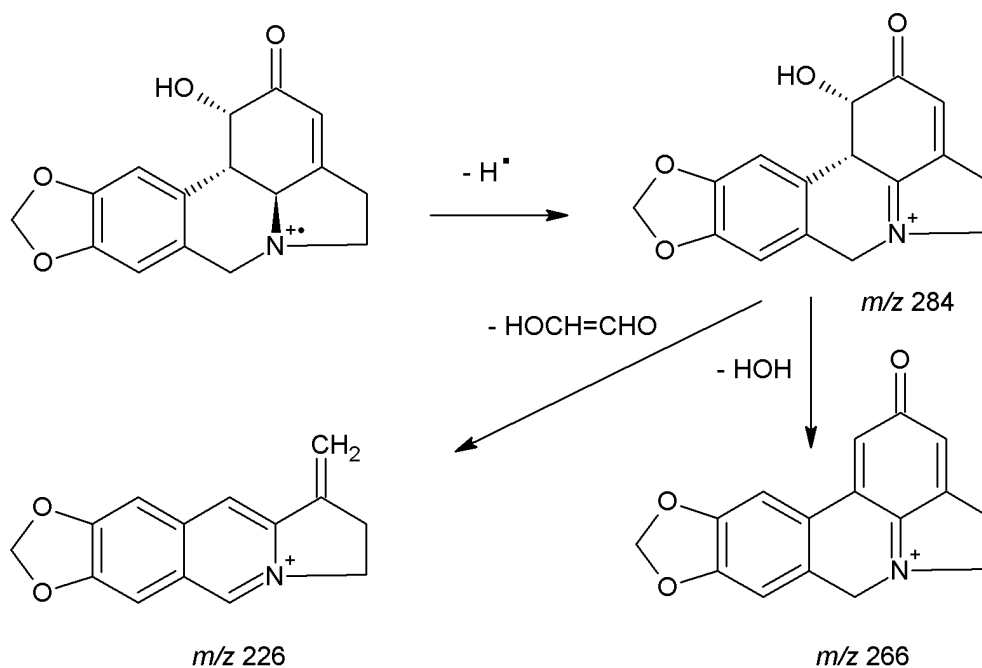


Fig. 3. Proposed electron impact mass spectral fragmentation of 2-oxolycorine

found to reveal higher anti-HCV activity compared to lycorine [18]. Thus, by proving available the microbial transformation of lycorine we suggest an alternative approach for getting 2-oxolycorine. In this context, we are inclined to speculate that further studies on microbial transformation of alkaloids carried out by filamentous fungi from the rhizospheric microbiom of alkaloid producing plants may be successfully applied as an alternative approach for obtaining valuable derivatives as well as for studying human and mammalian metabolism of lycorine (and alkaloids in general). In long term perspective microbial transformations of alkaloids have a potential to become an indispensable part of drug production schemes as it is in the case of steroid drugs manufacturing [19,20].

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