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Monasnicotinic acid, a novel pyridine alkaloid of the fungus *Aspergillus cavernicola*: isolation and structure elucidation

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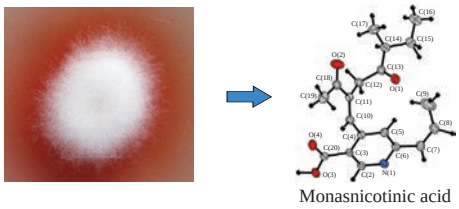
A novel pyridine alkaloid, monasnicotinic acid, was isolated from the culture liquid of the type strain of the fungus *Aspergillus amylovorus* (currently this species has been synonymized with *Aspergillus cavernicola*). The structure of monasnicotinic acid was established using mass spectrometry, multinuclear NMR spectroscopy and X-ray diffraction; its chemical properties were ascertained by ESI source H/D exchange (HDX) in negative and positive ion modes.



Monasnicotinic acid

Secondary metabolites of microscopic fungi possess a broad spectrum of antibacterial, antifungal, antitumor or lipid-lowering activities, and have already found application as drugs in medical practices.[1,2] Fungi of the genus *Aspergillus* are known to be especially capable of producing a diverse array of these compounds.[3–5]

Chemotaxonomic studies of the *Aspergillus* have shown that some species produce scarcely characterized metabolites including novel biologically active compounds. The profiles of most biosynthetic families of secondary metabolites are species-specific. However, individual secondary metabolite families can occur in other species, even those phylogenetically and ecologically unrelated to *Aspergillus*.[6]

The species *A. amylovorus* Panasenko 1964 ex Samson 1979 is attributed to the *Usti* section until 2016. In world microbial collections this species is represented by the single type strain VKM F-906 (= CBS 600.67T = ATCC 18351 = IMI 129961 = = MUCL 15648). At present, this species is synonymized with *Aspergillus cavernicola* Lörinczi 1969 and placed in the new section *Cavernicolus*.[7] The chemotaxonomic markers of this strain are phenolic polyketide asperugin, monascorubramin-like red pigments and several other metabolites of unknown structure,[8] which testifies to insufficient knowledge of the biosynthetic potential of this species. Monascorubramin is one of the pigments synthesized by the fungi genus *Monascus*. The occurrence of monascorubramin pigments in the phylogenetically different taxa like *A. cavernicola* and *Monascus sp*. allows one to regard the strain *A. cavernicola* as an interesting subject for detailed study of its secondary metabolites in order to discover novel biologically active compounds, as well as to expand knowledge in the field of fungal chemosystematics.

Here we present the determination of structure of isolated and purified new secondary metabolite of the fungus *A. cavernicola* VKM F-906, named as monasnicotinic acid.

To isolate the desired metabolite, the culture liquid filtrate of the fungus *A. cavernicola* grown on a mineral nutrient medium was extracted with chloroform.†

† The fungal strain *A. cavernicola* VKM F-906 was obtained from the fund of the All-Russian Collection of Microorganisms (VKM) of the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences. Stock culture of fungus was grown on wort agar slant for 14 days. Fermentation was carried out in shake flasks. The inoculum medium contained (g dm^{-3}): mannitol 50.0, succinic acid 5.4, $MgSO_4 \times 7H_2O$ 0.3 and KH_2PO_4 1.0; the pH was adjusted to 5.4 with NH_4OH and distilled H_2O to 1 dm^3. A 750-ml Erlenmeyer flask containing 150 ml of this medium was inoculated with aqueous conidial suspension [$(1–2) \times 10^7$ conidia ml^{-1}] of the cultures. The flasks were incubated at 24 ± 1 °C on a rotary shake at 220 rpm for 12 days. The mycelium was separated from the culture liquid by filtration through a cotton filter. The filtrate (3.9 dm^3) was acidified with a 3% solution of tartaric acid to pH 4 and extracted with $CHCl_3$ (v/v) at room temperature to afford a dark red extract. To remove the essential amount of pigments, the residue (807 mg) was subjected to the following purification. It was dissolved in $CHCl_3$ and extracted with H_2O and 25% NH_4OH (pH 9). The combined aqueous phase was acidified to pH 4 with a 3% solution of tartaric acid and extracted with $CHCl_3$. The $CHCl_3$ extract (490 mg) was subjected to silica gel column chromatography (Silicagel 60, 0.063–0.1 mm, Merck), using a gradient elution with $CHCl_3$/MeOH mixtures. The separation was monitored by TLC on silica gel plates (Silica gel F_{254}, Merck) in a $CHCl_3$–MeOH–25% NH_4OH system (80 : 20 : 0.2). The metabolite was detected by absorption of UV light (λ = 254 nm) with R_f = 0.18 and by the reaction with Ehrlich's reagent (violet staining). To obtain a highly purified material, the eluates containing target metabolite were recrystallized from MeOH.

Compound **1** was isolated as a yellowish powder (mp 168–169 °C). Its UV spectrum (MeOH) has absorption bands at λ_{max} (lg ε) = 254 (3.46) and 291 sh. (3.11) nm, what indicates the conjugation between chromophores (carbonyl, alkene and pyridyl groups) in a molecule. The similar bands have been observed earlier for pyridine derivatives, monasnicotinates A–D,[9] however, in the case of compound **1** the absorption bands are somewhat bathochromically shifted indicating the elongation of conjugation. This fact is fully confirmed by the further structure determination. The IR spectrum possesses several characteristic bands for the carbonyl group (hydrogen bonded, 1699 cm^{-1}; and 1673 cm^{-1}, conjugated with C=C), alkenyl double bonds (2925, 2968, 2880, 2853 cm^{-1}) and carboxylic acid (3407 cm^{-1}). The exact molecular mass was established by electrospray ionization (ESI) Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS) operated in positive ion mode, which gave the molecular ion, $[M+H]^+$ = 330.17007 (calc. for $C_{19}H_{24}NO_4$, *m/z*: 330.16998).

Electron impact mass spectrum of **1** revealed the pronounced fragmentation pattern. Most abundant peaks were attributed to molecular ion M^+ (*m/z* 330) and its characteristic fragments, *i.e.* $[M+H-CO_2]^+$ (*m/z* 286), $[M-Bu]^+$ (*m/z* 272), $[A = M-Bu+H-CO]^+$ (*m/z* 245) and $[M-CO_2-Et(Me)CH]^+$ (*m/z* 227), $[A-C_3H_5]^+$ (*m/z* 202). According to these data, the metabolite molecule contains *sec*-butyl, one aromatic carboxylic group and a CH=CH(Me) fragment.

According to ^{1}H NMR, **1** contains four Me groups [δ 0.84 (t, $^3J_{HH}$ 7.4 Hz), 1.06 (d, $^3J_{HH}$ 6.7 Hz), 2.09 (dd, $^3J_{HH}$ 7.4 Hz, $^4J_{HH}$ 1.7 Hz), 2.49 (s)], one CH [δ 2.58–2.53 (m)], and two CH_2 [δ 3.35 (2d, $^2J_{HH}$ 17.0 Hz), 1.71–1.65, 1.40–1.36 (2 m)]. Herewith, the protons of the second CH_2 group are diastereotopic, *i.e.* the CH_2 groups are bonded to the chiral centre, namely, *sec*-butyl group [CH(Me)Et, δ 0.84 (t, 3 H, $^3J_{HH}$ 7.4 Hz), 1.06 (d, 3 H, $^3J_{HH}$ 6.7 Hz), 1.71–1.65, 1.40–1.36 (2 m, each 1H), 2.58–2.53 (m, 1H)]. The chemical shift of the CH group [δ 2.58–2.53 (m)] indicates the bond with carbon substituents (not with heteroatoms). The signals at δ 6.49 (dd, 1H, $^3J_{HH}$ 11.9 Hz, $^4J_{HH}$ 1.7 Hz, 1H) and 6.22–6.13 (m, 1H) correspond to the (*Z*)-HC=CH fragment. Furthermore, the presence of the second multiplet clearly points to the occurrence of the terminal Me group [δ 2.09 (dd, 3 H, $^3J_{HH}$ 7.4 Hz, $^4J_{HH}$ 1.7 Hz)], which supports CH=CHMe fragment in mass spectrum. Three singlets (δ 9.32, 8.15 and 7.26) indicate separated protons bonded with sp^2-hybridized (aryl and/or alkenyl) carbon atoms. Thus, 22 protons connected with carbon atoms are unambiguously determined. The broad singlet at δ 4.93 can be identified as an acidic proton of the CO_2H group.

In ^{13}C NMR spectrum of **1**, seven signals for aliphatic C attributed to 2 CH_2 (δ 25.84 and 39.53), 4 Me (δ 35.48, 15.86, 15.49 and 11.53) and one CH (δ 48.30) groups were observed. Two signals of quaternary carbons at δ 211.99 and 198.77 can be identified as C=O carbons. In addition, there are five CH signals (δ 152.00, 140.63, 135.86, 127.86, 123.24) and five quaternary signals (δ 168.42, 160.37, 146.04, 137.15, 120.77), which correspond to aromatic or alkenyl CH= moieties. Taking into account that the molecule contains the (*Z*)-CH=CHMe fragment we conclude that the signals of this group (δ 127.86 and 123.24) are significantly shifted into the low field due to the conjugation with the electron acceptor heterocyclic core. The presence of a substituted pyridine cycle can be assumed.

Based on the data of mass spectrometry, ^{1}H and ^{13}C NMR spectroscopy, including special [DEPT and 2D (COSY, NOESY, HMBC)] experiments (for details, see Online Supplementary Materials), the disposition of some fragments determined above was performed in whole molecule of **1**. Using these data several structures may be proposed, the most probable structure of the isolated metabolite being shown in Figure 1.

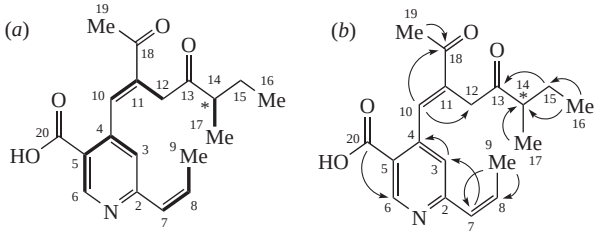


Figure 1 Chemical structure and key (*a*) ^{1}H–^{1}H COSY and (*b*) HMBC for compound **1**.

The proposed structure of metabolite **1** in solid state was ultimately confirmed by single crystal X-ray diffraction analysis (Figure 2). Single crystals were grown from a diluted MeOH solution under argon atmosphere. The crystal was chiral (space group $P2_1$). A chiral centre C^{14} (see Figure 1) is present in the molecule. Really, the isolated compound **1** can be characterized by polarimetry {$[\alpha]_D^{20}$ –15.0 (*c* 0.50, MeOH)}, and so **1** represents an enantiomerically pure compound isolated from the fungus. Unfortunately, the precise establishment of the absolute configuration of the C^{14} is not possible at this stage of work. The molecular structure of **1** described in the present work is a first example of pyridine metabolites investigated by single crystal X-ray analysis.‡

Thus, using the data obtained, the structure of **1** was determined as 4-[(1*E*)-2-acetyl-5-methyl-4-oxohept-1-en-1-yl]-6-[(1*Z*)-prop-1-en-1-yl]pyridine-3-carboxylic acid.

At the beginning of the establishment of structure of **1**, the determination of the quality of acidic and/or easily enolizable protons was performed by an ESI source H/D exchange (HDX)[10] in negative and positive ion modes (for details, see Online Supplementary Materials).

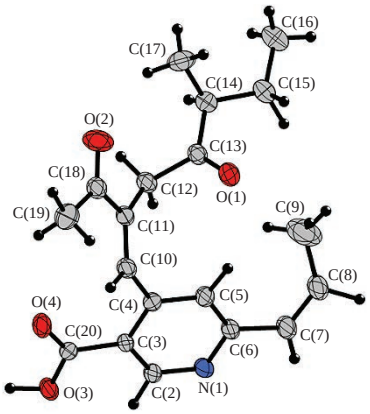


Figure 2 X-ray crystal structure of compound **1**.

‡ *Crystallographic data for* **1**: crystals of $C_{19}H_{23}NO_4$ (M = 329.38) are monoclinic, space group $P2_1$, at 293(2) K: a = 4.5664(2), b = 11.0804(5) and c = 17.9363(7) Å, β = 97.263(4)°, V = 900.25(7) Å^3, Z = 2, d_{calc} = = 1.215 g cm^{-3}, μ(MoKα) = 0.691 mm^{-1}, F(000) = 352. Total of 6082 reflections were measured and 2299 independent reflections (R_{int} = 0.0743) were used in a further refinement. The refinement converged to wR_2 = = 0.0882 and GOF = 0.848 for all independent reflections [R_1 = 0.0445 was calculated against F for 2299 observed reflections with $I > 2\sigma(I)$]. The measurements were made on a STADIVARI Pilatus diffractometer with graphite-monochromated MoKα radiation (λ = 0.71073 Å) using ω-scan mode. Absorption correction based on measurements of equivalent reflections was applied. The structures were solved by direct methods and refined by full matrix least-squares based on F^2 with anisotropic thermal parameters for all non-hydrogen atoms. All aromatic hydrogen atoms were placed in calculated positions. All H atoms were refined using a riding model.

CCDC 1545288 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre *via* http://www.ccdc.cam.ac.uk.

In the positive ion mode, we identified $C_{19}H_{24}NO_4^+$, which corresponds to protonated metabolite **1**. HDX experiment revealed two labile protons in the molecule: the first exchange took place in the protonation center; the second, in the functional group containing an acidic proton (COOH group).

In the negative ion mode, $C_{19}H_{22}NO_4^-$ was identified, which corresponds to a dissociated molecule of **1**. Surprisingly, at 200 °C on the desolvating capillary of the mass spectrometer two exchanges were observed, whereas three HDX were found at 400 °C. Since in the negative ESI mode the ionization is provided by the dissociation of a carboxyl group, we concluded that C–H bonds participating in keto-enol tautomerism undergo HDX. These reactive molecular sites are attributed to the protons situated in α-position to the carbonyl groups (C^{12}, C^{19} and C^{14}), *i.e*. groups bonded to the acceptors.[11] Using these data, we can affirm that the molecule contains three C–H acidic protons and can be regarded as a potential promising ligand for synthesis of various derivatives.

Compound **1** has a nitrogen-carbon composition and is characterized by the presence of pyridyl ring as a key framework and several functional groups (two keto groups and alkene fragment), which is similar to monasnicotinates A–D. At the same time compound **1** is significantly different from the latter [free carboxylic acid, (*Z*)-configuration of the double bond, the structure of one of the alkyl fragment C^{14}–C^{17}], which greatly complicated the structure determination. The isolated compound can be assumed to be a precursor of these alkaloids. For this reason, the metabolite was given a trivial name of monasnicotinic acid. Recently, new systems were developed for the identification of fungi, in which, along with the morphological and genetic characters, one makes use of secondary metabolites profiles.[7,8] Since monasnicotinic acid has been isolated from a typical strain of the fungus, this metabolite can be considered as a chemotaxonomic marker of the species *A. cavernicola*. Note that monasnicotinates as red azaphilone pigments are synthesized by *Monascus spp*. The presence of these two types of compounds was also observed in the case of the strain studied. Due to similarity in chemical structures of the pigments and monasnicotinates it is possible to propose the participation of common precursors in their synthesis. It is known that the acetate is an initial compound for carboxylate group in pigment biosynthesis;[12] therefore, one can assume that acetate participates in formation of the keto groups in monasnicotinates.

It is known that monasnicotinates are pharmacologically acceptable molecules for increasing the activity of peroxisome proliferator-activated gamma receptor (PPARγ) and can be used for the prevention and/or treatment of a disease related to insulin resistance, such as metabolic syndrome.[13] Using these data it is possible to assume that monasnicotinic acid also possesses antidiabetic efficiency. Pyridine alkaloids are very rare among the known natural fungal products. At the same time, these compounds attract significant attention due to their similarity with plant pyridine alkaloids, which are known to be pharmacologically active molecules. Therefore, monasnicotinic acid should be further investigated for other biological activities.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2018.01.018.

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