Folia Microbiologica (2024) 69:407–414
https://doi.org/10.1007/s12223-023-01113-4

SHORT COMMUNICATION

Biotransformation of progesterone by endophytic fungal cells immobilized on electrospun nanofibrous membrane

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Received: 14 May 2023 / Accepted: 14 November 2023 / Published online: 18 November 2023

Abstract

Biotransformation of steroids by fungi has been raised as a successful, eco-friendly, and cost-effective biotechnological alternative for chemical derivatization. Endophytic fungi live inside vegetal tissues without causing damage to the host plant, making available unique enzymes that carry out uncommon reactions. Moreover, using nanofibrous membranes as support for immobilizing fungal cells is a powerful strategy to improve their performance by enabling the combined action of adsorption and transformation processes, along with increasing the stability of the fungal cell. In the present study, we report the use of polyacrylonitrile nanofibrous membrane (PAN NFM) produced by electrospinning as supporting material for immobilizing the endophytic fungus *Penicillium citrinum* H7 aiming the biotransformation of progesterone. The PAN@H7 NFM displayed a high progesterone transformation efficiency (above 90%). The investigation of the biotransformation pathway of progesterone allowed the putative structural characterization of its main fungal metabolite by GC–MS analysis. The oxidative potential of *P. citrinum* H7 was selective for the C-17 position of the steroidal nucleus.

Keywords Biotransformation · GC–MS · Electrospinning · Endophytic fungus · Fungal immobilization · Steroids

Introduction

Despite the interesting bioactivities displayed by terpenes and steroids, such compounds are difficultly used by organic synthesis as starting materials due to their poor reactivity. Biotransformation by fungi is an efficient alternative and powerful tool to achieve selective transformation of steroidal and terpene scaffolds (Cruz de Carvalho et al. 2020; Hussain et al. 2020). Biotransformation characteristics are advantageous over transformation by traditional chemical approaches when low costs of reactants, little environmental pollution, and the high availability of many microorganisms are considered (Wenda et al. 2011). The transformations catalyzed by fungi are carried out at mild temperature, pressure, and pH that contribute to avoiding isomerization, racemization, epimerization, and rearrangement of reactants and products (Hegazy et al. 2015).

Many fungal species have shown potential for biotransforming numerous chemicals as they quickly secrete a complex set of enzymes that can act over various substrates (Purohit et al. 2018). Additionally, the use of whole fungal cells facilitates the natural regeneration of essential cofactors for biotransformation (Hawksworth 2001; Nassiri-Koopaei and Faramarzi 2015). Selective biotransformation of steroids by different fungi has attracted the efforts of our research group (Pereira dos Santos et al. 2022). Frequently, hydroxyl groups are inserted at steroid nuclei through selective biotransformation by fungi (Pereira dos Santos et al. 2020). Therefore, discovering fungi strains able to biotransform steroidal nuclei might help developing cost-effective and efficient methods to achieving new steroids. Endophytic fungi are capable of living inside vegetal tissues without

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causing any significant harm to the host plant (Arnold and Lutzoni 2007). Evidence suggests that the interaction between members of the endophytic community can play a crucial role in triggering the production of defense substances, such as metabolites and enzymes (Qi-he et al. 2009). Searching fungi from uncommon and underexploited habitats, like endophytes, may result in a successful strategy for discovering new enzymatic systems that will result in new biotransformation processes.

However, employing free fungal cells in biotransformation faces challenges that can hinder the process optimization. For instance, mechanical disturbances may disrupt mycelia growth, which may lead to operational issues such as clogging, nutrient addition, foaming, biomass aging, and microbial contamination (Mir-Tutusaus et al. 2018; Ahn et al. 2020; George et al. 2022). On the other hand, immobilizing fungi on suitable supports can be a sustainable strategy to overcome such drawbacks, as it prevents mycelial dispersion, allows for easier solid–liquid separation, and enhances the enzymatic activity of fungi (Beltrán-Flores et al. 2022; Alam et al. 2023). Electrospun nanofibrous membrane (NFM) offers several advantages as a support for immobilizing fungi, such as a large surface area, high porosity, excellent liquid permeability, and cost-effectiveness (Balusamy et al. 2019; Mercante et al. 2021).

Progesterone is a biologically active steroid biosynthesized from cholesterol, and its chemical structure has the characteristic cyclopentane-perhydrophenanthrene ring (Bexfield et al. 2019). Progesterone has been used in biotransformation studies to select fungi strains capable of catalyzing transformation at the steroidal nucleus (Virués-Segovia et al. 2023). The ability of various *Penicillium* strains to biotransform progesterone is known. For instance, the mechanism of side chain degradation of progesterone by *Penicillium lilacinum* to form testosterone, androst-4-ene-3,17-dione, and testololactone has been described (Carlström 1967). In addition, *Penicillium oxalicum* hydroxylated progesterone at C7 and C15 positions (de Paula et al. 2021).

In the present study, a polyacrylonitrile NFM produced by electrospinning was utilized as supporting material to immobilize endophytic fungal cells (*Penicillium citrinum* H7), yielding a nanohybrid platform (PAN@H7) suitable to be employed for progesterone biotransformation. The evaluation of PAN@H7 NFM biotransformation performance and the chemical characterization of metabolites from progesterone were carried out by GC–MS, revealing interesting transformation at the steroidal scaffold.

Materials and methods

Isolation and identification of the selected endophyte

The endophytic fungus was isolated from fresh leaves of *Handroanthus impetiginosus* (Mart. ex DC.) Mattos and identified, as described previously by our group (do Nascimento et al. 2020). The study with the isolated fungus was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under the code A6F76F0.

The genomic DNA of the endophytic strain was extracted with physical lysis of the mycelium using glass microspheres (425–600 µm in diameter, Sigma) (Aamir et al. 2015). The β-tubulin sequences were generated from the H7 strain, whose DNA was used as the template. Bt2a and Bt2b were used as the primers. After purification (GFX PCR DNA and Gel Band Purification Kit; GE Healthcare, Little Chalfont, UK), the sequencing was carried out in an ABI 3500XL Series (Applied Biosystems, Foster City, USA) automatic sequencing. The DNA sequences were analyzed by ClustalX (Thompson 1997) with the MEGA 6.0 program (Tamura et al. 2013). A phylogenetic tree based on the β-tubulin gene sequence was constructed using the Kimura model (Kimura 1980) and neighbor-joining (Saitou and Nei 1987) parameter modeling (1,000 bootstrap replications). The sequence was deposited in GenBank database under the accession number OQ909423. The strain was preserved in our laboratory.

Production of PAN@H7 NFM

Polyacrylonitrile (PAN, $M_w = 120{,}000$ g/mol) and *N,N*-dimethylformamide (DMF, anhydrous, 99.8%) were acquired from Sigma-Aldrich. The PAN NFM was prepared following the procedure described in previous work (Facure et al. 2022). Briefly, 500 mg of PAN was added into 500 mL of DMF and stirred for 6 h at room temperature. For the electrospinning process, the applied potential, the feed rate, and the distance between the tip of the syringe needle and the grounded collector were set (optimized conditions) at 12 kV, 0.5 mL/h, and 12 cm, respectively. The NFM was collected onto a drum collector covered with aluminum foil.

Then, the PAN@H7 membranes were prepared by adding 15.6 mg of PAN NFM and the endophytic strain in Petri dishes containing potato dextrose agar (PDA, Kasvi, Curitiba, Brazil) at 28 °C for 7 days. The fungal immobilization was confirmed by scanning electron microscopy analysis (SEM, JOEL JSM-6510).

Biotransformation assay

H7 free cells and PAN@H7 NFM were employed to investigate the biotransformation of progesterone (Sigma-Aldrich, ≥ 99%). For this, 10 disks (5 mm) containing mycelia and agar were added to 250-mL Erlenmeyer flasks containing 100 mL of a fermentative medium (pH 6.5) consisting of 0.18% glucose (Synth, São Paulo, Brazil), 0.06% peptone (Merck, Darmstadt, Germany), and 0.04% yeast extract (Acumedia, Baltimore, USA). Then, 250 µL of progesterone solution (40% m/v) in tetrahydrofuran (Synth, São Paulo, Brazil) was added to the Erlenmeyer flask. Biotransformation assays were carried out in triplicate at 28 °C for 8 days under stirring at 120 rpm (Tecnal TE-420, Piracicaba, Brazil). Appropriate control samples using progesterone-free and non-inoculated control mediums were also carried out. The culture medium's initial pH (pH = 6.5) was not changed during the biotransformation assay.

The mycelia were separated by filtration, and the fermentation broths were extracted using ethyl acetate (Synth, São Paulo, Brazil) three times. The solvent was evaporated under reduced pressure to yield crude extracts, which were analyzed by TLC and GC–MS.

Chromatographic analysis

Thin-layer chromatography (TLC) analysis was employed to monitor the biotransformation of progesterone and also to compare the extracts of biotransformation and controls. The crude extracts were dissolved in methanol and applied on TLC plates (Merck, Darmstadt, Germany). The mobile phase consisted of hexane/ethyl acetate 60:40 (v/v). After elution, the plates were observed under ultraviolet radiation (254 nm).

The crude extracts from the biotransformation assay were dissolved in ethyl acetate, filtered (PTFE; 0.45 µm), and analyzed by gas chromatography-mass spectrometry (GC–MS). GC–MS analyses were conducted on a Shimadzu GCMS-QP2010 SE (Shimadzu Corporation, Kyoto, Japan) system equipped with an AOC-20i autoinjector. The column consisted of DB-5 ms (Agilent Technologies, Palo Alto, USA) fused silica capillary (30-m length × 0.25-mm i.d. × 0.25-µm film thickness). The temperature of the column was programmed to rise from 80 to 200 °C at 40 °C/min and from 200 to 300 °C at 8 °C/min, remaining at 300 °C for 4.67 min. Helium (99.999%) was used as the carrier gas at a constant flow of 1.12 mL/min. The injection volume was 1.0 µL (splitless mode). The peak areas were expressed as the average of triplicate analysis and used to predict substrate conversion and product formation. The injector and the ion-source temperatures were set at 310 and 250 °C, respectively. Analyses were performed in SCAN mode (range from *m/z* 40 to 400). The electron impact ionization mode was used at 70 eV. The mass spectra were taken with a scanning interval of 0.3 s.

The main derivative of progesterone was identified based on computer matching with the NIST 11 spectral library, analysis of their fragmentation patterns, and comparisons with literature data.

Progesterone (**1**): EI (70 eV) *m/z* (rel. int.): 314 M + (20), 299 (5), 272 (26), 257 (2), 124 (95).

17-Methyltestosterone (**2**): EI (70 eV) *m/z* (rel. int.): 302 M + (26), 284 (5), 260 (59), 245 (11), 229 (9), 217 (8), 124 (31).

Results

Some endophytes isolated from *Handroanthus impetiginosus* leaves were assayed for biotransformation of progesterone (**1**). The endophytic strain coded as H7 has shown the best conversion rate of **1**, and it was identified by molecular technique as *Penicillium citrinum* H7. The phylogenetic tree was constructed by a neighbor-joining approach (Fig. 1), and β-tubulin sequences of the H7 strain were compared with type strains belonging to the *Penicillium* genus. The β-tubulin sequence of H7 showed 100% similarity with a sequence from *Penicillium citrinum* strain.

Next, a polyacrylonitrile nanofibrous membrane was produced by electrospinning and utilized as supporting material to immobilize endophytic fungal cells (H7), yielding a nanohybrid platform (PAN@H7) suitable to be employed for progesterone biotransformation. The biohybrid platform (H7@PAN NFM) was analyzed by scanning electron microscopy (SEM) shown in Fig. 2. Prior to the immobilization step, the PAN nanofibers (Fig. 2a) showed a smooth and bead-free surface and an average diameter of 198 ± 27 nm. Figure 2b and c revealed the presence of an extensive network between the substrate and aerial mycelia, and oval-shaped spores on the top of the polymeric NFM could be visualized. Two layers can be clearly distinguished from the cross-sectional image of PAN@H7 NFM shown in Fig. 2d, with a superior layer composed of H7 and an inferior one comprising the PAN NFM, confirming the successful fungal immobilization.

Finally, to confirm the efficacy of the nanohybrid platform for the biotransformation of steroid substrate and also to provide metabolites for chemical characterization, a biotransformation assay was carried out on progesterone (**1**) using PAN@H7 NFM. TLC experiments revealed that the PAN@H7 NMF efficiently transformed **1** into one main derivative after 4 days of incubation.

For refining our analysis, the crude extract of biotransformation of **1** by PAN@H7 NMF was analyzed by GC–MS. The total ion chromatogram (TIC) is shown in Fig. 3. As can be seen, two main peaks related to the residual substrate (**1**,

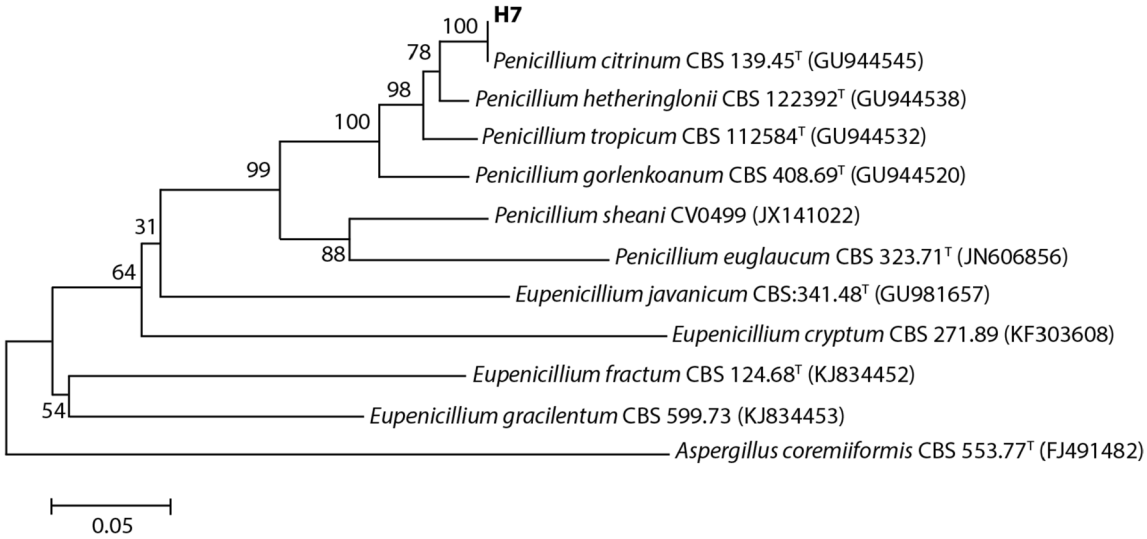


Fig. 1 Phylogenetic position of *Penicillium citrinum* H7 based on sequences of β-tubulin. The phylogenetic tree was constructed by the neighbor-joining approach with MEGA 6 software. Bootstrap analysis was performed using 1000 replications, and it is indicated at the nodes. The scale bars represent 0.05 substitutions per site

peak area 116,724 ± 812) and its main derivative (**2**, peak area 1,469,187 ± 812) were detected. Other peaks have been detected at TIC, but none of them correlate with progesterone. All other peaks are probably due to compounds from culture medium or fungal metabolism. Based on the peak areas from TIC, the biotransformation efficiency was estimated to be around 93%.

Figure 4 shows the mass spectra (EI-MS) of the substrate (**1**) and its derivative (**2**) obtained at a collision energy of

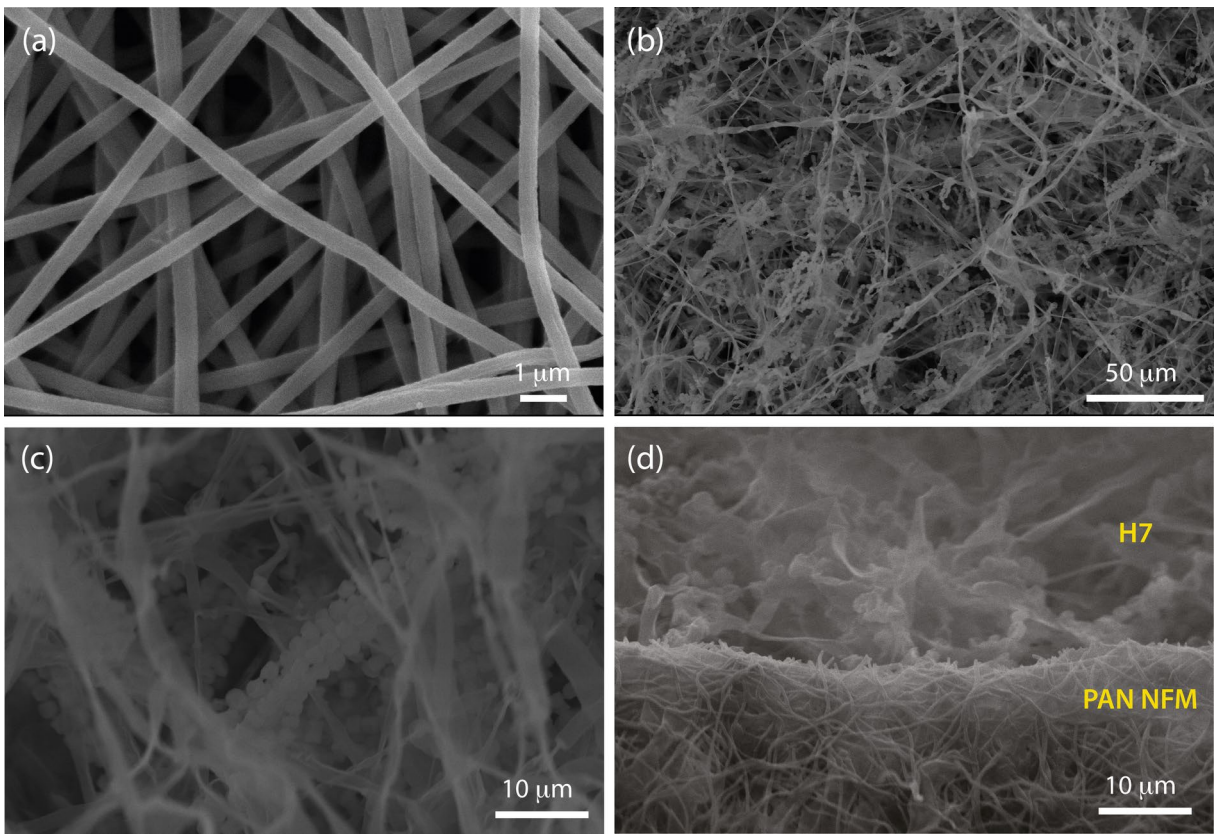


Fig. 2 SEM images of the NFMs: top view images of **a** PAN NFM and **b**, **c** PAN@H7 NFM. **d** Cross-sectional image of PAN@H7 NFM

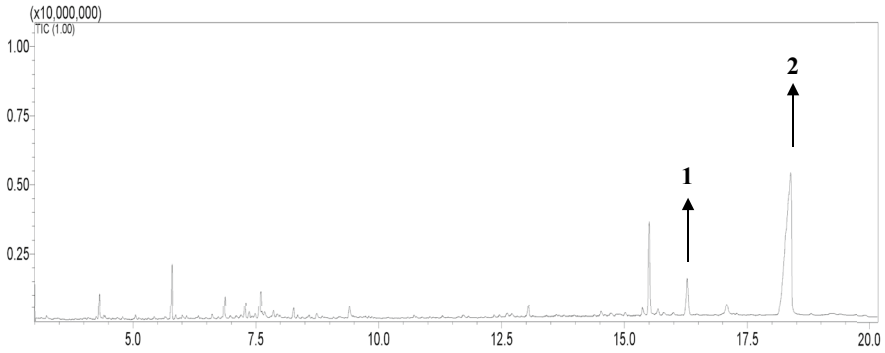
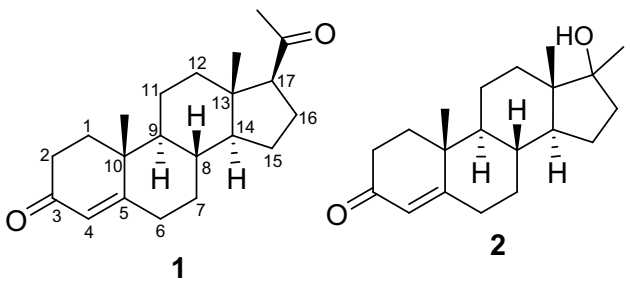


Fig. 3 Total ion chromatogram (TIC) from GC–MS analysis, SCAN mode, of the ethyl acetate extract of the biotransformation of progesterone (**1**) by PAN@H7 NMF. The derivative (**2**) was detected in the biotransformation of **1**

70 eV. Detailed analysis of EI-MS spectra provided useful structural fragmentation information that supported the putative annotation—identification level 2 (Creek et al. 2014)—of the chemical structure of **2** (Schemes 1 and 2).

Analysis of the spectrum shown in Fig. 4a allowed the proposition of fragmentation pathway and ion structures, confirming the presence of progesterone (**1**) in the biotransformation extract. The main fragmentation of the molecular ion of **1** (*m*/*z* 314) leads to the base peak at *m*/*z* 124 (A-ring fragment), which is characteristic of α,β-unsaturated 3-keto-steroids. The formation of the ion structure at *m*/*z* 124 started with an ionization at the carbonyl oxygen (C3) followed by homologous fission of the bond C9-C10 (Thevis and Schänzer 2007). Deuterium labeling at C8 has been reported to delineate fragmentation in Δ^4-3-ketosteroids and confirmed the migration of the respective hydrogen to carbon C10 (Brown and Djerassi 1980, 1981). Posterior McLafferty rearrangement led to the formation of the ion at *m*/*z* 124 (Thevis and Schänzer 2007), as depicted in the route on the right of Scheme 2. The ion formation at *m*/*z* 272 is shown in the route on the left of Scheme 2. It started with the removal of one π-electron from the α,β-unsaturated system, followed by the elimination of ketene (- 42 u), forming a 4-member ring (Thevis and Schänzer 2007). Loss of a methyl group (- 15 u) from the ion at *m*/*z* 272 gives rise to *m*/*z* 257. The ion at *m*/*z* 299 is formed after the loss of methyl radical from the molecular ion.



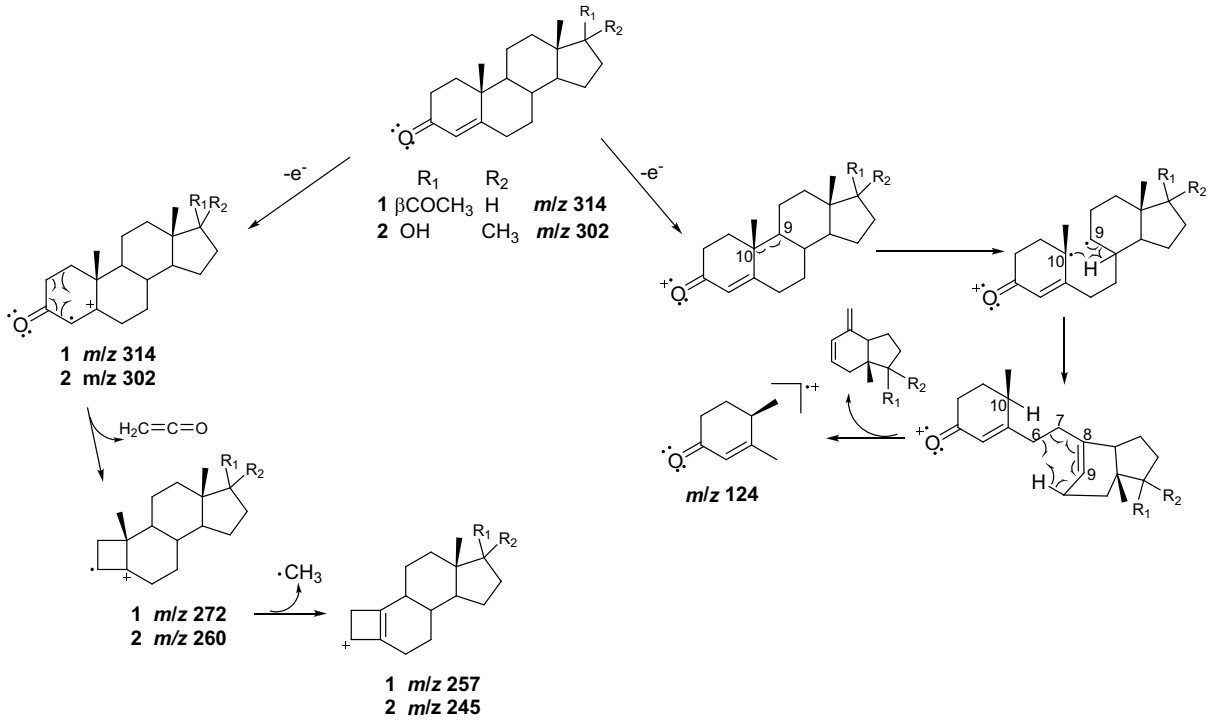
Scheme 1 GenBank database under the accession number

The chemical structure of derivative **2** (Scheme 1) was first proposed by GC–MS with NIST library match. The structural characterization was supported by the same rationale established for progesterone chemical identification. The EI-mass spectra of **2** (Fig. 4b) contained a base peak at *m*/*z* 124, like progesterone, and the fragmentation mechanism described for 3-keto-4-ene steroids (Scheme 2) confirms the presence of the ring A in **2**. The elimination of ketene (- 42 u) after the removal of one π-electron from the α,β-unsaturated system and the formation of a 4-member ring (Scheme 2) confirms the presence of rings B, C, and D at chemical structure of **2**, similar to the structure **1**. Additionally, the loss of methyl group from ion at *m*/*z* 260 explained the formation of ion *m*/*z* 245 (Scheme 2). However, the chemical structure of **2** differs from the **1** at C17. Fragment ions that contain rings B, C, and D in **1** and **2** spectra differ by 12 u. The proposition of hydroxyl and methyl groups at C17 of **2**, instead of the carboxyl group at **1**, explains the fragment ions seen at the mass spectrum of **2** and corroborates with the NIST library. Thus, derivative **2** was putatively identified as 17-methyltestosterone.

Discussion

Chemical characterization of microbial metabolites produced during biotransformation has attracted interest from biotechnologists for development of alternative derivatization processes. Fungi produce and secrete several enzymes that act by transforming xenobiotics through various chemical reactions that can be scalable under mild reaction conditions and at low cost. Since steroid biotransformation products are interesting for pharmaceutical industries, besides developing novel and efficient platforms, it is mandatory to comprehensively understand all chemical processes for future microbial biotransformation applications.

During our efforts to discover new natural biocatalysts for transforming steroidal nucleus, we selected the endophytic fungus *Penicillium citrinum* H7, which has been isolated



Scheme 2 Formation of the main ion structures observed in the EI-mass spectra of progesterone (**1**) and its derivative (**2**). Right: fragmentation route that confirms the ring A (α,β-unsaturated 3-keto) in **1** and **2**. Left: fragmentation route that confirms rings B, C, and D in **1** and **2**

from *Handroanthus impetiginosus* leaves, to carry out chemical transformation of progesterone, a biologically active steroid. Transformation by whole cell rather than isolated or immobilized enzymes is advantageous because the former allows for a great quantity of catalysts in small volumes and high turnover rates of enzymes and cofactors. To overcome drawbacks that could hinder the biotransformation by the fungus cells, a polyacrylonitrile nanofibrous membrane was produced by electrospinning and utilized as supporting material to immobilize *Penicillium citrinum* H7. PAN was chosen as immobilization matrix due to its nanofiber-forming ability and chemical stability (Yasar Mahlicli et al. 2012; Xiong et al. 2023). The nanohybrid platform (PAN@H7 NFM) was suitable to be employed for progesterone biotransformation and efficiently biotransformed it.

The crude extracts of the biotransformation of progesterone by PAN@H7 NFM was analyzed by TLC and GC–MS. The GC-EI-MS combination is an analytical technique successfully used in the putative characterization of biotransformation products, and several recent examples may be found in the current literature (Xu et al. 2022; Narayanan et al. 2022). Our mass data analysis showed that PAN@H7 NFM biotransformed almost completely progesterone and this finding may provide interesting guidance for further studies about biotransformation of progesterone by fungi. The pH monitoring revealed that the studied metabolism did not produce high amounts of acid

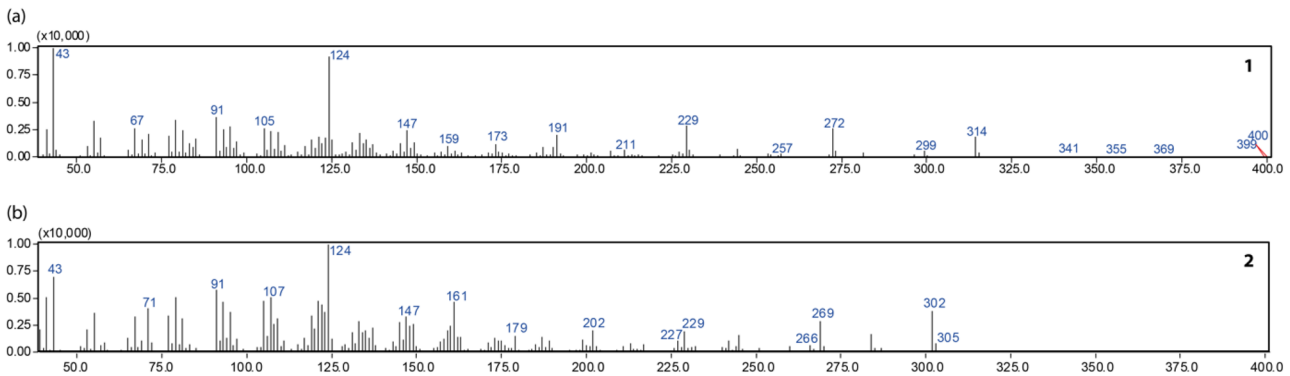


Fig. 4 EI-mass spectra (70 eV) of **a** progesterone (**1**) and **b** its derivative (**2**) obtained through biotransformation by PAN@H7 NFM

or basic compounds and the pH of the reactional medium was kept constant throughout biotransformation.

Understanding the chemistry involved in any biotransformation is mandatory for rational planning and prediction of the end products, which permit to guide its future application. Within this context, the chemical structure of main derivative obtained from biotransformation of progesterone was putatively annotated through GC-EI-MS analysis as 17-methyltestosterone (**2**). Moreover, the presence of residual substrate in the culture medium has also been confirmed through the analysis of its EI-MS. For estimating the efficiency of biotransformation as well as its selectivity, we provided a detailed analysis of all peak areas in GC chromatogram. Thus, the biotransformation process developed in the present study may be considered an efficient way to transform progesterone.

Conclusion

The endophyte *Penicillium citrinum* H7, isolated from *H. impetiginosus* leaves, was selected as a potential biocatalyst capable of biotransforming steroidal nucleus. Then, progesterone was employed as a model for mapping the biotransformation potential of fungus against the steroidal scaffold. The fungus was immobilized onto a PAN NFM to improve its transformation efficiency. The results showed that the PAN@H7 NFM displayed a great biotransformation efficiency of progesterone. The chemical structure of the main derivative achieved from biotransformation was identified based on the proposed fragmentation pathways and EI-mass data. Our results contribute to understanding the chemistry involved in the biotransformation of the steroidal scaffold, which is essential for rational planning of its derivatization, prediction of the end products, and guidance of future application of the methodology developed herein. The results of this study may impact the development of further approaches for optimizing enzymes, metabolic pathways, and organisms relevant to the efficient biotransformation of steroids.

Acknowledgements VHPS thanks the Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB) for his scholarship.

Funding This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, grant number: 445982/2020–9), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil)-código de financiamento 001, Rede Agronano (EMBRAPA), and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, grant number: 2018/22214–6).

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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