

Biotransformation of Digitoxigenin by *Cochliobolus lunatus*

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A reação de biotransformação da digitoxigenina (**1**) por *Cochliobolus lunatus* foi investigada. Foram realizados experimentos com duração de 4 dias, que resultaram no isolamento de quatro produtos, cujas estruturas químicas foram elucidadas como sendo 1 β -hidroxidigitoxigenina (**2**), 7 β -hidroxidigitoxigenina (**3**), 8 β -hidroxidigitoxigenina (**4**) e digitoxigenona (**5**). A obtenção desses produtos nas condições empregadas nunca foi anteriormente descrita. A produção da substância **4** em uma reação de biotransformação também é inédita.

The biotransformation of digitoxigenin (**1**) by *Cochliobolus lunatus* was investigated. The biotransformation reaction was carried out in a 4-day process, resulting in the isolation of four products, whose structures were elucidated as 1 β -hydroxydigitoxigenin (**2**), 7 β -hydroxydigitoxigenin (**3**), 8 β -hydroxydigitoxigenin (**4**) and digitoxigenone (**5**). The production of these derivatives under the employed conditions has never been described so far. This is also the first report on the production of compound **4** by a biotransformation reaction.

Keywords: digitoxigenin, *Cochliobolus lunatus*, 1 β -hydroxydigitoxigenin, 7 β -hydroxydigitoxigenin, 8 β -hydroxydigitoxigenin, digitoxigenone

Introduction

Enzymes are known to possess a wide substrate tolerance by keeping their exquisite catalytic properties with respect to chemo-, regio- and enantio-selectivity, playing an important role in biotransformations.¹ Biotransformation reactions can be accomplished at room temperature and in aqueous medium, presenting itself as a milder alternative to classical chemical reactions,²⁻⁴ being employed for the resolution of racemates and to introduce chiral centers in substrates, among other uses.⁵

Fungi are eukaryotic organisms that possess enzyme systems similar to those of mammals. They usually present highly flexible metabolism, thus accepting varied sources of carbon and nitrogen. The ecological relations of these organisms include the metabolism of different secondary compounds, a feature sustained by diversified

enzymatic systems, both intra and extra cellular, capable of carrying out numerous reactions.⁶⁻⁸ Such attributes suggest fungi as suitable organisms to perform biotransformation reactions.

The biotransformation of steroidal compounds by fungi has been extensively evaluated, including reactions with cardiac glycosides (Pádua *et al.*⁹ and references herein; Table 3 of the present work). Digoxin, a *Digitalis* cardenolide, is still the drug of choice for the treatment of congestive heart failure, acting as a selective inhibitor of the Na⁺,K⁺ ATPase enzyme. Biotransformation of cardenolides has been investigated either as a strategy to obtain new derivatives or to convert the A-type cardenolides into the corresponding C-type compounds, which have clinical relevance.⁹

The main reactions obtained so far for cardenolide biotransformation were hydroxylation in different positions of the steroidal skeleton, oxidation, glycosylation, epimerization and esterification of the hydroxyl

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group at C-3 (see Table 3 for references). Analysis of those data indicate that in general plant cell cultures hydroxylize position 5 β , whereas the major hydroxylation sites carried out by fungi and bacteria are 7 β and 12 β . Besides, solely plant cell cultures are capable of performing glycosylation reactions, as indicated by the reviewed literature (Table 3).

Hydroxylation reactions may be seen as a defense mechanism of fungi and are carried out by cytochrome P-450 monooxygenases found in their endoplasmatic reticulum. Difficulties in isolation and stabilization of those enzymes, associated with the necessity of recycling NAD(P)H co-factors, prevent the industrial use of isolated enzymes.^{7,10} Intact fungus organisms are therefore alternatively employed in biotransformation reactions.^{6,11}

The fungus *Cochliobolus lunatus* and its conidial anamorphous form *Curvularia lanata* are known for their capacity of hydroxylating Δ^{4-5} steroids.¹² The substrate induced 11 β -hydroxylation of steroids is a classical biotransformation reaction carried out by this fungus, which tolerates structure variation in substrates. Apart from 11 β position ("normal binding"), the major sites for *C. lunatus* hydroxylation are 14 α and 7 α resulting from inverted and reverse inverted binding of enzyme to steroid, respectively.¹³

Within this context, the main goal of the present work was to evaluate the biotransformation reaction of the cardenolide digitoxigenin by *C. lunatus* and to compare the hydroxylation sites with those previously described for other steroids.

Materials and Methods

General

¹H NMR, ¹³C NMR, ¹H-¹H COSY and HMQC spectra were recorded on a Bruker DRX-400 spectrometer (¹H 400 MHz and ¹³C 100 MHz) using TMS as internal standard for both nuclei. Chemical shifts (δ) are given in ppm and *J* couplings in Hertz (Hz). Optical rotations were measured with Perkin Elmer 341 polarimeter.

Chemicals

Acetonitrile chromatographic grade LiChrosolv and digitoxin were obtained from Merck (Germany). Water was purified using a Milli-Q⁵⁰ purification system (Millipore, USA). Digitoxigenin, $[\alpha]_D^{25} +197^\circ$ (CH₂Cl₂; c.0.14), employed in the biotransformation experiments, was obtained by hydrolysis of digitoxin as previously described by Pádua *et al.*⁹

Biotransformation experiments

The filamentous fungus *Cochliobolus lunatus* (CCT0271/ NRRL 2178) was obtained from Fundação André Tosello, Coleção de Culturas Tropicais (CCT), Campinas, SP, Brazil (<http://www.cct.org.br/sdms.cgi>). Before the biotransformation experiment, the fungus culture was incubated in malt agar 2%, pH 6.5 adjusted with 1 mol L⁻¹ NaOH, for 7 days, at room temperature.

A total of 14 erlenmeyer flasks (300 mL), each containing 100 mL of sterile medium (composition: 2.0% glucose, 0.5% peptone, 0.3% yeast extract and 0.5% KH₂PO₄, pH adjusted to 5.7), was inoculated with fresh fungal suspension. After 24 h incubation, digitoxigenin (**1**) (10 mg) was dissolved in DMF (1 mL) and added to each flask, following incubation at room temperature (24–26 °C), under stirring (200 rpm) for 4 days. The total amount of digitoxigenin (**1**) submitted to biotransformation was 140 mg. Control experiments containing medium plus substrate (C-1) and medium plus fungus culture (C-2) were carried out in each case. After removing the mycelium by filtration, the biotransformation products were sequentially extracted with chloroform (2 × 100 mL) and chloroform/2-propanol (3:1) (100 mL) in a separator funnel and the solvent was vacuum removed at 50 °C, until residue. The obtained residues were combined (296 mg) and analyzed by TLC (acetone:chloroform:dichlormethane; 50:35:15; Kieselgel 60G; 0.1 mm; 20 × 20 cm, Merck). In sequence, the combined residues were submitted to purification on a Shimadzu HPLC semi-preparative system (Japan) composed of pump SCL-8A and integrator C-R4A. Portions of the combined residues (20 mg) were dissolved in MeOH (1.0 mL) for the injection into the apparatus. An ODS column (250 × 20 mm i.d., Shimadzu, Japan) was employed at room temperature, eluted with 84% aqueous CH₃CN / H₂O (46:54), at a flow rate of 5.0 mL min⁻¹ and UV₂₂₀ detection. This procedure resulted in the isolation of compounds **2** (R_t: 20.18 min, 4.0 mg, 2.9 % yield) and **5** (R_t: 45.30 min, 5.0 mg, 3.6% yield), along with a mixture of **3** and **4**, eluted as partially superimposed bands (R_t:27.75 - 28.85 min, 11.0 mg). The mixture of **3** and **4** was further submitted to flash chromatography on a silica gel column (Pharmacia 100 × 10 mm i.d, silica gel 60, 0.040–0.063 mm, Merck), employing dichlormethane:acetonitrile (75:25) as eluent, at a flow rate of 2.3 mL/min, and resulted in the isolation of **3** (2.5 mg, 1.8% yield) and **4** (5.0 mg, 3.6% yield). All biotransformation products were isolated as colourless needles after crystallization from ACN:H₂O (9:1, compounds **2** and **5**) and CH₃OH (compounds **3** and **4**). Optical rotation was determined in CH₂Cl₂ solution, at

20 °C: compound **2** $[\alpha]_D^{20}$: +56° (c.0.04); **3** $[\alpha]_D^{20}$: +278° (c.0.195); **4** $[\alpha]_D^{20}$: +162° (c.0.36), **5** $[\alpha]_D^{20}$: +228° (c.0.195).

Results and Discussion

TLC analysis of the combined residues from digitoxigenin transformation (**1**) by *C. lunatus*, employing Kedde as spray reagent, showed spots with distinct R_f values of **1** solely for the biotransformation reaction, i.e., no product was observed for controls C1 and C2, as expected.

Chromatographic separation of the combined residues from biotransformation experiments of **1** resulted in the isolation of four compounds, along with the recovery of part of the starting material digitoxigenin (**1**). The structures of the products were defined based on spectroscopic analysis, using digitoxigenin (**1**) as model compound, and also by comparison with spectral data reported for the compounds or structurally related cardenolides.

The ¹H NMR spectrum of compound **2** showed a complex profile and the only signals easily assigned were those of H-3α (δ 4.13), H-21(δ 5.03 and 4.91), H-22

(δ 5.89), H-18 (δ 0.88) and H-19 (δ 1.09), thus confirming the presence of the C-3 hydroxyl group, the integrity of the α,β unsaturated lactone ring at C-17 and the methyl groups in the steroidal structure. The hydroxylation site was suggested by the paramagnetic shift observed for H-19 (δ 1.09) in comparison to digitoxigenin (**1**) (δ 0.96), pointing out the hydroxylation position close to C-19 (Table 1). The presence of a broad signal at δ 3.76 was also indicative of an additional hydroxyl group.

The HMQC spectrum obtained for **2** allowed determining ¹H/¹³C one-bond shift correlations of all hydrogen-bearing carbon atoms in the compound. Correlation spots between C-1 / C-2 (δ 29.6 / 27.9) and their corresponding hydrogens H-1α and H-1β (δ 1.49 and 1.49) / H-2α and H-2β (δ 1.53 and 1.53) were clearly observed in the HMQC spectrum of model compound **1**. On the other hand, those cross-peaks were absent in the equivalent region of HMQC spectrum of **2**, indicating their shift resulting from hydroxylation at C-1 or C-2.

The COSY spectrum was helpful for assigning ¹H NMR chemical shifts of **2** and also disclosed the precise site of hydroxylation. Hence, the sign of a methine hydrogen at

Table 1. ¹H NMR assignments of digitoxigenin (**1**) and its biotransformation products (**2-5**)

| Hydrogen | 1 | | 2 | | 3 | | 4 | | 5 | |
|----------|----------|--------------------------------|----------|--------------------------------|----------|--------------------------------|-------------------|--------------------------------|----------|--------------------------------|
| | δ / ppm | J / Hz | δ / ppm | J / Hz | δ / ppm | J / Hz | δ / ppm | J / Hz | δ / ppm | J / Hz |
| 1α | 1.49 | | 3.76 | $J_{1\alpha, 2\alpha} = 2.4$ | 1.51 | | 1.53 | | 1.59 | $J_{1\alpha, 2\beta} = 14.4$ |
| 1β | 1.49 | | - | | 1.48 | | 1.53 | | 1.46 | $J_{1\beta, 2\beta} = 5.5$ |
| 2α | 1.53 | | 1.74 | | 1.48 | | 1.45 | | 2.21 | |
| 2β | 1.53 | | 1.87 | $J_{2\beta, 1\alpha} = 2.4$ | 1.54 | | 1.63 | | 2.34 | $J_{2\beta, 2\alpha} = 14.4$ |
| 3α | 4.13 | | 4.13 | | 4.01 | | 4.04 | | - | |
| 4α | 1.89 | | 2.04 | | 1.82 | | 1.83 | | 2.13 | |
| 4β | 1.34 | | 1.46 | | 1.49 | | 1.35 | | 2.62 | $J_{4\beta, 4\alpha} = 14.2$ |
| 5β | 1.78 | | 2.05 | | 1.70 | | 1.75 ^a | | 1.84 | $J_{5\beta, 4\beta} = 14.2$ |
| 6α | 1.30 | | 1.36 | | 1.49 | $J_{6\alpha, 7\alpha} = 4.9$ | 2.21 | | 1.44 | |
| 6β | 1.87 | | 1.86 | | 1.90 | | 2.21 | | 1.86 | |
| 7α | 1.71 | | 1.82 | | 3.90 | $J_{7\alpha, 6\beta} = 10.6$ | 1.52 | | 1.82 | |
| 7β | 1.25 | | 1.25 | | - | | 1.38 | | 1.36 | |
| 8β | 1.56 | | 1.67 | $J_{8\beta, 9\alpha} = 12.0$ | 1.71 | $J_{8\beta, 7\alpha} = 10.4$ | - | | 1.63 | |
| 9α | 1.62 | | 1.60 | $J_{9\alpha, 11\alpha} = 12.0$ | 1.88 | | 1.82 ^a | | 1.76 | |
| 11α | 1.46 | | 1.44 | | 1.45 | | 1.33 | | 1.52 | |
| 11β | 1.46 | | 1.36 | $J_{11\beta, 9\alpha} = 4.4$ | 1.31 | | 1.70 | | 1.32 | |
| 12α | 1.40 | | 1.44 | | 1.48 | | 1.47 | | 1.47 | |
| 12β | 1.53 | | 1.50 | | 1.50 | | 1.53 | | 2.03 | |
| 15α | 2.13 | | 2.18 | | 2.24 | | 2.23 | | 2.15 | |
| 15β | 1.71 | | 1.74 | | 1.88 | | 1.80 | | 1.73 | |
| 16α | 2.16 | $J_{16\alpha, 17\alpha} = 4.8$ | 2.17 | $J_{16\alpha, 17\alpha} = 5.6$ | 2.19 | $J_{16\alpha, 17\alpha} = 5.2$ | 2.18 | $J_{16\alpha, 17\alpha} = 5.4$ | 2.18 | $J_{16\alpha, 17\alpha} = 5.4$ |
| 16β | 1.87 | | 1.87 | | 1.96 | | 1.83 | | 1.92 | |
| 17α | 2.79 | $J_{17\alpha, 16\beta} = 8.8$ | 2.82 | $J_{17\alpha, 16\beta} = 8.8$ | 2.85 | $J_{17\alpha, 16\beta} = 9.0$ | 2.83 | $J_{17\alpha, 16\beta} = 9.0$ | 2.80 | $J_{17\alpha, 16\beta} = 9.2$ |
| 18 | 0.88 | | 0.88 | | 0.90 | | 1.05 | | 0.91 | |
| 19 | 0.96 | | 1.09 | | 1.00 | | 1.11 | | 1.03 | |
| 21a | 5.00 | $J_{21a, 21b} = 18.1$ | 5.03 | $J_{21a, 21b} = 18.4$ | 5.10 | $J_{21a, 21b} = 18.3$ | 5.10 | $J_{21a, 21b} = 17.9$ | 4.99 | $J_{21a, 21b} = 18.0$ |
| 21b | 4.81 | | 4.91 | | 4.92 | | 4.93 | | 4.81 | |
| 22 | 5.87 | $J_{22, 21} = 1.3$ | 5.89 | $J_{22, 21} = 1.6$ | 5.88 | $J_{22, 21} = 1.5$ | 5.88 | $J_{22, 21} = 1.7$ | 5.90 | $J_{22, 21} = 1.6$ |

Compounds **1** and **5** were dissolved in CDCl₃ and **2**, **3**, **4** in CD₃OD. ^a Values may be interchangeable.

δ 3.76 showed cross-peaks with C-2 methylene hydrogens (δ 1.74 and 1.87), whereas C-3 methine proton (δ 4.13) showed correlation with both 2-CH₂ (δ 1.74 and 1.87) and 4-CH₂ (δ 2.04 and 1.46). These correlations clearly indicated the hydroxylation site at C-1.

Further evidence of the hydroxylation position was given by comparing carbon chemical shifts obtained for compound **2** and those previously reported for 1 β -hydroxy-17 β -H-digitoxigenin.¹⁴ The values showed close correspondence, except for the chemical shifts of C-12, C-16, C-17 and C-18, what was expected result since **2** and 1 β -hydroxy-17 β -H-digitoxigenin are epimers at C-17. Furthermore, carbon chemical shifts obtained for **2** were similar to those of digitoxigenin (**1**), with the exception of C-1 (δ 74.9), C-2 (δ 33.2), C-3 (δ 69.6), C-5 (δ 32.0) and C-19 (δ 19.6), which presented either paramagnetic or diamagnetic shifts, attributed to α -, β - and γ -effects, resulting from the hydroxylation at C-1.

The stereochemistry of the hydroxylation site was indicated by the vicinal coupling constant of H-1 (J = 2.4 Hz), which pointed out the axial position for the C-1 hydroxyl group. Based on these findings, the structure of compound **2** was defined as 1 β -hydroxydigitoxigenin, also named acovenosigenin A.¹⁵

Acovenosigenin A and its glycosides have been isolated from different plant species and also as a product of biotransformation reactions.¹⁶⁻¹⁹ NMR data previously reported showed good agreement with those of compound **2**. As a result of the small amount of **2** isolated in the present work, ¹³C NMR data were obtained indirectly by HMQC experiment. Therefore, it was not possible to attribute the resonances of non-hydrogenated carbons (Table 2).

¹³C NMR spectrum of compound **3** presented 23 signals, disclosed by DEPT-135 experiment as two methyl, nine methylene, seven methine and five non hydrogenated carbons. Compound **3** presents a methine signal at δ 71.3, not found in the spectrum of digitoxigenin (**1**), indicating that hydroxylation occurred at a methylene group. Besides, a signal with chemical shift typical of C-7 (δ 21.2) or C-11 (δ 21.3) in digitoxigenin (**1**) was absent in compound **3**. Compounds **1** and **3** present equivalent chemical shift values for C-12 (Table 2); hence, it is very unlikely that hydroxylation had occurred at C-11, since β effect would result in diamagnetic shift of C-12.

Therefore, compound **3** is the hydroxylation product of digitoxigenin at C-7, what can be confirmed by the chemical shift (δ 3.90) and coupling constants of H-7 (J = 10.6, 10.4 and 4.9 Hz), consistent with two *trans* diaxial and one axial-equatorial coupling. Altogether these data allowed identifying compound **3** as 7 β -hydroxydigitoxigenin. As

Table 2. ¹³C NMR assignments of digitoxigenin (**1**) and biotransformation products (**2-5**)

| Carbon | δ / ppm | | | | |
|--------|--------------------|----------|--------------------|--------------------|--------------------|
| | 1 | 2 | 3 | 4 | 5 |
| 1 | 29.6 | 74.9 | 30.4 | 28.9 | 39.8 |
| 2 | 27.9 | 33.2 | 28.3 | 28.9 | 37.1 |
| 3 | 66.8 | 69.6 | 67.3 | 67.6 | 212.3 |
| 4 | 33.3 | 34.6 | 35.2 | 34.3 | 42.1 |
| 5 | 36.0 ^b | 32.0 | 36.0 | 37.0 ^b | 43.6 |
| 6 | 26.5 | 27.6 | 37.7 | 23.8 | 26.5 |
| 7 | 21.2 ^c | 22.6 | 71.3 | 32.4 | 21.0 |
| 8 | 41.8 | 43.1 | 47.5 | 77.7 | 41.7 |
| 9 | 35.5 ^b | 38.9 | 38.3 | 38.0 ^b | 36.7 |
| 10 | 35.4 | n.a. | 36.3 | 36.5 | 35.2 |
| 11 | 21.3 ^c | 22.6 | 22.4 | 18.9 | 21.2 |
| 12 | 40.0 | 41.0 | 40.8 | 41.5 | 36.7 |
| 13 | 49.6 | n.a. | 50.6 | 52.0 | 49.6 |
| 14 | 85.5 | n.a. | 86.8 | 87.0 | 85.3 |
| 15 | 33.1 | 33.6 | 34.8 | 35.8 | 33.2 |
| 16 | 26.9 | 28.4 | 27.8 | 28.1 | 26.9 |
| 17 | 51.0 | 52.2 | 52.3 | 53.2 | 50.8 |
| 18 | 15.7 | 16.0 | 16.6 | 18.8 | 15.8 |
| 19 | 23.7 | 19.6 | 24.2 | 26.3 | 22.5 |
| 20 | 174.5 ^d | n.a. | 177.3 ^a | 177.2 ^a | 174.1 ^a |
| 21 | 73.4 | 75.5 | 75.4 | 75.3 | 73.4 |
| 22 | 117.6 | 118.0 | 117.8 | 117.9 | 117.9 |
| 23 | 174.4 ^d | n.a. | 178.4 ^a | 178.4 ^a | 174.3 ^a |

Compounds **1** and **5** were dissolved in CDCl₃ and **2**, **3**, **4** in CD₃OD.
^{a-d} Values may be interchangeable; n.a. = not assigned (see text for details).

expected, ¹H NMR spectrum of **3** was similar to that of digitoxigenin (**1**), apart from the diamagnetic shifts observed for H-7 α (δ 3.90), H-6 α (δ 1.49), H-6 β (δ 1.90) and H-8 β (δ 1.71), resultant from the vicinity of the 7 β -hydroxyl group (Table 1). Assignment of these hydrogens was confirmed by data of the HMQC spectrum.

Further confirmation of the hydroxylation site at C-7 was furnished by analysis of the COSY spectrum, which showed cross-peaks between H-7 α (δ 3.90) both with 6-CH₂ (δ 1.90 and 1.49) and C-8 methine proton (δ 1.71).

¹³C and DEPT-135 NMR spectra of **4** revealed two methyl, ten methylene, five methine and six non hydrogenated carbons. The signal at δ 77.7 in the spectrum of **1** disappeared in the DEPT-135 spectrum of **4**, suggesting the occurrence of hydroxylation at a methine group. Comparison of ¹³C NMR spectrum obtained for compound **4** and digitoxigenin (**1**) showed that the signal attributed to C-8 (δ 41.8) in the later, was absent in the first. Consequently, compound **4** was identified as 8 β -hydroxydigitoxigenin. As expected, ¹H NMR spectra of **4** and digitoxigenin (**1**) were similar, excluding the signals attributed to H-6 α (δ 2.21), H-6 β (δ 2.21), H-7 α (δ 1.52), H-7 β (δ 1.38), H-9 α (δ 1.75 or 1.82), H-11 α (δ 1.33), H-11 β (δ 1.70), H-18 (δ 1.05) and H-19 (δ 1.11), which showed distinct values, due to the influence of the

Table 3. Biotransformation reactions of digitoxigenin and digitoxin (Dt) carried out by cell cultures

| Cell cultures | Source | Oxidation (3β-OH→ 3-ceto) | Epimeration (3β-OH→ 3A-OH) | Hydroxylation | | | | | | Glycosylation | Isomerization (17β-17α lactone ring) | Esterification |
|---|---------------|---------------------------------|----------------------------------|---------------|----|----|-------|---------|-------|-----------------------|--|--|
| | | | | 1β | 4β | 5β | 7β | 12β | 16β | | | |
| 1. Plants | | | | | | | | | | | | |
| <i>Ammi visnaga</i> | [19] | - | - | + | - | + | - | - | - | - | - | - |
| <i>Cannabis sativa</i> | [27] | - | - | - | - | + | - | - | - | - | - | - |
| <i>Caspicum frutescens</i> | [28] | - | - | - | - | - | - | +(Dt) | - | +(Dt)(Glucose) | - | - |
| <i>Daucus carota</i> | [29-31] | - | - | - | - | + | - | - | - | - | - | - |
| <i>Digitalis cariensis ssp. trojana</i> | [27] | - | - | - | - | - | - | +(Dt) | - | +(Dt)(Glucose) | - | - |
| <i>Digitalis dubia</i> | [27] | - | - | - | - | - | - | - | - | +(Dt)(Glucose) | - | - |
| <i>Digitalis grandiflora</i> | [27] | - | - | - | - | - | - | - | - | +(Dt)(Glucose) | - | - |
| <i>Digitalis lanata</i> | [30, 32-35] | + | + | - | - | - | - | ++;(Dt) | - | +(Digitoxose/Glucose) | - | - |
| <i>Digitalis lutea ssp. lutea</i> | [27] | - | - | - | - | - | - | - | - | +(Dt)(Glucose) | - | - |
| <i>Digitalis metonensis</i> | [27] | - | - | - | - | - | - | - | - | +(Dt)(Glucose) | - | - |
| <i>Digitalis parviflora</i> | [27] | - | - | - | - | - | - | - | - | +(Dt)(Glucose) | - | - |
| <i>Digitalis purpurea</i> | [32, 36-38] | + | + | - | - | + | - | - | +(Dt) | ++; +(Dt)(Glucose) | - | - |
| <i>Ipomoea sp.</i> | [27] | - | - | - | - | + | - | - | - | - | - | - |
| <i>Nerium oleander L</i> | [39] | + | + | - | - | - | - | - | - | +(Glucose) | - | - |
| <i>Panax giseng</i> | [40] | - | + | + | - | + | - | - | - | +(Glucose/ Sorfrose) | - | +(C-3) (stearate/palmitate/ myristate/laurate) |
| <i>Pergularia tomentosa</i> | [19] | - | - | + | - | + | - | - | - | - | - | - |
| <i>Strophanthus gratus</i> | [14] | - | + | + | + | + | - | - | - | - | + | - |
| <i>Strophanthus amboensis</i> | [41] | - | + | - | - | + | - | - | - | +(Glucose) | - | - |
| <i>Strophanthus intermedius</i> | [42] | + | + | - | - | + | - | - | + | +(Glucose) | + | - |
| <i>Strophanthus divaricatus</i> | [43] | - | + | - | - | + | - | - | + | - | + | - |
| <i>Thevetia nerifolia</i> | [33] | + | - | - | - | - | - | - | - | +(Glucose) | - | - |
| 2. Fungi | | | | | | | | | | | | |
| <i>Absidia coerulea</i> ^a | [25, 44-47] | + | - | + | - | + | + | - | - | - | - | - |
| <i>Actinomyces elegans</i> ^b ATCC 6476 | [25, 48, 49] | + | - | - | - | + | + | - | - | - | - | - |
| <i>Aspergillus oryzae</i> | [50] | + | - | - | - | - | + | - | - | - | - | - |
| <i>Chaetomium globosum</i> MN-211 | [51] | - | - | - | - | - | +(Dt) | - | - | - | - | - |
| <i>Chrysosporium merdarium</i> MN-72 | [51] | - | - | - | - | - | +(Dt) | - | - | - | - | - |
| <i>Circinella muscae</i> MN-120/ ATCC 16008 | [51] | - | - | - | - | - | - | +(Dt) | - | - | - | - |
| <i>Cunninghamella blakesleeana</i> | [52, 53] | - | - | - | - | - | + | - | + | - | - | - |
| <i>Fusarium avenaceum</i> | [54*] | - | - | - | - | - | - | + | - | - | - | - |
| <i>Fusarium ciliatum</i> ^c | [9, 48] | + | - | - | - | - | - | + | - | - | - | - |
| <i>Fusarium oxysporum f. sp. Lini</i> ^d | [54, 55] | - | - | - | - | - | - | + | - | - | - | - |
| <i>Gibberella fujikuroi</i> | [52] | - | - | - | - | - | - | + | + | - | - | - |
| <i>Gibberella saubinettii</i> (Mont.) Sacc. | [56] | - | - | - | - | - | - | + | - | - | - | - |
| <i>Gongronella butleri</i> ^e | [51] | - | - | - | - | - | +(Dt) | - | - | - | - | - |
| <i>Psilocybe mexicana</i> Heim | [57] | + | - | - | - | - | + | + | - | - | - | - |
| <i>Psilocybe semperviva</i> | [57] | - | - | - | - | - | - | + | - | - | - | - |
| <i>Thammostylum piriforme</i> ^f | [52] | - | - | - | - | - | - | + | + | - | - | - |
| <i>Mortierella isabellina</i> MN-64 | [51] | - | - | - | - | - | - | +(Dt) | - | - | - | - |
| <i>Nigrospora sphaerica</i> | [48] | + | - | - | - | - | - | + | - | - | - | - |
| <i>Rhizopus oryzae</i> ^g ATCC 11145 | [25,45,50,58] | + | + | - | - | + | + | - | - | - | - | - |
| <i>Rhizopus stolonifer</i> ^h ATCC 6227b | [25] | - | - | + | - | - | + | - | - | - | - | - |
| <i>Trichocladium asperum</i> MN-37/ ATCC 16009 | [51] | - | - | - | - | - | - | +(Dt) | - | - | - | - |
| <i>Trichothecium roseum</i> Link ATCC 8685 | [50] | + | - | - | - | - | + | + | - | - | - | - |
| 3. Bacteria | | | | | | | | | | | | |
| <i>Streptomyces alboniger</i> MNG** 180 | [59] | - | - | - | - | - | +(Dt) | +(Dt) | - | - | - | - |
| <i>Streptomyces cellulosae</i> ATCC 3313 | [51] | - | - | - | - | - | +(Dt) | - | - | - | - | - |
| <i>Streptomyces diastatochromogenes</i> S-59 | [51] | - | - | - | - | - | - | +(Dt) | - | - | - | - |
| <i>Streptomyces griseoflavus</i> ATCC 12269 | [51] | - | - | - | - | - | +(Dt) | - | - | - | - | - |
| <i>Streptomyces lividans</i> | [60] | - | + | + | - | + | - | - | - | - | + | - |
| <i>Streptomyces owasiensis nov. sp</i> | [51] | - | - | - | - | - | - | +(Dt) | - | - | - | - |
| <i>Streptomyces praecox</i> MNG** 127 | [59,61] | - | - | - | - | - | +(Dt) | +(Dt) | - | - | - | - |
| <i>Streptomyces purpurascens</i> (MNG** 176-179) | [59] | - | - | - | - | - | +(Dt) | +(Dt) | - | - | - | - |
| <i>Streptomyces rimosus</i> NRRL 2234 | [51] | - | - | - | - | - | +(Dt) | - | - | - | - | - |

Notes: * results cited in literature as footnote; ** number of the Hungarian National Collection of Microorganisms (Budapest). Species formerly known as: ^a*Absidia orchidis* (Vuill.) Hagen, ^b*Mucor parasiticus*, ^c*Calonectria decora*, ^d*Fusarium lini*, ^e*Gongronella wceolifera*, ^f*Helicostylum piriforme* Bainier, ^g*Rhizopus arrhizus*, ^h*Rhizopus nigricans*.

8 β -hydroxyl group. Deshielding induced by this group imposed diamagnetic shifts to H-18 and H-19, due to 1,4 effect.²⁰

The COSY spectrum of **4** did not provide additional information on the hydroxylation site, since it occurred in a methine group. On the other hand, comparison of HMQC spectra recorded for **1** and **4** clearly indicated, in the last, the absence of a cross-peak between C-8 (δ 41.8) and H-8 β (δ 1.56) found in digitoxigenin (**1**). Comparison between NMR data assignments carried out for compound **4** and literature records for its glycoside²¹ has confirmed the structure of **4** as 8 β -hydroxydigitoxigenin, also named cerdollagenin.

The major differences between ¹³C NMR spectra of compound **5** and digitoxigenin (**1**) were the presence of a carbonyl carbon signal (δ 212.3) and the absence of the C-3 carbinol carbon signal (δ 66.8) in the first. In total, these data strongly suggest that compound **5** is digitoxigenone, a product from digitoxigenin oxidation at C-3. Aside from the diamagnetic shifts observed for C-1 (δ 39.8), C-2 (δ 37.1), C-4 (δ 42.1) and C-5 (δ 43.6) in compound **5**, as a result from deshielding effect of the carbonyl at C-3, ¹³C NMR chemical shifts observed for **5**

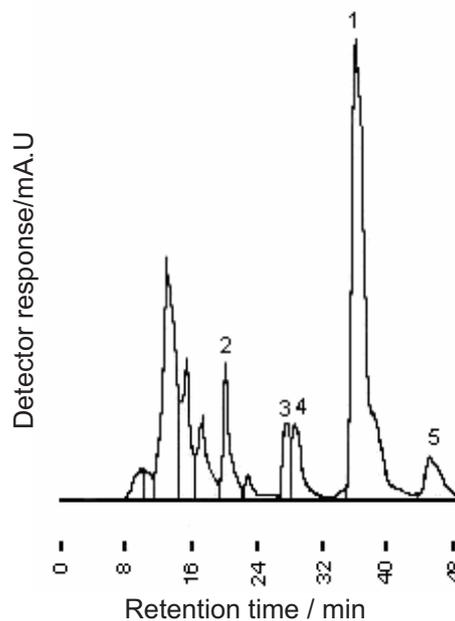


Figure 1. Semi-preparative RP-HPLC chromatogram obtained for the residue from digitoxigenin biotransformation. Identified peaks: 1 = digitoxigenin; 2 = 1 β -hydroxydigitoxigenin; 3 = 7 β -hydroxydigitoxigenin; 4 = 8 β -hydroxydigitoxigenin; 5 = digitoxigenone. Chromatographic conditions: see Materials and Methods.

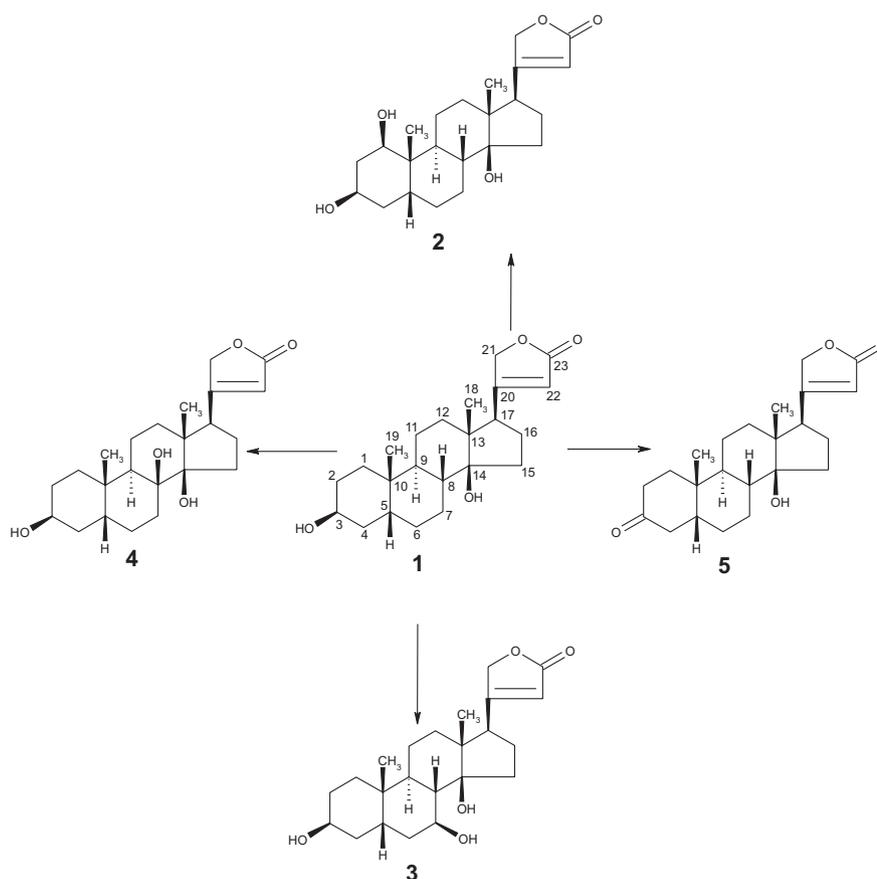


Figure 2. Chemical structures of biotransformation products from digitoxigenin (**1**) by *Cochliobolus lunatus*: 1 β -hydroxydigitoxigenin (**2**), 7 β -hydroxydigitoxigenin (**3**), 8 β -hydroxydigitoxigenin (**4**) and digitoxigenone (**5**).

and digitoxigenin were similar. Assignments of the above mentioned carbons were attested by HMQC spectrum, whereas COSY data allowed confirming the attribution of some hydrogens. Hence, H-2 β (δ 2.34 J = 14.4, 14.4 and 5.5 Hz) showed correlation spots with 1-CH₂ (δ 1.59 and 1.46) and H-2 α (δ 2.21), while H-4 β (δ 2.62 J = 14.2, and 14.2 Hz) showed cross-peaks with 5-CH (δ 1.84) and H-4 α (δ 2.13). Definitive confirmation of the structure of **5** was given by comparison with NMR data previously reported for digitoxigenone.²²

The biotransformation of digitoxigenin by *C. lunatus* is here reported for the first time and it afforded hydroxylated products at positions 1 β , 7 β and 8 β . These hydroxylation sites are distinct from those previously described for $\Delta^{4,5}$ steroids in reactions with the same fungus, that usually occur at the positions 11 β , 14 α and 7 α .^{7,23,24} Such differences may be explained by distinct enzyme/substrate interactions, arising from the uncommon configuration of digitoxigenin steroidal frame (*cis*, *trans*, *cis*), in comparison to other steroids, or due to the presence of the α,β -unsaturated lactone ring at C-17. Such hypotheses consider that the same hydroxylases do participate in reactions with digitoxigenin and other steroids. In this sense, Nozaki *et al.*²⁵ improved the 7 β hydroxylation of digitoxigenin by *Absidia coerulea*, *Rhizopus oryzae* and *Rhizopus stolonifer* after pre-incubation with progesterone and deoxycorticosterone. Such result demonstrates that monooxygenases induced by $\Delta^{4,5}$ steroids are also capable of hydroxylating digitoxigenin.

Hydroxylation of digitoxigenin at positions 1 β and 7 β , as well as oxidation of its hydroxyl group at C-3, have been previously reported for plant cell cultures and fungi (Table 3). It should be stressed, however, that this is the first report on compounds **2**, **3** and **5** as biotransformation products of digitoxigenin by *C. lunatus*. Hydroxylation at 1 β -position is of special interest considering that some glycosides of 1 β -hydroxydigitoxigenin have been reported to exhibit potent *in vitro* activity against ovarian adenocarcinoma and lung carcinoma.^{15, 16} Hence, such reaction may be employed for the future production of new bioactive cardenolide derivatives.

The 8 β -hydroxylation of digitoxigenin employing a cell culture, the fungus *C. lunatus*, is here described for the first time (Table 3). Some cardiac glycosides hydroxylated at 8 β -position have been isolated from the plant species *Nerium oleander*, *Cerbera manghas* and *Cerbera odalamm*.²¹ The 8 β -hydroxydigitoxigenin obtained in the present work is clear evidence that *C. lunatus* hydroxylases involved in the reaction are not affected by the steric hindrance of the 14 β -OH group. Therefore, it is feasible to obtain a product with two vicinal

hydroxyls at 8 β and 14 β -positions. Such reaction may present several synthetic applications in the future and can also lead to new bioactive cardenolides and steroid derivatives.

As a future perspective, the conditions for this biotransformation reaction have to be optimized to overcome the obstacles and to allow its application in large scale: the low aqueous solubility of digitoxigenin, which results in limited substrate accessibility to the biocatalyst, and toxicity of both substrate and product against fungus culture.²⁶ The use of surfactants and water-miscible or immiscible solvents is suggested by several authors as a strategy to diminish these difficulties.²⁶

Acknowledgments

This research was supported by grant CDS1203/98 from FAPEMIG (Brazil). CNPq (Brazil) is also acknowledged for research (F. C. B.) and graduate (R. M. P.) fellowships.

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Received: February 4, 2007

Web Release Date: October 8, 2007