Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 68 (2007) 834–839
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Biotransformation of betulinic and betulonic acids by fungi

Denise Z.L. Bastos [a], Ida C. Pimentel [b], Daniel A. de Jesus [a], Brás H. de Oliveira [a,*]

[a] Universidade Federal do Paraná, Departamento de Química, CP19081, 81531-970 Curitiba-PR, Brazil
[b] Universidade Federal do Paraná, Departamento de Patologia Básica, Curitiba-PR, Brazil

Received 9 August 2006; received in revised form 5 December 2006
Available online 26 January 2007

Abstract

Betulinic acid (**1**), a triterpenoid found in many plant species, has attracted attention due to its important pharmacological properties, such as anti-cancer and anti-HIV activities. The closely related, betulonic acid (**2**) also has similar properties. In order to obtain derivatives potentially useful for detailed pharmacological studies, both compounds were submitted to incubations with selected microorganisms. In this work, both were individually metabolized by the fungi *Arthrobotrys*, *Chaetophoma* and *Dematium*, isolated from the bark of *Platanus orientalis* as well as with *Colletotrichum*, obtained from corn leaves; such fungal transformations are quite rare in the scientific literature. Biotransformations with *Arthrobotrys* converted betulonic acid (**2**) into 3-oxo-7β-hydroxylup-20(29)-en-28-oic acid (**3**), 3-oxo-7β,15α-dihydroxylup-20(29)-en-28-oic acid (**4**) and 3-oxo-7β,30-dihydroxylup-20(29)-en-28-oic acid (**5**); *Colletotrichum* converted betulinic acid (**1**) into 3-oxo-15α-hydroxylup-20(29)-en-28-oic (**6**) acid whereas betulonic acid (**2**) was converted into the same product and 3-oxo-7β,15α-dihydroxylup-20(29)-en-28-oic acid (**4**); *Chaetophoma* converted betulonic acid (**2**) into 3-oxo-25-hydroxylup-20(29)-en-28-oic acid (**7**) and both *Chaetophoma* and *Dematium* converted betulinic acid (**1**) into betulonic acid (**2**). Those fungi, therefore, are useful for mild, selective oxidations of lupane substrates at positions C-3, C-7, C-15, C-25 and C-30.

Keywords: Betulinic acid; Betulonic acid; Biotransformation; Triterpene, *Arthrobotrys*; *Colletotricum*; *Chaetophoma*; *Dematium*

1. Introduction

Betulinic acid (**1**) and betulonic acid (**2**) are naturally occurring triterpenes found in many plants. For example, Betulinic acid (**1**) has been found in *Ziziphus* spp. (Su et al., 2002), *Syzygium* spp. (Chang et al., 1999), *Diospyros* spp. (Mallavadhani et al., 2004), *Paeonia* spp. (Lin et al., 1998), and *Doliocarpus* spp. (de Oliveira et al., 2002). However, one of the most widely reported sources of it is the birch tree (*Betula* spp.), where it can be found in high concentration (Yogeeswari and Sriram, 2005; Cichewicz and Kouzi, 2004). Both (**1**) and (**2**) are valuable compounds because they exhibit important biological properties. Betulinic acid (**1**) has been reported to have anti-melanoma, anti-neuroblastoma, anti-leukemia, anti-HIV, and anti-malaria properties, whereas betulonic acid (**2**) has anti-inflammatory, anti-melanoma and anti-viral activity properties (Yogeeswari and Sriram, 2005). Due to such properties, the preparation of derivatives of these compounds for structure–activity relationship studies, has attracted recent attention, with target positions being mainly the hydroxyl moiety at C-3 and the carboxyl group (Yogeeswari and Sriram, 2005; Cichewicz and Kouzi, 2004). The chemical procedures used, however, were limited to activated positions in the molecule.

In order to target positions more difficult to be functionalized by chemical methods, microbial procedures have also been used. On one occasion (Chatterjee et al., 1999), betulinic acid (**1**) was glycosylated by a species of *Cunninghamella*, and in another report (Chatterjee et al., 2000) oxidation at C-3 and hydroxylation at C-1, C-7, C-11 and C-15 by *Bacillus megaterium* was described. In yet another study, the use of *Bacillus megaterium*, *Cunninghamella elegans* and *Mucor mucedo* resulted in metabolites oxidized at

* Corresponding author. Tel.: +55 41 33613395; fax: +55 41 33613186.
E-mail address: bho@ufpr.br (B.H. de Oliveira).

0031-9422/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2006.12.007

C-1, C-3, C-6, C-7, C-11 and C-15 (Kouzi et al., 2000), whereas the methyl ester of betulinic acid (**1**) was obtained by incubation with *Nocardia* (Zhang et al., 2005). Betulonic acid (**2**) has also been subjected to biotransformation. It was transformed by *Chetomium longisrotre* to produce metabolites hydroxylated at C-7 and C-15, together with a derivative whose ring A was opened at C-3 (Akihisa et al., 2002).

Microbiological transformation of organic compounds can be useful in two ways during drug development. The derivatives may have improved properties when compared to the lead compound, or they may be identical to the metabolites of those compounds when they are administered to mammals. In this case, microbiological transformation can be a viable alternative to chemical synthesis for preparation of drug metabolites (Azerad, 1999). There are few reports on biotransformation by fungi of triterpenes in general, and of lupanes in particular. The objective of this work, therefore, was to obtain new derivatives of betulinic (**1**) and betulonic (**2**) acids, potentially useful for pharmacological studies, using the fungi *Arthrobotrys*, *Chaetophoma, Dematium*, and *Colletotrichum*.

2. Results and discussion

Betulinic acid (**1**) was isolated from the bark of *Platanus orientalis*, and betulonic acid (**2**) was prepared from it by oxidation with Jones' reagent. The spectroscopic data for both compounds were consistent with their structures and compatible with published data (Peng et al., 1998; Gonzalez et al., 1983). Both compounds were then submitted to biotransformation by fungi isolated from plants.

The use of epiphytic and endophytic microorganisms may be a useful strategy for the biotransformation of natural compounds found in the same plant source. The fungi *Arthrobotrys*, *Chaetophoma* and *Dematium* were isolated from *Platanus orientalis*, the same source of betulinic acid (**1**) used in this work, whereas *Colletotrichum* was from corn leaves (Bastos et al., 2004). *Colletotrichum* has been successfully used in the biotransformation of steroids giving more highly oxidized metabolites (Wilson et al., 1999).

Biotransformation of betulonic acid (**2**) with *Arthrobotrys* led to isolation of three metabolites. The spectroscopic data of the first two were compatible with those of the known 3-oxo-7β-hydroxylup-20(29)-en-28-oic acid (**3**) and 3-oxo-7β,15α-dihydroxylup-20(29)-en-28-oic acid (**4**), respectively (Akihisa et al., 2002). The third metabolite (**5**) had a molecular formula established as $C_{30}H_{46}O_5$ from its HRMS ($[M-H]^-$, m/z 485.3343). The ^{1}H NMR spectrum, when compared to the first metabolite, showed a new peak at 3.8 ppm integrating for one proton, indicating possible hydroxylation at a methylene carbon. This was confirmed by analysis of the ^{13}C NMR spectrum (Table 1), which showed nine methylenes (DEPT), instead of 10 for the parent compound, as well as an extra methine signal. The resonance corresponding to C-7 in the substrate (a methylene group at 33.7 ppm) was absent but another methine signal appeared instead at 72.8 ppm. The hydroxylation at C-7 was further confirmed by the paramagnetic shifts of 11.3 and 7.2 ppm of the neighboring C-6 and C-8 respectively (from to 19.7 and 40.7 to 31.0 and 47.9). The stereochemistry at C-7 was deduced from the multiplicity of corresponding C–H signal in the ^{1}H NMR spectrum (3.8 ppm). It was a double doublet ($J = 6.4$ and 15.6 Hz) compatible with an axial (α) position for that proton. The product, therefore, is 3-oxo-7β,30-dihydroxylup-20(29)-en-28-oic acid (**5**).

Table 1
^{13}C NMR spectroscopic data for 3-oxo-7β,15α-dihydroxylup-20(29)-en-28-oic acid (**5**), 3-oxo-15α-hydroxylup-20(29)-en-28-oic acid (**6**) and 3-oxo-25-hydroxylup-20(29)-en-28-oic acid (**7**) ($CDCl_3$; 100 MHz)

C	**5**	**6**	**7**
1	39.5	39.9	29.9
2	34.1	34.2	34.2
3	218.1	218.1	218.1
4	46.7	47.3	47.0
5	52.1	54.6	51.4
6	31.0	19.8	20.8
7	72.4	37.0	33.7
8	47.9	42.3	40.5
9	48.7	50.4	42.1
10	37.0	37.2	41.4
11	21.1	21.5	22.7
12	25.1	25.5	25.8
13	37.2	38.0	38.4
14	48.6	47.4	42.9
15	68.9	70.9	29.4
16	41.5	42.3	32.5
17	54.6	55.2	56.4
18	49.7	48.9	49.3
19	46.5	46.5	47.0
20	149.9	149.8	150.9
21	29.7	31.0	30.7
22	36.8	36.9	37.3
23	26.4	26.6	27.3
24	21.1	21.0	20.4
25	15.5	16.0	60.4
26	10.7	16.3	16.2
27	8.3	8.2	14.6
28	180.2	180.5	179.0
29	110.0	110.1	109.5
30	19.5	19.5	19.4

The fungus *Colletotrichum* was used for biotransformation of betulinic acid (**1**) to produce a more polar compound as determined by TLC. Its molecular formula was established as $C_{30}H_{46}O_4$ from its HRMS ($[M-H]^-$, m/z 469.3380). Its IR spectrum showed the expected carboxyl carbonyl absorption at 1694 cm^{-1} and also a new carbonyl absorption at 1706 cm^{-1}, indicating the formation of a ketone functionality, which was absent in the parent compound. The ^{1}H NMR spectrum of the metabolite was similar to that of the parent compound showing characteristic signals corresponding to six methyl groups. However, it lacked a resonance for H-3 indicating oxidation of the corresponding secondary alcohol to a ketone. Moreover, a new signal appeared at 4.05 ppm integrating for one pro-

ton, indicating possible hydroxylation at a methylene carbon. Those differences were also noted in the ^{13}C NMR spectrum (Table 1), where the resonance corresponding to C-3 of betulinic acid (**1**) (a methylene at 78.2 ppm) was absent and a new methine signal appeared at 70.9 ppm. The resonances of C-14 and C-16 were also subject to paramagnetic shifts from 42.6 and 32.4 to 47.2 and 42.3, respectively, indicating hydroxylation had occurred at C-15. An HMQC experiment established a connection between C-15 and H-15 (δ_C: 70.9 and δ_H: 4.05). Further evidence was provided by the signal of the methyl group (C-27). The γ effect provided by the hydroxyl at C-15 produced a shift from 14.8 ppm in betulinic acid (**1**) to 8.2 ppm in the metabolite. The stereochemistry at C-15 was inferred from the multiplicity of the corresponding resonance in the ^1H NMR spectrum (4.05 ppm). It was a double doublet ($J = 4.8$ Hz and 11.2 Hz) indicating an axial position for H-15, which otherwise would be a broad singlet. The equatorial (α) orientation for the hydroxyl is, therefore, the most likely, and the product is 3-oxo-15α-hydroxylup-20(29)-en-28-oic acid (**6**).

The biotransformation of betulonic acid (**2**) by *Colletotrichum* resulted in formation of two more polar compounds as determined by TLC. The first was identical to that of **6** produced from biotransformation of betulinic acid (**1**) by the same fungus. The second metabolite was more polar than the first, as determined by TLC. The spectroscopic data were, however, identical to that of **5** obtained from the biotransformation of betulonic acid (**2**) by *Arthrobotrys* as described above. It has also been described previously as a result of biotransformation of betulonic acid (**2**) by *Chetomium longirostre* (Akihisa et al., 2002).

The biotransformation of betulonic acid (**2**) by *Chaetophoma* resulted in formation of a more polar compound as determined by TLC. Its molecular formula was established as $C_{30}H_{46}O_4$ from its HRMS ($[M-H]^-$, m/z 469.3380). Its ^1H NMR spectrum, when compared to that of the substrate, showed five methyl signals, instead of six. The signal of C-25 was missing and a new methylenic group appeared at 4.10 ppm. Hydroxylation at C-25 was confirmed by ^{13}C NMR spectroscopic analysis (Table 1), which showed a resonance at 60.4 ppm, and shifts on the absorptions of the neighboring C-1, C-5 and C-9 (from 39.7, 55.0 and 49.9 to 29.9, 51.4 and 42.1, respectively). The product, therefore, is 3-oxo-25-hydroxylup-20(29)-en-28-oic acid (**7**).

The biotransformation of betulinic acid (**1**) by *Chaetophoma* and by *Dematium* resulted in the formation of betulonic acid (**2**). The same transformation was previously reported by the use of *Bacillus megaterium* (Kouzi et al., 2000). It is interesting to note the similarity of this 3-OH oxidation reaction to that of cholestanol compounds into cholestanones, catalyzed by microbial cholesterol oxidases (Motteran et al., 2001). Similar enzymes might also be present in plants because betulinic (**1**) and betulonic (**2**) acids have been found together in plants (Chiang et al., 2005).

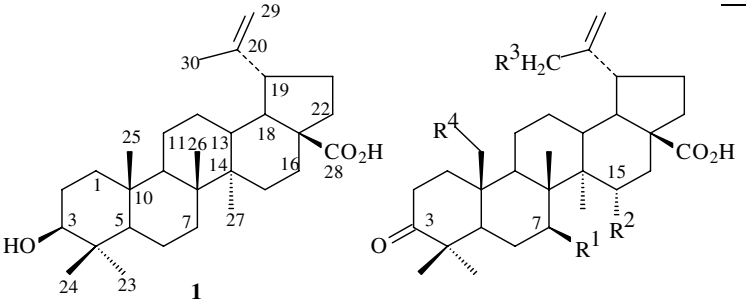
3. Concluding remarks

The results obtained with *Arthrobotrys*, *Chaetophoma* and *Dematium* showed that the use of microorganisms isolated from the same source as the substrate increase the likelihood of obtaining derivatives using this approach. The oxidation reactions with *Choletotrichum*, however, were similar to those obtained by biotransformation of steroids by the same fungus genus (Wilson et al., 1999) thus showing that its oxidases and monooxygenases have broad substrate specificity towards their polynuclear terpenes. The use of the fungi described in this work is, therefore, a viable procedure for obtaining derivatives of betulinic (**1**) and betulonic (**2**) acids. The derivatives obtained result from oxidation reactions, which are of normal occurrence during mammalian metabolism. Those compounds could potentially be useful in the study of the metabolism of betulinic (**1**) and betulonic (**2**) acids in mammals.

4. Experimental

4.1. General experimental details

Mps were determined in a Quimis apparatus, model Q340523. Optical rotations were measured using a Bellingham Stanley apparatus, model D, whereas IR spectra were recorded in KBr discs using a Bio-Rad spectrometer model FTS3500GX. NMR spectra were acquired with Bruker spectrometer models Avance DRX400 and Avance DPX 200 using TMS as internal standard; 1D and 2D experiments (DEPT, 1H-1H COSY, HMQC, HMBC, NOESY) were carried out for structure determinations. Mass spectra



1

(**2**) $R^1 = R^2 = R^3 = R^4 = H$
(**3**) $R^1 = OH$, $R^2 = R^3 = R^4 = H$
(**4**) $R^1 = R^2 = OH$, $R^3 = R^4 = H$
(**5**) $R^1 = R^3 = OH$, $R^2 = R^4 = H$
(**6**) $R^1 = R^3 = R^4 = H$, $R^2 = OH$
(**7**) $R^1 = R^2 = R^3 = H$, $R^4 = OH$

were recorded in an API 3000 System from Applied Biosystems/MDS Sciex, whereas high resolution mass spectra employed a Micromass Q-Tof from Waters. Solvents were distilled before use. TLC was performed on 0.2 mm thick silica prepared plates from Merck (art. # 5554). Spot visualizations were made by spraying with H_2SO_4/EtOH (1:1, v/v) followed by heating and inspection under UV light. Vacuum liquid CC was performed with silica Si-60 from Merck (art. # 7744), using appropriate mixtures of hexane/EtOAc. Planar centrifugal chromatography was performed with a Chromatotron (Harrison Research, Palo Alto, CA, USA) model 7924T. CC was performed on silica from Merck (art. # 7736) whereas preparative HPLC was carried out using a Rainin system with a pump (model SD-200), pump head 50 (50 ml/min part # R007101065), UV detector (UV-1) and Dynamax C_{18} column (21.4 × 250 mm, 8 μm, art # R00083221C). Flow rate used was 20 ml/min and eluate monitored at 210 nm.

4.2. Isolation of betulinic acid (*1*)

The bark of *Platanus orientalis* was collected in Curitiba (Brasil), with a voucher specimen deposited at the herbarium of UFPR (#47812). The dried pulverized bark (100.0 g) was continuously extracted with CH_2Cl_2 in a Soxhlet apparatus for 3 h. After solvent evaporation, a portion of the extract (4.0 g) was submitted to vacuum liquid CC on silica (80.0 g), with elution using hexane/EtOAc mixtures. A solid was obtained (0.69 g), which was characterized as betulinic acid (**1**) by spectroscopic methods and comparison with literature data (Peng et al., 1998).

4.3. Preparation of betulonic acid (*2*)

Betulinic acid (**1**) (1.0 g, 2.26 mmol) was dissolved in acetone (50 ml) and the mixture was cooled in an ice bath. Jones' reagent was then added slowly (10 ml), with stirring, and the reaction was allowed to proceed for 1.5 h. Then MeOH (25 ml) was added, followed by H_2O (40 ml). The solvents were evaporated under reduced pressure, and the aqueous residue was extracted with EtOAc (2 × 40 ml). The organic phase was dried, filtered and the solvent evaporated. The solid residue was then subjected to silica CC affording a solid, which was characterized as betulonic acid (**2**) by spectroscopic methods and comparison with literature data (Gonzalez et al., 1983).

4.4. Fungi

Isolation and identification of fungi are described elsewhere (Bastos et al., 2004). They were deposited at the culture collection of the Department of Basic Pathology of Universidade Federal do Paraná: *Arthrobotrys* (DPB134) *Colletotrichum* (DPB136), *Chaetophoma* (DPB125) and *Dematium* (DPB157).

4.5. General biotransformation procedure

Stock cultures of fungi were stored on potato dextrose agar slants, at 4 °C. Seed cultures of each fungus were obtained by transferring fungi from stock cultures to a liquid medium containing (g l^{-1}) glucose (10), corn steep liquor (8) and yeast extract (2). The experiments were conducted in conical flasks (1 l), containing the same liquid medium (200 ml) inoculated with the fungi. The flasks were shaken at 30 °C and 150 rpm for 48 h. The substrates, previously dissolved in a minimum volume (~2 ml) of acetone (betulonic acid, **2**) or DMSO (betulinic acid, **1**), were evenly distributed among the flasks, except one, kept as a control, and the reaction was allowed to proceed for 7 more days. The mycelium was then removed by filtration, and the biomass was extracted with EtOAc (100 ml), for 30 min, in an ultrasonic bath and filtered again. The broth was extracted with the same solvent (3 × 100 ml) and all extracts were combined and dried (anhydr. Na_2SO_4). After filtration, the solvents were evaporated under reduced pressure, and the crude extract was fractionated by chromatography. Controls were carried out in order to verify the action of the medium (without fungus) on the substrates and the presence of similar metabolites on the fungi cultures (without substrates).

4.6. Biotransformation of betulonic acid (*2*) by Arthrobotrys

The crude extract obtained from the biotransformation of **2** (1.280 g) was fractionated by CC on silica. Elution with hexane–EtOAc (2:1 v/v) led to the isolation of metabolites **3** (21 mg, 1.64% yield), **4** (8 mg, 0.62% yield) and **5** (17 mg, 1.33% yield).

4.6.1. 3-Oxo-7β-hydroxylup-20(29)-en-28-oic acid (*3*)

M.p. 165–168 °C; $[\alpha]_D^{25}$ + 17 (MeOH; c0.6); HRMS *m/z*: 469.3380 $[M-H]^-$ (calcd for $C_{30}H_{46}O_4$: 470.3396). ESI-MS −45 eV, *m/z* (rel. int.) 469.4 $[M-H]^-$ (100), 451 $[M-H-H_2O]^-$ (2.0), 424 $[M-H-COOH]^-$ (8.0), 407 $[M-H-OH-COOH]^+$ (2.0), 411 $[M-H-OH-C_3H_5O]^-$ (5.0); IR ν_{max}/cm^{-1}: 3438, 2956, 1694. ^1H NMR (400 MHz, $CDCl_3$) δ: 0.92 (3H, s, H-25), 1.02 (3H, s, H-26), 1.00 (3H, s, H-24), 1.07 (3H, s, H-27), 1.28 (3H, s, H-23), 1.69 (3H, s, H-30), 3.84 (1H, dd, $J = 5.2$ Hz and 15.2 Hz, H-7), 4.61 (1H, s, H-29), 4.77 (1H, s, H-29). ^{13}C NMR (100 MHz, $CDDl_3$): δ 39.3 (*t*, C-1), 34.0 (*t*, C-2), 218.0 (*s*, C-3), 46.6 (*s*, C-4), 52.4 (*d*, C-5), 32.5 (*t*, C-6), 74.4 (*d*, C-7), 48.9 (*s*, C-8), 49.8 (*d*, C-9), 36.9 (*s*, C-10), 21.4 (*t*, C-11), 25.5 (*t*, C-12), 37.2 (*d*, C-13), 48.6 (*s*, C-14), 68.9 (*d*, C-15), 41.5 (*t*, C-16), 54.6 (*s*, C-17), 49.7 (*d*, C-18), 46.5 (*d*, C-19), 149.9 (*d*, C-20), 29.7 (*t*, C-21), 36.8 (*t*, C-22), 26.4 (*q*, C-23), 21.1 (*q*, C-24), 15.5 (*q*, C-25), 10.7 (*q*, C-26), 8.3 (*q*, C-27), 180.2 (*s*, C-28), 110.0 (*t*, C-29), 19.5 (*q*, C-30).

4.6.2. 3-Oxo-7β,15α-dihydroxylup-20(29)-en-28-oic acid (4)

M.p. 280–282 °C; $[\alpha]_D^{25}$ + 20 (MeOH; c0.3); HRMS *m/z*: 485.3323 $[M-H]^-$ (calcd for $C_{30}H_{46}O_5$:486.3345). ESI-MS −45 eV, *m/z* (rel. int.) 485.4 $[M-H]^-$ (100), 467 $[M-H-H_2O]^-$ (55.9), 440 $[M-H-COOH]^-$ (26.1), 423 $[M-H-OH-COOH]^-$ (3.7), 411 $[M-H-OH-C_3H_5O]^-$ (3.8); IR ν_{max}/cm^{-1}: 3467, 2945, 1706, 1688; ^1H NMR (400 MHz, $CDCl_3$) δ: 0.93 (3H, s, H-25), 1.03 (3H, s, H-26), 1.04 (3H, s, H-24), 1.02 (3H, s, H-27), 1.10 (3H, s, H-23), 1.69 (3H, s, H-30), 3.80 (1H, dd, *J* = 6.4 Hz and 15.6 Hz, H-7), 3.90 (1H, dd, *J* = 6.8 Hz and 15.6 Hz, H-7), 4.63 (1H, s, H-29), 4.72 (1H, s, H-29). ^{13}C NMR (100 MHz, $CDDl_3$): δ 39.5 (*t*, C-1), 34.1 (*t*, C-2), 218.0 (*s*, C-3), 46.9 (*s*, C-4), 52.1 (*d*, C-5), 31.0 (*t*, C-6), 72.8 (*d*, C-7), 47.9 (*s*, C-8), 48.7 (*d*, C-9), 37.0 (*s*, C-10), 21.1 (*t*, C-11), 25.1 (*t*, C-12), 37.2 (*d*, C-13), 48.6 (*s*, C-14), 68.9 (*d*, C-15), 41.5 (*t*, C-16), 54.6 (*s*, C-17), 49.7 (*d*, C-18), 46.5 (*d*, C-19), 149.9 (*d*, C-20), 29.7 (*t*, C-21), 36.8 (*t*, C-22), 26.4 (*q*, C-23), 21.1 (*q*, C-24), 15.5 (*q*, C-25), 10.7 (*q*, C-26), 8.3 (*q*, C-27), 180.2 (*s*, C-28), 110.0 (*t*, C-29), 19.5 (*q*, C-30).

4.6.3. -Oxo-7β,30-dihydroxylup-20(29)-en-28-oic acid (5)

M.p.: 168–171 °C; $[\alpha]_D^{25}$ + 14.2 (MeOH; c0.3); HRMS *m/z*: 485.3343 $[M-H]^-$ (calcd for $C_{30}H_{46}O_5$: 486.3345). ESI-MS −45 eV, *m/z* (rel. int.) 485.4 $[M-H]^-$ (100), 440 $[M-H-COOH]^-$ (23.8), 423 $[M-H-OH-COOH]^-$ (52.4), 411 $[M-H-C_3H_5O]^-$ (38.0); IR ν_{max}/cm^{-1}: 3487, 2945, 1694; ^1H NMR (400 MHz, $CDCl_3$) δ: 0.92 (3H, s, H-25), 0.99 (3H, s, H-24), 1.02 (3H, s, H-26), 1.08 (3H, s, H-27), 1.27 (3H, s, H-23), 3.85 (1H, dd, *J* = 4.8 Hz and 15.6 Hz, H-7), 4.13 (2H, m, H-30), 4.92 (1H, s, H-29), 4.98 (1H, s, H-29); ^{13}C NMR spectrum, see Table 1.

4.7. Biotransformation of betulinic acid (1) by Colletotrichum

The extract obtained from the biotransformation of **1** (640 mg) was fractionated by planar centrifugal chromatography on a silica rotor (2 mm thick). Elution with hexane–EtOAc (8:2 v/v) produced a white solid (23 mg;), which was refractionated by prep. HPLC on a C_{18} column. Elution with CH_3CN-H_2O (4:1) provided a white solid (15 mg; 2.34% yield) which was characterized as 3-oxo-15α-hydroxylup-20(29)-en-28-oic acid (**6**). M.p. 274–276 °C; $[\alpha]_D^{25}$ − 11.5 (MeOH; c0.3); HRMS *m/z*: 469.3380 $[M-H]^-$ (calcd for $C_{30}H_{46}O_4$: 470.3396). ESI-MS −45 eV, *m/z* (rel. int.) 469.3 $[M-H]^-$ (100), 451 $[M-H-H_2O]^-$ (1.2), 424 $[M-H-COOH]^-$ (10.0), 407 $[M-H-OH-COOH]^+$ (3.0); IR ν_{max}/cm^{-1}: 2945, 1706, 1694; ^1H NMR (400 MHz, $CDCl_3$) δ: 0.94 (3H, s, H-25), 1.01 (3H, s, H-26), 1.01 (3H, s, H-24), 1.08 (3H, s, H-27), 1.26 (3H, s, H-23), 1.70 (3H, s, H-30), 4.05 (1H, dd, *J* = 4.8 Hz and 11.2 Hz, H-15), 4.64 (1H, s, H-29), 4.74 (1H, s, H-29); for ^{13}C NMR spectrum, see Table 1.

4.8. Biotransformation of betulonic acid (2) by Colletotrichum

The crude EtOAc extract obtained from the biotransformation of **2** (640 mg) was fractionated by planar centrifugal chromatography, and then by CC on silica gel, using mixtures of hexane–EtOAc (2:1 v/v) as eluent. The first metabolite isolated (**6**, 19 mg; 2.97% yield) was identical to that obtained from the biotransformation of betulinic acid (**1**) by *Colletotrichum*, and the second one (**5**, 11 mg; 1.72% yield) was the same as that obtained for biotransformation of betulonic acid (**2**) by *Arthrobotrys*.

4.9. Biotransformation of betulonic acid (2) by Chaetophoma

The crude extract obtained from the biotransformation of **2** (640 mg) was fractionated by vacuum liquid chromatography on silica. Elution with hexane and hexane/EtOAc produced a solid (27 mg; 4.22% yield) which was characterized as 3-oxo-25-hydroxylup-20(29)-en-28-oic acid (7). M.p. 215–218 °C; $[\alpha]_D^{25}$ − 12.7 (MeOH; c0.3); HRMS *m/z*: 469.3380 $[M-H]^-$ (calcd for $C_{30}H_{46}O_4$: 470.3396). ESI-MS −45 eV, *m/z* (rel. int.) 469.3 $[M-H]^-$ (100), 451 $[M-H-H_2O]^-$ (2.2), 424 $[M-H-COOH]^-$ (9.0), 407 $[M-H-OH-COOH]^+$ (5.0); IR ν_{max}3403, 2951, 1700, 1688 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.00 (3H, s,H-24), 1.01 (3H, s, H-27), 1.23 (3H, s, H-26), 1.28 (3H, s, H-23), 1.70 (3H, s, H-30), 3.12 (2H, m, H-25), 3.20 (2H, ddd, *J* = 4.4, 17, 26 Hz, H-12), 4.61 (1H, s, H-29), 4.76 (1H, s, H-29). For ^{13}C NMR spectrum, see Table 1.

4.10. Biotransformation of betulinic acid (1) by Chaetophoma

The crude extract from the biotransformation **1** (618 mg) was fractionated by vacuum liquid chromatography on silica. Elution with hexane and hexane/EtOAc produced a solid (5.8 mg; 0.94% yield) which was characterized as betulonic acid (**2**) by comparison with spectroscopic data identical to that prepared previously.

4.11. Biotransformation of betulinic acid (1) by Dematium

The extract from the biotransformation of **1** (640 mg) was fractionated by vacuum liquid chromatography on silica. Elution with hexane and hexane/dichloromethane produced a solid (4 mg; 0.62% yield), which was characterized as betulonic acid (**2**) identical to that prepared previously.

Acknowledgements

The financial support of FINEP and CAPES (Brazil) are greatly acknowledged.

References

Akihisa, T., Takamine, Y., Yoshizumi, K., Tokuda, H., Kimura, Y., Ukiya, M., Nakahara, T., Yokochi, T., Ichiishi, E., Nishino, H., 2002. Microbial transformations of two lupane-type triterpenes and anti-tumor-promoting effects of the transformation products. J. Nat. Prod. 65, 278–282.

Azerad, R., 1999. Microbial models for drug metabolism. In: Faber, K. (Ed.),. Advances in Biochemical Engineering/Biotechnology, vol. 63. Springer, Berlin/Heidelberg, pp. 169–218.

Bastos, D.Z.L., Pimentel, I.C., Dykstra, C., Gabardo, J., Oliveira, B.H., 2004. Fungos associados à casca do caule de *Platanus orientalis* L. Estudos de Biologia 26, 37–41.

Chang, C.W., Wu, T.S., Hsieh, Y.S., Kuo, S.C., Chao, P.D.L., 1999. Terpenoids of *Syzygium formosanum*. J. Nat. Prod. 62, 327–328.

Chatterjee, P., Pezzuto, J.M., Kouzi, S.A., 1999. Glucosidation of betulinic acid by *Cunninghamella* species. J. Nat. Prod. 62, 761–763.

Chatterjee, P., Kouzi, S.A., Pezzuto, J.M., Hamann, M.T., 2000. Biotransformation of the antimelanoma agent betulinic acid by *Bacillus megaterium* ATCC 13368. Appl. Environ. Microbiol. 66, 3850–3855.

Chiang, Y.M., Chang, J.Y., Kuo, C.C., Chang, C.Y., Kuo, Y.H., 2005. Cytotoxic triterpenes from the aerial roots of *Ficus microcarpa*. Phytochemistry 66, 495–501.

Cichewicz, R.H., Kouzi, S.A., 2004. Chemistry, biological activity and chemotherapeutic potential of betulinic acid for the prevention and treatment of cancer and HIV infection. Med. Res. Rev. 24, 90–114.

Gonzalez, A.G., Amaro, J., Fraga, B.M., Luis, J.G., 1983. 3-Oxo-6b-hydroxyolean-18-en-28-oic acid from *Orthopterygium huancuy*. Phytochemistry 22, 1828.

Kouzi, S.A., Chatterjee, P., Pezzuto, J.M., Hamann, M.T., 2000. Microbial transformations of the antimelanoma agent betulinic acid. J. Nat. Prod. 63, 1653–1657.

Lin, H.C., Ding, H.Y., Wu, Y.C., 1998. Two novel compounds from *Paeonia suffruticosa*. J. Nat. Prod. 61, 343–346.

Mallavadhani, U.V., Satyanarayana, K.V., Mahapatra, A., 2004. Quantitative evaluation of anticancer marker levels of an Ayurvedic preparation, ''Virala''. Pharm. Biol. 42, 338–341.

Motteran, L., Pilone, M.S., Molla, G., Ghisla, S., Pollegioni, L., 2001. Cholesterol oxidase from *Brevibacterium sterolicum* – the relationship between covalent flavinylation and redox properties. J. Biol. Chem. 276, 18024–18030.

de Oliveira, B.H., Santos, C.A.M., Espindola, A.P.D.M., 2002. Determination of the triterpenoid, betulinic acid, in *Doliocarpus schottianus* by HPLC. Phytochem. Anal. 13, 95–98.

Peng, C., Bodenhausen, G., Qiu, S.X., Fong, H.H.S., Farnsworth, N.R., Yuan, S.G., Zheng, C.Z., 1998. Computer-assisted structure elucidation: application of cisoc-sec to the resonance assignment and structure generation of betulinic acid. Magn. Reson. Chem. 36, 267–278.

Su, B.N., Cuendet, M., Farnsworth, N.R., Fong, H.H.S., Pezzuto, J.M., Kinghorn, A.D., 2002. Activity-guided fractionation of the seeds of *Ziziphus jujuba* using a cyclooxygenase-2 inhibitory assay. Planta Med. 68, 1125–1128.

Yogeeswari, P., Sriram, D., 2005. Betulinic acid and its derivatives: a review on their biological properties. Curr. Med. Chem. 12, 657–666.

Wilson, M.R., Gallimore, W.A., Reese, P.B., 1999. Steroid transformations with *Fusarium oxysporum* var. cubense and *Colletotrichum musae*. Steroids 64, 834–843.

Zhang, J., Cheng, Z.-H., Yu, B.-Y., Cordell, G.A., Qiuc, S.X., 2005. Novel biotransformation of pentacyclic triterpenoid acids by *Nocardia* sp. NRRL 5646. Tetrahedron Lett. 46, 2337–2340.