J Ind Microbiol Biotechnol (2011) 38:1493–1498
DOI 10.1007/s10295-010-0935-y

ORIGINAL PAPER

Biotransformation using *Mucor rouxii* for the production of oleanolic acid derivatives and their antimicrobial activity against oral pathogens

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Received: 7 July 2010 / Accepted: 23 December 2010 / Published online: 13 January 2011

Abstract The goal of this study is to produce oleanolic acid derivatives by biotransformation process using *Mucor rouxii* and evaluate their antimicrobial activity against oral pathogens. The microbial transformation was carried out in shake flasks at 30°C for 216 h with shaking at 120 rpm. Three new derivatives, 7β-hydroxy-3-oxo-olean-12-en-28-oic acid, 7β,21β-dihydroxy-3-oxo-olean-12-en-28-oic acid, and 3β,7β,21β-trihydroxyolean-12-en-28-oic acid, and one know compound, 21β-hydroxy-3-oxo-olean-12-en-28-oic acid, were isolated, and the structures were elucidated on the basis of spectroscopic analyses. The antimicrobial activity of the substrate and its transformed products was evaluated against five oral pathogens. Among these compounds, the derivative 21β-hydroxy-3-oxo-olean-12-en-28-oic acid displayed the strongest activity against *Porphyromonas gingivalis*, which is a primary etiological agent of periodontal disease. In an attempt to improve the antimicrobial activity of the derivative 21β-hydroxy-3-oxo-olean-12-en-28-oic acid, its sodium salt was prepared, and the minimum inhibitory concentration against *P. gingivalis* was reduced by one-half. The biotransformation process using *M. rouxii* has potential to be applied to the production of oleanolic acid derivatives. Research and antimicrobial activity evaluation of new oleanolic acid derivatives may provide an important contribution to the discovery of new adjunct agents for treatment of dental diseases such as dental caries, gingivitis, and periodontitis.

Keywords Biotransformation · *Mucor rouxii* · Oleanolic acid derivatives · Antimicrobial activity · Oral pathogens

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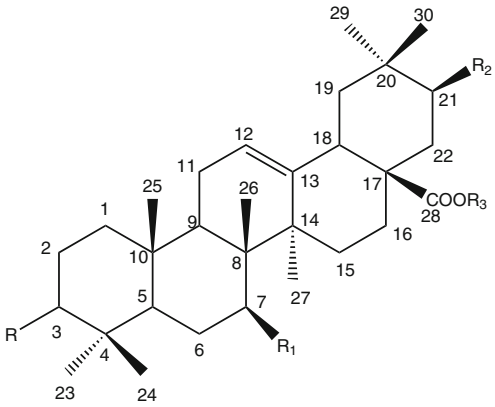
Introduction

Oleanolic acid (**1**) (Fig. 1) is a pentacyclic triterpene widespread in the plant kingdom in the form of free acid or aglycone for triterpenoid saponins [27]. It is reported to possess interesting bioactivities including anti-inflammatory [10], hepatoprotective [23], antitumor [21], antiviral [29], and antimicrobial activities [19]. Previous studies from our laboratory and others have shown that oleanolic acid and its derivatives display antibacterial activity against oral pathogens [8, 31, 34]. Oleanolic acid also suppressed in vitro adherence of cariogenic *Streptococcus mutans* biofilm [34] and inhibited insoluble glucan synthesis by *S. mutans* in the oral cavity [14].

Dental caries, gingivitis, and periodontal diseases may affect a person's overall health. Periodontal pathogens are capable of invading epithelial cells and connective tissue, causing periodontal inflammation and bleeding, which enable entry of oral flora into systemic circulation. Once in the blood, the bacteria are capable of interacting with platelets, forming platelet–bacterial aggregates, which can bind to heart valves or interact with sites of atherosclerosis [30]. Haraszthy and coworkers [13] reported that 19 of 27

Fig. 1 The structures of compounds **1–5**



Compound	R	R_1	R_2	R_3
1	OH	H	H	H
2	=O	OH	H	H
3	=O	H	OH	H
3S	=O	H	OH	Na^+
4	=O	OH	OH	H
5	OH	OH	OH	H

atheromas obtained from patients during endarterectomy were positive for bacterial DNA from periodontal pathogens. Therefore, oral bacteria may contribute to increased risk of cardiovascular disease [12], stroke [3], and preeclampsia [20].

The continuing search for new active compounds against oral pathogens might be justified by the side-effects of chlorhexidine, the most effective antimicrobial in oral use as mouthwash, including teeth staining, taste alteration, and calculus formation [5].

Oleanolic acid derivatives evaluated against oral pathogens have been obtained by means of synthetic approach and include oleanolic sodium salt, oleanolic aldehyde, derivatives with a hydroxyl group at C-6 or with an additional carboxyl group at C-23, and those obtained by a series of acylation and etherification reactions [8, 31].

Biotransformation processes have been used to target positions more difficult to functionalize by chemical methods. Microorganisms of the genus *Mucor* have been used to catalyze valuable reactions, including steroid hydroxylation [24]. Thus, in this work, production of oleanolic acid derivatives by biotransformation process using *M. rouxii* is described for the first time. Three obtained derivatives were found to be new compounds, and the antimicrobial activity of the substrate and its transformed products was evaluated against five oral pathogens.

Materials and methods

Substrate and chemicals

Oleanolic acid was purchased from Sigma–Aldrich (St. Louis, MO, USA). All reagents and solvents were of analytical or chromatographic grade, purchased from Grupo Química (São Paulo, Brazil) or Sigma–Aldrich (St. Louis, MO, USA), respectively.

Microorganisms and maintenance

Mucor rouxii strain NRRL 1894 was obtained courtesy of Dr. C.W. Hesseltine (Northern Utilization Research and Development Division, ARS, USDA, Peoria, IL, USA) and belongs to a collection of fungal cultures of the Biology Department of the School of Philosophy and Sciences of Ribeirão Preto, University of São Paulo. The microorganism is stored as a conidial suspension on silica gel (6–12 mesh, grade 40, desiccant activated) at 4°C and on slants of solid oatmeal baby food consisting of 0.4% (w/v) oatmeal and 1.8% (w/v) agar.

The following oral pathogens were obtained from the American Type Culture Collection (ATCC) and were used for antimicrobial evaluation: *Actinomyces naeslundii* (ATCC 19039), *Aggregatibacter actinomycetemcomitans* (ATCC 43717), *Enterococcus faecalis* (ATCC 4082), *Porphyromonas gingivalis* (ATCC 33277), and *Prevotella nigrescens* (ATCC 33563).

Biotransformation procedure

A two-step culture was performed for biotransformation reactions. An initial inoculum of a suspension of 4×10^6 conidia/ml was added into 15 Erlenmeyer flasks (250 ml) containing 50 ml seed medium [22]. The flasks were incubated for 48 h at 30°C on a rotary shaker operating at 120 rpm (orbital shaker with 2.5 cm throw). The resulting mycelia were harvested, rinsed, and transferred into 15 Erlenmeyer flasks (500 ml) containing 100 ml Koch's K1 medium [2] [glucose 0.18%, bacteriological peptone (Oxoid) 0.06%, and yeast extract (Oxoid) 0.04%]. Oleanolic acid (10 mg) was added to each flask as a solution in dimethyl sulfoxide (10 mg dissolved in 2 ml). Control flasks consisted of culture medium with fungus without oleanolic acid, culture medium with dimethyl sulfoxide without oleanolic acid and fungus, culture medium with fungus and dimethyl sulfoxide without oleanolic acid, culture

medium with oleanolic acid without fungus, and culture medium alone. Biotransformation experiments were carried out at 30°C for 216 h with shaking at 120 rpm.

Extraction and isolation of oleanolic acid derivatives

The culture broth obtained after filtration was extracted twice with ethyl acetate, and the solvent was evaporated under reduced pressure to yield a crude extract (86 mg), which was then submitted to a solid-phase extraction clean-up procedure using Strata C18-E cartridge (Phenomenex, Torrance, CA, USA) and elution with methanol. The eluate was evaporated to dryness, and the residue was dissolved in methanol and submitted to semipreparative high-performance liquid chromatography (HPLC) using a Synergi MAX-RP 80A column (250 × 4.6 mm id, 4 µm particle size; Phenomenex, Torrance, CA). Instrumentation consisted of a Shimadzu (SCL-10Avp, Kyoto, Japan) multisolvent delivery system, Shimadzu SPDM10Avp photodiode array detector, and an Intel Celeron computer for analytical system control, data collection, and processing. Semipreparative chromatography was carried out using the following gradient solvent system at flow rate of 3.0 ml/min: (1) 40% to 100% acetonitrile in water over 40 min, (2) 100% acetonitrile over 5 min, (3) 100% to 40% acetonitrile in water over 5 min, and (4) 40% acetonitrile over 5 min. The spectral data from the detector were collected over the 200–400 nm range of the absorption spectrum.

Structure determination of compounds

The structures of the compounds were determined by spectroscopic techniques, especially by detailed analyses of their ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra, including two-dimensional (2D) NMR techniques [heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond coherence (HMBC)], as well as mass spectrometry. NMR spectra were performed on a Bruker (Rheinstetten, Germany) DPX 500 spectrometer (500 MHz for ^{1}H, 125 MHz for ^{13}C). Samples were dissolved in deuterated $CDCl_3$ (compounds **2**–**4**) or CD_3OD (compound **5**), with tetramethylsilane (TMS) as internal reference. Deuterated solvents were purchased from Aldrich (Milwaukee, WI, USA). High-resolution mass spectra (HRMS) were recorded on a MICROMASS Q-TOF MICRO mass spectrometer using electrospray ionization time of flight (ESI-TOF).

Preparation of the sodium salt of 21β-hydroxy-3-oxo-olean-12-en-28-oic acid

The sodium salt of the derivative 21β-hydroxy-3-oxo-olean-12-en-28-oic acid was prepared according to Rivero-Cruz et al. [31] by treating the derivative with a molar equivalent of sodium hydroxide.

In vitro antimicrobial activity assessment assay

The minimal inhibitory concentration values (MIC; lowest concentration of the compound capable of inhibiting microorganism growth) and the minimal bactericidal concentration values (MBC; lowest concentration of the compound at which 99.99% or more of the initial inoculum was killed) of the substrate and its transformed products were determined in triplicate by using the microdilution broth method in 96-well microplates [6, 7]. The samples were dissolved in dimethyl sulfoxide (DMSO) at 1 mg/ml, followed by dilution in Schadler broth (Difco, Kansas City, MO) supplemented with hemin (5 µg/ml) and vitamin K1 (10 µg/ml) for anaerobic bacteria. *E. faecalis* and *A. actinomycetemcomitans* were diluted in tryptic soy broth (Difco, Kansas City, MO) as a facultative microorganism and microaerophilic, respectively. The final DMSO content was 5% (v/v), and this solution was used as negative control. The inoculum was adjusted for each organism to yield cell concentration of 5×10^5 colony forming units (CFU/ml), according to previous standardization by the Clinical Laboratory Standards Institute [6, 7]. One inoculated well was included, to allow control of the adequacy of the broth for organism growth. One noninoculated well, free of antimicrobial agent, was also included, to ensure medium sterility. Chlorhexidine dihydrochloride was used as positive control. The time necessary for growth was 24 h for *E. faecalis* and *A. actinomycetemcomitans*, and 96 h for anaerobic bacteria, incubated at 37°C in appropriate gaseous conditions (facultative in aerobic condition; microaerophilic in an atmosphere of 10% CO_2; anaerobic bacteria in the anaerobic workstation Don Whitley Scientific, Bradford, UK, in an atmosphere of 5–10% H_2, 10% CO_2, 80–85% N_2). After that, resazurin (30 µl) in aqueous solution (0.02%) was added to the microplates, to indicate microorganism viability [1]. Before addition of resazurin and to determine MBC, an aliquot of the inoculum was aseptically removed from each well showing no apparent growth, then plated onto Schadler agar (Difco, Kansas City, MO) supplemented with hemin (5 µg/ml), vitamin K1 (10 µg/ml), and sheep blood (5%) for anaerobic bacteria. *E. faecalis* and *A. actinomycetemcomitans* were plated in tryptic soy agar (Difco, Kansas City, MO) supplemented with 5% sheep blood. The plates were incubated as previously described.

Results and discussion

Compounds **2**–**5** were isolated in 1.2%, 3.2%, 6.3%, and 2.6% yield, respectively, based on weight relative to starting substrate.

The ESI mass spectrum of metabolite **2** obtained in positive ion mode showed the cationized molecule at *m/z* 493.3288 $[M + Na]^+$, which indicated an increment of 14 a.m.u. when compared with compound **1**. The molecular formula was deduced as $C_{30}H_{46}O_4$ and was supported by its NMR data. The ^1H NMR spectrum displayed characteristic signals of seven methyl groups, a proton geminal to an oxygen function at δ_H 3.92 (1H, dd, $J = 4.4$ Hz, $J = 11.4$ Hz), and an olefinic hydrogen at δ_H 5.35 (1H, t, $J = 3.5$ Hz). The ^{13}C NMR spectrum showed signals of seven methyls, nine methylenes, three methines, one oxygenated methine at δ_C 73.3, six quaternary carbons, a double bond (δ_C 122.3 and δ_C 143.6, C-12 and C-13), one oxo group at δ_C 217.3, and a carbonyl of an acid group at δ_C 181.0 (Table 1). Correlations of hydrogens of C-23 and C-24 methyl groups and of the hydrogens of C-2 with the oxo group were observed in the HMBC spectrum, which indicated that this function was at C-3. Hydroxylation at C-7 position was deduced from the HMBC correlations of hydrogens of C-26 methyl group with C-7 at δ_C 73.3, and its β-configuration was confirmed by the high-field shift of the resonance of C-26 due to the γ-gauche effect [28]. Therefore, the structure of compound **2** was determined as 7β-hydroxy-3-oxo-olean-12-en-28-oic acid. The obtained data of compound **2** were in part similar to those of camarin, 7α-hydroxy-3-oxo-olean-12-en-28-oic acid, isolated from *Lantana camara* Linn. [4], in which the C-26 did not show the high-field shift which would have resulted from the β disposition of the hydroxyl group.

The ESI mass spectrum of metabolite **3** obtained in positive ion mode showed the cationized molecule at *m/z* 493.3290 $[M + Na]^+$, which also indicated an increment of 14 a.m.u. when compared with compound **1**. The molecular formula was also deduced as $C_{30}H_{46}O_4$. The ^1H and ^{13}C NMR data of (**3**) closely resembled those of (**2**), but with a proton geminal to an oxygen function at δ_H 3.54 (1H, dd, $J = 4.6$ Hz, $J = 11.9$ Hz). HMBC correlations were also similar, but the hydroxylation position was deduced at C-21 from the HMBC correlations of hydrogens of C-29 and C-30 methyl groups with C-21 at δ_C 73.3 and on the basis of multiplicity of H-21 signal, while its β-configuration was confirmed by the high-field shift of the resonance of C-30 due to the γ-gauche effect [28]. Therefore, the structure of compound **3** was determined as 21β-hydroxy-3-oxo-olean-12-en-28-oic acid. This compound was previously obtained as a biotransformation product using *Penicillium chrysogenum* [15].

The ESI mass spectrum of metabolite **4** obtained in positive ion mode showed the cationized molecule at *m/z* 509.3237 $[M + Na]^+$, which indicated an increment of 30 a.m.u. when compared with compound **1**. The molecular formula was deduced as $C_{30}H_{46}O_5$. The ^1H and ^{13}C NMR spectroscopic data of (**4**) closely resembled those of (**2**) and (**3**), displaying two hydroxyl-bearing methine proton at δ_H 3.90 (1H, dd, $J = 4.8$ Hz, $J = 10.9$ Hz) and δ_H 3.54 (1H, dd, $J = 4.6$ Hz, $J = 11.9$ Hz), and an olefinic hydrogen at δ_H 5.36 (1H, t, $J = 3.5$ Hz). The ^{13}C NMR spectrum showed signals of two oxygenated methine at δ_C 73.0 and δ_C 73.3 (Table 1). The HMBC correlations observed for both (**2**) and (**3**) were observed for (**4**). Therefore, the structure of compound **4** was determined as 7β,21β-hydroxy-3-oxo-olean-12-en-28-oic acid, a new oleanolic acid derivative.

The ESI mass spectrum of metabolite **5** obtained in positive ion mode showed the cationized molecule at *m/z* 511.3397 $[M + Na]^+$, which indicated an increment of 32 a.m.u. when compared with compound **1**. The molecular formula was deduced as $C_{30}H_{48}O_5$. Among the NMR data, three hydroxyl-bearing methine proton signals resonated at δ_H 3.81 (1H, dd, $J = 4.9$ Hz, $J = 11.0$ Hz), δ_H 3.47 (1H, dd, $J = 4.9$ Hz, $J = 11.7$ Hz), and δ_H 3.14 (1H, dd, $J = 4.8$ Hz,

Table 1 ^{13}C NMR spectral data of compounds **2**–**5** (125 MHz; **2**–**4**, $CDCl_3$; **5**, CD_3OD)

Carbon	Compounds			
	2	**3**	**4**	**5**
1	39.0	39.2	39.1	38.2
2	34.1	34.1	34.2	27.9
3	217.3	217.6	217.6	79.4
4	36.8	47.5	36.7	39.5
5	53.7	55.7	53.7	53.9
6	27.6	19.5	26.9	26.1
7	73.3	32.1	73.0	73.9
8	37.8	39.1	37.9	39.7
9	47.4	46.8	47.2	48.2
10	37.2	36.7	37.1	37.1
11	23.3	24.2	23.7	24.6
12	122.3	123.4	123.0	124.2
13	143.6	142.7	142.7	143.9
14	43.7	41.7	41.1	41.1
15	28.0	27.7	26.9	29.5
16	23.6	27.7	24.7	24.6
17	46.5	46.3	46.1	46.2
18	41.2	40.7	43.2	43.0
19	46.4	39.1	38.8	38.4
20	31.0	36.0	35.0	36.3
21	34.2	73.3	73.3	73.8
22	32.0	39.8	38.8	36.9
23	26.1	26.4	26.3	27.9
24	21.5	21.4	21.5	15.9
25	16.0	15.0	14.9	15.9
26	9.7	16.9	9.7	10.6
27	25.7	25.6	25.7	25.8
28	181.0	180.8	180.6	180.8
29	33.0	28.8	28.8	28.7
30	23.4	16.9	16.8	16.3

Table 2 Antimicrobial activity of oleanolic acid and its derivatives against oral pathogens

Microorganisms	Minimum inhibitory concentration (μM/)/minimum bactericidal concentration (μM) Compounds						
	1	**2**	**3**	**3S**	**4**	**5**	**C**
Actinomyces naeslundii	2.1/2.1	53.1/53.1	13.2/13.2	13.2/13.2	821.8/>821.8	>818.4/>818.4	3.18/3.18
Aggregatibacter actinomycetemcomitans	13.1/26.2	849.8/>849.8	13.2/106.2	12.7/101.4	205.4/410.9	204.6/409.2	12.7/12.7
Enterococcus faecalis	26.2/87.5	>849.8/> 849.8	>849.8/>849.8	>849.8/> 849.8	>821.8/>821.8	>818.4/>818.4	6.3/6.3
Porphyromonas gingivalis	87.5/131.3	53.1/53.1	13.2/13.2	6.6/6.6	410.9/410.9	818.4/818.4	1.5/1.5
Prevotella nigrescens	437.9/>875.8	>849.8/>849.8	849.8/849.8	811.8/811.8	410.9/410.9	818.4/>818.4	1.5/1.5

C chlorhexidine dihydrochloride

J = 11.4 Hz). The three oxygenated methine carbons were observed at δ_C 73.9, δ_C 73.8, and δ_C 79.4, respectively, in the ^{13}C NMR spectrum. The oxo group at C-3 of (**4**) was replaced by a hydroxyl group in compound **5**. Hydroxylation at C-3 position was deduced from the HMBC correlations of hydrogens of C-23 and C-24 methyl groups with C-3 at δ_C 79.4. The β configuration was assigned for C-3 hydroxyl group on the basis of the high-field shift of the resonance of C-24, as well as by the characteristic couplings of H-3 [28]. Therefore, the structure of compound (**5**) was determined as 3β,7β,21β-trihydroxyolean-12-en-28-oic acid, another new oleanolic acid derivative.

The antimicrobial activity of the substrate and its transformed products was evaluated against five oral pathogens. The values of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) are presented in Table 2.

The derivative 21β-hydroxy-3-oxo-olean-12-en-28-oic acid (**3**) displayed the strongest activity against *P. gingivalis* (MIC and MBC values of 13.2 μM), which is a primary etiological agent of periodontal disease and the main protagonist involved in initiation and progression of severe forms of periodontal disease [25]. In an attempt to improve antimicrobial activity of this derivative, its sodium salt (**3S**) was prepared, and the MIC and MBC values against *P. gingivalis* were reduced by one-half (Table 2).

P. gingivalis produces a broad array of potential virulence factors, such as proteases, lipopolysaccharide, fimbriae, and toxic products of metabolism, all of which enable this anaerobe to cause disease either directly or indirectly by activation of host cells to release inflammatory mediators [9]. Also, it has been proposed that *P. gingivalis* may be an important factor in the association between rheumatoid arthritis and periodontitis [16].

The most effective control of periodontal *P. gingivalis* seems to be achieved by employing a combination of respective periodontal surgery, systemic antibiotic therapy, and good oral hygiene, in which antiseptic mouthwash is recommended as a daily adjunct to mechanical cleaning [33]. Chlorhexidine mouthwash has many side-effects such as staining of teeth, impaired sense of taste, increased formation of supragingival calculus, and occasionally mucous membrane irritation and desquamation, and it is only recommended for short periods [11]. An alternative mouthwash with fewer unwanted side-effects may be an option mainly for elimination of *P. gingivalis*, which is capable of mediating initial adherence and colonization of the oral cavity [9].

The derivative 21β-hydroxy-3-oxo-olean-12-en-28-oic acid (**3**) and its sodium salt (**3S**) also displayed significant antimicrobial activity against *A. actinomycetemcomitans*, which is closely associated with destructive periodontal disease [32]. The MIC values of these derivatives against *A. actinomycetemcomitans* were similar to those of oleanolic acid and chlorhexidine dihydrochloride.

Previous studies have shown that the activity of the oleanolic acid sodium salt against *P. gingivalis* was about 100 times more potent than that of oleanolic acid [31]. In this study, the activity of the sodium salt of derivative **3** against *P. gingivalis* was only about two times more potent than that of derivative **3**. These results revealed that a greater number of compounds should be investigated to enhance understanding of structure–activity relationship.

Even though more than 100 triterpene skeletons have been reported in nature [35], hundreds of new derivatives have been synthesized to improve potency [17, 18]. Among them, two synthetic oleanane triterpenoids, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) and its methyl ester (CDDO-Me), are currently in phase I clinical trials for treatment of leukemia and solid tumors [26].

Likewise, research and antimicrobial activity evaluation of new oleanolic acid derivatives may provide an important contribution to the discovery of new adjunct agents for treatment of dental diseases such as dental caries, gingivitis, and periodontitis.

Acknowledgments This work was supported by the Research Support Foundation of the State of São Paulo (FAPESP proc. 05/59329-5),

CAPES and Ministerio de Ciencia e Innovación (MICIN, PHB2008-0067). We would like to thank Maria de Lourdes Teixeira de Moraes Polizeli and João Atílio Jorge (FFCLRP-USP) for providing fungal strain used in this work, Boniek Gontijo Vaz and Marcos Nogueira Eberlin (Unicamp) for mass spectra, and Flavio da Silva Emery for synthesis assistance.

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