Phytochemistry 117 (2015) 500–508

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Biotransformation of oleanolic and maslinic methyl esters by *Rhizomucor miehei* CECT 2749

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ARTICLE INFO

Article history:
Received 15 May 2015
Received in revised form 22 July 2015
Accepted 27 July 2015

Keywords:
Rhizomucor miehei
Biotransformation
Biohydroxylation
Triterpenoids
Oleanolic acid
Maslinic acid
Methyl oleanolate
Methyl maslinate

ABSTRACT

The pentacyclic triterpenoids methyl oleanolate, methyl maslinate, methyl 3β-hydroxyolean-9(11),12-dien-28-oate, and methyl 2α,3β-dihydroxy-12β,13β-epoxyolean-28-oate were biotransformed by *Rhizomucor miehei* CECT 2749. Microbial transformation of methyl oleanolate produced only a 7β,30-dihydroxylated metabolite with a conjugated 9(11),12-diene system in the C ring. Biotransformation of the substrate with this 9(11),12-diene system gave the same 7β,30-dihydroxylated compound together with a 7β,15α,30-trihydroxyl derivative. The action of this fungus (*R. miehei*) on methyl maslinate was more varied, isolating metabolites with a 30-hydroxyl group, a 9(11),12-diene system, an 11-oxo group, or an 12-oxo group. Microbial transformation of the substrate with a 12β,13β-epoxy function resulted in the isolation of two metabolites with 12-oxo and 28,13β-olide groups, hydroxylated or not at C-7β, together with a 30-hydroxy-12-oxo derivative. The structures of these derivatives were deduced by extensive and rigorous spectroscopic studies.

1. Introduction

Currently, plants, marine sources, and microorganisms represent excellent raw materials for natural products, which either could be used directly as drugs for treating human diseases, or could play a dominant role in the discovery of leads for drug development (Bauer and Brönstrup, 2014; Morrison and Hergenrother, 2014). This pharmacological significance has made triterpenoids one of the most studied families of natural products in recent decades (Hill and Connolly, 2015; Han and Peng, 2014; Nazaruk and Borzym-Kluczyk, 2014; Salvador et al., 2014; Parmar et al., 2013; Petronelli et al., 2009).

Oleanolic and maslinic acids are two naturally pentacyclic triterpenes, with oleanane skeleton, which possess numerous biological activities (Shanmugam et al., 2014; Camer et al., 2014; Lozano-Mena et al., 2014; Sanchez-Quesada et al., 2013; Pollier and Goossens, 2012; Wolska et al., 2010). Chemical modifications of these triterpenic acids have resulted in compounds that have improved the biological activities of their precursor products (Parra et al., 2014a,b; Liby and Sporn, 2012; Sporn et al., 2011). Some of these derivatives, i.e. 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO), and their C-28 methyl ester (CDDO-Me) and C-28 imidazole (CDDO-Im) derivatives, are currently under evaluation in the preclinical phase (Shanmugam et al., 2014; Wang et al., 2014).

The biotransformation of triterpenes under mild conditions appears as an attractive alternative to the traditional chemical methods, since it has an elevated chemo-, regio- and enantioselectivity. These processes do not generate toxic waste products, and the compounds achieved can be labelled as a "natural source" (Shah et al., 2014; Muffler et al., 2011; Parra et al., 2009; Simeo and Sinisterra, 2009).

Methyl oleanolate and methyl maslinate are two non-acidic triterpene derivatives of oleanolic and maslinic acid, respectively and, like these, possess numerous biological activities as anticancer (Siewert et al., 2013; Mallavadhani et al., 2013; Fu et al., 2005), anti-HCV (Yu et al., 2013), antibacterial (Weimann et al., 2002), anti-inflammatory (Bai et al., 2012), or cardiotonic and antidysrhythmic effects (Somova et al., 2004).

The present paper describes the biotransformation of methyl oleanolate, methyl maslinate, and other analogues by *Rhizomucor miehei* CECT 2749. Biocatalysis of these compounds have never before been described. This study complements the prior paper of biotransformation of oleanolic (**1**) and maslinic (**2**) acids by the same fungus (Martinez et al., 2013).

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http://dx.doi.org/10.1016/j.phytochem.2015.07.020
0031-9422/

2. Results and discussion

2.1. Biotransformation of methyl oleanolate (3)

Methyl oleanolate (**3**) was achieved treating oleanolic acid (**1**) with NaOH and CH_3I (Garcia-Granados et al., 2000) (Fig. 1). The biotransformation of this compound (**3**) with *R. miehei* for 13 days resulted in the recovery of 72% of the substrate (**3**), and some 15% of a new metabolite (**5**) (Fig. 2).

Metabolite **5** had a molecular formula of $C_{31}H_{48}O_5$, indicating that the microorganism had inserted two oxygen atoms onto the substrate (**3**), and two atoms of hydrogen were lost. The main difference of the ^1H NMR spectra of compounds **3** and **5** was the presence in the latter of a collapsed AB system signal (δ_H 5.60, 2H, s) due to the olefinic protons of a 9(11),12-conjugated diene system on the C ring. Also, appeared in this spectrum (**5**) a signal of an AB system (δ_H 3.60 and 3.54), which was due to the coupling of two geminal protons to a hydroxyl group placed on a primary carbon (C-30), together with another signal (δ_H 4.04, 1H, dd, J = 11.3, 5.4 Hz), which was due to a geminal proton to an equatorial hydroxyl group (C-7) (Table 1). The analysis of the HMBC spectrum for this metabolite (**5**) showed correlations between the signal of H-7 (δ_H 4.04) and C-6, C-8, C-14, and C-26, and between the signals of 2H-30 (δ_H 3.60 and 3.54) with C-19, C-20, C-21, and C-29 (Fig. 2). Also, its HMBC spectrum revealed a correlation between the signals of H-11 and H-12 (δ_H 5.60) with C-8, C-9, C-10, C-13, C-14, C-18, C-25, and C-26, which confirmed the conjugated diene system on the C ring. Therefore, metabolite **5** had a structure of methyl 3β,7β,30-trihydroxyoleana-9(11),12-dien-28-oate. These microbial hydroxylations at C-7β and C-30 were also brought about by the same fungus on oleanolic acid (**1**) (Martinez et al., 2013), but the 9(11)-double bond was not formed in that case.

A possible mechanism to explain the formation of this double bond in metabolite **5** could be the introduction of a hydroperoxyl group at C-11 by the microorganism, and the subsequent loss of a hydrogen peroxide molecule, giving the 9(11),12-conjugated diene system on the C ring (Martinez et al., 2013). The existence of an ester group at C-28 could prevent the attack of this group over C-13, and no 28,13β-olide group was formed in this case, as occurred in the biotransformation of maslinic acid (**2**) with the same microorganism (Martinez et al., 2013).

2.2. Biotransformation of compound 6

Biotransformation of the 9(11),12-diene derivative (**6**) was performed to check whether the formation of the double bond, in metabolite **5**, preceded the microbial hydroxylations at C-7 and C-30. This substrate (**6**) was achieved by treating of methyl oleanolate (**3**) with NBS/AIBN (Garcia-Granados et al., 2004). Thus, the microbial incubation of **6** with *R. miehei* for 13 days resulted in the recovery of 62% of the substrate (**6**), and the isolation of metabolite **5** (11%), and a new metabolite **7** (8%) (Fig. 2).

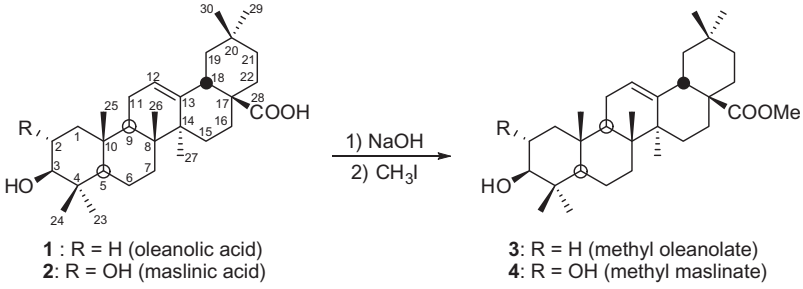

Fig. 1. Formation of methyl oleanolate (**3**) and methyl maslinate (**4**).

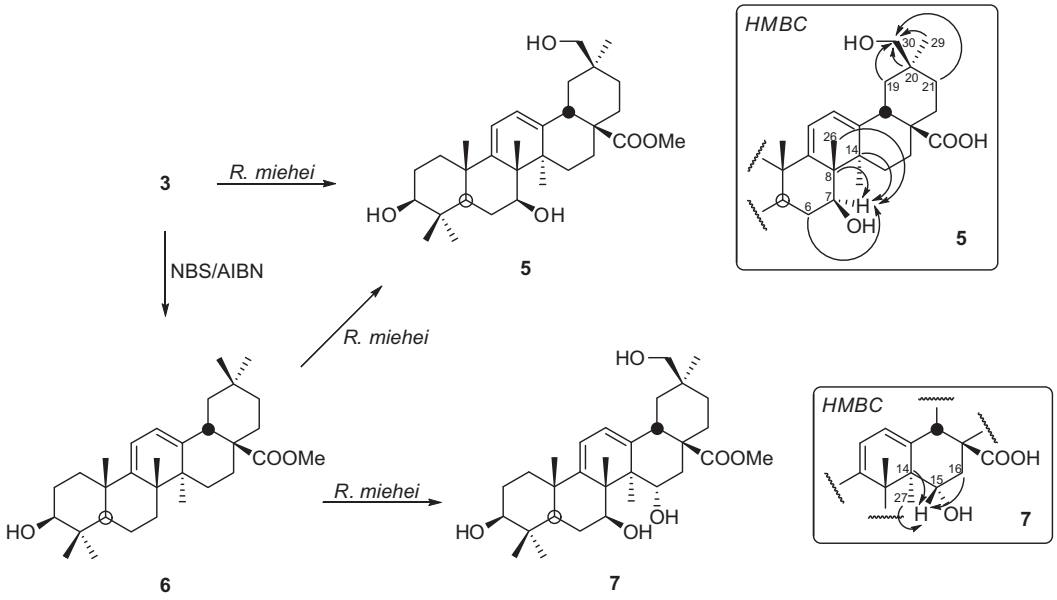

Fig. 2. Biotransformation of methyl oleanolate (**3**) and the diene derivative **6** with *Rhizomucor miehei*. Key HMBC (C → H) correlations in metabolites **5** and **7**.

Table 1
^{1}H NMR data (δ_H) for compounds **5**, **7**, **8**, **10**, **11** (*J* in Hz).[a]

Position	5	7	8	10	11
1	1.98	1.96	2.26	3.19 (dd, 12.9, 4.5)	1.95
	1.40	1.28	1.30	1.12	1.01
2	1.76	1.72	4.12 (ddd, 11.1, 9.4, 4.3)	5.22 (ddd, 10.6, 10.6, 4.5)	5.06 (ddd, 10.7, 10.7, 4.7)
	1.66	1.62			
3	3.23 (dd, 11.6, 4.6)	3.24 (dd, 7.2, 4.6)	3.41 (d, 9.4)	4.71 (d, 10.6)	4.73 (d, 10.7)
5	1.01	0.87	1.05	0.92	0.97
6	1.82	1.78	1.62	1.60	1.64
	1.54	1.60	1.49	1.39	1.46
7	4.04 (dd, 11.3, 5.4)	4.10 (dd, 11.5, 5.1)	1.51	1.60	1.45
			1.31	1.37	1.36
9			1.80	2.36	1.69
11	5.60 (s)	5.62 d (6.2)	1.96		2.19
12	5.60 (s)	5.67 d (6.2)	5.39 (dd, 3.7, 3.3)	5.63 (s)	
13					2.60 (d, 4.3)
15	1.86	4.20 (dd, 7.3, 4.8)	1.85	1.72	1.66
	0.94		1.15	1.26	1.08
16	1.92	2.10	2.15	2.05	1.90
	1.66	1.90	1.82	1.74	1.66
18	2.97 (dd, 14.0, 4.3)	2.91 (dd, 9.2, 4.4)	3.25 (dd, 12.4, 6.2)	2.97 (dd, 13.8, 3.9)	2.78 (ddd, 13.5, 4.3, 4.3)
19	1.56	1.55	1.76	1.62	1.93
	1.43	1.44	–	1.20	1.20
21	1.47	1.51	1.76	1.37	1.33
	1.34	1.30	1.40	1.30	1.20
22	1.61	1.62	1.94	1.78	1.80
	–	–	1.65	1.66	1.47
23	1.03 (s)	1.03 (s)	1.30 (s)	0.89 (s)	0.89 (s)
24	0.80 (s)	0.80 (s)	1.12 (s)	0.90 (s)	0.91 (s)
25	1.15 (s)	1.15 (s)	1.03 (s)	1.22 (s)	0.99 (s)
26	0.98 (s)	1.01 (s)	0.87 (s)	0.91 (s)	0.96 (s)
27	1.08 (s)	1.08 (s)	1.26 (s)	1.35 (s)	0.93 (s)
29	0.91 (s)	0.91 (s)	1.20 (s)	0.93 (s)	0.90 (s)
30	3.60 (d, 10.8)	3.57 (d, 10.8)	3.91 (d, 10.6)	0.94 (s)	0.97 (s)
	3.54 (d, 10.8)	3.53 (d, 10.8)	3.85 (d, 10.6)		
MeOCO	3.65 (s)	3.65 (s)	3.70 (s)	3.48 (s)	3.68 (s)
MeCOO				2.06 (s)	2.06 (s)
MeCOO				2.04 (s)	1.99 (s)
MeCOO				1.95 (s)	

The underlined term indicates the carbon atom to which the signal belongs in a functional group. **Me**COO corresponds to the signal of the methyl of the acetoxyl group. Me**CO**O corresponds to the signal of the carbonyl of the acetoxyl group. **MeO**CO corresponds to the signal of the methoxyl of the methoxycarbonyl group.

[a] Assignments were based on ^{1}H-^{1}H COSY, HSQC, HMBC, DEPT, and NOESY experiments. Overlapped ^{1}H NMR signals are reported without designated multiplicity.

Metabolite **7** had a molecular formula of $C_{31}H_{48}O_6$, with three extra oxygen atoms with respect to that of the substrate (**6**). In the ^{1}H NMR spectrum of metabolite **7** appeared an AB system signal (δ_H 3.57 and 3.53) and a double doublet signal (δ_H 4.10, 1H, dd, J = 11.5, 5.1 Hz), similar to those of metabolite **5**, suggesting the presence of a 30- and a 7β-hydroxyl groups in the molecule. Another double doublet signal at δ_H 4.20 (1H, dd, J = 7.3, 4.8 Hz) due to a geminal proton to an equatorial hydroxyl group (C-15) appeared in this spectrum (Table 1). The correlations observed in the HMBC spectrum of this metabolite (**7**) between this signal (H-15, δ_H 4.20) and C-14, C-16, and C-27, suggested that the new hydroxyl group was located at C-15 (Fig. 2). These data confirmed the structure of methyl 3β,7β,15α,30-tetrahydroxyoleana-9(11),12-dien-28-oate for metabolite **7**.

2.3. Biotransformation of methyl maslinate (*4*)

Methyl maslinate (**4**) was achieved by treating of maslinic acid (**2**) with NaOH and CH_3I (Garcia-Granados et al., 2000) (Fig. 1). Microbial transformation of this methyl maslinate (**4**) by *R. miehei* for 13 days produced a mixture of metabolites difficult to separate. Chromatography on a silica-gel column of this mixture resulted in the recovery of 63% of the substrate (**4**), and the isolation of metabolites **8** (12%) and **9** (7%). Acetylation of the rest of the fractions (8%) yielded the 2α,3β-diacetyl derivatives **10** and **11** (Fig. 3).

The molecular formula of the metabolite **8** ($C_{31}H_{50}O_5$) indicated that the microorganism had inserted a new oxygen atom onto substrate **4**. The basic difference of the ^{1}H NMR spectra of substrate **4** and metabolite **8** was the presence in the latter of an AB system signal (δ_H 3.91 and 3.85), which was due to two geminal protons to a hydroxyl group on a primary carbon (C-30) (Table 1). The position of this microbial hydroxylation was confirmed by the HMBC correlations of 2H-30 with C-19, C-20, C-21, and C-29. These data indicated that the structure of compound **8** was methyl 2α,3β,30-trihydroxyolean-12-en-28-oate.

The molecular formula of metabolite **9** ($C_{31}H_{48}O_4$) indicated that this metabolite had two hydrogen atoms less than the substrate (**4**). Its spectroscopic data were consistent with a structure of methyl 2α,3β-dihydroxyoleana-9(11),12-dien-28-oate, a compound previously synthesised by our group from compound **4** with NBS/AIBN (Garcia-Granados et al., 2004). A possible mechanism to explain the formation of the 9(11)-double bond of this metabolite (**9**) could be similar to that proposed for metabolite **5**.

The rest of the fractions from the chromatographic separation were combined and acetylated, yielding the 2α,3β-diacetyl derivatives **10** and **11**. Compound **10** had molecular formula of $C_{35}H_{52}O_7$. Its ^{1}H NMR spectrum showed a singlet signal (δ_H 5.63) corresponding to the olefinic proton at C-12 (Table 1) and, in its ^{13}C NMR spectrum, a signal of a carbonyl group (δ_C 199.8) appeared (Table 2). The correlations observed in the HMBC spectrum of this compound (**10**) between the signal of H-9 (δ_H 2.36) and C-11, and between the signal of H-12 (δ_H 5.63) and C-9, C-14, and C-18 (Fig. 3), suggested the presence of an α,β-unsaturated system on the C ring of the molecule, with a carbonyl group at C-11 and a double bond between C-12 and C-13. Therefore, compound **10** had a structure of methyl 2α,3β-diacetoxy-11-oxoolean-12-en-28-oate. A possible mechanism to explain the formation of this compound (**10**) could be, as for metabolite **5**, the initial introduction of a hydroperoxyl group on the allylic position at C-11, by the microorganism (Martinez et al., 2013). Then, there would be the loss of a molecule of H_2O to form a carbonyl group at this position. A similar compound, with the free carboxyl group at C-28, was previously achieved in the biotransformation of maslinic acid (**2**) with the same microorganism (Martinez et al., 2013).

Compound **11** had a molecular formula of $C_{35}H_{54}O_7$. In its ^{1}H NMR spectrum, no signals of olefinic protons were detected, but a doublet signal (δ_H 2.60) appeared due to a vicinal proton (H-13) to a carbonyl group (C-12) (Table 1). This signal (H-13β, δ_H 2.60) was correlated with C-12, C-14, C-19, and C-27 in its HMBC spectrum, suggesting that this proton was located at C-13 (Fig. 3). In its ^{13}C NMR spectrum appeared two signals (δ_C 211.1 and 52.0) owing to a carbonyl group (C-12) and to a methine carbon (C-13), respectively (Table 2). The stereochemistry of C-13 was determined by a NOE correlation between H-13 and 3H-26, suggesting a β-disposition for this proton (Fig. 3). These data confirmed the structure of methyl 2α,3β-diacetoxy-12-oxo-13*R*-oleanan-28-oate for compound **11**.

This compound (**11**) was semi-synthesised from the 2α,3β-diacetyl derivative of methyl maslinate with MCPBA, which exhibited moderate cytotoxic activity on different cancer-cells lines (Siewert et al., 2014). The formation of this compound (**11**) could be explained

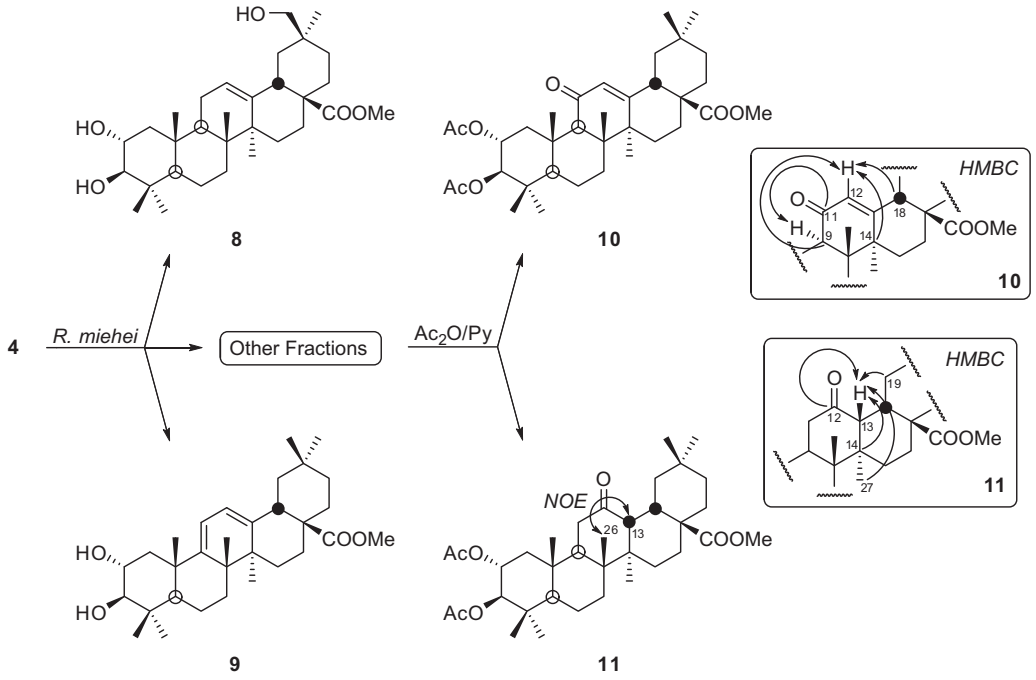

Fig. 3. Biotransformation of methyl maslinate (**4**) with *Rhizomucor miehei*. Key HMBC (C → H) correlations in compounds **10** and **11**.

Table 2
^{13}C NMR spectroscopic data (δ_C) for compounds **5**, **7**, **8**[a], **10**, **11**, **13**–**16**.

Position	5	7	8	10	11	13	14	15	16
1	37.5	37.6	48.1	44.2	43.6	46.1	43.9	43.7	43.6
2	28.9	27.9	68.9	69.2	69.8	68.9	69.6	69.5	69.7
3	78.6	78.5	84.2	80.8	80.4	83.9	80.2	79.8	80.4
4	38.8	38.8	40.2	39.5	39.5	39.3	39.5	39.3	39.5
5	48.0	48.5	56.2	54.8	54.9	55.6	54.8	51.7	55.0
6	29.4	28.2	19.2	17.4	18.3	17.9	17.5	24.5	18.3
7	71.8	71.5	33.4	32.8	31.8	34.2	32.9	75.6	31.8
8	48.5	49.3	40.1	42.2	41.4	41.3	42.7	47.1	41.5
9	154.7	154.7	48.4	61.5	49.8	46.5	51.0	51.2	49.9
10	39.2	39.4	38.9	38.2	38.1	38.5	38.4	38.5	38.1
11	117.2	117.4	24.2	199.8	38.7	21.7	37.5	36.5	38.7
12	121.2	122.4	123.3	128.0	211.1	56.0	205.5	204.8	210.6
13	145.1	145.1	144.5	169.0	52.0	67.2	91.0	91.0	51.8
14	41.9	47.7	42.4	43.6	42.0	40.3	44.1	45.7	42.1
15	27.9	66.7	28.5	27.9	27.7	25.5	26.0	28.3	27.6
16	24.3	33.0	24.1	22.7	22.8	23.8	20.8	21.0	23.1
17	46.0	45.4	47.3	46.1	47.5	46.1	43.8	43.6	47.2
18	39.5	40.1	41.8	41.5	32.1	44.4	44.1	44.1	31.7
19	41.5	41.1	41.9	44.6	36.3	40.5	37.4	37.5	32.1
20	35.3	35.2	36.1	30.8	30.8	30.7	31.7	31.8	34.0
21	30.1	28.8	29.7	33.8	34.6	34.2	34.3	34.2	30.0
22	31.6	31.4	33.0	31.7	33.1	32.9	27.4	27.2	32.5
23	28.3	28.3	29.7	28.5	28.4	28.7	28.4	28.3	28.4
24	15.7	15.8	18.1	17.7	17.6	20.4	18.6	14.5	16.2
25	25.0	25.4	17.2	17.5	16.5	16.7	17.4	17.5	17.5
26	13.4	12.9	17.6	19.3	16.3	16.5	17.0	17.0	16.5
27	20.6	13.3	26.6	23.7	20.7	22.1	18.2	18.0	20.6
28	178.1	177.7	178.4	182.5	178.5	178.4	178.4	178.2	177.9
29	27.4	27.4	28.7	33.0	33.5	33.2	33.3	33.3	28.4
30	66.3	66.1	65.7	23.5	23.3	23.5	23.9	23.9	67.4
MeOCO	52.0	52.2	52.0	51.0	52.0	51.8			52.1
MeCOO				171.1	170.9		178.4	170.7	171.6
MeCOO				170.4	170.6		170.5	170.5	170.9
MeCOO								170.3	170.5
MeCOO				21.2	21.3		21.2	21.8	21.2
MeCOO				21.0	21.1		21.0	21.2	21.1
MeCOO								21.0	21.0

The underlined term indicates the carbon atom to which the signal belongs in a functional group. **Me**COO corresponds to the signal of the methyl of the acetoxyl group. Me**CO**O corresponds to the signal of the carbonyl of the acetoxyl group. **MeO**CO corresponds to the signal of the methoxyl of the methoxycarbonyl group.

[a] ^{13}C NMR data for compound **8** are measured in C_5D_5N.

from an initial epoxidation of the double bond of the substrate (**4**), by the action of the fungus, and the subsequent rearrangement of this epoxy group to form a carbonyl group at C-12. The rearrangements of 12,13-epoxy oleananes to 12-oxo-13*R* derivatives have been achieved under mildly acidic conditions. These 12-oxo-13*R* derivatives have a less stable *cis* junction between rings C and D, and under conditions favouring keto–enol tautomerism tend to isomerise to the 12-oxo-13*R* derivatives, with a more stable *trans* junction between these rings (Farina and Pinza, 1987).

2.4. Biotransformation of compound **13**

The semi-synthesis of a 12,13-epoxy derivative, to check whether its biotransformation could lead to the formation of 12-oxo derivatives, was performed. A previous study states that the treatment of oleanolic acid (**1**) or methyl maslinate (**4**) with MCPBA does not produce epoxy derivatives (Siewert et al., 2014). Therefore, from maslinic acid (**2**) and through the 12α-bromo-28,13β-olide derivative (**12**), we produced the 12,13-epoxy derivative (**13**) (Fig. 4). Thus, treatment of maslinic acid (**2**) with Br_2/CCl_4 for 30 min originated compound **12** (82%) (Martinez et al., 2013). The subsequent reaction of this compound (**12**) with MeONa/MeOH (2%) gave the 12β,13β-epoxy derivative (**13**, 92%) (Fig. 4). In the ^{1}H NMR spectrum of this compound (**13**), no signal of geminal proton to a bromine atom was detected, but a doublet signal (δ_H 2.78) appeared due to the geminal proton to an epoxy group (C-12), and a singlet signal (δ_H 3.70) of a methoxyl group (C-28) (Table 3). This first signal (H-12, δ_H 2.78) was correlated with C-9, C-11, and C-13 in its HMBC spectrum, suggesting that this proton was located at C-12; and the second one (MeO, β_H 3.70) with C-28, indicating the disappearance of the lactone group, of its precursor (**12**), and the presence of a methyl ester group at this position (Fig. 4). In its ^{13}C NMR spectrum appeared two signals of oxygenate carbon atoms at δ_C 56.0 (C-12) and at δ_C 67.2 (C-13), characteristic of an epoxy group (Table 2). The stereochemistry of this epoxy group was determined by a NOE correlation between H-12 with H-11α and 3H-27, suggesting an α-disposition for this proton (Fig. 4). These data indicated that the structure of compound **13** was methyl 2α,3β-dihydroxy-12β,13β-epoxyoleanan-28-oate. A possible mechanism to explain the formation of this methyl 12β,13β-epoxyoleanan-28-oate derivative (**13**) could be an initial addition of a methoxide ion to the carbonyl group of the lactone ring of the 12α-bromo-28,13β-olide derivative (**12**). Then, the regeneration of the carbonyl group and the opening of the lactone ring could occur, forming an alkoxide ion at C-13. The subsequent attack of the alkoxide ion to C-12, by the β-face, with the output of the bromide ion would generate the 12β,13β-epoxy group (Martinez et al., 2015).

Table 3
^{1}H NMR data (δ_H) for compounds **13**–**16** (*J* in Hz).[a]

Position	**13**	**14**	**15**	**16**
1	1.88	2.04	2.05	1.93
	0.83	1.01	0.99	0.98
2	3.66 (ddd, 11.3, 9.5, 4.5)	5.06 (ddd, 10.4, 10.4, 4.7)	5.07 (ddd, 10.4, 10.4, 4.3)	5.05 (ddd, 10.4, 10.4
3	2.96 (d, 9.5)	4.71 (d, 10.4)	4.74 (d, 10.4)	4.71 (d, 10.4)
5	0.74	0.93	1.05	0.95
6	1.49	1.61	1.80	1.66
	1.43	1.52	1.60	1.43
7	1.41	1.51	5.13 (dd, 10.7, 5.1)	1.48
	1.23	1.34		1.34
9	1.38	1.57	1.51	1.67
11	1.89	2.72 (dd, 14.3, 14.3)	2.87 (dd, 14.3, 14.3)	2.20
	1.71	2.32 (dd, 14.3, 3.1)	2.33 (dd, 14.3, 2.9)	–
12	2.78 (d, 4.2)			
13				2.59 (d, 4.4)
15	2.24 (ddd, 13.0, 13.0, 4.7)	2.01	2.15	1.67
	1.10	1.29	1.14	1.08
16	1.92	2.12	2.09	1.89
	1.78	1.32	1.29	1.70
18	1.88	2.51 (dd, 8.3, 8.3)	2.51 (dd, 11.2, 4.8)	2.68 (m)
19	1.76	1.64	1.68	2.24
	1.24	–	–	1.18
21	1.34	1.32	1.31	1.48
	1.12	–	–	1.26
22	1.94	1.63	1.63	1.81
	1.38	–	–	1.46
23	1.01 (s)	0.88 (s)	0.88 (s)	0.89 (s)
24	0.80 (s)	0.90 (s)	0.89 (s)	0.90 (s)
25	0.91 (s)	1.04 (s)	1.06 (s)	0.98 (s)
26	0.97 (s)	1.30 (s)	1.46 (s)	0.95 (s)
27	1.17 (s)	0.94 (s)	1.05 (s)	0.93 (s)
29	0.93 (s)	0.96 (s)	0.98 (s)	0.93 (s)
30	0.89 (s)	0.94 (s)	0.95 (s)	4.18 (d, 11.1)
				3.96 (d, 11.1)
MeOCO	3.70 (s)		3.66 (s)	3.66 (s)
MeCOO		2.03 (s)	2.02 (s)	2.12 (s)
MeCOO		1.96 (s)	1.97 (s)	2.04 (s)
MeCOO			1.97 (s)	1.96 (s)

The underlined term indicates the carbon atom to which the signal belongs in a functional group. **Me**COO corresponds to the signal of the methyl of the acetoxyl group. Me**CO**O corresponds to the signal of the carbonyl of the acetoxyl group. **MeO**CO corresponds to the signal of the methoxyl of the methoxycarbonyl group.

[a] Assignments were based on ^{1}H-^{1}H COSY, HSQC, HMBC, DEPT, and NOESY experiments. Overlapped ^{1}H NMR signals are reported without designated multiplicity.

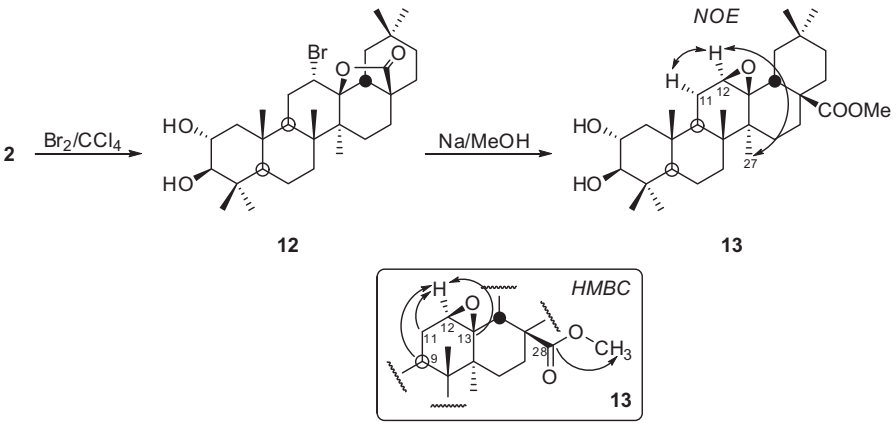

Fig. 4. Semi-synthesis of the epoxy derivative **13**. Key HMBC (C → H) correlations in compound **13**.

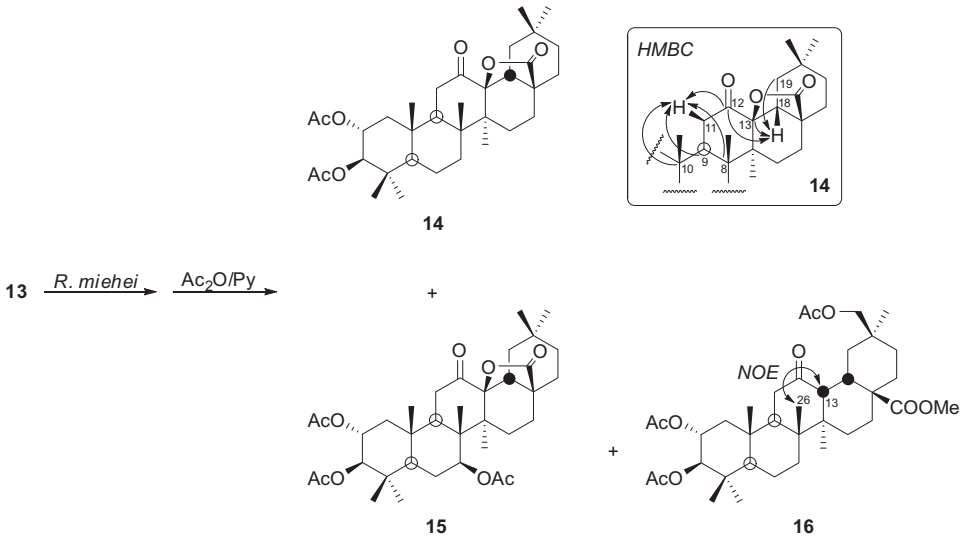

Fig. 5. Acetylated derivatives achieved from the incubation of the epoxy derivative **13** with *Rhizomucor miehei*. Key HMBC (C → H) correlations in compound **14**.

The microbial transformation of compound **13** by *R. miehei* for 13 days yielded a mixture of metabolites very difficult to separate. Chromatography on a silica-gel column of this mixture resulted in the recovery of 29% of the substrate (**13**). Acetylation of the rest of the fractions (49%) yielded the acetyl derivatives **14**, **15**, and **16** (Fig. 5).

The first acetyl derivative isolated (**14**) had a molecular formula of $C_{34}H_{50}O_7$. In its ^{1}H NMR spectrum, no signal of a geminal proton to an epoxy group was detected, but two double doublet signals (δ_H 2.72 and 2.32) appeared due to the vicinal protons (2H-11) to a carbonyl group (C-12) (Table 3). In the ^{13}C NMR spectrum of this compound (**14**) appeared a signal of a carbonyl group (δ_C 205.5, C-12) and a signal of carboxyl group (δ_C 178.4) together with a signal of a totally substituted oxygenated carbon (δ_C 91.0), these latter being characteristic of a lactone group between C-28 and C-13 (Table 2). The analysis of the HMBC spectrum of **14** showed a correlation between H-11β (δ_H 2.72) with C-8, C-9, C-10, and C-12, and between H-18 (δ_H 2.51) with C-12, C-13, and C-19 (Fig. 5). Then, compound **14** had a structure of 2α,3β-diacetoxy-12-oxooleanan-28,13β-olide.

The molecular formula ($C_{36}H_{52}O_9$) of the second acetyl derivative (**15**) indicated that this compound had one more acetoxyl group than compound **14**. The ^{1}H NMR spectra of these compounds (**14** and **15**) were very similar, the main difference being the presence, in the latter, of a signal of the geminal proton to the hydroxyl group at C-7 (δ_H 5.13, 1H, dd, J = 10.7, 5.1 Hz) (Table 3). Then, compound **15** had a structure of 2α,3β,7β-triacetoxy-12-oxooleanan-28,13β-olide (Fig. 5).

A possible mechanism to explain the formation of these compounds (**14** and **15**) could be an initial addition of oxygen at C-12 of substrate **13**, by the microorganism, with the formation of either an epoxide-hemiketal or a 12-hydroperoxy-13-ol intermediate that evolved towards a 13β-hydroxy-12-oxo group, as occurred in the biosynthesis of (+)-lycosantanolol from epoxy-lycosantalene (Zi et al., 2014). In the literature, other microbial transformations of an epoxide group to a α-hydroxyketone group are also shown (Gliszczynska and Wawrzenczyk, 2008; Yamada et al., 1992). This 13β-hydroxyl group could then attack the carboxymethyl group at C-28, probably due to their 1,3-diaxial dispositions, giving a 28,13β-lactone group.

The third acetyl derivative isolated (**16**) had a molecular formula of $C_{37}H_{56}O_9$. Its ^{1}H NMR spectrum was very similar to that of compound **11**, except for the presence in the first (**16**) of an AB system signal (δ_H 4.18 and 3.96), which was due to the coupling of two geminal protons to an acetoxyl group at C-30. The stereochemistry of C-13 was again determined by a NOE correlation between H-13 and 3H-26, suggesting a β-disposition for this proton (Fig. 5). Then, compound **16** had a structure of methyl 2α,3β,30-triacetoxy-12-oxo-13*R*-oleanan-28-oate. The formation of this 12-oxo group and the β-disposition for H-13, could also be explained through a rearrangement of the 12β,13β-epoxy group of the substrate **13**, in the same way as for compound **11**.

3. Concluding remarks

The filamentous fungus *R. miehei* can perform remarkable functionalization on these methyl ester derivatives of natural triterpene compounds. This microorganism has triggered hydroxylation reactions at non-activated positions, such as C-7, C-15, and C-30, this being difficult to accomplish by chemical synthetic means. Also, this fungus has produced derivatives with an extra double bond, or with an 11-oxo or a 12-oxo group, and derivatives with a 28,13β-lactone group from the carboxymethyl group present in these triterpene compounds. For all the metabolites isolated, we propose suitable mechanisms to explain the formation of these compounds.

4. Experimental

4.1. General experimental procedures

Measurements of NMR spectra were made in a VARIAN Inova unity (300 MHz ^{1}H NMR), and a VARIAN direct drive (400, 500, and 600 MHz ^{1}H NMR) spectrometers. Assignments of ^{13}C NMR chemical shifts (Tables 1 and 2) were made with the aid of distortionless enhancement by polarization transfer (DEPT) using a flip angle of 135°. Several programs were used for COSY, HSQC, HMBC, and NOE experiments. IR spectra were recorded in a MATTSON SATELLITE FT-IR spectrometer. High-resolution mass spectra were made in a WATERS LCT Premier XE spectrometer or by LSIMS (FAB) ionization mode in a MICROMASS AUTOSPEC-Q spectrometer (EBE geometry). Melting points (mp) were determined using a Kofler (Reichert) apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer 431 polarimeter at 25 °C. Incubations of substrates with the microorganism

were shaken in CERTOMAT R B. Braun orbital shakers. Commercially available reagents were used without further purification. Merck silica-gel 60 (0.040–0.063 mm, Ref. 1.09385) was used for flash chromatography. CH_2Cl_2 (Fisher, Ref. D/1852/17) or n-hexane (Merck, Ref. 1.04374), with increasing amounts of Me_2CO (Fisher, Ref. A/0600/17), MeOH (Fisher, Ref. M/4000/17), or AcOEt (Fisher, Ref. E/0900/17), were used as eluents (all the solvents had an analytical reagent grade purity). Merck silica-gel 60 aluminium sheets (Ref. 1.16835) were used for TLC, and spots were rendered visible by spraying with H_2SO_4–AcOH, followed by heating to 120 °C, and also visualized under UV at 254 nm.

4.2. Organism, media and culture conditions

R. miehei (CECT 2749) was obtained from the Spanish Type Culture Collection (CECT), Departamento de Microbiología, Universidad de Valencia, Spain. Medium PDA containing 4 g/l of potato peptone, 20 g/l of glucose, 2 g/l of agar, at pH 5.6, was used to store the microorganisms. In all microbial transformation experiments, a medium of potato dextrose broth (Scharlau 02-483) was used for microorganism proliferation. Erlenmeyer flasks (250 ml) containing 90 ml of sterilized medium were inoculated with 1 ml of microorganism suspended in saline suspension (9%). Incubations were maintained at 28 °C with gyratory shaking (150 rpm) for 6 days, after which the substrates (5–10%) in EtOH were added.

4.3. Isolation of oleanolic (**1**) and maslinic (**2**) acids

Oleanolic (3β-hydroxyolean-12-en-28-oic acid, **1**) and maslinic (2α,3β-dihydroxyolean-12-en-28-oic acid, **2**) acids were isolated from solid wastes resulting from olive-oil production, which were extracted in a Soxhlet with hexane and EtOAc successively (Garcia-Granados, 1998). Both acids were purified from these mixtures by column chromatography over silica gel, eluting with a $CHCl_3$/MeOH or CH_2Cl_2/acetone mixtures of increasing polarity (Martinez et al., 2013).

4.4. Esterification of oleanolic acid (**1**)

Oleanolic acid (**1**, 7.1 g, 15.3 mmol) was dissolved in THF (25 ml) and then, 14.2 ml of a solution of NaOH 5 N were added. The reaction was kept for 3 h at reflux. Then, 2.84 ml of CH_3I were added to the reaction mixture, keeping it under reflux 2 h. Afterwards, the reaction mixture was washed with water, neutralized with a HCl solution, and extracted repeatedly with CH_2Cl_2. The organic phase was dried with dry Na_2SO_4, and evaporated under reduced pressure. After chromatographic purification of the mixture, 6.5 g of methyl 3β-hydroxyolean-12-en-28-oate (methyl oleanolate, **3**, 90%) were isolated (Garcia-Granados et al., 2000).

4.5. Esterification of maslinic acid (**2**)

Maslinic acid (**2**, 12.3 g, 26 mmol) was dissolved in THF (25 ml) and then, 24.6 ml of a solution of NaOH 5 N were added, keeping the reaction for 3 h at reflux. Then, 4.92 ml of CH_3I were added to the reaction mixture, keeping it under reflux 2 h more. This mixture was treated as described above in the esterification of oleanolic acid (**1**) to give 11.4 g of methyl 2α,3β-dihydroxyolean-12-en-28-oate (methyl maslinate **4**, 90%) (Garcia-Granados et al., 2000).

4.6. Incubation of methyl oleanolate (**3**) with R. miehei

Methyl oleanolate **3** (150 mg, 0.32 mmol) was dissolved in EtOH (10 ml), distributed among 5 Erlenmeyer flask cultures (*R. miehei*), and incubated for 13 days. Then, the cultures were filtered and pooled, the cells were washed thoroughly with water, and the liquid was saturated with NaCl and extracted continuously with CH_2Cl_2 for four 6-h periods. Dry fungal cells were washed repeatedly with CH_2Cl_2. Both extracts were pooled, dried with dry Na_2SO_4, and evaporated under reduced pressure. The resulting mixture of compounds was chromatographed on a silica-gel column to provide 108 mg of starting material (**3**, 72%), and 24 mg of methyl 3β,7β,30-trihydroxyoleana-9(11),12-dien-28-oate (**5**, 15%).

4.6.1. Methyl 3β,7β,30-trihydroxyoleana-9(11),12-dien-28-oate (**5**)

Syrup; $[\alpha]_D^{25}$ +70 (*c* 1, $CHCl_3$); IR (film) ν_{max} 3457, 2947, 1723, 1240, 760 cm^{-1}; for ^1H NMR (400 MHz, $CDCl_3$) see Table 1; for ^{13}C NMR (100 MHz, $CDCl_3$) see Table 2; HRESIMS *m*/*z* 501.3567 $[M+1]^+$ (calcd. for $C_{31}H_{49}O_5$, 501.3580).

4.7. Treatment of methyl oleanolate (**3**) with NBS

Methyl oleanolate (**3**, 1 g, 2.12 mmol) was dissolved in CCl_4 (25 ml), and NBS (378 mg, 2.12 mmol) and a catalytic amount of AIBN, were added. The reaction was kept for 1 h at reflux. Then, the reaction mixture was treated with an aqueous solution of $NaHCO_3$, and the organic phase was extracted repeatedly with CH_2Cl_2, dried with dry Na_2SO_4, and evaporated under reduced pressure. The chromatographic purification gave 785 mg of methyl 3β-hidroxyoleana-9(11),12-dien-28-oate (**6**, 79%) (Garcia-Granados et al., 2004).

4.8. Incubation of **6** with R. miehei

Product **6** (500 mg, 1.07 mmol) was dissolved in EtOH (16 ml), distributed among 8 Erlenmeyer flask culture (*R. miehei*) and incubated for 13 days. Then, we proceeded as described above in the incubation of substrate **3** with the same microorganism. After chromatographic purification by flash column, 310 mg of starting material (**6**, 62%), 59 mg of metabolite **5** (11%), and 44 mg of methyl 3β,7β,15α,30-tetrahydroxyoleana-9(11),12-dien-28-oate (**7**, 8%), were isolated.

4.8.1. Methyl 3β,7β,15α,30-tetrahydroxyoleana-9(11),12-dien-28-oate (**7**)

White solid; mp 134 °C; $[\alpha]_D^{25}$ +5 (*c* 1, $CHCl_3$); IR (KBr) ν_{max} 3360, 1722, 1374, 1243, 756 cm^{-1}; for ^1H NMR (500 MHz, $CDCl_3$) see Table 1; for ^{13}C NMR (125 MHz, $CDCl_3$) see Table 2; HRESIMS *m*/*z* 517.3521 $[M+1]^+$ (calcd. for $C_{31}H_{49}O_6$, 517.3529).

4.9. Incubation of methyl maslinate (**4**) with R. miehei

Methyl maslinate (**4**, 600 mg, 1.27 mmol), was dissolved in EtOH (40 ml), distributed among 20 Erlenmeyer flask culture (*R. miehei*) and incubated for 13 days. Next, we proceeded as described above in the incubation of substrate **3** with the same microorganism. The resulting mixture of compounds was chromatographed on a silica-gel column to provide 378 mg of starting material (**4**, 63%), 74 mg of methyl 2α,3β,30-trihydroxyolean-12-en-28-oate (**8**, 12%), 41 mg of methyl 2α,3β-dihydroxyoleana-9(11),12-dien-28-oate (**9**, 7%) (Garcia-Granados et al., 2004). The rest of the fractions (50 mg, 8%) were acetylated with Ac_2O (2 ml) in pyridine (4 ml). After the reaction was maintained for 24 h at room temperature, it was diluted with cold H_2O, extracted with CH_2Cl_2, washed with saturated aqueous $KHSO_4$, and dried over dry Na_2SO_4. Chromatography on silica gel of the reaction mixture gave 28 mg of methyl 2α,3β-diacetoxy-11-oxoolean-12-en-28-oate (**10**) and 16 mg of methyl 2α,3β-diacetoxy-12-oxo-13*R*-oleanan-28-oate (**11**).

*4.9.1. Methyl 2α,3β,30-trihydroxyolean-12-en-28-oate (**8**)*

White solid; mp 223 °C with decomposition; $[\alpha]_D^{25}$ +19 (*c* 1, $CHCl_3$:MeOH 2:1); IR (KBr) ν_{max} 3454, 2980, 2873, 2342, 1387, 1219, 772 cm^{-1}; for ^1H NMR (500 MHz, C_5D_5N) see Table 1; for ^{13}C NMR (125 MHz, C_5D_5N) see Table 2; HRESIMS *m*/*z* 503.3720 $[M+1]^+$ (calcd. for $C_{31}H_{51}O_5$, 503.3736).

*4.9.2. Methyl 2α,3β-diacetoxy-11-oxoolean-12-en-28-oate (**10**)*

White solid; mp 83 °C; $[\alpha]_D^{25}$ +34 (*c* 1, $CHCl_3$); IR (KBr) ν_{max} 2925, 1739, 1367, 1246, 756 cm^{-1}; for ^1H NMR (500 MHz, $CDCl_3$) see Table 1; for ^{13}C NMR (125 MHz, $CDCl_3$) see Table 2; HRESIMS *m*/*z* 585.3792 $[M+1]^+$ (calcd. for $C_{35}H_{53}O_7$, 585.3791).

*4.9.3. Methyl 2α,3β-diacetoxy-12-oxo-13R-oleanan-28-oate (**11**)*

White solid; mp 79 °C; $[\alpha]_D^{25}$ +27 (*c* 1, $CHCl_3$); IR (KBr) ν_{max} 2925, 1738, 1368, 1246, 755 cm^{-1}; for ^1H NMR (600 MHz, $CDCl_3$) see Table 1; for ^{13}C NMR (150 MHz, $CDCl_3$) see Table 2; HRESIMS *m*/*z* 587.3932 $[M+1]^+$ (calcd. for $C_{35}H_{55}O_7$, 587.3948).

*4.10. Bromination of maslinic acid (**2**)*

Maslinic acid (**2**, 500 mg) was dissolved in CCl_4 (5 ml), and 15 ml of a solution of bromine dissolved in CCl_4 (8.7×10^{-2} M) were added. The mixture was kept for 30 min at room temperature, and then the solvent was evaporated and purified on column, yielding 478 mg of 12α-bromo-2α,3β-dihydroxyoleanan-28,13β-olide (**12**, 82%) (Martinez et al., 2013).

*4.11. Treatment of **12** with MeONa/MeOH*

Product **12** (300 mg, 0.54 mmol) was dissolved in 25 ml of a solution of sodium metal in MeOH (2%). The reaction mixture was kept for 12 h at room temperature. Then, 200 ml of H_2O were added to the reaction mixture, which was extracted five times with CH_2Cl_2 (50 ml). The organic phase was dried with dry Na_2SO_4 and evaporated under reduced pressure. After chromatographic purification 252 mg of methyl 2α,3β-dihydroxy-12β,13β-epoxyoleanan-28-oate (**13**, 92%), were isolated.

*4.11.1. 2α,3β-dihydroxy-12β,13β-epoxyoleanan-28-oate (**13**)*

White solid; mp 217 °C; $[\alpha]_D^{25}$ +28 (*c* 1, $CHCl_3$); IR (KBr) ν_{max} 2947, 1719, 1367, 1241, 863, 759 cm^{-1}; for ^1H NMR (300 MHz, $CDCl_3$) see Table 3; for ^{13}C NMR (75 MHz, $CDCl_3$) see Table 2; HRESIMS *m*/*z* 503.3720 $[M+1]^+$ (calcd. for $C_{31}H_{51}O_5$, 503.3736).

*4.12. Incubation of **13** with R. miehei*

Product **13** (150 mg, 0.30 mmol) was dissolved in EtOH (10 ml) and distributed among 5 Erlenmeyer flask cultures (*R. miehei*) and incubated for 13 days. Then, we proceeded as described above in the incubation of substrate **3** with the same microorganism. The resulting mixture of compounds was chromatographed in a silica-gel column to provide 44 mg of starting material (**13**, 29%). The rest of the fractions (49%) were acetylated with Ac_2O (1 ml) in pyridine (2 ml). After the reaction was maintained for 24 h at room temperature, it was diluted with cold H_2O, extracted with CH_2Cl_2, washed with saturated aqueous $KHSO_4$, and dried over dry Na_2SO_4. Chromatography on silica gel of the reaction mixture gave 12 mg of 2α,3β-diacetoxy-12-oxooleanan-28,13β-olide (**14**), 45 mg of 2α,3β,7β-triacetoxy-12-oxooleanan-28,13β-olide (**15**), and 21 mg of methyl 2α,3β,30-triacetoxy-12-oxo-13*R*-oleanan-28-oate (**16**).

*4.12.1. 2α,3β-Diacetoxy-12-oxooleanan-28,13β-olide (**14**)*

Syrup; $[\alpha]_D^{25}$ −31 (*c* 1, $CHCl_3$); IR (film) ν_{max} 3431, 2951, 2359, 1368, 1253, 755 cm^{-1}; for ^1H NMR (300 MHz, $CDCl_3$) see Table 3; for ^{13}C NMR (75 MHz, $CDCl_3$) see Table 2; HRESIMS *m*/*z* 571.3638 $[M+1]^+$ (calcd. for $C_{34}H_{51}O_7$, 571.3635).

*4.12.2. 2α,3β,7β-Triacetoxy-12-oxooleanan-28,13β-olide (**15**)*

White solid; mp 210 °C; $[\alpha]_D^{25}$ +35 (*c* 1, $CHCl_3$); IR (KBr) ν_{max} 2949, 2866, 1735, 1370, 1246, 769 cm^{-1}; for ^1H NMR (300 MHz, $CDCl_3$) see Table 3; for ^{13}C NMR (75 MHz, $CDCl_3$) see Table 2; HRESIMS *m*/*z* 629.3699 $[M+1]^+$ (calcd. for $C_{36}H_{53}O_9$, 629.3690).

*4.12.3. Methyl 2α,3β,30-triacetoxy-12-oxo-13R-oleanan-28-oate (**16**)*

White solid; mp 151 °C; $[\alpha]_D^{25}$ −5 (*c* 1, $CHCl_3$); IR (KBr) ν_{max} 2958, 2359, 1790, 1735, 1372, 1240, 772 cm^{-1}; for ^1H NMR (300 MHz, $CDCl_3$) see Table 3; for ^{13}C NMR (75 MHz, $CDCl_3$) see Table 2; HRESIMS *m*/*z* 645.4011 $[M+1]^+$ (calcd. for $C_{37}H_{57}O_9$, 645.4003).

Acknowledgements

This work was financially supported by Grants from the "Consejería de Innovación, Ciencia y Empresa" of the "Junta de Andalucía" (FQM–7372), and the "Plan Propio" of the University of Granada. We thank David Nesbitt for reviewing the English of the manuscript.

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