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Lipase-catalysed preparation of acyl derivatives of the germacranolide cnicin

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ABSTRACT

Several acyl derivatives of cnicin were obtained through lipase-catalysed acylation and alcoholysis reactions. In most reactions lipases showed a regioselective behaviour affording only one product. Longer chain acyl derivatives were prepared at lower temperature than the used in lipase-catalysed reactions, to preclude side products formation. The enzymatic approach let to prepare a family of novel acetyl and fatty acid derivatives of cnicin which are not obtainable following traditional organic synthetic procedures.

1. Introduction

Sesquiterpene lactones are a class of secondary metabolites found in some plant families, mainly in several genera of Asteraceae, such as *Centaurea* [1]. This genus contains about 1000 species and the aerial parts of several species are used in the popular medicine and, in some cases, evidence for antimicrobial [2], antibacterial [3], cytotoxic and phytotoxic [4] activities has been pointed out.

The most important metabolites in genus *Centaurea* are acetylenic compounds, flavonoids and terpenoids, mainly sesquiterpenes with guaiane, germacrane, elemane and eudesmane skeletons. Often, among the sesquiterpenes, the most abundant one is the germacranolide cnicin (**1**), sometimes isolated in grams quantity and whose antibacterial cytotoxic and antifungal properties have been investigated (Scheme 1) [5–7].

Regarding its antibacterial activity, it is interesting to observe that the presence of an ester at C-8 of the germacranolide is important for the activity, being the esterified derivatives more active against Gram-positive bacteria [3]. Cnicin is a potent and irreversible inhibitor of the bacterial enzyme Mur A (EC 2.5.1.7) which is responsible for the first step in the cytoplasmatic biosynthesis of peptidoglycan precursor molecules. The Mur-A-dependent metabolites are of vital importance for bacteria, and the enzyme is therefore in the focus of several drug development projects in academic and industrial groups [8]. The first explanation of the antibacterial mode of action of cnicin on a molecular basis takes into account the α,β-unsaturated carbonyl system in the side chain of the molecule. A Michael addition on the electrophilic double bond of cnicin by the thiol group of Cys115 of Mur-A, which results in a stable binding, is proposed. As a consequence of the alkylation of this important residue, the enzyme closes that active site completely.

Recently, we reported a study on the chemical reactivity of cnicin towards oxidative processes [9]. We have also studied the structure–cytotoxic activity relationships of cnicin, and confirmed that an α-exo-methylene-γ-lactone is a necessary feature for the cytotoxic effect of cnicin and other sesquiterpene lactones [10].

The presence in cnicin (**1**) of three free hydroxyl groups prompted us to synthesize various esters. The position of the ester group could influence the numerous biological activities displayed by cnicin. Due to the high reactivity of the germacranolide nucleus, it was not possible to achieve significant results by means of conventional synthetic methods, and a mixture of products was always obtained. Therefore, we decided to apply an enzymatic approach.

It is well known that lipases are efficient as biocatalysts for chemo-, regio- and stereoselective reactions under mild conditions [11,12]. They can be used in a wide variety of organic solvents and do not require a coenzyme for activity [13].

In previous works, we reported the regioselective acylation of natural products as polyhydroxy compounds by means of alkyl carboxylates or carboxylic acids under the catalysis of enzymes in

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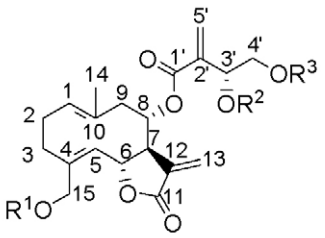


Compound	R^1	R^2	R^3
1	H	H	H
2	H	H	Ac
3	Ac	H	Ac
4	H	H	$CH_3(CH_2)_4CO$
5	H	H	$CH_3(CH_2)_8CO$
6	H	H	$CH_3(CH_2)_{12}CO$
7	H	H	*cis*-$CH_3(CH_2)_7CH{=}CH(CH_2)_7CO$
8	Ac	Ac	Ac
9	Ac	Ac	H
10	Ac	H	H

Scheme 1.

anhydrous organic solvents [14,15]. In the steroid field, enzyme catalysis can play an important role for the mild and selective interconversion of functional groups via regio- and stereoselective transformations [16,17]. Studies carried out in our laboratory on the esterification and transesterification of polyfunctionalyzed steroids, have shown that lipases can act on substituents either on A-ring or on the D-ring [18–20]. Taking into account these properties we have prepared series of fatty acid derivatives of dehydroepiandrosterone [21] and 3,17-β-estradiol [22] and alkyl succinates of a series of pregnanes [23].

Regarding terpenoids, lipases catalysed the regioselective acylation of polyhydroxylated sesquiterpenoids [24] and, by using both lipases and esterases, we performed regio- and stereoseletive acetylation and alcoholysis reactions of *ent*-kauranes [25]. Here we report the use of lipases in the preparation of acyl derivatives of the germacranolide cnicin (**1**) (Scheme 1).

2. Experimental

2.1. General remarks

All solvents and reagents were reagent grade and used without purification. Lipase from *Candida rugosa* CRL (0.905 U/g solid), and type II crude from porcine pancreas (190,000 U/g protein) were purchased from Sigma Chemical Co.; *Candida antarctica* lipase A: Chirazyme L-5, c.-f. lyo (400 U/g) and *C. antarctica* lipase B: Chirazyme L-2, c.-f., C3, lyo (6300 U/g) were purchased from Roche Diagnostics GmbH; Lipozyme RM 1M (7800 U/g) was a generous gift of Novozymes Latinoamerica Ltda.; *Pseudomonas* lipase: Lipase PS Amano PSL (33,200 U/g) was purchased to Amano Pharmaceutical Co. All enzymes were used "straight from the bottle".

Enzymatic reactions were carried out on a Sontec incubator shaker (Scientifica, Bs. As., Argentina) at 10, 33 and 55 °C and 200 rpm. Enzymatic transesterifications were followed by TLC on Merck silica gel 60F-254 aluminium sheets (0.2 mm thickness). For column chromatography, Merck silica gel 60 (70–230 mesh) was used. IR spectra were obtained on a Shimadzu FTIR-8300 spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded at 500 MHz with Bruker AM 500 spectrometer. Chemical shifts are reported in δ units relative to tetramethylsilane (TMS) set at 0 δ, and coupling constants are given in Hz. Solvents are indicated. Elemental analysis was carried out with a CE-440 Elemental Analyzer. Optical purities of products were determined by specific rotation with PerkinElmer 343 and Jasco P-1010 polarimeters. Solvents are indicated. Microwave reactions were carried in a SEM Discover monomode reactor using a closed vessel with magnetic stirring.

2.2. Molecular modelling

Conformational search was performed using AM1 semi empirical method integrated on HyperChem 7.5. The whole structure was first minimized and then the Conformational Search algorithm integrated on the same software was employed to find local minima. The torsions to vary were C5–C4–C15–O and C4–C15–O–H. Fig. 1 was captured from the graphical interface of HyperChem 7.5.

2.3. Isolation of (6R,7R,8S,1″R)-[(1″,2″-dihydroxyethyl)acryloyl]-15-hydroxygermacra-1 (**10**),4,11 (**13**)-trien-6,12-olide (cnicin) (**1**)

Cnicin (**1**) was isolated from *Centaurea sphaerocephala* and *Centaurea napifolia* both collected in June 2006 at Lascari, 60 km east of Palermo, Italy following the purification procedures reported previously [26,27]. ^{1}H and ^{13}C NMR: Tables 1 and 2.

2.4. Chemical and microwave assisted acetylation

2.4.1. 8-α-(3′,4′-diacetoxy-2′-methylene-butanoyloxy)-15-acetyl-11,13-dehydromelitensine (**11**)

50 mg (0.13 mmol) of cnicin were dissolved in 2 ml of acetic anhydride and heated under microwave irradiation (70 W) at 100 °C for 30 min. The reaction was then quenched with methanol and the solvent was evaporated. A colorless oil was obtained (63 mg, 93% yield). $[\alpha]_D^{25} = +67.8$ (c = 0.02, $CHCl_3$, IR (film) ν_{max} = 2931, 1777, 1743, 1718, 1369, 1222, 1136, 1044, 966 cm^{-1}. ^{1}H and ^{13}C NMR: Tables 1 and 2. Anal. calcd. for $C_{26}H_{32}O_{10}$: C 61.90%; H 6.39%. Found: C 61.79; H 6.47%.

2.4.2. Cnicin 4′,15-diacetate (**3**) and cnicin triacetate (**8**)

They were prepared according to a procedure previously reported. Physical and spectral data are according to those previously reported [28].

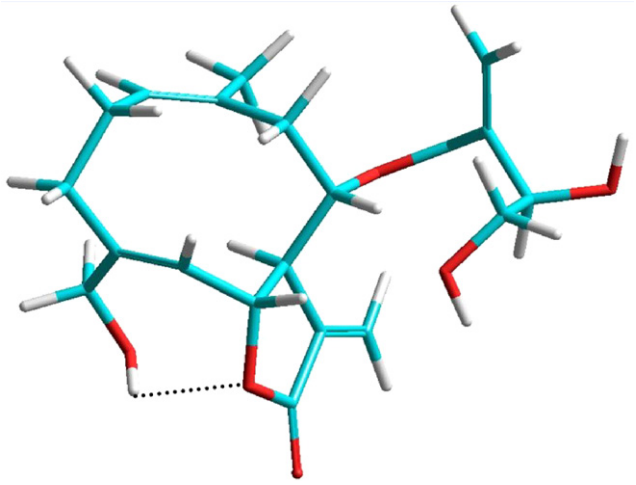


Fig. 1. Structural representation of a conformer (local minimum) of cnicin. A hydrogen bonding (black dotted line) between hydrogen on hydroxyl 15 and lactone oxygen, forms a seven-membered ring. Bond distance is 2.2 Å.

Table 1
^{1}H NMR data of compounds **1**, **4**–**7**, **9**–**11**

H no.	1	4	5	6	7	9	10	11
1	5.07 brd (7.0)	5.00 brd (7.0)	5.00 brd (7.0)	5.00 brd (7.0)	5.00 brd (7.0)	5.00 brd (7.0)	5.04 brd (7.0)	5.76 dd (17.4, 11.0)
2a	2.23 m	2.21 m	2.21 m	2.23 m	2.22 m	2.22 m	2.27 m	5.07 brd (11.0)
2b	2.23 m	2.21 m	2.21 m	2.23 m	2.22 m	2.22 m	2.27 m	5.02 brd (17.4)
3a	2.55 m	2.58 m	2.58 m	2.60 m	2.60 m	2.52 m	2.54 m	5.43 brs
3b	2.01 m	1.97 m	1.97 m	1.97 m	1.97 m	2.06 m	2.09 m	5.04 brs
5	4.95 d (10.0)	4.81 d (10.0)	4.81 d (10.0)	4.82 d (10.0)	4.82 d (10.0)	4.93 d (10.0)	5.06 d (10.0)	2.47 d (12.2)
6	5.16 dd (10.0, 8.5)	5.11 dd (10.0, 8.5)	5.11 dd (10.0, 8.5)	5.11 dd (10.0, 8.5)	5.12 dd (10.0, 8.5)	4.90 dd (10.0, 8.5)	5,14 dd (10.0, 8.5)	4.22 t (11.2)
7	3.12 m	3.09 m	3.09 m	3.09 m	3.09 m	3.11 m	3.26 m	2.95 dddd (11.2, 10.8, 3.0, 2.8)
8	5.21 brdd (12.0, 11.5)	5.17 brdd (12.0, 11.5)	5.17 brdd (12.0, 11.5)	5.19 brdd (12.0, 11.5)	5.19 brdd (12.0, 11.5)	5.17 brdd (12.0, 11.5)	5.08 brdd	5.31 td (10.8, 4.6)
9a	2.65 brd (11.5)	2.60 brd (11.5)	2.60 brd (11.5)	2.60 brd (11.5)	2.60 brd (11.5)	2.61 brd (11.5)	2.58	2.09 o.s.
9b	2.59 dd (12.0, 12.0)	2.49 dd (12.0, 12.0)	2.49 dd (12.0, 12.0)	2.49 dd (12.0, 12.0)	2.49 dd (12.0, 12.0)	2.51 dd (12.0, 12.0)	2.52	1.69 dd (12.8, 10.8)
13a	6.39 d (3.5)	6.27 d (3.5)	6.27 d (3.5)	6.28 d (3.5)	6.28 d (3.5)	6.32 d (3.5)	6.25 d (3.5)	6.15 d (3.0)
13b	5.81 d (3.0)	5.75 d (3.0)	5.75 d (3.0)	5.75 d (3.0)	5.75 d (3.0)	5.74 d (3.0)	5.84 d (3.0)	5.57 d (2.8)
Me-14	1.54 s	1.50 s	1.50 s	1.50 s	1.50 s	1.51 s	1.55 s	1.17 s
15 a	4.27 d (15.0)	4.29 d (15.0)	4.29 d (15.0)	4.30 d (15.0)	4.30 d (15.0)	4.63 d (16,0)	4.72 s	4.51 brs
15 b	4.01 d (15.0)	4.09 d (15.0)	4.09 d (15.0)	4.10 d (15.0)	4.10 d (15.0)	4.59 d (16,0)		
3′	4.53 dd (10.0, 8.5)	4.70 dd (7.0, 3.5)	4.70 dd (7.0, 3.5)	4.70 dd (7.0, 3.5)	4.70 dd (7.0, 3.5)	5.62 dd (7.0, 3.5)	4.55 dd (7.0, 3.5)	5.83 t (4.8)
4′a	3.71 dd (11.5, 3.5)	4.28 dd (11.5, 3.5)	4.28 dd (11.5, 3.5)	4.29 dd (11.5, 3.5)	4.29 dd (11.5, 3.5)	3.88 dd (11.5, 3.5)	3.76 dd (11.5, 3.5)	4.29 d (4.8)
4′b	3.47 dd (11.5, 7.0)	4.21 dd (11.5, 7.0)	4.21 dd (11.5, 7.0)	4.21 dd (11.5, 7.0)	4.21 dd (11.5, 7.0)	3.79 dd (11.5, 7.0)	3.51 dd (11.5, 7.0)	
5′a	6.39 brs	6.38 brs	6.38 brs	6.38 brs	6.38 brs	6.39 brs	6.41 brs	6.40 brs
5′b	6.09 brs	6.10 brs	6.10 brs	6.09 brs	6.09 brs	5.98 brs	6.13 brs	5.96 brs
OR^{1}	–	–	–	–	–	2.11 s	2.12 s	2.12 s
OR^{2}	–	–	–	–	–	2.13 s	–	2.09 s
OR^{3}	–	2.32 t (7.5), 1.61 m, 1.30 m, 0.89 t (7.5)	2.33 t (7.5), 1.61 m, 1.25 m, 0.87 t (7.5)	2.32 t (7.5), 1.61 m, 1.25 m, 0.87 t (7.5)	5,34 m; 2.34 t (7.5), 1.61 m, 1.30 m, 0.91 t (7.5)	–	–	2.05 s

Solvent: **1**: CD_3OD; **4–9** and **11**: $CDCl_3$; **10**: $CDCl_3/CD_3OD$.

Table 2
^{13}C NMR of compounds **1**–**11**

C no.	1	2	3	4	5	6	7	8	9	10	11
1	129.74	129.81	129.63	129.81	129.81	129.83	129.82	129.71	129.66	130.72	145.29
2	26.91	26.29	26.14	26.30	26.33	26.35	26.32	26.25	26.20	25.91	117.12
3	35.19	34.68	34.84	34.69	34.72	34.73	34.72	34.98	34.80	34.54	113.45
4	142.31	143.96	138.92	143.91	143.93	143.87	143.89	138.86	138.91	139.18	138.35
5	130.84	128.39	130.61	128.41	128.41	128.44	128.43	130.63	130.70	129.52	51.35
6	78.70	76.52	76.35	76.46	76.44	76.43	76.43	73.36	76.26	77.13	78.16
7	54.05	52.93	52.82	52.94	52.96	52.96	52.95	52.84	52.80	52.69	52.26
8	71.96	73.29	73.07	73.29	73.29	73.31	73.31	69.42	73.26	72.94	69.58
9	49.35	48.59	48.44	48.60	48.61	48.61	48.61	48.42	48.45	48.03	44.82
10	133.24	132.26	132.09	132.32	132.32	132.38	132.41	132.28	132.12	132.11	41.92
11	172.11	135.40	135.00	135.41	135.40	135.42	135.27	135.00	134.91	135.32	136.45
12	137.45	169.75	169.39	169.69	169.67	169.63	169.65	169.42	169.33	170.46	169.80
13	125.26	125.38	125.20	125.27	125.28	125.27	124.34	125.32	125.50	125.05	120.22
14	17.14	16.73	16.80	16.73	16.73	16.76	16.74	16.86	16.70	16.30	18.64
15	60.83	61.41	61.66	61.48	61.52	61.58	61.54	61.73	61.64	65.47	67.31
1′	166.54	164.54	164.43	164.52	164.50	164.50	164.51	163.57	164.10	165.10	163.75
2′	145.50	138.71	138.60	138.74	138.71	138.71	138.71	136.31	136.60	140.37	136.04
3′	74.48	69.49	69.33	69.66	69.69	69.75	69.71	69.42	72.40	70.56	69.80
4′	66.70	67.26	67.20	67.08	67.09	67.11	67.11	63.90	63.81	65.47	64.00
5′	127.29	127.75	127.72	127.70	127.70	127.70	127.70	128.06	127.68	126.57	128.34
OR[1]	–	–	20.84, 170.59	–	–	–	–	20.89, 170.59	20.96, 170.72	20.18, 171.21	20.91, 170.47
OR[2]	–	–	–	–	–	–	–	20.89, 170.40	21.04, 169.74	–	20.95, 169.55
OR[3]	–	20.79, 171.30	20.73, 171.18	34.05, 174.20, 31.22, 24.54, 22.26, 13.86	174.22, 31.83, 29.39, 29.22, 29.09, 29.04, 24.88, 24.70, 22.63, 14.08	174.22, 34.10, 31.90, 29.63, 29.24, 29.10, 24.89, 23.72, 22.96, 22.67, 14.10, 29.59, 29.44, 29.33, 29.24, 29.10, 24.89, 23.72, 22.96, 22.67, 14.10	174.18, 130.00, 129.70, 33.80, 31.90, 29.74, 29.65, 29.49, 29.29, 29.14, 29.12, 29.07, 29.04, 29.01, 27.20, 27.14, 22.65, 14.09	20.70, 171.52	–	–	20.70, 170.61

Solvent **1**: CD_3OD; **2**–**9** and **11**: $CDCl_3$; **10**: $CDCl_3/CD_3OD$.

2.5. Enzymatic acylation

2.5.1. Acetate derivatives of cnicin

*2.5.1.1. Cnicin 4′-acetate (**2**).* Cnicin (**1**) (25 mg, 0.07 mmol) was suspended in ethyl acetate (10 ml) and the amount equivalent to 1500 units of the indicated lipase was added. The mixture was shaken overnight at 33 or 55 °C at 200 rpm and the progress of the reaction was monitored by TLC. Once completed, the enzyme was filtered off and the solvent evaporated and the crude residue purified by flash chromatography (eluent:hexane-AcOEt), yielding a colorless oil (25.6 mg, yield 92%). Physical data according to those previously reported [29,30]. ^{13}C NMR: Table 2.

*2.5.1.2. Cnicin 4′,15-diacetate (**3**).* Cnicin (**1**) (40 mg, 0.11 mmol) was dissolved in acetonitrile (25 ml), and vinyl acetate (1 ml, 865 mmol) and CAL B (400 mg) were added. The mixture was shaken overnight at 55 °C at 200 rpm. Once completed, the enzyme was filtered off and the solvent evaporated. Two products were separated by flash chromatography (AcOEt:hexane 3:7): 29.8 mg of **2** (81% yield) and 12.1 mg of **3** (17% yield) were isolated. Physical and spectral data according to those previously reported [28]. ^{13}C NMR: Table 2.

2.5.1.3. Long chain carboxylic acid derivatives of cnicin: general procedure. Cnicin (**1**) (40 mg, 0.11 mmol) was dissolved in acetonitrile (25 ml). The acylating agent, the corresponding carboxylic acid or ethyl ester (0.20 mmol) and the enzyme (amount equivalent to 1500 units of the indicated lipase) were added. The mixture was shaken for 5 days at the indicated temperature at 200 rpm. Enzyme was filtered off and the solvent evaporated. Products were separated by flash chromatography (ethyl acetate:hexane 1:9).

*2.5.1.4. Cnicin 4′-hexanoate (**4**).* 72% yield; colorless oil; $[\alpha]_D^{25}$ = +115.8 (c = 0.02, $CHCl_3$, IR (film) ν_{max} = 3446, 2932, 2865, 1760, 1740, 1716, 1456, 1386, 1276, 1153, 1090, 1000, and 954 cm^{-1}. ^{1}H and ^{13}C NMR: Tables 1 and 2. Anal. calcd. for $C_{26}H_{36}O_8$: C 65.53%; H 7.61%. Found: C 65.36%; H 7.78%.

*2.5.1.5. Cnicin 4′-decanoate (**5**).* 68% yield; colorless oil; $[\alpha]_D^{25}$ = +71.9 (c = 0.02, $CHCl_3$, IR (film) ν_{max} = 3445, 2954, 2826, 1735, 1713, 1458, 1408, 1277, 1144, 1086, 1019, 997, and 816 cm^{-1}. ^{1}H and ^{13}C NMR: Tables 1 and 2. Anal. calcd. for $C_{30}H_{44}O_8$: C 67.64%; H 8.33%. Found: C 67.74%; H 8.41%.

*2.5.1.6. Cnicin 4′-tetradecanoate (**6**).* 70% yield; colorless oil; $[\alpha]_D^{25}$ = +70.0 (c = 0.02, $CHCl_3$, IR (film) ν_{max} = 3434, 2929, 2851, 1743, 1707, 1454, 1383, 1274, 1149, 1080, 1021, 994, 952, and 813 cm^{-1}. ^{1}H and ^{13}C NMR: Tables 1 and 2. Anal. calcd. for $C_{34}H_{53}O_8$: C 69.36%; H 8.90%. Found: C 69.30%; H 8.99%.

*2.5.1.7. Cnicin 4′-cis-9-octadecenoate (**7**).* 66% yield; colorless oil; $[\alpha]_D^{25}$ = +68.6 (c = 0.02, $CHCl_3$, IR (film) ν_{max} = 3448, 3001, 2920, 2856, 1740, 1713, 1454, 1402, 1388, 1277, 1149, 1086, 1049, 1019, 996, 949, and 814 cm^{-1}. ^{1}H and ^{13}C NMR: Tables 1 and 2. Anal. calcd. for $C_{38}H_{58}O_8$: C 71.00%; H 9.01%. Found: C 69.81%; H 8.98%.

2.5.2. Enzymatic alcoholysis

*2.5.2.1. Cnicin 3′,15-diacetate (**9**).* Cnicin triacetate (**8**) (20 mg, 0.04 mmol) was suspended in acetonitrile or alcohol (8 ml). In the case of acetonitrile as solvent, the corresponding alcohol (1.71 mmol) was added. Finally CAL B (200 mg) was added and the mixture shaken at 55 °C and 200 rpm. After 36 h, enzyme was filtered off and the solvent evaporated. Product was purified by flash chromatography (Ethyl acetate:hexane 3:7). 28.5 mg (yield 99%). Colorless oil. $[\alpha]_D^{25}$ = +102.1 (c = 0.03, $CHCl_3$, IR (film) ν_{max} = 3475, 2956, 2918, 2845, 1741, 1369, 1224, 1144, 1041, 966, 816 cm^{-1}. ^{1}H and ^{13}C NMR: Tables 1 and 2. Anal. calcd. for $C_{24}H_{30}O_9$: C 62.33%; H 6.54%. Found: C 62.24%; H 6.50%.

2.5.2.2. Cnicin 15-acetate (10). **3** (20 mg, 0.04 mmol) was suspended in acetonitrile or alcohol (8 ml). In the case of acetonitrile as solvent, ethanol (0.1 ml, 1.71 mmol) was added. Finally 200 mg of CAL B were added, and the mixture shaken at 55 °C at 200 rpm. After 36 h enzyme was filtered off and the solvent evaporated. Product was purified by flash chromatography (ethyl acetate:hexane 3:7). 21.8 mg (yield 98%). Colorless oil. $[\alpha]_D^{25}$ = +49.5 (c = 0.03, isopropanol, IR (film) ν_{max} = 3412, 2934, 2868, 1760, 1732, 1718, 1444, 1377, 1263, 1230, 1144, 1075, 1030, 997, 958, and 821 cm^{-1}. ^{1}H and ^{13}C NMR: Tables 1 and 2. Anal. calcd. for $C_{22}H_{28}O_8$: C 62.85%; H 6.71%. Found: C 62.70%; H 6.80%.

3. Results and discussion

3.1. Acylation reactions

3.1.1. Enzymatic acetylation

The presence of the three hydroxyl groups in cnicin (**1**) makes this compound an interesting model for enzymatic transformation. Therefore, we began investigating the behaviour of lipases from several sources in the acetylation of this germacranolide. Accordingly, cnicin (**1**) was dissolved in ethyl acetate, working as acylating agent and solvent. Then, the enzyme was added and the suspension shaken at 33 and 55 °C for 24 and 36 h.

At both temperatures, the six lipases afforded compound **2** as a single product (Table 3, entries 1–6). (Scheme 2).

The product was identified as cnicin-4′-acetate (**2**). ^{1}H NMR spectral data was according to literature [29]. Acylation of cnicin on hydroxyl of methylene 4′ can be confirmed by the chemical shift of protons belonging to methylene 4′. This product can be isolated from aerial parts of *Centaurea* sp. [25], and it is less abundant than cnicin [27].

The enzymatic reaction allowed us to obtain this product from cnicin in quantitative yield and under simple and mild reaction conditions. In this case, the lipase behaviour was highly regioselective since primary hydroxyl moiety on carbon 15 did not react at all under the mentioned conditions.

Since the reaction with ethyl acetate only afforded the monoacylated product in the position 4′, we decided to try the enzymatic acetylation using vinyl acetate and isopropenyl acetate as acylating agents, well-known as active reagents in lipase-catalysed acetylations (Table 3, entries 7–12). In this case, we performed reactions with CAL B and PSL, which had shown the best performance, working with ethyl acetate and various solvents.

As solvent effect is very important in biocatalysis [13,31], we tried to test the influence of the reaction media in yield and selectivity of the enzymatic transesterification. Using isopropenyl acetate in acetonitrile both enzymes gave the same product (**2**) after 24 h in high yields, being CAL B (95%, entry 7) more efficient than PSL (84%, entry 8).

Longer reaction times did not promote any change. In acetone, vinyl acetate proved to be a good acylating agent with both CAL B and PSL, obtaining **2** in 82 and 80% yield, respectively (entries 9 and 10). Finally, CAL B was not active enough using the system vinyl acetate and diisopropyl ether since the product **2** was obtained in only 16% yield (entry 12). In almost all these reaction conditions, lipases showed a decrease in regioselectivity. Only when vinyl acetate, CAL B and acetonitrile were employed to acetylate cnicin, the compound **2** was accompanied by a second product: cnicin-15,4′-diacetate (**3**), obtained in 17% yield (entry 11).

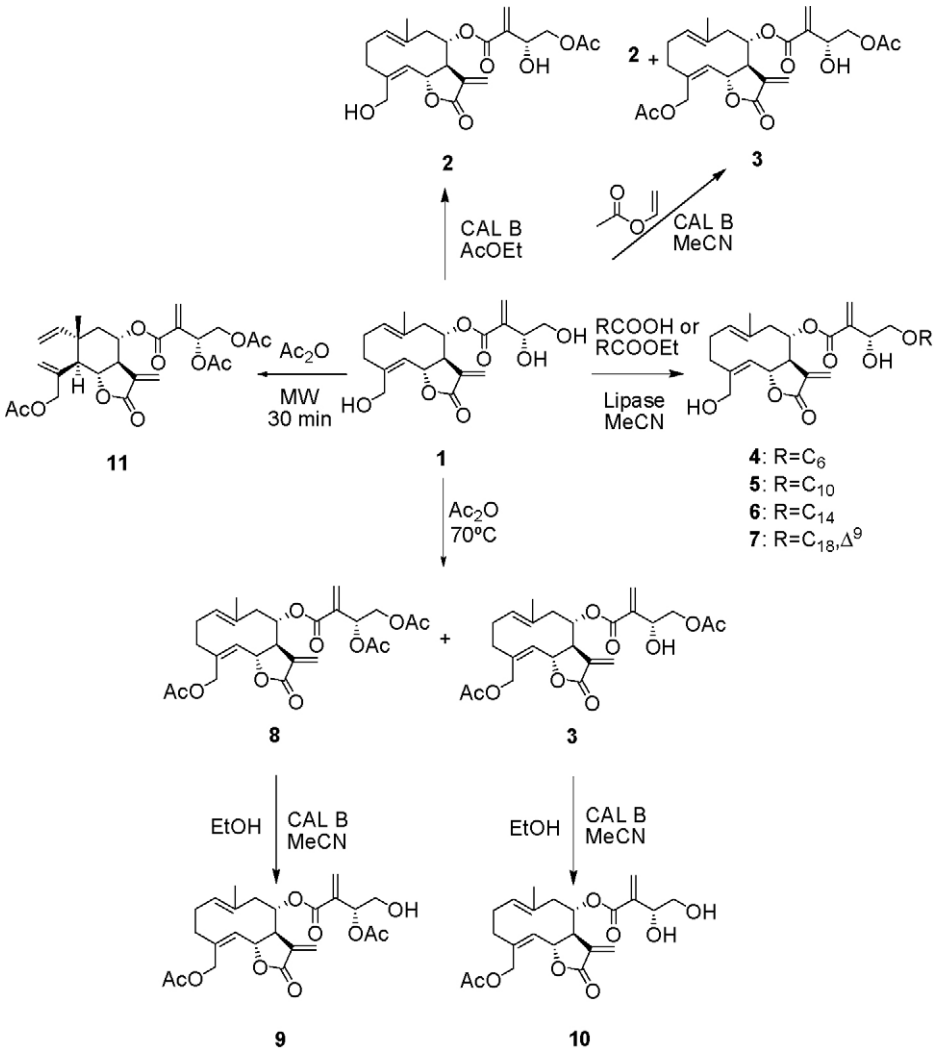
Table 3
Lipase-catalysed acetylation of cnicin (**1**)

Entry	Enzyme	Acylating agent	Solvent	Time (h)	Product 2 yield (%)	
					33 °C	55 °C
1	CAL A	Ethyl acetate	AcOEt	36	20	22
2	CAL B	Ethyl acetate	AcOEt	36	43	97
3	CRL	Ethyl acetate	AcOEt	36	23	29
4	LIP	Ethyl acetate	AcOEt	36	13	17
5	PPL	Ethyl acetate	AcOEt	36	9	9
6	PSL	Ethyl acetate	AcOEt	36	34	64
7	CAL B	Isopropenyl acetate	MeCN	24	–	95
8	PSL	Isopropenyl acetate	MeCN	24	–	84
9	CAL B	Vinyl acetate	Acetone	24	–	82
10	PSL	Vinyl acetate	Acetone	24	–	80
11	CAL B	Vinyl acetate	MeCN	24	–	81[a]
12	CAL B	Vinyl acetate	DIPE	24	–	16

[a] Product **2** is accompanied by product **3** that was obtained in 17% yield.

Acylation of hydroxyl on methylene 15 was confirmed by ^{1}H NMR. In **1**, protons belonging to methylene 15 appear as two doublets centered at 4.09 and 4.29 ppm. When the substituent on this methylene becomes an acetate group, both signals collapse to a singlet. In this case, its chemical shift is 4.61 ppm. This change might be caused by the absence of a conformational restriction of methylene 15, probably due to hydrogen bonding between hydroxyl on methylene 15 and the oxygen atom of the lactone on methyne 6 (Fig. 1).

When acetate is present this interaction is not longer possible. Moreover, a second singlet arises at 2.11 ppm, which belongs to acetate protons. Fig. 1 shows a structural representation of a conformer (local minimum) of cnicin. Two stable torsion angles found for C5–C4–C15–O can be attributed to hydrogen bonding between



Scheme 2.

hydrogen on hydroxyl 15 and the lactone oxygen, due to the distance between hydroxyl hydrogen and the oxygen of the lactone is no longer than 4 Å.

3.1.2. Enzymatic acylation

Encouraged with the previous results, we decided to apply the same enzymatic strategy in the preparation of acyl derivatives of cnicin having longer chain acyl moieties. It is well-known that the presence of medium and long chain fatty acid in derivatives of bioactive compounds often enhance their absorption into the cell, thus increasing their activity compared to the original natural compounds [32].

In previous work we have observed that lipases show the same regioselective behaviour, acting on the same position in the substrate structure, such in the esterification as in the hydrolysis of esters [14]. Therefore it is highly probable that the acyl derivatives obtained through lipases catalysis are more likely to be hydrolyzed in a biological environment. This fact could lead to a controlled release of active molecules.

We began the screening using three enzymes (CAL B, PSL, LIP), acetonitrile as solvent and ethyl caproate as acylating agent (Table 4, entries 2–4).

Neither acetone nor acetonitrile as solvents, in reactions carried out at 55 °C, afforded satisfactory yields after 24 h. Longer reaction time did not improve the results. Furthermore, after 72 h cnicin began to decompose giving a mixture of products. As this problem can be attributed to thermally induced rearrangements in the germacranolide skeleton of cnicin (**1**) [9], we decided to reduce the reaction temperature.

The acylation of cnicin at 10 °C, using ethyl caproate as acylating agent, CAL B as biocatalyst and acetonitrile as solvent afforded the compound **4** in a slightly higher yield, 47% in comparison with 34% at 55 °C (Table 4, entry 5). Even better results were obtained in experiments performed in the same conditions but using caproic acid as acylating agent: **4** was obtained in 72% yield (Table 4, entry 7). The other fatty acid derivatives of cnicin (**5**, **6** and **7**) were obtained in high yield (66–70%). In every case, it was observed that the free acids proved to be more effective than ethyl esters in the acylation reactions.

Moreover, the results showed that CAL B maintained the high selectivity towards the acylation of hydroxyl on carbon 4′ in every case. Reactions made at 10 °C with CAL B gave the best results after 120 h, as monoacylation products on hydroxyl group of carbon 4′ were obtained as the only product in high yields. The remarkable behaviour of *C. antarctica* lipase, keeping its activity at low temperature, allowed us to achieve a transformation minimizing undesirable products as a consequence of thermally induced reactions. Therefore, we could obtain a series of fatty acid derivatives of cnicin not previously reported in literature.

3.2. Enzymatic alcoholysis

As cnicin possesses two ester moieties, we found interesting to test the ability of lipases to make transformations on these groups. We attempted to carry out the enzymatic alcoholysis of cnicin using the same enzymes tested on the acetylation reactions; ethanol, *n*-butanol and octanol as nucleophiles and acetonitrile, acetone, diisopropyl ether and ethanol as solvents. Under these conditions, cnicin remained unaltered. We also tried a hydrolysis of cnicin catalysed by CAL B in a mixture of phosphate buffer and tetrahydrofuran but we obtained the same unsatisfactory results.

In order to complete this study, we decided to apply the enzymatic alcoholysis on polyacetylated derivatives of cnicin. Peracetylation of cnicin was not easy to achieve. Attempts were made with acetic anhydride and pyridine, *N*,*N*-dimethylaminopyridine and Lewis acid catalysts, such as trimethylsilyl trifluoromethanesulfonate, but all of them were unsuccessful.

We also tried a microwave-aided acetylation with acetic anhydride. In this case we obtained the novel elemanolide: 8-α-(3′,4′-diacetoxy-2′-methylene-butanoyloxy)-15-acetyl-11,13-dehydromelitensine (**11**), product of peracetylation and Cope rearrangement of cnicin. Product **11** was completely identified by spectroscopic methods. Its structure was evident from its NMR spectra. In fact, the signals at 5.76, 5.07 and 5.02 ppm (H-1, H-2a and H-2b; δC 145.29 and 117.12, C-1 and C-2) and the two broad singlets at 5.43 and 5.04 ppm (H-3a and H-3b; δC 113.45 and 138.35, C-3 and C-4) were typical of two vinyl groups, monosubstituted and disubstituted, respectively. The presence of three acetyl groups was clearly indicated by the singlets at 2.12, 2.09 and 2.05 and by the downfield shifts of the signals of H-15 protons (δH 4.51, brs, 2H), H-3′ (δH 5.83, brt, 1H) and H-4′ protons (δH 4.29, brd, 2H) with respect to the data reported in literature for the trideacetyl derivative of **11**, isolated from *Centaurea cineraria* subsp. *Umbrosa* [28]. The stereochemistry of the new stereocenters (C-5 and C-10) was unambiguously determined by the chemical shift of protons in CH3-14 and H-5 at δH 1.17 and 2.47 ppm, respectively. Furthermore, the value of the coupling constant of H-5 signal (12.2 Hz) was in full agreement with a diaxial coupling with H-6.

Finally, the best results in peracetylation were obtained when we treated cnicin with acetic anhydride at 70 °C [28]. The reaction was not selective and instead of the desired triaceyl derivative of cnicin as the only product, we obtained a mixture of this compound cnicin-3′,4′,15-triacetate (**8**) and cnicin-4′,15-diacetate (**3**) in 18 and 76% yield, respectively (Scheme 2).

Table 4
Lipase-catalysed acylation of cnicin (**1**) with longer chain carboxylic acids

Entry	Enzyme	Acylating agent	Solvent	Temperature (°C)	Product (yield%)
1	CAL B	Ethyl caproate	Acetone	55	4 (16)
2	CAL B	Ethyl caproate	MeCN	55	4 (34)
3	PSL	Ethyl caproate	MeCN	55	4 (27)
4	LIP	Ethyl caproate	MeCN	55	4 (16)
5	CAL B	Ethyl caproate	MeCN	10	4 (47)
6	CAL B	Caproic acid	MeCN	55	4 (61)
7	CAL B	Caproic acid	MeCN	10	4 (72)
8	CAL B	Ethyl caprate	MeCN	10	5 (35)
9	CAL B	Capric acid	MeCN	10	5 (68)
10	CAL B	Ethyl myristate	MeCN	10	6 (38)
11	CAL B	Myristic acid	MeCN	10	6 (70)
12	CAL B	Ethyl oleate	MeCN	10	7 (44)
13	CAL B	Oleic acid	MeCN	10	7 (66)

Reaction time 120 h.

Table 5
Lipase-catalysed alcoholysis of cnicin-3′,4′,15-triacetate (**8**) and cnicin-4′,15-diacetate (**3**)

Entry	Substrate	Nucleophile	Solvent	Product (yield%)
1	8	EtOH	MeCN	9 (99)
2	8	EtOH	EtOH	9 (72)
3	8	*n*-BuOH	MeCN	9 (23)
4	8	*n*-OcOH	MeCN	n.d.
5	3	EtOH	MeCN	10 (98)
6	3	EtOH	EtOH	10 (75)

Enzyme: CAL B; temperature: 55 °C; time: 36 h. n.d.: non detected.

The identity of the products was confirmed by ^{1}H NMR. Proton on carbon 3′ of **1** appears as a double doublet at 4.52 ppm. When the substituent is acetate this signal shows a downfield shift to 5.62 ppm. Methyl protons belonging to acetate in this position arises as a singlet at 2.12 ppm.

Both compounds **3** and **8** were tested on enzymatic alcoholysis using CAL B as biocatalyst and various alcohols as nucleophiles. The results are presented in Table 5.

From these data we can conclude that best results can be achieved when employing CAL B as catalyst and acetonitrile as solvent. Under these conditions, it was possible to obtain cnicin-15,3′-diacetate (**9**) from **8** and cnicin-15-acetate (**10**) from **3** in quantitative yield (Table 5, entries 1 and 5) (Scheme 2). The site of alcoholysis in every case was unambiguously established by NMR spectroscopic analysis.

Attempts to use ethanol both as nucleophile and solvent showed a decrease in products yield of about 20%. Higher alcohols such as *n*-butanol and *n*-octanol did not show a good performance (Table 5, entries 3 and 4).

In addition, these results proved that the enzyme kept the same regioselectivity that it was shown in the acylation reactions. The lipase catalysed both reactions, acylation and alcoholysis, in the same position of the germacranolide molecule: the oxygen atom on carbon 4′. This fact is in agreement with previous work performed in our laboratory using pyridoxine and steroids as substrates [14,20,21] in which CAL B was active at the same position both in alcoholysis and acylation reactions.

4. Conclusions

This work describes the application of enzymes to the preparation of acyl derivatives of cnicin in a highly regioselective way. Lipases from different sources exhibited good performance as catalysts both in alcoholysis and acylation reactions. *Candida antartica* B lipase gave the best results in both reactions. By enzymatic acetylation and deacetylation reactions, various mono- and diacetylated derivatives of cnicin have been regioselectively obtained. Some of these products have not been reported early in literature. Derivatives of cnicin with longer chain carboxylic acids, not reported early in literature, were also obtained in high yields. Through the enzymatic reaction, carried on at low temperature, CAL B lipase was able to successfully catalyse the acylation and secondary products were not obtained in any of the examples tested.

Alcoholysis reactions afforded good results in the presence of short chain alcohols such as ethanol and allowed us to obtain complementary derivatives of cnicin. Taking into account the current results showed by CAL B it could be assumed that the active site of the enzyme acts in the same position of the germacranolide skeleton both in acylation and alcoholysis reactions. In contrast with these results, the chemical methodology uses conventional acylating agents, such as acetic anhydride that do not let to acetylate regioselectively one of the primary hydroxyl moieties of cnicin. Moreover, the germacranolide skeleton of cnicin seems to be sensitive to pyridine and Lewis acid catalysts such as trimethylsilyl trifluoromethanesulfonate. Regarding microwave irradiation, this new methodology allowed to obtain, in high yield and selectivity, an elemanolide not previously described in literature.

Due to the mild conditions required for the biocatalysts, enzymatic reactions allowed us to obtain a family of novel germacranolides which cannot be prepared through traditional synthetic methods.

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