ISSN: 0974 - 7516 Volume 10 Issue 4

Organic CHEMISTRY

An Indian Journal

Full Paper

OCAIJ, 10(4), 2014 [140-144]

Bioreduction of (-)-achalensolide using pure strains of *Penicillium* sp. and *Rhizopus* sp.

Daniela Alicia Bustos[1], Romina Salem[1], Patricia Varela[2] Adriana Pacciaroni[3], Virginia Sosa[3], Daniel Bustos[1]*

[1]Basic Sciences Institute, National University of Saint John, Saint John, (ARGENTINE)

[2]Biotechnology Institute, National University of Saint John, Saint John, (ARGENTINE)

[3]Organic Chemistry Department, School of Chemical Sciences, National University of Córdoba, Multidisciplinary Institute of Plant Biology (CONICET-UNC), Córdoba, (ARGENTINE)

E-mail: idbustos@ffha.unsj.edu.ar

ABSTRACT

Natural products and related structures are an important source of compounds with biological activity. Biotransformations of organic molecules provides the ability to obtain new compounds which can be tested. This technique has the advantage of enabling functionalized organic compounds using enzymes in microorganisms through simple reactions, in a regio-and stereoselective way, in mild conditions and avoiding polluting the environment. In order to look for new active compounds, pure strains of phytopatogenic fungi *Penicillium* sp. and *Rhizopus* sp., were used to transform the sesquiterpene lactone (-)-achalensolide in a regio and streoselective way to obtain one reduced metabolite, 11β,13-dihydroachalensolide. A two stages standard protocol was used and after isolation and purification of product, IR, ^{1}H and ^{13}C NMR spectroscopy allowed to confirm its structure. 1,1-diphenyl-2-picrylhydrazyl and *Artemia salina in vitro* assays were done to test starting material and product obtained. They showed weak similar activity when compared to reference compounds, so structural modification had no effect on it.

KEYWORDS

Biotransformation;
Reduction;
(-)-achalensolide;
Fungi;
11β;
13-dihydroachalensolide;
Biological activity.

INTRODUCTION

The sesquiterpene lactones are an important group of natural compounds, knowing far more than 3000 structures. They are present in fungi and bryophytes. Except for a few drugs that contain them, and artemisinin derivatives, they have almost no therapeutic uses. The biological properties of these lactones includes antibacterial and antifungal activities, antiparasitic, anthelmintic, anti-inflammatory and cytotoxic[1-3]. Some of these lactones isolated from different *Asteraceae* species have also shown aromatase inhibitory activity[4].

A useful method to obtain new molecules with potential biological activity is the conversion of organic compounds of known structure, using the enzyme potential of microorganisms such as fungi and bacteria, and also plant cells. This technique is known as biotransformation, and enzymes act as catalysts, with the advantage that they are friendly to the environment, operate under mild conditions and have a high tolerance to a

variety of natural and unnatural substrates[5]. The reactions involved in these transformations include hydrolysis, reduction, oxidation, carbon-carbon bond formation, addition and elimination, halogenation and dehalogenation[6].

Continuing with our goal to find related natural products compounds with cytotoxic or antioxidant activity, we have chosen (-)-achalensolide **(1)** a sesquiterpene lactone isolated from *Stevia achalensis*[7]. This compound was never before biotransformed, and has only been tested showing aromatase inhibitory activity[4].

At this time we used the phytopathogenic fungi *Penicillium* sp. and *Rhizopus* sp. as a source of enzymes to modify the structure of compound **(1)**. These fungi have already demonstrated their ability to transform terpenes[8-13].

Here we report the transformation of (-)-achalensolide **(1)** to the reduced metabolite 11β,13-dihydroachalensolide **(2)** by the aforementioned fungi and antioxidant and cytotoxic activities of both compounds as compared to reference substances.

EXPERIMENTAL

Microorganism and media

Penicillium sp. and *Rhizopus* sp. from the culture collection of the Laboratory of Mycology, Department of Microbiology and Immunology, National University of Río Cuarto, (Córdoba, Argentina) were used for screening experiments.

The microorganisms were stored on Sabouraud dextrose agar (Britania Laboratories, Buenos Aires, Argentina) slants at 10°C. Liquid medium for screening and preparative-scale (scale up) experiments was potato-dextrose broth (PDB) (Britania Laboratories, Buenos Aires, Argentina) sterilized at 121°C for 20 minutes.

Equipment

Hydrogen-1 (^{1}H) and carbon-13 (^{13}C) nuclear magnetic resonance (NMR) spectra were recorded on an Avance™ II AV400 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) in deuterated chloroform with tetramethylsi-lane as the internal standard. Thin layer chromatography was performed on Kieselgel 60 GF254 precoated plates (Merck and Co, Inc, Whitehouse Station, NJ) DCM:Me_2CO (9.5:0.5 volume/volume [v/v]) as elution solvent mixture. Detection was achieved by spraying with p-anisaldehyde:EtOH:AcOH:H_2SO_4 (0.1:17:2:1 v/v/v/v) followed by heating at 150°C. Column chromatographies were run using Kieselgel 60 H (Merck) as the stationary phase. All organic solvents were distilled prior to use. The starting material achalensolide **(1)** was isolated from *Stevia achalensis*, as a white solid as previously described[7].

Biotransformation

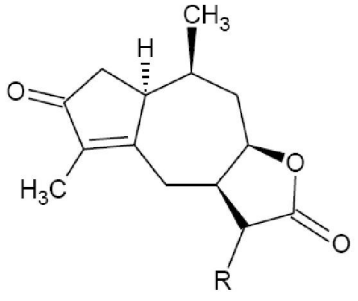
Screening experiments were done following a standard two-stage protocol, previously described[14].

The preparative scale transformation was performed in five 1 L Erlenmeyer flasks containing 400 mL of sterilized liquid medium and then incubated at 25-30°C. (-)-Achalensolide **(1)** was added to a final concentration of 0.2 mg/mL as a 1.5% solution in DMSO:EtOH (5:1, v/v) and the fermentation was continued for 12 days. The starting material disappearance was detected by TLC.

Extraction and purification

After the time above mentioned, extraction was carried out as described before[15], giving an oily residue (429.7 mg).

After purification by column chromatography using DCM:Me_2CO (100:0 to 89:11v/v) as mobile phase a mixture of starting material **(1)** and the compound **(2)** was obtained (214.8mg). Further purification by PTLC using DCM:Me_2CO (9:1; v/v) as mobile phase was carried out. The compound 11β,13-dihydroachalensolide (2) was obtained as white needle shape crystals (26.5mg), mp 120-123°C (crude). IR (KBr) νcm^{-1}: 1775 (C=O, γ-lactone), 1680 (C=O, α,β-unsaturated ketone), 1440, 1410, 1375, 1360, 1310,



1 R = $=CH_2$

2 R = $\cdots CH_3$

Figure 1 : Structure of (-)-Achalensolide (1) and the metabolite (2)

1230, 1120, 1060, 1020, 910, 870, 750, 500, 400. The physical properties were in accordance to bibliographical data[7] allowing to assign the structure showed in figure 1, with β stereochemistry for proton at carbon-11 based on ^{1}H NMR (400.3 MHz, deuterated chloroform) and ^{13}C NMR (100.03 MHz, deuterated chloroform) spectra.

These chemical shifts are listed in TABLE 1.

TABLE 1 : ^{1}H (400.3 MHz) and ^{13}C (100.03 MHz) NMR spectral data of compound 2

Carbon	δ_H (*J*)	δ_C (ppm) (HSQC)	HMBC
1	2.91, brs	46.9	C_4-C_5-C_7-C_8
2	1.98, dd (4.0;18.1) 2.38, dd (6.0; 18.1)	37.1	C_1-C_5
3		207.5	
4		140.0	
5		168.8	
6	2.58, dd (4.7; 14.0) 2.80, dd (4.7; 14.0)	26.58	C_{13}-C_{10}-C_9-C_{11}-C_7-C_1 C_7-C_1-C_{11}
7	2.14, m	44.62	
8	4.53, ddd (4.5;7.5;12.1)	80.0	C_7-C_{10}-C_{11}-C_{12}
9	1.57, m 1.24, m	33.4	C_1-C_7-C_8-C_{10}-C_{14}
10	2.11, m	27.4	
11	2.15, m	35.45	
12		178.12	
13	1.24, d (7.1)	13.6	C_7-C_{11}-C_{12}
14	0.97, d (7.1)	19.6	C_1-C_9-C_{10}
15	1.65, d (2.1)	8.5	C_3-C_4-C_5

Note: *J* values in Hz; spectra in deuterated chloroform with tetramethylsilane as the internal standard.
Abbreviations: δ chemical shift; HSQC heteronuclear single quantum correlation; HMBC homonuclear multiple-bond correlation

Antioxidant activity

The antioxidant activities of substrate **(1)** and its metabolite **(2)** were tested using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical method in the same way as described previously[15]. Concentrations of 0.005, 0.01, 0.015 and 0.02 % (w/v) were used and compared to a reference compound (ascorbic acid). Results are shown in TABLE 2.

Citotoxic activity

The citotoxic avtivity of starting material and the compound obtained was determined by *Artemia salina* method[15]. Achalensolide **(1)** and 11β,13-dihydroachalensolide **(2)** were tested at 100, 75, 50, 25 and 10 ppm.

TABLE 2 : Antioxidant activity of compounds 1 and 2 by 1,1-diphenyl-2-picrylhydrazyl method[a]

Compound	Concentration (%)	A at 510nm	Scavenging activity(% inhibition DPPH)	EC_{50}[b] (mg/mL)	AE = $1/EC_{50}$
1	0.02	0.193	43.6	0.25	4
	0.015	0.196	43		
	0.01	0.2	40		
	0.005	0.205	39		
2	0.02	0.416	36	0.35	2.85
	0.015	0.420	35		
	0.01	0.428	32		
	0.005	0.433	29		
Ascorbic acid	0.02	0.012	99	0.02	50
	0.015	0.017	98		
	0.01	0.025	97		
	0.005	0.035	96		
Control		0.289			

Notes: [a]Values expressed are mean ± standard deviation of three parallel measurements; [b]concentration of substrate that causes 50% loss of 1,1-diphenyl-2-picrylhydrazyl activity.

Results are expressed as mortality percentage given by the formula: Mortality(%) = ([%mortality of test-%mortality control]/[100-%mortality control]) x 100 and compared to caffeine (see TABLE 3).

RESULTS AND CONCLUSIONS

The target compound (-)-achalensolide **(1)** was screened in small scale to get its transformation with two pure strains of *Penicillium* sp. and *Rhizopus* sp. After that only one metabolite was seen in both cases when checked by TLC. Then biotransformation was scaled up on PDB at 25-30°C and after the time and purification technique above mentioned the pure metabolite **(2)** (6.5% yield) was obtained. IR spectrum showed a peak at 1680 cm^{-1} from carbonyl of α,β-unsaturated ketone and 1775 cm^{-1} due to carbonyl of saturated γ-lactone. No band due to hydroxyl group was seen showing that lactone ring was not hydrolyzed. The ^{1}H-NMR spectrum showed a signal at δ = 2.15 ppm as multiplet assigned to proton-11 and a doublet

TABLE 3 : *Artemia salina* test of compounds 1 and 2

Compound	Concentration (ppm)	% mortality	LD_{50} (ppm)[a]
	100	34	
	75	33	
1	50	27	22.69
	25	14	
	10	7	
	100	33	
	75	20	
2	50	17	24.64
	25	9	
	10	4	

Note: [a]Concentration of compound that causes 50% death of *Artemia salina* nauplii.
Abbreviation: LD_{50}, median lethal dose.

at δ = 1.24 ppm (*J* = 7.1) assigned to protons of carbon-13 methyl group. These are new signals when compared with (-)-achalensolide **(1)** spectrum where it can be seen at δ = 5.71 and 6.39 ppm two doublets due to carbon-13 methylene group[7], absents in the spectrum of product **2**. The signals of ^{13}C-NMR were assigned through the heteronuclear single quantum correlation spectrum. A signal at δ = 35.45 ppm was attributed to carbon-11 and the new methyl group of carbon-13 showed a signal at δ = 13.6 ppm. Signal at δ = 207.5 ppm due to carbonyl group of cyclic ketone was not modified, and signal at δ = 178.12 ppm due to carbonyl group of lactone ring was downfield, when compared with the same functional groups of starting compound **(1)**. Signals at δ = 138.4 and 123.4 ppm due to exocyclic carbon-carbon double bond of starting compound are absent. Stereochemistry of the new quiral carbon-11 was determined comparing the ^{13}C NMR spectrum with those of the same compound and its diastereomer[7]. Considering the signals at δ = 44.62 ppm (carbon-7), 33.4 ppm (carbon-9), 27.4 ppm (carbon-10), 35.45 ppm (carbon-11) and basically 13.6 ppm (carbon-13), close to those of the same stereoisomer already published, we can ensure methyl group of carbon-11 has the α configuration.

The presence of the new carbon-13 methyl group was corroborated by heteronuclear multiple-bond correlation spectrum since these protons (δ = 1.24 ppm) showed important correlations with carbon-7 (δ = 44.62 ppm), carbon-11 (δ = 35.45 ppm) and carbon-12 (δ = 178.12 ppm). See figure 2.

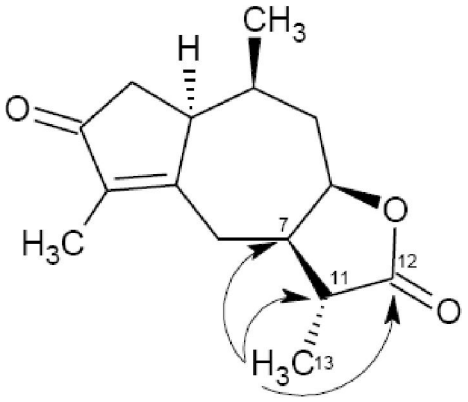


Figure 2 : Heteronuclear multiple-bond correlation for compound 2

From the results obtained, it can be seen that the enzymes expressed in the used microorganism are enoate reductases, which have allowed the reduction of the exocyclic double bond of the lactone ring with the stereochemistry shown.

Antioxidant activity assay showed the product **(2)** has slightly less activity than the substrate **(1)**, which suggests that the structural change introduced is responsible for the decrease.

By contrast, in the brine shrimp test product **(2)** showed slightly higher toxicity than the starting compound **(1)**, due to the reduction of the exocyclic double bond of the lactone ring.

We can conclude that both microorganisms reduced (-)-achalensolide **(1)** in a regio and stereoselective manner, since only the exocyclic double bond of the lactone ring was hydrogenated and the single stereoisomer 11β,13-dihydroachalensolide **(2)** was detected, compared to natural product previously isolated[7]. Moreover it is important to note that enzymatic reduction of **(1)** is verified by the more hindered α side like the chemical one, whereas the catalytic reduction is produced by the opposite β side[7]. Then it could be assumed that the enzyme which catalyzes the reaction allows anchoring the substrate masking the β face, so that only the α side is exposed to the hydride transfer[16].

This is the first report of the biotransformation of (-)-achalensolide **(1)** and the first time an activated carbon-carbon double bond was reduced with pure strains of fungi of the genus *Rhizopus* and *Penicillium*. However, the structural modification achieved in the product, did not improve biological activities tested here.

Full Paper

ACKNOWLEDGEMENTS

The authors would like to thank Universidad Nacional de San Juan, CONICET, Agencia Córdoba Ciencia, and SECyT-UNC for the financial support. Also, thanks to Jorge W. Romero for his help on chromatography and Rafael Fava for his technical assistance.

REFERENCES

[1] J.Bruneton; Pharmacognosy: Phytochemistry, Medicinal Plants. 2nd Edition, Lavoisier Publishing Inc.; Paris, **(1999)**.

[2] A.Ghantous, H.Gali-Muhtasib, H.Vuorela, N.A.Saliba, N.Darwiche; Drug Disc.Today, **15**, 668 **(2010)**.

[3] M.Maas, H.Hensel, F.B.de Costa, R.Brun, M.Kaiser, T.J.Schmidt; Phytochemistry, **72**, 635 **(2011)**.

[4] J.G.Blanco, R.R.Gil, C.I.Alvarez, L.C.Patrito, S.Genti-Raimondi, A.Flury; FEBSLett., **409**, 396 **(1997)**.

[5] K.Faber; Biotransformations in Organic Chemistry. A Textbook, 3rd Edition, Springer; New York, **(1997)**.

[6] M.Maurs, R.Azerad, M.Cortés, G.Aranda, M.B.Delahaye, L.Ricard; Phytochemistry, **52**, 291 **(1999)**.

[7] J.C.Oberti, V.E.Sosa, W.Herz, J.S.Prasad, V.L.Goedken; J.Org.Chem., **48**, 4038 **(1983)**.

[8] G.G.deSouza, T.S.Oliveira, J.A.Takahashi, I.G.Collado, A.J.Macías-Sánchez, R.Hernández-Galán; Org.Biomol.Chem., **10**, 3315 **(2012)**.

[9] C.C.R.deCarvalho, M.R.daFonseca; Biotechnol.Adv., **24**, 134 **(2006)**.

[10] I.A.Parshikov, A.I.Netrusov, J.B.Sutherland; Biotechnol. Adv., **30**, 1516 **(2012)**.

[11] M.I.Choudhary, M.Y.Mohammada, S.G.Musharraf, I.Onajobi, A.Mohammad, I.Anis, M.R.Shah, A.-ur-Rahman; Phytochemistry, **90**, 56 **(2013)**.

[12] S.Taveepanich, N.Muangsin, S.Saithong, C.Pakawatchai, N.Chaichit, S.Roengsumran, A.Petsom; Nat.Prod.Res., **24**, 1050 **(2010)**.

[13] D.A.M.Glenroy, W.F.Reynoldsb, P.B.Reese; Phytochemistry, **65**, 701 **(2004)**.

[14] J.A.Allendes, D.A.Bustos, A.V.Pacciaroni, V.E.Sosa, D.Bustos; Biocatal. Biotransform., **29**, 83 **(2011)**.

[15] D.A.Bustos, A.V.Pacciaroni, V.E.Sosa, D.Bustos; Reports in Organic Chemistry, **2**, 1 **(2012)**.

[16] T.Matsuda, R.Yamanaka, K.Nakamura; Tetrahedron: Asymmetry, **20**, 513 **(2009)**.