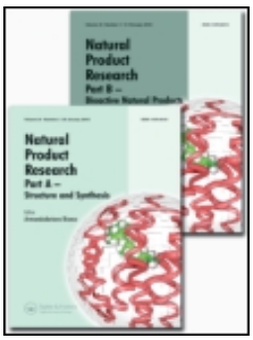

Natural Product Research

ISSN: 1478-6419 (Print) 1478-6427 (Online) Journal homepage: http://www.tandfonline.com/loi/gnpl20

Rearrangement and oxidation of β-amyrin promoted by growing cells of Lecanicillium muscarinium

Leonardo Ribeiro Martins & Jacqueline Aparecida Takahashi

To cite this article: Leonardo Ribeiro Martins & Jacqueline Aparecida Takahashi (2010) Rearrangement and oxidation of β-amyrin promoted by growing cells of Lecanicillium muscarinium , Natural Product Research, 24:8, 767-774, DOI: 10.1080/14786410903262865

To link to this article: http://dx.doi.org/10.1080/14786410903262865

Natural Product Research
Vol. 24, No. 8, 10 May 2010, 767–774

Rearrangement and oxidation of β-amyrin promoted by growing cells of *Lecanicillium muscarinium*

Leonardo Ribeiro Martins and Jacqueline Aparecida Takahashi*

Departamento de Química, Instituto de Ciências Exatas, Universidade Federal de Minas Gerais UFMG, Av. Presidente Antonio Carlos, 6627, CEP 31.270-901, Belo Horizonte, MG, Brazil

(*Received 17 February 2009; final version received 17 August 2009*)

Microbial transformation of β-amyrin by growing cells of the fungus *Lecanicillium muscarinium* (former *Cephalosporium aphidicola*) was successfully accomplished after 15 days of incubation with orbital shaking at 120 rpm. Two products purified by column chromatography were identified by ^{1}H and ^{13}C mono- and bi-dimensional nuclear magnetic resonance as 3β-hydroxy-olean-12-en-11-one and 11α,12α-oxidotaraxerol; these later formed new compounds formed by an interesting oxidative rearrangement of the starting material, probably via an initial hydroxylation at C-11.

Keywords: microbial transformation; β-amyrin; *Lecanicillium muscarinium*; rearrangement

1. Introduction

Bioreactions or biotransformations have been showing great potential for modifying the chemical structures of organic molecules (Holland, 1992) since the first report on their use in the early 1950s (Peterson & Murray, 1952) followed by their progressive industrial use some years later when pharmaceutical industries patented many industrially useful biotransformation protocols. Competitive biotransformations are only representative when they present advantages over the well-established conventional organic synthesis. Nature seems to be very prodigious in furnishing high yields of non-polar molecules; however, more functionalised (and therefore, more polar) molecules are frequently associated with an increased bioactivity. Therefore, a great number of biotransformations aim at the functionalisation of inactivated carbons, especially in mono, sesqui and diterpenes (Hanson & Truneh, 1996) and steroids (Boynton, Hanson, & Hunter 1997; Rahman, Choudhary, Asif, Farooq, & Yaboob, 1998).

It is interesting to point out that, while biotransformations of steroids and diterpenes are well reported, triterpenes have not been widely targeted by microbial transformations, even though they are abundantly found in many plant species and hundreds of biological activities have been associated with them (Dzubak et al., 2006; Tanachatchairatana, Bremner, Chokchaisiri, & Suksamrarn, 2008). Betulin, a

*Corresponding author. Email: jat@qui.ufmg.br

ISSN 1478–6419 print/ISSN 1029–2349 online

DOI: 10.1080/14786410903262865
http://www.informaworld.com

lupane-type triterpene, is one of the few triterpenes successfully microbiologically metabolised into more polar derivatives; in this case, it was featured by the fungus *Chaetomium longirostre*; interestingly, the biotransformation products were more active than the natural starting materials (Akihisa et al., 2002). Betulinic acid (Bastos, Pimentel, De Jesus & De Oliveira, 2007) and oleanoic acid (Choudhary et al., 2008) were also subjected to fungal biotransformations, but the literature reports that fungal enzymatic transformation of triterpenes is a difficult task (Collins, Ruddock, de Grasse, Reynolds, & Reese, 2002).

In this way, we have been screening a series of filamentous fungi in order to find species able to accept and chemically modify triterpene molecules. One of the tested fungal species was *Lecanicillium muscarinium* (formerly *Cephalosporium aphidicola*), notorious by its versatility to perform oxidative biotransformations in the skeleton of several classes of monoterpenes (Farooq & Hanson, 1995) and diterpenes of several different classes, such as stemodane (Hanson, Reese, Takahashi, & Wilson, 1994), kaurane (Hanson et al., 1995a) and aphidicolane (Hanson et al., 1995b). *Lecanicillium muscarinium* was successfully incubated with β-amyrin, an oleanane triterpene known for its anti-inflamatory and gastro-protective activities (Aragão et al., 2006). We report herein the interesting chemical modifications accomplished by *L. muscarinium* on the skeleton of β-amyrin.

2. Experimental

2.1. *General*

Vanillin and solvents were purchased from Synth (Labsynth Produtos para Laboratórios Ltda, Diadema, SP, Brazil). Silica gel for column chromatography was obtained from Merck (63–200 μm) (Darmstadt, Germany) and Sigma (40–63 μm) (Sigma Chemical Co., St. Louis, MO, USA). Neutral alumina was purchased from Macherey-Nagel Gmbh & Co. (Duren, Germany). Reagents for fungal growth were obtained from BIOBRAS (BIOBRAS S.A., Montes Claros, MG, Brazil), Sigma and Synth.

^{1}H, ^{13}C and bi-dimensional COSY, HMBC, HMQC and NOESY nuclear magnetic resonance (NMR) spectra were recorded on a Bruker model DRX-400 (400 MHz) Avance spectrometer (Departamento de Química, ICEx, UFMG) using tetramethylsilane (TMS) as the internal reference. Deuterated chloroform ($CDCl_3$), purchased from Aldrich Chemical Company Inc. (Milwaukee, WI, USA), was used as a solvent. β-Amyrin (**1**) was obtained from a series of plants previously studied in our laboratories. For its purification, it was recrystallised in methanol and then it was fully identified by NMR (Table 1) (Vieira Filho, Duarte, Silva, Howarth, & Lula, 2003).

2.2. *Thin-layer chromatography*

Thin-layer chromatography (TLC) plates were revealed by spraying a solution of vanillin (100 mg) in 100 mL of a methanol–water–sulphuric acid solution (45 : 45 : 10 v/v) and by heating the plates at 100°C for 5 min.

Table 1. ^{1}H and ^{13}C NMR assignments ($CDCl_3$) for starting material **1** and biotransformation products **2** and **3.**

	1		**2**		**3**	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	0.97 1.65	38.6	0.98 s	39.2	1.25 1.87	38.2
2	0.73 1.65	26.9	0.96 s 1.71	26.4	1.08 1.73	26.9
3	3.22 (dd, *J* 5.0 and 4.5 Hz)	79.0	3.25	78.3	3.25	78.5
4	–	38.7	–	39.3	–	38.7
5	0.73	55.2	0.70 s	55.0	0.76	54.7
6	1.40 1.56	18.4	0.84 s 1.61	17.5	1.50 1.62	18.9
7	1.35 1.50	32.6	0.90 1.25	32.8	0.90 1.02	33.1
8	–	39.8	–	43.4	–	38.9
9	1.56	47.6	2.34 s	61.8	0.96	51.9
10	–	36.9	–	37.1	–	37.5
11	1.85	23.5	–	200.2	3.12 (t, *J* 5.2 Hz)	53.6
12	5.18 (t, *J* 3.5 Hz)	121.7	5.58 s	128.3	2.81	58.2
13	–	145.2	–	170.4	–	36.6
14	–	41.7	–	45.4	–	157.2
15	0.97 1.10	26.1	0.96 s 1.18	26.4	5.55 (dd, *J* 8.3 Hz and 3.3 Hz)	118.8
16	0.97 1.60	27.2	1.02 s 1.66	27.3	1.13 1.49	35.2
17	–	32.5	–	32.3	–	35.3
18	1.65	47.2	2.13	47.6	1.22	48.1
19	0.97 1.58	46.8	1.08 s 1.69	45.2	1.31 2.08	40.4
20	–	31.0	–	31.0	–	28.7
21	1.10 1.32	34.7	1.16 1.35	34.4	1.18 1.50	36.5
22	1.25 1.45	37.1	1.13 1.49	36.5	1.73 2.00	38.4
23	0.98	28.1	0.99	28.7	1.08	28.0
24	0.79	15.5	1.13	16.4	1.08	16.9
25	0.93	15.5	0.84	15.7	0.84	15.6
26	0.95	16.8	1.67	18.7	1.02	27.0
27	1.13	26.0	1.35	23.4	0.86	30.2
28	0.83	28.4	1.02	28.2	0.99	29.9
29	0.87	33.3	1.29	33.0	1.03	33.6
30	0.87	23.7	0.92	23.5	0.86	19.5

2.3. *Microorganism and feeding conditions*

The *Lecanicillium muscarinium* strain used in this experiment belongs to the Mycological Collection of LaβB (Biotechnology and Bioassays Laboratory, Chemistry Department, Minas Gerais Federal University, Brazil). The fungus was maintained in potato dextrose agar slopes. In the two-stage feeding experiment, the fungus was grown on a liquid medium containing ($g\,L^{-1}$): D-glucose (240), KH_2PO_4

(4.8), $MgSO_4$ (2.4), KCl (4.8), glycin (4.8) and trace elements solution (4.8) (Vieira, Takahashi, & Boaventura, 2002). A pre-culture (stage 1) of *L. muscarinium* was prepared in a conical flask containing 200 mL of this liquid medium. Incubation took place for 48 h at room temperature (25°C) on an orbital shaker at 120 rpm. Five millilitres of the pre-culture was added to each of 24 conical flasks and incubated under the same conditions (stage 2). After 72 h, fully grown mycelia were observed and the substrate was added (β-amyrin, 20 mg mL^{-1} in chloroform solution per flask) and again incubated for 15 days.

2.4. *Extraction and purification of the products*

After the incubation period, the contents of all flasks were vacuum filtered and the aqueous layer was extracted using ethyl acetate (3×200 mL). Mycelia were also extracted with the same solvent. Both extracts were combined, dried with anhydrous magnesium sulphate and the solvent was removed on a rotator evaporator. The resulting extract (1.2 g) was subjected to column chromatography eluted by a gradient of hexane, ethyl acetate and methanol in crescent polarities. Fraction 6 (40.4 mg) was found to be nearly pure by TLC and was filtered over neutral alumina with hexane and chloroform solution (2 : 8 v/v) to furnish 31 mg of a compound (6.45% yield) seen as a single round spot by TLC (revealed by an acidic vanillin solution) with $Rf = 0.49$ (hexane–chloroform 2 : 8 v/v) and $Rf = 0.42$ (hexane–ethyl acetate 1 : 9 v/v).

3. Results and discussion

Despite the repeated observation of a single spot by TLC using different solvent systems, the biotransformation product, recovered after silica gel chromatography column, was shown to be a mixture of two compounds because the presence of 60 carbons was observed in the ^{13}C NMR spectrum. Attempts to find suitable conditions and solvent systems for their separation were unsuccessful but a careful NMR spectra study enabled us to identify both products, initially codified as **2** and **3**. Attribution of ^{13}C NMR signals of compound **2**, in comparison to starting material chemical shifts, showed that no considerable changes occurred in most of the carbons, except by the resonance of C-11 (δ_C 23.5 in the starting material) that was found to be missing. On the other hand, a carbon signal at δ_C 200.2, present in the ^{13}C NMR spectrum of the product, indicated the presence of a carbonyl group in the molecule, which was assigned to C-11. An H-12 signal, a triplet (J 3.5 Hz) in the starting material became a singlet (δ_H 5.58) in compound **2**, due to the absence of hydrogens at C-11. Therefore, compound **2** was identified as 3β-hydroxy-olean-12-en-11-one (**2**), and the NMR data for this compound is in full accordance with the literature (Agrawal & Jain, 1992) (Table 1).

Concerning to compound **3**, the observation of the ^{13}C NMR spectrum showed that the chemical shifts of carbons belonging to rings A, B and E were not significantly shifted. In the olefin signals region, two chemical shifts were observed but their values were surprising, leading to the conclusion that a double bond would be present but it was not the Δ^{12-13} double bond present in the starting material. Therefore, two sp^2 carbon signals remained unassigned in the spectrum. Their values,

δ_C 157.2 and 118.8, were characteristic of taraxeran triterpenes (Tanaka & Matsunaga, 1988), compounds possessing a double bound between C-14 and C-15 and a methyl group at C-13. Therefore, a migration of the C-27 methyl group from C-14 to C-13 was considered and showed to be consistent with the ^3J correlation observed between C-27 (δ_C 30.2) and H-12 (δ_H 2.81) and between C-13 (δ_C 36.6) and H-27 (δ_H 0.86). Carbon resonances at δ_C 53.6 and δ_C 58.2 were compatible with the presence of an epoxide in C-11–C-12. Those data led to the proposal of the structure of 11α,12α-taraxerol oxide for compound **3** (Figure 1), formed from a rearrangement of β-amyrin. NMR data for **3** was compared to that of its acetylated derivative found in the literature (Agrawal & Jain, 1992), showing complete accordance.

Assignments for compound **3** were corroborated by correlations found in the NOESY and COSY correlations maps. By COSY, there were found correlations between H-11 and H-12 (δ_H 3.12 and 2.81) and between H-11 and H-9 (δ_H 3.12 and 0.96). In addition, the NOESY correlations map showed spatial proximity between H-12 (δ_H 2.81) and both H-11 and the methyl group C-26 (δ_H 3.12 and 1.02, respectively), showing that both H-11 and H-12 are in beta position. NOESY-type correlations found for H-11 were also supporting, since there were observed correlations between H-11, H-1β and H-26 (Figure 2).

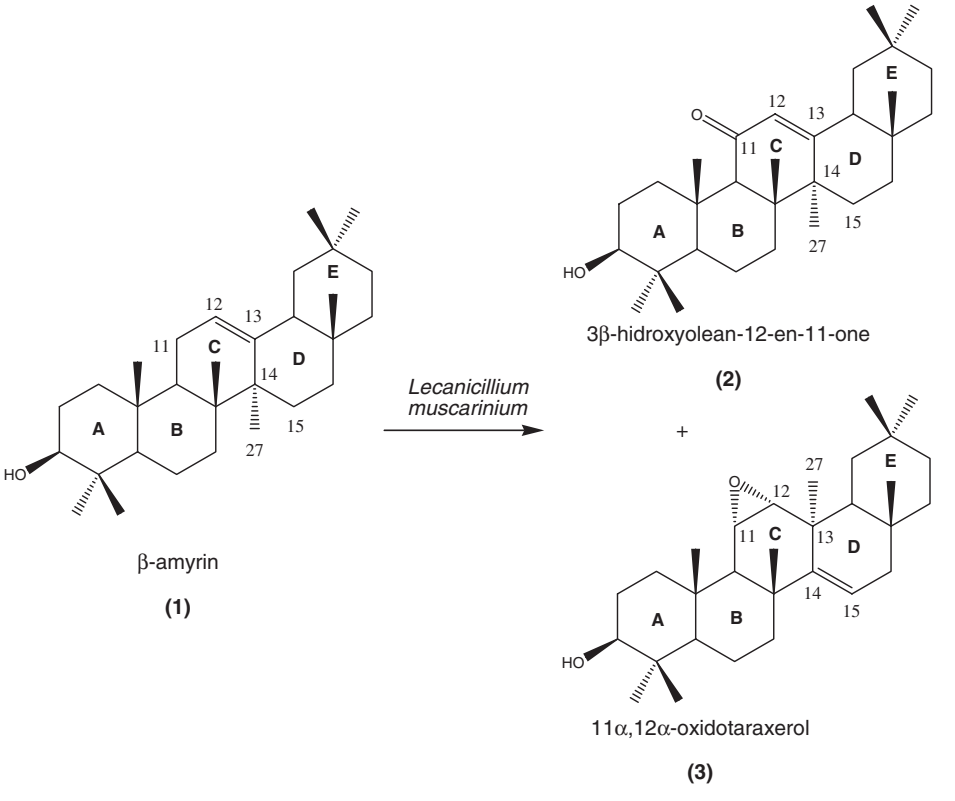

Figure 1. Structure of products (**2** and **3**) obtained from the biotransformation of β-amyrin (**1**) by *L. muscarium*.

A mechanism for this rearrangement was proposed (Figure 3) based on the concomitant isolation of compound **2** from the same experiment, where an oxidation of C-11 was performed by *L. muscarinium*. C-11 hydroxylation as the starting route to form product **3** is also supported by a series of previous biotransformations already reported for *L. muscarinium*, where C-11 is one of the most often hydroxylated positions (Hanson et al., 1995a, 1998). We therefore propose that, starting from a product oxidised at C-11 by a first acting enzyme, a second enzyme would be able to abstract a proton from C-15, directing to the formation of a double bound Δ^{14-15}. This would imply a migration of C-27 methyl group at C-14 to a neighbouring position; in this case, C-13 would concomitantly direct electrons at the p-orbital of C-12 to displace the enzyme still bounded to the oxygen previously inserted at C-11 position (Figure 2) to form an epoxide. This oxidative enzymatic rearrangement is similar to the rearrangement described by Agata et al. (1965), which states that this kind of molecular structural modification is 'powered by the oxidation of C-11', synthetically promoted in their experiments.

Oxidation of β-amyrin was previously described by using *Rhodobacter sphaeroids*, with hydroxylation of methyl groups (Yang et al., 2008). The current transformations

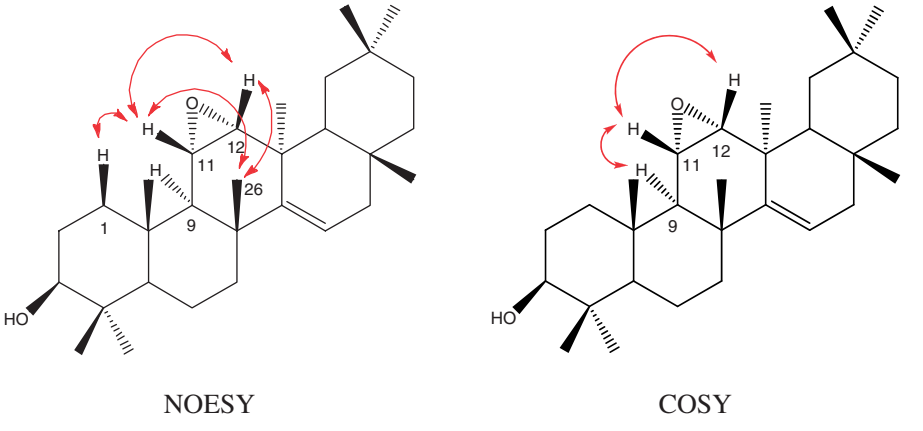

Figure 2. Selected NOESY and COSY key correlations found for compound **3**.

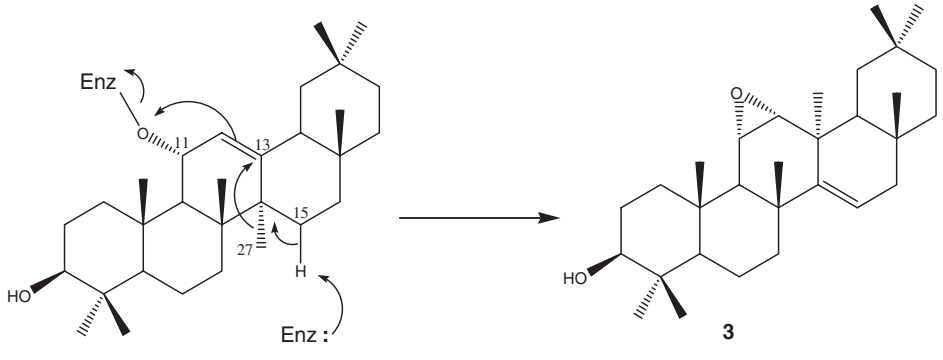

Figure 3. Proposed mechanism for oxidative rearrangement of β-amyrin into compound **3**.

of β-amyrin, to the best of our knowledge, have not yet been described in the literature. Interestingly, another type of microorganism that promoted triterpene rearrangement was already described (Cheng et al., 2004; Zhang, Cheng, Yu, Cordell, & Qiu, 2005), where an ursane-type triterpene was converted into an oleanane-type triterpene by a species of *Nocardia*.

Biotransformations such as the present reaction accomplished by *L. muscarinium* and those described by Cheng et al. (2004) and Zhang et al. (2005) constitute a neat alternative choice for the synthesis of some key biologically active compounds starting from less polar natural triterpenes, although, as for most fungal-performed reactions described for the first time, yield improvement is still a challenge (Ye & Guo, 2008).

Acknowledgements

The authors thank CNPq for scholarships (307846/2006-7 and 141551/2007-0) and FAPEMIG (APQ-6629-5.02/07) and IFS (F/3564-2) for financial help.

References

Agata, I., Corey, E.J., Hortmann, A.G., Klein, J., Proskow, S., & Ursprung, J.J. (1965). Oxidative rearrangements of pentacyclic triterpenes. A method for the synthesis of certain naturally occuring triterpenes from α- and β-amyrin. *Journal of Organic Chemistry, 30*(16), 1698–1710.

Agrawal, P., & Jain, D.C. (1992). ^{13}C NMR spectroscopy of oleanane triterpenoids. *Progress in NMR Spectrospopy, 24*, 1–90.

Akihisa, T., Takamine, Y., Yoshizumi, K., Tokuda, H., Kimura, Y., Ukiya, M., et al. (2002). Microbial transformations two lupine-type triterpenes and anti-tumor-promoting effects of the transformation products. *Journal of Natural Products, 65*(3), 278–282.

Aragão, G.F., Carneiro, L.M.V., Junior, A.P.F., Vieira, L.C., Bandeira, P.N., Lemos, T.L.G., et al. (2006). A possible mechanism for anxiolytic and antidepressant effects of alpha- and beta-amyrin from *Protiun heptaphyllum* (Aubl.) March. *Pharmacology. Biochemistry and Behavior, 85*, 827–834.

Bastos, D.Z.L., Pimentel, I.C., De Jesus, D.A., & De Oliveira, B.H. (2007). Biotransformation of betulinic and betulonic acids by fungi. *Phytochemistry, 68*(6), 834–839.

Boynton, J., Hanson, J.R., & Hunter, A.C. (1997). The hydroxylation of some 13α-methylsteroids by *Cephalosporium aphidicola*. *Phytochemistry, 45*(5), 951–956.

Cheng, Z.H., Yu, B.Y., Cordell, G.A., & Qiu, S.X. (2004). Biotransformation of quinovic acid glycosides by microbes: Direct conversion of the ursane to the oleanane triterpene skeleton by *Nocardia* sp. NRRL 5646. *Organic Letters, 6*(18), 3163–3165.

Choudhary, M.I., Batool, I., Khan, S.N., Sultana, N., Shah, S.A.A., & Atta-Ur-Rahman (2008). Microbial transformation of oleanolic acid by *Fusarium lini* and α-glucosidase inhibitory activity of its transformed products. *Natural Product Research, 22*(6), 489–494.

Collins, D.O., Ruddock, P.L.D., de Grasse, J.C., Reynolds, W.F., & Reese, P.B. (2002). Microbial transformations of cadina-4,10(15)-dien-3-one, aromadendr-1(10)-en-9-one and methyl ursolate by *Mucor plumbeus* ATCC 4740. *Phytochemistry, 59*(5), 479–488.

Dzubak, P., Hajduch, M., Vydra, D., Hustova, A., Kvasnica, M., Biedermann, D., et al. (2006). Pharmacological activities of natural triterpenoids and their therapeutic implications. *Natural Product Reports, 23*, 394–411.

Farooq, A., & Hanson, J.R. (1995). The microbiological hydroxylation of some pinane monoterpenoids by *Cephalosporium aphidicola*. *Phytochemistry, 40*(3), 815–817.

Hanson, J.R., Hitchcock, P.B., & Takahashi, J.A. (1995a). Biotransformation of *ent*-16β, 19-dihydroxykaurene by *Cephalosporium aphidicola*. *Phytochemistry, 40*(3), 797–800.

Hanson, J.R., Jarvis, A.G., Laboret, F., & Takahashi, J.A. (1995b). The incubation of 3-alpha, 16-beta-dihydroxyaphidicolane with *Cephalosporium aphidicola*. *Phytochemistry, 38*(1), 73–75.

Hanson, J.R., Reese, P.B., Takahashi, J.A., & Wilson, M.R. (1994). Biotransformation of some stemodane diterpenoids by *Cephalosporium aphidicola*. *Phytochemistry, 36*(6), 1391–1934.

Hanson, J.R., & Truneh, A. (1996). The biotransformation of Ambrox® and sclareolide by *Chephalosporium aphidicola*. *Phytochemistry, 42*(2), 1021–1023.

Holland, H.L. (1992). *Organic synthesis with oxidative enzymes*. Cambridge, UK: VCH Publishers Inc.

Peterson, D.H., & Murray, H.C. (1952). Microbial oxygenation of steroids at carbon 11. *Journal of the Americam Chemical Society, 74*, 1871–1872.

Rahman, A.U., Choudhary, M.I., Asif, F., Farooq, A., & Yaboob, M. (1998). Microbial transformations of testosterone. *Natural Product Research, 12*(4), 255–261.

Tanachatchairatana, T., Bremner, J.B., Chokchaisiri, R., & Suksamrarn, A. (2008). Antimycobacterial activity of cinnamate-based esters of the triterpenes betulinic, oleanolic and ursolic acids. *Chemical & Pharmaceutical Bulletin, 56*(2), 194–198.

Tanaka, R., & Matsunaga, S. (1988). Triterpene constituents from *Euphobia supine*. *Phytochemistry, 27*(11), 3579–3584.

Vieira Filho, S.A., Duarte, L.P., Silva, G.D.F., Howarth, O.W., & Lula, I.S. (2003). 3β-(Stearyloxy)olean-12-ene from *Austroplenckia populnea:* Structure elucidation by 2D-NMR and quantitative ^{13}C-NMR spectroscopy. *Helvetica Chimica Acta, 86*(10), 3445–3449.

Vieira, H.S., Takahashi, J.A., & Boaventura, M.A.D. (2002). Novel derivatives of *ent*-17, 19-dihydroxy-16βH-kaurane obtained by biotransformation with *Verticillium lecanii*. *Journal of Agricultural and Food Chemistry, 50*, 3704–3707.

Yang, G., Zhang, Z., Bai, H., Gong, J., Wang, Y., Li, B., et al. (2008). Biotransformation of b-amyrin acetate by *Rhodobacter sphareoides*. *Journal of Bioscience and Bioengineering, 105*(5), 558–561.

Ye, M., & Guo, D.A. (2008). New bufadienolide obtained from the biotransformation of cinobufagin by *Alternaria alternata*. *Natural Product Research, 22*(1), 26–30.

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