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Biotransformation of 5α-hydroxy-14-eudesm-11-en-3-one by *Rhizopus nigricans*, *Cunninghamella elegans* and *Mucor plumbeus*

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Abstract

5α-Hydroxy-14-eudesm-11-en-3-one (**1**) was metabolised by the filamentous fungi *Rhizopus nigricans*, *Cunninghamella elegans* and *Mucor plumbeus* to produce four metabolites. Regioselective hydroxylation at C-6 and C-11 were detected. These 11-hydroxylated compounds can be chemically transformed into α-agarofuran.

Keywords: Eudesmanes; Sesquiterpenes; Biotransformation; Microbiological hydroxylation

1. Introduction

There is an increasing body of information about the use of biocatalysis for selective transformations of synthetic and natural products. The principal advantages of these biocatalytic processes are their relatively mild conditions, environmental safety, and especially high selectivity. Special attention has been paid to filamentous fungi because they are able of catalysing regio and stereoselective hydroxylation [1,2]. The enzymatic systems involved are monoxygenases capable of providing one of two oxygens molecules. Among these monoxygenases, the key enzyme is cytochrome P450, which is contained within the microorganism [3,4].

Eudesmane sesquiterpene are common in nature, and the biotransformation with different type of filamentous fungi of this type of compounds has been carried out several times, producing a hydroxylated product, among others, that is difficult to achieve by classical chemical means [5]. α-Cyperone and two of its isomers has been biotransformed. The metabolites obtained by bioconversion of these three stereoisomers of α-cyperone show that the predominant transformation of the side chain included a vinyl group. Other studies with eudesmane derivates show similar results [6–12].

The eudesmane sesquiterpene derivative is a pathway in the chemical synthesis of natural agarofurans (epoxyeudesmane), which is isolated from the Celastraceae family. Several of these compounds exhibit insecticide, insect anti-feedant, cytotoxic, anti-tumour and immunosuppressive activities [13–22]. It has been reported that when the amount of hydroxyl groups is increased in the molecule, an increment of biological activity can be observed [23]. From the several routes for synthetic agarofurans production [24–26], we employed Robinson annelation combined with microbiological hydroxylation in the synthesis of hydroxylated agarofuran [24]. This route used 7-epi-cyperone as starting material because this compound has an angular methyl group and the three-carbons side chains *trans*, which are necessary to synthesise agarofurans compound (Scheme 1).

This work deals with the metabolism of the sesquiterpene eudesmane with different microorganisms. We studied the biotransformation of 5α-hydroxy-14-eudesm-11-en-3-one with *Rhizopus nigricans*, *Cunninghamella elegans* ATCC 35112 and *Mucor plumbeus* ATCC 4740 (Scheme 2). The structures of these metabolites were fully elucidated with spectroscopic experiments. Derivatives **2** and **3** are useful as intermediates in synthetic agarofuran formation because total agarofuran synthesis can be reduced to only two steps [24,27,28].

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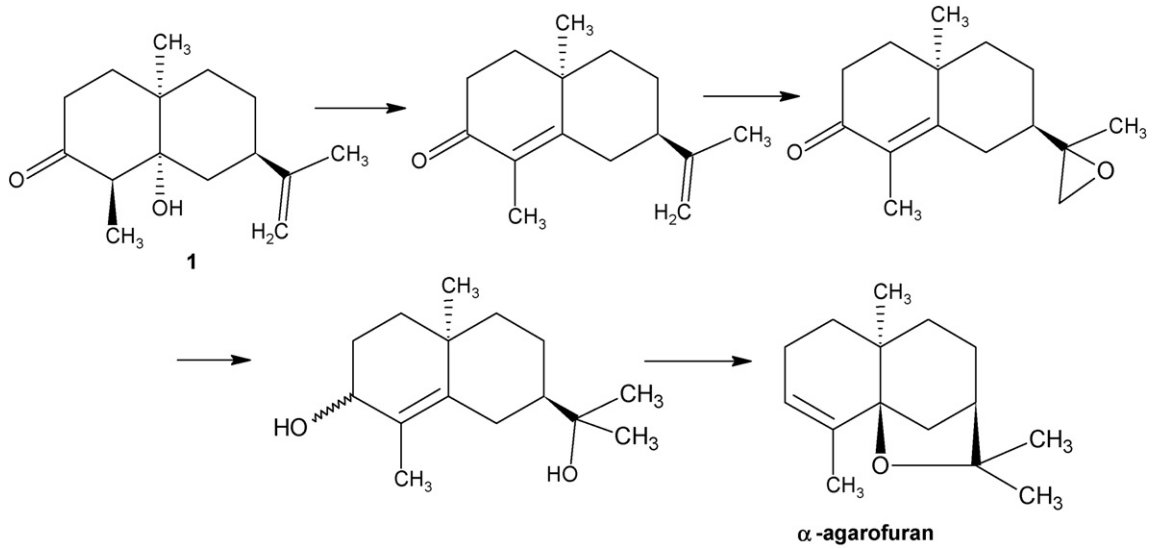
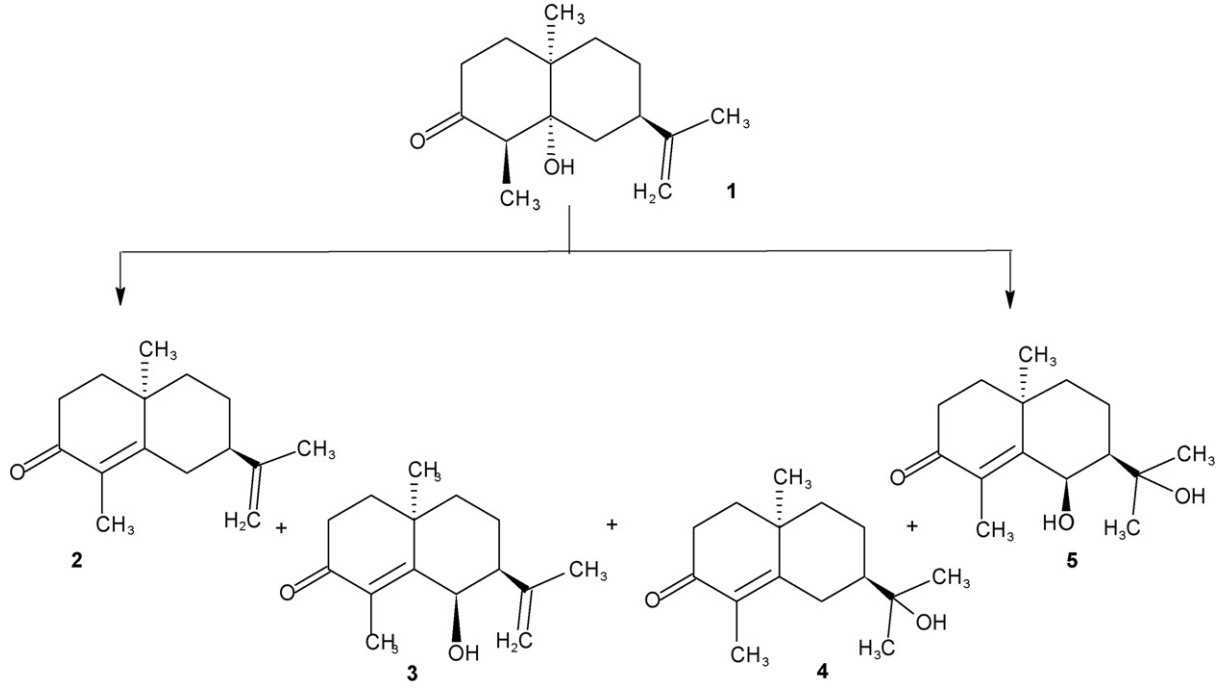


Scheme 1. Classical synthesis of agarofurans from 5α-hydroxyeudemane-3-one (**1**).

2. Material and methods

2.1. General experimental procedures

The NMR spectra (250 MHz ^{1}H and 65 MHz ^{13}C) were measured in $CDCl_3$ (which also provided the lock signal) with Bruker spectrometers (AC-250). The ^{13}C chemical shift was assigned with distortion-free enhancement of polarization transfer (DEPT) using a flip angle of 135°. NOESY experiment was used by determining the stereochemistry of OH group at C6. IR spectra were recorded on Shimadzu FTIR-8400. Optical rotations were measured in chloroform at 25 °C. The GC and GC–MS analyses were made on a gas chromatography–mass selective detector (GC: Shimadzu GC-17A, injection temperature 270 °C, split/splitless injector, MS: Shimadzu QP5050A). Helium (1.2 ml/min) was used as carrier gas. Capillary column DB-1 (30 m × 0.25 mm i.d., film thickness 0.25 μm) was used in the GC, the oven temperature was programmed at 120 °C for 1 min and then increased 10 °C/min to 270 °C. Merck 60 silica gel (40–60 μm) was used for flash chromatography. Hexane and AcOEt containing increasing amounts of MeOH were used as eluents. Analytical plates (silica gel, Merck 60G) were rendered visible by spraying with H_2SO_4–AcOH followed by heating to 120 °C.



Scheme 2. Modification by microbiological biotransformation of 5α-hydroxyeudemane-3-one (**1**).

2.2. Synthesis of 5α-hydroxy-14-eudesm-11-en-3-one (**1**)

Compound **1** was prepared from (+)-dihydrocarvone by a Robinson annelation reaction with ethyl vinyl ketone [28] (Scheme 1). The structure of compound **1** was determined by NMR spectroscopy. $[\alpha]_D{}^{15} = +50^\circ$ IR (thin film) ν 3506, 2931, 2869, 1701, 1643, 1454, 1438, 1377, 1138, 1056, 1022, 991, 960, 887, 794 cm^{-1}. MS: *m/z* (relative intensity) = 236 (M^+, 25), 218 (8), 203 (4), 175 (2), 161 (4), 152 (80), 137 (39), 123 (35), 109 (100), 95 (35), 81 (24), 55 (73). ^1H NMR (250 MHz, $CDCl_3$, δ in ppm, respect to TMS). δ = 4.68–4.65 (d, 2H, J = 8.0 Hz), 2.86 (q, 1H, J = 6.4 Hz), 2.65–2.5 (m, 1H), 2.37–2.28 (m, 1H), 2.26–2.15 (m, 1H), 2.14–2.00 (m, 1H), 1.95–1.80 (m, 1H), 1.68 (s, 3H), 1.63–1.51 (m, 3H), 1.49–1.35 (m, 3H), 1.23 (s, 3H), 1.13–1.06 (d, 1H, J = 13.1 Hz) and 1.03 (d, 3H, J = 6.7 Hz). ^{13}C NMR (62.4 MHz, $CDCl_3$, δ in ppm, respect to TMS). δ = 210.62, 149.26, 109.03, 77.94, 51.73, 39.70, 37.64, 37.54, 35.38, 33.39, 31.56, 25.76, 21.67, 20.79 and 6.52.

3. Organism, media and culture conditions

Native *R. nigricans* was obtained from the Colección de Cultivos, Laboratorio de Síntesis y Biotransformación de Productos Naturales, Facultad de Ciencias, Universidad del Bío-Bío, Chillán, Chile. *C. elegans* ATCC 35112 and *M. plumbeus* ATCC 4740 were obtained from the American Type Culture Collection, Rockville, MD. USA. The microorganisms were kept in Hagen medium containing $CaCl_2$ (0.005%), KH_2PO_4 (0.0025%), $(NH_4)_2HPO_4$ (0.025%), $MgSO_4{\cdot}7H_2O$ (0.015%), $FeCl_3$ (0.0012%), malt extract (0.3%) and glucose (1%) in H_2O at pH 6.5. Erlenmeyer flasks (250 ml) containing 125 ml of medium were inoculated with microorganism before 5 days of preculture. The cultures were incubated with shaking (100 rpm) at 25 °C for 12 days, after which a solution of the substrate in ethanol was added.

3.1. Biotransformation of **1**

The substrate **1** (300 mg) was dissolved in 3 ml ethanol and the solution was added in three equal portions to the three Erlenmeyer flask cultures, each containing 125 ml of medium. After incubation for 12 days in a shaker at room temperature, the cultures were filtered over filter paper, the cells were washed thoroughly with EtOAc, and the filtrates were pooled and extracted with EtOAc. The combined extracts were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to give a mixture of compound (310 mg). Chromatography on silica gel using *n*-hexane: EtOAc mixes produced the yields indicated in Table 1.

3.2. Spectroscopy data of metabolite **2**

$[\alpha]_D^{20} = -208^\circ$ IR (0.6, $CHCl_3$) ν 2924, 2870, 1662, 1450, 1091, 1118 and 887 cm^{-1}. MS: *m/z* (relative intensity) = 218 (M^+, 33), 203 (35), 190 (37), 175 (56), 161 (73), 147 (80), 132 (88), 119 (76), 105 (73), 91 (100), 79 (91), 67 (71) and 55 (82). ^1H NMR (250 MHz, $CDCl_3$, δ respect to TMS). δ = 4.75 (s, 1H), 4.56 (s, 1H), 1.76 (s, 3H), 1.67 (s, 3H) and 1.19 (s, 3H). ^{13}C NMR (62.4 MHz, $CDCl_3$, δ respect to TMS). δ = 198.61, 162.92, 147.26, 129.00, 110.89, 40.82, 37.43, 35.76, 35.72, 33.83, 30.99, 22.98, 22.93, 22.47 and 10.84.

Table 1
Hydroxylation of *5α-hydroxy-14-eudesm-11-en-3-on* using different fungal strain as biocatalyst

Fungi	Time (days)	Transformation	Metabolites formed (%)			
		Compound **1** (%)	**2**	**3**	**4**	**5**
R. nigricans	12	98	23	15	38	22
C. elegans	12	91	21	23	35	12
M. plumbeus	12	59	12	15	32	0

3.3. Spectroscopy data of metabolite **3**

IR (thin film) ν 3421, 2924, 2924, 2854, 1647, 1450, 987, 887 and 737 cm^{-1}. MS: *m/z* (relative intensity) = 234 (M^+, 12), 219 (10), 205 (28), 191 (29), 177 (17), 164 (24), 145 (18), 135 (57), 123 (67), 107 (57), 93 (57), 79 (63), 67 (58) and 55 (100). ^1H NMR (250 MHz, $CDCl_3$, δ, respect to TMS). δ = 4.91 (s, 1H), 4.81 (s, 1H), 4.37 (s, 1H), 1.87 (s, 3H), 1.73 (s, 3H) and 1.38 (s, 3H). ^{13}C NMR (62.4 MHz, $CDCl_3$, δ, respect to TMS). δ = 199.91, 160.07, 145.26, 132.02, 111.37, 69.53, 47.71, 38.81, 35.10, 35.02, 34.13, 25.66, 22.90, 18.60 and 10.38.

3.4. Spectroscopy data of metabolite **4**

IR (thin film) ν 3440, 2927, 2866, 1647, 1458, 1377, 1184, 1088, 929, 752 cm^{-1}. MS: *m/z* (relative intensity) = 236 (M^+, 25), 218 (8), 203 (4), 175 (2), 161 (4), 152 (80), 137 (39), 123 (35), 109 (100), 95 (35), 81 (24), 55 (73). ^1H NMR (250 MHz, $CDCl_3$, δ in ppm) δ = 2.67–2.45 (m, 2H), 2.44–2.24 (m, 2H), 2.20–1.91 (m, 1H), 1.80 (s, 3H), 1.76–1.60 (m, 2H), 1.58–1.50 (m, 3H), 1.41–1.25 (m, 2H) 1.23 (s, 3H), 1.21 (s, 3H), 1.17 (s, 3H). ^{13}C NMR (62.4 MHz, $CDCl_3$, δ in ppm), 198.63, 164.87, 129.80, 73.43, 44.47, 37.29, 36.36, 35.89, 34.05, 28.75, 27.45, 26.83, 24.64, 21.30 and 11.26.

3.5. Spectroscopy data of metabolite **5**

IR (thin film) ν 3417, 2931, 2866, 1651, 1454, 1377, 1326, 1184, 1018, 921, 860, 818 cm^{-1}. MS: *m/z* (relative intensity) = 251 (M^+, 3), 234 (95), 221 (69), 203 (100), 177 (79), 175 (59), 163 (73), 161 (55), 147 (18), 133 (31), 119 (29), 107 (39), 105 (47), 91 (59), 75 (53), 57 (42), 55 (44). ^1H NMR (250 MHz, $CDCl_3$, δ in ppm) δ = 4.99 (d, 1H, J = 5 Hz), 2.89–2.51 (m, 4H), 1.92 (s, 3H), 1.89–1.70 (m, 2H), 1.65–1.57 (m, 3H), 1.55–1.42 (m, 2H), 1.39 (s, 3H), 1.32 (s, 3H), 1.14 (s, 3H). ^{13}C NMR (62.4 MHz, $CDCl_3$, δ in ppm), 199.92, 161.13, 133.15, 73.52, 69.60, 53.81, 37.92, 37.75, 35.09, 34.26, 30.05, 28.00, 25.10, 21.02 and 11.18.

3.6. Time-course experiment

Spores of filamentous fungi *R. nigricans*, *C. elegans* ATCC 35112 and *M. plumbeus* ATCC 4740 (about 6×10^5 spores)

were transferred to a 250 ml Erlenmeyer flask containing 125 ml Hagen medium, and cultured with continuous shaking for 7 days at room temperature. The compound **1** (100 mg) was added to the suspension cultures and incubated at room temperature in rotary shaker (100 rpm). At a regular time interval of 3 days, three Erlenmeyer flask of the incubation mixture was taken out under sterile conditions for 12 days and then extracted with EtOAc. The yields of the product were determined based on the peak area from GC–MS.

4. Results and discussion

Compound **1** was obtained by a Robinson annelation reaction of (+)-dihydrocarvone with ethyl vinyl ketone as a white powder, very soluble in ethanol with a fusion point of 105 °C, and strong absorptions at 3506 and 1701 cm^{-1} in its I.R. spectra. The ^{1}H NMR, ^{13}C NMR and its additional experiments, permitted us to elucidate its chemical structure as 5α-hydroxy-14-eudesm-11-en-3-one (**1**). The incubation of **1** with *R. nigricans*, *C. elegans* AT35112 and *M. plumbeus* ATCC 4740 produced four metabolites that were identified as *14-eudesm-4,11-dien-3-one* (**2**), *6β-hydroxy-14-eudesm-4,11-dien-3-one* (**3**), *11-hydroxy-14-eudesm-4-en-3-one* (**4**), and *11,6β-dihydroxy-14-eudesm-4-en-3-one* (**5**). By contrast, *M. plumbeus* produced only metabolites **2**, **3** and **4**.

Metabolite **5** contained additional oxygen, as was deduced from its molecular ion peak (*m*/*z* 251). The hydroxylation configuration at C-6 was spectroscopically characterised. The stereochemistry of **3** was established with the 2D-NOESY experiment. The most relevant dipolar coupling was observed between H-6 and H-15. These features show that the hydroxylation occurred on the β-face, indicating the stereoselectivity of the biotransformation process. Furthermore, the proton–proton coupling constant between H-6 and H-7 proton was 5 Hz. This value is greater that the *J* value (1.3 Hz) reported for α-hydroxylated sesquiterpen eudesmane derivative previously synthesized by chemical methods [29].

The metabolites obtained could be formed in two stages. The first stage corresponds to a dehydration of the C-5 position of the starting compound, producing metabolite **2** and subsequent hydroxylation of this metabolite in the position C-6, C-11 and both positions. This interpretation is confirmed by a kinetic study of the metabolism where the concentration of **2** is greater, until the third day, when the concentration of the other metabolites begins to increase (Figs. 1–3).

The exocyclic double-bond elimination performed by *R. nigricans*, *C. elegans* and *M. plumbeus,* which produced the hydroxylated product at the C-11 position, could occur via an intermediate epoxide, although this species was not observed. The formation of an intermediate epoxide has also been reported in limonene [30] and other sesquiterpene biotransformations [8]. Furthermore, it is possible that an epoxide intermediate was not observed due to its high instability in aqueous systems [31–33]. Regioselective hydroxylations in both "A" and "B" rings, on C-2 and C-8, were detected when was used 4α-hydroxylated eudesmane as substrate, but the main hydroxylating action was directed to the isopropenyl moiety, mainly

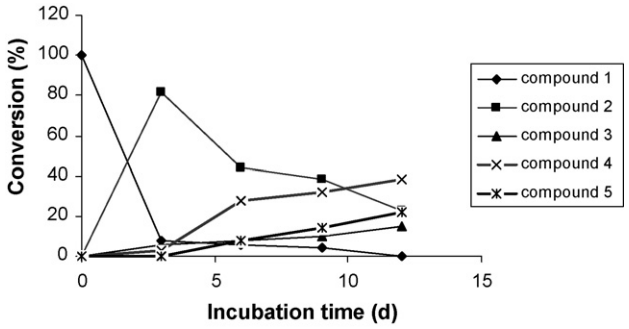


Fig. 1. Kinetics of compound **1** metabolism by *Rhizopus nigricans*.

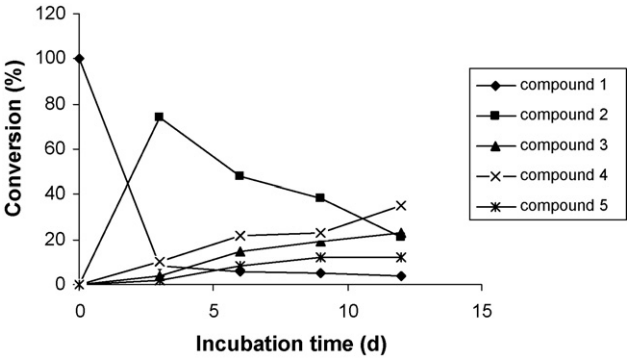


Fig. 2. Kinetics of compound **1** metabolism by *Cunninghamella elegans* ATCC 35112.

on C-11[]. Biotransformation of (−)-10-epi-α-cyperone yielded three stereoisomers, and one of them present hydroxylation on C-6 [34]. Biotransformation of 5α-hydroxyeudesmanolides and yours kinetic have not previously been reported.

The hydroxylation in C-11 can play a very important role, since the 11-hydroxyeudesmane compounds by means of acid catalysis give agarofurans compounds. The results show that the obtained yields are greater than the reported in literature for the chemical route of obtention of these compounds (Table 1) [28].

The present work newly confirms the use of biohydroxylation catalysis as a promising tool to achieve the semi-synthesis of sesquiterpenolides from their sesquiterpene starting material.

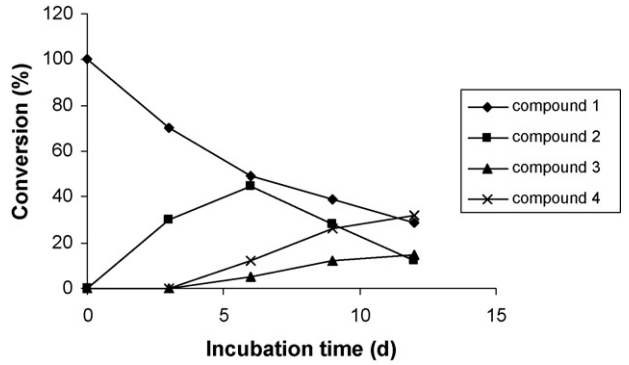


Fig. 3. Kinetics of compound **1** metabolism by *Mucor plumbeus* ATCC 4740.

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References

[1] H.L. Holland, Stereoselective Hydroxylation Reaction, Marcel Dekker, New York, 2000, p. 131.
[2] H.L. Holland, H.K. Weber, Curr. Opin. Biotechnol. 11 (2000) 547.
[3] H.J.M. van den Brink, R.F.M. van Gorcom, C.A.M.J.J. van den Hondel, P.J. Punt, Fungal Genet. Biol. 23 (1998) 1.
[4] R. Bernhanrdt, J. Biotechnol. 124 (2006) 128.
[5] B.M. Fraga, Nat. Prod. Rep. 17 (2000) 483 (and references therein).
[6] V. Lamare, R. Furstoss, Tetrahedron 46 (1990) 4109.
[7] A. García-Granados, M.C. Gutiérrez, F. Rivas, J. Mol. Catal. B: Enzym. 27 (2004) 133.
[8] A. García-Granados, M.C. Gutiérrez, A. Parra, F. Rivas, J. Nat. Prod. 65 (2002) 1011.
[9] A. García-Granados, M.C. Gutiérrez, F. Rivas, J.M. Arias, Phytochemistry 58 (2001) 891.
[10] A. García-Granados, E. Melguizo, A. Parra, F.L. Perez, Y. Simeó, B. Viseras, J.M. Arias, Tetrahedron 56 (2000) 6517.
[11] A. García-Granados, E. Melguizo, A. Parra, Y. Simeó, B. Viseras, J.A. Dobado, J. Molina, J.M. Arias, J. Org. Chem. 65 (2000) 8214.
[12] A. García-Granados, A. Martínez, A. Parra, F. Rivas, M.E. Honorato, J.M. Arias, Tetrahedron 49 (1993) 1091.
[13] Y.H. Kuo, M.L. King, C.F. Chen, C.H. Chen, K. Chen, K.H. Lee, J. Nat. Prod. 57 (1994) 263.
[14] Y. Takishi, K. Ujita, H. Tokuda, H. Nishino, A. Iwashima, T. Fujita, Cancer Lett. 65 (1992) 19.
[15] Y.L. Zheng, L. Xu, L.J.F. Liu, Acta Pharm. Sin. 24 (1989) 568.
[16] A.G. González, I.A. Jiménez, A.G. Ravelo, J.A. González, J. Lloria, Biochem. Syst. Ecol. 25 (1997) 513.
[17] A.G. González, I.A. Jiménez, A.G. Ravelo, X. Bellés, M.D. Piulachs, Biochem. Syst. Ecol. 20 (1992) 311.
[18] J. Alarcón, J. Becerra, M. Silva, Bol. Soc. Chil. Quim. 43 (1998) 65.
[19] F. Boyer, C.L. Descoins, C. Descoins, T. Prangé, P. Ducrot, Tetrahedron Lett. 43 (2002) 8277.
[20] F. Boyer, T. Prangé, P. Ducrot, Tetrahedron Asymm. 14 (2003) 1153.
[21] A. Spivey, M. Weston, S. Woodhead, Chem. Soc. Rev. 31 (2002) 43.
[22] C. Lee, P. Floreancig, Tetrahedron Lett. 45 (2004) 7193.
[23] X. Chen, S.C. Shao, T.S. Li, Y.L. Li, Synthesis (1992) 1061.
[24] J. Alarcón, S. Águila, Z. Naturforsch 59c (2004) 215.
[25] X. Wu-Jiong, S. Liang-Dong, S. Lei, Z. Shu-Yu, T. Yong-Qiang, Chin. J. Chem. 22 (2004) 377.
[26] J. White, H. Shin, T. Kim, N. Cutshall, J. Am. Chem. Soc. 119 (1997) (2004).
[27] J.W. Huffman, R.C. Desai, J. Org. Chem. 47 (1982) 3254.
[28] J. Alarcón, J. Alderete, M.G. Peter, J. Becerra, M. Silva, Bol. Soc. Chil. Quim. 43 (1998) 325.
[29] F. Aladro, F. Guerra, F. Moreno-Dorado, J. Bustamante, Z. Jorge, G. Massanet, Tetrahedron 57 (2001) 2171.
[30] K. Kieslich, W.R. Abraham, B. Stumpf, B. Thede, P. Washausen, in: E.J. Walter (Ed.), Progress in Essential Oil Research, de Gruyter and Co., Berlin, 1986, p. 367.
[31] Z. Li, J. Beilen, W. Duetz, A. Schimid, Curr. Opin. Chem. Biol. 6 (2002) 136.
[32] S. Prichanont, D. Leak, D. Stuckey, Enz. Microb. Technol. 27 (2000) 134.
[33] G. Steinig, A. Livingston, D. Stuckey, Biotechnol. Bioeng. 70 (2000) 553.
[34] H. Hikino, C. Konno, Y. Ikeda, N. Izumi, T. Takemoto, Chem. Pharm. Bull. 23 (1975) 1231.