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Microbial hydroxylation of natural drimenic lactones

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Abstract

Incubation of confertifolin and isodrimenin with *Mucor plumbeus*, *Aspergillus niger* or *Rhizopus arrhizus* gave in good yields the corresponding 3β-hydroxy derivatives. From isodrimenin, the known natural 7α-hydroxy derivative (futronolide) was also obtained and its structure was definitely established by X-ray crystallographic study of its acetate derivative.

Keywords: *Mucor plumbeus*; *Aspergillus niger*; *Rhizopus arrhizus*; Biotransformation; Hydroxylation; Confertifolin; Isodrimenin

1. Introduction

Microbial hydroxylations have been frequently employed to achieve the functionalization of unactivated saturated carbon atoms, a difficult challenge using chemical methods (Holland, 1992). Such hydroxylation of terpenoid compounds has been occasionally but repeatedly reported (Krasnobajew, 1984; Lamare & Furstoss, 1990), mainly in view of the use of the resulting derivatives as fragrance or flavor agents (Krasnobajew, 1984; Hagedorn & Kaphammer, 1994). However, the pool of natural terpenoids represents a unique and inexpensive source of structural diversity for the preparation of asymmetric synthons, hemisynthesis intermediates and chiral auxiliaries, provided selective functionalizations could be realized.

Much attention has been paid to the synthesis of hydroxylated drimanic compounds due to their wide range of biological activities and possible industrial applications. The usual 3β-hydroxylation, a high yielding microbial reaction commonly observed with most 4,4-dimethyl terpenoid compounds (Hollinshead et al., 1983; Aranda, Hammoumi, Azerad, & Lallemand, 1991; Aranda et al., 1991, 1992; Ramirez, Cortes, & Agosin, 1993), would yield 3β-hydroxy drimanic derivatives, sometimes naturally encountered in small amounts in plants or fungi. Nevertheless, hydroxylation at the 1α-position, a distinctive feature of several bioactive terpenic compounds, such as forskolin for example, cannot be achieved by this method. However, starting from 3β-hydroxy derivatives, we have previously shown that a simple reaction sequence results in a functionalization transfer which allows the preparation in good yields of 1α-hydroxylated compounds (Aranda et al., 1994, 1997, 1998).

Earlier results with sclareolide (**1**) (Aranda et al., 1991; Hanson & Truneh, 1996; Atta-Ur-Rahman, Farooq, & Choudhary, 1997) incubated with filamentous fungi showed low or moderate yields of hydroxylation products, and apparent degradation of the substrate. On the contrary, good yields of hydroxylated product (**2**) from cinnamolide (**3**) have been

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Table 1
Biotransformation of drimane lactones from *Drimys winteri*. Confertifolin and isodrimenin were added to the grown cultures as EtOH solutions and incubations were continued (see Section 3) during 48 h

Microorganism	Confertifolin, **4**	Isodrimenin, **8**
Mucor plumbeus ATCC 4740	about 90–95% 3β-hydroxylated metabolite (**5**); 80–90% yield after crystallization of the crude biotransformation extract	very slow transformation
Aspergillus niger ATCC 9142	”	3β-OH (**9**), 85–90% yield after crystallization of the crude biotransformation extract
Rhizopus arrhizus ATCC 11145	”	66% of a 4:1 mixture of 3β-OH (**9**) and 7α-OH (**11**), separated as their acetate esters

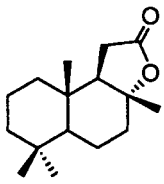
reported (Hollinshead et al., 1983), indicating that the B-ring unsaturation could have stabilized the lactonic ring toward hydrolysis during fungal incubation.

As a continuation of our interest in the microbial hydroxylation of terpenoid compounds for hemisynthesis, we report here our results with two 8,9-unsaturated lactonic drimane derivatives, confertifolin **4** and isodrimenin **8**, isolated from the bark of *Drimys winteri* Forst. (Winteraceae) (Appel, Connolly, Overton, & Bond, 1960), a South American tree commonly found in Chile and Argentina.

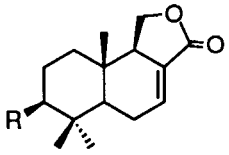
2. Results and discussion

Confertifolin **4** and isodrimenin **8** (0.5 g l^{-1}) were separately incubated with 65 h-old shake cultures of *Mucor plumbeus*, *Aspergillus niger* and *Rhizopus arrhizus*.The results obtained are summarized in Table 1.

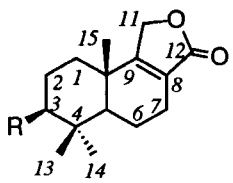
With all three strains, confertifolin **4** was converted in high yield into one major metabolite (**5**), $C_{15}H_{22}0_3$ (as established by EI-HRMS) which was identified as a secondary equatorial alcohol: the ^1H



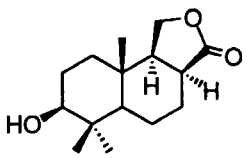
1



2: R = OH
3: R = H



4: R = H
5: R = OH
6: R = OAc



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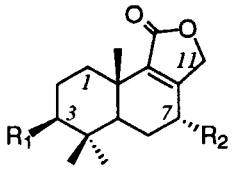

8: $R_1 = R_2 = H$
9: $R_1 = OH$; $R_2 = H$
10: $R_1 = OAc$; $R_2 = H$
11: $R_1 = H$; $R_2 = OH$
12: $R_1 = H$; $R_2 = OAc$

NMR spectrum of **5** exhibited a signal at $\delta = 3.27$ ppm (dd, J = 4.9 and 9.9 Hz) indicating a –C*H*OH– group adjacent to methylene protons. The ^{13}C NMR spectra of **5** and its acetate (**6**) were characterized by new signals at 78.3 and 79.9 ppm, respectively, corresponding to odd multiplicity, and replacing a methylene signal at 41.7 ppm attributed to C-3 in the confertifolin spectrum. The chemical shifts and patterns of new signals in ring A were in agreement with all reference data from the literature (Hollinshead et al., 1983; Aranda et al., 1991, 1992; Ayer & Trifonov, 1992; Hanson & Truneh, 1996; Atta-Ur-Rahman et al., 1997) assigned to 3β-OH structures. Furthermore, substituent effects of the acetate group in the acetylated product **6** (Ac_2O/pyridine) resulted in the expected shielding of C-2 and C-4, and deshielding of C-3, compared to the values observed in the ^{13}C NMR spectrum of the unesterified derivative (Breitmaier & Voelter, 1987). Thus metabolite **5** was identified as 3β-hydroxyconfertifolin.

Catalytic hydrogenation of 3β-hydroxyconfertifolin (**5**) obtained by microbial transformation afforded the dihydroderivative **7**, identical to a natural drimanic lactone previously isolated from the fungus *Peniophora polygonia* (Ayer & Trifonov, 1992).

Minor hydroxylation products (< 1%) were additionally detected in the crude extracts of the incubation supernatants, probably corresponding to C-13 or C-14 hydroxylated products, characterized in ^1H NMR by an AB-system centered at 3.2 ppm ($J_{AB} = 11$ Hz).

On incubation of isodrimenin (**8**) with *A. niger* and *M. plumbeus*, a major metabolite (**9**), $C_{15}H_{22}0_3$ (as established by EI-HRMS) was obtained, which was similarly identified by ^1H and ^{13}C NMR as a 3β-hydroxyderivative and easily converted to its acetate (**10**). However, on incubation with *R. arrhizus*, isodrimenin afforded two isomeric hydroxylated metabolites, one of them being identical to 3β-hydroxyisodrimenin (**9**). The ^1H NMR spectrum of the new one (**11**) exhibited a one-proton doublet at 4.48 ppm (broad, J = 4.5 Hz). The ^{13}C NMR spectra of this new alcohol and its acetate (**12**) were characterized by –CH(OH)– or –CH(OAc)– signals at 62.4 and 64.9 ppm, respectively, corresponding to the disappearance of the original methylene signal at 25.3 ppm, attributed to C-7 in the isodrimenin spectrum. Substituent effects of the acetate group showed the expected upfield shifts of C-6 and C-8 (Breitmaier & Voelter, 1987). The presence of an AB system at 4.75 ppm (2H, $J_{AB} = 18.5$ Hz) in the ^1H NMR spectrum of **11**, attributed to a shifted C-11 methylene signal, confirmed hydroxylation at C-7. The (pseudo axial) α-stereochemistry of the hydroxyl group was deduced from the low coupling constant (4.5 Hz) observed for the CH(OH) hydrogen. Thus metabolite **11** was 7α-hydroxyisodrimenin. An identical structure was earlier assigned to a natural product, futronolide, isolated in very small amount (0.001%) from the stem bark of a South American tree, *Capsicodendron dinisii* (Canellaceae) (Mahmoud, Kinghorn, Cordell, & Farnsworth, 1980). The reported physical data (m.p., $[\alpha]_D$) are in relative agreement with those of metabolite **11**, but serious discrepancies appear between the reported ^1H and ^{13}C NMR data of futronolide and our measurements. On the other hand, our NMR data are in good agreement with those of other reported natural drimenic lactones such as 7α-hydroxyconfertifolin, previously isolated from *Peniophora polygonia* (Ayer & Trifonov, 1992). Moreover, the acetate ester (**12**) of metabolite **11** could be obtained as a crystallized product, suitable for X-ray crystallography (Fig. 1), ensuring without ambiguity our preceding identification.

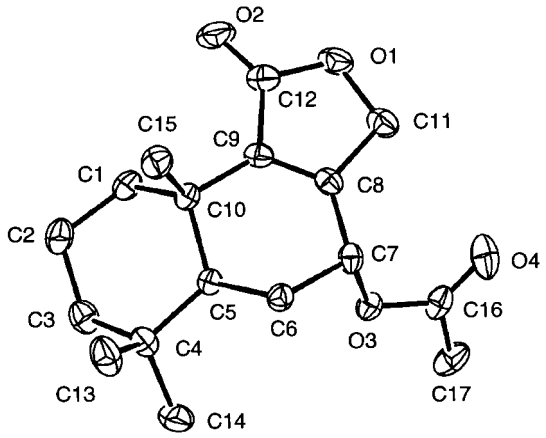

Fig. 1. X-ray molecular structure of 7α-acetoxyisodrimenin (**12**).

Minor hydroxylation products were also detected in the crude extracts of the incubation supernatants of isodrimenin, characterized in ^1H NMR by an AB-system centered at 3.5 ppm ($J_{AB} = 11$ Hz), shifted towards 4.15 ppm after acetylation, and probably corresponding to C-13- or C-14 hydroxylated products.

We have shown that microbial hydroxylation of drimenic lactones easily provides in high yield the 3β-hydroxy derivatives. In one case, incubation of isodrimenin with *R. arrhizus*, an additional product hydroxylated at C-7 (an allylic position) could be obtained. Such regio- and stereoselectively functionalized compounds are of interest because they often correspond to minor natural products usually isolated in very small amounts. Moreover, they can constitute new and valuable starting materials for various hemisynthetic strategies which are underway in our laboratory (Aranda et al., 1997, 1998), leading to new derived structures potentially active as cytotoxic, antibacterial or antifungal compounds.

Table 2
^{13}C Chemical shifts. Multiplicity was determined by DEPT experiments. Assignments indicated (*) may be interchanged. (50.323 MHz,δ ppm of indicated compounds in $CDCl_3$)

Carbon number	**4**[a]	**5**	**6**	**8**[a]	**9**	**10**	**11**	**12**
1	36.2	34.2	33.9	34.5	32.7	32.5	34.0	34.0
2	18.4	27.1	23.6	18.3*	27.3	23.7	18.3	18.3
3	41.7	78.3	79.9	41.8	78.6	80.3	41.7	41.7
4	33.3	39.0	38.0	33.1	38.9	37.9	32.7	32.8
5	51.4	50.6	50.7	52.3	51.6	51.8	46.6	47.7
6	18.1	18.1	18.0	18.1*	18.1	18.1	28.8	25.7
7	21.5	21.6	21.5	25.3	25.5	25.4	62.4	64.9
8	123.5	123.8	124.0	159.2	159.3	159.1	157.8	153.5
9	170.8	170.0	169.5	135.5	135.3	135.2	137.8	140.9
10	36.7	36.4	36.3	34.9	34.6	34.6	35.9	35.6
11	68.3	68.2	68.1	70.6	70.7	70.7	69.7	70.0
12	175.0	174.7	174.1	172.4	172.3	172.1	172.4	172.3
13	21.5	15.6	16.7	21.4	15.5	16.6	21.4	21.3*
14	33.3	28.2	28.2	33.4	28.3	28.3	33.1	33.1
15	21.0	20.9	21.0*	20.0	20.0	20.1	18.4	18.4
CH_3CO	–	–	21.2*, 170.8	–	–	21.3, 170.8	–	21.0*, 170.8

[a] Attributions were derived from ^1H–^{13}C chemical shift correlations.

3. Experimental

3.1. General

General experimental methods have been earlier described (Aranda et al., 1991). High resolution mass spectrometry (HRMS) was performed on a JEOL MS700 spectrometer. EI- and CIMS were performed on a Hewlett-Packard 5989B instrument. The incubation course was monitored by GC–MS, using a 25 m × 0.2 mm Ultra 2 (Hewlett-Packard) capillary column (temp. programmed 110 to 270°C at 8°C min^{-1}). Column chromatography was performed on silicagel Merck 60H (70–230 mesh).

3.2. Starting materials

Lactones **4** and **8** from the natural extract mixture of *Drimys winteri* were separated by column chromatography on silicagel, using increasing amounts of ethyl ether in cyclohexane. Both lactones were crystallized twice from CH_2Cl_2–pentane mixtures. Confertifolin **4**: m.p. 150–151°C, $[\alpha]_D^{22}$ +71.7° ($CHCl_3$; *c* 1.8); isodrimenin **8**: m.p. 130–131.5°C, $[\alpha]_D^{22}$ +89.6° ($CHCl_3$; *c* 1.83); IR and ^1H-NMR data in agreement with earlier literature results (Appel et al., 1960; Nakano & Maillo, 1981; Hueso-Rodriguez & Rodriguez, 1989).

3.3. Microorganisms, culture and incubation conditions

Mucor plumbeus ATCC 4740, *Aspergillus niger* ATCC 9142 and *Rhizopus arrhizus* ATCC 11145 were grown at 27°C in orbitally shaken 250 ml-conical flasks containing 100 ml of liquid medium. One litre of medium contained: corn steep liquor (Solulys L, Roquette, France), 10 g; glucose, 30 g; KH_2PO_4, 1 g; K_2HPO_4, 2 g; $NaNO_3$, 2 g; KCl, 0.5 g; $MgSO_4{\cdot}7H_2O$, 0.5 g; $FeSO_4{\cdot}7H_2O$, 0.02 g. After 65 h-growth, the substrate (250 mg) in EtOH (5 ml) was evenly distributed between 5 flasks and incubation was continued in the same conditions. Samples (1 ml) were aseptically withdrawn every day, saturated with sodium chloride and extracted with ethyl acetate for TLC and GC–MS analysis. Most transformations were continued until no further increase of metabolite(s) was observed (usually 2 days).

3.4. Isolation and purification of biotransformation products

Extraction of metabolites and residual substrate was performed by diluting the cultures with CH_2Cl_2 (2 vol.) and shaking at room temperature for 24 h. Such extraction was repeated twice. Alternatively, continuous extraction with CH_2Cl_2 for 36–48 h was used. The organic phase was dried with K_2CO_3 and the solvent was removed under vacuum. When a crystalline residue containing one major derivative was obtained (see Table 1), two crystallizations of the crude hydroxylation product from CH_2Cl_2–pentane were sufficient to obtain it pure. The mixture of 3β-hydroxy and 7α-hydroxyisodrimenin (see Table 1) could be separated only after acetylation and silicagel chromatography. Acetates were hydrolyzed in dilute aqueous KOH solution–dioxane at room temperature and the resulting alcohols were recrystallized from a CH_2Cl_2–pentane mixture.

3.5. (+)-3β-Hydroxyconfertifolin (**5**)

M.p. 182–183.5°C. $[\alpha]_D^{22}$ +61.4° ($CHCl_3$; *c* 1.01). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3714, 3636, 3520, 2958, 2933, 2908, 1748, 1672, 1029. ^1H NMR (200 MHz, $CDCl_3$): δ 0.84, 1.03, 1.19 (9H, 3s, 13-, 14- and 15-CH_3), 3.27 (1H, dd, *J* = 9.9 and 4.9 Hz, H-3α), 4.56, 4.64, 4.68 and 4.77 (2H, AB part of an ABX_2 system, J_{AB} = 17 Hz, J_{AX} = 2.8 Hz, J_{BX} = 1.6 Hz, H-11). ^{13}C NMR, see Table 2. EI-HRMS (70 eV): calculated for $C_{15}H_{22}O_3$ $[M]^+$ 250.1569, found 250.1568.

3.6. (+)-3β-Acetoxyconfertifolin (**6**)

M.p. 254°C. $[\alpha]_D^{22}$ +47.3° ($CHCl_3$; *c* 0.54). IR $\nu_{max}^{CCl_4}$ cm^{-1}: 2968, 2948, 2929, 1748, 1729 (sh.), 1671, 1374, 1249, 1028. ^1H NMR (200 MHz, $CDCl_3$): δ 0.96 (6H, s, 2 CH_3), 1.20 (3H, s, CH_3), 2.08 (3H, s, $COCH_3$), 4.53 (1H, dd, *J* = 11 and 4.8 Hz, H-3α), 4.59, 4.68, 4.72 and 4.81 (2H, AB part of an ABX_2 system, J_{AB} = 18 Hz, J_{AX} = 2.7 Hz, J_{BX} = 1.9 Hz, H-11). ^{13}C NMR, see Table 2. CI-HRMS (CH_4): calculated for $C_{17}H_{25}O_4$ $[M+1]^+$ 293.1753, found 293.1754.

3.7. (−)-8*S*,9*S*-Dihydro-3β-hydroxyconfertifolin (**7**)

(−)-8*S*,9*S*-Dihydro-3β-hydroxyconfertifolin (**7**) was obtained by catalytic hydrogenation of **5** (100 mg) in the presence of Adams catalyst (8 mg) in acetic acid, under pressure (60 psi) at 60°C during 10 h. M.p. 177–179°C, after crystallization from CH_2Cl_2–pentane; $[\alpha]_D^{24}$ −1.5° (MeOH, *c* 1.31), −5.9° ($CHCl_3$, *c* 1.31). ^1H and ^{13}C NMR identical to data from the literature (Ayer & Trifonov, 1992).

3.8. (+)-3β-Hydroxyisodrimenin (**9**)

M.p. 170–171°C. $[\alpha]_D^{22}$ +87.6° ($CHCl_3$; *c* 1). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3692, 3615, 3477, 1742, 1666, 1448, 1344, 1030. ^1H NMR (200 MHz, $CDCl_3$): δ 0.86, 1.06, 1.14 (9H,3 s, 13-, 14- and 15-CH_3), 2.60 (1H, dt, *J* = 13 and 3.5 Hz, H-1β), 3.28 (1H, dd, *J* = 11,1 and 5.3 Hz, H-3α), 4.57 (2H, br. s, H-11). ^{13}C NMR, see Table 2. EI-HRMS (70 eV): calculated for $C_{15}H_{22}O_3$ $[M]^+$ 250.1569, found 250.1571.

3.9. (+)-3β-Acetoxyisodrimenin(**10**)

M.p. 167–168°C. $[\alpha]_D^{22}$ +77.4° ($CHCl_3$; *c* 1.27). ^1H NMR (200 MHz, $CDCl_3$): δ 0.93 (6H, s, 2 CH_3), 1.16 (3H, s, CH_3), 2.06 (3H, s, $COCH_3$), 2.61 (1H, dt, *J* = 13.5 and 3.4 Hz, H-1β), 4.54 (1H, dd, *J* = 11.2 and 5.3 Hz, H-3α), 4.58 (2H, br. s, H-11). ^{13}C NMR, see Table 2. CIMS (NH_3) *m*/*z* 293 $[M+H]^+$.

3.10. (+)-7α-Hydroxyisodrimenin (**11**)

M.p. 217–218°C. $[\alpha]_D^{21}$ +109° ($CHCl_3$; *c* 0.46). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3596, 3010, 2955, 2930, 2584, 1754, 1744, 1661, 1378, 1140, 1066, 1008. ^1H NMR (200 MHz, $CDCl_3$): δ 0.90, 0.95, 1.11 (9H, 3 s, 13-, 14- and 15-CH_3), 2.56 (1H, dt, *J* = 13 and 4.1 Hz, H-1β), 4.48 (1H, br. d, *J* = 4 Hz, H-7β), 4.58, 4.67, 4.84 and 4.93 (2H, AB part of an AB system, J_{AB} = 18.5 Hz, H-11). ^{13}C NMR, see Table 2. EI-HRMS: calculated for $C_{15}H_{22}O_3$ $[M]^+$ 250.1569, found 250.1562.

3.11. (+)-7α-Acetoxyisodrimenin (**12**)

M.p. 123.5–125°C. $[\alpha]_D^{22}$ +189° ($CHCl_3$; *c* 0.47). IR $\nu_{max}^{CCl_4}$ cm^{-1}: 2962, 2931, 2858, 2930, 1768, 1744, 1742, 1370, 1239. ^1H NMR (200 MHz, $CDCl_3$): δ 0.90, 0.94, 1.14 (9H,3 s, 13-, 14- and 15-CH_3), 2.10 (3H, s, $COCH_3$), 2.08 (1H, dt, *J* = 13.5 and 3.5 Hz, H-1β), 4.64 (2H, br. s, H-11), 5.46 (1H, br.d, *J* = 5 Hz, H-7β). ^{13}C NMR, see Table 2. CIMS (NH_3) *m*/*z* 293 $[M+H]^+$.

3.12. X-ray crystallographic data and structure determination of (**12**)

Crystals of **12**, $C_{17}H_{24}O_4$, were grown from a mixture of CH_2Cl_2/pentane. Data were collected at 123 ± 0.5 K on an Enraf Nonius CAD4 diffractometer using Mo Kα (λ = 0.71073 Å) radiation and a graphite monochromator. The crystal structure was solved and refined using the Enraf Nonius MOLEN package. The compound crystallises in space group $P2_12_12_1$ (19), *a* = 8.189(1) Å, *b* = 10.356(1) Å, *c* = 18.404(2) Å; *V* = 1560.72(51) $Å^3$; *Z* = 4; d_{calc} = 1.244 g/cm^3; μ = 0.8 cm^{-1}; *F*(000) = 632. A total of 2924 unique reflexions were recorded in the range $2° \leq 2\theta \leq 60.0°$ of which 1060 were considered as unobserved ($F^2 < 3.0s(F^2)$), leaving 1864 for solution and refinement. Direct methods yielded a solution for all atoms. The hydrogen atoms were refined with isotropic temperature factors in the final stages of least-squares while using anisotropic temperature factors for all other atoms. A non-Poisson weighting scheme was applied with a *p* factor equal to 0.05. The final agreement factors were *R* = 0.031, R_w = 0.039, G.O.F. = 1.06. The crystallographic data have been deposited with the Cambridge Crystallographic Data Centre.

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