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MICROBIAL TRANSFORMATION OF TETRACYCLIC DITERPENES: CONVERSION OF *ENT*-BEYERENES BY *RHIZOPUS NIGRICANS* CULTURES

ANDRÉS GARCÍA-GRANADOS*, ANTONIO MARTINEZ, M. ESTHER ONORATO,

Departamento de Química Orgánica, Universidad de Granada, Granada 18071, Spain

and JOSE M. ARIAS

Departamento de Microbiología, Facultad de Ciencias, Universidad de Granada, Spain

ABSTRACT.—Microbial transformation of *ent*-7α,18-diacetoxy-14β-hydroxybeyer-15-ene (**2**) was carried out with *Rhizopus nigricans*. The fungus produced an *ent*-15α,16α-epoxidation and/or *ent*-3β-hydroxylation. The structures of resulting products were established by chemical and spectroscopic methods.

Occasionally, microbial transformations constitute an alternative for introducing chemical functions into inaccessible sites of molecules. Thus, a great number of microbial transformations of steroids have been performed for this purpose (1).

Microbial transformations have been also applied to nonsteroid compounds (2, 3). On certain occasions, the products formed by this procedure can be easily obtained by chemical reactions. When transformations are made on natural substrates, the introduction of functions into biogenetically favorable sites is a frequent phenomenon (4).

As far as we know, only one paper on the microbial transformation of *ent*-beyerenes by hydroxylating fungi has been published (5): *Aspergillus ochraceous* catalyzes a reduction at C-3 on *ent*-3-ketobeyer-15-en-17-oic acid to give an axial alcohol at this position, and *Calonectria decora* catalyzes hydroxylation at C-6, giving an equatorial hydroxyl group. No transformation were observed using *Rhizopus nigricans* cultures. Incubation of a 19-norderivative of this substrate with *C. decora* cultures produced an equatorial hydroxylation at C-6 of this substrate. Incubation of this latter substrate with *A. ochraceous* and *R. nigricans* yielded no metabolites.

As indicated above, reports on microbial transformations of *ent*-beyerenic systems are scarce, and no information is available on the transformation of 14-hydroxybeyerenes.

As a continuation of our studies on microbial transformations of tetracyclic diterpenes (6, 7), we now report the action of hydroxylating fungi (*R. nigricans* and *A. ochraceous*) on *ent*-beyer-15-enic substrates with an *ent*-14β hydroxylation.

MATERIAL AND METHODS

PHYSICAL ANALYSES.—Mps were determined in a Kofler apparatus and are uncorrected. ^{1}H-nmr spectra were obtained with a Hitachi-Perkin Elmer R-20B (60 MHz) or a Bruker HX-90E (90 MHz) spectometer, using TMS as internal standard and $CDCl_3$ as solvent. ^{13}C-nmr spectra were determined on the latter apparatus (25.4 MHz) or a Bruker WM-360 (90.6 MHz) spectrometer, also in $CDCl_3$ solution (which also provided the lock signal), with TMS added as internal reference. Assignments of ^{13}C chemical shifts were made with the aid of broad-band proton decoupling and SFOR experiments, setting the decoupler frequency in the middle of the proton range in the former and 2 ppm to the right of the TMS in the latter. Mass spectra were determined on a Hewlett-Packard 5930A instrument (70 eV, direct inlet). Ir spectra were recorded on a Pye Unicam SP-1000 grating ir spectrometer. The rotatory powers were measured on a Perkin Elmer 240 polarimeter.

ISOLATION OF *ent*-7α-ACETOXY-14β,18-DIHYDROXYBEYER-15-ENE (7-ACETYLPUSILLATRIOL, **1**).—The 7-acetylpusillatriol used in these experiments was isolated from *Sideritis pusilla* spp. *flavovirens* (8).

Acetylation of 7-acetylpusillatriol (**1**).—7-Acetylpusillatriol (1.5 g) was acetylated in Ac_2O/pyridine (15/50 ml) at 0° for 1 h, after which time the resulting mixture was placed on a silica gel column (7729) and

eluted with $CHCl_3$. In this form, 1 g of pure *ent*-7α,18-diacetoxy-14β-hydroxybeyer-15-ene (7,18-diacetylpusillatriol, **2**) was isolated. Mp 156-158° (from hexane/$CHCl_3$); $[\alpha]^{20}D=58.10°$ (c 1, $CHCl_3$); ir ν max (KBr) 3450, 1735, 1640, 1250, and 740 cm^{-1}. ^1H nmr (δ, 60 MHz) 5.5 (2H, collap. Q_{AB}, H-15 and H-16); 5.08 (1H, m, W½=7 Hz, H-7); 3.65 (2H, collap. Q_{AB}, 2H-18); 3.15 (1H, s, H-14); 2.01 (6H, s, 2 AcO); 1.05 (3H, s, Me group) and 0.83 (6H, s, 2 Me groups). ^{13}C nmr (90.6 MHz) See Table 1; ms *m/z* (%) 344 (36, M-60), 326 (39), 315 (93), 284 (33), 271 (20), 269 (51), 266 (30), 255 (100), 254 (66), 253 (67), 251 (34), 241 (26). (Found: C, 71.43; H, 8.89; $C_{24}H_{36}O_5$ requires: C, 71.26; H, 8.97%).

Reduction of 7-acetylpusillatriol (**1**).—7-Acetylpusillatriol (**1**) (1.1 g) was dissolved in EtOH (50 ml) and hydrogenated [Parr apparatus, 5 atm, 500 mg Pd/$BaSO_4$ (10%), 5 h] to give 1.1 g of the 15,16-dihydroderivative(**6**): MP 203-205° (from hexane/$CHCl_3$); $[\alpha]^{20}D=50.3°$ (c 0.51, $CHCl_3$); ir ν max (KBr) 3450, 1725, and 1260 cm^{-1}. ^1H nmr (δ, 60 MHz) 5.15 (1H, m, W½=7 Hz, H-7); 3.25 (1H, bs, H-14); 3.25 (Q_{AB}, J=11 Hz, 2H-18); 2.12 (3H, s, AcO); 1.05 (6H, s) and 0.78 (3H, s) (Me groups at C-17, C-19, and C-20). (Found: C, 72.66; H, 10.01. $C_{22}H_{36}O_4$ requires: C, 72.49; H, 9.95%).

ORGANISM, MEDIA, AND CULTURE CONDITIONS.—*Rhizopus nigricans* CECT-2672 and *Aspergillus ochraceous* CECT-2069, from Colección Española de Cultivos Tipo were used in these studies (9).

Medium YEPGA containing 1% yeast extract, 1% peptone, 2% glucose, 2% agar, pH 5 was used for storage of *R. nigricans*. In all transformation experiments, a medium of the following composition was used: 0.1% peptone, 0.1% corn steep solids, 0.1% beef extract and 0.5% glucose in H_2O.

Erlenmeyer flasks (250 ml) containing 100 ml of medium were inoculated with a very dense suspension of *R. nigricans*. Incubation was maintained at 28° with gyratory shaking (120 rpm) for 6 days after which substrates, in EtOH, were added.

INCUBATION OF *ENT*-7α,18-DIACETOXY-14β-HYDROXYBEYER-15-ENT (7,18-DIACETYLPUSILLATRIOL, **2**) WITH *R. NIGRICANS*.—1 g of substrate **2** was dissolved in EtOH (20 ml) and distributed among 20 Erlenmeyer flask cultures and incubated for 30 h, after which the cultures were filtered and pooled; the cells were washed twice with H_2O. The liquid was saturated with NaCl and extracted with CH_2Cl_2. Both extracts were jointly dried with $MgSO_4$ and evaporated at 40° in vacuo, giving 0.6 g. The mixtures were placed on silica gel columns (Merck 7729) and eluted stepwise with $CHCl_3$ containing increasing amounts of Me_2CO. Fractions containing homogenous material (demonstrated by tlc), were pooled. These tlc plates (silica gel 0.25 mm, Merck G) were developed with CH_2Cl_2-Me_2CO (2:1). Starting materials and products were detected with a H_2O-H_2SO_4-AcOH (32:10:160) spray, followed by heating at 120°. With this method, 44 mg of metabolite **3**, 30 mg of substrate **2**, 20 mg of metabolite **4**, and 15 mg of metabolite **5** were isolated.

Ent-7α,*18-diacetoxy-14β-hydroxy-15α,16α-epoxibeyerane* (**3**).—Mp 128-130° (from hexane/$CHCl_3$); $[\alpha]^{20}D=61.90°$ (c 1, $CHCl_3$); ir (ν max (KBr) 3550, 1750, 1270, 1250, 880, and 870 cm^{-1}. ^1H nmr (δ, 90 MHz) 5.42 (1H, m, W½=9 Hz, H-7); 3.75 (2H, collap. Q_{AB}, 2H-18); 3.66 (1H, dd, J_1=3, J=1.5 Hz) and 3.33 (1H, dd, J_1=3, J_2=2 Hz) (H-15 and H-16); 2.98 (1H, m, W½=6 Hz, H-14); 2.09 and 2.06 (3H each, 2 AcO); 1.21, 1.00, and 0.88 (3H each, s, Me groups at C-17, C-19 and C-20). ^{13}C nmr (90.6 MHz) see Table 1. Ms *m/z* (%) 360 (23, M-60), 345 (10), 342 (30), 332 (25), 327 (7), 314 (30), 300 (26), 289 (45), 285 (43), 282 (22), 272 (35), 270 (35), 268 (51), 253 (100), 244 (38), 242 (62), 240 (75), 229 (100). (Found: C, 68.20; H, 8.83. $C_{24}H_{36}O_6$ requires: C, 68.55; H, 8.63%).

Ent-3β,*14β-dihydroxy-7α,18-diacetoxy-15α,16α-epoxibeyerene* (**4**).—Mp 180-182° (from hexane/$CHCl_3$); $[\alpha]^{20}D=88.04°$ (c 1, $CHCl_3$); ir ν max (KBr) 3500, 1730, 1270, 1250, 880 cm^{+1}. ^1H nmr (δ 90 MHz): 5.43 (1H, m, W½=8 Hz, H-7); 4.38 and 3.59 (Q_{AB}, J=12 Hz, 2 H-18); 3.61 (1H, dd, J_1=3, J_2=1.5 Hz); 3.33 (1H, dd, J_1=3, J_2=2 Hz) (H-15 and H-16); 2.98 (1H, m, W½=6 Hz, H-14); 2.10 and 2.06 (3H each, 2 AcO); 1.19, 0.98, and 0.79 (3H each, s, Me groups at C-17, C-19 and C-20). ^{13}C nmr (90.6 MHz) see Table 1. Ms *m/z* (%) 376 (60, M-60), 358 (70), 348 (45), 347 (55), 343 (30), 331 (100), 303 (38), 302 (60), 298 (95), 283 (60). (Found: C, 65.82; H, 8.43. $C_{24}H_{36}O_7$ requires: C, 66.03; H, 8.31%).

Ent-3β,*14β-dihydroxy-7α,18-diacetoxybeyer-15-ene* (**5**).—Mp 144-145° (from hexane/$CHCl_3$); $[\alpha]^{20}D=88.80°$ (c 0.5, $CHCl_3$); ir (ν max (KBr) 3400, 3060, 1740, 1640, 1260, 740 cm^{-1}. ^1H nmr (δ, 90 MHz) 5.57 (nearly collap. Q_{AB}, H-15 and H-16); 5.18 (1H, m, W½=8 Hz, H-7); 4.33 and 3.60 (Q_{AB}, J=12 Hz, 2 H-18); 3.33 (1H, m, W½=18 Hz, H-3); 3.26 (1H, s, H-14); 2.12 and 2.10 (3H each, s, 2 AcO); 1.12, 0.83, and 0.79 (3H each, s, Me groups at C-17, C-19 and C-20). ^{13}C nmr (20.15 MHz) see Table 1. Ms *m/z* (%): 360 (15, M-60), 342 (9), 332 (17), 327 (11), 313 (9), 300 (6), 282 (52), 267 (66), 253 (100). (Found: C, 68.31: H, 8.89. $C_{24}H_{36}O_6$ requires: C, 68.55; H, 8.63%).

INCUBATION OF *ENT*-7α,18-DIACETOXY-14β-HYDROXYBEYERANE (**6**) WITH *R. NIGRICANS*.—One g of substrate **6** was dissolved in EtOH (20 ml), distributed into 20 Erlenmeyer flask cultures and incubated for 96 h after which, substrate **6** was recovered unchanged.

TABLE 1. ^{13}C nmr Chemical Shifts of Compounds **2**, **3**, **4**, **5** and **7**

Atom	Compound				
	2	**3**	**4**	**5**	**7**
C-1	38,31[a]	38,50	37,21	37,08	38,94
C-2	17,69	18,54	25,80	25,88	17,64
C-3	35,53	35,36	71,50	71,80	35,41
C-4	36,09	36,06	42,11	42,08	36,04
C-5	41,60	41,68	39,90	39,83	41,66
C-6	24,79	24,75	24,39	24,32	24,74
C-7	71,25	68,96	69,09	71,39	75,51
C-8	57,56	51,75	51,78	57,52	52,28
C-9	48,08	51,62	51,60	48,03	51,09
C-10	37,17	37,37	37,37	37,08	37,68
C-11	19,23	17,63	18,63	19,29	19,28
C-12	31,18	34,12	34,13	32,19	37,97
C-13	49,10	43,49	43,68	49,10	43,36
C-14	89,88	82,62	82,59	89,85	86,76
C-15	130,60	56,67	56,55	130,34	28,39
C-16	135,65	61,68	61,68	135,88	34,45
C-17	19,23	16,83	16,82	19,21	21,55
C-18	72,44	71,17	65,88	66,14	72,67
C-19	17,69	17,68	12,05	12,59	17,64
C-20	15,37	15,59	15,83	15,58	15,76
Me-COO	21,33	21,26	21,25	21,28	21,55
Me-COO	21,05	20,95	20,96	21,00	21,05
Me-*C*OO	170,92	170,85	171,55	171,65	170,98
Me-*C*OO	170,35	170,10	169,92	170,31	170,98

[a]The ^{13}C-chemical shifts are given in ppm relative to TMS.

INCUBATION WITH *A. OCHRACEOUS* (CONIDIA).—*A. ochraceous* (conidia) were obtained as described in (10). A total of 540 mg of substrate **2** was dissolved in EtOH (50 ml) and distributed into 10 Erlenmeyer flask cultures and incubated for 96 h after which time only residual substrate **2** was recovered.

EPOXIDATION OF 7,18-DIACETYLPUSILLATRIOL (**2**).—A total of 100 mg of product **2** was dissolved in $CHCl_3$ (10 ml) and epoxidized with *m*-chloroperoxybenzoic acid (MCPBA) (250 mg) for 48 h at 0°. After column chromatography, 92 mg of *ent*-7α,18-diacetoxy-14β-hydroxy-15α,16α-epoxibeyerane, showing physical and spectroscopical properties identical to those of metabolite **3**, was isolated.

EPOXIDATION OF *ENT*-4β,14β-DIHYDROXY-7α,18-DIACETOXYBEYER-15-ENE (7,18-DIACETYLPUSILLATETROL, **5**).—7,18-Diacetylpusillatetrol (**5**) (50 mg) isolated from a *Sideritis* was dissolved in $CHCl_3$ (5 ml) and epoxidized with MCPBA (125 mg). After column chromatography, 37 mg of *ent*-3β,14β-dihydroxy-7α,18-diacetoxy-15α,16α-epoxibeyerane was obtained, which showed physical and spectroscopical properties identical to those described for metabolite **4**.

REDUCTION OF 7,18-DIACETYLPUSILLATRIOL (**2**).—A total of 100 mg of product **2** was hydrogenated as described for product **1** to give 100 mg of the dihydroderivative (**7**): Mp 173-176° (from hexane/$CHCl_3$). $[\alpha]^{20}D = 55.2°$ (c 1, $CHCl_3$); ir (ν max (KBr) 3450, 1730, 1250 cm^{-1}. ^1H nmr (δ, 60 MHz) 5.20 (1H, m, W½=7 Hz, H-7); 3.70 (2H, collap. Q_{AB}, 2 H-18); 3.20 (1H, s, H-14); 2.10 (6H, s, 2 AcO); 1.0 (6H, s, 2 Me groups) and 0.82 (3H, s, Me group). ^{13}C nmr (25.4 MHz) see Table 1.

RESULTS AND DISCUSSION

Several incubations of 7,18-diacetylpusillatriol (**2**), with *A. ochraceous* (conidia) and *R. nigricans*, have been carried out. In the former case, only unaltered substrate **2** was recovered after 96 h of incubation. However, *R. nigricans* incubation produced some transformation products.

Tlc evaluation of later incubation times indicates that, after 24 h, one-half of substrate **2** was consumed. After 30 h, the transformation did not progress. At this point, the incubation was stopped as indicated in the Material and Methods section.

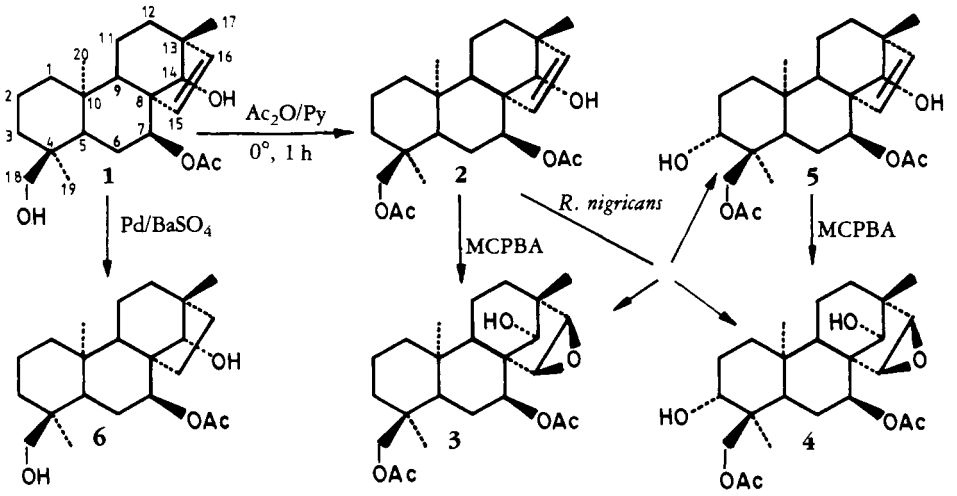

FIGURE 1. Microbial transformation of product **2** by *Rhizopus nigricans* and chemical correlations of metabolites isolated.

The main metabolite (**3**) isolated from the incubation exhibited a higher mobility (tlc) than the substrate (**2**), with an estimated molecular weight of 420, in accordance with a molecular formula of $C_{24}H_{36}O_6$ (substrate **2**, $C_{24}H_{36}O_5$). From ir and ^{1}H nmr spectra of product **3**, the absence of Δ^{15} and the presence of a 15,16 epoxide group could be deduced.

From analyses of ^{1}H-nmr signals due to H-15 and H-16, an *ent*-15α,16α configuration was assigned. In addition to a vicinal coupling constant (J=3 Hz), a W type constant J=1.5 Hz for one of the protons and J=2 Hz for the other one) can be observed. The W coupling between H-14 and H-15 and H-16 indicates *ent*-β disposition of H-15 and H-16 because such coupling between H-14 and *ent*-α H-15 and H-16 is not possible. In addition, ^{13}C nmr experiments confirm *ent*-α configuration for the 15,16-epoxide group of product **3** (see Table 1).

The α-effects, deduced from comparison of assigned chemical shifts of C-15 and C-16 of products **2** and **3**, were consistent with the 15,16-epoxidation. In addition, a similar deshielding was observed for C-8 and C-13.

The γ-effect observed for C-7 and C-17 was also consistent with the *ent*-15α,16α-epoxy group, this γ-effect being very appreciable on the C-14 chemical shift of product **3** (δ 82.62). On the other hand, the observed C-9, C-11, and C-12 chemical shifts for product **3** are consistent with those observed for the synthetical product **7** (*ent*-7α,18-diacetoxy-14-hydroxybeyerane) which has tetrahedral carbons at the 15 and 16 positions. The chemical shift for C-20 was similar for substrate **2** and metabolite **3**, which is in accordance with the proposed configuration for epoxide group at **3**.

We have also confirmed the structure of metabolite **3** by a semisynthesis. The epoxidation of substrate **2** with MCPBA gives a product identical to metabolite **3**. As epoxidation of this type of compound involves an approach from only the *ent*-α side (11), we conclude that the structure of metabolite **3** is *ent*-7α,18-diacetoxy-14β-hydroxy-15α,16α-epoxibeyerane.

Another metabolite (**4**) isolated from this incubation presents a molecular formula $C_{24}H_{36}O_7$. From its spectroscopic data, an epoxide group and a new hydroxyl group may be deduced. Thus, in the ^{1}H-nmr spectrum of this metabolite, in addition to similar signals to those of metabolite **3**, there exists a broad signal at δ 3.33 (1H, W½=18 Hz) partially superimposed on one of the two signals due to the protons on the epoxide group. The last described signal is similar to one of those observed in the ^{1}H-nmr spec-

trum of a more polar metabolite (**5**) also isolated from this incubation, which still has Δ^{15} group. This metabolite **5** was shown to be identical to a minoritary product isolated from a *Sideritis* (8), which structure was *ent*-3β,14β-dihydroxy-7α,18-diacetoxybeyer-15-ene. From these data, a structure of *ent*-3β,14β-dihydroxy-7α,18-diacetoxy-15α,16α-epoxibeyerane could be assigned to metabolite **4**. The epoxidation of product **5** with MCPBA gives a product identical to metabolite **4**.

In order to confirm the structures of metabolites **4** and **5**, we have obtained ^{13}C-nmr spectra of metabolites **4** and **5** (see Table 1), which are consistent with an *ent*-3β-hydroxylation at C-3 of both metabolites (12). Thus, the α-effect is in accordance with an equatorial disposition of this hydroxyl group at C-3. In the same way, γ-effects at C-1 and C-5 are moderate values, and γ-gauche effects of both C-18 and C-19 confirm the equatorial disposition.

We conclude that the action of *R. nigricans* results in oxidation at C-15/C-16 and also at C-3, in a similar way to that found by us in the case of *ent*-kaur-16-enes (6) using the same strain of microorganism. However, transformation of *ent*-7α-acetoxy-14β,18-dihydroxybeyerane (**6**) was unsuccessful in contrast to that found for *ent*-kauranols studied by us (7).

Hydroxylation on the *ent*-β side at C-3 of substrate **2** indicates that the enzymatic action occurs on the *ent*-β side, in accordance with what was previously observed for 3-keto derivatives of *ent*-kaurenes (6) and *ent*-kauranes (7), although the reduction of this keto group on the *ent*-β side logically gives an axial (*ent*-α) alcohol group.

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