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MICROBIAL TRANSFORMATION OF TETRACYCLIC DITERPENES: CONVERSION OF KAURANOLS AND KAURANONES BY *RHIZOPUS NIGRICANS*

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ABSTRACT.—Microbial transformation of *ent*-3β,7α-dihydroxy-18-acetoxy-16(S)kaurane (**2**) are carried out with *Rhizopus nigricans*. The fungus produces an *ent*-16β-hydroxylation in the substrate, which is isolated and characterized by nmr and ms analyses; its structure is verified by chemical correlation. Under the same conditions, incubation of *ent*-18-acetoxy-16(S)kauran-3,7-dione (**9**) gives *ent*-3α,16β-dihydroxy-18-acetoxykauran-7-one (**10**), as main product. The structure of this product is established by chemical and spectroscopic methods.

Despite the great number of diterpenoids isolated from nature, there are few microbiological transformations that have been carried out with this type of compound (1). Those carried out with the fungus *Giberella fujikuroi* are, however, of great importance for elucidating the biogenetic route of giberellines formation. These studies have been made starting from compounds with gibane skeletons (2) or their biogenetic precursor, the *ent*-kaur-16-enic system (3).

On the other hand, there are several publications that record the microbiological transformation of diterpenoids with the fungi whose enzymatic systems are not selective as regards the substrate. A tricentric action similar to that which occurs in steroids (4) has been observed and seems also to be present in tetracyclic diterpenes, though, in this last case, definitive conclusions still can not be made (5-8).

In a previous study (9), we proved that *Rhizopus nigricans* behaves in a similar manner when faced with both 3,7-diketoandrostanic and *ent*-kaur-16-ene-3,7-diketonic substrates.

This study is part of our research into the action of hydroxylating fungi to several types of tetracyclic diterpenoids. We are attempting to find out whether there exists a parallel between the action of the fungi on steroidal systems and on tetracyclic diterpenoids with similar chemical functions.

MATERIAL AND METHODS

ORGANISM.—*Rhizopus nigricans* CECT (2672) from Colección Española de Cultivos Tipo was used in these studies.

MEDIA.—Medium YEPGA containing 1% yeast extract, 1% peptone, 2% glucose, and 2% agar (pH 5) was used for storage. In all transformation experiments, a semisynthetic medium of the following composition was used: 0.1% peptone, 0.1 corn steep, 0.1% beef extract, and 0.5% glucose in water.

CULTURE CONDITIONS.—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 d. Substrate 2, 1.8 g in ethanol (40 ml), was distributed between 36 flasks, and the incubation was continued for 24 h. For the incubation of substrate 9, 775 mg in ethanol (30 ml) were distributed among 15 flasks, and the incubation was performed for the same period of time.

RECOVERY AND PURIFICATION.—After removing the cells by filtration, the cultures were filtered and pooled; the cells were washed twice with water. The liquid was saturated with NaCl and extracted with CH_2Cl_2. These extracts were dried with $MgSO_4$ and evaporated at 40° in a vacuum. The mixtures were placed on silica gel columns (Merck 7729) and eluted stepwise with chloroform containing increasing amounts of acetone. Fractions that contained homogeneous material on tlc plates were pooled. These tlc

plates (Silica gel 0.25 mm Merck G) were eluted with mixtures of CH_2Cl_2-acetone (2:1). Starting materials and transformed zones were detected with a H_2SO_4-AcOH spray at 120°.

ISOLATION OF *ENT*-3β,7α-HYDROXY-18-ACETOXYKAUR-16-ENE (LINEAROL, **1**).—The linearol used in these experiments was isolated from *Sideritis arborescens* (arborescens) Salzm (10).

PHYSICAL ANALYSES.—Proton magnetic resonance (pmr) spectra were obtained with a Hitachi-Perkin-Elmer R-20 B (60 MHz) or a Brüker HX-90E (90 MHz), using tetramethylsilane as internal standard and $DCCl_3$ as solvent. The ir spectra were obtained using a Pye-Unicam SP-1000; ms spectra were obtained with a Hewlett-Packard 5390 A, with direct inlet and operating at 70 eV. The melting points (uncorrected) were obtained on a Kofler apparatus. The optical rotations were determinated with a Perkin-Elmer 141 polarimeter.

SYNTHESES OF SUBSTRATES **2**(*ENT*-3β,7α-DIHYDROXY-18-ACETOXY-16(S)KAURANE) and **9** (*ENT*-18-ACETOXY-16(S)KAURAN-3,7-DIONE).—Linearol (**1**) (3.37 g) was dissolved in EtOH (50 ml), and Pd on $BaSO_4$ (5%) (3 g) was added. The hydrogenation was carried out at 5 atm. The tlc controls indicated that the reaction had concluded after 1 h. The reaction mixture was filtered and washed with EtOH. The filtrate was concentrated to give 3.1 g of product **2**: m.p. 179-181°, $[\alpha]^{20}D=6.02°$ (c 1.3 $CHCl_3$); ms, *m/z* (%): 364(1), 362(1), 360(1), 346(8), 329(4), 316(4), 304(24), 286(50), 273(34), 268(72), 256(100), 255(62), 241(52), 235(89), 229(45), 227(40), 217(49), 213(60), 200(52); ir [ν max (KBr), cm^{-1}]: 3400, 1720, and 1250; pmr (δ, 60 MHz): 4,0 (2H, Q_{AB} collap.), 3.5-3.8 (2H, m), 2.10 (3H, s), 1.10 (6H, s), 0.82 (3H, s).

Found: C, 71.92; H, 10.20. $C_{22}H_{36}O_4$ requires: C, 72.49; H, 9.95%.

Product **2** (1 g) was dissolved in acetone (6 ml) and oxidized with Jones' reagent (11). After column chromatography, 775 mg of product **9** were obtained: ms, *m/z* (%): 360(9), 318(14), 300(100), 287(62), 272(31), 257(31), 244(28), 229(21), 217(31); ir [ν max (neat), cm^{-1}]: 1735, 1695, 1235, 1030; pmr (δ, 60 MHz): 4.05 (2H, Q_{AB}, $J=12$ Hz), 2.0 (3H, s), 1.25 (3H, s), 1.0 (6H, s).

Found: C, 72.85; H, 9.22. $C_{22}H_{32}O_4$ requires: C, 73.30; H, 8.95%.

SYNTHESIS OF *ENT*-3β,7α,18-TRIACETOXY-16β-HYDROXYKAURANE (**7**) FROM LINEAROL (**1**).—Linearol (**1**) (500 mg) was epoxidized with *m*-chloroperoxybenzoic acid (MCPBA) (350 mg) as usual, and yielded a mixture (540 mg) from which 320 mg of main product **5** (*ent*-3β,7α-dihydroxy-16β,17-epoxi-18-acetoxykaurane) was isolated; $[\alpha]^{25}D=-18.9°$ (c 1.1 $CHCl_3$); ms, *m/z* (%): 378(9), 360(5), 342(10), 318(45), 301(65), 282(100); ir [ν max (neat), cm^{-1}]: 3460, 1715, 1270; pmr (δ, 60 MHz): 3.94 (2H, s), 3.2-3.7 (2H, m), 2.75 (2H, Q_{AB}, $J=5$ Hz), 2.09 (3H, s), 1.07 (3H, s), 0.77 (3H, s).

Found: C, 69.70; H, 9.33. $C_{22}H_{34}O_5$ requires: C, 69.81; H, 9.05%.

Product **5** (100 mg) in Et_2O, was reduced with $LiAlH_4$ (135 mg) as usual, yielding product **6** (*ent*-3β,7α,16β,18-tetrahydroxykaurane) (90 mg), which was acetylated (Ac_2O-Py, reflux for 2 h) to give a mixture that was purified by column chromatography. Product **7** (*ent*-3β,7α,18-triacetoxy-16β-hydroxykaurane) (45 mg) was isolated: m.p. 166-168°; $[\alpha]^{25}D=24.89°$ (c 3.1 $CHCl_3$); ms, *m/z* (%): 464(0.5), 446(0.5), 404(31), 348(8), 344(12), 328(4), 314(4), 312(2), 304(6), 303(7), 302(4), 285(26), 284(100), 271(23), 269(24), 267(15), 253(23), 251(16); pmr (δ, 60 MHz): 4.6 and 4.95 (2H, m), 3.8 (2H, Q_{AB}, $J=12$ Hz), 2.1 (9H, s), 1.45 (3H, s), 1.2 (3H, s), 0.9 (3H, s).

Found: C, 66.95; H, 8.73. $C_{26}H_{40}O_7$ requires: C, 67.22; H, 8.68%.

Ent-3β,7α,16β-trihydroxy-18-acetoxykaurane (product **3**) gave: m.p. 210-212° $[\alpha]^{25}D=1.84°$ (c 1.9 $CHCl_3$); ms, *m/z* (%): 380(3), 363(28), 344(19), 327(19), 316(19), 304(42), 298(47), 287(91), 274(58), 269(93), 256(100), 235(81); ir [ν max (KBr), cm^{-1}]: 3450, 1715, 1250; pmr (δ, 90 MHz): 4.03 (2H, Q_{AB}, $J=11$ Hz), 3.5-3.8 (2H, m), 2.08 (3H, s), 1.38 (3H, s), 1.06 (3H, s), 0.75 (3H, s).

Found: C, 69.05; H, 9.45. $C_{22}H_{36}O_5$ requires: C, 69.44; H, 9.54%.

Ent-7α,16β,18-trihydroxy-3β-acetoxykaurane (product **4**) gave: m.p. 224-5°. $[\alpha]^{25}D=3.42$ (c 1.18 $CHCl_3$); ms, *m/z* (%): 380(3), 363(45), 304(15), 302(15), 298(15), 286(30), 284(40), 273(100).

Found: C, 69.07; H, 9.87. $C_{22}H_{36}O_5$ requires: C, 69.44; H, 9.54%.

Ent-18-acetoxy-16β-hydroxykauran-3,7-dione (product **8**) gave: m.p. 148-150°. $[\alpha]^{25}D=12.57°$ (c 2.1 $CHCl_3$); ms, *m/z* (%): 376(18), 362(28), 359(22), 344(22), 334(52), 319(100), 298(49), 278(78), 259(74); pmr (δ, 60 MHz): 4.1 (2H, Q_{AB}, $J=12$ Hz), 2.05 (3H, s), 1.45 (3H, s), 1.4 (3H, s), 1.1 (3H, s).

Found: C, 69.72; H, 8.97. $C_{22}H_{32}O_5$ requires: C, 70.18; H, 8.57%.

Ent-3α,16β-dihydroxy-18-acetoxykaurane-7-one (product **10**) data were: Oil. $[\alpha]^{25}D=-0.86°$ (c 1.4 $CHCl_3$); ms, *m/z* (%): 378(13), 364(22), 360(9), 346(9), 337(47), 322(91), 318(27), 300(33), 288(25), 261(47), 257(41), 247(59), 245(100), 240(42), 226(52), 216(63); pmr (δ, 60 MHz): 4.0 (2H, Q_{AB}, $J=12$ Hz), 3.75 (1H, $W½=7$ Hz), 2.08 (3H, s), 1.45 (3H, s), 1.25 (3H, s), 0.95 (3H, s).

Found: C, 70.06; H, 9.12. $C_{22}H_{34}O_5$ requires: C, 69.81; H, 9.05%.

Ent-16β,18-dihydroxy-3α-acetoxykauran-7-one (product **11**) gave: Oil. $[\alpha]^{25}D=11.47°$ (c 1.2 $CHCl_3$); ms, *m/z* (%): 378(50), 364(88), 350(40), 346(45), 337(100), 322(96), 303(70), 300(88),

284(24), 288(88), 286(66), 277(82), 271(78); pmr (δ, 60 MHz): 4.95 (1H, m, $W_{1/2}$=7 Hz), 3.40 (2H, Q_{AB} collap.), 2.08 (3H, s), 1.45 (3H, s), 1.25 (3H. s), 0.95 (3H, s).

Found: C, 69.56; H, 8.93. $C_{22}H_{34}O_5$ requires: C, 69.81; H, 9.05%.

Ent-18-acetoxy-16β-hydroxykauran-3,7-dione (product **12**) data were: Oil. $[\alpha]^{25}D = -11.32°$ (c 1.08 $CHCl_3$); ms, *m/z* (%): 376(26), 362(35), 359(24), 344(5), 334(62), 319(100), 298(50), 278(68), 259(73); pmr (δ, 60 MHz): 4.1 (2H, Q_{AB}, J=12 Hz), 2.05 (3H, s), 1.45 (3H, s), 1.4 (3H, s), 1.1 (3H, s).

Found: C, 69.83; H, 8.72. $C_{22}H_{32}O_5$ requires: C, 70.18; H, 8.57%.

RESULTS

The microbial and chemical transformations appearing in this work are summarized in Figure 1.

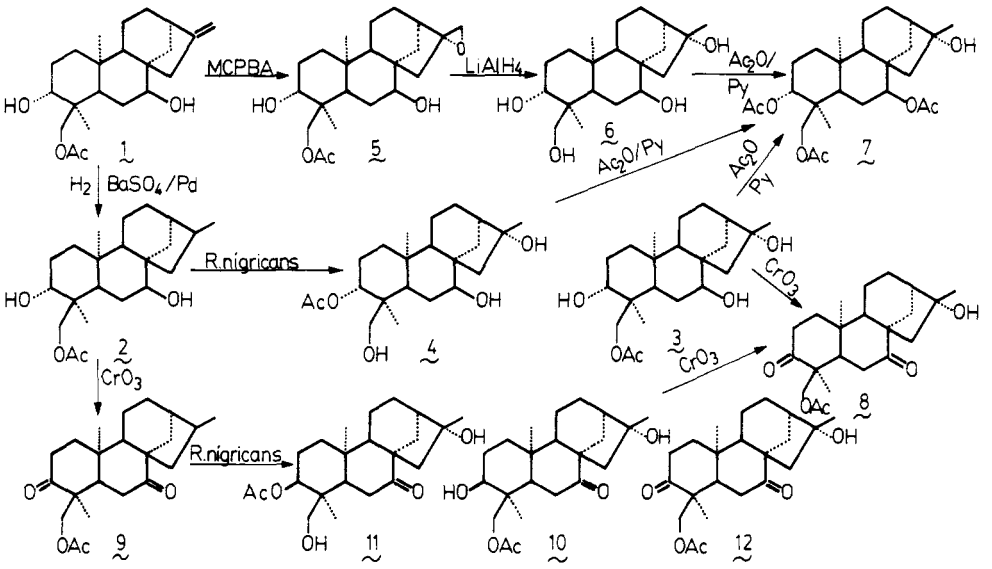

FIGURE 1. Microbial transformation of producs **2** and **9** by *Rhizopus nigricans* and chemical correlations of metabolites isolated.

Product **2** (1.8 g, *ent*-3β,7α-dihydroxy-18-acetoxy-16 (S)kaurane) was incubated as previously described. Product **3** (475 mg) and product **4** (150 mg) were isolated. The acetylation of products **3** and **4** led to product **7**, identical to synthetic *ent*-3β,7α,18-triacetoxy-16β-hydroxykaurane.

The main metabolite isolated from the substrate **2** incubation was product **3**. Its molecular weight (380) indicates that oxygenation occurred. The pmr spectrum shows the presence of a methyl group at low field (δ 1.38, 3H). This displacement is consistent with the presence of a methyl group geminal to a hydroxyl group. The spectroscopic data of this product indicates that an *ent*-16β-hydroxylation occurred; this was proved by correlating product **3**, after acetylation, with product **7** obtained by chemical means using reactions of known stereochemistry.

Another product isolated from this incubation (product **4**) was also transformed into product **7** by acetylation. Thus, we suppose product **4** to be acetoxy position isomer to product **3**. In systems of this type, the usual acetoxy group transposition is known to be between positions 3 and 18. Spectroscopic study of product **4** effectively indicates an acetoxy transposition product from C-18 to C-3. Products **3** and **4** are converted from one to the other after a few hours of dissolving in $HCCl_3$.

Product **9** (*ent*-18-acetoxy-16(S)kauran-3,7-dione) (775 mg) was also incubated as previously described; 210 mg of product **10**, 60 mg of product **11**, and 40 mg of prod-

uct **12** were isolated. The oxidation of product **10** gave product **8**, identical to that obtained by oxidation of product **3**.

The main metabolite, product **10**, had a molecular weight of 378, which shows an increase of 18 units compared with substrate **9**. In its pmr spectrum, signals attributable to methyl singlets were observed at δ 0.95 (3H), 1.25 (3H), and 1.45 (3H). The chemical shift of this last methyl group led us to suppose that, as in metabolite **2**, an *ent*-16β-hydroxylation had occurred. A signal corresponding to an acetoxy group at δ 2.08 (3H) was also detected which, along with a Q_{AB} centered at δ 4.0 (J=12 Hz), seems to indicate that the acetoxy group is maintained at C-18. Partially overlapping the already mentioned Q_{AB}, a narrow multiplet at δ 3.75 (1H, W½=7 Hz) was detected, similar to that observed in the corresponding spectrum of the main metabolite from the *ent*-18-acetoxykaur-16-en-3,7-dione incubation (9) and which was due to an equatorial proton situated at C-3, and geminal to a hydroxyl group.

In the case of metabolite **10**, a transposition of the acetoxy group was also observed. The product of this conversion is identical to another metabolite isolated from this incubation (product **11**). Metabolite **11**, also with a molecular weight of 378, does not have its original Q_{AB} centered at δ 4.0 in its pmr spectrum; nonetheless, a colapsed Q_{AB} centered at 3.40 (2H) appeared. A narrow multiplet, similar to that found in product **10**, but centered at δ 4.95, was observed. The oxidation of metabolite **10** yielded product **8**, identical to that obtained by the oxidation of metabolite **3**. This correlation proves that products **3** and **10** have functional groups at the same skeletal positions. Product **8** turned out to be identical to the other metabolite (product **12**) isolated from the incubation of product **9**.

We may, thus, conclude that the action of *R. nigricans* on substrate **9** produces an *ent*-16β-hydroxylation and a reduction of the keto group on C-3, yielding the corresponding axial alcohol. The product of the *ent*-16β-hydroxylation was also isolated as a minority metabolite (product **12**). On no occasion were the keto groups at C-7 of the isolated metabolites altered.

These results are in accord with those we have previously obtained: the incubation of *ent*-kaur-16-enes, similarly functionalized, produces an *ent*-16β,17-epoxidation as well as a reduction of the keto group at C-3, yielding an axial alcohol group at this carbon (9).

The microbial action apparently is produced through a tricentric system that would include the positions C-3, C-7, and C-16 of the *ent*-kauranic system. It is worth noting that distances between the mentioned carbons are similar to those existing between carbon of the same numeration in 5α-androstanic systems.

In our *ent*-kaur-16-enic and *ent*-kauranic systems, the enzymatic action of *R. nigricans* was directed to the *ent*-β side at C-3 as well as at C-16, although it must be noted that the substrate centers (C-3 and C-16) were not situated on the same plane.

In the case of 3,7-diketoandrostanic systems incubated with *R. nigricans* cultures, the respective hydroxylations and reductions occur at opposite sides (4).

The results we have obtained are parallel with those published on *ent*-17-norkauranone and 17-norkauranone (7), although the resulting configurations at C-3 are logically contrary, taking into account that both are of the same skeletal stereochemistry. In the already mentioned *ent*-17-norkauranone, the *ent*-β-hydroxylation at C-3 is produced by the substitution of an *ent*-β hydrogen by a hydroxy group. This process must occur with the retention of configuration (this result also occurs in the hydroxylation at C-16 of substrates **2** and **9**). However, the *ent*-β reduction of the keto group at C-3 of substrate **9** must lead to the formation of an *ent*-α-OH group at C-3.

We believe that the described transformations of 16(S)kauranes, carried out under conditions comparable to those done on similarly functionalized steroids, are of special

interest, and suggest that previous studies in the steroids field will be applicable to the design of transformations of tetracyclic diterpenoids.

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