Journal of Natural Products
Vol. 49, No. 1, pp. 126-132, Jan-Feb 1986

MICROBIAL TRANSFORMATION OF TETRACYCLIC DITERPENES: CONVERSION OF *ENT*-KAURENONES BY *ASPERGILLUS NIGER*

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ABSTRACT.—Microbial transformations of *ent*-18-acetoxykaur-16-en-3,7-dione (**2**) were carried out with *Aspergillus niger*. After 48 h of incubation, only one metabolite (**3**) was isolated as a result of the hydrolysis of the acetoxy group. Incubation for 6 days produced a series of metabolites: *ent*-16β,18-dihydroxykauran-3,7-dione (**4**), *ent*-17,18-dihydroxykauran-3,7-dione (**5**), *ent*-16α,17,18-trihydroxykauran-3,7-dione (**6**), and *ent*-16β,17,18-trihydroxykauran-3,7-dione (**7**). Structures of these products were established by chemical and spectroscopical methods.

Microbial transformations are a tool for semisynthesis in organic chemistry. In the field of natural product chemistry, microbial transformations are especially used for bioconversion of steroids (1), although microbial transformations have been also applied to nonsteroid compounds (2,3).

For diterpenic compounds, in addition to those carried out with the fungus *Gibberella fujikuroi* for elucidating the biogenetic route of gibberellin formation (4,5), several microbial transformations of kaurenic compounds have been performed (6-11). Microbial transformations on beyeranic (12,13) and trachylobanic (14) diterpenoids have also been accomplished.

As a continuation of our studies on microbial transformations of tetracyclic diterpenes (15-17), we now report the action of *Aspergillus niger* on *ent*-18-acetoxykaur-16-en-3,7-dione (**2**).[1] In a previous paper (15), we reported the conversion of substrate **2** by *Rhizopus nigricans*, which produced the reduction of the keto group at C-3 (to give an axial hydroxy group) and the *ent*-16β,17-epoxidation of the double bond.

MATERIAL AND METHODS

PHYSICAL ANALYSES.—Mps were determined in a Kofler apparatus and are uncorrected. ^{1}H-nmr spectra were measured at 80 MHz ($CDCl_3$ with TMS as internal standard) in a Bruker WP8OSY spectrometer. ^{13}C-nmr spectra were determined at 20.13 MHz also in $CDCl_3$ (which also provided the lock signal) with TMS added as internal reference, equipped with a 3-clock multipulser. Assignments are made with the aid of distortionless enhancement by polarization transfer (DEPT), using a "flip angle" of 135°. Ir spectra were recorded on a Pye Unicam SP-1000 grating infrared spectrometer. The rotatory powers were measured on a Perkin-Elmer 240 polarimeter.

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

SYNTHESIS OF SUBSTRATE **2** (*ENT*-18-ACETOXYKAUR-16-EN-3,7-DIONE).—Linearol (**1**) (2.10 g) was dissolved in Me_2CO (60 ml) and oxidized with Jones's reagent (19). After column chromatography, 2 g of product **2** was obtained (15). ^{13}C nmr see Table 1.

ORGANISM.—*A. niger* (Strain No. 2091) from Colección Española de Cultivos Tipo was used in these studies (20).

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

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[1]Nomenclature of compounds is based on J.W. Rowe, "The Common and Systematic Nomenclature of Cyclic Diterpenes," 3rd revision, U.S. Forest Product Laboratory, Madison, WI, 1969.

TABLE 1. ^{13}C-nmr Chemical Shifts of Compounds **2, 3, 8, 9, 12, 13,** and **14**.

Atoms	Compounds						
	2	**3**	**8**	**9**	**12**	**13**	**14**
C-1	37.37[a]	37.83	37.41	37.60	37.14	37.41	37.79
2	34.68	35.07	34.69	34.74	34.75	34.62	35.07
3	212.41	216.49	212.44	212.43	212.34	212.23	216.41
4	50.00	52.33	50.02	50.11	50.07	50.05	52.32
5	45.94	45.32	46.17	46.08	46.01	46.19	45.45
6	37.01	37.20	37.07	37.17	37.14	36.99	36.97
7	211.37	211.97	210.92	211.43	210.78	210.29	211.75
8	57.07	56.79	58.34	57.45	56.66	57.75	55.45
9	54.14	54.40	54.84	55.46	55.15	54.68	55.12
10	38.07	38.09	38.08	38.13	38.21	38.13	38.10
11	18.38	18.28	18.15	18.85	18.67	18.36	18.34
12	32.33	32.39	25.98	25.19	25.83	25.36	25.50
13	42.32	42.06	47.57	36.72	40.70	44.85	43.40
14	38.52	38.25	37.07	39.72	37.14	36.60	36.97
15	41.16	42.06	49.10	35.25	44.08	44.68	44.80
16	153.01	153.55	78.89	39.01	78.38	79.68	86.56
17	105.05	105.19	24.43	65.26	70.81	68.22	76.78
18	67.29	66.23	67.37	67.33	67.36	67.32	66.23
19	16.10	16.17	16.24	16.21	16.10	16.25	16.13
20	17.26	16.64	17.16	17.22	17.27	17.16	16.54
CH_3	20.77		20.80	20.99	20.91	20.80	26.21
CH_3				20.88	20.91	20.90	27.51
CO			170.31	171.29	171.38	171.15	
CO				170.39	170.41	170.28	
$(CH_3)C$							109.27

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

CULTURE CONDITIONS.—Erlenmeyer flasks (250 ml), containing 100 ml of medium, were inoculated with a dense suspension of *A. niger* in distilled H_2O prepared from slants. Incubations were maintained at 28° with gyratory shaking (120 rpm) for 6 days. Substrate **2**, 500 mg in EtOH (10 ml) was distributed among ten culture flasks and incubated for 48 h, after which four flasks were separated from incubation. For the other six flasks, incubation was maintained for 6 days.

RECOVERY AND PURIFICATION.—*48-H incubation.*—Cultures were filtered and pooled, and the cells were washed twice with H_2O. The liquid was saturated with NaCl and extracted repeatedly with CH_2Cl_2. These extracts were dried with $MgSO_4$ and evaporated at 40° in vacuo, giving 80 mg of crude products. The mixture was placed on a silica gel column (7 g of silica gel Merck 7729 was utilized for each 100 mg of mixture to separate) and eluted stepwise with $CHCl_3$ containing increasing amounts of Me_2CO (to obtain a rapid gradient of polarity). Fractions that contained homogeneous material on tlc plates (silica gel 0.25 mm, Merck G, developed with CH_2Cl_2-Me_2CO, 2:1) were pooled. Starting material and products were detected with a H_2O-H_2SO_4-HOAc (3:1:16) spray, followed by heating at 120°. With this method, 5 mg of substrate **2** and 58 mg of metabolite **3** (*ent*-18-hydroxykaur-16-en-3,7-dione) were isolated.

6-Day incubation.—Proceeding as indicated for 48 h incubations, 20 mg of metabolite **4**, 70 mg of metabolite **6**, and 11 mg of metabolite **7** were isolated. Another mixture of products (12 mg) was acetylated and chromatographed to give 6 mg of diacetate **9**.

ENT-18-HYDROXYKAUR-16-EN-3,7-DIONE (**3**).—Mp 148-150° (from hexane/$CHCl_3$); $[\alpha]^{20}D = -25.7°$ (c 1, $CHCl_3$); ir ν max (KBr) cm^{-1} 3400, 3060, 1695, 1650, 1030, and 750; ^{1}H nmr (δ) 4.90 (2H, m, $W_{1/2}$=7 Hz, 2H-17), 3.69 and 3.30 (2H, Q_{AB}, J=12 Hz, 2H-18), 1.35 and 1.02 (3H each, s, C-Me groups at C-19 and C-20). ^{13}C nmr see Table 1. (Found: C, 75.63; H, 8.98. $C_{20}H_{28}O_3$ requires: C, 75.91; H, 8.92%).

ENT-16β,18-DIHYDROXYKAURAN-3,7-DIONE (**4**).—Mp 202-204° (from hexane/$CHCl_3$); $[\alpha]^{20}D = -34.8°$ (c 1, EtOH); ir ν max (KBr) cm^{-1} 3500, 1710, 1360, and 1020; ^{1}H nmr (δ) 3.70 and

3.30 (2H, Q_{AB}, J=12 Hz, 2H-18), 1.41, 1.37, and 1.00 (3H each, s, Me groups C-17, C-19, and C-20). (Found: C, 71.51; H, 8.77. $C_{20}H_{30}O_4$ requires: C, 71.82; H, 9.04%).

ENT-16α,17,18-TRIHYDROXYKAURAN-3,7-DIONE (**6**).—Mp 168-170° (from hexane/$CHCl_3$); $[\alpha]^{20}D=-41.6°$ (c 1, EtOH); ir ν max (KBr) cm^{-1} 3500, 1710, 1350, and 1020; ^1H nmr (δ) 3.70 and 3.30 (2H, Q_{AB}, J=12 Hz, 2H-18), 3.50 (2H, Q_{AB} collap., 2H-17), 1.35 and 1.00 (3H each, s, Me-groups C-19 and C-20). (Found: C, 68.10; H, 8.77. $C_{20}H_{30}O_5$ requires: C, 68.54; H, 8.63%).

ENT-16β,17,18-TRIHYDROXYKAURAN-3,7-DIONE (**7**).—Gum; ir ν max neat, cm^{-1} 3560, 1710, 1360, and 1030; ^1H nmr (δ) 3.70 and 3.30 (2H, Q_{AB}, J=12 Hz, 2H-18), 3.70 and 3.42 (2H, Q_{AB}, J=12 Hz, 2H-17), 1.25 and 1.00 (3H each, s, Me-groups C-19 and C-20).

ACETYLATION OF METABOLITE **3**.—Metabolite **3** (10 mg) was acetylated with pyridine-Ac_2O (1:0.5) for 2 h at 0°. After column chromatography, 7 mg of a product identical to substrate **2** was isolated.

ACETYLATION OF METABOLITE **4**.—Metabolite **4** (12 mg) was acetylated with pyridine-Ac_2O (1:0.5) for 2 h at 0°. After column chromatography, 9 mg of *ent*-16β-hydroxy-18-acetoxykauran-3,7-dione (**8**) was isolated. This product is identical to one described in a previous report (16). ^1H-nmr (δ, 80 MHz) 4.18 and 3.80 (2H, Q_{AB}, J=12 Hz, 2H-18), 2.00 (3H, s, AcO), 1.42, 1.36, and 1.05 (3H each, s, Me-groups C-17, C-19, and C-20). ^{13}C nmr see Table 1.

ISOLATION OF METABOLITE **9**.—Acetylation of the 12 mg fraction of the 6-day incubation gave 5 mg of product **8** and 6 mg of *ent*-17,18-diacetoxy-16(*S*)-kauran-3,7-dione (**9**): gum; $[\alpha]^{20}D=-18.6°$ (c 1, $CHCl_3$); ir ν max, neat, cm^{-1} 3060, 1740, 1710, 1360, 1230, 1020; ^1H nmr (δ) 4.19 and 3.81 (2H, Q_{AB}, J=12 Hz, 2H-18), centered at 4.10 (2H, m, 2H-17), 2.06 and 2.00 (3H each, s, AcO groups), 1.32 and 1.05 (3H, s, Me-groups C-19 and C-20); ^{13}C nmr see Table 1.

ACETYLATION OF METABOLITE **6**.—Metabolite **6** (25 mg) was acetylated with pyridine/Ac_2O (2:1) for 12 h at room temperature. After column chromatography, 20 mg of *ent*-17,18-diacetoxy-16α-hydroxy kauran-3,7-dione (**12**) was isolated: gum; $[\alpha]^{20}D=-16.3°$ (c 1, $CHCl_3$); ir ν max, neat, cm^{-1} 3560, 3060, 1740, 1710, 1360, 1230, and 1020; ^1H nmr (δ) 4.17 and 3.81 (2H, Q_{AB}, J=12 Hz, 2H-17), 4.11 and 3.94 (2H, Q_{AB}, J=12 Hz, 2H-18), 2.10 and 1.98 (3H each, s, AcO groups), 1.33 and 1.04 (3H each, s, Me-groups C-19 and C-20); ^{13}C nmr see Table 1.

ACETYLATION OF METABOLITE **7**.—Metabolite **7** (11 mg) was acetylated with pyridine/Ac_2O (2:1) for 2 h at 0°. After column chromatography, 10 mg of *ent*-17,18-diacetoxy-16β-hydroxykauran-3,7-dione (**13**) was isolated: gum; $[\alpha]^{20}D=-25°$ (c 1, $CHCl_3$); ir ν max, neat, cm^{-1} 3560, 3060, 1740, 1710, 1360, 1230, and 1020; ^1H nmr (δ) 4.37 and 4.17 (2H, Q_{AB}, J=12 Hz, 2H-17), 4.17 and 3.81 (2H, Q_{AB}, J=12 Hz, 2H-18), 2.10 and 1.98 (3H each, s, AcO groups), 1.33 and 1.04 (3H each, s, Me-groups C-19 and C-20); ^{13}C nmr see Table 1.

HYDROBORATION OF SUBSTRATE **2**.—Substrate **2** (100 mg) was dissolved in toluene (5 ml), and 80 mg of borane-trimethylamine complex was added; the mixture was warmed in a sealed tube at 120° for 3 h, after which a solution of 3M NaOH and H_2O_2 (30%) (1 ml) was added. After 1 h, the toluene layer was washed with H_2O, diluted HCl, and again with H_2O. The organic layer was dried with $MgSO_4$ and concentrated in vacuo. After column chromatography, 42 mg of a polar product (**10**) was isolated; *ent*-3β, 17-dihydroxy-18-acetoxy-16(*S*)-kauran-7-one (**10**): gum; $[\alpha]^{20}D=-53.2°$ (c 1, $CHCl_3$); ir ν max, neat, cm^{-1} 3540, 3060, 1730, 1700, 1450, 1360, 1230, and 1020; ^1H nmr (δ) 4.05 (2H, Q_{AB} collap., 2H-18), 3.75 (2H, d, J=8 Hz, 2H-17), 3.46 (1H, m, $W_{1/2}$=16 Hz, H-3), 2.02 (3H, s, Aco), 1.11 and 1.02 (3H each, s, Me-groups at C-19 and C-20).

ACETYLATION OF PRODUCT **10**.—Product **10** (32 mg) was acetylated with pyridine/Ac_2O (1.5:0.7) for 1 h at 0°. After column chromatography, 18 mg of *ent*-17,18-diacetoxy-3β-hydroxy-16(*S*)-kauran-7-one (**11**) was obtained: gum; $[\alpha]^{20}D=-59°$ (c 1, $CHCl_3$); ir ν max, neat, cm^{-1} 3500, 1730, 1710, 1450, 1360, 1230, and 1020; ^1H nmr (δ) 4.16 (2H, m, $W_{1/2}$=10 Hz, 2H-17), 4.04 (2H, Q_{AB} collap., 2H-18), 3.46 (1H, dd, J_1=10 Hz, J_2=6 Hz, H-3), 2.06 and 2.02 (3H each, s, AcO groups), 1.11 and 1.02 (3H each, s, Me groups at C-19 and C-20).

OXIDATION OF PRODUCT **10**.—Product **10** (18 mg) was dissolved in 2 ml of Me_2CO, and Jones' reagent (19) was added. After column chromatography, 13 mg of a product, identical to acetate **9**, was obtained.

OSMILATION OF SUBSTRATE **2**.—Substrate **2** (200 mg) was dissolved in 4 ml of Me_2CO and 1 ml of anhydrous Et_2O, after which 0.5 ml of H_2O_2 (30%) and 0.5 ml of t-BuOH containing 0.5% w/w of osmium tetroxide (21) were added, and the mixture was stirred for 2 h at room temperature. After concentration in vacuo, the mixture was extracted repeatedly with CH_2Cl_2, dried with $MgSO_4$, and concentrated

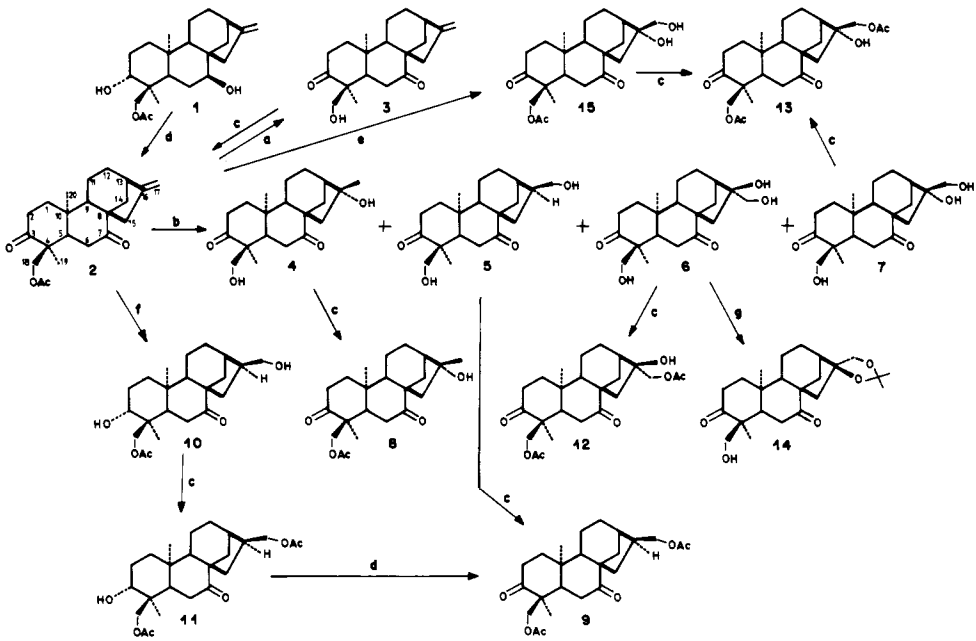
in vacuo. After column chromatography, 96 mg of a polar product was obtained (**15**) (*ent*-16β,17-dihydroxy-18-acetoxykaur-3,7-dione): gum; $[\alpha]^{20}D = -11.2°$ (c 1, $CHCl_3$); ir ν max, neat, cm^{-1} 3560, 3060, 1740, 1700, 1370, 1230, and 1025; ^1H nmr (δ) 4.17 and 3.81 (2H, Q_{AB}, $J = 12$ Hz, 2H-18), 3.75 (2H, Q_{AB} collap., 2H-17), 2.00 (3H, s, AcO), 1.36 and 1.06 (3H each, s, Me group at C-19 and C-20).

ACETYLATION OF PRODUCT **15**.—Product **15** (50 mg) was acetylated with pyridine/Ac_2O (2:1) for 2 h at 0°. After column chromatography, 40 mg of a diacetate, which was identical to the previously described product **13**, was isolated.

ISOPROPYLIDENEDIOXY DERIVATIVE OF METABOLITE **6**.—Metabolite **6** (40 mg) was dissolved in 5 ml of 2,2-dimethoxypropane, and 10 mg of pyridinium *p*-toluensulfonate was added. The solution was refluxed for 2 h, then concentrated in vacuo, dissolved in CH_2Cl_2 and washed with H_2O, dried with $MgSO_4$, and concentrated in vacuo. After column chromatography, 33 mg of *ent*-16α,17-isopropylidenedioxy-18-hydroxykauran-3,7-dione (**14**) was isolated: gum; $[\alpha]^{20}D = -17.6°$ (c 1, $CHCl_3$); ir ν max, neat, cm^{-1} 3450, 1710, and 1050; ^1H nmr (δ) 3.85 and 3.71 (2H, Q_{AB}, $J = 8$ Hz, 2H-17), 3.70 and 3.30 (2H, Q_{AB}, $J = 12$ Hz, 2H-18), 1.39 (3H), 1.36 (6H), 1.02 (3H) (s, Me groups at C-19, C-20 and isopropylidenedioxy group); ^{13}C nmr see Table 1.

RESULTS AND DISCUSSION

Incubations of *ent*-18-acetoxykaur-16-en-3,7-dione (**2**) with *A. niger* cultures were carried out, as described, for 48 h or for 6 days. The microbial and chemical transformations appearing in this work are summarized in Scheme 1.



SCHEME 1. Microbial transformation of product **2** by *Aspergillus niger* and chemical correlations of metabolites isolated. **a**, *A. niger* (48 h); **b**, *A. niger* (6 days); **c**, Ac_2O/pyridine; **d**, Jones's oxidation; **e**, OsO_4/H_2O_2; **f**, $BH_3 \cdot TMA/H_2O_2 \cdot OH^-$; **g**, 2,2-dimethoxy propane/H^+.

Incubation of substrate **2** for 48 h yielded a more polar (tlc) metabolite (**3**) than substrate **2**. From ir and ^1H nmr spectra of this metabolite (**3**), the absence of the acetyl group at C-18 can be deduced. However, double bond bands in both spectra indicate that Δ^{16} was unaltered. Signals attributable to a hydroxymethylenic group were also evidenced (see Material and Methods section). Table 1 shows the ^{13}C-nmr spectra of both substrate **2** and metabolite **3**. These data confirm that metabolite **3** only differs from substrate **2** in the absence of the acetyl group. Surprisingly, several attempts of chemical saponification of this acetate group on product **2** were unfruitful, and a considerable mixture of substances was obtained in such experiments. However, correla-

tion of metabolite **3** with substrate **2** was easily carried out by acetylation of metabolite **3**. Thus, we conclude that a 48-h incubation of substrate **2** leads to deacetylation at C-18 of this substrate.

When substrate **2** was incubated for 6 days, four metabolites were isolated. Their structures have been elucidated by physical methods and chemical correlations. Substrate **2** was not isolated after 6 days of incubation. These metabolites are named in order of increasing polarity in tlc. The most apolar metabolite (**4**) (Rf 0.8) shows a ^{1}H-nmr spectrum where the transformation of a double bond of substrate **2** and the presence of a methyl group at low field (δ 1.42, 3H) can be observed. This displacement is consistent with the presence of a methyl group geminal to a hydroxyl group. On the other hand, absence of the signal at δ 1.90 together with the presence of a Q_{AB} system with doublets centered at 3.70 and 3.30 in the ^{1}H-nmr spectrum of metabolite **4** indicates that, as in incubations for 2 days (metabolite **3**), loss of the acetate group at C-18 occurs. Acetylation of metabolite **4** produces a monoacetate (**8**), which was studied by ^{13}C nmr. Comparison of ^{13}C-nmr spectra of substrate **2** and acetate **8** indicates clearly that the new hydroxyl group is situated at C-16 (δ 78.89), with β-effects at C-13 (Δδ=5.25) and C-15 (Δδ=7.94), and γ-effects at C-12 (Δδ=−6.35) and C-14 (Δδ=−1.45). As this monoacetate **8** was identical to a metabolite isolated from the incubation of the *ent*-18-acetoxy-16(*S*)-kauran-3,7-dione (16), we concluded that metabolite **4** isolated in the present incubation with *A. niger* has the structure of *ent*-16β,18-dihydroxykauran-3,7-dione.

Another metabolite (**5**) (Rf 0.3) was isolated as the diacetate **9** after acetylation of a mixture that was difficult to separate (see Material and Methods section). Both acetoxy groups of product **9** were introduced by this artificial acetylation because the ir spectrum of the original mixture did not show acetoxy bands. In the ^{1}H-nmr spectrum of product **9**, in addition to two methyl group signals at δ 1.32 and 1.06, a Q_{AB} system (δ 4.19 and 3.81, J=12 Hz), attributable to an acetoxy group at C-18, partially overlapped to another complex signal (2H), and two acetoxy signals at δ 2.06 and 2.00 (3H each) were observed. This spectroscopic behavior suggested the presence of acetoxy groups at C-17 and C-18 in diacetate **9**. ^{13}C-nmr spectral data of product **9** confirm this hypothesis because, in addition to two ketonic (δ 211.43 and 212.43) and two acetoxy groups (δ 170.39 and 171.29), two oxygenated carbons at δ 65.26 and 67.33 are present which, in the spectrum obtained by DEPT using a "flip angle" of 135°, appeared to have a methylenic character. One of these carbons (δ 67.33) can be assigned to C-18 by comparison with the corresponding carbons at substrate **2** and acetate **8** (see Table 1). The other signal (δ 65.26) could be attributed to C-17 because signals attributable to C-19 and C-20 appeared to be very similar to those of reference compounds **2** and **8**. Configuration at C-16 is too difficult a question to be elucidated by spectroscopic means, and hence, we have correlated diacetate **9** with substrate **2** in a chemical way. Hydroboration of substrate **2** leads to product **10**, which presents, in its ^{1}H-nmr spectrum, some signals similar to those described in linearol (**1**) (18,22) for protons at C-3 and C-18 as well as a doublet signal at δ 3.75 (2H, d, J=8 Hz) attributable to C-17 hydroxymethylenic protons. A configuration 16*S* was assumed for compound **10** in view of the well-known tendencies of reagents to attack the less hindered face of *ent*-kaur-16-enic compounds (23,24). Mild acetylation of product **10** gives a diacetate **11** which, by Jones's oxidation (19), was transformed into a diketocompound identical to diacetate **9**, obtained by acetylation of metabolites **5**, as described above. Thus, we conclude that metabolite **5** produced by incubation of substrate **2** with *A. niger* is *ent*-17,18-dihydroxy-16(*S*)-kauran-3,7-dione.

The next metabolite (**6**) isolated (Rf 0.2) from incubation of substrate **2** is the main metabolite of this incubation. ^{1}H nmr and chromatographic behavior was very similar

to that observed for the last metabolite isolated (product **7**). Acetylation of metabolites **6** and **7** rendered diacetates **12** and **13**, respectively. The absence of double bonds can be deduced from the ^{1}H-nmr spectra of metabolites **6** and **7**. In both metabolites, dihydroxylation at the C-16 and C-17 positions could have occurred; Q_{AB} systems in both metabolites [doublets at δ 3.70 and 3.30 (J=12 Hz)] can be assigned to hydroxymethylenic groups at C-18; a collapsed Q_{AB} at δ 3.50 for metabolite **6** and a Q_{AB} system (doublets centered at δ 3.70 and 3.42, J=12 Hz) for metabolite **7** can be observed. Treatment of metabolite **6** with 2,2-dimethoxypropane gave a isopropylidenedioxy derivative (**14**), which proves the presence of two spatially vicinal hydroxy groups. ^{1}H-nmr spectra of both diacetates **12** and **13** showed an acetoxymethylenic group at C-18 (Q_{AB}, doublet centered at δ 4.17 and 3.81, J=12 Hz), but differed in the other acetoxymethylenic group [Q_{AB}, δ 4.11 and 3.94 (J=12 Hz) for diacetate **12** and δ 4.37 and 4.17 (J=12 Hz) for diacetate **13**]. ^{13}C-nmr spectra of products **12, 13,** and **14** showed (see Table 1), apart from similar chemical shifts in all cases for C-18, the presence of functionalized C-16 (δ 78.38, 79.68, and 86.56, respectively) and C-17 (δ 70.81, 68.22, and 76.78, respectively). It seems obvious that metabolites **6** and **7** differ only in the configuration at C-16. Acetate **13** derived from **7** gives signals that are more in accord with an *ent*-16β-hydroxy configuration (25). In addition, chemical shifts of C-13 could be conclusive for this assignation by comparison with those described, but we prefer a chemical correlation for the deduction of configuration at C-16. For this purpose, we have treated substrate **2** with OsO_4/H_2O_2 reagent (21), which produces an *ent*-β hydroxylation (23,24) (product **15**). Acetylation of product **15** yielded a diacetate identical to product **13**, obtained as reported above by acetylation of the minor metabolite **7**. Thus, metabolite **7** is *ent*-16β,17,18-trihydroxykauran-3,7-dione, and hence, the main metabolite **6** of this incubation, has the structure of *ent*-16α,17,18-trihydroxykauran-3,7-dione. This later metabolite has a 16,17-functionalization similar to those described for two diterpenoid acids isolated from immature seeds of evening-glory (*Calonyction aculeatum*) (24).

As a result of incubation of *ent*-18-acetoxykaur-16-en-3,7-dione (**2**) with *A. niger* cultures, we conclude that the fungus produces, first, a deacetylation at C-18 of the substrate (which is not possible in a chemical basic medium) and, later on, hydrations to give *ent*-16β-hydroxykauranes and *ent*-17-hydroxy-16(*S*)-kauranes as well as hydroxylations of double bond of substrate rendering both 16,17-glycol epimers at C-16. In any case, no alterations of keto groups at C-3 and C-7 have been detected. Behavior of *A. niger* with respect to the *ent*-kaurenic double bond could be explained as a series of nonregio and stereoselective actions or, in contrast, as *trans*-hydrations and *trans*-hydroxylations at the *ent*-β side, without regioselectivity, although these results are very open to speculations. For this same substrate **2**, *R. nigricans* cultures produced reductions of the C-3 keto group, to give a 3*S*-hydroxy group, and *ent*-β epoxidation of the 16,17 double bond [15]. *R. nigricans* is more selective than *A. niger* used in the present study.

ACKNOWLEDGMENTS

We wish to thank Dr. D. Gonzalez-Pacanowska for correcting the English version of the manuscript. This work is supported by a grant from Comisión Asesora de Investigación Científica y Técnica.

LITERATURE CITED

1. W. Charney and H.L. Herzog, "Microbial Transformations of Steroids," Academic Press, Inc., New York, 1967.
2. K. Kieslich, "Microbial Transformations of Nonsteroid Cyclic Compounds," John Wiley and Sons, Inc., New York, 1976.
3. O.K. Sebek and K. Kieslich, "Alkanes, Alyciclic, Terpenes and Alkaloids," in: "Annual Reports on Fermentations Processes," vol. 1. Ed. by D. Perlman, Academic Press, New York, 1977.

4. J.R. Bearder, J. McMillan, and B.O. Phinney, *J. Chem. Soc. Perkin I*, 721 (1975).
5. J.R. Bearder, V.M. Frydman, P. Gaskin, I.K. Hatton, W.E. Harney, and J. McMillan, *J. Chem. Soc. Perkin I*, 178 (1976).
6. J.R. Hanson, G. Savona, and M. Siverns, *J. Chem. Soc. Perkin I*, 2001 (1974).
7. G. Ellanes and J.R. Hanson, *J. Chem. Soc. Perkin I*, 2773 (1976).
8. A.B. Anderson, R. McCrindle, and J.K. Turnbull, *Can. J. Chem.*, **53**, 1181 (1975).
9. A.B. Anderson, R. McCrindle, and J.K. Turnbull, *J. Chem. Soc. Perkin I*, 1202 (1975).
10. J.R. Hanson, M.G. Melchor, and B.M. Fraga, *Phytochemistry*, **17**, 812 (1978).
11. E.L. Ghisalberti, P.R. Jefferies, and M.A. Sefton, *J. Chem. Res. Synop.*, **1**, 8 (1982).
12. J.P. Beilby, E.L. Ghisalberti, P.R. Jefferies, M.A. Sefton, and P.M. Sheppard, *Tetrahedron Lett.*, **28**, 2589 (1973).
13. B.M. Fraga, A.G. Gonzalez, M.G. Hernandez, and A. San Martin, *Tetrahedron Lett.*, **24**, 1945 (1983).
14. J.R. Hanson, F.Y. Sarah, B.M. Fraga, and M.G. Hernandez, *Phytochemistry*, **18**, 1875 (1979).
15. A. García-Granados, E. Onorato, A. Saenz de Buruaga, and J.M. Arias, *Anal. Quim.*, **78 C**, 287 (1982).
16. J.M. Arias, A. García-Granados, A. Martínez, and E. Onorato, *J. Nat. Prod.*, **47**, 59 (1984).
17. A. García-Granados, A. Martínez, E. Onorato, and J.M. Arias, *J. Nat. Prod.*, **48**, 371 (1985).
18. A. García-Granados, F. Lizana, A. Martínez, E. Onorato, A. Saenz de Buruaga, and J.M. Saenz de Buruaga, *Anal. Quim.*, **79 C**, 460 (1983).
19. R.G. Curtis, I. Heilbron, E.H.R. Jones, and Q.F. Woods, *J. Chem. Soc.*, 457 (1953).
20. Colección Española de Cultivos Tipo (CECT). Departamento de Microbiología, Facultad de Ciencias, Universidad de Valencia, Spain.
21. R. Daniels and J. Fischer, *J. Org. Chem.*, **28**, 320 (1963).
22. T.G. de Quesada, B. Rodriguez, S. Valverde, and S. Huneck, *Anal. Quim.*, **69**, 757 (1973).
23. J.R. Hanson, *Tetrahedron*, **22**, 1453 (1966).
24. N. Murofushi, T. Yokota, and N. Takahashi, *Tetrahedron Lett.*, **10**, 789 (1973).
25. K. Yamasaki, H. Kohda, T. Kobayashi, R. Kasai, and D. Tanaka, *Tetrahedron Lett.*, **13**, 1005 (1976).

Received 18 July 1985