Journal of Microbiology and Biotechnology
Vol. 2, No. 92-97, 1992

Microbial Transformation of Bioactive Diterpenoids from *Acanthopanax koreanum* by *Fusarium oxysporum*

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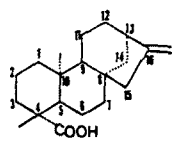
Received 7 May 1992 / Accepted 19 June 1992

Microbial transformation of (−)kaur-16-en-19-oic acid and (−)pimara-9(11),15-dien-19-oic acid from *A. koreanum* was investigated. Throughout the screening of the capability of metabolizing these bioactive diterpenoids, two microorganisms have chosen among various fungi and streptomycetes tested. Scale-up fermentation with *Fusarium oxysporum* KCTC 6051 produced two metabolites related to the precursor diterpenoids. The two metabolites were isolated by column chromatography and identified by chemical and spectroscopic methods as 2β,16α-dihydroxy kauran-19-oic acid and 16α-hydroxy kauran-19-oic acid. However any microorganisms capable to transform (−) pimara-9(11),15-dien-19-oic acid was not screened in this condition.

Recently (−)kaur-16-en-19-oic acid{**1**} and (−)pimara-9(11), 15-dien-19-oic acid{**4**} were isolated as major constituents of *Acanthopanax koreanum*. Another three diterpenoids such as ent-16β,17-dihydroxy kauran-19-oic acid, (−)pimara-9(11),15-dien-19-ol, (−)pimara-9(11),15-dien-19-ol 19-acetate were also indentified, along with other compounds (12, 13). (−)Kaur-16-en-19-oic acid is an important compound having diverse biological functions. These functions include antihepatotoxic activity (25), antimicrobial activity (18), antiinflammatory activities (9, 14), larval growth inhibition (10) and an intermediate of gibberellin biosynthesis (5). (−)Pimara-9(11),15-dien-19-oic acid also exhibited analgesic and potent antiinflammatory activities due to inhibition of prostaglandin E_2 and leucotriens biosynthesis (16). Many terpenoids show important physiological activities and their structural diversities have attracted for chemical synthesis and modification of these compounds. Often, the special activities of terpenoids depend on the absolute configuration of the molecules, so the synthesis or modification of these substances requires highly regio- and stereospecific reactions. Biotransformation of complex natural products, like terpenoids, is valuable in these respects and numerous applications have already been reported (1, 2, 6, 8, 11, 15, 17). As a part of our studies on the microbial metabolism of natural bioactive constituents we investigated the microbial transformation of these bioactive diterpenoids with the hope to obtain new hydroxylated metabolites. Among twenty strains of microorganisms tested for their conversion activity, *Fusarium oxysporum* KCTC 6051 was found to produce two transformed metabolites from (−)kaur-16-en-19-oic acid {**1**}. These metabolites were isolated by column chromatography and identified as 2β,16α-dihydroxy kauran-19-oic acid {**3**}, which is a new derivative of {**1**}, and 16α-hydroxy kauran-19-oic acid {**2**}. Their isolation and structure elucidation are described herein.



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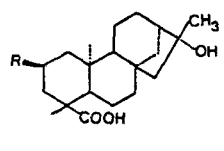

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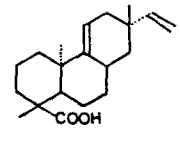
3 R=OH



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Key words: Microbial transformation, (−)kaur-16-en-19-oic acid, 2β,16α-dihydroxy kauran-19-oic acid, 16α-hydroxy kauran-19-oic acid

MATERIALS AND METHODS

General Experimental Procedures

Melting points were determined on an electrothermal melting-point apparatus, and are reported in uncorrected form. IR spectra were recorded with an Analect 5X-6160 FT-IR spectrophotometer using KBr disks. ^{1}H-and ^{13}C-NMR spectra were obtained in C_5D_5N on a Bruker AM-300 NMR spectrometer at 300 MHz and 75 MHz, respectively. Chemical shifts are presented as δ values relative to TMS as an internal standard. Mass spectra were measured with a Hewlett Packard Model HP5985B GC/MS system at an ionization voltage of 70 eV. The substrate was isolated from the root bark of *Acanthopanax koreanum* using earlier procedures (13), as mixture of (−)kaur-16-en-19-oic acid and (−)pimara-9(11),15-dien-19-oic acid (ratio, 1:2), which was identified based on the ^{1}H-NMR spectrum (data not shown), and used without further purification.

Microorganisms

Microbial cultures were obtained from the Korean Collection for Type Cultures, the Genetic Engineering Research Institute, Korea Institute of Science and Technology, Taejon. The cultures used for preliminary screening are listed in Table 1.

Media

All preliminary screening and scale-up experiments were carried out in YMPG media consisting of 3 gr of yeast extract, 20 gr of malt extract, 10 gr of peptone, 10 gr of glucose in 1*l* of distilled water, or PD media consisting of 24 gr of potato-dextrose in 1*l* of distilled water.

Table 1. Microorganisms used for biotransformation of compounds 1 and 4

Microorganism	KCTC No.	Media	Conversion activity
Absidis spinosa	6004	YMPG	−
A. coerulea	1219	PD	−
Aspergillus carbonarius	1237	〃	−
A. niger	1225	〃	−
Botryspherica dothidea	6068	〃	−
Chaetomium globosum	2121	YMPG	−
C. indicum	6059	〃	−
Cladosporum resine	6019	〃	−
C. resine f. avellaneum	1735	PD	−
Cunninghamella echinulata	1702	YMPG	+
Fusarium oxysporum	6051	PD	++
Gibberella fujikuroi	1249	YMPG	−
Pseudomonas fluoresence	1645	〃	−
Rhizopus delemar	1272	PD	−
R. oligosporus	1778	〃	−
R. stolonifer	6062	〃	−
R. oryzae	1277	〃	−
Streptomyces rimosus	1077	YMPG	−
S. hygroscopicus s. angustmyceticus	1089	〃	−
Zygorrynchus sp.	6014	〃	−

Fermentation and Purification

The screening experiments were conducted in 50 m*l* baffled flasks containing 10 m*l* of media. Cultures were incubated at 25℃ under moderate shaking in a KMC-8480S shaking incubator (Vision Scientific Company, 250-4, Towha-Dong, Mapo-ku, Seoul, Korea). The substrate was added to 48 hour old cultures at a concentration of 0.1 mg/m*l* of culture media using one % ethanol solution. Culture controls were consisted of fermentation blanks in which microorganisms were grown under identical conditions without substrate addition. After five days the cultures were removed from the incubator and extracted with ethylacetate. These extracts were analyzed on TLC plates (sillica gel GF 254) which was developed in chloroform:methanol (10:1) and visualized by spraying with *p*-anisaldehyde-acetic acid-sulfuric acid (1:100:2) followed by heating.

Scale-up experiments with *Fusarium oxysporum* were conducted in Sakaguchi flasks (500 m*l* capacity), each containing 100 m*l* of PD medium. After three days cultivation at 25℃, 50 mg of substrate in 1 m*l* ethanol was added to each flask. A sample of culture broth was taken every 24 hours and extracted with ethylacetate. After evaporation of the solvent the residue was examined for extent of the microbial conversion of the substrate by TLC. Fermentation was continued until any more increase in amount of metabolite was not detected. After 8 days, the entire incubation broths were combined and mycelia were separated from the culture broth by centrifugation. The supernatants were extracted with ethylacetate three times. All the extracts were combined and evaporated *in vacuo*, giving a slightly yellow residue.

Isolation of 16α-Hydroxy Kauran-19-oic Acid {2} and 2β,16α-Dihydroxy Kauran-19-oic Acid {3}

The slightly yellow residue was purified on a silica gel column using chloroform-methanol (20:1) as an eluent. A total of 6 fractions (FO-1∼FO-6) was collected. Fraction FO-2 was further purified on a Sephadex LH-20 column with chloroform-methanol (2:1) followed by preparative TLC (benzene-ethanol 10:1) to give 16α-hydroxy kauran-19-oic acid {**2**}. Another fraction (FO-4) containing the metabolite was evaporated to dryness *in vacuo* and the residue was recrystallized from aqueous methanol to give 2β,16α-dihydroxy kauran-19-oic acid {**3**} as white cubic crystals.

16α-Hydroxy Kauran-19-oic Acid {2}

This compound was formed as a slightly yellow amorphous powder, mp 283∼286℃, ^{1}H-NMR (300 MHz, C_5D_5N) δ: 1.25, 1.37, 1.56 (-CH_3), ^{13}C-NMR: Table 2; MS

m/z (relative intensity %) : 302 (M^+-H_2O, 9.5), 287 (9.3), 259 (16.7), 247 (4.8), 149 (21.4).

2β,16α-Dihydroxy Kauran-19-oic Acid {3}

This compound was formed as white cubic crystal, mp 265~267℃, IR ν_{max} (KBr) (cm^{-1}): 3450 (-OH), 1680 (C=0); ^{1}H-NMR (300 MHz, C_5D_5N) δ: 1.30, 1.46, 1.56 (-CH_3), 2.62 (1H, H-1β, dd J=12.2, 4.4), 3.11 (1H, H-3β, dd, J=12.3, 4.4), 4.84 (1H, H-2, tt, J=11.0, 4.4 Hz); ^{13}C-NMR: Table 2; MS m/z (relative intensity %): 336 (M^+, 1.2), 318 (M^+-H_2O, 8.3), 300 (M^+-$2H_2O$, 7.6), 278 (6.5), 260 (33.3), 187 (16.2), 159 (18.6), 147 (32.7).

RESULTS AND DISCUSSION

Among the 20 strains of microorganisms investigated, the biotransformation activity of *Fusarium oxysporum* and *Cunninghammella echinulata* produced new metabolite spots of related to precursors on a TLC plate. Large-scale fermentation of *F. oxysporum* produced mixture of several metabolites, two of which were purified through column chromatography and preparative TLC, or recrystallization.

The first metabolite, mp 283~286℃, Rf=0.6 on TLC with chloroform-methanol (10:1), was isolated as a slightly yellow amorphous powder. The ^{1}H-NMR spectrum of this compound showed three methyl groups at δ1.25, 1.35 and 1.56, but all the olefinic signals related to the substrate (δ 4.86, 4.93, 5.41 and 5.83 due to compound **4**, δ4.75 due to compound **1**) were absent. The methyl signal at δ1.56 as a singlet and the absence of the olefinic signals indicated that one molecule of water was added to the exocyclic double bond of compound **1** to generate the methyl group bound to oxygen-carrying carbon (C-16). This was confirmed from the ^{13}C-NMR spectrum which the chemical shift of C-16 was moved from δ155.6 to δ78.0. The ^{13}C-NMR spectrum also showed 20 carbon signals with chemical shifts in good agreement with those of 16α-hydroxy kauran-19-oic acid from the literature (7, 22, 24). The Mass spectrum of the metabolite was devoid of molecular ion (M^+), however a fragment ion at m/z 302 could be interpreted as being produced by dehydration of one molecule of water (M^+-H_2O). Based on these chemical and spectroscopic comparisons the first metabolite was identified as 16α-hydroxy kauran-19-oic acid {**2**} that was derived by addition of water to the compound **1**.

The second metabolite, mp 265~267℃, was isolated as white cubic crystals. The mass spectrum showed molecular ion at m/z 336 [M^+], which indicated a 32 mass unit difference from the precursor. It also showed fragment ion peaks at m/z 318 [M^+-H_2O], 300 [M^+-$2H_2O$], and 285 [M^+-$2H_2O$-CH_3], among others, which were characteristic of dihydroxy kauranoid. Therefore, this metabolite was seemed to be one of dihydroxylated compounds to have one additional oxygen on the first metabolite. The ^{1}H-NMR spectrum (Fig. 1) of this second metabolite showed three methyl signals and no olefinic signal, but the appearance of a new proton signal at δ4.84 (1H, triplet-triplet, J=11.0 and 4.4 Hz) was characteristic. This signal could be interpreted as a proton bound to oxygen-carrying carbon. It was confirmed from the ^{1}H-^{13}C correlation spectrum (Fig. 2) that this proton (triplet triplet at δ4.84) was correlated with the tertiary carbon at δ63.8 in ^{13}C-NMR spectrum. Murakami group reported several 2β-hydroxy kauranoids, such as pterokauran (23), 2β,15α,16α,17-tetrahydroxy-(−)-kauran (19), 2β,16α-dihydroxy-(−)-kauran (3) and Substance C (4). Based upon comparisons of chemical shifts and coupling constants with 2β,16α-dihydroxy kauranoids, we concluded that this metabolite had a secondary equatorial hydroxyl group (β-configulation) at the C-2 position. As discussed for the compound **2**, a methyl proton signal at δ1.56 on the ^{1}H-NMR spectrum of this metabolite indicated the presence of another hydroxyl group. The ^{13}C-NMR spectrum (DEPT) revealed that this metabolite had three methyl, eight methylene, four methine and five quarternary carbons. The ^{13}C-NMR signals

Table 2. Comparison of ^{13}C-NMR spectral data of 16α-hydroxy kauran-19-oic acid (19), compound 2, compound 3 and 2β, 16α-dihydroxy kauran (20)(C_5D_5N, δ).

C	16α-hydroxy kauran 19-oic acid	compound **2**	compound **3**	2β,16α-dihydroxy kauran
1	41.0	41.8	51.0	50.4
2	19.7	20.6	63.8	63.9
3	38.6	40.0	48.7	52.3
4	43.8	44.7	45.6	34.9
5	56.3	57.8	56.8	56.0
6	22.8	23.4	22.8	20.5
7	42.6	43.2	42.7	42.4
8	45.4	45.8	45.3	45.4
9	57.0	56.7	56.4	57.1
10	39.9	40.2	41.4	41.2
11	18.6	18.8	18.8	18.5
12	27.2	27.4	27.3	27.3
13	49.0	49.3	49.2	49.3
14	37.9	38.2	38.1	38.1
15	58.4	58.9	58.6	58.7
16	77.9	78.0	77.9	77.8
17	25.0	25.1	25.1	25.0
18	29.3	30.6	29.5	33.9
19	179.8	183.4	180.2	22.6
20	16.0	16.4	17.3	19.1

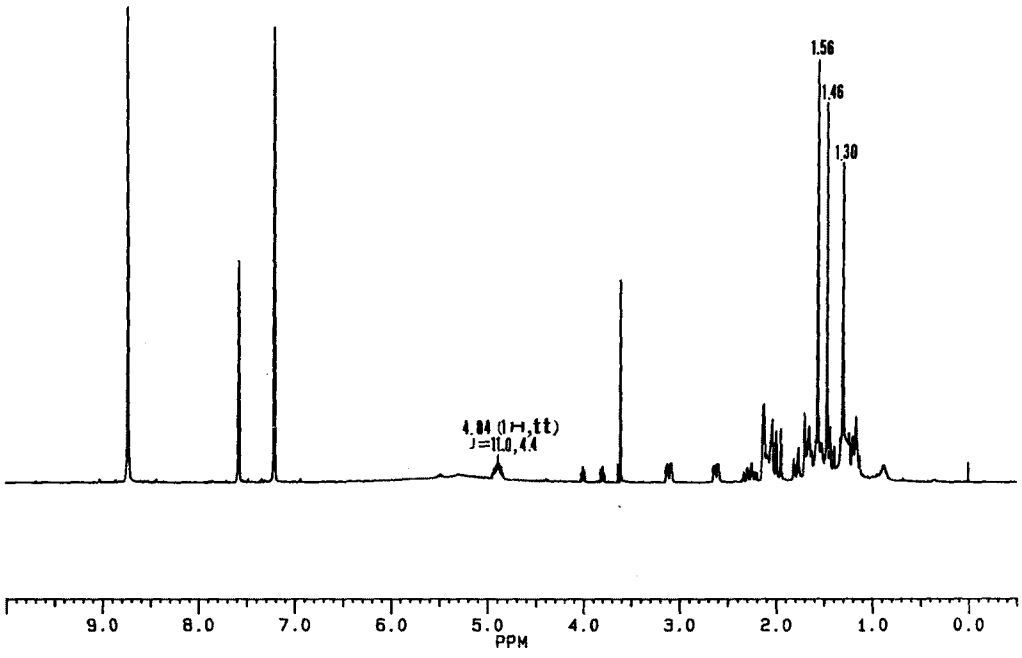

Fig. 1. ^{1}H-NMR spectrum of compound 3.

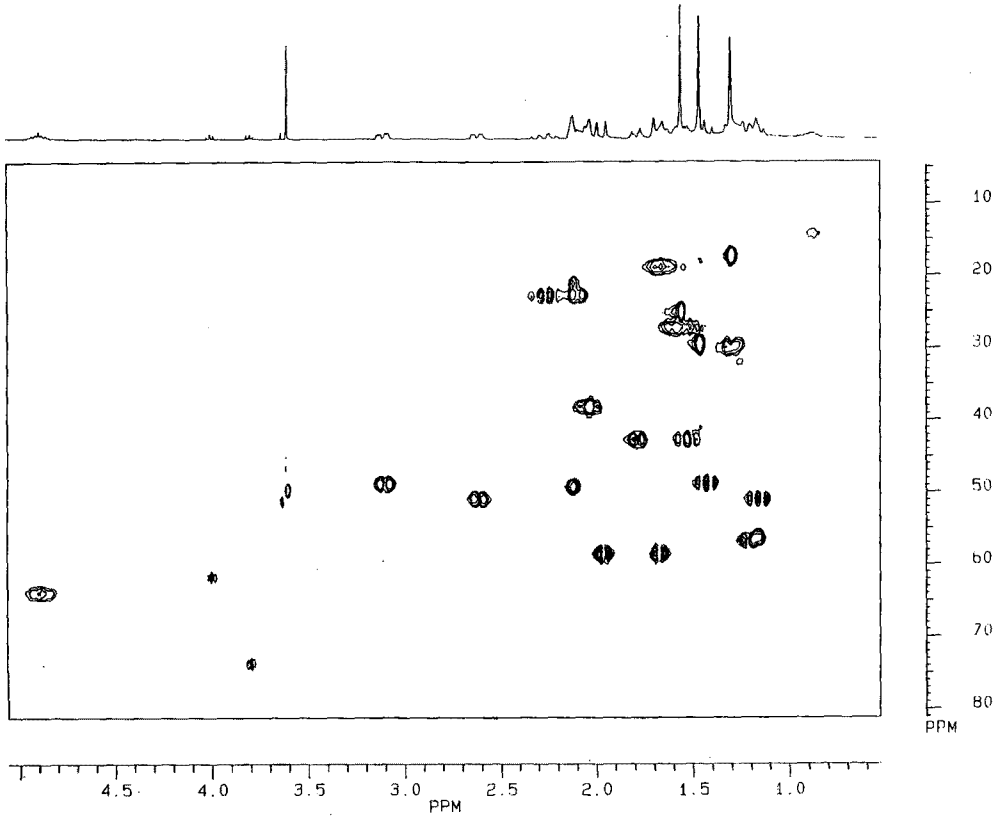

Fig. 2. ^{1}H-^{13}C COSY spectrum of compound 3.

due to C-16 and C-17, found at δ77.9 and δ25.1, respectively, indicated the presence of a tertiary hydroxyl group at C-16. In comparison with known compounds (21), the chemical shift values are close to those of 16α, 19-dihydroxy-ent-kaurane (δ77.7, 25.0), rather than those of 16β,19-dihydroxy-ent-kaurane (δ76.3, 33.1). This indicates that the hydroxyl group at C-16 is oriented in the α-configuration. All other chemical shifts of the metabolite were also compared with those of the derivatives of kauran type diterpenoids from the literature (Table 2). We identified all of the proton-proton and proton-carbon couplings of this compound, based on the ^{1}H-^{1}H COSY and ^{1}H-^{13}C COSY spectra. All the evidence indicated that the second metabolite was 2β,16 α-dihydroxy kauran-19-oic acid {**3**}, which was a new derivative of compound **1**.

Large scale fermentation of *C. echinulata* produced a small amount of various different metabolites. We isolated one metabolite and, based on the MS(M^+, 318) and ^{1}H-NMR spectra, this compound appeared to be another hydroxylated kauranoid. Further studies are continuing in our laboratory. Surprisingly a metabolite of compound **4** was not detected in the culture broths of any microorganisms used in this study.

Acknowledgement

We wish to express our gratitude to Dr. J. Kim, Sun Business Corp., for measuring ^{1}H, ^{13}C-NMR. Our appreciation is also extended to H.J. Kim for sending us a strain of *A. koreanum*.

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