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THE BIOTRANSFORMATION OF METHYL *ENT*-15-OXOKAUR-16-EN-19-OATE BY *RHIZOPUS STOLONIFER* AND *MUCOR PLUMBEUS*

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Key Word Index—*Rhizopus stolonifer*; *Mucor plumbeus*; diterpenoid; methyl *ent*-15-oxokaur-16-en-19-oate; microbiological transformation.

Abstract—Incubation of methyl *ent*-15-oxokaur-16-en-19-oate with *Rhizopus stolonifer* and *Mucor plumbeus* gave methyl *ent*-7β,11α-dihydroxy-15-oxokauran-19-oate and methyl *ent*-7β,16β-dihydroxy-15-oxokauran-19-oate, respectively.

INTRODUCTION

There are a large number of *ent*-kaurenoid tetracyclic diterpenoids which have been isolated from Chinese medicinal plants of the family Rabdosia (Isodon) [1]. Many of these compounds (e.g. oridonin (**1**)) show tumour-inhibitory properties. Although this activity is associated with the presence of an α,β-unsaturated methylene ketone on ring D, it has been proposed that additional remote functionality has a synergistic effect on the biological activity [2]. In some previous work [3], we have endeavoured to make compounds of this type from the diterpenoid, fujenal (**2**) by chemical means. Whilst the introduction of the 15-ketone adjacent to a 16-ene is relatively easy by chemical means, the introduction of more remote functionality may be better performed by microbiological means. In this paper we describe the biotransformation of methyl *ent*-15-oxokaur-16-en-19-oate (**3**) by *Rhizopus stolonifer* (*nigricans*) and *Mucor plumbeus* which reveal both the potential of the approach and a serious limitation. The ester (**3**) is easily obtained from xylopic acid (**4**) [4, 5] which is available from a number of plant sources amongst the Annonaceae. *Rhizopus* and *Mucor* species have been widely used in biotransformations including those of the diterpenoids [6].

RESULTS AND DISCUSSION

Incubation of methyl *ent*-15-oxokaur-16-en-19-oate (**3**) with *R. stolonifer* for ten days gave one major metabolite (**5**), $C_{21}H_{35}O_5$ (20% conversion). The ^{1}H NMR spectrum showed methyl group signals at δ_H 0.82, 1.15 and 1.32 (doublet) and a methoxyl signal at δ_H 3.61. The alkene resonances of the starting material had been replaced by the methyl group doublet (δ_H 1.32, J = 7 Hz) which was coupled to a pentuplet (J = 7 Hz) at δ_H 2.25 assigned to the system –CH(OH)–CH(OH)–CH_3. The location of the two hydroxyl groups (δ_H 3.96 and 3.98) at C-7 and C-11 followed from changes in the ^{13}C NMR spectrum (see Table 1). In particular, the signals assigned to the methylenes at C-7 and C-11 in the substrate had been replaced by CH(OH) signals and those assigned to C-6, C-8, C-9 and C-12 had moved downfield. The β-stereochemistry of the hydroxyl group at C-11 followed from an NOE experiment in which the signal at δ_H 3.96 was enhanced (4.6%) on irradiation of the 20-H signal (δ_H 0.82). There was no enhancement of the H-7 signal. This appeared as a double:doublet (δ_H 3.98, J = 4 and 12 Hz). The multiplicity of this signal is typical of a 7α-hydroxykaurene. Thus the metabolite was methyl *ent*-7β,11α-dihydroxy-15-oxokauran-19-oate (**5**). The overall structure and stereochemistry of this metabolite, particularly that at C-16, was established by X-ray crystallography (see Fig. 1).

The transformation using *Mucor plumbeus* was less successful and a single metabolite (**6**) was obtained in low yield. The compound possessed ^{1}H NMR signals for tertiary methyl groups at δ_H 0.89, 1.20 and 1.32 and a methoxyl signal at δ_H 3.65. Again the alkene resonance had been replaced, this time by a –C(OH)–CH_3 grouping. Changes in the ^{13}C NMR spectrum (see Table 1) when compared to the starting material revealed the presence of hydroxyl groups at C-7 and C-16. The CH(OH) resonance (δ_H 3.86) was, as previously, a doublet (J = 12 Hz) of

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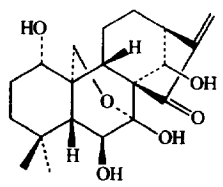

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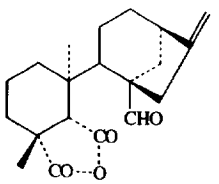

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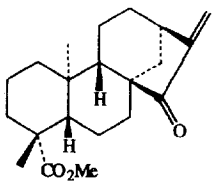

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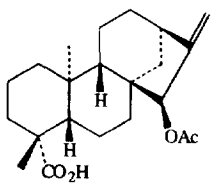

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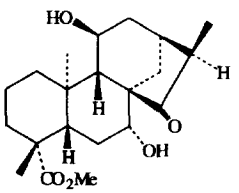

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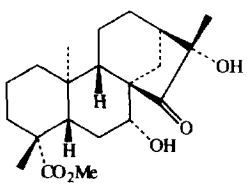

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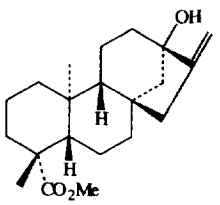

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Table 1. ^{13}C NMR Data for Compounds **3**, **5** and **6** determined in $CDCl_3$ at 125 MHz

Carbon atom	3	5*	6
1	39.9	39.4	39.5
2	18.9	19.0	18.6
3	37.9	38.2	37.8
4	43.8	43.8	43.5
5	56.2	53.5	53.2
6	20.1	29.3	25.8
7	33.7	71.3	71.4
8	52.9	57.4	58.5
9	51.6	61.5	51.5
10	40.1	38.1	39.3
11	18.4	64.3	18.1
12	32.2	34.7	27.6
13	38.1	34.7	41.0
14	36.6	30.3	29.5
15	210.6	221.4	220.8
16	149.6	50.5	77.3
17	114.4	11.6	17.9
18	28.7	28.7	28.5
19	177.8	177.8	177.5
20	15.4	15.5	15.3
OMe	51.1	51.4	51.3

*Determined in $CDCl_3$–C_5D_5N.

doublets (J = 4 Hz) indicating that it was an axial proton and hence there was a 7α-alcohol. The stereochemistry at C-16 was tentatively assigned by analogy with the metabolite **5**. Thus the biotransformation product was methyl *ent*-7β,16β-dihydroxy-15-oxokauran-19-oate (**6**).

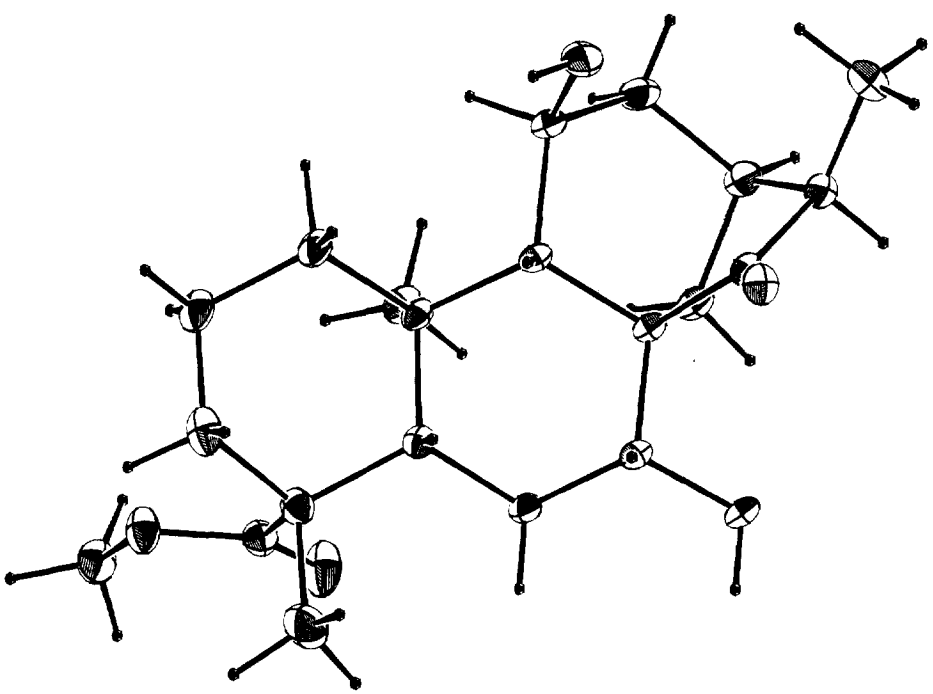

Fig. 1. Molecular structure of methyl ent-7β,11α-dihydroxy-15-oxokauran-19-oate (**5**).

The formation of these diols is of interest. Firstly it reveals the ability of these organisms to introduce functionality onto rings B and C, but it shows that the methyleneketone on ring D is particularly sensitive to bioreduction. The double-bond was not reduced when there was no carbonyl group at C-15. Thus, incubation of steviol methyl ester (**7**) with *R. stolonifer* afforded the 7β and 9β-hydroxylation products [7]. There was a difference in the stereochemistry of hydroxylation at C-7 as well. The susceptibility of this unsaturated ketone to biochemical reduction and, in the case of *Mucor plumbeus*, to further hydroxylation correlates with facile nucleophilic addition and the possible mode of biological activity of this moiety [1]. It is also of interest to note that there are a number of situations where kauran-15-ones co-occur with kaur-16-en-15-ones (e.g. *Eupatorium album* [8]). The reduction of the ring D methyleneketone implies a limitation on this approach to the biologically active diterpenoids.

EXPERIMENTAL

General experimental details. ^{1}H and ^{13}C NMR spectra were determined at 500 and 125 MHz, respectively. IR spectra were determined as Nujol mulls; Silica gel for chromatography was Merck 9385. Petrol refers to petrol bp 60–80°. Extracts were dried over Na_2SO_4.

Incubation of methyl ent-15-*Oxokaur*-16-*en*-19-*oate* (**3**) *with* Rhizopus stolonifer. *Rhizopus stolonifer* was grown in shake flasks (100 ml medium per 250 ml conical flask). The medium comprised (per l); glucose (20 g), peptone (5 g), yeast extract (3 g), KH_2PO_4 (5 g) and the pH was adjusted to 5.6. The substrate (**3**, 631 mg) (prepared as described previously [5]) in DMSO (20 ml) and EtOH (5 ml) was evenly distributed between 50 flasks 2 days after inoculation. The metabolites were isolated after a further 10 days by extraction with EtOAc. The extract was dried and the solvent evaporated to give a semi-solid residue. Crystallization from EtOAc gave methyl *ent*-7β,11α-dihydroxy-15-oxokauran-19-oate (**5**) (71 mg) as rods, mp 234–235°. (Found: C, 69.2; H, 8.85. $C_{21}H_{32}O_5$ requires C, 68.9; H, 8.81%). IR ν_{max} cm^{-1}: 3450 (br), 1730 (br). ^{1}H NMR δ_H (C_5D_5N–$CDCl_3$): 0.82 (3H, *s*, 20-H), 1.15 (3H, *s*, 18-H), 1.32 (3H, *d*, J = 7 Hz, 17-H), 3.61 (3H, *s*, OMe), 3.96 (*m*) and 3.98 (*dd*, J = 4 and 12 Hz) (2H, overlapping signals, 7- and 11-H). Chromatography of the residue on silica gel in a gradient of EtOAc–petrol gave a further 67 mg of **5**.

Incubation of Methyl ent-15-*Oxokaur*-16-*en*-19-*oate* (**3**) *with* Mucor plumbeus. *Mucor plumbeus* was grown on the above medium for 1 day. The substrate (500 mg) in EtOH (25 ml) was evenly distributed between 60 shake flasks (100 ml medium per flask) and the fermentation was continued for a further 7 days. The metabolites were extracted with EtOAc and the solvent was evaporated to give a gum. Chromatography on silica gel in a gradient of EtOAc–petrol gave the starting material (150 mg) and a more polar fraction which was rechromatographed to give methyl *ent*-7β,16β-dihydroxy-15-oxokauran-19-oate (**6**) (31 mg) as a gum, MS: *m/z* 346 (M-18). IR ν_{max} cm^{-1} 3510, 3398, 1730 (br). ^{1}H NMR δ_H ($CDCl_3$): 0.89 (3H, *s*, 20-H), 1.20 (3H, *s*, 18-H), 1.32 (3H, *s*, 17-H), 3.65 (3H, *s*, OMe), 3.86 (1H, *dd*, J = 4 and 12 Hz, 7-H).

X-ray crystallographic data and structure determination of (**5**). $C_{21}H_{32}O_5$, M 364.5, monoclinic, space group $P2_1$ (no 4), a = 11.142 (4), b = 7.381 (6), c = 11.910 (2), α = 90°, β = 103.91 (2), γ = 90°, U = 950.8 Å^3, Z = 2, D_{calc} = 1.27 g cm^{-3}, F (000) 396, monochromated CuKα radiation, λ = 1.5418 Å, μ = 6.8 cm^{-1}

Data were collected using a crystal *ca* 0.4 × 0.15 × 0.08 mm on an Enraf–Nonius CAD4 diffractometer in the θ–2θ mode. A total of 2074 reflections were measured for $2 < \theta < 75°$ and $h0 \rightarrow 13$, $k0 \rightarrow 9$, $l{-}14 \rightarrow +14$. There were 1988 unique reflections and 1775 significant reflections with $|F^2| > 2\sigma(F^2)$ were used in the refinement, where $\sigma(F^2) = \{\sigma^2(I) + (0.04\,I)^2\}^{1/2}/L_p$. There was no crystal decay and no correction was made for absorption.

The structure was solved by direct methods using SHELXS-86 and non-hydrogen atoms were refined anisotropically by full-matrix least squares using programs from the Enraf–Nonius MoLEN package. The hydrogen atoms were freely refined isotropically. With a weighting scheme of $w = 1/\sigma^2(F)$, the refinement converged with R = 0.037 and R' = 0.048. The absolute stereochemistry was taken as that known on chemical grounds. The crystallographic data have been deposited with the Cambridge Crystallographic Data Centre.

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