Pergamon 0031-9422(95)00289-8 *Phytochemistry*, Vol. 40, No. 2, pp. 439–442, 1995
Elsevier Science Ltd
Printed in Great Britain
0031-9422/95 $9.50 + 0.00

THE BIOTRANSFORMATION OF *ENT*-15-OXOKAUR-16-EN-19-OIC ACID AND ITS METHYL ESTER BY *CEPHALOSPORIUM APHIDICOLA*

ALAIDE B. DE OLIVEIRA,* JAMES R. HANSON† and JACQUELINE A. TAKAHASHI*

School of Molecular Sciences, University of Sussex, Brighton, Sussex, BN1 9QJ, U.K.

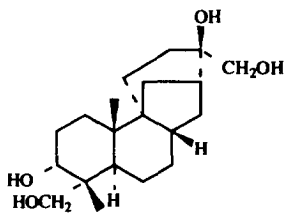
(*Received in revised form* 16 *February* 1995)

Key Word Index—*Cephalosporium aphidicola*; fungus; *ent*-15-oxokaur-16-en-19-oic acid; aphidicolin; biotransformation.

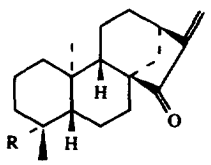
Abstract—*ent*-15-Oxokaur-16-en-19-oic acid and its methyl ester are transformed to *ent*-11α,16β-dihydroxy-15-oxokauran-19-oic acid and its methyl ester, respectively, by *Cephalosporium aphidicola*.

INTRODUCTION

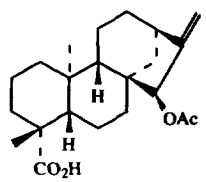
The extent to which the substrate flexibility of biosynthetic pathways may be used for biosynthetically-directed biotransformations requires delineation. Useful biosynthetic information has accrued from the explorations of the gibberellin:kaurenolide pathway in *Gibberella fujikuroi* [1]. We have been examining the biosynthetic pathways [2] in *Cephalosporium aphidicola* that lead to aphidicolin (**1**) in these terms [3]. In a previous publication [4] we suggested that a C-4α hydroxymethyl group on a tetracyclic diterpenoid served as a 'local' directing group favouring a biosynthetically directed hydroxylation at C-3α. We observed this in the biotransformation of *ent*-19-hydroxy-kaur-16-en-15-one (**2**). In this paper we are concerned with the effect of replacing this directing group by a carboxylic acid and ester as in (**3**) and (**4**). The acid (**3**) has recently attracted interest because of its anti-HIV activity [5].



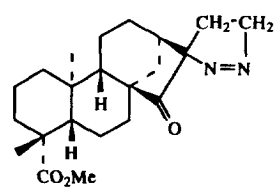
1



2 R = CH_2OH
3 R = CO_2H
4 R = CO_2Me



5

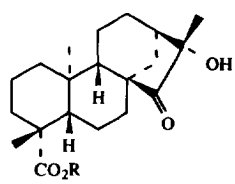


6

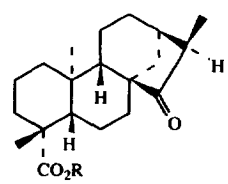
RESULTS AND DISCUSSION

The substrates were prepared from xylopic acid (**5**) [6]. Alkaline hydrolysis of the acetate proved unpredictable and was achieved more satisfactorily by brief treatment with lithium aluminium hydride in ether at room temperature. The 15-hydroxyl group was oxidized with chromium trioxide to afford the 15-ketone. Methylation with diazomethane gave the pyrazolone (**6**). The methyl ester (**4**) was obtained by treatment of (**3**) with caesium fluoride and methyl iodide in dimethylformamide [7].

The substrates (**3**) and (**4**) were incubated with *C. aphidicola* for 11 days and 14 days respectively. The metabolites were then separated by chromatography. A number of metabolites were obtained from the carboxylic acid (**3**). Their structure followed from an examination of their ^1H and ^{13}C NMR spectra (see Table 1 for ^{13}C NMR data).



7 R = H
8 R = Me



9 R = H
10 R = Me

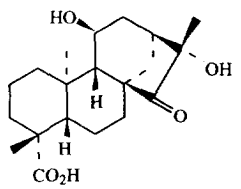
*Present address: Departamento de Quimica, Universidade Federal de Minas Gerais, 31270 Belo Horizonte, Minas Gerais, Brazil.

†Author to whom correspondence should be addressed.

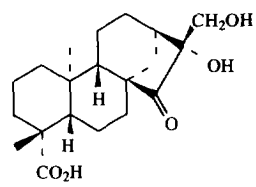
Table 1. ^{13}C NMR data for 15-oxokauranes determined in $CDCl_3$ at 125 MHz

Carbon atom	3	4	7	8	10	11	12	13	14
1	39.9	39.9	40.1	39.9	39.6	39.3	39.6	39.5	39.3
2	18.8	18.9	18.7	18.8	18.8	18.6	18.7	18.7	18.2
3	37.7	37.9	37.6	37.9	37.9	37.5	37.6	37.7	31.8
4	43.6	43.8	43.7	43.8	43.7	43.6	43.6	43.6	49.6
5	56.9	56.2	56.0	56.1	56.1	55.9	55.9	55.8	50.6
6	20.1	20.1	20.1	20.3	20.3	19.9	20.1	20.0	19.9
7	32.2	33.7	34.0	34.0	34.2	33.1	33.9	34.7	33.8
8	52.5	52.9	52.4	52.4	52.6	50.1	53.4	50.3	52.0
9	51.6	51.6	52.0	52.0	51.5	62.6	51.5	64.1	51.9
10	40.3	40.1	39.7	39.8	39.8	38.6	39.9	38.5	39.5
11	18.4	18.4	18.4	18.4	18.2	65.2	18.5	65.3	18.3
12	33.7	32.2	27.1	27.2	24.7	34.4	25.3	33.3	27.0
13	38.1	38.1	41.3	41.3	34.9	34.7	32.9	41.1	41.3
14	36.6	36.6	34.4	34.5	37.3	37.1	37.4	35.8	34.5
15	210.7	210.6	222.1	221.4	224.8	223.0	225.6	220.5	221.3
16	149.5	149.6	77.3	77.3	47.8	49.6	54.6	78.1	77.3
17	114.5	114.4	18.3	18.3	10.0	11.1	60.6	19.7	18.3
18	28.9	28.7	28.9	28.7	28.7	28.8	28.9	28.6	70.8
19	192.6	177.8	183.8	177.8	177.9	183.4	183.1	177.8	175.9
20	15.6	15.4	15.4	15.2	15.2	15.6	15.5	15.2	15.3
OMe		51.1		51.2	51.2	(in C_5D_5N)		51.3	51.5

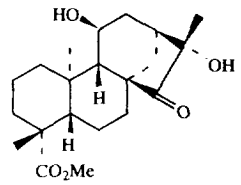
The ketol (**7**) lacked the alkene resonances of the substrate, possessing instead a new quaternary carbon resonance (δ_C 77.3 ppm) and a new tertiary methyl group (δ_H 1.33; δ_C 18.3 ppm). It was assigned the same stereochemistry at C-16 as the corresponding metabolite obtained from *ent*-19-hydroxykaur-16-en-15-one for which the stereochemistry was established by X-ray crystallography [4]. The dihydro-acid (**9**) was purified as its methyl ester (**10**) and identified by comparison with an authentic sample prepared by the acid-catalysed rearrangement of desacetylxylopic acid [8]. The location of the hydroxyl group in the hydroxy-acid (**11**) followed from changes in the ^{13}C NMR spectrum. The signal (δ_C 18.4 ppm) assigned to C-11 in the substrate was replaced by a CH(OH) signal at δ_C 65.2, whilst those assigned to C-9 and C-12 had shifted downfield. The stereochemistry of the hydroxyl group was established by a significant NOE enhancement (2.9%) of the C<u>H</u>(OH) resonance (δ_H 3.93) on irradiation of the 20-H-signal at δ_H 0.94 ppm. This metabolite, *ent*-11α-hydroxy-15-oxokauran-19-oic acid (**11**) has been isolated previously from *Eupatorium album* [9], *Adenostemma lavenia* [10], and *Pteris dispar* [11]. The final metabolite (**12**) possessed additional ^1H NMR signals attributable to a primary alcohol in place of the alkene signals of the substrate. The signal at δ_H 4.03 was a double-doublet, J = 8.1 and 11.1 Hz whilst that at δ_H 3.70 was a doublet (J = 6.3 Hz) of doublets (J = 11.1 Hz) in accord with the structural fragment $-\overset{|}{C}H-CH_2OH$. The stereochemistry of the primary alcohol followed from a series of NOE experiments. Decoupling experiments showed that the primary alcohol (17-H_2) was coupled to a double-doublet δ_H 2.53 (16-H). An NOE experiment based on irradiating δ_H 1.01 (20-H) produced a 7.6% enhancement of a doublet, δ_H 2.45, J = 12.2 Hz, which was assigned to the 14α-H. A spin decoupling experiment based on this signal revealed that it was coupled to a doublet at δ_H 1.43 which was therefore assigned to the 14β-H. Whereas NOE experiments based on irradiating the 17-H_2 signals enhanced the 16-H resonance (1.7 and 2.0%), irradiation of the 16-H signal enhanced the 14β-H doublet (2.5%). Hence the stereochemistry of the primary alcohol is assigned as in (**12**) and it is identical to that of the methyl ketone at C-16.



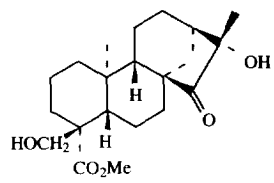
11



12



13



14

Incubation of the methyl ester (**4**) with *C. aphidicola* gave the methyl esters (**8**) and (**13**) which were separated by chromatography. The structure of the latter followed from the changes in the ^{13}C NMR spectrum in which the C-9, C-11 and C-12 resonances now appeared at δ_C 64.1, 65.3 and 33.3 ppm whilst the C-16 and C-17 signals appeared at δ_C 78.1 and 19.7 ppm respectively. Irradiation of the 20-H resonance (δ_H 0.80) produced a 4.9% enhancement of the CH(OH) resonance at δ_H 3.91. Hence the hydrogen atom of the secondary alcohol at C-11 has the α-configuration and the hydroxyl group is β. The final metabolite (**14**) of the keto-ester (**4**) to be eluted from the column, also possessed NMR signals associated with a $C(OH)CH_3$ grouping in place of the alkene. In addition one of the tertiary methyl groups had been replaced by a primary alcohol (δ_H 3.45 and 3.89, doublet, $J = 10.3$ Hz; δ_C 70.8 ppm). The location of this group followed from the changes in the ^{13}C NMR spectrum (see Table 1). In particular the C-4 resonance had moved downfield whilst the resonances assigned to C-3 and C-5 showed a typical γ-upfield shift, which had been noted previously in 18-hydroxykaurenes [12].

In contrast to the biotransformation of the 19-hydroxykaurene (**2**) [4], there were no detectable metabolites arising from hydroxylation at C-3. The major metabolites instead possessed a hydroxyl group at C-11. The conversion of the unsaturated ketone on ring D to an α-ketol which had been observed previously, was also noted here. The dihydro compounds (**9**, **10**) may be intermediate in this transformation. On the other hand (**12**) might arise by a Michael type of hydration. It is interesting to note that (**9**) and (**10**), have the same configuration at C-16.

EXPERIMENTAL

General experimental details. ^1H NMR spectra were determined at 360 and 500 MHz; ^{13}C NMR spectra were determined at 125 MHz; IR spectra were determined as nujol mulls; Silica for chromatography was Merck 9385. *Cephalosporium aphidicola* (IMI 68689) was grown on shake culture in conical flasks (250 cm^3) on a medium (100 cm^3) as described previously [3].

Preparation of the substrates. Xylopic acid (**5**) (1.5 g) in Et_2O (50 cm^3) was treated with $LiAlH_4$ (400 mg) at room temp. for 1 hr. H_2O and dil. HCl were cautiously added and the desacetylxylopic acid (1.2 g), mp 203–205° (lit., [6] 204–206°) recovered. Desacetylxylopic acid (200 mg) in acetone (10 cm^3) was treated with the Jones' reagent (1 cm^3) for 30 min. MeOH and dil. HCl were added and the soln was concd. The product was recovered in EtOAc to give *ent*-15-oxokaur-16-en-19-oic acid (195 mg), mp 198–201° (lit., [6] 197–201°).

Methylation of ent-15-*oxokaur*-16-*en*-19-*oic acid.* (a) The above keto-acid (200 mg) in EtOH (20 cm^3) was cooled in ice and treated with CH_2N_2 in Et_2O until the yellow colour persisted. The solvent was evapd to give the pyrazolone (**13**), mp 109–114° (Found: C, 70.9; H, 8.6; N, 7.5. $C_{22}H_{32}N_2$ requires C, 70.6; H, 8.5; N, 7.8%), δ_H ($CDCl_3$) 0.95 (3H, *s*, 20-H), 1.21 (3H, *s*, 18-H), 2.62 (2H, *m*), 3.68 (3H, *s*, OMe), 4.66 (2H, *m*). (b) A mixture of the above keto-acid (640 mg), CsF (227 mg), MeI (213 mg) and dry DMF (15 cm^3) was stirred at room temp. for 5 days. The mixt. was poured into aq. $NaHCO_3$ (50 cm^3) and the product recovered in EtOAc to give methyl *ent*-15-oxokaur-16-en-19-oate (424 mg), mp 143–145° (Found: C, 76.0; H, 9.3. $C_{21}H_{30}O_3$ requires C, 76.3; H, 9.15%), ν_{max} 1741, 1720, 1665 cm^{-1}, δ_H ($CDCl_3$) 0.91 (3H, *s*, 20-H), 1.19 (3H, *s*, 18-H), 3.65 (3H, *s*, OMe), 5.25 and 5.94 (each 1H, *br s*, 17-H).

Incubation of 15-*oxokaur*-16-*en*-19-*oic acid* (**3**) *with C. aphidicola.* The acid (500 mg) in DMSO (25 cm^3) and chlorocholine chloride (CCC) (300 mg) in EtOH (5 cm^3) were evenly distributed between 50 flasks of *C. aphidicola* 5 days after inoculation. The broth was filtered after a further 11 days and the metabolites were recovered in EtOAc. The resultant gum was chromatographed on Si gel. Gradient elution with EtOAc:petrol bp 60–80°, gave *ent*-16β-hydroxy-15-oxokauran-19-oic acid (**7**) (32 mg), mp 258–261° (Found: C, 71.9; H, 8.8. $C_{20}H_{30}O_4$ requires C, 71.8; H, 9.0%), ν_{max} 3402, 1748, 1698 cm^{-1}, δ_H ($CDCl_3$) 1.02 (3H, *s*, 20-H), 1.27 (3H, *s*, 18-H), 1.33 (3H, *s*, 17-H). Further elution gave a mixt. containing (**7**) which was further purified as its Me ester (prepared with CH_2N_2) to give methyl *ent*-15-oxokauran-19-oate (**8**) (9 mg), mp 117–118°, δ_H ($CDCl_3$) 0.89 (3H, *s*, 20-H), 1.10 (3H, *d*, $J = 7$ Hz, 17-H), 1.18 (3H, *s*, 18-H). The sample was identical to the Me ester of *ent*-15-oxokauran-19-oic acid (**7**) prepared by treatment of desacetylxylopic acid with acid [8]. Further elution of the original column gave *ent*-11α-hydroxy-15-oxokauran-19-oic acid (**9**) (15 mg), mp 210–212° (lit., [9] 215–217°), ν_{max} 3584, 3406, 1715, 1686 cm^{-1}, δ_H (C_5D_5N) 0.95 (3H, *s*, 20-H), 1.20 (3H, *s*, 18-H), 1.28 (3H, *d*, $J = 7$ Hz, 17-H), 3.93 (1H, *d*, $J = 5.5$ Hz, 11-H). Further elution gave *ent*-17-hydroxy-15-oxokauran-19-oic acid (**12**) (45 mg), mp 203–205° (Found: C, 70.6; H, 8.75. $C_{20}H_{30}O_4 \cdot H_2O$ requires C, 70.0; H, 9.0%), ν_{max} 3580 (*br*), 1730, 1698 cm^{-1}, δ_H ($CDCl_3$) 1.01 (3H, *s*, 20-H), 1.27 (3H, *s*, 18-H), 3.70 (1H, *dd*, $J = 6.3$ and 11.1 Hz, 17-H), 4.03 (1H, *dd*, $J = 8.1$ and 11.1 Hz, 17-H).

Incubation of methyl ent-15-*oxokaur*-16-*en*-19-*oate* (**4**) *with* C. aphidicola. The ester (310 mg) in DMSO (25 cm^3) and CCC (300 mg) in EtOH (5 cm^3) and 4 drops Tween 80, were evenly distributed between 25 flasks of *C. aphidicola* 3 days after inoculation. After a further 14 days the broth was extracted with EtOAc and the metabolites were separated by chromatography on Si gel using a gradient of EtOAc:petrol (bp 60–80°). Methyl *ent*-16β-hydroxy-15-oxokauran-19-oate (**8**) (8 mg) was obtained as a gum, EIMS, *m/z* 330 (M-18), ν_{max} 3467, 1737, 1712 cm^{-1}, δ_H ($CDCl_3$) 0.89 (3H, *s*, 20-H), 1.19 (3H, *s*, 18-H), 1.33 (3H, *s*, 17-H), 3.66 (3H, *s*, OMe). Further elution gave methyl *ent*-11α,16β-dihydroxy-15-oxokauran-19-oate (**13**) (37 mg), mp 231–232° (Found: C, 69.1; H, 8.8. $C_{21}H_{32}O_5$ requires C, 69.2; H, 8.8%), ν_{max} 3510, 3406, 1720 (*br*) cm^{-1}, δ_H ($CDCl_3$) 0.80 (3H, *s*, 20-H), 1.18 (3H, *s*, 18-H), 1.51 (3H, *s*, 17-H), 3.63 (3H, *s*, OMe), 3.91 (1H, *d*, $J = 5.4$ Hz, 11-H). Further elution gave

methyl *ent*-16β,18-dihydroxy-15-oxokauran-19-oate (**14**) (26 mg), mp 161–162 (Found: C, 68.6; H, 8.8. $C_{21}H_{32}O_5$ requires C, 69.2; H, 8.8%), ν_{max} 3438, 3309 (*br*), 1738, 1700 cm^{-1}, δ_H ($CDCl_3$) 0.91 (3H, *s*, 20-H), 1.31 (3H, *s*, 17-H), 3.68 (3H, *s*, OMe), 3.45 and 3.89 (each 1H, *d*, J = 10.3 Hz, 18-H).

Acknowledgement—We thank CNPq (Brazil) for the grant number 202268/91-4 to Mrs J. A. Takahashi.

REFERENCES

1. Hanson, J. R. (1992) *Natural Product Reports* **9**, 139.
2. Ackland, M. J., Gordon, J. F., Hanson, J. R., Yeoh, B. L. and Ratcliffe, A. H. (1988) *J. Chem. Soc. Perkin Trans. 1*, 1477.
3. Hanson, J. R., Jarvis, A. G. and Ratcliffe, A. H. (1992) *Phytochemistry* **31**, 3851.
4. Boaventura, M. A. D., Hanson, J. R., Hitchcock, P. B. and Takahashi, J. A. (1994) *Phytochemistry* **37**, 387.
5. Gustafson, K. R., Munro, M. H. G., Blunt, J. W., Cardellina, J. H., McMahon, J. B., Gulakowski, R. J., Cragg, G. M., Cox, P. A., Brinen, L. S., Clardy, J. and Boyd, M. R. (1991) *Tetrahedron* **47**, 4547.
6. Ekong, D. E. U. and Ogan, A. U. (1968) *J. Chem. Soc. (C)* 311.
7. Sato, T., Otera, J. and Nozaki, H. (1992) *J. Org. Chem.* **57**, 2166.
8. Cannon, J. R., Chow, P. W., Jefferies, P. R. and Meehan, G. V. (1966) *Aust. J. Chem.* **19**, 861.
9. Herz, W. and Sharma, R. P. (1976) *J. Org. Chem.* **41**, 1021.
10. Cheng, P. C., Hufford, C. D. and Doorenbos, N. J. (1979) *J. Nat. Prod.* **42**, 183.
11. Murakami, T., Tanaka, N., Hata, M., Saiki, Y. and Chen, C.-M. (1976) *Chem. and Pharm. Bull. (Japan)* **24**, 549.
12. Gonzalez, A. G., Fraga, B. M., Hernandez, M. G. and Hanson, J. R. (1981) *Phytochemistry* **20**, 846.