Phytochemistry, 1967, Vol. 6, pp. 1151 to 1154. Pergamon Press Ltd. Printed in England

SHORT COMMUNICATION

PLANT HORMONES—VI.*

ISOLATION OF (−)-KAUR-16-EN-19-OIC ACID FROM THE MYCELIUM OF *GIBBERELLA FUJIKUROI*

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(*Received* 11 *January* 1967)

Abstract—(−)-Kaur-16-en-19-oic acid, recently shown[1] to act as a precursor for the biosynthesis of gibberellins in *Gibberella fujikuroi*, has been found in the mycelium of the fungus. Other detected mycelial constituents include: gibberellins A_4, A_7 and A_9, fujenal, 17-hydroxykaurenolide, cerotic, tetracosanoic and behenic acids, and an unidentified liquid, $C_{20}H_{36}O_8$.

A RECENT paper,[1] describing the utilization of (−)-kaur-16-en-19-oic acid (1) as a precursor for the biosynthesis of gibberellins in *Gibberella fujikuroi*, prompts us to record the isolation of this acid from the mycelium of the fungus.

In contrast to the culture filtrates, the mycelium of *Gibberella fujikuroi* has received little attention. Yabuta *et al.*[2] have reported oxalic acid, adenine and betaine, ergosterol and probably fungisterol among the mycelial constituents while D-mannitol and D-arabitol were isolated by Borrow *et al.*[3]

In the present work, an ethanolic extract of the moist mycelium of *G. fujikuroi* ACC 917 (mutant M 419) was fractionated in the usual way. (−)-Kaur-16-en-19-oic acid (1) was obtained as the sparingly soluble sodium salt in an aqueous sodium hydroxide extract. The m.p. (155–158°) of the free acid was lower than the reported values of 165–7,[4] 169–171[5] and 179–181,[5] but the infrared spectrum of a chloroform solution was identical to that of an authentic specimen and the proton magnetic resonance spectrum of deuterochloroform and deuteropyridine solutions showed the appropriate[5, 6] features. The methyl ester was obtained as an oil although previously reported as a crystalline solid, m.p. 71–73°[4] and 88–89°.[5] The liquid ester appeared to be homogeneous by gas liquid chromatography on a 2% QF-1 column with the same retention time as the methylation product from a sample of the authentic acid and it gave the known[5] (−)-kaur-16-en-19-ol (2) on lithium aluminium hydride reduction.

The occurrence of (−)-kaur-16-en-19-oic acid in *G. fujikuroi*, together with the results of Geissman *et al.*[1] suggest that the acid is an obligatory intermediate in the fungal biosynthesis of the gibberellins. The acid may play a similar role in higher plants; it has been isolated from *Ricinocarpus stylosus*[5] and *Phebalium rude* Bartl.[6] and has been detected[7] by TLC in the grain of *Hordeum vulgare*, known[8] to contain gibberellin A_3.

* Part V. B. D. CAVELL, J. MACMILLAN, R. J. PRYCE and A. C. SHEPPARD, *Phytochem.* **6**, 867 (1967).

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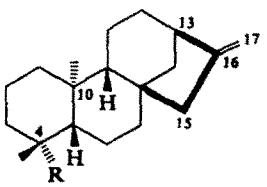
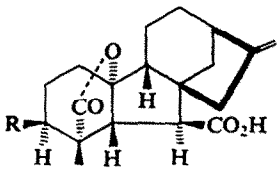
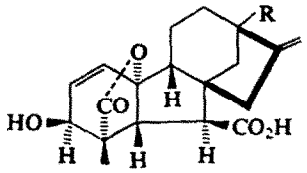


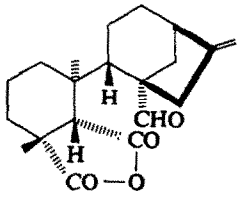
(1) R=CO_2H
(2) R=CH_2OH



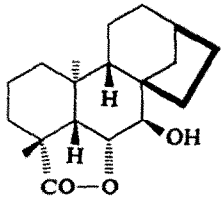
(3) R=OH
(4) R=H



(5) R=H
(6) R=OH



(7)



(8)

Gibberellin A_4(3),[9-11] gibberellin A_7(5),[9] gibberellin A_9(4),[9] fujenal (7)[12] and 7-hydroxykaurenolide (8),[13] known diterpenoid metabolites from culture filtrates of *G. fujikuroi*, were also found in the mycelial extract. Although gibberellin A_3 (6) is the major diterpenoid in the culture filtrate, none was detected in the mycelium.

A mixture of cerotic (10%), tetracosanoic (45%) and behenic (45%) acids was also isolated together with an unidentified liquid, $C_{20}H_{36}O_8$.

EXPERIMENTAL

Melting points, determined on a Kofler hot-stage, are corrected. Unless stated otherwise, i.r. spectra were determined for chloroform solutions on a Unicam SP.200 and p.m.r. spectra for $CDCl_3$ solutions, with tetramethyl silane as internal standard, on a Varian A60 spectrometer. Kieselegel G. (E. Merck) was used for TLC (0·3 mm) and PLC (1·0 mm). In GLC a column of 2% QF-1 on Gas Chrom. A (80–100 mesh) was used, at the temperatures indicated in the text, with a Pye 104 Model 24 instrument.

Extraction of the mycelium. The moist mycelium (3·24 kg) from *Gibberella fujikuroi* ACC 917 (mutant M 419), kindly provided by Pharmaceuticals Division, I.C.I. Ltd., was soxhlet-extracted for 20 hr with ethanol. Concentration of the aqueous ethanolic extract to 500 ml under vacuum and at 40–45° gave an oily solid which was collected and worked-up as described below. The filtrate was extracted with ether (10 × 85 ml) which was concentrated (500 ml) and successively extracted with 2% HCl (6 × 50 ml), 5% $NaHCO_3$ (5 × 20 ml)

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and 5% NaOH (5 × 20 ml). These extracts gave respectively the following fractions: (A) an oil (40 mg) containing many compounds (TLC) and not examined further; (B) an acidic gum (890 mg); and (C) a complex mixture (TLC) of weak acids (104 mg) which was discarded. Recovery of the ether solution gave (D) a neutral oil (990 mg).

The oily solid was washed with ether and the insoluble solid m.p. > 300° (Found: C,0·0; H, 1·6%) was discarded. The ether washings (750 ml) were extracted in turn with 2% HCl, 5% $NaHCO_3$, 5% Na_2CO_3 (emulsions), and 5% NaOH to give, respectively, (*a*) an oil (140 mg) which was discarded, (*b*) an acidic gum (510 mg) which was combined with fraction B, (*c*) a gum (5·5 g, Fraction E) and (*d*) a fraction F consisting of an insoluble sodium salt (170 mg) and a gum (245 mg). A neutral oil (6·6 g, fraction F) was recovered from the extracted ether solution.

Isolation of (−)-*kaur*-16-*en*-19-*oic acid.* Acidification of an aqueous solution of the sodium salt, ν_{max} (nujol mull) 1640 and 1625 (C=O), 1650 and 860 (=CH_2)cm^{-1}, gave (−)-kaur-16-en-19-oic acid, recovered in ether and crystallized from aqueous methanol in needles m.p. 155–158°, not raised by sublimation and/or repeated recrystallization. The i.r. spectrum, ν_{max} 1685, 1655, and 882 cm^{-1}, was identical with that of an authentic specimen. τ values, 9·05 (10-CH_3), 8·75 (4-CH_3), 7·94 (15-H_2), 7·38(13-H) and 5·24 (17·H_2); pyridine) 8·91, 8·70, 7·99, *ca.* 7·50 and 5·23. A further 125 mg of the acid m.p. 155–158° was obtained by chromatography of the gum (245 mg) from fraction E on silica (14 × 2·5 cm) and elution with light petroleum, b.p. 60–80°, containing 10–20% ethyl acetate.

Methylation of the acid with CH_2N_2 gave a gum, purified by molecular distillation at 80° and 0·1 mm pressure, ν_{max} (liquid film) 3020, 1723, 1658 and 880 cm^{-1} τ values, 9·16 (10-CH_3), 8·83 (4-CH_3), 7·93 (15-H_2), *ca.* 7·40 (13-H) and 5·24 (17-H_2). The retention time (10·5 min) at 149° was identical with that of the methylation product of a sample of the authentic acid.

Reduction of the ester (65 mg) in ether (10 ml) with lithium aluminium hydride (110 mg) as described[5] gave (−)-kaur-16-en-19-ol (12 mg) m.p. 138–140° (recorded m.p. 141–142°) with a retention time in GLC at 174° identical to that of an authentic specimen. Both specimens contained an impurity of shorter retention time, possibly the endo isomer, (−)-kaur-15-en-19-ol.

Isolation of Fujenal and Identification of Gibberellins A_4, A_7, and A_9

The combined fractions B (1·4 g) were chromatographed on a column (40 × 3 cm) of celite (60 g): charcoal (30 g) as described.[14] Elution in 200 ml fractions, first with water then with water containing acetone increasing in 5 per cent steps gave 21 fractions.

Fraction 16, eluted with 75% acetone, was rechromatographed on silica (15 × 2 cm). Elution with chloroform gave a gummy solid (12 mg), ν_{max} 1762, 1720, 1655 and 885 cm^{-1}, shown to contain gibberellin A_9 by TLC comparison with an authentic specimen in the solvent system, di-isopropyl ether: acetic acid (95:5)[15] and by GLC[16] of the methylation product at 200° which showed a single symmetrical peak with the same retention time as gibberellin A_9 methyl ester. Elution of the silica column with chloroform containing 80–90% ethyl acetate gave a solid (10 mg), ν_{max} 3400, 1760, 1705, 1655 and 880 cm^{-1}, shown to contain gibberellin A_4 and A_7 (*a*) by the comparison with authentic specimens in the solvent systems[15] di-isopropyl ether:acetic acid (95:5) and benzene:acetic acid:water (8:3:5) and (*b*) by GLC[16] of the methylation product at 200° which showed two peaks with the correct retention times for the methyl esters of gibberellins A_4 and A_7.

Fraction 19, eluted from the charcoal:celite column, with 90% acetone gave a gum (120 mg) which, after chromatography on silica and elution with chloroform, gave fujenal (93 mg), as needles m.p. 166–8° from ethanol, ν_{max} 2700, 1850, 1782, 1712, 920, 900, 885 and 865 cm^{-1} $[\alpha]_D^{22}$-68° (C, 0·17 in acetone); TLC. *R* value 0·85 in di-isopropyl ether:acetic acid (95:5).

Identification of Behenic, Tetracosanoic and Cerotic Acids

The oil (5·5 g) in fraction E was recrystallized from acetone or methanol in needles, m.p. 73–74°, ν_{max} 1705, 725, and 715 cm^{-1} shown to be a mixture of behenic (45%), tetracosanoic (45%) and cerotic (10%) acids by GLC of the methylation product at 186·5° and co-chromatography with samples of the authentic methyl esters. The mass spectrum of the mixture of acids showed the appropriate parent ions.

Isolation of 7-Hydroxykaurenolide and the Neutral Oil $C_{20}H_{36}O_8$

The neutral oil (990 mg) from fraction D was chromatographed on silica (12 × 2 cm). Elution with benzene: ethyl acetate (9:1) gave an oil (780 mg) which was molecularly distilled at 118° and 0·2 mm pressure. (Found: C, 59·5, 59·7; H, 8·3, 8·6%; M/e, 404. $C_{20}H_{36}O_8$ requires C, 59·4; H, 9·0%; M, 404) ν_{max} (liquid film) 2950 and 1745 cm^{-1}.

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Elution of the silica column with benzene:ethyl acetate (4:1) gave a gum (134 mg) which was purified by PLC in benzene:ethyl acetate (7:3); elution of the silica from R_f value 0·45–0·55 gave 7-hydroxykaurenolide (30 mg) m.p. 180–3° raised to 187·5–188·5° by recrystallization from acetone:light petroleum (b.p. 60–80°) ν_{max} 3595, 3495, 1760, 1650 and 885 cm^{-1}.

Fraction F (6·6 g) consisted mainly of the neutral oil $C_{20}H_{36}O_8$.

Acknowledgements—We thank Dr. R. H. B. Galt for a specimen of (−)-kaur-16-en-19-oic acid. One of us (B.D.C.) thanks the S.R.C. for a Research Studentship.