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DETAILED ANALYSIS OF METABOLITES FROM MEVALONIC LACTONE IN *GIBBERELLA FUJIKUROI**

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Key Word Index—*Gibberella fujikuroi* strain GF-1a, fungus, diterpenoid metabolites, gibberellins

Abstract—Methods for the detailed analysis of diterpenoid metabolites from *Gibberella fujikuroi* are described The metabolites from 2-[^{3}H]-mevalonic acid lactone are separated by partition chromatography on Sephadex LH20 columns, detected by radio-counting and characterised by gas-chromatography directly linked to radio-counting and MS From one fermentation, 72 compounds were detected Of these, 25 known diterpenes, including 15 gibberellins, were identified and 7 new products were assigned tentative structures

STRAIN GF-1a of *Gibberella fujikuroi* is a fast growing producer of gibberellins (GAs) from which mutants blocked for GA-synthesis have been produced by B. O. Phinney (unpublished work). As a prelude to biochemical studies with these mutants a detailed examination of the metabolites of the parent strain, GF-1a, was undertaken.

To facilitate the detection of terpenoid metabolites the fungus was cultured on a medium containing 2-[^{3}H]-mevalonic acid lactone (MVL). The crude mixture of metabolites, extracted by EtOAc, was subjected to partition chromatography on Sephadex LH20 using the wide range and narrow range columns, described by MacMillan and Wels.[1] Fractions were monitored by liquid scintillation counting and combined in groups according to radioactivity. Thus from the wide range column combined fractions A–W were obtained and from the narrow range column combined fractions A′–Q′ were obtained Each of these combined fractions was derivatised and analysed by combined gas chromatography–radio-counting (GC–RC) and combined GC–MS.

The method used for GC–RC was a modification of that described by Belham and Neal[2] in which the tritiated water from the FID was mixed with scintillation fluid and the mixture then cooled by an ice-water bath. The recovery of [^{3}H]-label as tritiated water was constant throughout each GLC run in the range 75–90%. The percentage recovery depended upon the cleanliness of the FID and the flow-rate of H_2 and air. This GC–RC method is sufficiently sensitive to obtain comparative specific activities and there is little loss of GLC resolution. In a later modification the ice-water condenser was replaced by a short tube packed with glass beads and is probably the method of choice

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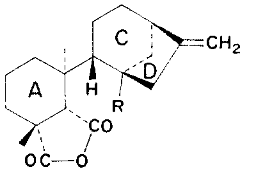
* Part X in the series "Fungal Products" For Part IX see (1973) *J Chem Soc Perkin I*, 2824

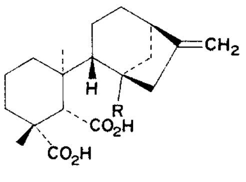
[1] MACMILLAN, J and WELS, C M (1973) *J Chromatog* **87**, 271

[2] BELHAM, J E, and NEAL, G. E (1972) *Anal Biochem* **45**, 6

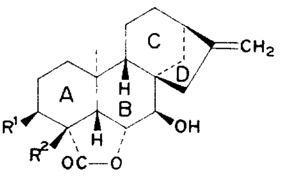
although it has not been as thoroughly evaluated This modification has also been used for [^{14}C]-labelled compounds by including 2-phenylethylamine in the scintillation fluid with recoveries as $^{14}CO_2$ of 50–60%.



(1) R = CHO
(2) R = H
(3) R = CO_2H



(4) R = CHO
(5) R = CO_2H



(6) $R^1 = R^2 = H$
(7) $R^1 = H, R^2 = OH$
(8) $R^1 = OH, R^2 = H$

The radioactive compounds identified by direct comparison of the GC–MS spectra with reference spectra are listed in Table 1 for the wide range Sephadex LH20 column. In addition, from the narrow range column, 7β-hydroxykaurenolide (**6**) and GA_9 were present in combined fractions F′ and H′. The co-occurrence of fujenal (**1**) and the corresponding diacid (**4**) in the widely separated fractions C and I shows that these two compounds are readily interconverted. All the presently known fungal GAs were detected with the exception of GA_{11} and, notably, the hydrates of GA_4(GA_2), GA_9 (GA_{10}), GA_{13} (GA_{41}) and GA_{14} (GA_{42}). The latter two hydrates are new gibberellins which have recently been isolated[3] from strain TP 70 of *G. fujikuroi*. The production of GA_{37} in the same strain has also been demonstrated.[3]

TABLE 1 COMPOUNDS IDENTIFIED BY GC–MS IN FRACTIONS FROM SEPHADEX LH20 WIDE RANGE COLUMN

Fraction	dpm × 10^3	Compound	Fraction	dpm × 10^3	Compound
A	867	—	M	190	GA_7 (trace), iso GA_7, GA_{25}, GA_{37}
B	812	—	N	349	(**13**), GA_{14}
C	2317	(**1**), (**2**), (**3**), (**4**)	O	545	GA_{14}, (**3**), (**5**), (**14**), (**15**)
D	1217	(**1**), (**6**)	P	2104	MVL
E	594	(**6**)	Q	677	GA_{16}, GA_{36} (**16**)
F	810	GA_9, GA_{12}, GA_{15} (trace), (**9**)	R	465	GA_{13} (trace), GA_{16} (trace), (**16**) (**17**)
G	264	(**8**) (**9**)	S	1342	GA_1, GA_3, GA_{13}
H	860	GA_{24} (trace), (**7**), (**8**)	T	4981	GA_1 (trace), GA_3, GA_{13}, (**10**)
I	1277	GA_4, GA_{24}, (**1**), (**3**), (**4**), (**7**), (**8**)	U	979	GA_3 (trace), (**12**),
J	3084	GA_4 (**1**), (**4**)	V	188	(**12**), (**18**)
K	1209	GA_7, (**4**)	W	86	(**10**)
L	637	GA_7, GA_{25} GA_{37}			

Thus in this investigation 25 metabolites were identified providing the most detailed analysis to date of the metabolites of *G. fujikuroi*. In addition to the identified metabolites (Table 1), well-defined GC–MS spectra of another 47 radio-active GLC peaks were obtained Of these, the following compounds were tentatively identified from the MS data presented in the Experimental: the nor-fujenal (**2**), the GA_{14} isomer (**13**), the

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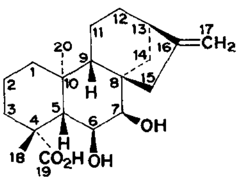
[3] BEARDER J R and MACMILLAN J (1973) *J. Chem. Soc. Perkin I*, 2824.

diene (**14**); the 1β-epimer (**15**) of GA_{16}; 2α-hydroxy epimer of GA_{34} (**16**); the 13-hydroxy-diacid (**17**), and the epoxide (**18***a* or *b*).



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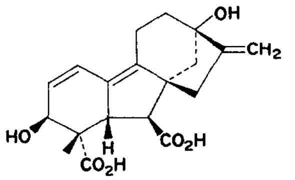

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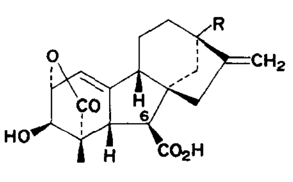
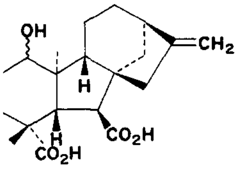


(**11**) R = H
(**12**) R = OH

The norfujenal (**2**), present in fraction C, gave an MS similar to that of fujenal (**1**) with a parent ion (*m*/*e* 302) at 28 a.m.u. lower. Both spectra contained ions at *m*/*e* 109 (**19**) and 153 (**20**) derived from ring A; they also contained an ion at *m*/*e* 121(**21**) from rings C and D which was the base peak in the MS of the norfujenal (**2**). The 1-hydroxy GA_{12} structure (**13**) for a compound in fraction N was inferred from the MS of the Me TMS derivative which showed a close similarity to the MS of Me GA_{14} TMS including an ion characteristic of a 1- or 3-TMS ether.[4]



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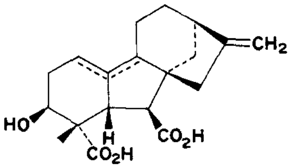

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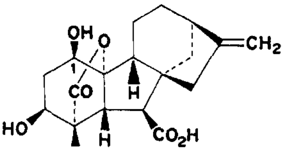
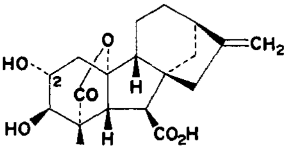
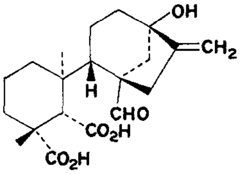


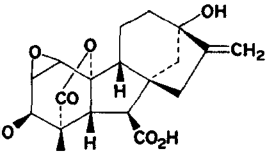
(**15**)



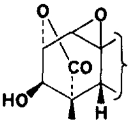
(**16**)



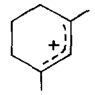
(**17**)



(**18a**)



(**18b**)


(**19**)

The MS of the Me TMS derivative of a component of fraction O was consistent with structure (**14**); typical fragmentations of two -CO_2Me groups were present and the two most intense ions at *m*/*e* 282 and 223 correspond formally to the aromatic structures (**22**) and (**23**). Fraction O also contained a compound whose Me TMS derivative gave an MS very like that of Me GA_{16} TMS; both spectra contained ions at M^+-116 (see **24**) and at *m*/*e* 147. Fragmentation ions at *m*/*e* 223 and *m*/*e* 217 in both spectra are assigned the respective structures (**23**) and (**25**). From these MS data this compound in fraction O is considered to be the 1β-epimer (**15**) of GA_{16}, the greater intensity of the *m*/*e* 147 ion[5] from the 1,3-*cis*-TMS ether than from the 1,3-*trans* TMS ether of GA_{16} being consistent with this structural assignment.

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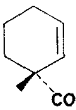
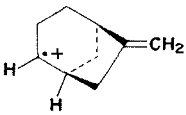
[4] BEARDER, J R, MACMILLAN, J and PHINNEY, B. O (1973) *Phytochemistry* **12**, 2173

[5] BINKS, R, MACMILLAN, J and PRYCE, R J (1969) *Phytochemistry* **8**, 271

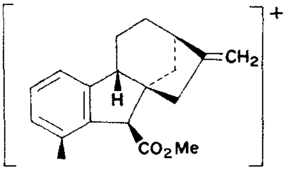
The MS of the Me and Me TMS derivatives of a component of fraction R were very like the MS of these derivatives of GA_{34}. However, the GLC retention times were slightly different from those of the GA_{34} derivatives and this compound may be the 2α-epimer (**16**) of GA_{34}. By GC–RC the specific activity of this compound was *ca* 10 times that of the other metabolites. Fraction R also contained a compound which as the Me TMS derivative showed the same MS fragmentation of ring A as shown by the di-Me ester of (**4**) including ions at *m/e* 195 (**26**), 167 (**27**), and 107 (**28**). The presence of an ion at *m/e* 237, corresponding to the *m/e* 207/208 ions typical of 13-TMS ethers of 13-hydroxytetracyclic diterpenes[5] with an additional CHO group, indicates structure (**17**)



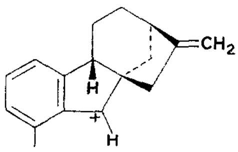
(**20**)



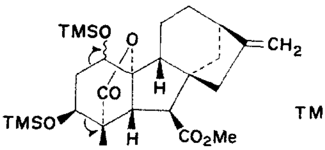
(**21**)



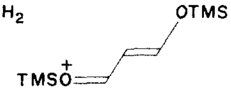
(**22**)



(**23**)



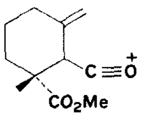
(**24**)



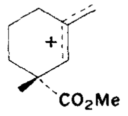
(**25**)

Finally fraction T contained a compound which, as the Me TMS derivative had M^+ at *m/e* 520, shown by high resolution to be $C_{26}H_{40}O_7Si_2$ and contained intense ions at *m/e* 207/208 indicating the presence of a 13-TMS ether. An intense ion at *m/e* 303, with the molecular composition $C_{17}H_{23}O_3Si$ is also shown by Me GA_6 TMS and by Me GA_{29} TMS and is assigned structure (**29**). The epoxy GA_3 (**18a**) or epoxyiso GA_3 (**18b**) structures are tentatively suggested for this compound.

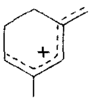
Several new mono- and di-hydroxykaurenoids were also detected but the position of the hydroxy groups could not be determined.



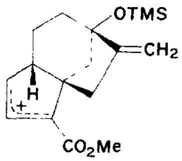
(**26**)



(**27**)



(**28**)



(**29**)

EXPERIMENTAL

Feed of 2-[^{3}H]-MVL to Gibberella fujikuroi Strain GF-la, a fast growing strain supplied by Professor B O Phinney, U C L A, was maintained on potato-dextrose-agar and stored at −5° Flasks and P.D L medium[6] were sterilised by autoclaving at 121° for 20 min Incubations were made at 25° in 100 ml conical flasks, shaking at 220 orbits min^{-1} Mycelium from a slant was incubated for 5 days A portion (1 ml) was

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[6] Spector, C and Phinney, B O (1968) *Plant Physiol* **21**, 127

transferred to another flask and incubated for 24 hr Two flasks, each containing 2-[^{3}H]-MVL (100 μCi, 500 mCi mmol^{-1}) added in C_6H_6 to the hot medium, were inoculated with portions (1 ml) of this culture and incubated for 5 days. The total medium was acidified to pH 2 5 and extracted with EtOAc (2×200 ml). Each extract was washed with pH 2 5-H_2O (50 ml), combined and evaporated to dryness (recovery 57×10^6 dpm, 26% of R-MVL fed)

Partition chromatography on Sephadex LH20 The procedures of MacMillan and Wels[1] were used (a) *Wide range column* Four-fifths ($45 6 \times 10^6$ dpm) of the extract were applied in the aqueous phase (200 μl) of the biphasic system PE-EtOAc-AcOH-MeOH-H_2O (100 80 50 40 7) to a Sephadex LH20 column ($145 \times 1 5$ cm) made up in aqueous phase The column was eluted with organic phase (195×10 ml) and then with aqueous phase (25×10 ml) Fractions were monitored by radioactive scintillation counting of aliquots (100 μl), recovery was $43 9 \times 10^6$ dpm (96%) Fractions were pooled according to radioactivity to give combined fractions A-W and evaporated to dryness after addition of toluene to azeotrope the AcOH (recovery, $25 5 \times 10^6$ dpm, 56%) The loss in radioactivity on evaporating the combined fractions is ascribed to the volatility of MVL (b) *Narrow range column* One-fifth ($11 4 \times 10^6$ dpm) of the total extract was chromatographed as described in (a) except that the solvent system PE-EtOAc-AcOH-MeOH-H_2O (50 15 10 10 2) was used. Combined fractions A′-Q′ were obtained (Recovery, $6 4 \times 10^6$ dpm, 56%).

GC-RC Me esters (CH_2N_2) and Me TMSi derivatives (prepared with Sweeley's reagent[7] at 80° in a sealed tube) were used for GLC on two columns (a) for less polar fractions, silanised glass column ($152 5$ cm $\times$ 3 2 mm i.d), 2% SE33 on Gas Chrom Q (80–100 mesh), N_2-carrier gas 30 ml min^{-1}, (b) for more polar fractions, silanised glass column ($152 5$ cm $\times$ 6·4 mm i d), 3% QF1 on Gas Chrom Q (80–100 mesh), N_2-carrier gas flow rate, 70 ml min^{-1} The effluent from the FID was collected by a modification of Belham and Neal's method [2] The gas exit port from the FID of a Pye Unicam 104 gas chromatogram was sealed off and the gases were led through a modified cap and a heated PTFE tube (0 2 mm i d) to a heated T-junction where they were mixed with scintillation fluid composed of butyl-PBD (1g), toluene (160 ml) and 2-methoxyethanol (40 ml) The flow-rate of fluid (8 ml min^{-1}) from the reservoir was controlled by a needle valve The mixed gases and scintillation fluid were cooled in an ice-bath and fractions were collected manually at 0 25, 0 5, or 1 min intervals In a later modification the ice-bath was replaced by a glass tube ($6 \times 1 25$) filled with glass beads (20 mm dia) For [^{14}C], 2-phenylethylamine(5%) was added to the scintillation fluid The recovery was 75–90% for [^{3}H] and 50–60% for [^{14}C]

GC-MS The conditions were as described in Part VII[8] except for the GLC conditions 2% GFI or 2% SE33 on Gas Chrom Q (80–100 mesh) in glass columns($152 5$ cm $\times$ 3 2 mm i d) with He-flow rate, 30 ml min^{-1} either isothermally at 200° then temp programmed at 5° min^{-1} or from 150° at 2° min^{-1} respectively The GC-MS data [*m/e* (% base peak)] for Me TMS derivatives upon which tentative identifications are based are. (a) the norfujenal (**2**) 302 (M^+,7), 258 (2), 153 (4), 149 (7), 121 (100), 109 (44), 93 (32), and 79 (38); (b) 1-hydroxy GA_{12} (**8**) 448 (M^+,10), 416 (57), 388 (6), 287 (49), 259 (88), 231 (65), 173 (100), 129 (57), and 73 (>100), (c) the diene (**14**) 432 (M^+,0), 417 (4), 372 (6), 342 (15), 310 (33), 282 (58), 223 (100), and 73 (67), (d) the 1β-epimer (**15**) of GA_{16} 506 (M^+,38), 462 (10), 447 (6), 416 (13), 390 (52), 300 (19), 241 (27), 223 (42), 217 (100), 147 (48), 73 and 75 (both >100), (e) the epimer (**16**) of GA_{34} 506 (M^+,100), 475 (3), 459 (7), 431 (5), 416 (4), 217 (30), 147 (24), 129 (30), and 73 (>100), (f) the 13-hydroxydi-acid (**17**) 464 (M^+,0), 449 (6), 237 (69), 227 (42), 195 (100), 167 (69), 107 (94), and 73 (>100), (g) the epoxide (**18**) 520 (M^+,100), 505 (10), 461 (11), 403 (7), 375 (9), 347 (8), 303 (48), 237 (13), 208 (52), 207 (27), and 73 (>100)

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[7] SWEELEY, C C., BENTLEY, R, MAKITA, M and WELLS, M M (1963), *J Am Chem Soc* **85**, 2497

[8] BEARDER, J R, MACMILLAN, J and PHINNEY, B O (1973) *Phytochemistry* **12**, 2655