Structure and Biosynthesis of Phomenoic Acid, an Antifungal Compound Isolated from *Phoma Lingam* Tode

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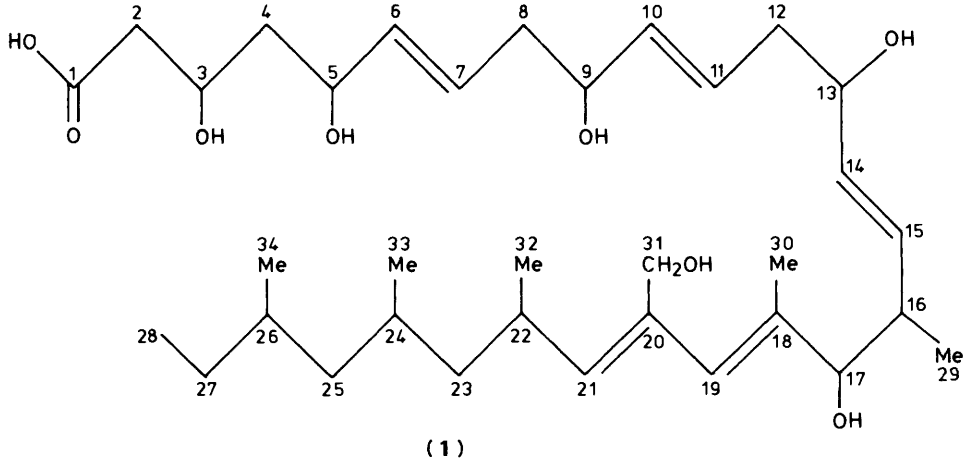
On the basis of ^{1}H and ^{13}C n.m.r. studies, ozonolysis, and biosyntheses from [^{13}C]acetates and [*methyl*-^{13}C]methionine, structure (**1**) is proposed for phomenoic acid, an antifungal compound isolated from the mycelium of the fungus *Phoma lingam* Tode.

Systematic searches for biologically active metabolites produced by the fungus *Phoma lingam* Tode, a common pest of *Cruciferae* species, have led to the isolation of compounds such as the sirodesmins PL,[1] phomamide,[2] and more recently to PLM I and II,[3] two metabolites that are now named phomenoic acid and phomenoic acid δ-lactone respectively. Antifungal properties have been established[3] for the last two compounds, and phomenoic acid has shown modest antibiotic effects; however, its full range of activity is still unclear. We present here the structure elucidation of this polyfunctionalized long-chain carboxylic compound and its biosyntheses with [^{13}C]acetates and [*methyl*-^{13}C]methionine.

Both fungal metabolites were extracted from the mycelium of *Phoma lingam* with ethanol and then partitioned between ethyl acetate and water. The crude compounds were obtained from the concentrated ethyl acetate residue by silica gel flash chromatography. Final purification of the compound by reversed phase high pressure liquid chromatography (h.p.l.c.) afforded phomenoic acid as the main product. The later eluted, less polar and minor substance PLM II, was shown to be the δ-lactone of phomenoic acid. The structure and properties of this lactone will be reported later, together with those of a series of other cyclized and dehydrated derivatives.

Phomenoic acid (**1**) showed an (M + H) ion at m/z 595 (FAB mass spectrum) and gave a microanalysis in agreement with the molecular formula $C_{34}H_{58}O_8$. Extensive ^{1}H and ^{13}C n.m.r. studies of phomenoic acid (**1**) and of its derivatives (see Tables 1 and 2) allowed the main structural features to be assigned, as follows.

The carboxylic nature of phomenoic acid was readily deduced from the ^{13}C n.m.r. signal at 180.4 p.p.m. and by the formation of a methyl ester [(M + H) at m/z (FAB) 609, $\nu_{max.}$ 1730 cm^{-1}] on treatment with MeOH–BF_3–Et_2O (classical diazomethane esterification only gave a mixture of products). The ^{13}C n.m.r. spectrum also revealed ten olefinic carbon atoms, six belonging to three disubstituted, isolated, (*E*)-double bonds (J 15 Hz; $\nu_{max.}$ 960 cm^{-1}) as shown by a careful examination (including multiple decoupling experiments) of the best resolved vinyl proton resonances of the per(dimethyl-t-butylsilyl) acid methyl ester (Table 1). Two trisubstituted unsaturated bonds were also shown to be present, presumably in a conjugated diene chromophore [$\lambda_{max.}$ 236 nm; δ_H 5.06 (21-H, d, 1 H, J 10 Hz,) and 5.84 (s, 1 H, for 19-H). The presence of five secondary hydroxy functions and of one isolated primary hydroxy group was established from the ^{1}H and ^{13}C n.m.r. spectra. As no saturated quaternary carbon atoms were found it was deduced that this CH_2OH [AB system for 31-H_2 at C-20, δ 4.09 (d) and 4.22 (d, J 11 Hz)] is located on one of the two trisubstituted carbons of the diene chromophore and that the other bears a methyl group [δ 1.68 (s, C-30 at C-18 (Table 1)]. Finally, four additional highfield methyl signals were assigned on the basis of the ^{1}H n.m.r. spectra, the first belonging to the terminal CH_3CH_2 group and the others to three CH_3CH groups.



(**1**)

From these data, phomenoic acid was shown to be a branched aliphatic, polyfunctional carboxylic acid with five unsaturated bonds, six hydroxy groups, and six methyl groups. In order to elucidate fully the structure we needed other analytical tools at this stage, and we therefore carried out both ozonolysis and ^{13}C-incorporations in biosynthetic experiments.

The ozonolysis was carried out in ethyl acetate at −40 °C on the peracetylphomenoic acid methyl ester, followed by reduc-

Table 1. ^{1}H N.m.r. spectrum of per(dimethyl-t-butylsilyl)phomenoic acid methyl ester[a]

2-H 2.56 (dd, 14.5), 2.40 (2 H, dd, 14.5, 8)	13-H 4.05—4.15 (1 H, m)
3-H 4.27 (1 H, m)	14-H 5.42 (1 H, dd, 15.5, 7)
4-H 1.58 (ddd, 14, 8.5, 4.5), 1.78 (2 H, ddd, 14, 8.5, 4.5)	15-H 5.70 (1 H, dd, 15.5, 6)
5-H 4.05—4.15 (1 H, m)	16-H 2.31 (1 H, ddq, 6, 7, 6.5)
6-H 5.43 or 5.45 (1 H, dd, 15.5, 7)	17-H 3.69 (1 H, d, 7)
7-H 5.56 (1 H, dt, 15.5, 7)	19-H 5.84 (1 H, s)
8-H 2.20 (2 H, m)	21-H 5.06 (1 H, d, 10)
9-H 4.05—4.15 (1 H, m)	22-H 2.62 (1 H, m)
10-H 5.43 or 5.45 (1 H, dd, 15.6, 7)	23-H to 28-H 0.77—0.88 (11 H)
11-H 5.50 (1 H, dt, 15.5, 7)	29-H (coalescence, 3 H)
12-H 2.20 (2 H, m)	30-H 1.68 (3 H, s)
	31-H 4.09 (d, 11), 4.22 (2 H, d, 11)
	32-H 0.96 (3 H, d)
	33- and 34-H 0.77—0.88 (6 H)

[a] Recorded on a Bruker 400 MHz instrument for solutions in $CDCl_3$, with Me_4Si as internal standard. δ in p.p.m.; multiplicities and J values (Hz) given in parentheses.

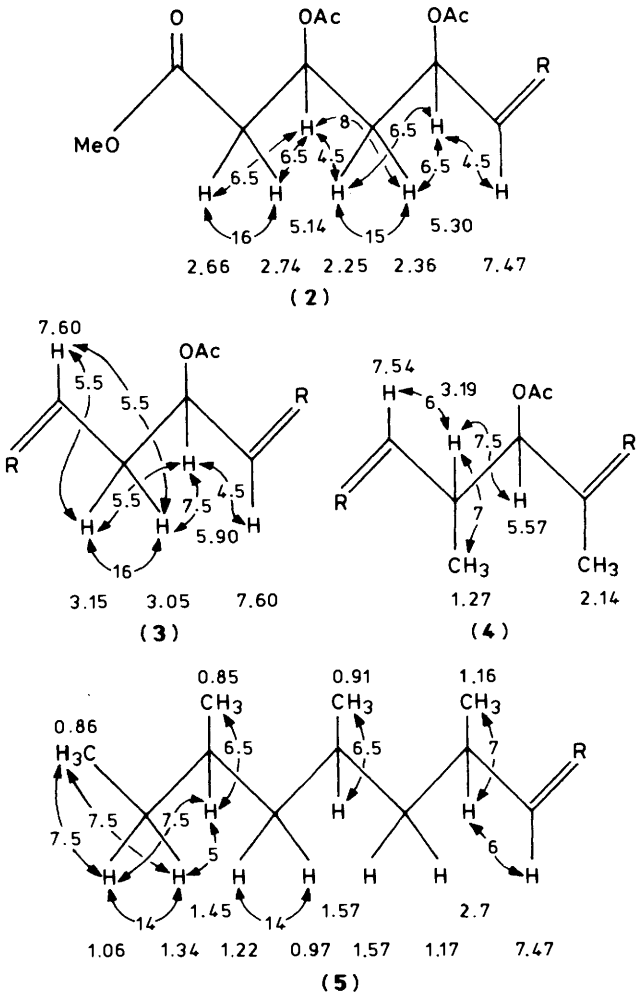
tion of the ozonides with PtO_2–H_2. The resulting ozonolysis fragments were purified as their 2,4-dinitrophenylhydrazones by h.p.l.c. on a Lichrosorb silica gel semi-preparative column. In this way, four main products were isolated and identified as follows.

The first dinitrophenylhydrazone eluted (microcrystals) was identified as fragment (**5**) [C(22)—C(28) in (**1**)] from its ^{1}H and ^{13}C n.m.r. spectra, in agreement with its electron impact (e.i.) mass spectrum, M^+ at m/z 350. The observed signals in the ^{1}H and ^{13}C n.m.r. spectra resembled those reported for the root-growth stimulant radiclonic acid.[4] The presence of the 1,3-methylated pattern was later confirmed by [^{13}C]acetate and [*methyl*-^{13}C]methionine incorporations in biosynthetic experiments, as described below.

The ^{1}H n.m.r. spectrum of the second eluted fragment (**4**) [m/z (c.i.) 533 $(M + 1)^+$ showed the presence of three different methyl groups in this C_4 unit, corresponding to CH_3CO_2CH, CH_3CH, and CH_3C=, in addition to two double bonds (C=N); the structure (**4**) was finally established by decoupling experiments.

The third fragment obtained was (**2**); this showed a peak at m/z 440 (M^+) in the e.i. mass spectrum, corresponding to a mono(dinitrophenylhydrazone) and its ^{1}H n.m.r. spectrum showed a carboxymethyl group as well as two secondary hydroxy groups. The remaining ^{1}H n.m.r. signals of this polar end of the chain in compound (**1**) were unambiguously assigned by comparison with the spectra of the peracetyl or per(dimethyl-t-butylsilyl) methyl esters (Table 1). Exhaustive double irradiation experiments have been carried out and the results compared with the signals reported for compactic acid and mevinolinic acid, the open forms of the antifungal lactones compactin and mevinolin.[5]

The chemical ionization (c.i.) mass spectrum of the most polar bis(dinitrophenylhydrazone) fragment (**3**) exhibited a $(M + 1)^+$ ion at m/z 445 which is in agreement with the structure proposed on the basis of its ^{1}H n.m.r. spectrum. A careful examination of the ^{1}H n.m.r. spectrum of phomenoic acid (**1**) and of its derivatives, in particular the per(dimethyl-t-butylsilyl) acid methyl ester, showed doubled signal integrations for 7-H at δ 5.56 (2 H instead of 1 H), 8-H at δ 2.20 (4 H instead of 2 H), and 9-H at δ 4.09—4.15 (2 H instead of 1 H), the latter coinciding with a vinyl proton indicating that this C_3 unit is probably duplicated in the molecule; thus the position of the fifth secondary hydroxy group at C-13 (or at C-9) was confirmed and the sequence C(7)—C(10) and C(11)—C(14) completed.



R = 2,4-Dinitrophenylhydrazone fragments (**2**)—(**5**) isolated after ozonization of the peracetylated phomenoic methyl ester, showing ^{1}H n.m.r. (p.p.m.) and coupling constants (Hz)

Hence, the different fragments obtained after ozonolysis lead to a partial formula, $C_{31}H_{54}O_7$, for phomenoic acid (**1**). The missing portion C_3H_4O was attributed to the two central carbon atoms of the conjugated diene and to the primary hydroxy group on C-31 at C-20, and it was assumed that this part of phomenoic acid is too fragile to give a stable fragment on ozonolysis, or that it is lost during extraction. On this basis we then looked at the connection pattern of these six different fragments in order to build up the formula of phomenoic acid to that represented in structure (**1**).

The sequence C(15)—C(22) in compound (**1**) was established on the basis of ^{1}H n.m.r. data for the olefinic protons in the persilylated methyl ester. The singlet signal observed for 19-H at δ 5.84 is consistent with it being in the vicinity of the vinylic methyl substituted olefinic carbon atom C-18, and the C(21)—C(22) sequence was deduced from decoupling experiments [δ 5.06 (J_{21-22} 10 Hz) and 2.62 (m) respectively]. As the ester bearing fragment (**2**) must have arisen from the polar head of the molecule, the only point remaining to be elucidated was the orientation of the two identical C_3 units C(7)—C(10) and C(11—C(14), isolated as the same dinitrophenylhydrazone derivative (**3**). However, owing to the coalescence of the resonances of the corresponding vinyl protons we were unable to establish the correct connections from the ^{1}H n.m.r. data.

A series of biosynthetic experiments involving the incorporation of ^{13}C-precursors in phomenoic acid were therefore

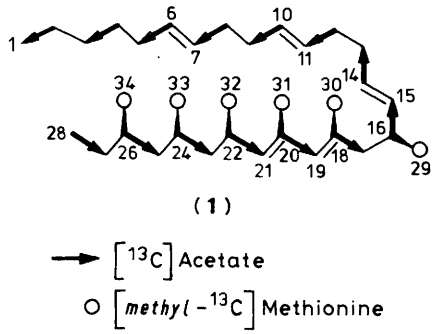
Table 2. ^{13}C N.m.r. spectra of phomenoic acid methyl ester after biosyntheses with [^{13}C]acetates and [*methyl*-^{13}C]methionine (% enrichments[6])

			% Enrichment		
	δ_C (p.p.m.)	$J^{1,2}$-(^{13}C–^{13}C) (Hz)	[2-^{13}C]-Acetate–[methyl-^{13}C]-methionine	[1,2-$^{13}C_2$]-Acetate	[1-^{13}C]-Acetate–[2-^{13}C]-acetate
C-1	172.86			5.0	14.3
C-2	41.97	57.3	6.1	3.0	6.1
C-3	67.37	36.4		9.2	6.5
C-4	42.63	37.7	5.5	2.7	7.3
C-5	72.43	39.4		*	*
C-6	136.07	47.4	*	*	*
C-7	129.78	*		*	7.3
C-8	40.48	41.1	6.2	3.1	8.1
C-9	72.79	40.8		*	9.7
C-10	135.46	46.7	7.6	3.7	8.3
C-11	129.44	*		7.0	12.6
C-12	40.11	43.2	5.0	2.6	6.0
C-13	72.43	39.8		*	*
C-14	134.27	47.2	8.3	3.7	11.7
C-15	136.04	53.0		*	*
C-16	40.90	42.6	4.3	4.4	9.6
C-17	82.69	37.8		3.2	11.6
C-18	139.39	45.1	4.4	3.1	6.4
C-19	129.60	73.0		*	10.8
C-20	133.89	54.3	8.7	5.1	15.0
C-21	137.86	80.0		2.3	6.7
C-22	30.05	42.4	7.8	4.2	10.4
C-23	45.24	34.1		3.1	6.8
C-24	28.30	35.0	6.4	2.6	5.5
C-25	45.50	35.2		3.1	10.1
C-26	31.63	34.9	5.6	3.0	6.6
C-27	29.37	34.6		2.9	8.0
C-28	11.32	34.8	5.7	4.0	6.8
C-29	20.13	$J_{16,29}$ 34.3	32.3		
C-30	11.87	$J_{18,30}$ 44.6	30.7		
C-31	60.52	$J_{20,21}$ 45.7	26.5		
C-32	22.48	$J_{22,32}$ 34.3	31.7		
C-33	19.78	$J_{22,33}$ 35.0	31.4		
C-34	17.58	$J_{26,34}$ 35.0	27.6		
CO_2Me	51.75				

* Signals present but not assigned.

carried out in order to establish finally the structure. Preliminary ^{14}C-incorporations enabled the conditions of the biosyntheses to be fixed and also established that labelled acetate and methionine were efficiently incorporated into phomenoic acid, whereas [^{14}C]-mevalonate and -propionate are not, in agreement with the polyacetic origins of the linear portion of such metabolites. On this basis, three different experiments were carried out using: i, [*methyl*-^{13}C]methionine–[2-^{13}C]acetate (1:1); ii, [1-^{13}C]acetate–[2-^{13}C]acetate; and iii, [1,2-$^{13}C_2$]acetate. After each feeding experiment, the major metabolite, phomenoic acid, was purified and converted into its methyl ester. The ^{13}C n.m.r. spectrum of the enriched product recovered from experiment i revealed, as expected, a significant enlargement of the signals due to the 14 even-numbered carbon atoms of the chain and of the attached hydroxymethyl and five methyl groups. The five carbon atoms corresponding to the secondary hydroxy groups were labelled to a much smaller extent, and therefore do not result from the labelled C-atoms of the above precursors. It was deduced from difference spectroscopy, and confirmed later, that C-1 of the acetate was located at the odd carbon atoms of the chain and, in particular, at the two hydroxy-bearing atoms of the duplicated central unit; these could be thus unambiguously assigned as C-9 and C-13, establishing the structure of the C(1)—C(14) part of the skeleton.

The ^{13}C n.m.r. signals of the six enriched carbon atoms corresponding to the methyl groups and of C-16, -18, -20, -22, -24, and -26 (Table 2) appeared as a symmetrical pair of satellites, with the natural abundance peaks appreciably enhanced. This clearly demonstrates a 1,3-substitution pattern with these substituents located on the even centres resulting from C-2 of the acetate, and the observed vicinal $J^{1,2}$(^{13}C–^{13}C) values for C-20 and -31 (45.67 Hz) and for C-18 and -30 (44.00 MHz) are in agreement with the postulated dienic chromophore.



(1)

→ [^{13}C]Acetate

○ [*methyl*-^{13}C]Methionine

^{13}C-Incorporations from ^{13}C-labelled acetates and [*methyl*-^{13}C]methionine in phomenoic acid (**1**)

These results have been further confirmed by two incorporation experiments, with [1,2-^{13}C]acetate and [1-^{13}C]-acetate–[2-^{13}C]acetate, which allowed definitive assignments of most of the ^{13}C n.m.r. signals in phomenoic acid to be made. The ^{13}C n.m.r. analysis of the vicinal carbon coupling constants was in full agreement with the preceding deductions about the structure of compound (**1**). The five double bonds were shown to occur at the junctions between the acetate units and the C(13)—C(16) sequence, already deduced from the preceding n.m.r. studies of the C(1)—C(14) and C(15)—C(28) parts of the molecule, was confirmed, resolving the ambiguities remaining after the ^1H n.m.r. double irradiation experiments.

Thus the structure of phomenoic acid is definitely established on being (**1**). However, the configurations of the nine asymmetric centres and the stereochemistry of the diene chromophore remain to be clarified, so far we have been unable to obtain suitable crystals of phomenoic acid or its derivatives for an *X*-ray crystallographic analysis.

Experimental

Ether refers to diethyl ether throughout.

Isolation of Phomenoic Acid.—Phoma lingam Tode was cultivated by the previously reported method[3] and, after 3 weeks, the filtered mycelium was ground in a mortar and then extracted with ethanol. After concentration of the ethanolic extract under reduced pressure and addition of water to the residue, the product was extracted three times with ethyl acetate, dried (Na_2SO_4), and concentrated. A crude fraction containing phomenoic acid (70—90%) was obtained by adding ether to a concentrated solution of the above extract. Pure phomenoic acid was finally prepared by h.p.l.c. on reversed phase Lichrosorb RP 18 (semipreparative column), eluting with a methanol–water (3:1) in 0.5% acetic acid. The average yield of phomenoic acid by *Phoma lingam* Tode mycelium from this method was 50 mg/l of culture medium.

Physical Properties of Phomenoic Acid.—Phomenoic acid is a microcrystalline white powder, m.p. 113—115 °C (ethyl acetate), $[\alpha]_D^{20}$ +73 °C (methanol, *c* 3); $\lambda_{max.}$(MeOH) 236 nm [ε 1 1500; Δε (228 nm) + 4.50 (in MeOH 0.4 × 10^{-3}M)]; $\nu_{max.}$(KBr) 3 320—3 380 cm^{-1} (OH), 2 920—2 950 (CO_2H), 1 735 (C=O), 1 560—1 660 (C=C), 1 050—1 100 (C–OH), 960 [C=C (*E*)], and 880 cm^{-1} (trisubstituted C=C); *m/z* (FAB) 617 (*M* + Na)$^+$ and 595 (*M*H)$^+$ (Found: C, 68.3; H, 10.2; O, 21.5. Calc. for $C_{34}H_{58}O_8$: C, 68.66; H, 9.83; O, 21.15%; *M* 594.81).

δ_H (CD_3OD; 250 MHz; 50 °C, uncompletely resolved) (proposed assignments obtained by comparison with the best resolved signals of the methyl dimethyl-t-butylsilylphomenoate, Table 1) 5.93 (1 H, s, carboxy H), 5.75—5.48 (6 H, m, 6-, 7-, 10-, 11-, 14-, 15-H), 5.14 (1 H, d, *J* 9.4 Hz, 19-H), 4.00—4.32 (6 H, m, 3-, 5-, 9-, 13-H), 3.72 (1 H, d, *J* 8.1 Hz, 17-H), 2.70 (1 H, m, 16-H), 2.20—2.42 (6 H, m, 2-, 8-, 12-H), 1.76 (3 H, d, *J* 1.2 Hz, 30-H), 1.00—1.74 (11 H, m, 4-, 23-, 25-, 27-H), 0.97 (3 H, d, *J* 6.7 Hz, CH_3), 0.90 (3 H, d, *J* 6.7 Hz, CH_3), 0.87 (3 H, d, *J* 7 Hz, CH_3), 0.85 (3 H, t, 28-H), and 0.83 (3 H, d, *J* 6.7 Hz, CH_3).

δ_C (CD_3OD; 250 MHz; 50 °C) (proposed assignments confirmed by comparison with the best resolved signals obtained after ^{13}C-incorporations in biosyntheses, Table 2) 180.40 (C-1), 139.85 (C-18), 136.72 (C-21), 136.66 (C-6), 136.45 (C-15), 135.96 (C-10), 135.78 (C-14), 134.27 (C-20), 129.68 (C-7), 128.65 (C-19 and -11), 83.53 (C-17), 73.59 (C-9), 73.34 (C-5), 72.09 (C-13), 68.75 (C-3), 61.29 (C-31), 46.72 (C-23 and -25), 45.42 (C-4), 45.15 (C-2), 41.63 (C-16), 41.57 (C-8), 41.45 (C-12), 32.98 (C-26), 31.34 (C-22), 30.46 (C-27), 29.55 (C-24), 22.79 (C-32), 20.72 (C-29), 20.27 (C-33), 17.81 (C-34), 13.50 (C-30), and 11.53 (C-28).

Phomenoic Acid Methyl Ester.—Phomenoic acid (200 mg, 0.34 mmol) in methanol (50 ml), was treated with boron trifluoride–ether (0.5 ml) for 1 h at 0 °C; the resulting mixture was then neutralized with solid $NaHCO_3$, filtered, and the solvent evaporated under reduced pressure giving the methyl ester in quantitative yield, m.p. 126—130 °C (ethyl acetate), white microcrystals, $\nu_{max.}$(KBr) 3 360 (OH), 1 730 (C=O), 1 665—1 630, 1 170 (OCH_3), 1 020, 1 010, 965, and 875 cm^{-1}; *m/z* (FAB) 631 (*M* + Na)$^+$ and 609 (*M*H)$^+$; the best resolved ^1H n.m.r. spectrum of per(dimethyl-t-butylsilyl)phomenoic acid methyl ester is presented in Table 1, and the ^{13}C n.m.r. spectrum of the methyl ester in Table 2.

Ozonolysis of the Peracetylphomenoic Acid Methyl Ester.—The peracetylated methyl ester was prepared by the usual procedure, from a solution of the methyl ester in dry pyridine, to which a slight excess of acetic anhydride had been added (quantitative yield). Ozone was bubbled for 20 min at −40 °C through a solution of this peracetylphomenoic ester (200 mg, 0.23 mmol) in ethyl acetate (100 ml), then the excess of ozone was removed by a stream of nitrogen and the ozonides were converted into the corresponding oxo derivatives by treatment with Pt/H_2 (50 mg PtO_2) for 1 h at room temperature. After filtration, the solution was poured into 1M-HCl (400 ml) containing 2,4-dinitrophenylhydrazone (500 mg, 2.5 mmol) and the mixture was vigorously stirred for about 1 h. The organic phase was washed with water, dried (Na_2SO_4), concentrated under reduced pressure to give a crude mixture of dinitrophenylhydrazones (650 mg), and subjected to preparative h.p.l.c. on a Si 60-10 Lichrosorb SiO_2 column. Elution was carried out with an increasing gradient of ethyl acetate in hexane (5% per min, u.v. detection at 360 nm). The four main 2,4-dinitrophenylhydrazones were obtained as crystals, and are described now in order of elution.

Compound (**5**) (18.5 mg), m.p. 79—81 °C, δ_H in Figure 1; δ_C (15.08 MHz; $CDCl_3$) 11.02 (C-28), 18.69, 19.65, 20.08 (C-32, -33, 34), 27.88 (C-24), 29.03 (C-27), 31.47 (C-26), 34.58 (C-22), 41.96 (C-23), 44.92 (C-25), 116.61, 123.49, 129.94 (C-aromatic), and 156.97 p.p.m. (C-2); *m/z* (e.i.) (%) 350 (*M*$^+$, 21), 279 (15), 238 (62), 220 (100), 203 (100), 117 (62), and 97 (49).

Compound (**4**) (14.4 mg), m.p. 104—108 °C, δ_H in Figure 1; *m/z* (c.i., isobutane) (%) 533 [7, (*M* + 1)$^+$], 473 {14, [(*M* + 1) − AcOH]$^+$}, 290 (100), 261 (98), 243 (100), 199 (70), 184 (100), 154 (76), 110 (64), and 109 (100).

Compound (**2**) (10.5 mg), m.p. 130—133 °C δ_H in Figure 1; *m/z* (e.i.) (%) 440 (5, *M*$^+$), 380 [5, (*M* − AcOH)$^+$], 320 [10, (*M* − 2AcOH)$^+$], 303 (17), 261 (40), 247 (100), 177 (80), and 127 (100).

Compound (**3**) (13.4 mg), 194—196 °C; δ_H in Figure 1; *m/z* (c.i., isobutane) (%) 445 {4, [(*M* + 1) − AcOH]$^+$}, 391 (21), 209 (54), 180 (55), 177 (100), and 139 (61).

Biosynthesis of Phomenoic Acid.—The following procedure has been established on the basis of preliminary experiments carried out with [^{14}C]acetate and [*methyl*-^{14}C]methionine. *Phoma lingam* was cultivated in Roux bottles each containing culture medium (200 ml), by dissolving the precursors in sterile water (500 µl) and introducing 250 µl on the seventh day and the rest on the eighth day. The mycelium was harvested on the twelfth day by filtration through filter paper. The product was extracted with ethanol and the concentrated residue re-extracted with ethyl acetate as reported above. The precursors used in these biosyntheses were from the C.E.A., Saclay, France. [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]-Acetic acid sodium salts and L-[*methyl*-^{13}C]methionine were all enriched at *ca.* 90% of their labelled C-atoms at the corresponding sites.

Phomenoic acid was isolated by SiO_2 flash chromatography (elutions with CH_2Cl_2–ethanol 8:2, ethanol, and methanol). A second chromatography was performed as reported above, with h.p.l.c. of the methyl ester prepared by treatment with BF_3 in methanol at 0 °C.

(*a*) Sodium [2-^{13}C]acetate (200 mg) [CH_3-^{13}C]-L-methionine (200 mg) were introduced into culture medium (0.8 l) giving ethyl acetate extract (143 mg) and methyl phomenoate (2 mg).

(*b*) Sodium [1-^{13}C]acetate (300 mg) and sodium [2-^{13}C]acetate (300 mg) were introduced into the culture medium (1.2 l) gave 170 mg of total extract from ethyl acetate and phomenoic acid methyl ester (17 mg).

(*c*) Sodium [1,2-$^{13}C_2$]acetate (200 mg) was introduced into the culture which had been diluted with natural acetate (600 mg) in order to avoid the direct incorporation of adjacent [^{13}C]acetate units leading to disturbances[6] in the n.m.r. spectrum. In this case 1.6 l of culture medium was used, giving 238 mg of total extract from ethyl acetate and 10 mg of phomenoic acid methyl ester after esterification and isolation.

The percentages of the ^{13}C-incorporations as reported in Table 2 were obtained by comparing the integrations of the signals with the integration of the methyl ester carbon atom as reference, and using the previously proposed[6] formula

$$\frac{\text{observed } ^{13}C \text{ abundance}}{\text{natural } ^{13}C \text{ abundance}} - 1.1 = \%$$

Acknowledgements

Thanks are due to Professor D. H. R. Barton, Gif sur Yvette, for his interest and for discussions. We also wish to thank Drs. B. C. Das and C. Girard for mass spectrometric determinations and Mrs L. Quaino for efficient laboratory technical assistance.

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Received 9th February 1984; *Paper* 4/193