Metabolites from Microbially Infected Potato. Part 1. Structure of Phytuberin

By **David T. Coxon,* Keith R. Price, Barbara Howard,** and **R. Frank Curtis,** A.R.C. Food Research Institute, Colney Lane, Norwich NR4 7UA

Phytuberin (1) was obtained from bacterially rotted potato tubers and shown to be a novel tricyclic sesquiterpene acetate. Two heterocyclic rings, a dihydrofuran ring which was hydrated under acid conditions to give the lactol (6) and readily oxidised to the γ-lactone (8), and a relatively inert tetrahydrofuran ring, were shown to be present. N.m.r. studies, including the use of lanthanide shift reagents, established an overall constitution for phytuberin {8-(1-acetoxy-1-methylethyl)-5,5a,6,7,8,9-tetrahydro-3a,5a-dimethyl-3a*H*-furo[3,2-*c*]isobenzofuran} and showed that the third ring was a cyclohexane held rigidly in a chair conformation by the two fused heterocyclic rings. Catalytic hydrogenation of phytuberin provided a dihydro-derivative (2) which was used in an independent *X*-ray crystallographic structure determination.

Our interest in metabolites produced by diseased potato tubers was aroused by the work of Lyon,[1] who showed that substantial quantities of two terpenoid compounds were formed in potato tubers inoculated with the soft rot bacterium *Erwinia carotovora* var. *atroseptica*. One of these had been identified as rishitin, a norsesquiterpene phytoalexin, $C_{14}H_{22}O_2$, first isolated [2] from potato tuber tissue infected by an incompatible race of the late blight fungus *Phytophthora infestans*. The structure of rishitin has been reported [3] and has been confirmed by its partial synthesis [4] in optically active form from (—)-α-santonin. The second compound, $C_{17}H_{26}O_4$ (determined by high resolution mass spectrometry), had properties in agreement with those described for phytuberin, a phytoalexin obtained [5] from potato tubers inoculated with an incompatible race of *P. infestans*. Varns [6] has described in detail the isolation and characterisation of phytuberin. He established by chemical means that phytuberin was the acetate of a tertiary alcohol and that the molecule also contained a vinyl ether function. On the basis of the limited information he obtained from a small quantity of material he was unable to put forward a definitive structure for phytuberin but his data suggested that the phytuberin molecule was tricyclic and possessed five methyl substituents including the acetate group. Varns [6] and later Kuc [7] put forward several tentative structures for phytuberin based on this information but none of these was related to any known sesquiterpene group, nor could any of them be derived from any obvious biosynthetic route. The availability of relatively large quantities of phytuberin from bacterially inoculated potato tubers enabled us to carry out a physicochemical examination of phytuberin and finally to prepare derivatives for *X*-ray crystallographic examination. We have also obtained phytuberin from potato tubers inoculated with *Phoma exigua* var. *foveata* or *Fusarium avenaceum*.[8] We now report chemical and physicochemical data in support of structure (1) for phytuberin.[9] The constitution and stereostructure (1) of phytuberin have been confirmed by *X*-ray crystallographic studies.[10,11]

An extract from the rotted tissue produced by ' deep wounds ' [1] in potato tubers inoculated with the bacterium *E. carotovora* var. *atroseptica* contained a complex mixture of terpenoid and other compounds from which three pure compounds were isolated by chromatography. Their molecular formulae suggested that two of the compounds were rishitin and phytuberin. The third, $C_{15}H_{24}O_3$, was eventually shown to be deacetylphytuberin (3), previously obtained as a degradation product of phytuberin.[9] When freshly harvested potato tubers of certain varieties (*e.g.* Pentland Dell and Pentland Crown) were used, a fourth compound, $C_{15}H_{22}O$ [spirovetiva-1-(10),11-dien-2-one], given the trivial name solavetivone, was also isolated.[12] Our rishitin sample was identical spectroscopically and chromatographically with natural rishitin.

Phytuberin was obtained as an oil shown to be homogeneous by t.l.c. in several solvent systems and by g.l.c. on polar and non-polar columns. It was a neutral compound giving no reactions for hydroxy-, ketone, or aldehyde groups and exhibiting no u.v. absorption above 210 nm. On thin-layer chromatograms an intense purple colour developed after spraying with vanillin–sulphuric acid reagent and heating to 120 °C, and a pale orange colour was obtained after spraying with a saturated solution of antimony trichloride in chloroform and heating at 100 °C.

N.m.r. spectra were useful in identifying isolated structural features of the phytuberin molecule. The ^1H n.m.r. spectrum (Table 1) showed the presence of **5** methyl groups (all singlet signals), two olefinic protons (doublets with J 2.8 Hz), and an AB methylene group (quartet, J_{AB} 8.5 Hz); an unresolved multiplet accounted

[1] G. D. Lyon, *Physiol. Plant Pathol.*, 1972, **2**, 411.
[2] K. Tomiyama, T. Sakuma, N. Ishizaka, N. Sato, N. Katsui, M. Takasugi, and T. Masamune, *Phytopathology*, 1968, **58**, 115.
[3] N. Katsui, A. Murai, M. Takasugi, K. Imaizumi, T. Masamune, and K. Tomiyama, *Chem. Comm.*, 1968, 43.
[4] A. Murai, K. Nishizakura, N. Katsui, and T. Masamune, *Tetrahedron Letters*, 1975, 4399.
[5] J. L. Varns, J. Kuc, and E. B. Williams, *Phytopathology*, 1971, **61**, 174.
[6] J. L. Varns, Ph.D. Thesis, Purdue University, Lafayette, Indiana, 1970.
[7] J. A. Kuc, *Teratology*, 1973, **8**, 333.
[8] K. R. Price, B. Howard, and D. T. Coxon, *Physiol. Plant Pathol.*, 1976, **9**, 189.
[9] For an account in which a preliminary structure for phytuberin was proposed see D. T. Coxon, R. F. Curtis, K. R. Price, and B. Howard, *Tetrahedron Letters*, 1974, 2363.
[10] D. L. Hughes and D. T. Coxon, *J.C.S. Chem. Comm.*, 1974, 822.
[11] D. L. Hughes, *J.C.S. Perkin I*, 1976, 1338.
[12] D. T. Coxon, K. R. Price, B. Howard, S. F. Osman, E. B. Kalan, and R. M. Zacharius, *Tetrahedron Letters*, 1974, 2921.

for the remaining 7 protons. The olefinic protons were present in a vinyl ether group and the coupling constant and δ values for these protons placed them in a five-membered ring.[13]

The natural abundance ^{13}C n.m.r. spectrum (Table 2) obtained with proton noise decoupling exhibited 16 resolved singlet signals. The multiplicities of these signals determined under single-frequency off-resonance decoupled conditions together with chemical shift considerations,[14] indicated that the 17 carbon atoms of phytuberin were present in the following skeletal units: one $-C(=O)-O-$, one $-O-CH=$, one $-CH=$, three $\geqslant C-O-$, one $-CH_2-O-$, one $\geqslant C-$, one $\geqslant C-H$, three $-CH_2-$, and five $-CH_3$.

The methyl ^1H n.m.r. signal at δ 1.99 was assigned to an acetate group, which could be removed either by alkaline hydrolysis with refluxing methanolic sodium hydroxide or by reduction with lithium aluminium hydride to give the tertiary alcohol deacetylphytuberin (3), $C_{15}H_{24}O_3$. Only three distinct methyl signals appeared in the ^1H n.m.r. spectrum of (3), representing four methyl groups. The methyl groups of the hydroxyisopropyl side chain were magnetically equivalent (δ 1.18), whereas they were not so (δ 1.44 and 1.47) in the acetoxyisopropyl side chain of phytuberin. The average upfield shift of δ 0.28 for the two methyl groups after removal of the acetate group was consistent with this structural feature.[15] Catalytic hydrogenation of phytuberin over reduced Adams catalyst gave the dihydroderivative (2), as crystals with the space group $P2_1$, which were used for an *X*-ray crystallographic study by direct methods.[11] Phytuberin underwent acid-catalysed hydration at room temperature when dissolved in 0.4N-sulphuric acid in aqueous dioxan. The product was an epimeric mixture of lactols from which the major epimer, 2α-hydroxydihydrophytuberin (6) was isolated by fractional crystallisation. Oxidation of the lactol with Collins reagent [16] or direct oxidation of phytuberin with

TABLE 1

Chemical shifts (δ) for phytuberin and its derivatives [a]

Compound	1-H_A	1-H_B	2-H	3-H	12-H, 13-H	14-H	15-H	17-H	*J*/Hz
Phytuberin (1)	3.41 (d)	3.28 (d)	6.44 (d)	4.68 (d)	1.44 1.47	1.02	1.56	1.99	$J_{1A,1B}$ 8.5 $J_{2,3}$ 2.8
Deacetylphytuberin (3)	3.37 (d)	3.23 (d)	6.41 (d)	4.64 (d)	1.18	0.99	1.53		$J_{1A,1B}$ 8.5 $J_{2,3}$ 2.8
Dihydrophytuberin (2)			3.92—3.24 (m)		1.42 1.50	1.01	1.49	1.94	
Deacetyldihydrophytuberin (9)			3.92—3.24 (m)		1.18	1.01	1.50		
2α-Ethoxydihydrophytuberin (4)	3.42 (d)	3.32 (d)	4.96 (dd)	3-H_A 2.30 (dd) 3-H_B 2.00 (dd)	1.41 1.36	0.95	1.50	1.93	$J_{1A,1B}$ 8.0 $J_{2,3A}$ 5.5 $J_{2,3B}$ 2.5 $J_{3A,3B}$ 14.0
2β-Ethoxydihydrophytuberin (5)	3.99 (d)	3.38 (d)	4.96 (t)	2.13 (d)	1.40 1.37	0.99	1.41	1.92	$J_{1A,1B}$ 8.0 $J_{2,3}$ 3.5
2α-Hydroxydihydrophytuberin (6)	3.43	3.43	5.45 (dd)	3-H_A 2.39 (dd) 3-H_B 2.00 (dd)	1.42 1.39	0.98	1.52	1.92	$J_{2,3A}$ 5.5 $J_{2,3B}$ 4.5 $J_{3A,3B}$ 14.0
Deacetylhydroxydihydrophytuberin (7)	3.44	3.44	5.45 (dd)	3-H_A 2.40 (dd) 3-H_B 2.00 (dd)	1.16	0.99	1.54		$J_{2,3A}$ 5.5 $J_{2,3B}$ 4.5 $J_{3A,3B}$ 14.0
Phytuberin lactone (8) in $CDCl_3$	3.58	3.58		2.68 (d)	1.44 1.38	1.06	1.50	1.94	
in C_6D_6	3.44 (d)	3.25 (d)		3-H_A 2.54 (d) 3-H_B 2.19 (d)	1.25	0.88	1.30	1.68	$J_{1A,1B}$ 9.0 $J_{3A,3B}$ 18.5
Phytuberin triol (10)	3.75 (d)	3.47 (d)	3.75 (m)		1.25 1.22	1.08	1.47		$J_{1A,1B}$ 8.5

[a] Unless otherwise stated all signals are singlets.

TABLE 2

^{13}C N.m.r. shifts [a] for phytuberin (1) and deacetylphytuberin (3)

(1)	(3)	Carbon type	Assignment	(1)	(3)	Carbon type	Assignment
16.5	16.4	$-CH_3$	C-14 or -15	45.0	45.0	$\geqslant C-$	C-10
21.6	21.6	$-CH_3$	C-15 or -14	73.6	73.7	$-CH_2-O-$	C-1
22.2		$-CH_3$	C-17	83.8	72.2	$\geqslant C-O-$	C-11
23.1	26.6	$-CH_3$	C-12 or -13	93.0	93.2	$\geqslant C-O-$	C-4 or -5
23.1	23.2	$-CH_2-$	C-8				
23.4	27.1	$-CH_3$	C-13 or -12	94.4	94.8	$\geqslant C-O-$	C-5 or -4
29.0	29.5	$-CH_2-$	C-6 or -9	104.4	104.4	$=CH-$	C-3
34.3	34.5	$-CH_2-$	C-9 or -6	146.2	146.6	$=CH-O-$	C-2
43.7	46.2	$>CH-$	C-7	170.0		$>C=O$	C-16

[a] In p.p.m. downfield from internal Me_4Si.

[13] L. M. Jackman and S. Sternhell, 'Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry,' Pergamon, Oxford, 1969, pp. 184—191, 195—200, 280—304.

[14] G. C. Levy and G. L. Nelson, 'Carbon-13 Nuclear Magnetic Resonance for Organic Chemists,' Wiley–Interscience, New York, 1972.

[15] R. Misra and S. Dev, *Tetrahedron Letters*, 1972, 4865.

[16] J. C. Collins, W. W. Hess, and F. J. Frank, *Tetrahedron Letters*, 1968, 3363.

chromium trioxide in acetic acid provided the γ-lactone (8). The ^{1}H n.m.r. spectrum of this lactone (Table 1) in hexadeuteriobenzene showed two methylene AB quartets associated with the methylene group adjacent to the carbonyl of a γ-lactone (δ 2.19 and 2.54, J_{AB} 18 Hz) and a further CH_2·O group (δ 3.25 and 3.44, J 9 Hz).

The simple chemical transformations so far described established that an acetate group present in phytuberin was part of an acetoxyisopropyl side chain, and confirmed that the vinyl ether group of phytuberin was associated with a dihydrofuran ring. Since phytuberin possesses only one carbon–carbon double bond and only one carbon–oxygen double bond (in the acetate group) the molecular formula requires it to possess a tricyclic structure, and the oxygen atom which is unaccounted for can only be present in a saturated ether ring system. The observed δ values and coupling constants for the protons of the CH_2·O group in both phytuberin and the lactone (8) indicated the presence of a saturated five-membered ether ring [13] in phytuberin.

A further chemical transformation involved the dihydrofuran ring. When a crude phytuberin extract was chromatographed on silica gel in AnalaR chloroform (containing 2% ethanol as stabiliser) as eluant, no phytuberin was detected in the eluates but a mixture of two compounds of similar polarity to phytuberin and giving the same colour reactions on t.l.c. plates was obtained. The products were 2α- (4) and 2β-ethoxydihydrophytuberin (5), arising from the silica-gel-catalysed addition of ethanol across the 2,3-double bond.

Phytuberin lactone was remarkably resistant to oxidative and reductive cleavage. It was inert to oxidation by chromium trioxide in aqueous acetic acid at room temperature and on attempted oxidation with alkaline permanganate gave only deacetylphytuberin lactone (8; but $R^1 = H$) as a partially characterised product (g.l.c.), *m/e* 253 ($M - CH_3$), 250 ($M - H_2O$), and 150 [base peak; *cf.* phytuberin lactone *m/e* 150 (50%) and 43 (base peak)]. Reduction of phytuberin lactone with lithium aluminium hydride in ether gave only deacetyl-2-hydroxydihydrophytuberin (7) and did not cleave the lactone ring. Reduction of the lactone (8) or further reduction of (7) with an excess of lithium aluminium hydride in refluxing tetrahydrofuran did cleave the ring system and provided the partially characterised triol (10).

None of the structural data yet presented gives any indication of the sesquiterpene class to which phytuberin belongs. In an attempt to obtain some information on the nature of the carbon skeleton present ^{1}H n.m.r. data were obtained on deacetylphytuberin (3) in the presence of the deuteriated lanthanide shift reagents [$^2H_{27}$]Eu(fod)$_3$ and [$^2H_{27}$]Pr(fod)$_3$.[17] The spectrum of deacetylphytuberin in the presence of 0—1.0 mol. equiv. of [$^2H_{27}$]Eu(fod)$_3$ showed downfield shifts of all signals which were approximately linear with respect to Eu(fod)$_3$ concentration. In the Eu(fod)$_3$-shifted spectrum five new multiplets (denoted A,C,E,F, and G in Table 3) were observed at all values between 0.4 and 1.0 mol equiv. of shift reagent, and a sixth multiplet (denoted B) was resolved in the presence of 1 mol. equiv. of shift reagent. The remaining proton signal (denoted D) was observed in the Pr(fod)$_3$-shifted spectrum. The relationship between the protons giving rise to these multiplets was established by examination of their multiplicities (Table 3) and by spin-decoupling experiments carried out on the spectrum in the presence of Eu(fod)$_3$ (Table 4). The observed pattern of proton signals was consistent with that expected from a 1,3,3,4,4-substituted cyclohexane ring in a rigid chair conformation with the hydroxyisopropyl group as an equatorial 1-substituent. In terms of the phytuberin numbering system, the cyclohexane ring protons whose signals were resolved in the Eu(fod)$_3$-shifted spectrum were 6-H$_a$, 7-H$_a$, 8-H$_a$, 8-H$_e$, 9-H$_a$, and 9-H$_e$. The 6-H$_e$ signal predicted as a double doublet, $J_{6a,6e}$ 13, $J_{6e,7a}$ 4 Hz, was resolved and observed

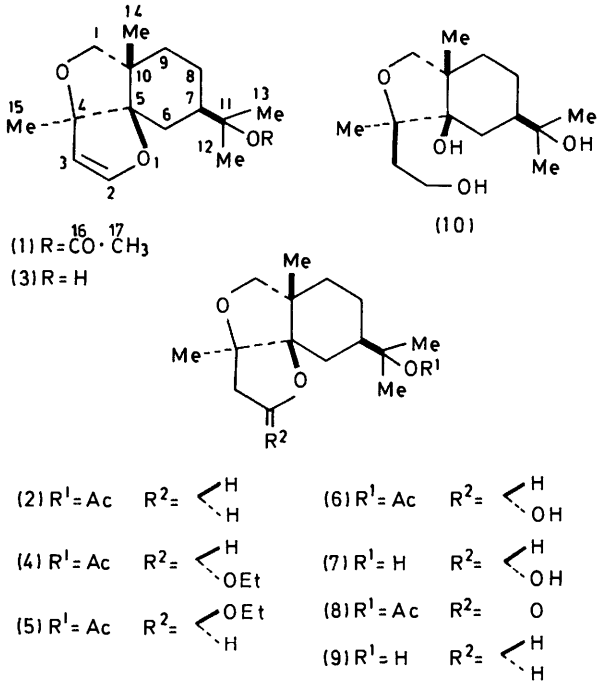


TABLE 3

Signals revealed in the n.m.r. spectrum of deacetylphytuberin (3) on the addition of shift reagent [Eu(fod)$_3$ or Pr(fod)$_3$].

Signal [a]	Observed multiplicity	No. of coupled protons	J_{obs}/Hz	Assignment
A	t of t	4	13, 13, 4, 4	7-H$_a$
B	v br d	4	13, 4, 4, 4	8-H$_e$
C	t	2	13, 13	6-H$_a$
D [b]	dd	2	13, 4	6-H$_e$
E	q of d	4	13, 13, 13, 4	8-H$_a$
F	t of d	3	13, 13, 4	9-H$_a$
G	d of t	3	13, 4, 4	9-H$_e$

[a] Multiplets A—G are in decreasing order of induced shift in the Eu(fod)$_3$-shifted spectrum. [b] This signal not resolved in the Eu(fod)$_3$-shifted spectra but appeared in Pr(fod)$_3$-shifted spectra.

[17] R. E. Rondeau and R. E. Sievers, *J. Amer. Chem. Soc.*, 1971, **93**, 1522.

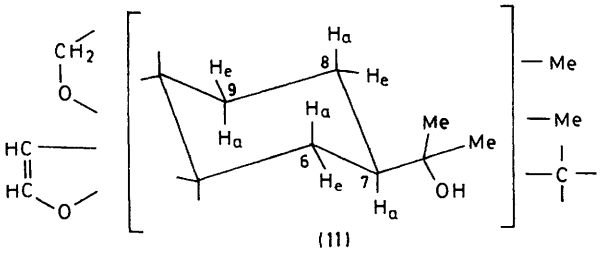
as such at δ −1.00 in the spectrum of deacetylphytuberin in the presence of 0.2 mol. equiv. of $Pr(fod)_3$. The praseodymium-shifted spectrum was in general far less useful than the europium-shifted spectrum for this compound. However, use of both shift reagents enabled the signals for the seven protons which were coincident in the normal spectrum of deacetylphytuberin to be resolved and assigned.

TABLE 4

N.m.r. spectrum of deacetylphytuberin (3) in the presence of $[^2H_{27}]Eu(fod)_3$ (0.40 mol. equiv.); spin decoupling observations.

Proton (δ)						Apparent multiplicity change	Splitting(s) decoupled (Hz)	
Irradiated			Observed					
A	(7-H_a)	6.12	E	(8-H_a)	4.13	qd → br t	13	$J_{7a,8a}$
			C	(6-H_a)	4.64	t → d	13	$J_{6a,7a}$
B	(8-H_e)	4.78	G	(9-H_e)	2.68	dt → dd	4	$J_{8e,9e}$
			F	(9-H_a)	3.81	td → t	4	$J_{8e,9a}$
C	(6-H_a)	4.64	A	(7-H_a)	6.12	tt → dt	13	$J_{6a,7a}$
E	(8-H_a)	4.12	A	(7-H_a)	6.12	tt → dt	13	$J_{7a,8a}$
			G	(9-H_e)	2.68	dt → dd	4	$J_{8a,9e}$
F	(9-H_a)	3.78	G	(9-H_e)	2.68	dt → t	13	$J_{9a,9e}$
G	(9-H_e)	2.68	F	(9-H_a)	3.81	td → br d	13	$J_{9a,9e}$
			E	(8-H_a)	4.13	qd → q	4	$J_{8a,9e}$

Observation of signal F whilst irradiating frequency of proton E and *vice versa* was not possible owing to saturation effects from the decoupling signal.



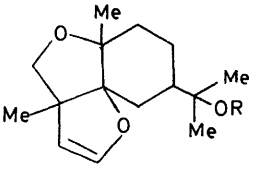
(11)

Partial structure for deacetylphytuberin. The two units on the left hand side of the bracket must each be included in a five-membered ring system in the final structure.

Thus deacetylphytuberin could be represented by the partial structure (11). Of the six possible structures based on this partial structure which seemed to fit all the ^{13}C and ^1H n.m.r. data we originally chose structure (12) for deacetylphytuberin. A biosynthetic relationship between the structures of rishitin and phytuberin was not recognised, but the correct structure (3) based on the *X*-ray crystallographically determined structure of dihydrophytuberin (2) shows a close relationship to that of rishitin. We speculate that rishitin and phytuberin are formed biosynthetically from a common eudesmane-related intermediate (14), which in the case of rishitin (15) becomes oxygenated at C-2 and C-3 and loses the C-14 methyl group. In the case of phytuberin, oxygenation at C-1 and C-2 followed by cleavage of the C–C bond and appropriate recyclisation could lead to the tricyclic phytuberin structure (1). On this basis we have numbered the phytuberin carbon skeleton according to that of the eudesmane precursor from which it is assumed to be derived.

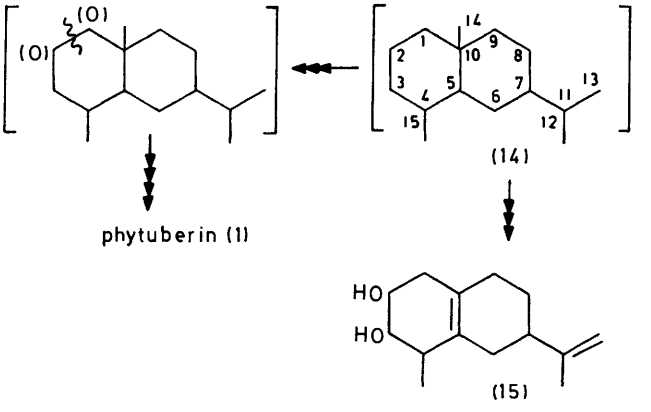
The substitution pattern and rigid conformation of the cyclohexane ring in phytuberin as determined by the n.m.r. experiments was confirmed by the *X*-ray crystallographic study.[11] Investigation by *X*-ray was unable to provide the absolute configuration of phytuberin. However, if we assume that rishitin and phytuberin are closely related biogenetically and have the same absolute configuration at C-7 then the absolute configuration of phytuberin can be represented as in (1).

The mass spectral fragmentation of phytuberin was studied in detail by high resolution mass measurements and metastable scanning. A simplified fragmentation diagram showing breakdown pathways indicated by metastable observations is given in the Scheme. It is now clear that the data provide evidence against the earlier proposed phytuberin structure (13) [9] and in favour of the correct structure (1). It is difficult to conceive how structure (13) could give rise to the $C_{11}H_{17}$ fragment *m/e* 149 whereas mechanisms can be drawn for the formation of such a fragment from structure (1).



(12) R = H

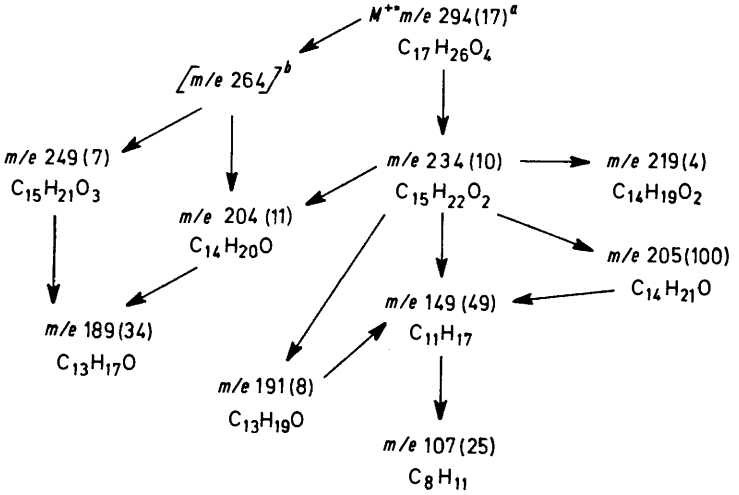
(13) R = Ac



The antimicrobial activity of phytuberin has been

studied in some detail. The early work of Varns on the accumulation of rishitin and phytuberin in potato tuber slices inoculated with incompatible races of *Phytophthora infestans* or with non-pathogens [6,18] raised the question of whether rishitin and phytuberin might have some role in resistance against the incompatible fungus. The antifungal activity of phytuberin against a series of ten potato pathogens and three non-pathogens has been studied *in vitro* by Harris and Dennis.[19] They found that phytuberin only showed appreciable activity against the germination of zoospores of *P. infestans* and then only in the absence of any organic nutrients. It therefore seems unlikely that phytuberin has any relevance to the resistance of potato tubers to fungal infection. It has also been reported [20,21] that phytuberin has no significant activity against the bacterium *Erwinia carotovora* var. *atroseptica*.

The occurrence of phytuberin in infected potato tuber tissue led Renwick [22] to include this compound, along with others, as a possible causal factor in a hypothesis linking the consumption of potatoes with the incidence of spina bifida and anencephaly. In view of this interest some preliminary testing of the mammalian toxicity of phytuberin was carried out. Four mice (two females and two males) were given phytuberin (250 mg kg^{-1}, in 50% aqueous propane-1,2-diol) by intraperitoneal injection for 5 consecutive days. No harmful effects were observed. After sacrifice on the eighth day no abnormalities of heart, lungs, stomach, liver, kidneys, gut, spleen, or pancreas were observed by histological studies.



SCHEME Major fragmentation processes in the mass spectrum of phytuberin

[a] Figures in parentheses indicate intensities relative to base peak, *m/e* 205. [b] This fragment not observed.

EXPERIMENTAL

Unless otherwise stated, i.r. spectra were measured for solutions in chloroform, u.v. spectra and optical rotations for solutions in ethanol, and n.m.r. spectra for solutions in deuteriochloroform (tetramethylsilane as internal standard). Mass spectra were determined with an A.E.I. MS 902 spectrometer. Samples were introduced *via* the direct insertion probe or from the effluent of a Pye 104 gas liquid chromatograph *via* a silicone rubber membrane.

Silica gel used for column chromatography was Mallinckrodt SilicAR CC7, and alumina was Woelm neutral alumina, activity 1. Ethanol-free chloroform was prepared by elution of AnalaR chloroform through a column of Laporte type H basic alumina (activity 1). Merck Kieselgel G was used for t.l.c. During isolation processes the appropriate combination of fractions was determined by their t.l.c. behaviour. Thin-layer chromatograms were examined by exposure to iodine vapour and then spraying with (*a*) a solution of vanillin (3 g) in ethanol (100 ml) acidified with sulphuric acid (0.5 ml), (*b*) a saturated solution of antimony trichloride in chloroform, or (*c*) a saturated solution of cerium(IV) sulphate in 2N-sulphuric acid followed by heating in all cases.

Analytical g.l.c. was carried out with a Pye 104 chromatograph [5 ft × 4 mm i.d. glass column packed with 5% OV17 on Diatomite CQ (80—100 mesh) operating isothermally at 200 °C and equipped with a flame ionisation detector; carrier gas argon at 45 ml min^{-1}]. Retention times were measured relative to that of an internal standard of methyl stearate and are quoted as relative retentions (R_t).

All evaporations were carried out under diminished pressure (water pump) with a rotary film evaporator operating at 40 °C or below. M.p.s were determined with a Kofler hot-stage apparatus.

Isolation of Metabolites.—Four hundred mature potato tubers (*Solanum tuberosum* var. King Edward; 45 kg) were washed with water, dipped in ethanol, and air dried. The tubers were each inoculated by the deep wound method [1] with 0.3 ml of a suspension (10^9 cells ml^{-1}) of *E. carotovora* var. *atroseptica*. Inoculated tubers were stored under

[18] J. L. Varns, W. W. Currier, and J. Kuc, *Phytopathology*, 1971, **61**, 968.
[19] J. E. Harris and C. Dennis, *Physiol. Plant Pathol.*, 1976, **9**, 155.
[20] G. D. Lyon, B. M. Lund, C. E. Bayliss, and G. M. Wyatt, *Physiol. Plant Pathol.*, 1975, **6**, 43.
[21] G. D. Lyon and C. E. Bayliss, *Physiol. Plant Pathol.*, 1975, **6**, 177.
[22] J. H. Renwick, *Brit. J. Prev. Soc. Med.*, 1972, **26**, 67.

aerobic conditions at 10 °C and 95—100% relative humidity for 14 days. The rotted tissue scraped from the tubers was lyophilised, and the dry tissue (2 kg) was extracted (8 h) with methanol (Soxhlet). The concentrated methanolic extract (1.5 l) was diluted with water (1 l) and extracted with chloroform (3 × 2.5 l). The combined chloroform layers were concentrated (to 2 l), dried (Na_2SO_4), and evaporated to dryness. The crude extract (24 g) was chromatographed on neutral alumina (800 g) with cyclohexane–ethyl acetate (1 : 1 v/v). Phytuberin-rich fractions were combined and evaporated to dryness. Deacetylphytuberin and rishitin were present in two other combined fractions from this column.

Phytuberin. The crude phytuberin fraction (3.6 g) was chromatographed on silica gel (150 g) and eluted with ethanol-free chloroform in the dark. The eluate was monitored by t.l.c. and appropriate fractions were combined and evaporated to give *phytuberin* (1) as an oil (1.1 g) (Found: M^+, 294.184 9. $C_{17}H_{26}O_4$ requires M, 294.183 1); $[\alpha]_D$ −35.9° (*c* 1.34); $\nu_{max.}$ (film) 3 080, 1 728, 1 620, 1 260, 1 088, and 735 cm^{-1}; for n.m.r. spectrum see Table 1; t.l.c. (single spot) R_F 0.65 (cyclohexane–ethyl acetate, 1 : 1), or 0.80 (chloroform–acetic acid–methanol, 85 : 2 : 13); g.l.c. R_t 0.51.

Deacetylphytuberin. A fraction (320 mg) containing crude deacetylphytuberin was chromatographed on silica gel and eluted with cyclohexane–ethyl acetate (3 : 1) to give *deacetylphytuberin* (3) as an oil (70 mg), identical with the product obtained by partial synthesis from phytuberin (see later).

Rishitin. A fraction (5.3 g) containing rishitin was chromatographed on silica gel and eluted with cyclohexane–ethyl acetate (4 : 1) to give a product (1.7 g) which was subjected to preparative g.l.c. [6 ft × 7 mm i.d. glass column packed with 20% OV17 on Diatomite CQ (60—70 mesh); 200 °C; argon flow rate 200 ml min^{-1}]. A 100 : 1 split ratio outlet was used on the column, and a flame ionisation detector and a ' total trapping ' system as described by Swoboda [23] were used. Chromatographically pure rishitin (480 mg) was obtained as an oil which crystallised on cooling; m.p. 64—66° (Found: M^+, 222.161 6. Calc. for $C_{14}H_{22}O_2$: M, 222.162 0); $[\alpha]_D$ −30.1° (*c* 2.5); $\nu_{max.}$ (KBr) 3 380, 3 076, 1 647, 1 087, and 890 cm^{-1}; δ_H 1.15 (d, *J* 6.5 Hz, 14-H_3), 1.74 (s, 13-H_3), 2.67br (s, OH), 3.23 (t, *J* 9 Hz, 3-H), 3.65 (dt, *J* 7, 9, and 9 Hz, 2-H), 4.65 (m) and 4.75 (m) (12-H_2); δ_C 16.3 (q), 20.8 (q), 26.2 (t), 29.4 (t), 30.8 (t), 38.2 (t), 40.1 (d), 41.3 (d), 71.2 (d), 79.0 (d), 108.9 (t), 124.9 (s), 129.0 (s), and 147.2 (s); t.l.c. R_F 0.25 (cyclohexane–ethyl acetate, 1 : 1) or 0.60 (chloroform–acetic acid–methanol, 85 : 2 : 13), g.l.c. R_t 0.61.

Dihydrophytuberin (2).—Phytuberin (33 mg) dissolved in glacial acetic acid (15 ml) was hydrogenated over freshly reduced Adams catalyst (23 mg). After 12 h the solution was filtered, diluted with water (60 ml), and extracted with ethyl acetate. Evaporation of the dried (Na_2SO_4) extract gave a solid residue which was crystallised from petroleum (b.p. 30—40 °C) at −20 °C to give *dihydrophytuberin*, m.p. 65—66° (Found: C, 68.5; H, 9.3. $C_{17}H_{28}O_4$ requires C, 68.9; H, 9.5%); *m/e* 296 ($M^{+\cdot}$ v. weak) and 236 (M − 60, loss of acetic acid), $[\alpha]_D$ +41.1° (*c* 1.27); $\nu_{max.}$ (KBr) 1 724, 1 255, 1 117, and 1 049 cm^{-1}; for n.m.r. spectrum see Table 1; t.l.c. R_F 0.55 (cyclohexane–ethyl acetate, 1 : 1), g.l.c. R_t 0.63.

Deacetylphytuberin (3).—Lithium aluminium hydride (100 mg) dissolved in anhydrous ether (30 ml) was filtered under slight vacuum. The filtrate was added dropwise to a solution of phytuberin (191 mg) in anhydrous ether (20 ml) and the mixture stirred overnight at room temperature and diluted with water (50 ml). The organic layer was separated and yielded a gum (135 mg) which was chromatographed [silica (10 g); cyclohexane–ethyl acetate (3 : 1)]. The major fraction provided *deacetylphytuberin* (3) (87 mg) as an oil (Found: M^+, 252.171 0. $C_{15}H_{24}O_3$ requires M, 252.172 6), $[\alpha]_D$ −36.9° (*c* 2.66), $\nu_{max.}$ (film) 3 440, 3 080, 1 624, 1 153, 1 088, 1 040, 1 027, and 735 cm^{-1}; for ^1H n.m.r. spectrum see Table 1; for ^{13}C n.m.r. spectrum see Table 2; t.l.c. (single spot) R_F 0.28 (cyclohexane–ethyl acetate, 1 : 1); g.l.c. R_t 0.36.

Alkaline Hydrolysis of Phytuberin.—A solution of phytuberin (16 mg) in methanol (5 ml) and 2N-sodium hydroxide solution (0.5 ml) was heated (12 h) under reflux, cooled, diluted with water, and extracted with chloroform. The extract yielded deacetylphytuberin (3) (16 mg) as an oil. A similar reaction carried out at room temperature gave very little conversion of phytuberin into (3).

Deacetyldihydrophytuberin (9).—Dihydrophytuberin (55 mg) was reduced with lithium aluminium hydride (50 mg) under the conditions used for conversion of phytuberin into deacetylphytuberin. On work-up the ethereal extract gave an oil (50 mg) which was chromatographed [silica (10 g); cyclohexane–ethyl acetate (1 : 1)]. The major fraction provided *deacetyldihydrophytuberin* (9) (35 mg) as an oil (Found: M^+, 254.190 4. $C_{15}H_{26}O_3$ requires M, 254.188 2), $[\alpha]_D$ +29.4° (*c* 1.47), $\nu_{max.}$ (film) 3 440, 1 125, 1 061, 1 045, and 1 033 cm^{-1}; for n.m.r. spectrum see Table 1; t.l.c. (single spot) R_F 0.20 (cyclohexane–ethyl acetate, 1 : 1), g.l.c. R_t 0.45.

Hydrogenation of Deacetylphytuberin.—Deacetylphytuberin (8 mg) in ethanol (5 ml) was hydrogenated (9 h) over 5% palladium–charcoal (15 mg) at room temperature. The catalyst was removed (filtration through Celite) and the solution evaporated. Deacetyldihydrophytuberin (5 mg) was obtained by preparative t.l.c. of the residue [cyclohexane–ethyl acetate (1 : 1)].

2α-Hydroxydihydrophytuberin (6).—Phytuberin (100 mg) was dissolved in dioxan (4 ml) and 2N-sulphuric acid (1 ml) and kept at room temperature for 4 h. Dilution with water and extraction of the aqueous mixture with ether (3 × 20 ml) gave the crude product (109 mg), which was applied to two 0.5 mm preparative thin-layer plates, which were developed in cyclohexane–ethyl acetate (1 : 1). The main product band, R_F 0.26, was scraped off and eluted with acetone. Crystallisation of the product from hexane gave *2α-hydroxydihydrophytuberin* as fine needles. m.p. 119—122° (Found: C, 65.0; H, 8.9. $C_{17}H_{28}O_5$ requires C, 65.4; H, 9.0%), $[\alpha]_D$ +55.8° (*c* 0.86), $\nu_{max.}$ (KBr) 3 460, 1 711, 1 265, 1 115, and 999 cm^{-1}; for n.m.r. spectrum see Table 1; t.l.c. (single spot) R_F 0.24 (cyclohexane–ethyl acetate, 1 : 1); g.l.c. R_t 1.28. The 2α-isomer (6) was the major product formed in the hydration but the epimer was present in the crude product. The two isomers could be distinguished by their n.m.r. spectra or by g.l.c. of their trimethylsilyl derivatives. The trimethylsilyl derivative of (6) had R_t 0.82. G.l.c. of the trimethylsilylation product of material from the mother liquors gave two peaks with R_t 0.82 and 1.13 and areas in the ratio 9 : 1, respectively.

2-Ethoxydihydrophytuberin.—A crude extract (16.5 g) from *Erwinia*-inoculated potato tissue was chromatographed

[23] P. A. T. Swoboda, *Nature*, 1963, **199**, 31.

on a column of silica gel (600 g) and eluted with chloroform containing 2% ethanol (as stabiliser). Among the fractions separated a crude mixture (860 mg) of the phytuberin transformation products (4) and (5) was obtained. The α- and β-epimers were roughly separated by preparative t.l.c. on silica plates [cyclohexane–ethyl acetate (1 : 1)] and were purified by preparative t.l.c. on silica plates [two elutions with hexane–acetone (9 : 1)]. *2α-Ethoxydihydrophytuberin* (4) (109 mg) was obtained as a mobile oil, *m/e* 340 ($M^{+\cdot}$ absent), 280 (M − 60, loss of acetic acid), and 234 (M − 60 − 46, loss of acetic acid and ethanol), $[\alpha]_D$ +89.5° (*c* 1.03), $\nu_{max.}$ (film) 1 743, 1 260, 1 110, 1 050, and 1 000 cm^{-1}; ^1H n.m.r. (220MHz) δ_A 3.78 (dq), δ_B 3.35 (dq), δ_{Me} 1.13 (t) [J_{AB} 9.5, $J_{AMe} = J_{BMe} = 7$ Hz, O·C(H_AH_B)Me]; for further n.m.r. data see Table 1; t.l.c. (single spot) R_F 0.58 (cyclohexane–ethyl acetate, 1 : 1), g.l.c. R_t 0.82. *2β-Ethoxydihydrophytuberin* (5) (68 mg) was obtained as a mobile oil, *m/e* 340 ($M^{+\cdot}$ absent), 294 (M − 46, loss of ethanol), and 234 (M − 46 − 60, loss of ethanol and acetic acid); $[\alpha]_D$ −4.8° (*c* 1.01); $\nu_{max.}$ (film) 1 731, 1 260, 1 130, 1 053, and 1 000 cm^{-1}; ^1H n.m.r. (220 MHz) δ_A 3.72 (dq), δ_B 3.36 (dq), δ_{Me} 1.18 (t) [J_{AB} 9.5, $J_{AMe} = J_{BMe} = 7$ Hz, O·C(H_AH_B)-Me]; for further n.m.r. data see Table 1; t.l.c. (single spot) R_F 0.51 (cyclohexane–ethylacetate, 1 : 1); g.l.c. R_t 1.23.

Oxidation of 2-Hydroxydihydrophytuberin (6): *Formation of Phytuberin Lactone* (8).—2-Hydroxydihydrophytuberin (45 mg) dissolved in anhydrous dichloromethane (6 ml) was added to a solution of Collins reagent (230 mg) in dichloromethane (5 ml) and kept at room temperature for 1 h. 5% Sulphuric acid (10 ml) was then added and the layers were separated. The organic layer was washed with water, dried (Na_2SO_4), and evaporated. The residue was redissolved in ether, filtered through a small Celite column, and applied to thin-layer silica plates, which were developed in cyclohexane–ethyl acetate (1 : 1). The product band R_F 0.50 was scraped off and eluted with acetone. Evaporation of the solvent gave *phytuberin lactone* (8) (41 mg) as a gum, *m/e* 310 ($M^{+\cdot}$ absent) and 250 (M − 60, loss of acetic acid), $[\alpha]_D$ +48.0° (*c* 1.00), $\nu_{max.}$ (film) 1 776, 1 731, 1 260, 1 144, 1 045, and 1 023 cm^{-1}; for n.m.r. spectrum see Table 1; t.l.c. (single spot) R_F 0.50 (cyclohexane–ethyl acetate, 1 : 1); g.l.c. R_t 1.98.

Oxidation of Phytuberin with Chromic Acid: Direct Formation of Phytuberin Lactone.—A filtered solution of chromium trioxide (150 mg) in glacial acetic acid (8 ml) and water (2 ml) was added to a solution of phytuberin (104 mg) in glacial acetic acid (10 ml) and the mixture stirred overnight at room temperature. Dilution with water (150 ml) and extraction with ether (3 × 50 ml) gave the crude lactone (8), which was purified on a column of silica gel eluted with cyclohexane–ethyl acetate (3 : 1) to give phytuberin lactone (69 mg).

Deacetyl-2-hydroxydihydrophytuberin (7).—A solution of lithium aluminium hydride (20 mg) in anhydrous ether (10 ml) was added to a solution of phytuberin lactone (35 mg) in ether and the mixture was heated (1 h) under reflux and then stirred (12 h) at room temperature. The mixture was then diluted with water (20 ml) and after separation of the layers the aqueous layer was repeatedly extracted (6 × 20 ml) with ether. The combined ethereal extracts yielded a gummy product (24 mg), which was purified by preparative t.l.c. on silica eluted with ethyl acetate. The main product band gave *deacetyl-2-hydroxydihydrophytuberin* (7) as a gum (18 mg), *m/e* 270 ($M^{+\cdot}$ v. weak) and 252 (M − 18, loss of water) [Found: $(M - 18)^+$ 252.170 7. $C_{15}H_{24}O_3$ requires (M − 18) 252.172 5], $\nu_{max.}$ (film) 3 400, 1 128, and 1 023 cm^{-1}; for n.m.r. spectrum see Table 1; t.l.c. (single spot) R_F 0.07 (cyclohexane–ethyl acetate, 1 : 1) or 0.26 (ethyl acetate); g.l.c. R_t 0.94.

Formation of the Triol (10).—A solution of the lactol (7) (16 mg) in anhydrous tetrahydrofuran (distilled from calcium hydride) was added dropwise to a solution of lithium aluminium hydride (20 mg) in tetrahydrofuran (10 ml) heated under reflux. After the addition more lithium aluminium hydride (20 mg) was added and heating was continued (4 h). The excess of lithium aluminium hydride was destroyed by addition of water and 10N-sodium hydroxide and the solution was then extracted with ether (6 × 20 ml). The extract yielded a gum which was purified by column chromatography (silica). Elution with ethyl acetate–acetone (9 : 1) gave phytuberin triol (10 mg), m.p. 85—86°, *m/e* 272 ($M^{+\cdot}$ absent) and 254 (M − 18, loss of water), $[\alpha]_D$ +16.2° (*c* 2.47); $\nu_{max.}$ (film) 3 360, 1 121, 1 086, and 1 051 cm^{-1}; for n.m.r. spectrum see Table 1; t.l.c. (single spot) R_F 0.03 (cyclohexane–ethyl acetate, 1 : 1), or 0.19 (ethyl acetate); g.l.c. R_t 2.24.

Microanalyses and 100 MHz ^1H and 25.2 MHz ^{13}C n.m.r. spectra were kindly determined by A. W. R. Saunders and P. Haylett of the University of East Anglia. Mass spectrometric data were provided by the Mass Spectrometry and Computer Groups, Food Research Institute.

The inoculum of *E. carotovora* var. *atroseptica* was provided by Mrs. C. E. Bayliss of the Institute.

We thank Dr. F. M. Sullivan, Department of Pharmacology, Guy's Hospital Medical School, London for the results of toxicity tests in mice and Professor T. Masamune, Hokkaido University, Sapporo, Japan, for a sample of rishitin.

[6/1008 *Received*, 26*th May*, 1976]