Biosynthesis of PR Toxin by *Penicillium roqueforti*

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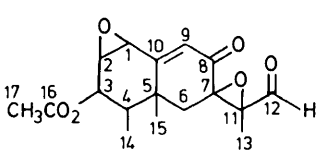
The biosynthesis of PR toxin, a metabolite produced by *Penicillium roqueforti*, has been studied utilizing both ^{14}C- and ^{13}C-labelled precursors. The results indicate that PR toxin is formed *via* an isoprene biosynthetic pathway.

PR TOXIN (1) is the major toxin produced by strains of *Penicillium roqueforti*, a fungus isolated from mouldy grains and silage.[1] The fungus was toxogenic when grown on corn or a yeast extract–sucrose medium and extracts of the pure cultures were lethal to rats by either intraperitoneal or oral administration.[2] The pure



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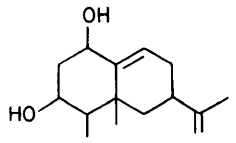

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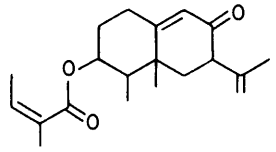

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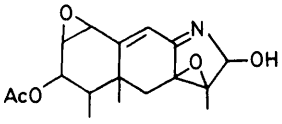
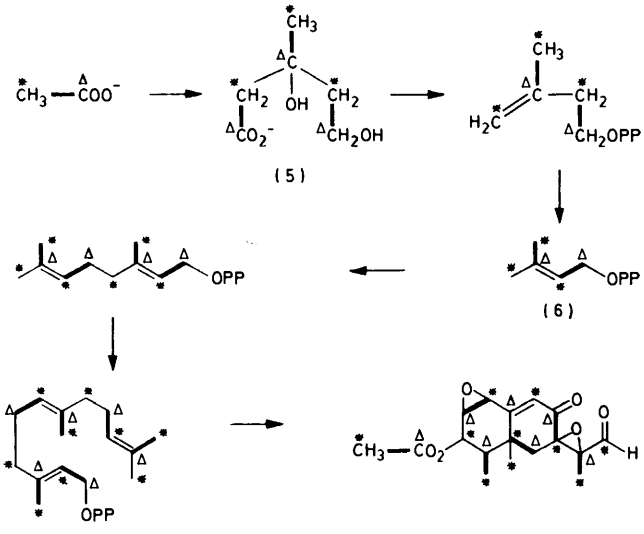


(4)

toxin was found to inhibit RNA and protein syntheses in eucaryotes.[3] The structure of PR toxin was determined by chemical and spectral techniques [4] and the absolute configuration assigned by *X*-ray anomalous dispersion.[5]

The biosynthetic origins of the molecule are indicated by the structural similarity with the sesquiterpenes capsidiol (2) and petasin (3), both of which have been the subject of biogenetic investigations.[6-9] By analogy, PR toxin should arise from acetate *via* an isoprenoid biosynthetic pathway, comprising mevalonate (5), dimethylallyl pyrophosphate (6), and farnesyl pyrophosphate (7), with concomitant distribution of the label as shown in the Scheme. Subsequent cyclization yielded initially a germacrene intermediate and a second cyclization proceeded *via* an eudesmane carbocation.[8] The C(15) methyl group originated by migration from C(10) to C(5) [6] and the process was accompanied by a hydride shift from C(5) to C(4).[8] We are investigating the biosynthesis of PR toxin and present here our initial results which detail the incorporation of some labelled precursors into the metabolite.

Four strains of *P. roqueforti* were examined for PR toxin production, ATCC 6987, 6989, 9295, and 10110; the results are in agreement with those reported previously.[10] All four strains formed PR toxin although the yields and the period of maximum production were different. In all cases a decrease in the amount of PR toxin after 15 d was associated with an increase in the pH of the medium. A detailed description of this investigation and the development of a high-performance liquid-chromatographic analysis for PR toxin will be presented elsewhere.[11] Based on these results strain ATCC 9295 was selected for the biosynthetic investigations; the cultures were grown on a yeast extract–sucrose medium and a labelled precursor was added periodically from day



(5) (6) (7)

SCHEME Possible biosynthetic route to PR toxin (OPP = pyrophosphate)

5 to day 11. The cultures were harvested on day 13 and the PR toxin was extracted and purified.

Preliminary feeding experiments with sodium [1-^{14}C]-acetate (Table 1) established the feasibility of ^{13}C studies, although the dilution value obtained (115.3) * indicated that acetate is not an efficient probe.

The complete assignment of the natural abundance ^{13}C n.m.r. spectrum of PR toxin was achieved by consideration of the characteristic chemical shifts,[12] and by

* The dilution value obtained from the ^{14}C-precursor experiments is defined by equation (1) where m and n are the appropriate number of labelled sites.

$$D = [\text{specific activity (precursor)}]/[\text{specific activity (product)}] \times [m(\text{product})]/[n(\text{precursor})] \quad (1)$$

TABLE 1

Specific radioactivities and dilution factors of PR toxin derived from ^{14}C-labelled precursors

^{14}C-precursor	Labelled precursor	Carrier (mg) [a]	Specific activity of precursor (μCi mmol)$^{-1}$	Yield of PR toxin (mg) [a]	Incorporation (%)	Specific activity of PR toxin (μCi mmol^{-1})	Dilution factor
Sodium [1-^{14}C]acetate	250	246	83.33	166.1	1.05	5.06	11.3
D,L-Sodium [2-^{14}C]mevalonate	50	139.6	46.56	148.1	5.02 [b]	2.71	51.6

[a] Values relative to 0.51 of culture medium. [b] Calculated for incorporation of L-mevalonate.

examination of the single-frequency off-resonance decoupled (SFORD) spectrum and the coupling patterns which arose in the ^{1}H-coupled and selectively ^{1}H-decoupled spectra. The assigned chemical shifts, with multiplicities in the ^{1}H-coupled spectrum, and $^1J_{CH}$ and some apparent long-range J_{CH} values are given in Table 2. Two previous assignments of the ^{13}C chemical shifts of PR toxin have been published.[4,5] These differ with respect to the resonances assigned to C(13) and C(15) and the earlier assignment does not differentiate between C(1) and C(2).[4] Our assignments for these ambiguous carbon atoms, based on long-range coupling patterns and selective decouplings, are in complete accord with those of Moreau *et al.*[5] obtained for C(13) and C(15) from labelling experiments. Selective irradiation at the position of the H(1) doublet (δ 3.65) [4] with a power (γH_2) of 500 Hz collapsed the resonance at δ 55.9 to a singlet and the resonance at δ 55.6 to a doublet with a residual coupling (J_R) of 13 Hz. This J_R value is close to the value calculated from the equation of Anderson and Freeman [13] which relates J_R to $^1J_{CH}$ (185 Hz), γH_2 (500 Hz), and the decoupler offset from resonance (26 Hz). In the ^{1}H-coupled spectrum of PR toxin the fine structure of the methyl quartet resonance of C(15) should be complex because of the three protons available for three-bond coupling, whereas the C(13) resonance can, at most, show only a doublet fine-structure from coupling with the aldehyde proton, which allows these resonances to be distinguished.

The coincident resonances of C(7) and C(11) were not separated by the addition of the Yb(fod)$_3$ shift-reagent (fod = 1,1,1,2,2,3,3-heptafluoro-7,7-dimethyloctane-4,6-dionate) to a 0.19 molar solution of PR toxin, even at a level of 0.12 mmol ml^{-1}. The relative isotropic shifts measured for one addition are given in Table 2 and although interpretation is complicated by complexation to different degrees at the various oxygen atoms the shifts corroborate the assignments.

In separate feeding experiments, cultures of *P. roqueforti* were pulsed every 24 h with [1-^{13}C]- and [2-^{13}C]-acetate. The proton-noise-decoupled (p.n.d.) ^{13}C n.m.r. spectrum of [1-^{13}C]acetate-derived PR toxin (Figure 1) showed six enhanced signals due to C(2), C(4), C(6), C(8), C(10), and C(16). In addition, the coincident resonances of C(7) and C(11) showed an overall enrichment; a subsequent feeding experiment with

TABLE 2

^{13}C N.m.r. data of PR toxin

Carbon atom	δ [a] PR toxin	Multiplicities [b] and J_{CH}	Relative shifts induced by Yb(fod)$_3$ [c]	Enrichment factors [d] [1-^{13}C]acetate	Enrichment factors [d] [2-^{13}C]acetate	$^1J_{CC}$
1	55.9	D(182)d(10)	14	0.88	1.25	*f*
2	55.5	D(185)s	22	3.37	1.43	*f*
3	70.0	D(148)t(5)	37	1.03	2.31	(38.6) [g]
4	42.8	D(120)m	16	2.50	0.94	35.7
5	38.1	Sm	12	0.84	1.50	34.2, (33.2) [j]
6	41.6	T(125)m	8	3.23	1.12	34.6 [h]
7	67.4	S	10	1.67, [e] (0.48) [i]	1.87 [e] (1.24) [i]	51.6
8	191.7	Sd(5)	6	1.86	0.82	51.4
9	129.9	D(165)d(4)	9	0.98	1.63	(64.1) [g]
10	164.7	Sm	9	1.85	1.00	
11	67.4	S	10	1.67 [e] (1.61) [i]	1.87 [e] (0.94) [i]	46.0
12	198.5	D(192)d(2)	18	1.00	2.24	(51.1) [g]
13	13.6	Q(129)s	11	0.96	2.13	45.2
14	10.1	Q(131)d(8)d(2)	12	1.08	2.53	35.7
15	21.9	Q(130)m	7	0.98	1.47	(33.2) [j]
16	170.6	Sm	100	2.40	0.79	58.6
17	20.6	Q(133)s	50	0.84	1.39	58.8

[a] Relative to internal Me_4Si. [b] S,s = singlet; D,d = doublet; T,t = triplet; Q = quartet; m = multiplet. Capital letters refer to directly bonded CH coupling, small letters to long-range CH coupling. Coupling constants are bracketed at the appropriate place. [c] Yb(fod)$_3$ (60 mg) was added to 60 mg ml^{-1} of a PR toxin sample. [d] Ratios between peak heights of the observed resonances of ^{13}C-enriched and natural-abundance PR toxin recorded under identical conditions. [e] Overall enrichment of degenerate signals. [f] These carbon atoms form an AB spin system for which the coupling constants cannot be observed or calculated (see the text). [g] Anomalous (C,C) coupling constants. [h] Calculated from equation (2). [i] Enrichment factors obtained from PR toxin imine (7). [j] Coupling constants obtained from the [2-^{13}C]acetate feeding experiment.

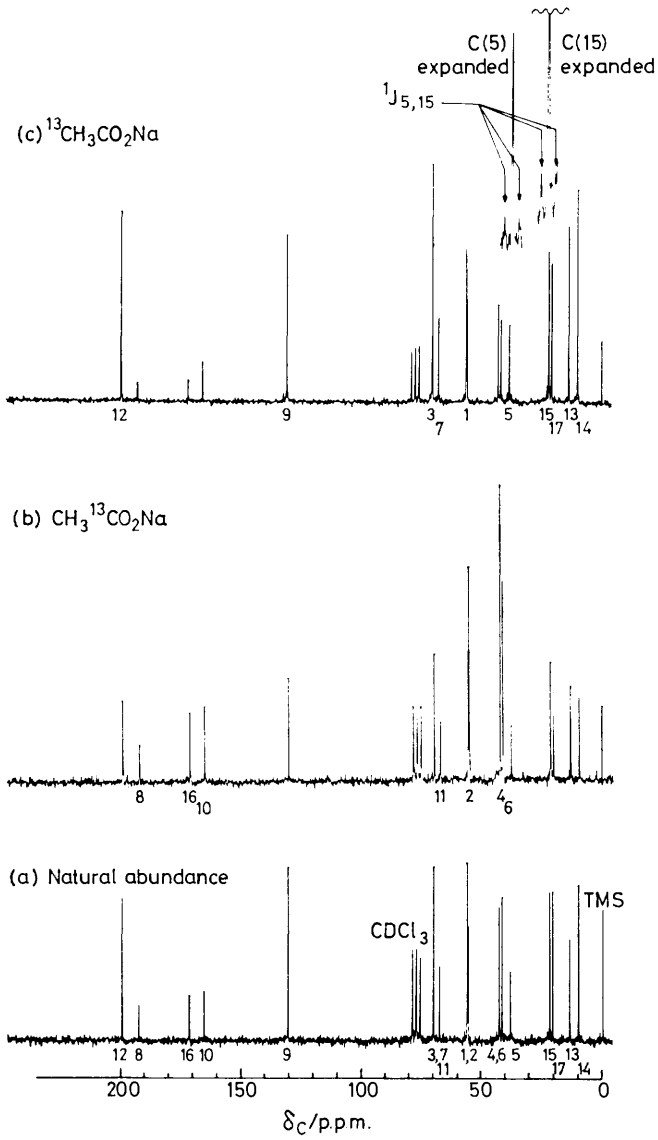


FIGURE 1 P.n.d. ^{13}C n.m.r. spectra (20 MHz) of PR toxin (1); (a) natural abundance, (b) derived from [1-^{13}C]acetate, (c) derived from [2-^{13}C]acetate

[1,2-$^{13}C_2$]acetate (see below) proved that this overall enrichment arose from the label at C(11). The carbons C(7) and C(8) originated from an intact acetate unit, and as C(8) is labelled by [1-^{13}C]acetate, C(7) must therefore come from C(2) of the acetate and C(11) from C(1) of the acetate. This was confirmed by treatment of the PR toxin, produced from [1-^{13}C]- and [2-^{13}C]-acetate feeding experiments, with ammonia to yield the imine (4).[2] The resonances assigned to C(7) and C(11) were then separated and appear at δ 67.8 and 72.1, respectively,[5] and their individual enrichments were calculated (Table 2). The p.n.d. ^{13}C n.m.r. spectrum of [2-^{13}C]-acetate-derived PR toxin (Figure 1) showed nine enhanced signals, *viz.* C(1), C(3), C(5), C(9), C(12), C(13), C(14), C(15), and C(17). In addition, the coincident resonances of C(7) and C(11) showed an overall enrichment due to an enhanced C(7) signal. The anomalous spread in the value of the individual enrichment factors is a common problem in biosynthetic work [14-16] and apparently has no significant biosynthetic implication. The results obtained are consistent with the acetate–mevalonate origin of PR toxin detailed in the Scheme. The apparent enrichment of labelled sites is lower with [2-^{13}C]acetate than with [1-^{13}C]acetate, and C(2) shows an unexpectedly high enrichment factor in the [2-^{13}C]acetate feeding experiment. Such anomalies have, however, been encountered in other biosynthetic investigations and it has been proposed that randomization of the [2-^{13}C]acetate label is possible *via* operation of the Krebs Cycle.[17] In the spectrum of [2-^{13}C]acetate-derived PR toxin a one bond carbon–carbon coupling of J 33.2 Hz is observed between C(5) and C(15), which confirms that C(15) arose from C(2) of the acetate and supports the postulated methyl migration from C(10) to C(5) (Figure 1).

To confirm that an isoprene biosynthetic pathway does, indeed, operate the incorporation of [1,2-$^{13}C_2$]-acetate into PR toxin was studied. In this experiment the precursor was added with a peristaltic pump, coupled to a timer, which delivered 7.3×10^{-3} mmol of 90%-enriched precursor to each growing culture every hour from day 5 to day 11. In this way it was hoped to obviate the high dilutions encountered in the previous feeding experiments. The results obtained from the ^{13}C n.m.r. spectrum of PR toxin derived from [1,2-$^{13}C_2$]acetate (Figure 2) are detailed in Table 2.

The chemical shifts of C(1) and C(2) are separated only marginally (δ 55.9 and 55.5) and the resultant AB spectrum is observed as a single line at δ 55.7 which is not resolved into its two components and cannot, therefore, yield a value for J(AB), but does indicate the presence of an intact acetate unit. Similarly, the resonances of C(5) and C(6) are close enough to be described as an AB system, which in this case gives rise to two strong inner lines with the outer line of C(6) obscured by the C(4) resonance and the outer line of C(5) too weak for precise measurement. The properties of an AB system [18] dictate the equality of the separations of each inner line from each chemical-shift position; the latter can be taken as the chemical shifts of the natural-abundance signals and the observed values [C(5) J 13.3 and C(6) 13.1 Hz] are in adequate agreement. A value for ^1J was calculated from equation (2) where $\Delta\nu$(AB) is the chemi-

$$J(\mathrm{AB}) = \{[\Delta\nu(\mathrm{AB})]^2 - x^2\}/2x \qquad (2)$$

cal-shift difference (Hz) and x the separation (Hz) of the inner lines, and the value obtained (34.6 Hz) is close to that measured from the weak outer C(5) line. The outcome of the analysis of the spectrum of the [1,2-$^{13}C_2$]-acetate-derived material is that C(1)–C(2), C(4)–C(14), C(5)–C(6), C(7)–C(8), C(11)–C(13), and C(16)–C(17) are derived from intact units.

This arrangement of intact acetate units is in accordance with that predicted from an isoprenoid biosynthetic pathway as outlined in the Scheme. A feeding experiment with sodium [2-^{14}C]mevalonic acid was used as an additional probe for this pathway and the more effective

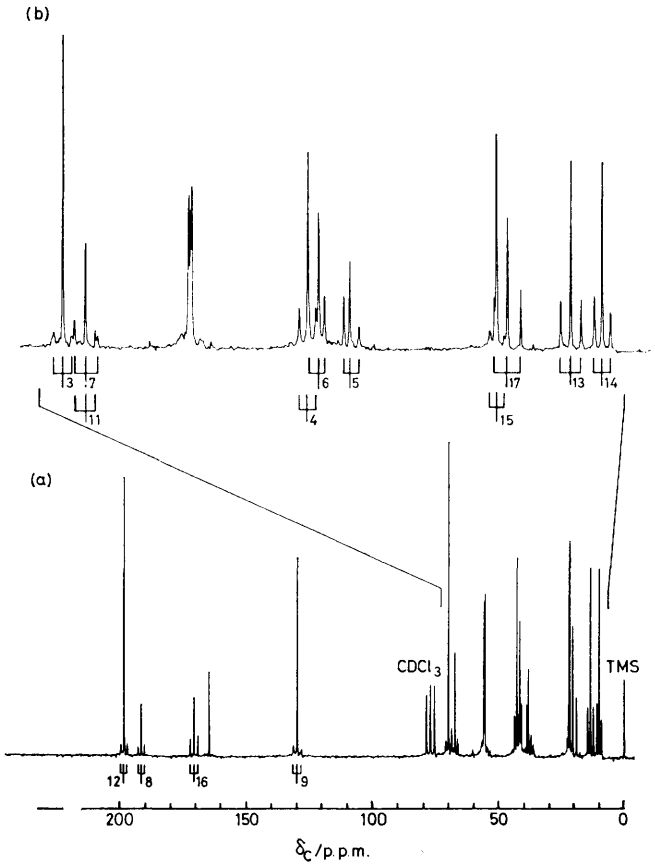


FIGURE 2 P.n.d. ^{13}C n.m.r. spectra (20 MHz) of PR toxin (1) derived from [1,2-$^{13}C_2$]acetate; (a) spectral width 5 000 Hz, (b) spectral width 1 400 Hz

incorporation of mevalonic acid into PR toxin (Table 1) confirmed that PR toxin arose from acetate *via* mevalonate, presumably through a farnesyl pyrophosphate intermediate. The pattern observed is the same as that determined by ^{13}C n.m.r. for rishitin [19] and, by assumption, the same as that of germacrene C.[20] The complex nature of the hydrogen migrations necessary to produce the metabolite cannot be deduced from these initial experiments; studies are underway to establish these migrations. The mode of folding of the farnesyl pyrophosphate is as shown in the Scheme. The carbon atom C(15) arose *via* migration of a carbon atom from C(10) to C(5). Consequently, an intact acetate unit was cleaved in this migration and a two-bond coupling was generated between C(10) and C(15), but this coupling could not be detected, presumably because the value $^2J_{CC}$ was close to zero.

In addition to the couplings arising from intact acetate units, three carbon atoms, C(3), C(9), and C(12) which are not part of an intact unit, also showed evidence of one-bond coupling. This phenomenon has been encountered previously in biosynthetic studies and two explanations have been proferred to account for the anomalous labels. Simpson *et al.*[21] have proposed that the additional coupling arises from multiple labels with consequent observation of inter-acetate and intra-mevalonate couplings. The multiple labels of the metabolite in question, wortmannin, are considered to arise by production, over a limited period, mainly from exogenous labelled acetate. The low, overall enrichments encountered are then explained by subsequent dilution with wortmannin produced from unlabelled endogenous material.[21] Alternatively, Baker and Brooks [7] have interpreted this anomalous coupling in terms of an 'abnormal' isopentenyl pyrophosphate unit, which arises from a *cis*-isopentenyl pyrophosphate isomerase, as postulated by Holloway and Popjak.[22] Studies are in progress to resolve this conundrum.

EXPERIMENTAL

Mass spectra were recorded on a Varian spectrometer and ^{13}C n.m.r. spectra on a Varian CFT-20 spectrometer. Radioactivity was measured on a Packard 3003 liquid-scintillation spectrometer and samples were calibrated by internal standardization with [1-^{14}C]n-hexadecane. For column chromatography Merck silica, particle size 0.063—0.200 mm, was used.

Incorporations of Sodium [1-^{14}C]-, [1-^{13}C]-, *and* [2-^{13}C]-*Acetate.*—Preliminary experiments on cultures of *P. roqueforti* (ATCC 9295) grown in static culture in the dark on a medium which consisted of yeast extract (20 g l^{-1}) and sucrose (150 g l^{-1}) showed that production of PR toxin commenced on day five and reached a maximum on the thirteenth day after inoculation.[11]

Typical procedure. To five 5-day old cultures of *P. roqueforti* on the above medium (100 ml) was added a solution (3 ml) of [1-^{13}C]acetate (600 mg, 90% enriched) in water (105 ml) every 24 h from day five to day eleven. After a further 48 h the cultures were filtered off and the filtrate was extracted with chloroform (2 × 100 ml). The organic phase was dried (Na_2SO_4) and the PR toxin was purified by chromatography on silica gel (30 g) [ethyl acetate–hexane (1 : 1 v/v) as eluant]. On removal of the solvent from the fractions which contained the toxin, PR toxin was left as an oil which crystallized with time (63 mg), m.p. 155—156 °C (lit.,[2] 155—157 °C).

In separate experiments, the following precursors were added as described above: [2-^{13}C]acetate (600 mg, 90% enriched) to give PR toxin (103 mg); [1-^{14}C]acetate (250 μCi) and sodium acetate (246 mg) to give PR toxin (166.1 mg) (specific activity 5.1 μCi $mmol^{-1}$, absolute incorporation 1.05%); [2-^{14}C]mevalonic acid lactone (50 μCi) and mevalonic acid lactone (139.6 mg) hydrolysed with sodium hydroxide (87 mg) to give PR toxin (148.1 mg) (specific activity 2.7 μCi $mmol^{-1}$, absolute incorporation 5.02%).

Incorporation of Sodium [1,2-$^{13}C_2$]*Acetate.*—A Watson–Marlow peristaltic pump was used to add the [1,2-$^{13}C_2$]acetate solution (0.1375 ml, 0.049M) to ten samples of *P. roqueforti*, grown as above, for 3 min every hour from day five to day eleven. After work-up, PR toxin (202 mg) was obtained.

Preparation of PR Toxin Imine (7).—To a solution of PR toxin (50 mg) in methanol (2 ml) and water (0.35 ml) was added ammonia solution (0.15 ml). After 15 min at 20 °C the solvent was removed in a stream of nitrogen and the residue was purified by chromatography on silica gel [chloroform–methanol (96 : 4 v/v) as eluant], to yield PR toxin imine (7) (36 mg) with properties identical with those reported.[2]

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