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THE STRUCTURE OF PR TOXIN, A MYCOTOXIN FROM *PENICILLIUM ROQUEFORTI*

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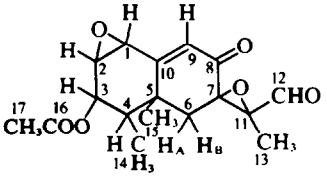
Abstract—An earlier report from our laboratories[1] described the isolation, partial characterization, and biological activity of a new mycotoxin from cultures of *Penicillium roqueforti*, and the name "PR toxin" was tentatively assigned to the compound. On the basis of further chemical and spectral evidence we propose that PR toxin has the structure shown in formula **1**.†

PR toxin was purified as a colorless, crystalline substance, m.p. 155–157°, $C_{17}H_{20}O_6$. In the earlier report[1] several functionalities of the toxin were identified: (1) The IR (1735 cm^{-1}) and PMR (singlet at δ 2·16) spectra, plus base hydrolysis of PR toxin (**1**) to alcohol **2** (Fig 1) established an acetoxy function. Acetylation of alcohol **2** regenerated **1**. (2) An aldehyde attached to a tertiary carbon was characterized by absorption at 1720 cm^{-1} and by a one-proton singlet at δ 9·75 in the PMR spectrum. (3) UV absorption at 249 nm, a band at 1680 cm^{-1} in the IR and a one-proton singlet at δ 6·43 gave evidence for an α,β-unsaturated ketone. Reduction ($NaBH_4$) of **1** to a diol **4**, (MW 324) which lacked the UV absorption and the ketone and aldehyde IR bands of **1**, but exhibited a doublet at δ 4·37 (J = 3·5 Hz) due to a secondary alcohol (–CHOH), a doublet at δ 6·0 (J = 3·5 Hz) for the olefinic proton, and two doublets, one at δ 3·83 (J = 12 Hz) and the other at δ 4·07 (J = 12 Hz) due to the two primary alcohol protons ($-CH_2OH$), substantiated this interpretation.



Formula I. PR toxin, $C_{17}H_{20}O_6$.

†The chemical name, 7 - acetoxy - 5,6 - epoxy - 3,5,6,7,8,8a - hexahydro - 3',8,8a - trimethyl - 3 - oxospiro[naphthalene - 2(1H),2' - oxirane] - 3' - carboxaldehyde is one of four suggested by the Chemical Abstracts Service for a substance of this structure. However, inasmuch as the basic ring structure is identical with that of eremophilane and its derivatives[2] it appears preferable to name the toxin as a derivative of one of these sesquiterpenes. Since the stereochemistry of the toxin is not yet established the name "PR toxin" is being retained for the present.

The two previously unassigned O atoms in PR toxin have now been demonstrated to be in epoxide form by the following results: (a) the IR spectrum of **1** which showed no band in the region of 3,400 cm^{-1} indicated the absence of free OH groups; (b) since compound **5** (Fig 1), derived from **1** by sodium borohydride reduction followed by saponification, contained no CO groups (IR spectrum), no lactone structure was present; (c) **1** gave a positive reaction in the thiosulfate test of Ross[3] for epoxides; (d) reaction of **1** with $NaBH_4$ led to a diol **4** (MW 324, MW of acetate 408), but further treatment of this tetrahydro derivative (**4**) with LAH gave a mixture of isomeric pentahydroxy compounds (**6**) of molecular weight 286. Pentaol **6** formed a pentatrimethylsilyl ether derivative (MW 646) and a tetraacetate (MW 454). These results establish the presence of two epoxide functions in toxin, of which one gives rise to a tertiary OH group by LAH reduction. Together with the observation of two oxirane protons in the PMR spectrum, which are coupled to each other and are part of the same oxirane system (see below), the data lead to the postulate of a disubstituted and a tetrasubstituted epoxide.

In the PMR spectrum (Fig 2) of PR toxin, 14 of the 20 protons can be assigned in a straighforward manner: one secondary Me (Cl4, δ 1·03, d, J = 6·7 Hz), two tertiary Me (Cl5, δ 1·45, showing fine splitting with J = 0·8 Hz, and Cl3, δ 1·49, s), one acetoxy Me (Cl7, δ 2·16, s) one isolated ethylenic proton (H9, δ 6·43, s), and one isolated aldehyde proton (Hl2, δ 9·75, s). Decoupling experiments and PMR data of chemical transformation products led to the following assignment of the six remaining protons.

A one-proton doublet of doublets (appearing as a triplet, since both coupling constants, J = 5 Hz) at δ 5·16 must be assigned to H3, since this signal is shifted upfield to δ 4·1 in the spectrum of saponification product **2**, and is not observed in the spectrum of ketone **3**. Proton H3 is coupled (J = 5Hz) to a proton centered at δ 1·79 (H4) which in turn is coupled (J = 6·7 Hz) to the protons of a Me group (Cl4, δ 1·03). H4 thus gives rise to a complex octet pattern (top scan, Fig 2), partly obscured also by the resonances of a

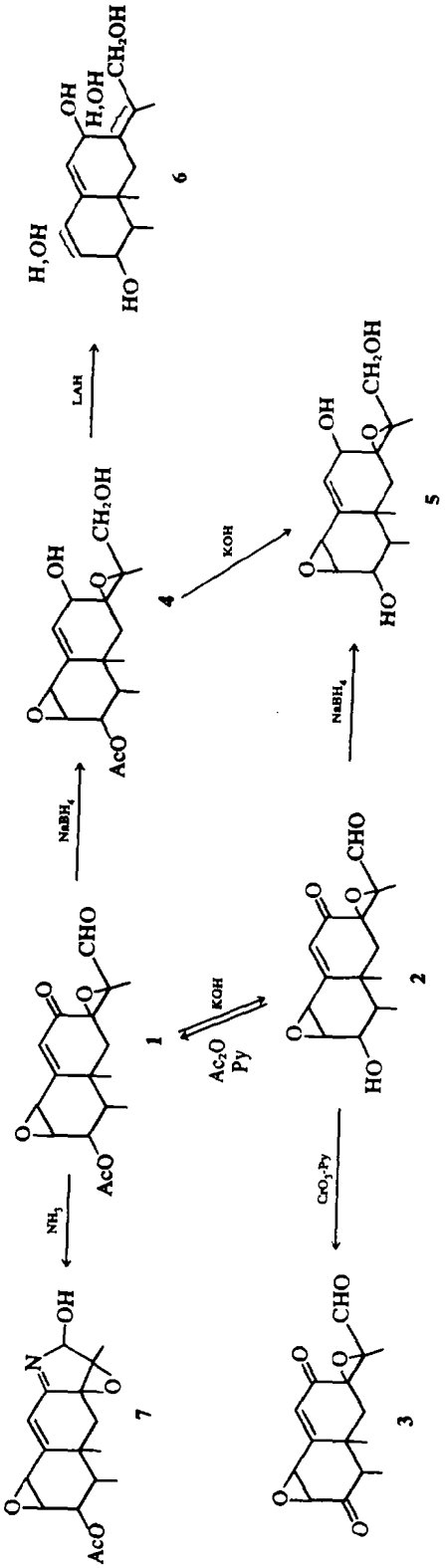


Fig 1. Chemical transformations and derivatives of PR toxin(1).

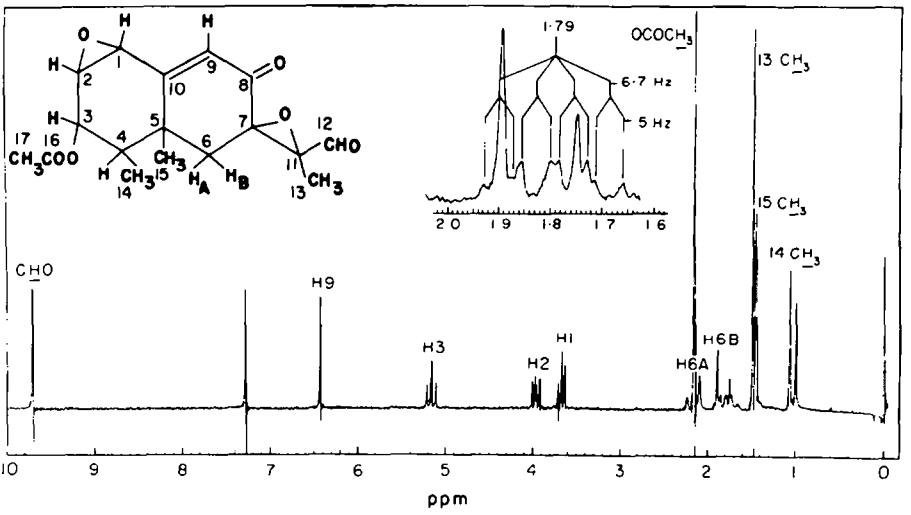
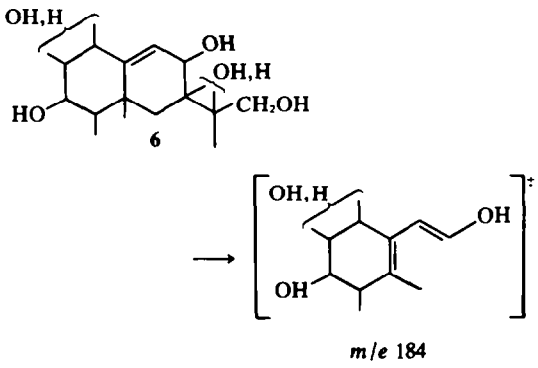


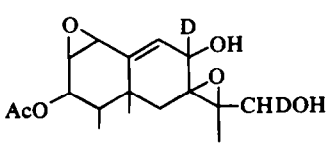
Fig 2. The 100 MHz PMR spectrum of PR toxin (1) in $CDCl_3$ with tetramethylsilane as internal standard. Peak at δ 7·28 due to $CHCl_3$. Expanded scale of signals between 1·6 and 2·0 ppm is shown in the top scan.

methylene group (C6). H3 is also coupled (J = 5 Hz) to one of the oxirane protons (H2, δ 3·96) which appears as a doublet of doublets due to additional coupling (J = 3·5 Hz) with the other oxirane proton (H1, δ 3·65, J = 3·5 Hz). Decoupling experiments fully support these assignments. Thus irradiation of H4 (δ 1·79) collapses the signal for H3 into a doublet (J = 5 Hz) and the Me signal at δ 1·03 (Cl4) appears as a broad singlet. Irradiation of H2 likewise produces a doublet (J = 5 Hz) for H3, but leaves the H4 and Cl4 resonances unchanged. Irradiation of H3 leads to a quartet for H4 (J = 6·7 Hz) and a doublet (J = 3·5 Hz) for H2. In the spectra of the borohydride product **4** and the imine **7** the resonance signals for the H1–H4 and Cl4 systems remain unchanged, and differences noted in the case of alcohol **2** involve only the expected upfield shift of H3, the disappearance of the acetate signal (Cl7) and an additional resonance at δ 1·8 due to the OH proton (exchangeable with D_2O). The spectrum of ketone **3** exhibits no signal for H3 but shows a doublet (δ 3·96, J = 3·5 Hz) for H2 and a quartet (δ 1·79, J = 6·7 Hz) for H4. These results establish the substitution pattern of carbons 1–4 as shown in structure **1**. From the fact that H1 is coupled only to H2, and the change of the H4 resonance from an octet in the case of **1** to a quartet in the case of **3**, the quaternary nature of both C10 and C5 also follows. Two one-proton doublets at δ 1·81 and 2·16, are interpreted as an AB-system (J = 14 Hz) due to an isolated ring methylene group ($H6_B$ and $H6_A$, respectively). The Cl5-methyl group (δ 1·45) is assigned to a carbon adjacent to this methylene to account for the observed coupling (J = 0·8 Hz) between that methyl substituent and proton $H6_A$. Finally the singlet resonance for the olefinic proton (H9, δ 6·43) which in the spectrum of the borohydride reduction product **4** has changed to a doublet (δ 6·00, J = 3·5 Hz) requires a β-disubstituted, α,β-unsaturated ketone, and from the observation that the proton at C8 of diol **4** (δ 4·37, J = 3·5 Hz) appears coupled only to H9, a quaternary center at C7 can be inferred.

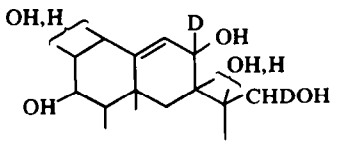
The nature of ring B in PR toxin, in particular the relationship between the aldehydic function and the second (tetrasubstituted) oxirane system, is further defined by the mass spectra of PR toxin and various hydride reduction products (Fig 3). The spectrum of alcohol **6**, for example, exhibited an ion at *m/e* 184 (base peak) which can be rationalized as the product of a retro-Diels–Adler reaction:



This interpretation is supported by the spectrum of **6**-tetraacetate (MW 454) in which this peak is shifted to *m/e* 310 (184 + 3 Ac) and the spectrum of the penta-TMS derivative of **6** (MW 646) which exhibits an intense peak at *m/e* 400 (184 + 3 TMS). Furthermore, reduction of PR toxin with $NaBD_4$ to the dideuterio-diol **4a** (MW 326, showing a prominent peak due to M–CHDOH at *m/e* 294), and subsequent treatment with LAH gave dideuteriopentaol **6a** (MW 288), with an ion of *m/e* 185 as the base peak of its mass spectrum.



4a



6a

The elimination of a $C_5H_{10}O_2$ fragment in the spectrum of **6** (and $C_5H_9DO_2$ in the case of **6a**), therefore, includes the tetrasubstituted epoxide and aldehyde functions of PR toxin, a result which, combined with the PMR data, fixes the structural relationship of all B-ring functionalities.

Formation of Schiff base **7** upon treatment of PR toxin with methanolic ammonium hydroxide establishes the orientation of the aldehyde group. The ammonia adduct (MW = 319) contains a chromophore (247 nm), and the original acetate ester (1739 cm^{-1}), but the IR absorptions due to the conjugated ketone and saturated aldehyde of PR toxin (1680, 1720 cm^{-1}) are replaced by a new, fairly strong band at 1631 cm^{-1} (conjugated imine). Instead of the aldehydic proton, a sharp one-proton singlet at δ 5·40 and a

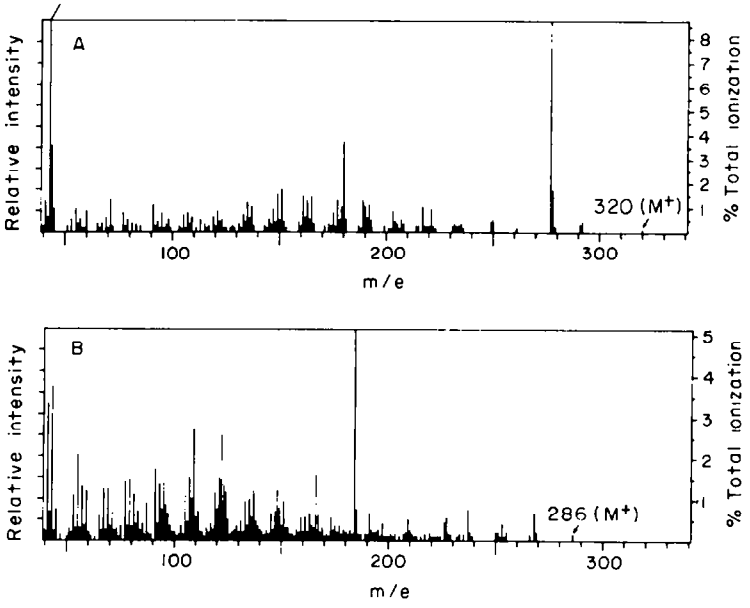


Fig 3. Mass spectra. A. PR toxin (**1**), B. Octahydro-PR toxin (**6**).

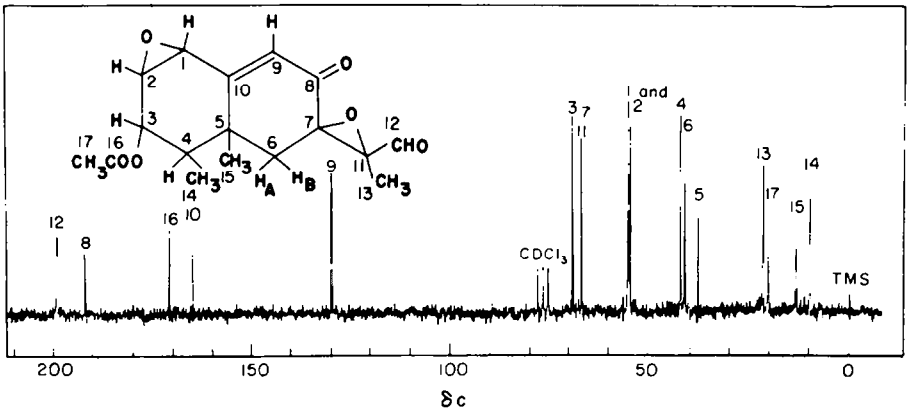
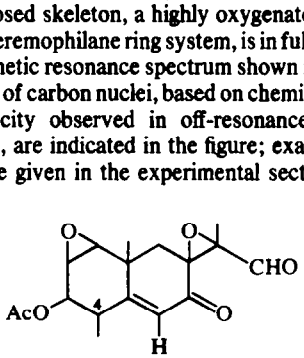


Fig 4. The 22·63 MHz ^{13}CNMR spectrum of PR toxin (**1**) in $CDCl_3$.

broad signal at δ 4·93 (exchangeable with D_2O) was observed in the PMR spectrum From these data, structure **7** can be derived for this product and from this assignment the apposition of ketone and aldehydic functions as given in structure **1** then necessarily follows.

The proposed skeleton, a highly oxygenated derivative of the basic eremophilane ring system, is in full accord with the ^{13}C-magnetic resonance spectrum shown in Fig. 4. Our assignments of carbon nuclei, based on chemical shifts and the multiplicity observed in off-resonance decoupling experiments, are indicated in the figure; exact resonance positions are given in the experimental section.



8

All evidence presented thus far, however, is equally consistent with an alternative—though biogenetically far less likely—carbon skeleton, structure **8**. Our decision in favor of structure **1** for PR toxin is based on the following observations: (a) elimination of acetic acid from **8** would be expected to be facile and lead to an extended conjugated system, but all attempts to detect the expected dienone upon base treatment of PR toxin under various conditions were unsuccessful; (b) for structure **8** a nuclear Overhauser interaction between the secondary Me group at C4 (see **8**) and the olefinic proton could be expected, but no such effect was observed, in contrast to (c) the pronounced interaction of the hydrogen at C–1 and the olefinic proton (structure **1**). Irradiation of Hl (Fig 2) produced a 25% enhancement of the signal due to the olefinic proton (H9), and irradiation of the olefinic proton produced a 16% enhancement of the Hl doublet. The nuclear Overhauser effects are fully in accord with the spatial relationship between these hydrogens as defined by structure **1**, but clearly inconsistent with that required by structure **8**. The smaller enhancement of the Hl proton resonance is a consequence of the larger number of relaxation pathways available to it.

EXPERIMENTAL

Microanalyses were by Micro-Tech Laboratories, Inc. Skokie Illinois. Mass spectra were determined on an Associated Electrical Industries, Ltd., MS-9 mass spectrometer. Optical rotations were measured on a Perkin–Elmer polarimeter, Model 141, in a one decimeter micro cell. UV and IR spectra were determined with Beckman DB and IR-5 spectrophotometers, respectively. PMR spectra were measured on Varian T-60 and XL-100 NMR spectrometers. C-13 NMR spectra and nuclear Overhauser effects were measured on a Bruker Spectrospin, Model HX90E. All NMR spectra were measured in $CDCl_3$.

The NOE experiments were carried out in the pulsed-Fourier transform mode. Sample preparation and basic experimental procedures were as previously reported.[4] Soon to be reported[5] will be a direct comparison between continuous wave NOE experiments, by far the most common method to date, and the pulsed-FT method. It suffices here to say that the two methods give comparable results if the f_1 (observing frequency) pulse width is sufficiently narrow.

Analytical and preparative TLC was done with silica gel G and H (Brinkmann Instruments, Inc.), respectively, and the plates were developed in one of the following solvent systems: A, MeOH:$CHCl_3$ (4:96 v/v); B, MeOH:$CHCl_3$ (10:90 v/v); C, MeOH:$CHCl_3$ (15:85 v/v). Spots were detected under ultraviolet light (Mineralight UVS 11, Ultra-Violet Products, Inc. San Gabriel, Calif.) or by spraying with 50% H_2SO_4 followed by charring at 230°.

PR toxin (**1**). Colorless crystalline PR toxin was isolated from *Penicillium roqueforti* cultures as previously described[1]: m.p. 155–157°, $[\alpha]_D^{25}$ +290° (*c* 1·34 in $CHCl_3$), λ_{max}^{EtOH} 249 nm (ϵ 15,278). Yields were *ca* 0·3 g/l of culture filtrate. Elemental analysis and mass spectral data established the molecular formula $C_{17}H_{20}O_6$ (Found: C, 63·11; H, 6·52; 0, 29·20; N, < 0·1; M.W. 320; $C_{17}H_{20}O_6$ requires; C, 63·74; H, 6·29; 0, 29·97%; M.W. 320), ν_{max} 1735, 1720, 1680 cm^{-1}, PMR spectrum (100 MHz): δ 1·03 (3H, d, J ~ 6·7 Hz, 14-CH_3); 1·45 (3H, d, J ~ 0·8 Hz, 15-CH_3); 1·49 (3H, s, 13-CH_3); 1·79 (1H, octet, J ~ 6·7, 5 Hz, *H*4); 1·81 (1H, d, J ~ 14 Hz, $H6_B$); ~2·16 (1H, d, J ~ 14 Hz, $H6_A$; 2·16 (3H, s, OCOCH_3); 3·65 (1H, d, J ~ 3·5 Hz, *H*1); 3·97 (1H, dd, J ~ 3·5, 5 Hz, *H*2); 5·16 (1H, dd, J ~ 5, 5 Hz, *H*3); 6·43 (1H, s, *H*9); 9·75 (1H, s, C*H*O). CMR spectrum (22·63 MHz): δ_C 198·5 (C12, d); 191·6 (C8, s); 170·5 (C16, s); 164·6 (C10, s); 129·9 (C9, d); 69·9 (C3, d); 67·3 (C7 and C11, s); 55·9 (Cl or C2, d); 55·6 (Cl or C2, d); 42·7 (C4, d); 41·5 (C6, t); 38·0 (C5, s); 21·9 (C13, q); 20·7 (C17, q); 13·6 (C15, q); 10·1 (C14, q). Mass spectrum, Fig. 3A.

PR alcohol (**2**). Saponification of PR toxin (50 mg) gave the crystalline alcohol (28 mg) as previously described[1]; m.p. 113·5–115°; M.W. 278; λ_{max}^{EtOH} 247 nm. The IR spectrum lacked the CO band at 1,735 cm^{-1} shown by the parent compound; ν_{max} 3440, 1720, 1675 cm^{-1}. Mass spectrum, *m/e* (rel. int.) 278 (1·2, M^+), 250 (2·4), 235 (100). PMR spectrum (60 MHz): δ 1·09 (3H, d, J ~ 6·7 Hz), 1·45 (3H, s), 1·49 (3H, s); 1·6–2·2 (3H, m); 3·65 (1H, d, J ~ 3·5 Hz); 3·97 (1H, dd, J ~ 3·5, 5 Hz); 4·15 (1H, dd, J ~ 5,5 Hz); 6·43 (1H, s); 9·75 (1H, s).

PR ketone (**3**). The alcohol **2** (73 mg) in dichloromethane (8·7 ml) was mixed with dipyridine chromium oxide (438 mg)[6] at room temp and allowed to stand for 15 min. The mixture was poured through *ca* 4 g of active neutral aluminum oxide (activity I, Brinkmann Instruments, Inc.) on a small Buchner funnel and eluted with acetone. The pale yellow eluate was evaporated *in vacuo* to dryness (52 mg). This residue was applied to 3 preparative TLC plates (20 × 20 cm, 0·5 mm thickness) which were developed in solvent system A. The edges of the plates were charred by H_2SO_4 and heat to locate the bands. The bands at R_f 0·50 were removed, combined, and eluted with MeOH. Evaporation of the solvent gave an oil (15 mg). Mass spectrum, *m/e* (rel. int.) 276 (0·5, M^+), 233 (100). PMR spectrum: δ 1·20 (3H, d, J ~ 6·7 Hz); 1·45 (3H, s); 1·49 (3H, s); 1·79 (1H, q, J ~ 6·7 Hz); 1·81 (1H, d, J ~ 14 Hz); 2·16 (1H, d, J ~ 14 Hz); 3·65 (1H, d, J ~ 3·5 Hz); 3·97 (1H, d, J ~ 3·5 Hz); 6·56 (1H, s); 9·75 (1H, s).

Tetrahydro-PR toxin (**4**). $NaBH_4$ reduction of PR toxin (193 mg) gave the diol **4** (130 mg) as previously described[1]. Attempts to crystallize this compound were unsuccessful. It showed R_f 0·55 in solvent system C, molecular ion peak at *m/e* 324, and no fluorencence or UV absorption in the 247 nm region. It is insoluble in CCl_4; ν_{max} 3405, 1735 cm^{-1}; mass spectrum, *m/e* (rel. int.) 324 (l, M^+), 306 (2), 293 (36), 176 (100). PMR spectrum: δ 1·03 (3H, d, J ~ 6·7 Hz); 1·20 (3H, s); 1·46 (3H, s); 1·6–2·2 (3H, m); 2·16 (3H, s); 2·48 (2H, broad s, exchangeable with D_2O); 3·65 (1H, d, J ~ 3·5 Hz); 3·97 (1H, dd, J ~ 3·5, 5 Hz); 3·8–4·2 (2H, m); 4·37 (1H, d, J ~ 3·5 Hz); 5·16 (1H, dd, J = 5, 5 Hz); 6·03 (1H, d, J = 3·5 Hz).

A small amount of deuterium-labeled compound **4** was similarly prepared with $NaBD_4$ instead of $NaBH_4$. It showed a molecular ion peak at *m/e* 326, and *m/e* 294 (M-CHDOH).

Tetrahydro-PR alcohol (**5**). Compound **4** (16 mg) in MeOH (0·64

ml) was mixed with 0·1 N KOH (0·16 ml). The mixture was kept at 40° for 4 h and was then applied to a 1·8 × 6 cm column packed with 4 g of silica gel and eluted with 20 ml of $CHCl_3$. Most of the unreacted starting material was washed out at this stage. The column was then eluted with 20 ml of MeOH, the eluate evaporated and applied to a preparative TLC plate (20 × 20 cm) developed by solvent system B. The edge of the plate was charred with H_2SO_4 and heat. Two bands, R_f 0·42 and 0·27 were observed. The bands were removed, eluted with MeOH, and evaporated to yield unreacted **4**, (R_f 0·42) 1·1 mg, and compound **5**, (R_f 0·27) 4 mg. Mass spectrum, *m/e* 282 (M^+). The IR spectrum lacked carbonyl bands; ν_{max} 3440 cm^{-1}. Compound **5** was also obtained by $NaBH_4$ reduction of compound **2**.

Octahydro-PR alcohol (**6**). Compound **4** (10 mg) in dry ethyl ether (15 ml) was mixed with LAH (40 mg) and the soln refluxed for 4 h. EtOAc (5 ml) was added and the mixture shaken with saturated aqueous potassium tartrate soln (10 ml). The ether layer was dried with Na_2SO_4 and evaporated to dryness. The product had R_f 0·16 in solvent system B and showed a molecular ion peak at *m/e* 286; mass spectrum, Fig 3B.

Compound **6** was also obtained from the direct reduction of PR toxin by LAH. PR toxin (30 mg) in dry ethyl ether was refluxed with LAH for 36 h. The mixture was worked up as above. The crude product showed four components by TLC (R_f 0·62, 0·46, 0·31 and 0·16) in solvent system B. The mixture was subjected to preparative TLC, the band at R_f 0·16 was removed, eluted with MeOH and evaporated to yield 4 mg of **6**.

PR-imine (**7**). Reaction of PR toxin (50 mg) with methanolic ammonium hydroxide gave the crystalline imino- compound (35 mg) as previously described[1]; m.p. 85–88°; λ_{max}^{EtOH} 247 nm; ν_{max} 3300–3200 (broad), 1739, 1631 cm^{-1}; mass spectrum, *m/e* (rel. int.) 319 (29, M^+), 290 (23), 276 (23), 216 (76), 188 (82), 160 (100). PMR spectrum: δ 0·99 (3H, d, J ~ 6·7 Hz); 1·34 (3H, s); 1·59 (3H, s); 1·6–2·2 (3H, m); 2·16 (3H, s); 3·65 (1H, d, J ~ 3·5 Hz); 3·97 (1H, dd, J ~ 3·5, 5 Hz); 4·93 (1H, broad s, exchangeable with D_2O); 5·16 (1H, dd, J ~ 5, 5 Hz); 5·40 (1H, s); 6·70 (1H, s).

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