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ISOLATION AND STRUCTURE OF A CHLOROSIS-INDUCING TOXIN OF *PSEUDOMONAS PHASEOLICOLA*

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Key Word Index—*Pseudomonas phaseolicola*; halo blight; bean; chlorosis; toxin; phaseolotoxin; sulfate ester; phosphate ester; peptide; N-P compound; carbamyl phosphate; sulphamyl phosphate; phosphosulphate.

Abstract—A toxin, named phaseolotoxin, which causes leaf-chlorosis in bean leaves has been isolated from liquid cultures of *Pseudomonas phaseolicola*, and purified. Its structure is (N^{δ}-phosphosulphamyl)ornithylalanylhomoarginine.

INTRODUCTION

Of the numerous phytopathogenic *Pseudomonas* species, five are documented [1] as causing a chlorotic region within the host plant as part of the overall disease symptom. This chlorosis is thought to be caused by extracellular toxins released by the bacteria as they grow within the plant tissue [2]. I have initiated a study of chlorosis-inducing toxins of *Pseudomonas* species and here report the isolation, purification, and structural elucidation of a toxin obtained from a defined liquid culture medium in which *P. phaseolicola* has grown.

The purification and chemical structure of only one other *Pseudomonas* toxin has been reported, that being tabtoxin produced by *P. tabaci* in the wildfire disease of tobacco [3]. In addition there is good evidence for the existence of a serine analogue of tabtoxin [4]. Tabtoxin has also been isolated from cultures of a *Pseudomonas* species attacking *Phleum pratense* (timothy grass) [4] and is believed to be present in toxin extracts produced by *P. coronafaciens* [5] and *P. garcae* [6]. The toxin produced by *P. phaseolicola*, however, has clearly been established to be different from tabtoxin [5]. Although other workers have described various purification procedures [7,8], they have not reported either the complete purification or the successful characterization of this toxin. Nonetheless, the active principle has been given the name "phaseotoxin". Early conflicting reports [8,9] on the nature of "phaseotoxin" (polysaccharide vs peptide) have recently been clarified: a polysaccharide structure has been refuted and "phaseotoxin" has been reported to be a low MW peptide yielding 5 amino acids upon acid hydrolysis [10]. The compound that I have isolated and purified contains only 3 amino acids and contains both phosphate and sulphate. It is distinct from "phaseotoxin" even though it is produced by the same organism and shows the same general characteristics. Because of the unusual nature of its peptide and non-peptide structural components, a preliminary note has been published [11].

RESULTS

A. *Purification.* The major problem encountered with previous attempts to study the *P. phaseolicola* toxin has been to obtain adequate purification; no completely successful procedure has been reported. I found it necessary to make extensive investigations before suitable steps were devised for purifying toxin from bulk quantities of growth medium. In these early studies, the presence of toxin was detected by the ability of the extract to induce chlorosis in young primary leaves of bean plants. After more had been learned about the structure of the toxin, chemical and physical detection methods were also employed.

From early studies I established that the toxin had both anionic and cationic characteristics, was readily adsorbed onto charcoal [7], could be partitioned in methanol–chloroform–water mixtures, and travelled as a single discrete band during both PC and paper electrophoresis (towards the cathode at pH 2 and towards the anode at pH 7.9). The toxin was unstable if it was exposed to acidic buffers and solvents for prolonged periods. In a two dimensional thin layer electrophoretic (TLE)/TLC separation system, the toxin (identified by its biological activity) was localised in a small area coincident with a ninhydrin-reacting substance. The colour produced from the reaction of 5 μg of toxin with ninhydrin was an orange shade which aided recognition. Because TLE took only 20 min, breakdown of the toxin was not a major problem.

Charcoal adsorption was a valuable first step in the recovery of the toxin from a large volume of medium, as it gave an initial 100-fold purification with very little loss in activity when the toxin was eluted with methanol–chloroform–aqueous ammonia solution. The second purification stage, ion exchange column chromatography on QAE sephadex, was devised after discovery of the anionic nature of the toxin at neutral pH. A single such column eluted by an ionic strength gradient of ammonium bicarbonate gave a further 20-fold purifica-

Table 1. Major steps in the purification of phaseolotoxin

Purification step	Initial wt (g)	Final wt (g)	Purification factor
Charcoal adsorption	98	0.9–1.3	100×
QAE Sephadex chromatography	2.5*	0.10–0.13	20×
LH20 Sephadex chromatography	0.12	0.012–0.015	10×

* Combined charcoal extracts from two 9 l. batches.

tion. The final purification (*ca* 10×) was achieved by partition chromatography on Sephadex LH20 utilizing methanol–chloroform–aqueous ammonia as solvent (Table 1). The product thus obtained was a colourless non-crystalline glass in which the toxin was the only major ninhydrin-reacting substance present.

Purification stages were monitored throughout by bioassay and by ninhydrin following TLE/TLC separations. Increase in the sp. act. of the extract was always accompanied by an increase in the relative proportion of the ninhydrin-reacting substance in the extract. Subsequent results made it clear that the ninhydrin-reacting substance was responsible for the biological activity of the final product.

B. *Structure.* Strong acid hydrolysis of the toxin gave three amino acids (alanine, ornithine, and homoarginine) in equimolar amounts. Identity of the two non-protein basic amino acids was confirmed by the MS of their *N*-trifluoroacetyl butyl ester derivatives.

Mild acid hydrolysis of the toxin yielded a single new ninhydrin-detectable product, compound **1** (pink–purple colour with ninhydrin). Strong acid hydrolysis of compound **1** liberated the same three amino acids as obtained from the toxin. End group analysis of compound **1** with 1-fluoro-2,4-dinitrobenzene (FDNB) gave N^{α},N^{δ}-di-dinitrophenyl (DNP) ornithine as the only major amino acid derivative, showing that compound **1** has a *N*-terminal ornithine. When reacted with Carboxypeptidase B (CPB), compound **1** yielded homoarginine (showing that homoarginine is C-terminal) plus a product which, when derivatised and examined by GC–MS, was conclusively identified as ornithylalanine. Compound **1** is therefore the tripeptide ornithylalanylhomoarginine; and the same amino acid sequence must exist in the toxin.

When the intact toxin was reacted with FDNB and the product hydrolysed, N^{α}-DNP ornithine was the only major amino acid derivative produced, demonstrating that the δ-*N* of the ornithyl residue, unbonded in compound **1**, is bonded in the toxin. Because homoarginine is readily liberated from the toxin by CPB enzyme (CPB requires a free α-carboxyl group on the C-terminal amino acid of a peptide in order to be able to act on the adjacent peptide bond), the carboxyl group of the homoarginyl residue is free and not involved in cyclic bonding with the δ-*N* of the ornithyl residue. Thus the evidence demonstrates that the toxin is a N^{δ}-substituted ornithylalanylhomoarginine.

Information on the nature of the N^{δ}-substituent group was obtained during a study of the peptidase degradation products of the intact toxin. These products still retained the unknown group linked through the δ-*N* of the ornithyl residue. Thus CPB enzyme selectively cleaved homoarginine from the C-terminus of the toxin, yielding compound **2**, which had a C-terminal alanine. End group analysis of purified compound **2** with FDNB produced only N^{α}-DNP-ornithine, showing that the bonding to the δ-*N* of the ornithyl residue was still intact. When compound **2** was in turn reacted with CPA enzyme, alanine and compound **3** were the products. Compounds **2** and **3** each gave a positive bioassay. Compound **3** was also produced directly from the toxin by reaction with the enzyme leucine amino peptidase (LAP) (which requires a free α-amino group on the *N*-terminal amino acid in order to be able to hydrolyse the adjacent peptide bond). Compound **3** contained a single amino acid, ornithine, which was liberated by mild acid hydrolysis. These enzymatic reaction products are consistent with the N^{δ}-substituted ornithyl-alanylhomoarginine structure* for the toxin. A further conclusion can be drawn from the enzyme reactions. LAP is specific for cleavage of amino acids having the L-configuration [12]. The ready hydrolysis of the toxin by LAP is good evidence that the amino acid components all have the L-configuration.

Compound **3** possesses unusual electrophoretic characteristics for an ornithine-containing compound. At pH 2 ornithine migrates as a strong cation, but in contrast compound **3** migrates as an anion. This unusual behaviour, shared by such amino acid derivatives as *O*-phosphoserine and cysteic acid, can only be satisfactorily explained by the compensating presence of strong acid groups in compound **3**. Carboxyl groups would not have been sufficient. I therefore postulated the presence of phosphate or sulphate groups in the N^{δ}-substituent of the toxin.

When tested by specific detection procedures for phosphate, compound **3**, compound **2**, and the toxin, each gave a positive reaction. The phosphorus content of the toxin was confirmed when *P. phaseolicola* was grown in a ^{32}P-enriched medium; ^{32}P was incorporated into the toxin. Two observations led me to suspect the additional presence of sulphate in the toxin. One of the acid hydrolysis products was a molybdate-reacting compound that had chromatographic characteristics unlike those of any common phosphate ester but identical with inorganic sulphate [13]. In addition, the ^{32}P product from mild acid hydrolysis of the toxin was a stronger anion than expected for a compound with a single phosphate group attached. *P. phaseolicola* was therefore grown in a ^{35}S-enriched medium; ^{35}S was incorporated into the toxin.

A study of the time-course of mild acid hydrolysis of ^{32}P-toxin (Fig. 1) and ^{35}S-toxin gave further information, and eliminated the possibility that a mixture of a phosphate- and a sulphate-containing toxin was being studied. The toxin was rapidly hydrolysed to yield ornithylalanylhomoarginine (compound **1**) and an intermediate, compound **4**, which contained both P and S. Compound **4** hydrolysed at a much slower rate yielding inorganic

* In studies with the original preparation of toxin, lysine was a consistent minor component of the hydrolysate; end group analysis of the toxin with FDNB gave N^{ε}-DNP-lysine as a minor product, but not when compound **2** was so treated. More exhaustive purification of the toxin reduced the lysine component. Together, the data suggests the presence of a proportion (10–20%) of an analogue in which lysine replaced homoarginine.

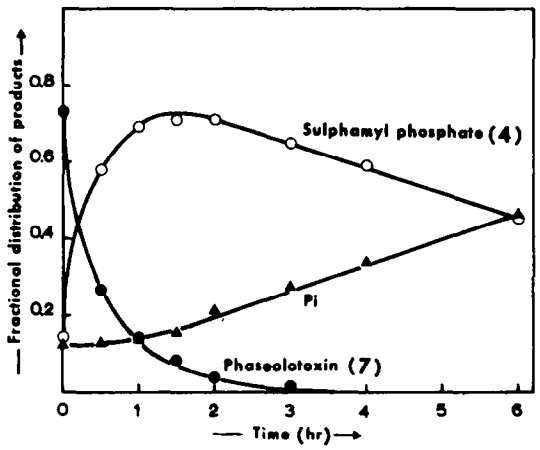

Fig. 1. Time-course of phaseolotoxin [^{32}P] hydrolysis in 0.025 M HCl at 40°. A separate study with phaseolotoxin [^{35}S] indicated that sulphamic acid (5) appeared at the same rate as Pi.

phosphate (Pi) and a new product, compound 5, which did not contain any P but which still contained S. Inorganic sulphate (SO_4^{2-}) was liberated from compound 5 under extended mild acid hydrolysis. Neither 4 nor 5 reacted with either phosphatase or sulphatase.

To aid the study of compound 5, ^{14}C-labelled toxin was prepared from growth medium that had been supplied with an extract from beans which had utilized $^{14}CO_2$ photosynthetically for 2 hr (*ca* 90% of the ^{14}C, 4.5 mCi, was in the form of glucose and fructose). Mild acid hydrolysis of the ^{14}C-toxin and purification of the products by preparative TLE gave ^{14}C-ornithylalanylhomoarginine (15 C-atoms), but no other ^{14}C-labelled product could be detected. Assuming the toxin was uniformly labelled, compound 5 should have been detectable with as few as 2 carbon atoms in the structure. On the assumption that compound 5 contained not more than 2 carbon atoms I then compared it with ^{35}S sulphate esters prepared by known synthetic procedures. It migrated electrophoretically and chromatographically to a characteristic position which was very close to those of glycolic and lactic acid *O*-sulphates*. Since these two sulphates had characteristic PMR spectra, I prepared sufficient compound 5 (6.5 mg from ^{35}S toxin) for PMR study. Whereas the standard compounds gave strong PMR signals, a similar quantity of compound 5 gave no detectable signals. This result supported the implication from the ^{14}C study that compound 5 contained one or no carbon atoms (and hence no non-exchangeable protons). An estimate of a MW of 120 for compound 5 was made from the yield data obtained during the preparation of ^{35}S-labelled compound 5 used for the PMR study. Assuming an ammonium salt, the MW contribution of the known part of compound 5, $-SO_2O^-{}^+NH_4$ (98 a.m.u.), left a choice for the remainder of the molecule, between –OH (ammonium hydrogen sulphate), –OMe (ammonium methyl sulphate) and $-NH_2$ (ammonium sulphamate) in order to approximate a MW of 120. Since the first two possibilities had already been eliminated, the presence of $-NH_2$ was tested (MW ammonium sulphamate = 114). Compound 5 and sulphamic acid were found to have identical TLE and TLC characteristics. When reacted with ice-cold dilute nitrous acid, SO_4^{2-} was liberated from each. Thus compound 5 was sulphamic acid.

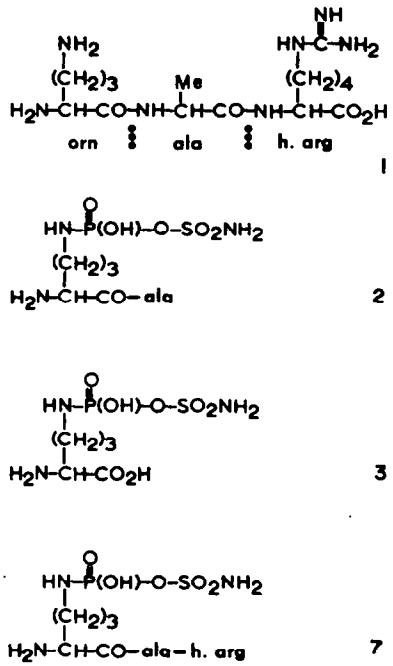

Fig. 2. Structures.

How were sulphamic acid and phosphate linked together in compound 4? During the preparation of compound 5 for PMR study, the amount of Pi liberated was estimated, and found to be one mol per mol toxin. Thus the hydrolysis of compound 4 to compound 5 which is the Pi-releasing step, involves the loss of one Pi. The exact structural relationship between compounds 4 and 5 was found by reacting compound 4 with cold dilute nitrous acid (generated in acetic acid). The new product, compound 6, contained both P (phosphate detection reagent) and sulphate (^{35}S). Under more acidic conditions (e.g. HNO_2 generated in dil HCl) compound 6 was not stable and only SO_4^{2-} and Pi were observed. This is consistent with compound 4 being sulphamyl phosphate $(HO)_2P(O)–O–SO_2–NH_2$ and with compound 6 being $(HO)_2P(O)–O–SO_2–OH$, a hitherto unreported inorganic species analogous to pyrophosphate. Such a structure would be expected to be readily hydrolysed to Pi and SO_4^{2-} under very mild acidic conditions [14]. Thus compound 3 is (N^{δ}-phosphosulphamyl)ornithine, compound 2 is (N^{δ}-phosphosulphamyl)ornithylalanine and the toxin is (N^{δ}-phosphosulphamyl)ornithylalanylhomoarginine (7). I propose the trivial name phaseolotoxin for this compound.

DISCUSSION

Although there have been several publications concerning the structure of the bean halo blight toxin, the information tends to be inconsistent. Patil has reported that acid hydrolysis of an active fraction "phaseotoxin" yielded glycine, serine, valine, and two unidentified

* Other *O*-sulphates, e.g. methyl and ethyl sulphate, methyl glycolate sulphate, methyl lactate sulphate, and glyceric acid sulphate migrated quite differently.

amino acids [10]. This fraction produced chlorosis in bean leaves but apparently did not cause the characteristic accumulation of ornithine [15,16] which occurred in the disease situation [17], even though it did appear to inhibit ornithine transcarbamylase [18].

In contrast, phaseolotoxin contains only three amino acids, none of which is apparently contained in "phaseotoxin". Furthermore, the chlorosis-inducing activity of the purified phaseolotoxin accounted for at least 80% of the chlorosis-inducing activity of the original culture medium; and the same degree of ornithine accumulation was produced in each case. There are also other discrepancies between the two sets of results. One important difference concerns the bacterial-isolate used: I isolated phaseolotoxin from a race 1 bacterial-isolate [19], whereas "phaseotoxin" was obtained from a race 2 bacterial-isolate. I have therefore examined the products from a race 2 isolate, but have found that it too produced phaseolotoxin in an amount comparable to that obtained from the race 1 isolate. Thus the differences are likely to be isolate-related rather than race-related.

In the present state of our knowledge there is no good reason to believe that the toxin which I have studied is the same as that being investigated by Patil and his colleagues. Thus the new trivial name, phaseolotoxin, is for the toxin that is produced by *P. phaseolicola* PDDCC 3612 (race 1) and 3603 (race 2), that gives symptoms resembling halo-bright when applied to bean leaves, and that has been completely characterised as the compound (N^{δ}-phosphosulphamyl)ornithyl-alanyl-homoarginine.

For the above reasons it cannot be assumed that the properties of phaseolotoxin are the same as those described in the literature for "phaseotoxin". Phaseolotoxin causes a marked accumulation of ornithine in bean leaves, and although its action on ornithine transcarbamylase (OTC) has not been tested, its structure is such as to make it a potential competitive inhibitor of OTC. Degradation products of phaseolotoxin that retain the ornithyl N^{δ}-P bond are all fully potent as toxins. In sharp contrast, whenever the N^{δ}-P bond is broken no trace of biological activity can be found when the products are tested at 10× the molar concentration at which phaseolotoxin is effective (Table 2). So the effective part of phaseolotoxin is the N^{δ}-substituted ornithine group.

There is an additional feature of phaseolotoxin which makes it a potential competitive inhibitor of OTC. OTC catalyses the condensation of ornithine with carbamyl phosphate. It may therefore be of significance that the substituent group in phaseolotoxin, sulphamyl phosphate, can be regarded as a simple analogue of carbamyl phosphate in which $>C=O$ has been replaced by $>SO_2$.

Exact knowledge of the chemical nature of phaseolotoxin presented here will now facilitate studies leading to an eventual understanding of the pathological phenomena in the bean halo-blight disease: such as the mechanism of ornithine accumulation and chlorosis production. Studies towards these aims are in progress.

EXPERIMENTAL

Culture. P. phaseolicola (PDDCC* 3612, race 1; PDDCC 3603, race 2) was grown in 9 l. of a defined medium (as in ref. [7] with glucose concentration reduced to 1/5) using 15 × 2 l. flasks on a rotary shaker for 4 days at 18°. Race 1 was used for most studies. When $^{35}SO_4^{2-}$ or $^{32}PO_4^{3-}$ (2 mCi in 4 flasks) was added to the medium, SO_4^{2-} or Pi concentration was reduced to 1/5 of the stated level. Bacteria were removed by centrifugation (16000 *g*) and discarded, and toxin was isolated from the supernatant.

Bioassay. Bean seeds were allowed to germinate at 25° in the dark and then grown at 25° in continuous light (*ca* 2000 lx) on stainless steel mesh in inorganic nutrient [20] medium. Best results were obtained by giving the seedlings an 8 hr dark period and then keeping them in a humid chamber for 3 hr to open the stomata prior to use. Test solns were then applied as a fine spray (Vilbiss atomiser) to a 1–1.5 cm diam. circular area on the under surface of the leaf towards the leaf-tip so that the area became water-injected (dark and translucent). A minimum of two plants (one leaf on each) was used per test. The nutrient medium was changed, and after a further 3 hr in the humid chamber the plants were placed in a glasshouse. Toxin-containing solns caused the sprayed area to become chlorotic within 2 days. High concns caused chlorotic symptoms in secondary leaves, presumably arising from translocated toxin. The concn of toxin was estimated by diluting solns until they gave a just-positive reaction. In the text "activity" and "active fraction" refer to fractions that caused chlorosis on bean leaves.

Thin layer separations were accomplished by 2D-TLE (pH 2)–TLC on cellulose layers. With amino acids or peptides the method was that of ref. [21]. With peptides the second dimension (TLC) employed a different solvent, BuOH–HOAc–C_5H_5N–H_2O (5:1:4:4) [22]. On some occasions TLE was at pH 7.9, when conditions used were 20 min at 600 V in 0.15 M NH_4HCO_3 at 15°. L-Aspartic acid-[U-^{14}C] in tracer amounts (10 nCi, 6 ng) was used as an internal reference marker, and its position was established by autoradiography for 16 hr. Visualisation was with ninhydrin/Cd $(OAc)_2$ [23] spray; spot positions (R_E for electrophoresis, R_C for chromatography) are relative to aspartic acid. Phosphate- and sulphate-containing compounds were examined by TLE (pH 3.6 with-

* Plant Diseases Division Culture Collection.

Table 2. Structure/activity relationship between phaseolotoxin and its degradation components

Compound	Structural name (isolated as ammonium salt)	Chlorosis inducing*	Orn. accumulation
phaseolotoxin (7)	(N^{δ}-phosphosulphamyl)orn-ala-h. arg	+	+
compound **1**	orn-ala-h. arg.	−	not tested
compound **2**	(N^{δ}-phosphosulphamyl)orn-ala	+	+
compound **3**	N^{δ}-phosphosulphamylornithine	+	+
compound **4**	ammonium phosphosulphamate	−	not tested
compound **5**	ammonium sulphamate	−	not tested

* + Was a positive assay at 1.3 μM concentration; − was a negative assay at 13 μM concentration.

out EDTA) and TLC on cellulose by the methods of ref. [24], and phosphates were visualised with ammonium molybdate spray [25].

Amino acid DNP-derivatives (from Sigma or prepared by a published method [26]) were examined by TLC on Si gel (GF254) using standard methods [26] and several solvents. *GLC* of amino acid derivates (*N*-trifluoroacetyl, butyl ester) was by the methods of ref. [27] using EGA and OV-17 glass columns (2 m × 3 mm) in a Varian 1520 series instrument. *GC–MS* of amino acid and peptide derivatives utilized GLC as above, with an OV-17 column in a Varian 2700 GC coupled via a membrane separator to an AEI MS 30 mass spectrometer. Spectra were recorded at 20 eV. Standard amino acids had fragmentation patterns in agreement with published data [28].

Preparative paper electrophoresis (PE) or PC used sheets of Whatman 3 MM paper. PE was carried out on a Savant FF22A electrophoresis apparatus (at pH 2, 2000 V for 1 hr; at pH 7.9, 1500 V for 1.5 hr) and used the same buffers as in TLE. PC used a single development with the peptide solvent as in TLC. Developed and dried papers were cut into appropriate strips and the substances eluted from them with H_2O.

Partition column chromatography. Sephadex LH20 (100 g) was allowed to swell (24 hr) in the appropriate MeOH–$CHCl_3$–H_2O (MCW) solvent then equilibrated with fresh solvent and packed into a column. The Sephadex bed (46 × 3.5 cm) was washed with 500–600 ml MCW before use. In later expts the H_2O component of MCW was replaced with 0.25 M aq. ammonia (→ MCA) in order to minimize breakdown of the toxin. By eluting the column with solvent of the one composition, a total recovery of sample weight was obtained.

Ion-exchange column chromatography. QAE sephadex was allowed to swell in 0.8 M $KHCO_3$ for 48 hr (soln changed several times during this period), then equilibrated with 4 vol of 0.05 M NH_4HCO_3 for 5 hr and packed into a column (28 × 2.8 cm). The Sephadex bed (21 × 2.8 cm) was washed with 200 ml 0.05 M NH_4HCO_3 before use.

Concentration of samples. All eluates and fractions, whether from columns, PE, or PC; were evaporated on a rotary evaporator with bath temp < 40°, and then dried for 12 hr (*ca* 20°) at 5 × 10^{-3} τ. Fractions containing NH_4HCO_3 were evaporated then taken up in *ca* 0.5 ml H_2O, and this soln was left to stand for several hr then dried down again to remove residual NH_4HCO_3; compounds thus obtained were as their ammonium salts.

Radioactivity was measured on a Packard scintillation counter. Each sample, contained in 1 ml H_2O, was mixed with 10 ml Triton X-100 based scintillation fluid [29].

Preliminary studies. Supernatant from the bacterial culture (7.2 l) was concentrated in a Cyclone evaporator (1τ/35–40°/pH *ca* 7) to 0.18 l. and then MeOH (250 ml) was added with stirring. The final concn was critical: 57% MeOH precipitates inorganic salts without loss of toxin, but above 60% MeOH, some toxin is coprecipitated. Inorganic salts were filtered off, then the filtrate was evaporated to near dryness, residue dissolved in H_2O (150 ml), and the soln slowly added to rapidly stirring MeOH–$CHCl_3$ (1350 ml, 1:1)(final MCW ratio 9:9:2). Stirring was continued for 2 hr, and the two phases were then allowed to separate. The upper organic phase was discarded and the thick aq. syrup, which retained all the activity, was then partitioned into 1 l. of MCW (9:9:2). The second aq. syrup was dried, the residue dissolved in H_2O (55 ml) and the soln stirred while MeOH (145 ml) was slowly added. Stirring was continued for 2 hr and the resulting ppt. was then filtered off. The filtrate was stirred while 50 ml $CHCl_3$ was added (final MCW ratio 29:10:11). Stirring was continued for 1 hr then the two phases were allowed to settle. The upper organic phase was evaporated to yield the major active fraction. This was redissolved in 50 ml MCW (29:10:11): 72% dissolved, including 90% of the toxin. This soln was column chromatographed on Sephadex LH 20 (200 g) with MCW (29:10:11). The first material to elute from the column corresponded with elution of the first coloured band. From the start of this coloured band 46 ml was collected, then the activity eluted and was collected in 3 fractions totalling 64 ml. The combined active fraction was re-chromatographed on a similar column, and was then ready for PE and PC purification. After PE at pH 2, activity was in a 2.5 cm wide strip centred 6 cm from the origin (cathode side); after PE at pH 7.9, it was in a 2 cm wide strip centred 8.4 cm from the origin (anode side); after PC for 11 hr it was in a 1.4 cm wide strip centred 17.1 cm from the origin (R_f 0.43). The major active fraction was purified by PE (pH 7.9) followed by PC. Samples of this purified fraction were separated on duplicate TLE/TLC plates (peptide conditions). Application of ninhydrin detection procedures showed an intense orange spot (R_f 1.04, R_C 0.80). On the unsprayed plate the position of the major component was located relative to the aspartic acid [^{14}C] reference marker and this area was removed and extracted with H_2O (3 × 10 ml); the extract gave a characteristic bioassay response while no activity was detected on other parts of the TLC plate. When it was hydrolysed (6 M HCl, 100°, 18 hr) and then subjected to TLE/TLC (amino acid conditions) there were 3 major ninhydrin-positive products.

Main isolation. Information obtained in the preliminary studies enabled the purification procedure to be considerably simplified and improved. Reducing the sugar content of the growth medium to 1/5 helped in isolating the toxin without reducing its yield. Results of the simplified procedure are summarized in Table 1. Supernatant from each 9 l. batch was stirred (5 min) with activated charcoal (40 g, acid-washed). The charcoal was filtered off, washed with H_2O (filtrate and washings discarded), and extracted twice with 200 ml MCA (11:4:5 of 0.5 M ammonia). Each extract was recovered by filtration and the filter-cake was washed with MCA (2 × 100 ml). Combined yellow filtrate was evaporated, yielding 1–1.3 g residue per batch. Residues from 2 batches were combined, dissolved in 100 ml 0.05 M NH_4HCO_3 (pH 7.8) and applied to a column of QAE Sephadex. The column was washed with 100 ml 0.05 M NH_4HCO_3 and then eluted with a linear concentration gradient of the same buffer (1 l. 0.05 → 0.5 M). Fractions of 20 ml were taken and toxin eluted in 3 fractions after 560 ml of buffer-gradient had been collected. Each fraction was bioassayed (0.2% of fraction dissolved in 10 ml) and examined by TLC and TLE. Active fractions were combined, dried, dissolved in 10 ml MCA (3:1:1) and chromatographed on Sephadex LH 20. The column was eluted with MCA, the first solids appearing after 150 ml eluate had been collected. Activity was recovered in *ca* 25 ml vol after a total of 170 ml had passed. The toxin now travelled as a single compound on TLE/TLC (R_E 1.08, R_C 0.82) as determined with ninhydrin or phosphate-ester spray detection, or ^{32}P or ^{35}S-labelled material. If further purification was necessary, this was accomplished by repetition of QAE Sephadex chromatography as above.

Compound **1**; *ornithylalanylhomoarginine.* Mild acid hydrolysis of phaseolotoxin with 0.025 M HCl, 100°, 6 hr gave a single ninhydrin-reacting product which was purified by QAE Sephadex ion-exchange chromatography (as for phaseolotoxin). It eluted from the column with 0.05 M NH_4HCO_3 (before gradient elution was commenced), and on TLE/TLC had R_E 2.5, R_C 1.05. The same hydrolysis conditions were used in other steps requiring mild acid hydrolysis.

Strong acid hydrolyses of phaseolotoxin and compound **1**. Strong acid hydrolysis with 6 M HCl, 100°, 16 hr was in sealed glass tubes. Product from phaseolotoxin or compound **1**, evaporated *in vacuo* over conc H_2SO_4 and NaOH pellets, had 3 major amino acids by TLE/TLC. Alanine was identified by TLE/TLC and GLC procedures, and was confirmed by co-chromatography (TLE/TLC) with ^{14}C ala; ornithine and homoarginine were identified by GLC and confirmed by GC–MS with authentic samples. Study by automatic amino acid analyser confirmed the above identities and showed a 1:1:1 molar ratio.

Reaction of compound **1** *with CPB and GC–MS identification of product.* To compound **1**, 0.4 mg in 200 μl 0.25 M NH_4HCO_3, CPB enzyme (10 μl, 3.5 units) was added and the reaction mixture kept at 38°, 24 hr. TLE/TLC showed a peptide product (R_E 2.5, R_C 1.2) and homoarginine, along with much smaller amounts of lysine and arginine. The product was derivatised and examined by GC–MS; the peptide component of the product was identified as ornithylalanine, *m/e* 451 (M^+), 377 ($M^+ - n$-BuOH), 350 ($M^+ - CO_2n$-Bu), 307 ($M^+ - NHCH(CH_3)CO_2n$-Bu), 280($M^+ - CONHCH(CH_3)CO_2n$-Bu), and 166 by comparison with GC–MS of derivatized authentic lysylalanine which had *m/e* at 14 mass units higher than the above values. The homoarginine derivative had *m/e* 532 (M^+), 477, 463, 431, 407, 318, 306, 180 at 14 m.u. higher than arginine derivative.

Compound **2**; *(N$^\delta$-phosphosulphamyl)ornithylalanine.* Phaseolotoxin (*ca* 35 mg) in 0.25 M NH_4HCO_3 soln (1.3 ml) containing CPB (50 μl, 17.5 units) was incubated at 38° for 28 hr. TLE/TLC examination of the product showed homoarginine and one major peptide component, compound **2**, which was purified by QAE Sephadex chromatography as above, and eluted after 900 ml of buffer-gradient had been collected; detected as an orange (like phaseolotoxin) spot (R_E 0.14, R_C 0.88); hydrolysis in 6 M HCl/100°/16 hr liberated ornithine and alanine; hydrolysis in 0.02 M HCl/100°/6 hr yielded ornithylalanine.

Compound **3**; *N$^\delta$-phosphosulphamylornithine.* (i) From reaction of compound **2** with CPA. CPA (10 μl, 10 units) was diluted with 0.5 M NH_4HCO_3 (15 μl) then 2 μl (0.8 units) was added to compound **2** (*ca* 80 μg) in 0.25 M NH_4HCO_3 (50 μl) and the reaction mixture kept at 38°. After 24 hr the reaction was *ca* 70% complete, and after 48 hr TLE/TLC examination showed alanine and one new major ninhydrin detectable product, compound **3** (R_E −0.25, R_C 0.6). (ii) From reaction of phaseolotoxin with LAP. Phaseolotoxin (*ca* 100 μg) in 0.25 M NH_4HCO_3 (50 μl) was incubated at 37.5° with LAP (10 μg, 1.3 units) for 1 hr. TLE/TLC examination of the product showed alanine, homoarginine and compound **3**. Compound **3** was purified by QAE Sephadex chromatography: it eluted after 520 ml gradient was collected (cf phaseolotoxin, 560 ml); 0.02 M HCl/40°/6 hr liberated ornithine.

Compound **4**; *sulphamyl phosphate.* An aq. soln (1.6 ml) of ^{35}S-phaseolotoxin (1 mg) and 0.05 M HCl (0.4 ml) was kept at 40° for 1.5 hr then quickly evaporated. The residue was immediately purified on QAE Sephadex as above, and compound **4** eluted after 700 ml of buffer-gradient had been collected: 80% of the ^{35}S peak was collected in a 36 ml vol. Compound **4** in H_2O (40 μl) was cooled in an ice-bath and then ice-cold HNO_2 (10 μl, generated from equal vols. of cold 0.2 M $NaNO_2$ and 0.25 M HCl) was added. After 1 min the reaction was frozen (liquid N_2) and freeze-dried. TLE examination showed complete reaction with two products, $^{35}SO_4^{2-}$ and Pi, both not present in the starting material.

Compound **5**; *sulphamic acid.* Phaseolotoxin-[^{35}S] (43 mg) was hydrolysed in 0.5 M HCl (40°, 15.5 hr) and the product evaporated to dryness. Residue (58 mg), in 0.05 M NH_4HCO_3 (75 ml) was loaded on a QAE Sephadex column which was then washed with 300 ml 0.05 M NH_4HCO_3 (eluting cpd 1, 26 mg), and then eluted by a gradient as above; compound **5** (6.5 mg) was collected in 75 ml after 355 ml of buffer-gradient had passed, and then Pi was collected in 100 ml after 430 ml of gradient had passed (analysis [30] showed 13.5 mg as $(NH_4)_3PO_4.3H_2O$, calcd for 1 mol per mol phaseolotoxin: 15.3 mg); later fractions contained SO_4^{2-} and compound **4**. Fractions collected were all measured for radioactivity and examined by TLE. Compound **5** thus obtained reacted with cold HNO_2, as in the preparation of compound **6**, liberating $^{35}SO_4^{2-}$; co-chromatography and electrophoresis showed compound **5** was identical with authentic sulphamic acid; after exchanging H with D, PMR (D_2O) showed no sample signals (only HOD observed).

Compound **6**; *phosphosulphate.* To compound **4** in 40 μl H_2O in an ice bath was added 20 μl HNO_2 generated from mixture of equal vols of 0.2 M $NaNO_2$ and 0.4 M HOAc. The reaction released only minor amounts of $^{35}SO_4^{2-}$ and Pi; instead a new product was obtained which still contained ^{35}S and P (P-ester spray), with TLE (pH 3.6) mobility greater than starting material but slower than sulphamic acid. Attempts to isolate this product by both TLE and QAE Sephadex chromatography failed.

Derivatisations with 1-fluoro-2,4-dinitrobenzene (DNFB). Phaseolotoxin, or compound **1**, or compound **2** (each 100 μg) in aq. 1% Et_3N (200 μl) was kept for 1.5 hr at 20° (dark) with DNFB soln (400 μl 2.5% (w/v) in MeOH). MeOH was evaporated, H_2O (2 ml) and aq. 2% Et_3N (0.5 ml) were added, and the total washed with Et_2O (3 × 5 ml); the aq. phase was evaporated and hydrolysed with 6 M HCl/100°/16 hr. The hydrolysate was diluted with H_2O, extracted with Et_2O (2 × 5 ml) and then the Et_2O and aq. phases were studied by TLC. Only compound **1** yielded a DNP derivative from the Et_2O, di-DNP-orn, and the aq. phase had a small amount of ε-DNP-lysine. The aq. phase from phaseolotoxin had mainly α-DNP-ornithine with a small amount of ε-DNP-lysine. The aq. phase from compound **2** had only α-DNP-ornithine.

Time course of hydrolysis of ^{32}P-phaseolotoxin. Two solns of ^{32}P-phaseolotoxin, one in H_2O, one in 0.025 M HCl, were heated at 40°. At various times, samples (4 μl) from each vial were spotted on a TLE plate which was then immediately placed in an atmosphere of NH_3, to halt further reaction. After electrophoresis (pH 2), unreacted phaseolotoxin and the product spots, located by autoradiography, were removed from the plate [31] and their radioactivities were measured. The fraction of radioactivity in each spot was calculated and plotted vs time (Fig. 1). The same sequence was repeated with ^{35}S-toxin where compound **5** (which is unlabelled and not seen from ^{32}P-toxin) appeared at the same rate as Pi in Fig. 1.

Synthetic O-^{35}S-sulphate esters were prepared by the method of ref. [32] and will be the subject of a separate communication.

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