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Inhibitors of Ornithine Carbamoyltransferase from Pseudomonas syringae pv. phaseolicola. Revised Structure of Phaseolotoxin

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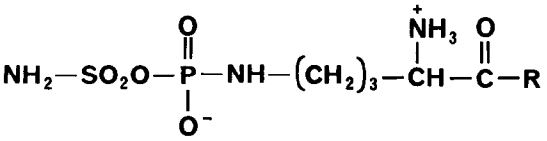
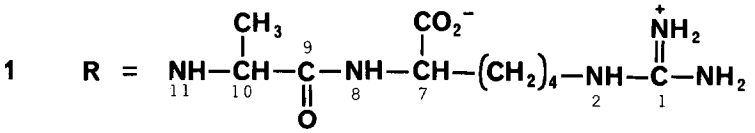
Abstract. The structure of phaseolotoxin, a toxin produced by cultured Pseudomonas syringae pv. phaseolicola, the causal agent of halo blight disease in bean plants is revised to 3. The structure of octicidin, isolated from leaves of bean plants infected with pv. phaseolicola, has been determined to be 4, a protease degradation product of 3 which is formed in the plant.

Pseudomonas syringae pv. phaseolicola,[1] the causal agent of halo blight disease in bean plants (Phaseolus vulgaris L.), produces a toxin in culture which mimics the pathogenic bacterium by generating symptoms of the disease (chlorosis and accumulation of ornithine) when injected into bean tissues. In 1976 Mitchell succeeded in isolating the toxin, which he named phaseolotoxin, from the culture medium of pv. phaseolicola and proposed 1 for its structure.[2,3] In the infected bean plant, however, it was suggested that another toxin 2, resulting from enzymatic degradation of phaseolotoxin in the host, was responsible for the disease conditions.[4] Both toxins were found to block the conversion of ornithine to citrulline, apparently by inactivation of ornithine carbamoyltransferase (OCT).[5] Chlorosis, the consequence of this irreversible inhibition of OCT, could be prevented or reversed by the administration of citrulline or arginine to the plant.[6]

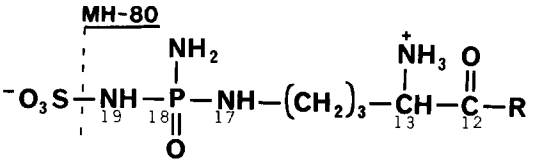
We reisolated phaseolotoxin from the culture medium of pv. phaseolicola (PDDCC 4612) by charcoal adsorption chromatography, QAE Sephadex ion exchange chromatography, and anion exchange (BioRad A-27, pH 6.6, 0.3 M NH_4OAc) and reverse phase (Altex ODS, pH 5, 2mM NH_4OAc in 0.4% MeOH/H_2O) HPLC using the inhibition of OCT assay to monitor the purification. ^1H, ^{13}C, and ^{31}P NMR studies in D_2O, 50% H_2O/D_2O, and 5% H_2O/DMSO-d_6 confirmed the structure of the tripeptide moiety and its point of attachment to the phosphorus.[7] We were not able, however, to observe NH-P and NH-S signals in the ^1H NMR spectrum in 50% D_2O/H_2O at pH 4.58 or 5% H_2O/DMSO-d_6, nor were we able to see ^{31}P-N^1H coupling in the corresponding ^{31}P NMR spectrum.





ala—harg

2 R = OH



3 R = ala—harg

4 R = OH

Mass spectral studies of phaseolotoxin indicated that the structure of the inorganic portion was incorrect and should be revised. The positive ion fast-atom bombardment mass spectrum (FABMS) showed a MH^+ ion peak at m/z 532.2069 ($C_{15}H_{35}N_9O_8PS$ requires 532.2067) and the negative ion FABMS showed a $(M-H)^-$ ion peak at m/z 530, leading to a molecular weight of 531 for phaseolotoxin. The molecular structure therefore possessed an additional NH and one less O. Structure 3 was immediately suggested from a high resolution measurement of the base peak in the positive ion FABMS, m/z 452.2499 ($C_{15}H_{35}N_9O_5P$ requires 452.2499), which represented the loss of SO_3 (not NH_2SO_2) from the MH^+ ion.

Conclusive proof that phaseolotoxin possessed a phosphorus directly bonded to three nitrogens was provided by a ^{15}N-labelling study. ^{15}N-labelled 3 (2 mg) was isolated from 9 l of culture medium of pv. phaseolicola grown on $(^{15}NH_4)_2SO_4$ (1 g/l, 99 atom %) as the sole source of nitrogen. In its proton-noise decoupled ^{31}P NMR spectrum in D_2O, an eight-line doublet of doublets of doublets was observed at 11.67 ppm, reflecting the coupling of ^{31}P to three ^{15}N atoms by 16.2, 28.1, and 31.9 Hz (signs undetermined).[8] The magnitudes of the three coupling constants were consistent with the ^{15}N-^{31}P coupling observed in $(Me_2N)_3PO$ (-26.9 ± -0.1 Hz).[9,10]

The major toxin, octicidin, generated in the leaves of bean plants infected with pv. phaseolicola (3 days after inoculation) was isolated[11] and shown to be identical in all respects with the toxin 4 resulting from enzymatic degradation of 3 with leucine

aminopeptidase, an enzyme that is specific for cleavage of peptides composed of L-amino acids.[12] Octicidin (4) proved to be 20 times more potent than 3 in inhibiting OCT.[4b] The positive ion FABMS of 4 exhibited a MH^+ ion peak at m/z 291 and a $(MH-SO_3)^+$ fragment ion peak at m/z 211.

Mitchell had proposed that P was attached to O in the sulfamyl group of phaseolotoxin based on acid hydrolysis (0.01 N HCl, 40°C, 1.5 hr) to a phosphosulfamic acid which in turn could be converted to another S and P containing compound on treatment with nitrous acid in aqueous acetic acid.[2] We repeated the acid hydrolysis with ^{15}N-labelled 3 and found that ^{15}N-phosphosulfamic acid [5, $HO_3S-^{15}NH-PO_3H_2$; ^{31}P NMR: δ -11.24 at pH 1.95 (br d) → δ 2.43 (br) on neutralization to pH 6.6 → δ -10.41 (d, $|J_{PN}|$ = 20 Hz) on reacidification to pH 1.88; ^{15}N NMR: δ 116.8 at pH 6.6] was produced in 80% yield at pH 1.95 and 25°C after 5 hr. N-Phosphosulfamic acid was stable at pH 6.6, but decomposed to sulfamic acid and phosphate at pH 1.95 after 1 day. When 5 in water at pH 1.95 was treated with aqueous sodium nitrite, N-nitroso-N-phosphosulfamic acid [6, $HO_3S-^{15}N(NO)-PO_3H_2$; ^{31}P NMR: δ -4.1 at pH 3.6 (br d, $|J_{PN}|$ = 15-20) → δ -3.0 at pH 7.1] was formed. Compound 6 appeared to be stable at pH 7, but very rapidly (< 15 min) decomposed to phosphate, sulfate, and nitrogen at pH 2.

The phosphorus atom in 3 and 4 is asymmetric, but it is not known whether it is R or S. To our knowledge 3 and 4 are the first examples of naturally-occurring phosphotriamides.

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References and Notes

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3. In an earlier paper [Patil, S. S.; Youngblood, P.; Christiansen, P.; Moore, R. E. Biochem. Biophys. Res. Commun. 1976, 69, 1019] we erroneously suggested that the major toxin in cultured pv. phaseolicola, which we named phaseotoxin A, was N-phosphoglutamate. Synthetic N-phosphoglutamate, however, was shown to lack the biochemical and biological properties of phaseolotoxin [Mitchell, R. E. Physiol. Plant Pathol. 1979, 14, 119. Smith, A. G.; Rubery, P. H. Physiol. Plant Pathol. 1979, 15, 269].
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7. Spectral data for 3: ^{1}H NMR (D_2O, p-dioxane as internal ref. = 3.747 ppm) δ 4.379 (q, 7.2 Hz, H on C-10), 4.143 (dd, 7.9 and 5.2 Hz, H on C-7), 4.028 (t, 6.8 Hz, H on C-13), 3.166 (t, 7.0 Hz, 2H on C-3), 2.990 (m, 11.7 for J_{HP} and 6.6 Hz, 2H on C-16), 1.967 (m, 2H on C-14), 1.785 (m, 1H on C-6), 1.690 (m, 1H on C-6), 1.595 (m, 4H on C-4 and C-15), 1.399 (d, 7.1 Hz, Me on C-10), 1.360 (quintet, 2H on C-5); ^{1}H NMR (50% H_2O/D_2O, pH 4.58) δ 8.686 (d, 5.9 Hz, H on N-11), 7.938 (d, 7.7 Hz, H on N-8), 7.118 (br t, H on N-2), 6.60 (br, 2 NH_2 on C-1). ^{13}C NMR (D_2O, p-dioxane as internal ref. = 67.4 ppm) δ 179.4(s), 174.2(s), 170.7(s), 155.0(s), 55.7(d), 53.7(d), 50.6(d), 41.7(t), 40.5(t), 32.0(t), 29.2(t), 28.2(t), 26.9 (td, J_{CP} = 6.0 Hz), 22.9(t), 17.3(q). ^{31}P NMR (D_2O), 85% H_3PO_4 as external ref. = 0 ppm) δ 11.78 (t, J_{PH} = 11.7 Hz). The ^{13}C NMR data are essentially identical to those in the literature: Mitchell, R. E. In "Toxins in Plant Diseases"; Durbin, R. D., Ed.; Academic Press: New York, 1981; pp. 259-293.

8. ^{15}N NMR (H_2O, proton noise decoupled, liquid NH_3 as external ref. = 0) δ 38.4 (s, NH_3^+ on C-13), 42.6 (d, $|J_{NP}|$ = 28.1 Hz, NH_2 on P-18), 51.6 (d, $|J_{NP}|$ = 31.9, N-17), 71.7 (br, 2 NH_2 on C-1), 86.5 (s, N-3), 112.5 (d, $|J_{NP}|$ = 16.2, N-19), 127.1 (s, N-8 or N-11), 127.7 (s, N-8 or N-11). Signals for H on N-19 and NH_3^+ on C-13 in undecoupled spectrum show no NH coupling due to rapid exchange; all other nitrogens show NH coupling of 85-95 Hz. Assignments are based on literature data [Posner, T. B.; Markowski, V.; Loftus, P.; Roberts, J. D. J. Chem. Soc. Chem. Commun. 1975, 769. Kanamori, K.; Cain, A. H.; Roberts, J. D. J. Am. Chem. Soc. 1978, 100, 4979].

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10. Gray, G. A.; Buchanan, G. W.; Morin, F. G. J. Org. Chem. 1979, 44, 1768. When the nitrogen has pure trigonal geometry, the ^{15}N-^{31}P coupling is calculated to be -39 Hz; when it has pure pyramidal geometry, J_{NP} is calculated to be -12 Hz.

11. Compound 4 was isolated from the leaves of diseased bean plants by extraction with 70% MeOH, gel filtration on Sephadex G-10 (H_2O), gradient chromatography on QAE Sephadex (0.02-0.7M NH_4HCO_3), anion exchange HPLC twice on BioRad A-27 (0.3M NH_4OAc, pH 7.0 for the first pass; 0.3M ammonium formate, pH 3.0, for the second pass), reverse-phase HPLC on ODS (2 mM NH_4OAc in 0.4% MeOH/H_2O), and ion-pair HPLC on ODS(0.02M tetrabutylammonium phospate) using the inhibition of OCT assay. ^{1}H NMR (D_2O): δ 3.752 (t, 6.1 Hz, C-2 H), 2.983 (m, $J_{H,P}$ = 11.3 Hz,2H on C-5), 1.912 (m, 2H on C-3), 1.585 (m, 2H on C-4).

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