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THE STRUCTURE OF TAGETITOXIN, A PHYTOTOXIN OF *PSEUDOMONAS SYRINGAE* PV. *TAGETIS**

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Key Word Index—*Pseudomonas syringae* pv. *tagetis*; marigold; phytotoxin; apical chlorosis; tagetitoxin; phosphate ester; hemithioketal.

Abstract—Tagetitoxin, a phytotoxin produced in liquid cultures of the plant pathogenic bacterium *Pseudomonas syringae* pv. *tagetis*, causes an apical chlorosis symptom when applied to stems of plants. Tagetitoxin was isolated from a liquid culture medium of the organism and purified to homogeneity by chromatography on DEAE Sephadex then LH-20 Sephadex. Chemical and spectroscopic studies allow the unambiguous assignment of the overall structure of tagetitoxin, but some stereochemical questions remain to be solved. Thus, a field desorption mass spectrum demonstrated a MW 435; other data showed that, in addition to oxygen, heteroatomic components were nitrogen in an amine, phosphorus in a phosphate ester, and sulphur. A radioactive double-labelling experiment established the phosphorus–sulphur ratio to be 1:1. The molecular formula was deduced to be $C_{11}H_{18}NO_{13}PS$ and a cyclic hemithioketal structure was derived from ^{1}H and ^{13}C NMR spectra. In accord with the hemithioketal structure, tagetitoxin was observed to be labile in aqueous dilute acidic conditions yielding a product mixture that gave a positive colour reaction for thiols, a property not shared with the parent toxin. The phosphate functionality was resistant to hydrolysis by phosphatase.

INTRODUCTION

Tagetitoxin is a phytotoxin produced in liquid cultures of the plant pathogenic bacterium *Pseudomonas syringae* pv. *tagetis* (Helmers 1955) Young, Dye and Wilkie 1978 [1]. Marigold or zinnia plants, when treated with as little as 20 ng of tagetitoxin, develop an apical chlorosis after 2–3 days, an effect which is also observed in marigold plants infected with toxin-producing strains of the bacterium. The apical chlorosis symptom caused by tagetitoxin, which is not specific to plant species, is thought to be the result of a disruption of the normal development of chloroplasts [2]. Knowledge of the structure of tagetitoxin may contribute to an understanding of the biochemical mode of action of tagetitoxin on plants. We here report data which allow the proposal of structure **1** for tagetitoxin.

RESULTS AND DISCUSSION

Tagetitoxin was obtained as a noncrystalline glassy residue in *ca* 40% recovery after purification of an extract of the culture medium by ion exchange chromatography on DEAE Sephadex and partition chromatography on LH-20 Sephadex [1]. It was soluble in water but had little apparent solubility in nonaqueous solvents. TLC and thin layer electrophoresis (TLE) procedures indicated a homogeneous preparation. An IR spectrum (Nujol) gave only limited information: it contained broad absorption at 2.85–3.20 μm typical of –O–H from the presence of water; broad $\geq$C=O absorption at 5.95–6.58 μm, probably due in part to carboxylate group(s); and broad absorption at 8.66–9.56 μm associated with a phosphate.

A field desorption mass spectrum demonstrated that tagetitoxin had a MW of 435 (FDMS *m/z* 435 for M^+). In addition to oxygen, heteroatomic components of tagetitoxin were nitrogen in an amine (purple colour with ninhydrin), phosphorus in a phosphate ester (blue colour with molybdate reagent, ^{31}P NMR) and sulphur (incorporation of ^{35}S). The sulphur was deduced (see later) to be present as a thiol or thioether. In the ^{31}P NMR spectrum, a doublet (J = 11.5 Hz) at δ 1.0 downfield from phosphate was consistent with the presence of a phosphate ester of a secondary alcohol function [3]. The ^{13}C NMR spectrum (Table 1) had signals from 11 carbons in total, of which three were present at carboxyl groups and five were monosubstituted with oxygen. The functionalities contained in these five carbon-substituents were deduced from ^{1}H and ^{13}C NMR chemical shifts to be one acetyl, one phosphate, and either three hydroxyl functions or one hydroxyl and one ether function. If the two unassigned carboxyl functions are assumed for the time being to belong to carboxylic acid groups, the two alternative assemblies of functionalities require either 12 or 13 oxygens. These data, for the case of 13 oxygens and a MW of 435, require the hydrogen complement of tagetitoxin to be 18. In contrast, if there were only 12 oxygens, the hydrogen complement would necessarily be 34, an untenable number for a compound with only 11 carbons. Therefore, these data suggest that the molecular formula of tagetitoxin is $C_{11}H_{18}O_{13}SNP$, and, furthermore, support the presence of three hydroxyl functions rather than the alternative combination of one hydroxyl and one ether function [4]. In accord with the molecular formula and the functional groups outlined, a fully saturated acyclic

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Table 1. ^1H and ^{13}C NMR data for tagetitoxin in D_2O

Carbon No.	^1H δ (multiplicity), J (Hz)	^{13}C δ (off-resonance multiplicity)
1	—	174.5* (*s*)
2	—	85.8 (*s*)
3	4.38 1H (*d*) 3.9	73.6 (*d*)
4	5.16 1H (*dd*) 3.9, 12	80.3 (*d*)
5	3.46 1H (*dd*) 12, 7.6	43.9 (*d*)
6	4.73 1H (*dd*) 7.6, 11.5	77.1† (*d*)
7	—	71.7† (*s*)
8	2.92, 3.32 2H (*q*) 14	33.7 (*t*)
9	—	174.1* (*s*)
10	—	171.5 (*s*)
11	1.98 3H (*s*)	23.2 (*q*)

Chemical shifts are relative to trimethylsilylpropane sulphonate in the ^1H spectrum and to 1,4-dioxane (δ 67.4 relative to TMS) in the ^{13}C spectrum.

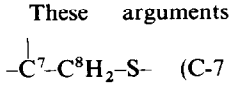
*The assignments to each of these two peaks is arbitrary only.

†In the proton broad band noise-decoupled spectra each of these two signals were doublets, J = 4.3 Hz.

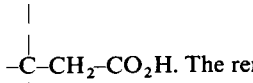
compound would have 20 hydrogens. Therefore, tagetitoxin has one unit of hydrogen deficiency and because the ^{13}C NMR spectrum showed there were no carbon–carbon double bonds present, tagetitoxin must accordingly contain one ring structure. These various arguments are based on the sulphur of tagetitoxin having an oxidation state of -1, such as a thiol or thioether and, thus, carrying no oxygenation. The following observations supported this assignment. The sulphur was not in a sulphate ester since strong acid hydrolysis of [^{35}S]tagetitoxin did not liberate sulphate (TLE, autoradiography), but multiple products were observed. Furthermore, reaction of tagetitoxin with sodium nitroprusside gave no colour reaction, but when the toxin was pretreated with dilute acid (0.05 M hydrochloric acid 20°, 1 hr) a positive colour test for thiols was obtained. There was indeed only one sulphur since a double-labelling experiment, performed by growing *P.s. tagetis* in the presence of $^{32}PO_4^{3-}$ and $^{35}SO_4^{2-}$, yielded a radiochemically pure tagetitoxin which demonstrated a phosphorus–sulphur ratio of 1:1.

The 270 MHz ^1H NMR spectrum of tagetitoxin displayed a single methyl group (acetyl –Me, see Table 1) and well resolved multiplets and, therefore, gave useful structural information about the entire carbon skeleton of tagetitoxin (Table 1). The two non-equivalent protons of a methylene group (AB*q*, δ 2.92, 3.32) were not coupled further, hence, there are no non-exchangeable protons on adjacent nuclei. The carbon bonded to this methylene must, therefore, be quaternary. The ^{13}C NMR chemical shift (δ 33.7) of the methylene carbon, identified from the off-resonance multiplicity (*t*) and also the ^1H NMR chemical shifts of the methylene protons are consistent with bonding of this methylene to both or either of sulphur and a quaternary carbon but not to either nitrogen or oxygen [4].

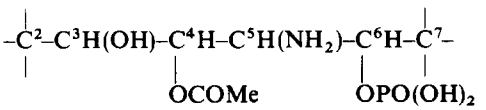
These arguments suggest the part-structure



(C-7 quaternary), or alternatively



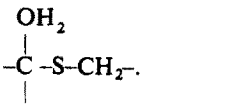
The remaining feature of the ^1H NMR spectrum was a series of 1H multiplets for single protons attached to each of four adjacent carbons (C-3–C-6 in (Table). Decoupling experiments confirmed the relationship between these four protons suggested by the coupling constants. The H-3 proton was a doublet, coupled only to H-4, thus indicating that C-2 is quaternary, and the H-4–H-6 protons each appeared as doublets of doublets. The H-6 proton had vicinal coupling to both H-5 ($J_{5,6}$ = 7.6 Hz) and phosphorus ($J_{6,P}$ = 11.5 Hz), since a doublet (J = 11.5 Hz) in the ^{31}P NMR spectrum collapsed to a singlet after proton decoupling and, thus, demonstrated the attachment of the phosphate ester at C-6. The absence of further coupling to H-6 indicated that C-6, like C-3, was adjacent to a quaternary carbon. Thus, a further part-structure from the ^1H NMR data is



where C-2 and C-7 are quaternary. The placement of a hydroxyl group at C-3 and an acetate at C-4 is based on the chemical shifts of H-3 and H-4 (Table 1) since the resonance of an acetate C–H generally occurs at lower field than a hydroxyl C–H [4]. Similarly, the placement of an amino group at C-5 is favoured on the basis of the H-5 chemical shift [4] and is substantiated by evidence discussed below.

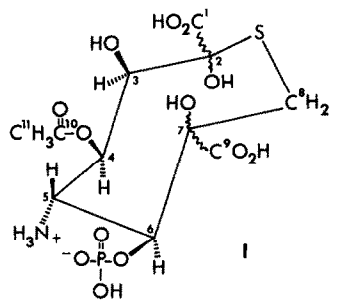
The presence of two quaternary carbons in tagetitoxin was confirmed from the ^{13}C NMR data. Five carbon signals in the broad band decoupled spectrum (three carboxy carbons at δ 174.5, 174.1 and 171.5, and two carbons at 85.8 and 71.7) were unchanged in the proton off-resonance decoupled spectrum (whereas the remaining six were multiplets). Thus, the carbons at δ 85.8 and 71.7 were quaternary. The structural location of the two quaternary carbons is determined on the basis of couplings observed between ^{13}C and ^{31}P in the broad band decoupled ^{13}C NMR spectrum; such couplings also add strong support to the structural sequence C-2–C-7 proposed above. In this part-structure, three adjacent carbons would be expected to show coupling to ^{31}P—one geminal and two vicinal to P. This is what was actually found. Because the quaternary carbon at δ 71.7 is a doublet (J

= 4.3 Hz) in the broad band decoupled spectrum it must, therefore, be the quaternary carbon (C-7) already deduced to be bonded to C-6 in the part-structure. The adjacent phosphate-bearing C-6 appears as a doublet (δ 77.1, J = 4.3 Hz) and the third carbon (C-5) of the trio, which appears as a broadened peak at δ 43.9 (and in one spectrum as a doublet, J = 2.4 Hz), is unambiguously assigned from its characteristic chemical shift to the amino carbon*. This provides conclusive evidence for amino and phosphate groups in a 1,2-relationship. The other ^{13}C peak characteristic of a quaternary carbon (at δ 85.8), which had no proximity to phosphorus, can now be assigned to C-2. The moderate downfield shift of this carbon is indicative of its structural environment: it is not large enough for double oxygen substitution, but is consistent with bonding to both oxygen and sulphur. Thus, we propose a hemithioketal part-structure at C-2:



In comparison, the hemithioacetal C-1 of 5-thio-D-glucose has δ 75.4 [5] and the thioketal C-1 in merosinigrin, 83.0 [6].

The structural components of tagetitoxin are now fully accounted for and correlated by the NMR spectral data and allow an eight-membered ring structure (**1**) (depicted for convenience in a crown conformation) to be proposed as the structure of the toxin, where the unassigned hydroxyl group and two carboxylic acid groups are placed at the two quaternary carbons, C-2 and C-7. There does not appear to be any alternative structure that adequately fits these data. An alternative proposal to accommodate a $-CH_2CO_2H$ functionality would require a seven-membered (or smaller) ring; however, this reduced ring size renders insurmountable difficulties in satisfactorily accommodating the remaining functionalities. Attempts have been made to gather further evidence to support structure **1** proposed for tagetitoxin, but they have not met with success. Thus, the phosphate group was unreactive to cleavage by phosphatase. However, this was to be expected, because the 1,2-amino phosphate of tagetitoxin would be expected to form an intramolecular zwitterionic association thereby imposing a degree of rigidity to this part of the eight-membered ring and, accordingly, a probable resistance to hydrolysis by phosphatase [Bieleski, R. L., personal communication]. Attempts to methylate the toxin (diazomethane) led to a loss of ninhydrin reactivity and this avenue was not pursued further. The toxin reacted readily with dilute hydrochloric acid (0.05 M) at ambient temperature. The reaction product, which had a different TLC migration, gave a positive colour test for thiols with sodium nitroprusside reagent, whereas tagetitoxin gave no such colour reaction. This result is considered to be positive evidence for the presence of the hemithioketal part-structure in tagetitoxin, which ring opens in dilute acid. However, further studies of the acid reaction product liberated from [^{35}S]tagetitoxin failed to yield additional structural information: the product was found to be a mixture of several components (TLE and autoradiography) [1].



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The usual flexibility expected of an eight-membered ring would undoubtedly be lowered in tagetitoxin by virtue of the number of bulky substituents and by further rigidity imparted by the zwitterionic amino phosphate. Thus, on the basis of coupling constants it should be possible to predict the relative stereochemistry, in particular between C-3 and C-6. A coupling of 12 Hz between H-4 and H-5 indicates a *trans* (i.e. diaxial) relationship, and, thus, the much smaller coupling of 3.9 Hz between H-3 and H-4 indicates a *cis* (i.e. axial–equatorial) relationship. The configuration of the phosphate group is not so easily assigned, but a 7.6 Hz coupling between H-5 and H-6 would favour assignment of a *trans* (i.e. diaxial) relationship. These relationships are proposed in **1**, but are only tentative on the basis of the assumed crown conformation.

The structure proposed for tagetitoxin must now await confirmation. Towards this goal, investigations are in progress to prepare a suitable crystal for an X-ray crystallographic structure determination. Our data shows that tagetitoxin, **1**, is a highly unusual and novel molecule synthesized by the organism *P. syringae* pv. *tagetis* and it contrasts markedly with other unusual chemicals from *P. Syringae* pathovars, such as the β-lactam, tabtoxin [7], the phosphosulphamyl tripeptide, phaseolotoxin [8] and the 1-carboxycyclopropyl-1-amide, coronatine [9].

EXPERIMENTAL

Radioactivity was measured by liquid scintillation spectrometry on a Packard TriCarb liquid scintillation spectrometer. MS were recorded by field desorption at the Space Sciences Laboratory, University of California, Berkeley. ^1H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker 90 instrument; the 270 MHz proton NMR was recorded in the Biochemistry Department, University of Wisconsin, Madison. Aq. samples, prior to measurement of NMR spectra, were passed through short columns (40 × 5.5 mm) of Bio-Rad Chelex 100 (Na^+ form) to remove paramagnetic impurities. Samples for proton NMR were then lyophilized several times from D_2O. All NMR spectra were recorded in D_2O in 5 mm tubes. TLC was on precoated 20 cm × 20 cm × 0.25 mm plates of cellulose MN 300 (Macherey–Nagel) and eluting solvents were either *n*-BuOH–HOAc–H_2O–pyridine (5:1:4:4) (BAWP) or the 'acid solvent' *n*-propyl acetate–90% HCO_2H–H_2O (11:5:3). TLE was also on cellulose MN 300, at pH 7.9 in 0.15 M NH_4HCO_3 at 12° for 20 min at 600 V. Radioactive components were detected on thin layers by autoradiography; non-radioactive components were otherwise detected with either 1% (w/v) ninhydrin in EtOH, or 1% (w/v) ammonium molybdate containing 42 ml/l. 70%

*It is noted that this chemical shift, δ 43.9, assigned to the amino carbon is probably at the upper field extremity for a secondary amine, but that it would be under the influence of an upfield shift by virtue of its β-relationship to the acetate function [4].

perchloric acid and 8 ml/l. conc. HCl. The latter spray could be used also as an overspray after visualization with ninhydrin.

Organisms, culture and bioassay. *Pseudomonas syringae* pv. *tagetis* DAR 26807 was used and cultured as described by Mitchell and Durbin [1]. Liquid culture for toxin production used a chemically defined medium as described in ref. [1]. A plant bioassay to monitor the early purification steps utilized the appearance or absence of apical chlorosis symptoms on zinnia, 2–3 days after the application of toxin-containing solns to their stems [1].

Isolation and purification of tagetitoxin. Tagetitoxin used in this study was produced in 6 l. batches; the culture supernatants from each batch were accumulated and stored frozen. Then toxin from two separate bulk quantities of culture supernatant, 16 and 27 l., was isolated and purified from concentrates as described in ref. [1], by MeOH pptn and sequential partitioning steps in MeOH–$CHCl_3$–H_2O, gel filtration on Biogel P-2, ion exchange chromatography on DEAE Sephadex utilizing gradient elution with NH_4HCO_3 and, finally, partition chromatography on LH-20 Sephadex utilizing MeOH–aq. 0.05 M NH_4OH (1:1). The yield of purified toxin thus obtained was *ca* 1 mg/l. of original culture medium, and was estimated by bioassay to be $< 40\%$ of that present in the original medium [1].

[^{35}S]- *and* [^{32}P/^{35}S]*tagetitoxin.* [^{35}S]Tagetitoxin was prepared from liquid cultures of *P.s. tagetis* containing $^{35}SO_4^{2-}$ as previously described [1]. For the preparation of [^{32}P/^{35}S]tagetitoxin, 1.7 mCi $^{35}SO_4^{2-}$ and 7.1 mCi $^{32}PO_4^{3-}$ was used for 3 l. regular medium which was sampled for radioactivity measurement, then dispensed equally into six 2.1. flasks. The sp. acts. of P and S at the conclusion of the expt (after 4 weeks) were, respectively, 61.96 and 504.6 μCi/mM. The medium was autoclaved then inoculated with *P.s. tagetis* and grown for 6 days at 25° [1]. The cell-free medium at harvest gave a good positive bioassay for toxin content. Toxin was isolated and purified from a concentrate of the culture supernatant by the method of ref. [1]. After passage through Biogel P-2 columns, toxin was located by bioassay and, after DEAE and LH-20 Sephadex CC, by TLC and autoradiography. Following this standard purification sequence, the toxin was not radiochemically pure (as determined by TLE and TLC) and was subjected to rechromatography on LH-20 Sephadex. Then a portion was finally preparatively purified by TLC (acid solvent) and the radioactivities in P and S quantitatively determined. Calculation gave an atomic ratio (μM) of P:S = 0.08634:0.07469 which corresponds to 1:0.87.

Acid hydrolysis and phosphatase reactions. These used [^{35}S]tagetitoxin and were carried out as described in ref. [1].

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