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Structural investigations and biological activity of inositol sphingophospholipids from *Phytophthora capsici*

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Inositol sphingophospholipids that protect pepper (*Capsicum annuum* c.v. Yolo Wonder) against pathogen have been isolated by chromatographic methods from the mycelium of *Phytophthora capsici*. The structure of the major compound was determined by chemical methods and mass spectrometry. Phosphodiester bond cleavage of the phospholipid by mild alkaline hydrolysis liberated a ceramide which contained a C_{16}-sphingosine. This long-chain base was identified by gas chromatography and mass spectrometry of its trimethylsilyl derivative. One of the amide-linked fatty acids was found to be 4-hydroxy-2 docosenoic acid. Fast-atom-bombardment mass spectrometry and fast-atom-bombardment collison-induced tandem mass spectrometry were used to characterize the ceramide as *N*(4-hydroxy-2-docosenoyl)C_{16}-sphingosine. These sphingolipids have a protective effect on cotyledons of young peppers against necrotic lesions induced by the pathogen *P. capsici*.

Various lipids from parasitic fungi have been found to be elicitors of plant defense reactions [1–4] and the polyunsaturated fatty acids, arachidonic and eicosapentaenoic acids, found in mycelial extracts of *Phytophthora infestans*, are highly active elicitors of the hypersensitive response in potato tuber tissue [5–7]. Creamer and Bostock [8] reported that triacylglycerols and phospholipids of the mycelium of *P. infestans*, containing arachidonic and eicosapentaenoic acids, elicit a hypersensitive response with sesquiterpenoid phytoalexins accumulation in potato tubers. Molot et al. [9, 10] isolated a mycelial extract from *Phytophthora capsici* which induced protection in cotyledons of pepper infected by a zoospores suspension. Lhomme et al. [11] found that the lipid components of this mycelial extract induced protection in pepper against the pathogen *P. capsici*.

The present paper describes the isolation and the characterization of the most active fraction as inositol sphingophospholipids.

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Abbreviations. HPTLC, high-performance thin-layer chromatography; GC/MS, gas chromatography/mass spectrometry; FAB-MS, fast-atom-bombardment mass spectrometry; FAB-MS/MS, fast-atom-bombardment tandem mass spectrometry; CID, collision-induced dissociation; MS-1 and MS-2, first and second of two high-resolution mass spectrometers in tandem; PtdSph, inositol sphingophospholipids from *Phytophthora capsici*; *m/z*, mass/charge ratio.

Enzyme. Alkaline phosphatase (EC 3.1.3.1).

MATERIALS AND METHODS

Reference compounds and chemicals

All chemicals and solvents were purchased from E. Merck (FRG) or Prolabo (France), silicic acid (100 mesh) from Mallinckrodt Ltd (USA), silicic acid Bio-Sil HA (325 mesh) from BioRad Laboratories (USA) and silica gel 60 F254 for high-performance thin-layer chromatography (HPTLC) glass plates from E. Merck (FRG). Alkaline phosphatase from *Escherichia coli*, natural D-sphingosine, D-sphingosine sulfate from bovine brain cerebrosides and standard phospholipids were purchased from Sigma Chemical Co. (USA). The silylating reagent *N*,*O*-bis(trimethylsilyl)trifluoracetamide was purchased from Serva (FRG).

Fungal cultures

Two strains of *Phytophthora capsici* have been used: 15-12 A (monozoospore strain) and 197. They were grown on unshaken potato dextrose medium during 6 weeks at 22 °C under 16 h of light by day. Mycelium was separated from medium by centrifugation.

Extraction and fractionation of lipids

The mycelium was treated with ethanol, *n*-butanol and hexane as previously described [1]. The extract (1 g) was treated with 150 ml chloroform/methanol/water (8:4:3, by vol.) and lipids were separated on a Mallinckrodt silicic acid/celite (2:1) column with chloroform and chloroform containing increasing concentrations of methanol. Inositol sphingo-

phospholipids were eluted with chloroform/methanol (75:25, by vol.) and purified by a second chromatography on a column of Bio-Sil HA silicic acid with chloroform/methanol (50:50, by vol.).

Thin-layer chromatography

Thin-layer chromatography of lipids was performed on HPTLC plates with the following solvents: chloroform/methanol/water (65:25:4, by vol.); methyl acetate/*n*-propanol/chloroform/methanol/0.25% aqueous KCl (25:25:28:10:7, by vol.) [12].

The phospholipids were analyzed by two-dimensional HPTLC with the solvent systems of Rouser et al. [13] and Owens [14]. They were detected by iodine vapor or by spray reagents: ninhydrin according to Russel [15], Dittmer and Lester reagent modified by Vaskovsky and Kostetsky [16], H_2SO_4 (10% by vol.) with vanilin. Sphingosine and related bases were stained with the ninhydrin reagent.

Analytical methods

Hydrolysis of phospholipids was performed with 4 M or 6 M HCl for 48 h at 100 °C; fatty acids were recovered from the hydrolysate by chloroform and esterified with diazomethane, inositol was acetylated with acetic anhydride/pyridine (1:1, by vol.) at 100 °C for 15 min and analyzed by gas chromatography.

Sphingosines were released by methanolysis according to Laine et al. [17] and their *N*-acetyl derivatives were prepared by addition of a mixture of methanol/acetic anhydride (4:1, by vol.), the reaction was allowed to proceed overnight at room temperature. The silylation of hydroxyl groups was performed with the silylating reagent for 30 min at 60 °C. The derivatives were analyzed by gas chromatography.

Phosphorus was measured by the method of Lowry et al. [18].

Enzymatic reaction

Alkaline phosphatase (10 µl) was added to inositol phosphate (100 µg) in 500 µl 0.2 M Tris/HCl pH 8.4. After incubation at 37 °C for 1 h, the mixture was assayed for free inositol.

Gas chromatography

Gas chromatography was carried out on an Intersmat IGC 120F apparatus fitted with ECNSS-M (3% on Gas Chrom Q, 80–100 mesh, 180 °C) in a glass column (0.6 × 150 cm) for analysis of hexaacetylinositol, with SE 30 (3% on Chromosorb W.A.W., 80–100 mesh, 230 °C) in a stainless steel column (0.3 × 150 cm) or with OV 17 (3% on Chromosorb W.A.W.-D.M.C.S., 80–100 mesh, 190 °C) in a glass column (0.3 × 200 cm) for analysis of *N*-acetyl-*O*-trimethylsilyl-sphingosines. The same device, which was fitted with a capillary SP 2100 column (0.22 mm × 25 m, 140–260 °C) was used for analysis of fatty acid methyl esters.

Combined gas chromatography/mass spectrometry (GC/MS) was performed on a Ribermag R10-10C apparatus (temperature 200 °C, ionisation potential 70 eV and current intensity 200 µA), which was connected to a gas chromatograph equipped with a capillary column SE 30 (0.22 mm × 25 m).

Mass spectra analyses

Fast-atom-bombardment mass spectrometry (FAB MS) of the phospholipid fraction was carried out using a VG Analytical ZAB-HF mass spectrometer. Xenon was used as the bombarding gas and the M-Scan atom gun was operated at 8–10 kV. Samples were dissolved in aqueous 5% acetic acid and a 1-µl sample loaded into a matrix composed of glycerol/thioglycerol (1:1). Spectra were recorded on oscillographic paper using a 300-s mass-controlled linear scan and assigned manually.

High-resolution (1:10000) FAB-MS measurements were made with a MAT 731 instrument with Ion Tech B11N neutral atom gun that produced a 6-kV xenon beam. Accelerating voltage was 8 kV; ion source temperature 35 °C. Peak-matching was performed against the glycerol cluster at m/z 645.3392 (G_7H^+).

Low-resolution FAB and FAB collision-induced decomposition (CID) tandem mass spectrometry (MS/MS) spectra were all obtained with a Jeol HX110/HX110 tandem mass spectrometer, operated at ± 10 kV accelerating voltage, with ± 18.5 kV post-acceleration at the detector. The Jeol DA-5000 data system was used for instrument control and data acquisition and processing.

For positive-ion FAB measurements, the sample was dissolved in dimethylsulfoxide/glycerol (1:1); for the negative-ion FAB spectra, a chloroform/methanol/triethanolamine solution was used. About 1 µg was used for each FAB analysis. For the FAB experiments, a 6-kV neutral xenon beam was produced with a Jeol gun; ion source temperature was 20 °C.

In FAB-MS/MS experiments, collisions took place on a cell located between the first (MS-1) and the second (MS-2) of two high-resolution mass spectrometers in tandem. The collision gas was helium, at a pressure sufficient to reduce the parent ion signal to 20% of its value. Both MS-1 and MS-2 were operated at more than unit resolution, usually 1:1000. MS/MS spectra were recorded during linked scans of MS-2 at a constant B/E ratio.

Elicitor activity assay

From young peppers (*Capsicum annuum* c. v. Yolo Wonder, susceptible to *P. capsici*), the cotyledons were removed and placed in water. On the lower side of the cotyledon, 25 µl elicitor fraction was added and, after 24 h, on the same site, the cotyledons were infected by a zoospores suspension (10 µl, 62550 zoospores/ml) which was produced as previously described [19]. After 4 days of incubation (22 °C at 16 h of light), the symptoms were noted according to the following rating: 0 = no symptom, 1 = necrosis which invades the whole cotyledon. The cumulated notes were expressed as a susceptibility percentage, 100% corresponding to the all cotyledons rated 1. Each lot involves 15–20 cotyledons.

RESULTS

Isolation and characteristics of inositol sphingophospholipids

Isolation and characteristics of inositol sphingophospholipids (PtdSph) were isolated from the total lipid extract by chromatography on Mallinckrodt silicic acid/celite followed by chromatography on Bio-Sil HA silicic acid (yield 0.01% from the wet mass of mycelium). Two-dimensional HPTLC chromatography of PtdSph is shown in Fig. 1; PtdSph gave

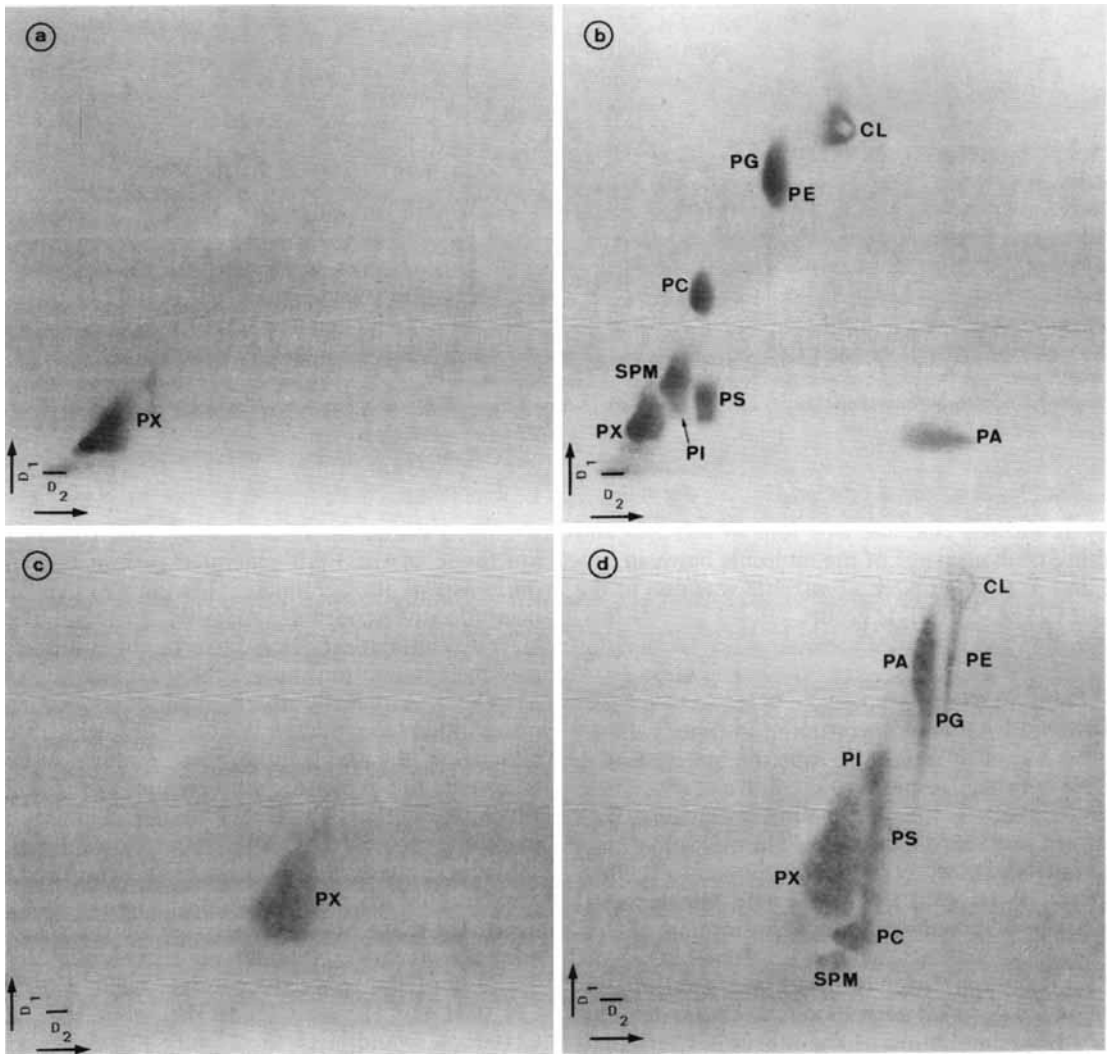


Fig. 1. *Two-dimensional HPTLC chromatography of inositol sphingophospholipids from* Phytophthora capsici *(a and c) and of inositol sphingophospholipids with standard phospholipids (b and d)*. Silica gel plates were developed with the solvent system of Rouser et al. [13] (a and b): i.e. in the first direction (D1) chloroform/methanol/28% aqueous ammonia (65:35:5, by vol.); in the second direction (D2), chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, by vol.), and with the solvent system of Owens [14] (c and d): i.e. in the first direction (D1) chloroform/methanol/water/acetic acid (65:43:3:1, by vol.); in the second direction (D2), chloroform/methanol/water (60:35:1, by vol.). Phospholipids were stained according to Vaskovsky and Kostetsky [16]. PA, phosphatidic acid; PI, phosphatidylinositol; PG, phosphatidylglycerol; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; CL, cardiolipin; SPM, sphingomyelin; PX, inositol sphingophospholipids

positive reactions with specific reagents for phosphorus and lipids but the reaction was negative with ninhydrin. Infrared spectrometry in KBr showed bands corresponding to CH_2 and CH_3 groups (2920 and 2850 cm^{-1}), hydroxyl groups (3400 cm^{-1}), secondary amide (1665 and 1530 cm^{-1}) and phosphate (1260 and 1090 cm^{-1}).

Chemical composition of PtdSph

Elemental analysis gave the following values: C, 62.20%; H, 9.89%; N, 1.65%; O, 22.61%; P, 3.65%; this corresponds to an N/P ratio of 1/1.

Hydrolysis of PtdSph with 4 M or 6 M HCl at 100 °C for 48 h gave inositol, identified by gas chromatography of the acetyl derivative, and fatty acids. The presence of inositol was further confirmed by mass spectrometry of PtdSph. No sugar and glycerol were found in the hydrolysate.

Gas chromatography of fatty acid methyl esters showed the following composition: $C_{18:0}$ (3%), $C_{18:1}$ (6%), $C_{16:0}$ (12%), $C_{22:0}$ (16%), $C_{22:1}$ (33%) and a minor amount of hydroxydocosenoic acid (5%). In fact these percentages did not express the actual composition of PtdSph as the major part of hydroxydocosenoic acid was destroyed by hydrolysis. The mass spectra of PtdSph and of the ceramide obtained from PtdSph showed that hydroxydocosenoic acid was the major fatty acid component of these sphingophospholipids.

The methanolysis of PtdSph according to Laine et al. [17] gave inositol, fatty acid methyl esters and a compound which comigrated on thin-layer chromatography in the solvent of Heape et al. [12] with standard sphingosine (R_F = 1.14). The characterization of this compound was performed by gas chromatography/mass spectrometry of the *N*-acetyl-O-trimethylsilyl derivative. The mass spectrum was similar to that of the C_{16}-sphingosine (R_F = 1.14) derivative previously

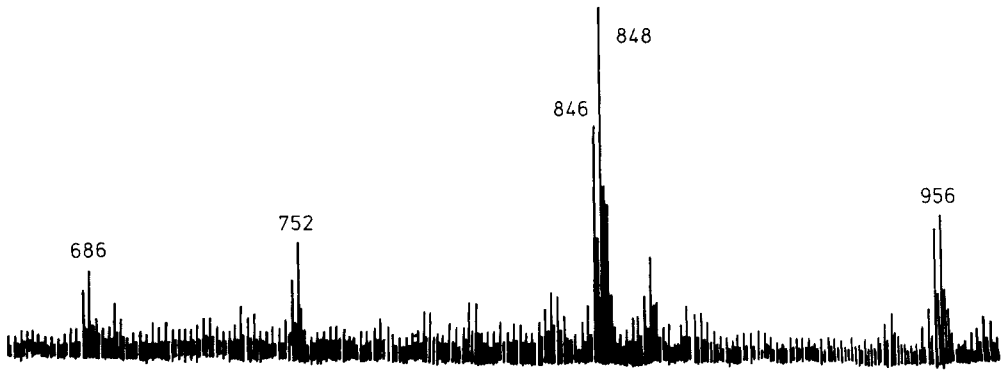


Fig. 2. *Negative-ion fast-atom-bombardment mass spectrometry spectrum of inositol sphingophospholipids from* P. capsici

described [20]. It showed characteristic ions at *m/z* 283 and *m/z* 174 resulting from cleavage of the molecule between carbon atoms 2 and 3. A small peak at *m/z* 103 was due to the loss of terminal CH_2-O-Si$(CH_3)_3$ group.

Structure of PtdSph

The negative-ion FAB mass spectrum of PtdSph is shown in Fig. 2. In the region of molecular ions the major peak is found at *m/z* 848 with smaller peaks at *m/z* 846 and 850. These values were confirmed in the positive-ion spectrum which displayed peaks at *m/z* 850, 848 and 852. The molecular mass of the major compound, M_r = 849, corresponds to the formula $C_{44}H_{84}NO_{12}P$, in good agreement with the elemental analysis; this compound contains equimolar amounts of C_{16}-sphingosine, hydroxydocosenoic acid, inositol and phosphate. The negative FAB mass spectrum shows a fragmentation triplet at *m/z* 684, 686, 688 which corresponds to ceramide phosphates produced by elimination of the inositol. The signals at *m/z* 956 are consistent with thioglycerol adducts on the molecular ions, which commonly form in FAB-MS of unsaturated fatty acids.

The ceramide was prepared by alkaline hydrolysis of PtdSph with 1 M potassium hydroxide at 37°C for 15 h according to Smith and Lester [21]. The water-soluble fraction of the hydrolysate was submitted to an acid hydrolysis and inositol was identified by gas chromatography of its peracetylated derivative. Inositol was also found in the water-soluble fraction after the action of the alkaline phosphatase. The chloroform-soluble fraction of the alkaline hydrolysate of PtdSph was submitted to methanolysis. Fatty acid methyl esters already found in the hydrochloric acid hydrolysate of PtdSph (see above) were identified by gas chromatography. In addition, another compound was identified as sphingosine by HPTLC in comparison with a standard. Thus the chloroform-soluble fraction was the ceramide moiety of PtdSph.

The determination of the structure of the ceramide was performed by FAB-MS and FAB-MS/MS. The mass spectra show the molecular ion $(M + H)^+$ at *m/z* 608 with positive-ion FAB and $(M - H)^-$ at *m/z* 606 with negative-ion FAB for the major component, M_r = 607. The exact mass of the $(M + H)^+$ ion was measured as *m/z* 608.5637 (calculated for $C_{36}H_{74}NO_4$, *m/z* 608.5617). The presence of three hydroxyl groups in the ceramide was confirmed by positive-ion FAB-MS of the acetylated ceramide. In the molecular ion region, the most abundant peak was observed at *m/z* 734, which corresponds to the $(M + H)^+$ of a triacetylated derivative.

Fig. 3 shows the CID MS/MS spectrum in the positive-ion mode of the FAB-generated protonated molecular ion (*m/z* 608) of the ceramide. This ion (Y) can undergo fragmentation as reported previously by Domon and Costello [22]: by elimination of one, two or three water molecules to give the product ions at *m/z* 590 (Y′) *m/z* 572 (Y″) and *m/z* 554 (Y‴). As usually observed for ceramides containing an unsaturated base, the ion W at *m/z* 272, which would arise by cleavage of the amide linkage and removal of the fatty acid, is absent, but peaks are observed at *m/z* 254 and *m/z* 236 which are derived therefrom by the elimination of one or two water molecules. This fragmentation characterizes a C_{16}-sphingosine base in the ceramide and confirms the previous results.

In the CID MS/MS spectrum of the $(M-H)^-$ ion at *m/z* 606 (Fig. 4), fragmentation between carbon atoms 2 and 3 of the sphingosine gives, by loss of the fragment $CH_3(CH_2)_{10}CH = CHCHOH$, or $CH_3(CH_2)_{10}CH = CHCH = O$ and H_2O, the T and S [22] peaks corresponding respectively to $(M\text{-}211\text{-}2H)^-$ at *m/z* 394 and to $(M\text{-}211\text{-}H_2O)^-$ at *m/z* 378. The T′ peak at *m/z* 376 and S′ peak at *m/z* 360 result from further loss of water. These sets of fragments indicate that an hydroxylated, monounsaturated fatty acid is present in the ceramide. The low-abundance peaks at *m/z* 283 and 353 result from cleavage on either side of the hydroxyl group at C4 in the carbon chain of the fatty acid. Thus the positions of the hydroxyl group and of the double bond in the fatty acid are determined.

All these results are in agreement with the formula shown in Fig. 5 for the most abundant component of *P. capsici* inositol sphingophospholipids. Other components differ in the nature of the fatty acyl group and/or the degree of unsaturation in the base. As noted above the minor amount of the main fatty acid, 4-hydroxy-2-docosenoic acid, found after total hydrolysis of the inositol sphingophospholipids was explained by the lability of this acid which was partially destroyed under the hydrolysis conditions. Variations in the surface activity of the different components also probably have some effect on the relative abundances of the molecular ions observed in the FAB mass spectra.

Biological activity

Inositol sphingophospholipids (PtdSph) induce protection in *Capsicum annuum* against *Phytophthora capsici*. PtdSph was applied on the *C. annuum* Yolo Wonder at increasing concentrations. The data (Fig. 6) show that the sensitivity of

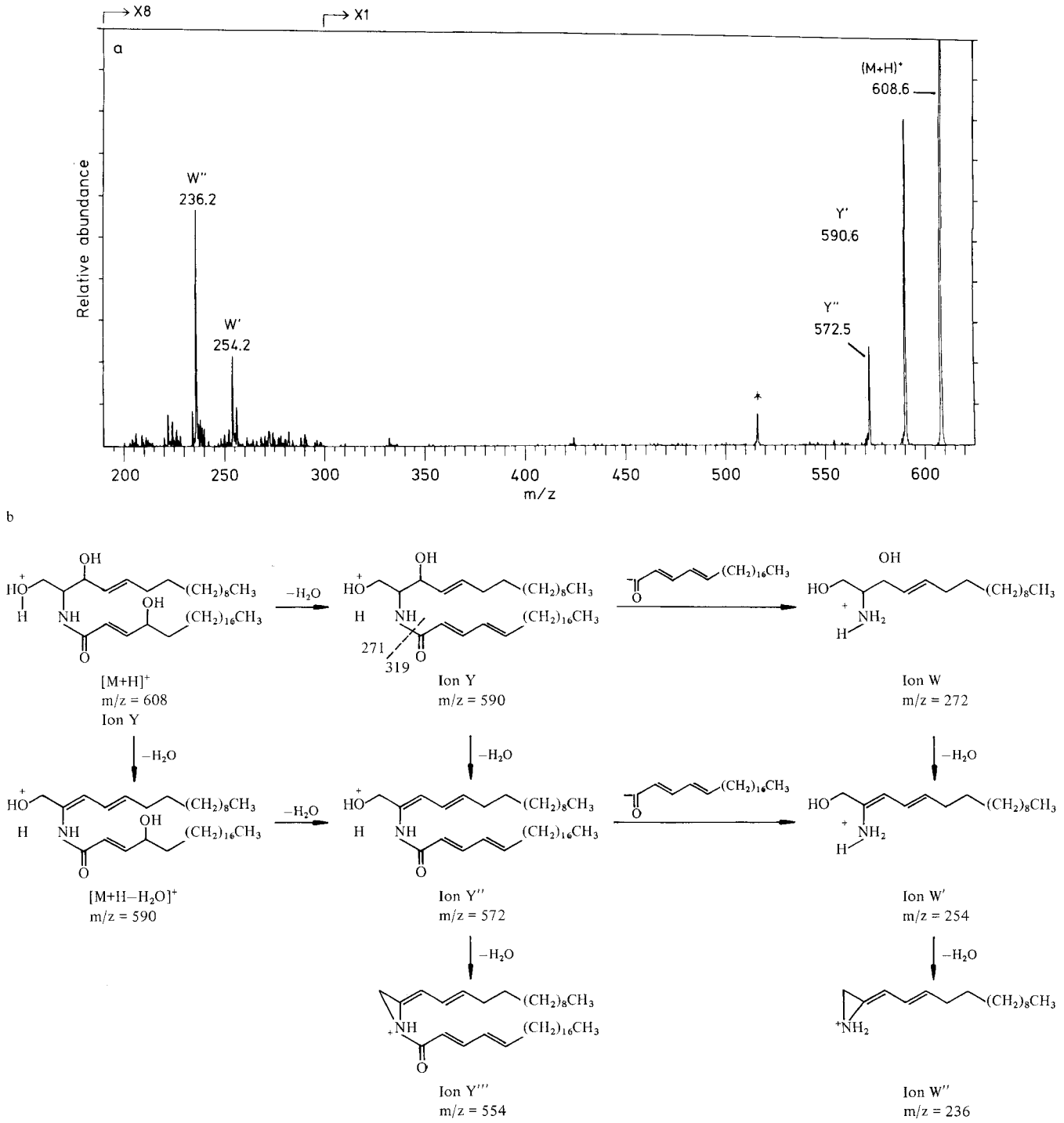


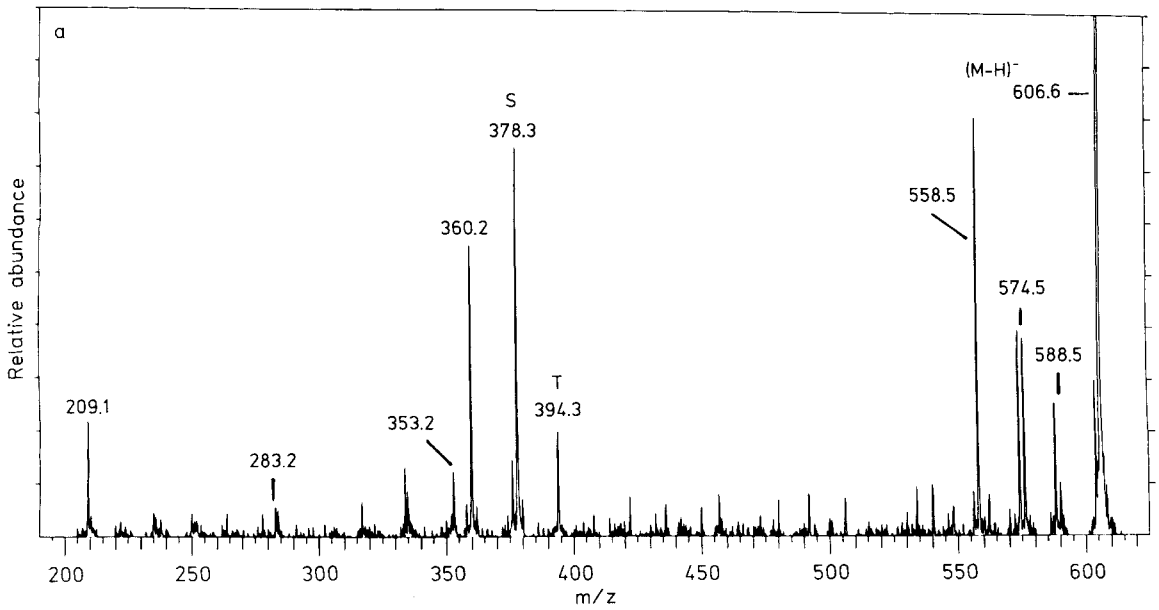
Fig. 3. *(a) Fast-atom-bombardment tandem mass spectrometry spectrum and (b) fragmentation pathways in the positive-ion mode of the ceramide moiety of inositol sphingophospholipids.* (a) Collision-induced dissociation of $(M + H)^+$, *m/z* 608. Peak marked with an asterisk (*) comes from a glycerol cluster

the *Capsicum* cotyledons against *P. capsici* decreases rapidly with an increase of the amount of PtdSph. The sensitivity of the *Capsicum* to its pathogen decreases by half after application of 7 μg/ml of PtdSph; the protective effect is maximal for doses of PtdSph varying between 50–250 μg/ml.

DISCUSSION

Our data establish the structure of the inositol sphingophospholipids in *P. capsici* (Fig. 5). The major compound contains a C_{16}-sphingosine linked to 4-hydroxy-2-docosenoic acid. The position of the inositol phosphate can be attributed to the C1 of the ceramide by analogy with other known inositol sphingophospholipids.

Sphingophospholipids containing inositol have been found in various species of plants, yeasts, bacteria and fungi [23]. However few structures were completely determined. In fungi, the long-chain base is generally represented by C_{18}-sphingosine or C_{18}-phytosphingosine and the most common fatty acids are 2- or 3-hydroxyhexacosanoic acids [21]. The



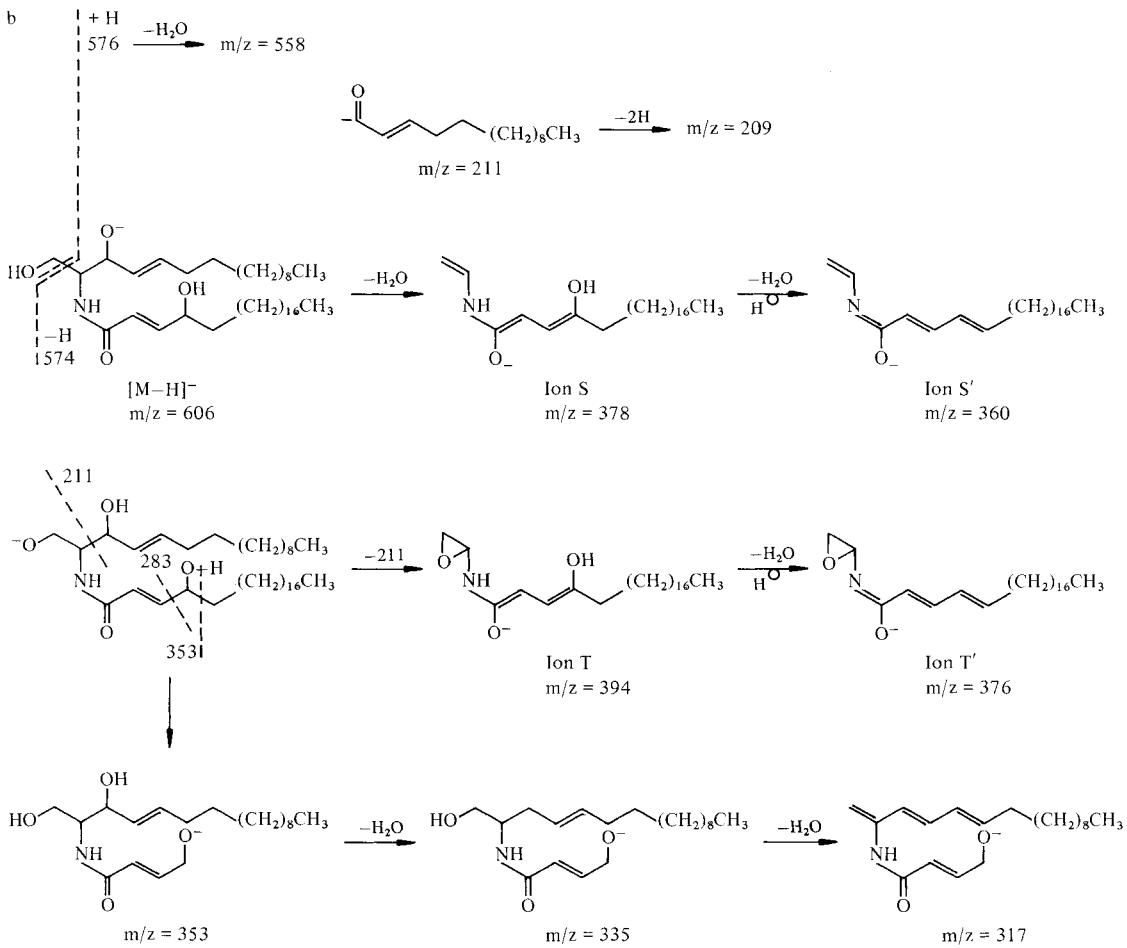
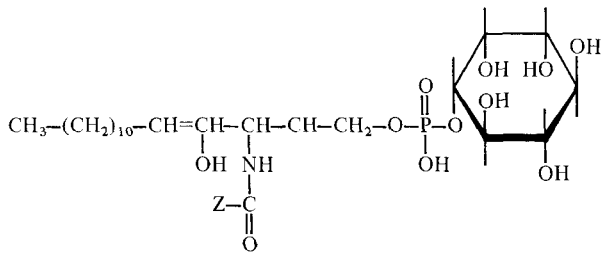


Fig. 4. *(a) Fast-atom-bombardment tandem mass spectrometry spectrum and (b) fragmentation pathways in the negative-ion mode of the ceramide moiety from inositol sphingophospholipids.* (a) Collision-induced dissociation of $(M-H)^-$, *m/z* 606



General formula of inositol sphingophospholipids (PtdSph) with Z=

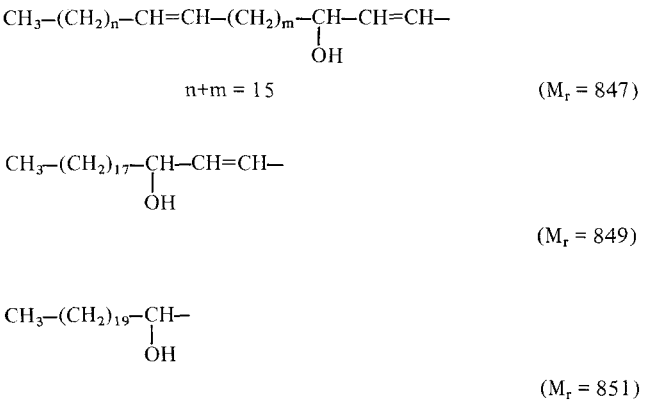


Fig. 5. *Structure of inositol sphingophospholipids (PtdSph) from* P. capsici

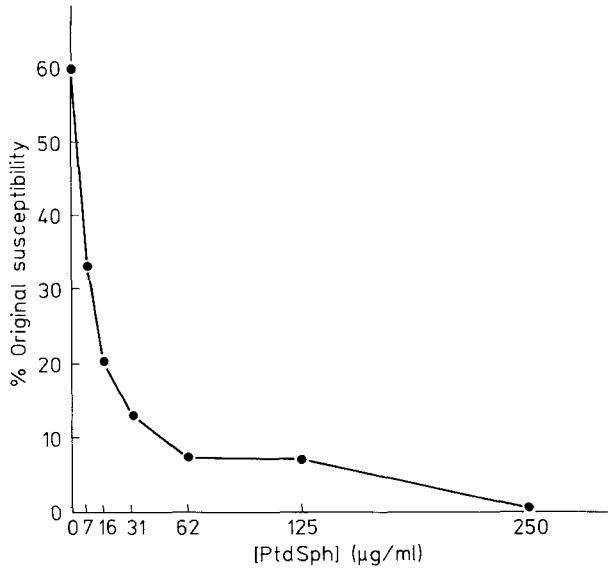


Fig. 6. *Biological activity of inositol sphingophospholipids (PtdSph) from* P. capsici. The volume of elicitor solution used was 25 µl/cotyledon

major fatty acid, 4-hydroxy-2-docosenoic acid, of the inositol sphingophospholipids from *P. capsici* does not appear to have been found previously in fungal lipids. Work on the sphingolipids of other species of *Phytophthora* are now in progress.

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