Thin Layer Chromatographic and Infra Red Spectral Evidence for the Presence of Phosphonolipids in *Cicada oni*

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Z. Naturforsch. **42c**, 1023–1026 (1987); received November 21, 1986/May 12, 1987

Phosphonolipids, Phosphono Analogues, Ceramide Aminoethyl Phosphonate

The phosphonolipids from whole *Cicada oni* have been isolated by preparative thin-layer chromatography (TLC) in methanol-water (2:1, v/v) and have been identified by TLC, infra-red spectroscopy, phosphono phosphorus determinations and elemental microanalyses. The presence has been confirmed of the phosphono analogues of phosphatidyl choline, phosphatidyl ethanolamine, sphingomyelin and of ceramide aminoethyl phosphonate.

Introduction

No evidence has been published for the presence of phosphonolipids in insects to this date. Experiments, however, have been carried out on the metabolism of phosphonolipids in insects and this can hardly be construed upon as providing evidence for the presence of phosphonolipids in insects [1].

In this present communication evidence is provided for the presence of phosphonolipids in *Cicada oni* of the genus Hemiptera of the Homoptera of the family of Cicadidae.

The phosphonolipids have been obtained by preparative TLC in methanol:water (2:1, v/v), free from phospholipids from the total phospholipid fraction, and have been identified by TLC, infra-red spectroscopy and elemental analyses on the individual phosphonolipids after silicic acid column chromatography.

Materials and Methods

The *Cicada oni* were collected during the summer in their natural environment at Agia Marina in Attica, an area free from air pollution.

Solvents for column chromatography and TLC were analytical reagent grade (Merck) and were distilled before use. Silicic acid for chromatography was purchased from Sigma (St. Louis, MO, USA).

The phosphono analogues of phosphatidyl choline, phosphatidyl ethanolamine and sphingomyelin were synthetic products. Phosphatidyl choline and phosphatidyl ethanolamine were purchased from Koch-Light (Colnbrook, U.K.).

IR spectra were recorded on a double beam Perkin-Elmer 197 spectrophotometer.

A glass column (35 × 1.6 cm I.D.) was employed for the separation of the phosphonolipids.

Preparative TLC was performed on glass plates coated with silica gel G to a thickness of 0.75 mm. The chromatograms were developed in methanol/water (2:1, v/v) as solvent (system A) with the run normally taking approx. 80 min for full development. The solvent system chloroform/methanol/water (65:25:4, v/v/v) (system B) was also used for identification purposes and for the quantitative isolation of the respective phosphonolipids.

TLC of neutral lipids was performed in system C, pet ether/ether/acetic acid (90:10:1, v/v/v); in system D, pet ether/ether/acetic acid (80:20:1, v/v/v) and in system E, isopropyl ether/acetic acid (96:4, v/v).

Visualisation was effected with iodine, ammonium molybdate, ninhydrin α-naphthol-sulphuric acid sprays and the Stillway and Harmon procedure [2]. Total phosphorus and phosphono-phosphorus were determined by the procedure of Kapoulas [3] and total nitrogen and lipid nitrogen by the procedures of Kjeldahl and Lea-Rhodes [4].

Procedure

The *Cicada oni* sample, which weighed 30.5 g, was homogenized in chloroform/methanol (2:1, v/v) with a Sorvall homogenizer. The lipids from the homogenized sample were extracted according to the procedure of Bligh-Dyer [5] and the solvents were evaporated under vacuum at 35 °C. The residue was redissolved in 100 ml of chloroform and rapidly extracted several times with water containing 5%

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0341–0382/87/0900–1023 $ 01.30/0

NaCl. The chloroform layer was filtered through anhydrous sodium sulphate and again evaporated to dryness. The total lipids were extracted exhaustively with acetone to constant phospholipid weight. The total neutral lipids and the total phospholipids were retained for further analyses and were kept in a vacuum desiccator over phosphorus pentoxide for 24 h at 7 °C.

The total neutral lipids were then subjected to qualitative TLC analysis for the identification of the inherent neutral lipids in solvent systems C, D and E.

The total phospholipids were dissolved in chloroform/methanol (2:1, v/v) and subjected to preparative TLC in solvent system A. The band whose R_f ranged from 0.80–0.98 was scraped off and the phosphonolipids were re-extracted from the silica gel using chloroform. The phospholipids were similarly re-extracted from the silica gel using chloroform/methanol (2:1, v/v).

Following this the phosphonolipids were checked for purity by rechromatographing a small sample in solvent system A, when no phosphorus or other lipid could be detected at the origin. The phosphonolipids were then subjected to TLC analysis to identify preliminarily the components present. They were chromatographed on glass plates coated with silica gel G to 0.25 mm thickness, in system B. The chromatograms were additionally tested for carbohydrates and amino-acids and the absence of both classes of compounds was confirmed.

After initial identification the phosphonolipids were fractionated on a silicic acid column as described in previous experiments [6]. Silicic acid, 10.7 g, was used for the chromatographic separation, the column was loaded to a height of 10.9 cm with a total volume of 26.0 ml. The flow rate was maintained at 1.7–1.8 ml per minute.

Results and Discussion

Cicada oni, 30.5 g, furnished, after extraction, 0.155 g of phospholipids, of which 3.6% were phosphonolipids.

Chromatography of the acetone soluble lipids, neutral lipids, in solvent systems C, D and E furnished the following results:

O-dialkyl glyceryl ethers at $R_F = 0.11$,
Long chain alcohols at $R_F = 0.16$,
and 1,2 diglycerides
Fatty acids at $R_F = 0.17$,
1,3 diglycerides at $R_F = 0.21$,
Triglycerides at $R_F = 0.34$ and $R_F = 0.45$,
Trialkyl glyceryl ethers at $R_F = 0.96$.

Chromatography of the total phospholipids in solvent system, chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1, v/v/v/v/v) gave the following results:

Sphingomyelin at $R_F = 0.25$,
Phosphatidyl serine at $R_F = 0.47$,
Cerebrosides at $R_F = 0.92$,

and of the phospholipid fraction in solvent system B gave similarly evidence for the presence of the following phospholipids:

Phosphatidyl serine at	$R_F = 0.107$, relative abundance:	2.9%
Sphingomyelin at	$R_F = 0.15$	5.8%
Phosphatidyl choline at	$R_F = 0.43$	33.0%
Phosphatidyl ethanolamine at	$R_F = 0.62$	32.0%
Lysophosphatidic acid or phosphatidic acid at	$R_F = 0.71$	15.8%
Phosphatidyl-N-methylethanolamine at	$R_F = 0.81$	5.7%
Cardiolipin at	$R_F = 0.84$	4.8%

The IR spectrum of the total phosphonolipid fraction is shown in Fig. 1.

Preliminary TLC study of the total phosphonolipid fraction in solvent system B furnished the following results:

$R_F = 0.44$, phosphono analogue of phosphatidylcholine;
$R_F = 0.60$, phosphono analogue of phosphatidylethanolamine;
$R_F = 0.70$, phosphono analogue of CAEP;
$R_F = 0.93$, phosphono sphingomyelin.

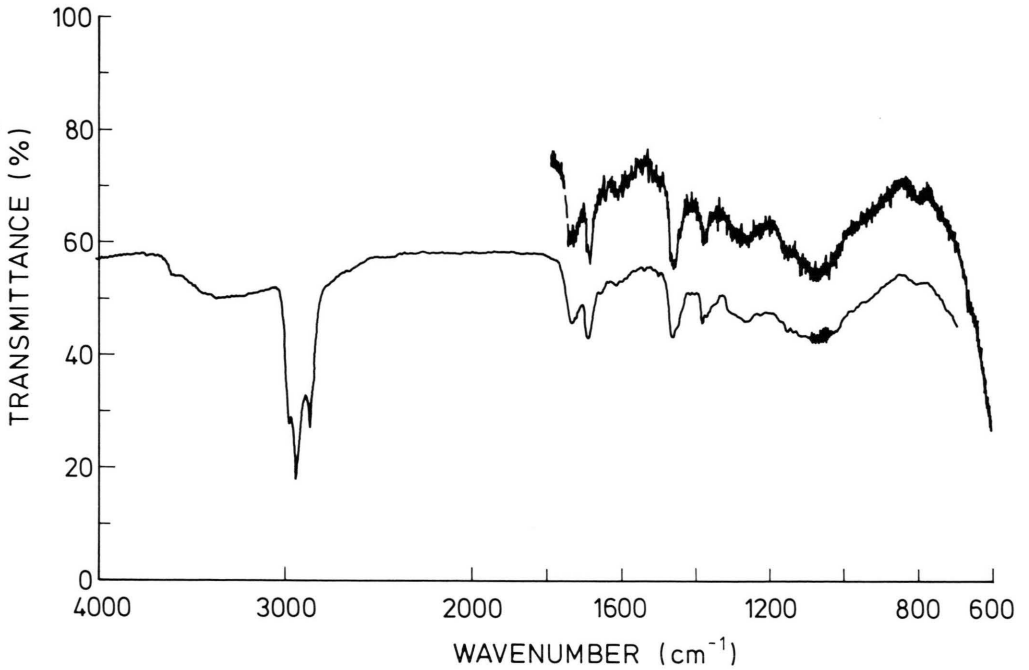

Fig. 1. IR spectrum of the total phosphonolipids isolated form *Cicada oni*, as a thin film from dry chloroform.

Table I provides information regarding the relative abundance of the various phosphonolipids in the fractions isolated; the fractionation pattern for the phosphonolipids is as shown in Fig. 2.

Information regarding the elution of the column is given in Table II and the composition of the fractions obtained by the column chromatography of the natural phosphonolipids is contained in Table III. During the course of these investigations no amino-acids or sugars were detected on the chromatograms examined.

Phosphono phosphorus determinations were carried, phospholipid and phosphonolipid fractions

Table I. Analytical data for the phosphonolipids isolated after column chromatographic separation and fractionation.

Phosphonolipid	% N	% P	% abundance
Phosphono-cephalin	2.61	4.42	24.8
Phosphono-lecithin	2.17	4.31	34.8
CAEP	4.19	5.01	20.3
Phosphono-sphingomyelin	3.79	4.49	9.5

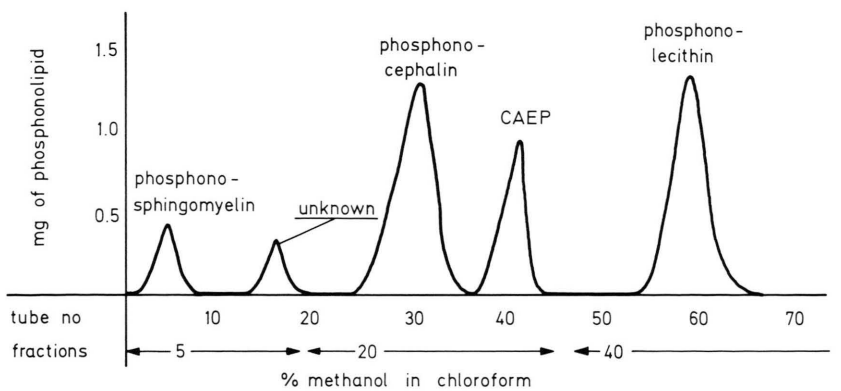

Fig. 2. General elution pattern of the natural phosphonolipids isolated from *Cicada oni*. Solvents used were various percentages of methanol in chloroform. The phosphonolipids were applied to the column in 3.0 ml of chloroform.

separated by preparative TLC and were shown to be free from interfering phosphonolipids and phospholipids respectively. Chromatography of the isolated phosponolipids in solvent system B provided evidence for the presence of the phosphono analogues of phosphatidyl choline, phosphatidyl ethanolamine, ceramide aminoethyl phosphonate and of shingomyelin.

Table II. Elution of the chromatographic column, 35 cm × 1.6 cm I.D., loaded with 10.7 silicic acid to a height of 10.9 cm and a total volume of 26.0 ml. Flow rate, 1.7–1.8 ml per minute. Fractions of approximately 4.5 ml were collected.

% Methanol in chloroform	Column volumes	Total ml of solvent	Fractions collected
5	3	75	1– 19
20	5	130	20– 46
40	7	180	47– 82
80	5	110	83–104

The fractionation pattern of the natural phosphonolipids closely resembles that of their synthetic counterparts [7–9]. Thus the experimental data indicates the presence of phosphonolipids in *Cicada oni*. Analysis and characterisation of the phosphonolipids was undertaken after their silicic acid column chromatographic separation.

An important observation is the occurrence of CAEP in the *Cicada oni* sample; experiments with insects fed with aminoethyl phosphonate failed to prove that CAEP was synthesized by insects. In the present case, however, CAEP has been shown to exist in insects. As far as is known this is the first instance that phosphonolipids are reported to occur in insects, which might prove to be of importance in the elucidation of the biochemistry of insects.

Table III. Composition of the fractions obtained by chromatography on silicic acid of the natural phosphonolipids isolated from *Cicada oni*. Total recovery was 99.4%.

Solvent	Fractions collected	TLC R_F system A	R_F system B	Component identified
5% methanol in chloroform	2– 9	0.94	0.93	phosphono-sphingomyelin
20% methanol in chloroform	24–34	0.85	0.60	phosphono-cephalin
	37–44	0.80	0.85	CAEP
40% methanol in chloroform	55–66	0.82	0.44	phosphono-lecithin

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