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CHARACTERIZATION OF PHOSPHONOMYCIN BY MICROCHROMATOGRAPHIC AND RELATED TECHNIQUES

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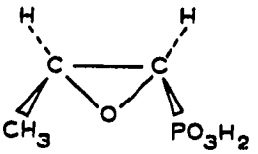
SUMMARY

Phosphonomycin has been characterized by R_F values and color reactions in a variety of chromatographic systems which include thin-layer and paper chromatography. Gas–liquid chromatography was carried out on the di-trimethylsilyl derivative. The gas–liquid chromatography peak was identified by mass spectrometry. Paper chromatography was adapted for purification of a crude concentrate and the purity monitored by gas–liquid chromatography.

INTRODUCTION

Phosphonomycin has been isolated from fermentation sources by ion-exchange and adsorption methods[1]. The antibiotic has been shown to have the following structure[2]:



This report describes microchromatographic and related techniques useful for characterization of the antibiotic. These procedures have been applied in monitoring the purity of concentrates and in establishing the identity of antibiotics present in fermentation broths or in samples of biological origin. Detection of small quantities on paper chromatography (PC) or thin-layer chromatography (TLC) is possible by bioautographic methods. In addition, chemical detection by reagents useful for phosphonates can be carried out using larger quantities of the compound. The procedure of BORST-PAUWELS[3] employing ΔR_m between two paper chromatographic systems was studied for the determination of the number of charged groups in the antibiotic using both crude and highly purified samples. Gas–liquid chromatography (GLC) and combined GLC–mass spectrometry (MS)[4,5] were employed to provide information concerning the composition and purity of samples of antibiotics.

MATERIALS

Reagent grade chemicals were used for the preparation of derivatives or detection reagents. Thin-layer plates were obtained from Anal. Tech. Whatman No. 1 paper was used for PC.

METHODS

PC and TLC

The antibiotic-containing samples were spotted as aqueous solutions containing 1–20 μg of phosphonomycin in up to 25 μl of solution. The paper strips were developed by descending chromatography until the solvent front traveled 20–25 cm. The procedure has been applied to fermentation broths, urine samples from laboratory animals and humans, and partially purified concentrates. For preparative PC crude phosphonomycin (30–50% pure) obtained after primary solvent partition purification[1] was streaked on paper sheets at a loading of about 1 mg/cm. The sheets were developed with system D. The zones located by bioautography were cut out and eluted with anhydrous methanol to obtain purified antibiotic.

Thin-layer plates were loaded with up to 150 μg of phosphonomycin in 2–5 μl and developed by the ascending technique. Mixed solvents were freshly prepared. Bioautographs were carried out by placing the dried plate on filter paper in contact with agar plates seeded with a sensitive organism.

The following solvent systems were used (volume ratios): System A, *n*-propanol–2 *N* methylamine (7:3); System B, *n*-propanol–2 *N* isopropylamine (7:3); System C, *n*-butanol–acetic acid–water (3:1:1); System D, *n*-butanol–acetic acid–water (4:1:1); System E, isopropanol–conc. ammonia–water (7:1:2); System F, methanol–water–triethylamine (80:20:5). The mobility of the antibiotic in the various systems is indicated in Table I.

TABLE I

R_F VALUES OF PHOSPHONOMYCIN IN VARIOUS SYSTEMS

Carrier	*System*					
	A	*B*	*C*	*D*	*E*	*F*
Paper	0.19	0.26	—	0.26	—	—
Cellulose thin layer	—	—	—	0.26	0.18	—
Silica Gel G thin layer	—	—	0.33	—	—	0.75

GLC and combined GLC–MS

The sample (preferably as the ammonium or benzylamine salt) was dissolved in bis-trimethylsilylacetamide (BSA, a powerful trimethylsilylating reagent[5,6]) and heated for 5 min at 60° or allowed to stand at room temperature for 30 min to form the trimethylsilyl (TMSi) derivative. Aliquots (1–2 μl of 0.5% solutions) of the reaction mixtures were used directly for analysis. Chromatography was carried out with a Barber-Colman Model 5000 instrument equipped with a 6 ft. × 4 mm glass U-tube; 4% F-60 (DC-560) coated over 1.5% SE-30 on 100–120 mesh acid-washed and silan-

ized[5] Gas-Chrom P; the temperature was programmed from 65° to 220° at 3°/min, and then held at the upper limit for 10 min; 15 p.s.i.; hydrogen flame ionization detection. Similar column conditions were employed with an LKB Model 9000 combined gas chromatograph–mass spectrometer. Mass spectra were obtained using 70 eV ionizing potential, 250° ion source temperature, 50 μA filament current, and 3.5 kV accelerating potential.

Detection of phosphonomycin

Bioautography

The antibiotic is readily detected at low levels (1–5 μg) by bioautographic techniques using agar seeded with a strain of *Proteus vulgaris* (MB-838). This procedure has been applied to both PC and TLC. Acidic systems must be well dried to eliminate acetic acid which affects the growth of the microorganism.

Phosphonic acid reagents

Molybdate reagent. Although phosphonomycin cannot be detected by molybdate reagents useful for phosphates, reagent containing 3% perchloric acid and 1% ammonium molybdate in 0.01 *N* hydrochloric acid readily reveals 5–30 μg of antibiotic as an intense blue zone after heating for 5 min at 85° (ref. 7).

Iron sequestering. In common with many phosphates, phosphonomycin sequesters iron. TLC or PC zones containing 20 μg or more of antibiotic can be readily detected using this property. The chromatogram is first sprayed with 0.1% ferric chloride ($FeCl_3 \cdot 6H_2O$) in 80% aqueous ethanol. After drying, the antibiotic is revealed as a white or light buff colored zone on a pinkish background by spraying with a 1% solution of sulfosalicylic acid in 80% ethanol.

Non-specific reagents. Positive tests are also obtained with less specific reagents such as dilute alkaline potassium permanganate or sulfuric acid–ammonium sulfate reagents. Impurities were sometimes revealed by ninhydrin spray or UV fluorescence.

RESULTS AND DISCUSSION

In order to ascertain the number of charged groups on phosphonomycin, the R_F values for a number of known compounds were determined by PC in systems A and B. The resulting R_F, R_M and ΔR_M values are tabulated in Table II. The system was originally investigated with crude material prepared by ion-exchange procedures.

TABLE II

MOBILITY OF MODEL COMPOUNDS AND PHOSPHONOMYCIN

Compound	*System A*		*System B*		
	R_F	R_M	R_F	R_M	ΔR_M
Tartaric acid	0.16	0.72	0.31	0.35	0.37
Lactic acid	0.51	−0.02	0.69	−0.23	0.21
Gluconic acid	0.21	0.58	0.25	0.48	0.10
Novobiocin	0.75	−0.48	0.86	−0.71	0.23
Phosphonomycin	0.19	0.63	0.33	0.31	0.32
Crude phosphonomycin	0.19	0.63	0.26	0.45	0.18

TABLE III

MOBILITY OF PHOSPHONOMYCIN IN URINE SAMPLES

Paper chromatography; human urine samples in systems A and B; bioautograph detection. Samples A–D from human source provided by Dr. I. FOLTZ. Sample E from dog (5–24 h).

Phosphonomycin content		*Amount spotted (μl)*		*Observed R_F*		ΔR_M
(μg/ml)		*Unknown*	*Control*	*System A*	*System B*	
Control	1000	—	0.25	0.21	0.31	0.23
A	1050	0.10	—	0.17	0.22	0.14
		0.25	—	0.17	0.23	0.16
		0.5	—	0.17	0.22	0.14
		—	0.5	0.18	0.27	0.23
		0.5	0.5	0.17	0.21	0.11
		—	0.25	0.18	0.27	0.23
B	1550	—	0.25	0.19	0.35	0.36
		0.1	—	0.16	0.25	0.24
		0.25	—	0.16	0.26	0.27
		0.5	—	0.17	0.27	0.23
		0.25	0.5	0.17	0.27	0.23
		—	0.25	0.17	0.32	0.36
C	400	—	0.25	0.19	0.29	0.24
		0.5	—	0.18	0.25	0.18
		1.0	—	0.19	0.24	0.13
		1.0	0.5	0.19	0.25	0.15
		—	0.25	0.23	0.32	0.20
D	90	—	0.25	0.19	0.30	0.26
		0.5	—	0.18	0.23	0.13
		1.0	—	0.18	0.27	0.23
		2.0	—	0.18	0.21	0.12
		2.0	0.5	0.17	0.20	0.23
		—	0.25	0.20	0.33	0.29
E	200	—	0.25	0.18	0.32	0.33
		1.0	—	0.20	0.34	0.31
		1.0	0.25	0.20	0.34	0.31

The ΔR_M value obtained indicated that phosphonomycin contained one PO_3H_2 group. This observation was consistent with the ion-exchange behavior of the material. With pure material the observed ΔR_M is somewhat greater but is still less than that usually obtained with dibasic acids.

It is recognized that chromatographic behavior of a compound may be affected by the presence of other substances. The paper chromatographic behavior of the antibiotic in urine samples was studied using systems A and B, with detection by bioautography. The data obtained from a series of five patients is tabulated in Table III. In these experiments all samples originating from a single individual were run on a single sheet. The data show clearly that extraneous substances influence the mobility of the antibiotic, and co-chromatography was required to show that the antibiotically-active substance present in the urine was indeed unchanged phosphonomycin.

Examination of Table III indicates considerable variation in observed ΔR_M which is primarily due to changes in the observed R_F in system B. Although some variation was obtained with system A, this may be due to the difficulty of reading the center of the bioautograph zone, which extends over 0.1 R_F equivalent or more depending on the sensitivity of the bioautograph and the amount loaded on the chromatogram. The reading was normally taken at the apparent zone center. However,

TABLE IV

SOLVENT DEMIXING IN SOLVENTS A AND B; EFFECT OF ADDED CALCIUM CHLORIDE
Bioautograph detection; 1 μl load sodium phosphonomycin (1 mg/ml).

Day	*Observed R_F*		*ΔR_M*
	System A	*System B*	
1, normal	0.22	0.35	0.28
2, normal	0.20	0.32	0.27
3, normal	0.20	0.30	0.23
5 cm below origin	0.17	0.26	0.24
+ 1 mg/ml $CaCl_2$	0.18	0.29	0.27
4, normal	0.22	0.34	0.26
5 cm below origin	0.21	0.29	0.19
+ 10 mg/ml CaCl	0.17	0.29	0.30
5, normal	0.25	0.30	0.11
5 cm below origin	0.23	0.28	0.12
+ 10 mg/ml $CaCl_2$	0.23	0.28	0.12

the presence of urine contaminants or extraneous solids affects the mobility in system B. Consequently, the systems were studied over several days to determine day to day variation and the effect of added calcium chloride. Solvent demixing was studied by spotting additional samples 5 cm below the origin. The data obtained (Table IV) indicate that added calcium chloride diminishes the R_F values in both systems and that solvent demixing can be important. The large day to day variation and the solvent demixing effect were indicative of a large effect of minor variation in the moisture content of the paper on the observed R_F value. This was confirmed by chromatography with systems containing 80:20 and 60:40 *n*-propanol–aqueous amine systems. The data tabulated in Table V also show the effect of added calcium chloride and solvent demixing on the R_F and ΔR_M values in the two systems.

It is clear that this chromatographic method of determining the number of charged groups requires careful attention to all experimental details as well as standardization of the reagents with known compounds. A cautious interpretation of the data is indicated.

TABLE V

R_F VALUES OF PHOSPHONOMYCIN (SODIUM SALT)
Solvent A: *n*-propanol–2 *N* methylamine; solvent B: *n*-propanol–2 *N* isopropylamine; both 80:20 and 60:40. Bioautograph detection.

System	*Observed R_F*		*ΔR_m*
	A	*B*	
System 80:20			
Normal	0.07	0.12	0.25
5 cm below origin	0.06	0.12	0.33
+ 10 mg/ml $CaCl_2$	0.03	0.12	0.64
System 60:40			
Normal	0.43	0.47	0.07
5 cm below origin	0.38	0.42	(−0.07)
+ 10 mg/ml $CaCl_2$	0.41	0.34	0.07

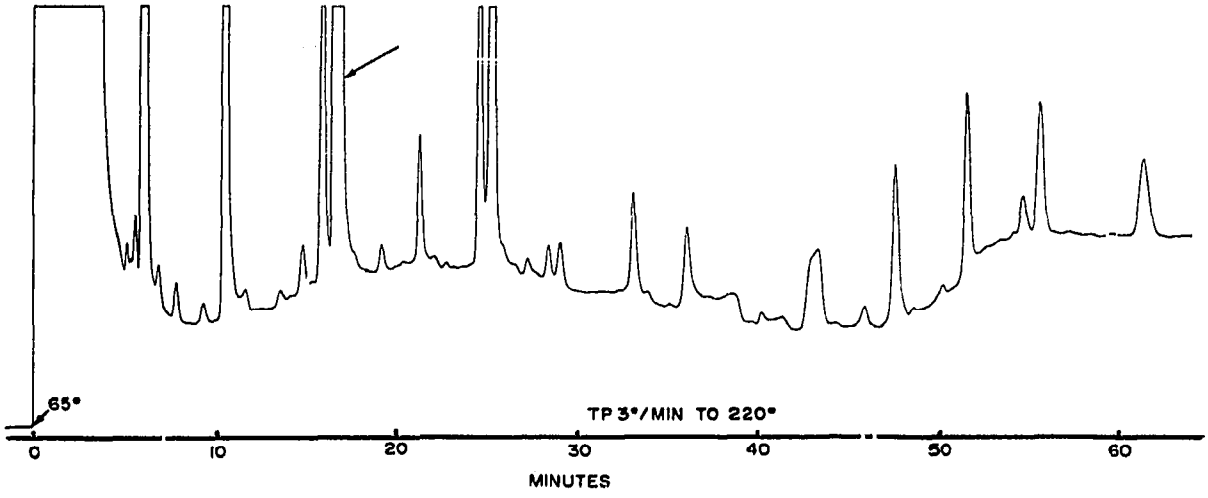


Fig. 1. GLC analysis of a phosphonomycin concentrate (before purification by PC)treated with bis-trimethylsilylacetamide. Conditions given under METHODS.

The value of GLC and combined GLC–MS in following the isolation and purification of a natural product is illustrated in Figs. 1 and 2. Fig. 1 shows the chromatogram resulting from the analysis of a sample of a phosphonomycin concentrate (before purification by PC) treated with BSA; the multi-component nature of the sample is evident. Preparative paper chromatography (System D) of the antibiotic concentrate yielded a product of increased biological activity which, following treatment with BSA, was subjected to GLC. As can be discerned from Fig. 2, this material is largely free from contamination. Note that the predominant peak in Fig. 2 possesses the same retention time as one of the larger peaks found in the non-paper chromatographically purified concentrate (see Fig. 1, arrow); the peaks also gave the same mass spectrum. GLC of the BSA-treated crystalline benzylamine salt of phosphonomycin yielded only two peaks. One corresponded in retention behavior (17 min) to the large peak from the paper chromatographically purified sample; combined GLC–MS of these 17 min

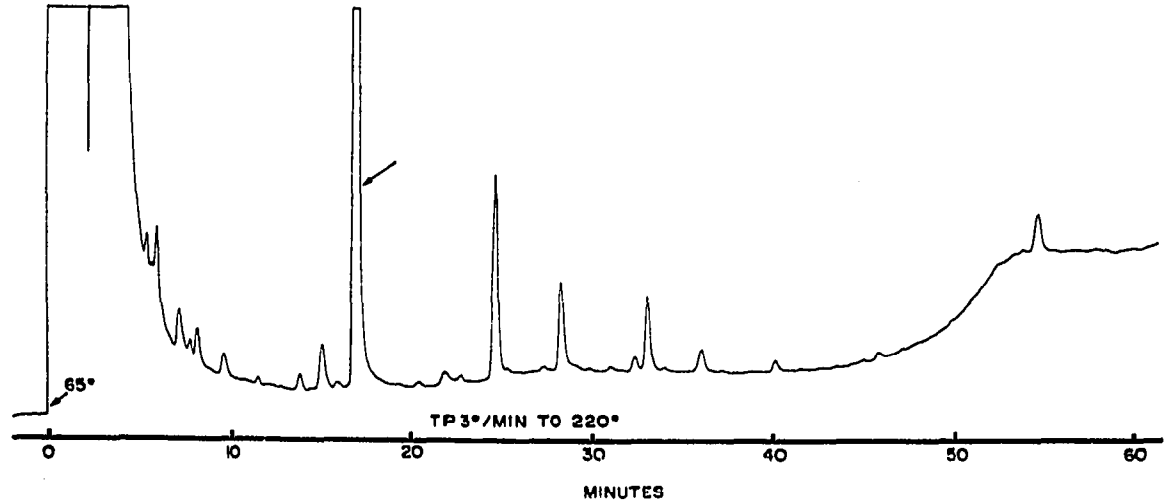


Fig. 2. GLC analysis of a phosphonomycin concentrate (after purification by PC) treated with bis-trimethylsilylacetamide. Conditions given under METHODS.

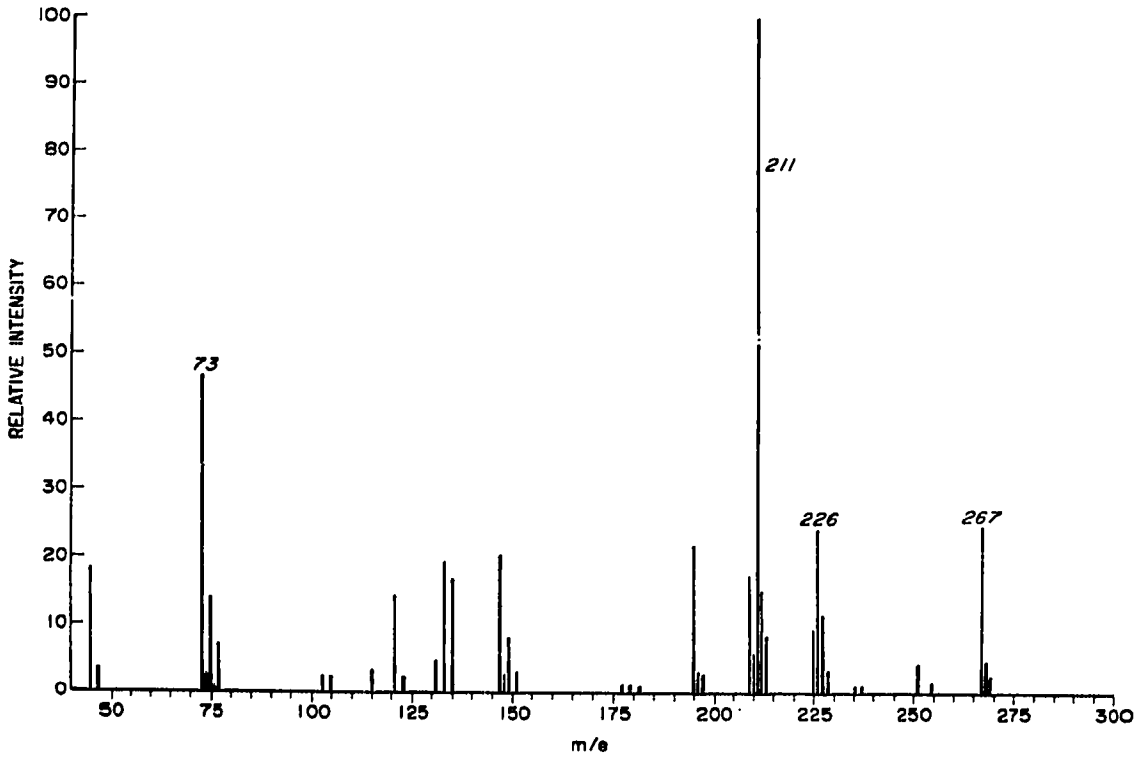


Fig. 3. Mass spectrum of the di-trimethylsilyl derivative of phosphonomycin obtained via combined GLC–MS. Conditions given under METHODS.

peaks confirmed the identical nature of the two compounds. The other peak (of shorter retention time) from the benzylamine salt was shown to be the TMSi derivative of benzylamine.

The mass spectrum of the di-TMSi ester of phosphonomycin is presented in Fig. 3. This compound (molecular weight 282) fails to display a molecular ion (M; *m/e* 282), but does produce a fragment ion of *m/e* 267, corresponding to loss of a methyl group (M-15), not uncommon for TMSi compounds[4,5]. Proposed structures for other major fragment ions are *m/e* 226, formally $(HOP(OSi(CH_3)_3)_2)^+$, resulting from scission of the carbon–phosphorus bond with transfer of a proton from the epoxide moiety to the phosphonate moiety; *m/e* 211, $(226\text{-}CH_3)$; and *m/e* 73, $(Si(CH_3)_3)^+$.

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