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GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF POLYAMINES AS THEIR N-ETHYLOXYCARBONYL DERIVATIVES AND IDENTIFICATION OF *sym*-HOMOSPERMIDINE AND *sym*-NORSPERMINE IN MOSSES AND FERNS

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SUMMARY

The N-ethyloxycarbonyl derivatives of eleven naturally occurring polyamines were investigated by combined gas chromatography–mass spectrometry using electron impact ionization. The mass spectra exhibited molecular ions of low but detectable intensity. The spectral data were used to identify the polyamines in some mosses and ferns, and the results showed that in addition to the well known polyamines *sym*-homospermidine was present in both of these plants and *sym*-norspermine exclusively in mosses.

INTRODUCTION

The polyamines putrescine, spermidine and spermine are widely distributed in microorganisms and all types of higher cells[1], and there are many reports on their function in processes of cell growth and proliferation[1–3]. In addition to these well known polyamines, several novel polyamines have been detected sporadically in certain organisms[4,5]. In recent years, interest has been focused on their chemotaxonomic or phylogenetic significance together with their function. In fact, the characteristic distribution of novel polyamines such as *sym*-homospermidine, *sym*-norspermidine and *sym*-norspermine has been shown to be valuable as a chemotaxonomic marker in green algae[6], nitrogen-fixing cyanobacteria[7], halophilic marine bacteria[8] and methanogenic bacteria[9]. These types of screening programmes involving large number of samples demand a simple and rapid method for the exact identification of polyamines.

The combination of gas chromatography and mass spectrometry (GC–MS) is well recognized as one of the most powerful tools for separating the individual components and then for obtaining unequivocal structural proof. However, polyamine analysis by GC–MS necessitates the preparation of volatile derivatives and common derivatizations have been carried out with trifluoroacetic anhydride or less frequently with trimethylsilyl chloride[10].

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In a previous paper[11], we reported a GC method for the determination of polyamines as their N-ethyloxycarbonyl (EOC) derivatives, which were prepared by reaction with ethyl chloroformate in an aqueous alkaline medium at room temperature. As the method was advantageous over conventional methods in terms of handling of the reagent, simplicity and reproducibility of the derivative preparation and stability of the derivatives, a GC–MS method employing the N-EOC derivatives was expected to be useful for the routine identification of polyamines in many kinds of samples. This paper describes the MS properties of the N-EOC derivatives of eleven naturally occurring polyamines (Table I) and the application of the spectral data to the identification of *sym*-homospermidine and *sym*-norspermine in some mosses and ferns.

TABLE I

NAMES AND STRUCTURES OF POLYAMINES INVESTIGATED

No.	*Name*	*Abbreviation*	*Structure*
1	Diaminopropane	Dap	$H_2N(CH_2)_3NH_2$
2	Putrescine	Put	$H_2N(CH_2)_4NH_2$
3	Cadaverine	Cad	$H_2N(CH_2)_5NH_2$
4	*sym*-Norspermidine	Nspd	$H_2N(CH_2)_3NH(CH_2)_3NH_2$
5	Spermidine	Spd	$H_2N(CH_2)_3NH(CH_2)_4NH_2$
6	3-Aminopropylcadaverine	Apc	$H_2N(CH_2)_3NH(CH_2)_5NH_2$
7	*sym*-Homospermidine	Hspd	$H_2N(CH_2)_4NH(CH_2)_4NH_2$
8	*sym*-Norspermine	Nspm	$H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$
9	Spermine	Spm	$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$
10	Thermospermine	Tspm	$H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_4NH_2$
11	Canavalmine	Can	$H_2N(CH_2)_4NH(CH_2)_3NH(CH_2)_4NH_2$

EXPERIMENTAL

Standards and reagents

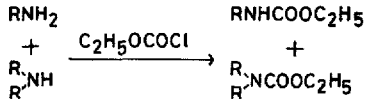
Dap, Put, Cad, Spd and Spm as hydrochloride salts were obtained from Nakarai Chemicals (Kyoto, Japan) and Nspd from Aldrich (Milwaukee, WI, U.S.A.). Hspd and Tspm as hydrochloride salts were prepared by the method of Okada *et al.*[12]; Apc and Nspm were kindly supplied by Dr. S. Matsuzaki (Institute of Endocrinology, Gumma University, Maebashi, Japan) and were converted into the respective hydrochloride salts; Can · 4HCl was provided by Dr. S. Fujihara (Department of Pharmacology, Nara Medical University, Kashihara, Japan).

Stock standard solutions (2.5 μmol/ml) of the individual amines were prepared with 0.1 *M* hydrochloric acid. Working standard solutions containing 125 nmol/ml of each amine were prepared by dilution with water. These solutions were stored refrigerated at 4°C.

Ethyl chloroformate as a derivatization reagent was obtained from Tokyo Kasei Kogyo (Tokyo, Japan) and was used after distillation. Uniport HP (100–120 mesh) as a support was purchased from Gasukuro Kogyo (Tokyo, Japan). A polyester liquid stationary phase, KT-300, was obtained from Japan Chromato Works (Tokyo, Japan). All other chemicals and solvents were of the highest purity commercially available.

Derivatization

The derivatization conditions were the same as those described previously[11] except that the total reaction volume without ethyl chloroformate was increased from 2 to 4 ml while maintaining the concentration of sodium hydroxide in the reaction mixture at 2%. The primary and secondary amino groups involved in polyamines were derivatized as shown in the following reaction scheme:



Gas chromatography–mass spectrometry

Combined GC–MS analyses were carried out by using a Shimadzu–LKB 9000 gas chromatograph–mass spectrometer operated in the electron impact mode. Polyamines were separated on a silanized 0.5 m × 3 mm I.D. glass column packed with 0.5% KT-300 on Uniport HP. The packing was prepared by using 1-butanol–chloroform (1:1) as a coating solvent according to the filtration technique[13]. The packed column was pre-conditioned at 280°C for 20 h with helium at a flow-rate of 30 ml/min. The gas chromatograph was programmed from 90 to 235°C at 4°C/min with injection at 250°C. The carrier gas was helium at a flow-rate of 40 ml/min. The separator oven was maintained at 240°C.

Mass spectra were obtained under the following conditions: trap current, 60 μA; ionizing voltage, 70 eV; accelerating voltage, 3.5 kV; and ion source temperature, 250°C. Spectra were recorded at a scan speed of 7 and were taken near the peak top, which was monitored by the total ion detector.

Extraction and isolation of polyamines from plant samples

Plant samples were collected in the summer of 1983 and were dried after being repeatedly washed with running water. Roots were removed from ferns. A known weight (1–2 g) of each sample was homogenized for 15 min with 70 ml of 4% $HClO_4$ and left to stand overnight to complete the extraction. The homogenate was centrifuged and the precipitate was washed twice with 10 ml of 4% $HClO_4$. The supernatant and washings were combined in a 100-ml calibrated flask and diluted to 100 ml with 4% $HClO_4$. Two 20-ml aliquots of this solution were applied separately to glass columns (9 mm I.D.) containing 3 ml of Amberlite CG-120 (H^+) resin (100–200 mesh). After each column had been washed successively with 30 ml of 1 *M* sodium phosphate buffer (pH 8) containing 0.1 *M* sodium chloride and with 30 ml of 0.1 *M* hydrochloric acid, the fraction eluted with 30 ml of 6 *M* hydrochloric acid was collected. The two eluates were combined and evaporated almost to dryness in a rotary evaporator at 70°C under vacuum. After being dissolved in 10 ml of 4% $HClO_4$, the residue was transferred quantitatively into another Amberlite column and treated as described above. To the 6 *M* hydrochloric acid eluate was added 1 ml of the internal standard solution (1,8-diaminooctane, 62.5 nmol) and the solution was evaporated. Finally, the residue was dissolved in 3.2 ml of water and transferred into a reaction vial (Pyrex, Code No. TST-SCR, 16 × 100 mm; Iwaki Glass, Tokyo, Japan) fitted with a PTFE-lined screw-cap for derivatization. This clean-up procedure

permitted the effective removal of many of the possible interfering materials. The recovery rates of amines added to the $HClO_4$ extract throughout the ion-exchange column chromatography followed by derivatization and GC analysis were between 91 and 106%.

Gas chromatography

A Shimadzu 4CM gas chromatograph with a flame-ionization detector equipped with a 0.5% KT-300 column was used to determine the polyamine contents of the plant samples. The gas chromatograph was operated under the following conditions: nitrogen flow-rate, 80 ml/min; injection port and detector temperatures, 285°C; column oven temperature, programmed from 130 to 280°C at 10°C/min. The N-EOC derivatives of polyamines obtained from the samples were finally dissolved in 0.2 ml of ethyl acetate and 2–4 μl of this solution were injected on to the gas chromatograph.

RESULTS AND DISCUSSION

From the results of preliminary experiments concerning the thermal stability of the liquid stationary phase and the GC properties of the derivatives, KT-300 was selected as being suitable for GC–MS analysis. With a 0.5% KT-300 column no significant interfering background peaks were observed in the mass spectra under the thermal conditions used. However, separation of the pairs of Apc–Hspd and Spm–Tspm was poor or nil, as shown in Fig. 1. In spite of carrying out further examinations, separation of these pairs could not be achieved with any of the liquid stationary phases tested.

Mass spectrometric properties

The fragmentation patterns of diamines are shown in Table II. In each instance the molecular ion (M) was easily recognizable (for simplicity the charge has been omitted). The characteristic ions representing the loss of C_2H_5OH from both the M

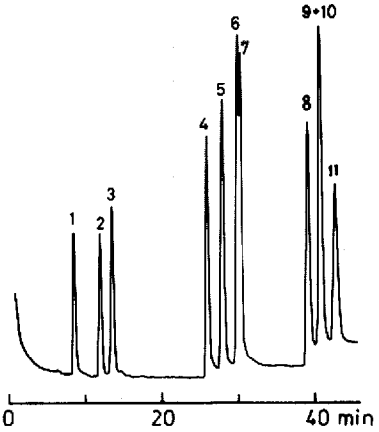


Fig. 1. Total ion current chromatogram of the N-EOC derivatives of a mixture of standard polyamines. A 100-nmol amount of each amine was derivatized; final volume, 100 μl; 4 μl injected. For peak identification see Table I.

TABLE II

PRINCIPAL IONS OBSERVED IN MASS SPECTRA OF N-EOC DERIVATIVES OF DIAMINES

Ion	*Dap*		*Put*		*Cad*	
	m/z	%*	*m/z*	%*	*m/z*	%*
M	218	12	232	6	246	7
Base ion	56	100	102	100	102	100
M − 45 (C_2H_5O)	173	16	187	14	201	13
M − 46 (C_2H_5OH)	172	12	186	14	200	4
M − 73 (C_2H_5OCO)	145	18	159	13	173	7
M − 91 (C_2H_5OH + C_2H_5O)	127	10	141	16	155	10
M − 119 (C_2H_5OH + C_2H_5OCO)	99	9	113	12	127	12
$(CH_2)_4NHCOOC_2H_5$	–		–		144	8
$(CH_2)_3NHCOOC_2H_5$	–		130	7	130	3
$(CH_2)_2NHCOOC_2H_5$	116	71	116	8	116	20
$CH_2NHCOOC_2H_5$	102	71	102	100	102	100
$(CH_2)_4NCO$	–		–		98	5
$(CH_2)_3NCO$	–		–		84	24
$(CH_2)_2NCO$	70	17	70	40	70	8
CH_2NCO	56	100	56	23	56	22
Others	129	36	142	52	156	18
	88	34			128	15

* Relative intensity.

ions and the fragmentation ions were observed. The ion at *m/z* 102 ($CH_2NHCOOC_2H_5$) was prominent in all instances, indicating easier cleavage of the bond between the C_1 and C_2 carbons. Loss of C_2H_5OH from the ion at *m/z* 102 gave an intense ion at *m/z* 56 (CH_2NCO), which was the base ion in Dap.

Table III shows the fragmentation patterns of triamines. These amines showed weak but perceptible M ions.

$$-CH_2\overset{a}{-}CH_2-N(COOC_2H_5)-CH_2\overset{b}{-}CH_2-$$

The fragmentation patterns show similarities and may be rationalized in terms of the cleavage of either the a or the b C–C bond together with elimination of C_2H_5OH. This fragmentation yielded a base ion for each of the triamines. Triamines having a propylamine moiety produce the base ion at *m/z* 185, whereas those having a butylamine or pentylamine moiety produce the ions at *m/z* 199 or 213, respectively, with high intensity. In fact, the ion at *m/z* 199 was the base ion in Hspd. Taking account of the relative intensities of these ions, their structures can be assigned as follows: *m/z* 185 in Nspd, Spd and Apc, [$CH_2N(COOC_2H_5)$ $(CH_2)_3NCO$]; *m/z* 199 in Spd and Hspd, [$CH_2N(COOC_2H_5)$ $(CH_2)_4NCO$]; and *m/z* 213 in Apc, [$CH_2N(COOC_2H_5)$ $(CH_2)_5NCO$]. The correctness of the structural assignment of these ions was confirmed by appropriate shifts in the masses of the ions from the corresponding derivatives prepared with *n*-propyl chloroformate in the same manner as with ethyl chloroformate. The other characteristic fragmentation attributable to cleavage of the C–C bonds described above probably includes one rearranged hydrogen to give the structurally significant ions at *m/z* 156, 142 and 128 with high

TABLE III

PRINCIPAL IONS OBSERVED IN MASS SPECTRA OF N-EOC DERIVATIVES OF TRIAMINES

Ion	*Nspd*		*Spd*		*Apc*		*Hspd*	
	m/z	%*	*m/z*	%*	*m/z*	%*	*m/z*	%*
M	347	6	361	7	375	3	375	4
Base ion	185	100	185	100	185	100	199	100
M − 45	302	4	316	4	330	3	330	2
M − 46	301	6	315	7	329	5	329	3
M − 73	274	11	288	9	302	7	302	4
M − 91	256	16	270	17	284	11	284	7
M − 102	245	8	259	4	–		–	
M − 116	231	9	245	8	259	3	–	
M − 119	228	19	242	29	256	25	256	13
M − 130	–		231	17	–		245	18
M − 144	–		–		231	14	–	
M − (102 + 46)	199	14	213	9	–		–	
M − (116 + 46)	185	100	199	70	213	44	–	
M − (130 + 46)	–		185	100	199	7	199	100
M − (144 + 46)	–		–		185	100	–	
$(CH_2)_4NHCOOC_2H_5$	–		144	13	144	5	144	14
$(CH_2)_3NHCOOC_2H_5$	130	34	130	20	130	17	130	3
$(CH_2)_2NHCOOC_2H_5$	116	63	116	70	116	65	116	8
$CH_2NHCOOC_2H_5$	102	34	102	32	102	28	102	12
$(CH_2)_4NCO$	–		98	46	98	14	98	41
$(CH_2)_3NCO$	84	16	84	31	84	24	84	35
$(CH_2)_2NCO$	70	25	70	48	70	22	70	34
CH_2NCO	56	59	56	54	56	44	56	23
Others	173	21	142	85	156	65	142	65
	139	40	128	25	128	23		
	128	41						

* Relative intensity.

intensity. The first two ions are characteristic of triamines having a pentylamine and butylamine moiety, respectively, and the last is characteristic of those having a propylamine moiety. These ions are provided by the loss of a 103 mass unit ($CH_2NHCOOC_2H_5$ + H) from the ions corresponding to M − 116 [$(CH_2)_2NHCOOC_2H_5$], M − 130 [$(CH_2)_3NHCOOC_2H_5$] and M − 144 [$(CH_2)_4NHCOOC_2H_5$].

Other abundant ions at *m/z* 144, 130, 116 and 102 arise from cleavage at different positions of the carbon chains, as indicated by a shift of 14 mass units. Of these, the ion at *m/z* 116 was the most prominent in the aminopropyl congeners. The ions at *m/z* 98, 84, 70 and 56 are possibly generated by elimination of C_2H_5OH from the ions at *m/z* 144, 130, 116 and 102 and in part by elimination of C_2H_5OH from the M ions followed by further C–C bond cleavages. A clear difference between Apc and Hspd was observed for the ions at *m/z* 185 and 199. These ions were useful for monitoring whether these pairs coexisted.

The fragmentation patterns of tetraamines are shown in Table IV. Each amine gave a small M ion, but an ion corresponding to M − 46 due to loss of C_2H_5OH was found with much higher intensity. The mode of fragmentation was, in many

TABLE IV

PRINCIPAL IONS OBSERVED IN MASS SPECTRA OF N-EOC DERIVATIVES OF TETRAAMINES

Ion	*Nspm*		*Spm*		*Tspm*		*Can*	
	m/z	%*	*m/z*	%*	*m/z*	%*	*m/z*	%*
M	476	2	490	2	490	6	504	3
Base ion	185	100	185	100	199	100	142	100
M − 46	430	11	444	10	444	13	458	5
M − (73 + 46)	357	9	371	20	371	11	385	8
M − (116 + 46)	314	3	328	8	328	9	342	2
M − (73 + 46 × 2)	311	14	325	22	325	34	339	7
M − (130 + 46)	300	2	314	16	314	7	328	4
M − (116 + 46 × 2)	268	16	282	19	282	14	–	
M − 231**	245	8	259	2	259	13	273	6
M − 245***	231	7	245	4	245	13	259	11
M − 259§	217	3	231	17	231	9	245	9
M − (231 + 46)	199	25	213	6	213	40	227	13
M − (245 + 46)	185	100	199	11	199	100	213	13
M − (259 + 46)	–		185	100	185	62	199	73
M − (245 + 46 × 2)	139	68	153	36	153	49	167	2
$(CH_2)_4NHCOOC_2H_5$	144	15	144	7	144	43	144	26
$(CH_2)_3NHCOOC_2H_5$	130	37	130	18	130	38	130	11
$(CH_2)_2NHCOOC_2H_5$	116	68	116	52	116	95	116	40
$CH_2NHCOOC_2H_5$	102	19	102	15	102	30	102	19
$(CH_2)_4NCO$	–		–		98	54	98	32
$(CH_2)_3NCO$	84	24	84	43	84	55	84	27
$(CH_2)_2NCO$	70	46	70	26	70	94	70	53
CH_2NCO	56	58	56	38	56	94	56	30
Others	213	32	183	11	197	49	197	31
	183	46	142	27	183	51	183	36
	142	31	128	20	156	23	156	6
	128	42			142	99	153	24
					139	53	142	100
					128	48		

* Relative intensity.

** $CH_2N(COOC_2H_5)(CH_2)_3NHCOOC_2H_5$ or $N(COOC_2H_5)(CH_2)_4NHCOOC_2H_5$.

*** $(CH_2)_2N(COOC_2H_5)(CH_2)_3NHCOOC_2H_5$ or $CH_2N(COOC_2H_5)(CH_2)_4NHCOOC_2H_5$.

§ $(CH_2)_3N(COOC_2H_5)(CH_2)_3NHCOOC_2H_5$ or $(CH_2)_2N(COOC_2H_5)(CH_2)_4NHCOOC_2H_5$.

respects, similar to that of triamines, although it became complicated. Considering the observation for triamines, it seems reasonable that the ions at *m/z* 199 and 185 would be derived from a combination of cleavage of the C–C bond between the two secondary amino groups and loss of C_2H_5OH. The ion at *m/z* 185 was also observed for Nspd, Spm and Tspm, which have a propylamine moiety. This ion was the base ion in both Nspm and Spm. The ion at *m/z* 199 was characteristic of Tspm and Can having a butylamine moiety, and constituted the base ion in Tspm. These results are indicative that these ions would possess the same structures as those of the corresponding ions observed in triamines. On the other hand, the ion at *m/z* 142, which was peculiarly observed for Spd and Hspd having a butylamine moiety, was prominent in Tspm and Can, and constituted the base ion in Can. Ions at *m/z* 144, 130,

116, 102, 98, 84, 70 and 56 were also observed with relatively high intensity. However, the structure of the ion at *m/z* 144 observed in Nspm and Spm is clearly different from that shown in Table IV. This ion in Nspm and Spm is probably due to another fragmentation product.

Application

This GC–MS method has been used in our laboratory for more than 1 year, and its practicability for the identification of polyamines has been ascertained by the analyses of halophilic marine bacteria[8] and aquatic plants[14]. The method was also successfully applied to the identification of polyamines present in mosses (Bryopsida) and ferns (Pteropsida), which have not previously been analysed for their polyamine distribution. The polyamine contents of some mosses and ferns determined by GC are shown in Table V, indicating that in addition to the well known polyamines Hspd is distributed in both of these plants but Nspm is exclusively in mosses. The identity of the polyamine peaks in each sample was confirmed by GC–MS analysis of the remains of the GC sample. Fig. 2 shows the representative gas chromatograms of the N-EOC derivatives of polyamines obtained from plant samples. Dap and Cad were identified as minor components in some samples of both phyla. Other unusual polyamines such as Nspd, Apc, Tspm and Can were not detected in any of the samples. As Nspd is supposed to be a biochemical precursor of Nspm, attempts were made to identify it in mosses. However, we were unable to detect appreciable amounts of this triamine in any of mosses investigated. This may suggest the rapid conversion of Nspd into Nspm.

The finding that these plants are different in their distributions of unusual polyamines is of great interest because there have been many discussions about the

TABLE V

POLYAMINE CONTENTS OF SOME MOSSES AND FERNS

Type	*Sample No.**	*Polyamine content (nmol/g)*				
		Put	*Spd*	*Hspd*	*Nspm*	*Spm*
Moss	1	89.9	206.6	118.3	41.9	31.7
	2	70.3	179.5	76.4	52.7	39.3
	3	52.2	184.0	42.6	11.3	10.1
	4	73.5	249.6	88.1	65.3	11.1
	5	243.5	233.6	163.1	49.8	46.0
	6	97.0	211.7	114.1	29.0	9.1
	7	131.8	110.3	170.7	28.3	75.7
Fern	1	37.5	119.9	24.5	N.D.**	63.0
	2	39.3	36.0	15.3	N.D.	20.0
	3	41.0	56.0	24.2	N.D.	65.6
	4	114.0	135.7	66.9	N.D.	55.9
	5	101.2	106.8	40.3	N.D.	115.1
	6	84.3	121.3	48.9	N.D.	139.1
	7	35.7	66.2	21.5	N.D.	68.8

* Samples are different species.

** Not detected.

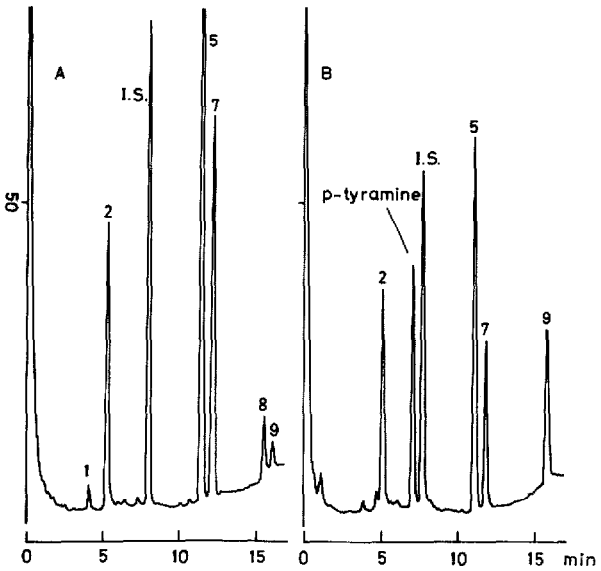


Fig. 2. Gas chromatograms of the N-EOC derivatives of polyamines obtained from (A) a moss and (B) a fern. I.S. = internal standard (1,8-diaminooctane) (62.5 nmol). For peak identification see Table I.

origins of the bryophytes and pteridophytes in conjunction with evolution of green algae[15–17] which contain Nspd and Nspm and sporadically Hspd[6,18]. In order to obtain the well defined phylogenetic significance related to the occurrence of the novel polyamines in these phyla, further studies on the polyamine distribution in other allied plants are currently being investigated using the present methodology.

In conclusion, the described GC–MS method in combination with GC determination should offer a convenient routine technique for polyamine analyses of a variety of samples. Although no interfering substances were found in the samples examined in this work, exact confirmation of the identity of polyamines in other samples will depend on the use of a clean-up procedure suitable for the samples. The method may have a limited utility for pentaamines, which have recently been found[19,20], owing to their low volatility.

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REFERENCES

1 S. S. Cohen, *Introduction to the Polyamines*, Prentice-Hall, Englewood Cliffs, NJ, 1971.
2 U. Bachrach, *Function of the Naturally Occurring Polyamines*, Academic Press, New York, 1973.
3 J. Jänne, H. Pösö and A. Raina, *Biochim. Biophys. Acta*, 473 (1978) 241.
4 T. Oshima, *Methods Enzymol.*, 94 (1983) 401; and references cited therein.
5 A. S. Dion and S. S. Cohen, *Proc. Nat. Acad. Sci. U.S.*, 69 (1972) 213.
6 E. Hegewald and H. Kneifel, *Arch. Hydrobiol., Suppl.*, 60 (1981) 313.
7 K. Hamana, K. Miyagawa and S. Matsuzaki, *Biochem. Biophys. Res. Commun.*, 112 (1983) 606.

8 S. Yamamoto, S. Shinoda, M. Kawaguchi, K. Wakamatsu and M. Makita, *Can. J. Microbiol.*, 29 (1983) 724.
9 P. Scherer and H. Kneifel, *J. Bacteriol.*, 154 (1983) 1315.
10 G. D. Daves, Jr., R. G. Smith and G. A. Valkenburg, *Methods Enzymol.*, 94 (1983) 48; and references cited therein.
11 S. Yamamoto, H. Itano, H. Kataoka and M. Makita, *J. Agr. Food Chem.*, 30 (1982) 435.
12 M. Okada, S. Kawashima and K. Imahori, *J. Biochem. (Tokyo)*, 85 (1979) 1235.
13 E. C. Horning, W. J. A. VandenHeuvel and B. G. Creech, *Methods Biochem. Anal.*, 11 (1963) 69.
14 S. Yamamoto, Y. Aoyama, M. Kawaguchi, A. Iwado and M. Makita, *Chem. Pharm. Bull.*, 31 (1983) 3315.
15 B. Lowry, D. Lee and C. Hébant, *Taxon*, 29 (1980) 183.
16 R. S. Chopra, *Misc. Bryol. Lichenol.*, 9 (1981) 183.
17 T. N. Taylor, *Taxon*, 31 (1982) 155.
18 K. Hamana and S. Matsuzaki, *J. Biochem. (Tokyo)*, 91 (1982) 1321.
19 T. Oshima, *J. Biol. Chem.*, 257 (1982) 9913.
20 T. Oshima and S. Kawahata, *J. Biochem. (Tokyo)*, 93 (1983) 1455.