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OCCURRENCE OF A NEW POLYAMINE, CANAVALMINE, IN THE SWORD BEAN *Canavalia gladiata*

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SUMMARY. A new tetraamine was detected in the seed of sword bean *Canavalia gladiata* and named canavalmine. The chemical structure was determined to be $NH_2(CH_2)_4NH(CH_2)_3NH(CH_2)_4NH_2$ (1,13-diamino-5,9-diazatridecane) based on gas chromatography-mass spectrometry after derivatization of polyamines with pentafluoropropionic anhydride. The proof of identity was established by comparison of infrared and ^{1}H-NMR spectra of the tetraamine isolated from sword bean with those of a synthetic compound.

Aliphatic polyamines, putrescine, spermidine and spermine are ubiquitously found in living cells. Their significance in the process of cell proliferation and cell development has been reviewed (1). In recent years several unusual polyamines, which differ from the well-known polyamines in the length of methylene chain in the amines, have been detected in a wide variety of living systems including bacteria (2-6), algae (7,8), plants (9,10), and animals (11-13). The physiological role of these polyamines, however, remains to be worked out.

During a routine analysis of polyamines in leguminous seeds by gas-liquid chromatography (GLC), we found two unknown peaks on the gas chromatogram of the extract from vegetable crop sword bean. From the analysis by gas chromatography-mass spectrometry (GC-MS) one was identified as 1,9-diamino-5-azanonane and the other as 1,13-diamino-5,9-diazatridecane. The former compound, usually referred to as sym-homospermidine, has been found in leaves of sandal wood (9), legume root nodules (10), thermophilic bacteria (5), green algae (7) and newt (13), but the latter was a new polyamine which has not been recognized in nature so far. This paper describes the isolation and identification of the unusual tetraamine. Referring to the scientific name of sword bean we propose the trivial name of canavalmine for this novel tetraamine.

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MATERIALS AND METHODS

Chemicals- Putrescine, cadaverine, spermidine and spermine were purchased from Nakarai Chemicals Ltd. in the form of the hydrochloride salts. Pentafluoropropionic anhydride, O-phthalaldehyde and activated Permutit were obtained from Wako Pure Chemical Industries, Ltd. Other organic and inorganic chemicals were the purest grade available from commercial sources.

Extraction of polyamines- Seeds of sword bean *Canavalia gladiata* (10g) were macerated in H_2O overnight, homogenized in a Waring blendor with 10 vols(V/W) of 0.2N $HClO_4$ and passed through 4 layers of cotton gauze. The filtrate was neutralized with 4N KOH and the resultant $KClO_4$ was removed by filtration. In order to separate polyamines from other acidic and neutral compounds, 10%(W/V) of activated Permutit was added to the solution and mechanically shaked for 5 min. The supernatant was discarded and the Permutit was washed twice with H_2O and once with 2% NH_4OH. Polyamines were freed from the Permutit particles by shaking with 10 vols(V/W) of 40% dimethylamine solution for 10 min. After sedimentation of Permutit, supernatant was collected and evaporated to dryness *in vacuo* to remove dimethylamine. The residue was dissolved in 1 ml of 1N HCl and subjected to the derivatization procedures described below.

Preparation of pentafluoropropionyl derivatives- The polyamines were converted to their volatile pentafluoropropionyl (PFP) derivatives for gas chromatographic analysis. The sample solution was dried under a stream of N_2 gas at 90°C in 3 ml reaction vials equipped with teflon lined screw caps. To the residue 0.2 ml of acetonitrile and 0.1 ml of pentafluoropropionic anhydride were added. The vial was capped and heated at 100°C for 15 min. The reaction mixture was evaporated to dryness under a stream of N_2 gas at room temperature and redissolved in 0.5 ml of diethyl ether. The ether solution was washed once with equal volume of saturated Na_2CO_3 solution. The ether phase was taken for the analysis by GLC or GC-MS.

Isolation of canavalmine- For the isolation and purification of the unusual tetraamine eluted after spermine on the gas chromatogram, extraction was carried out also on a large scale using 600g of dry sword beans. The extraction procedures used were basically similar to those described above. In order to separate the unknown polyamine from spermine and other polyamines, the extract after Permutit treatment was further fractionated by means of an ion exchange chromatography (IEC) using a column (0.9 x 10 cm) packed with Yanaco SCX 1001 cation-exchange resin. The column was eluted with 0.2M Na_2HPO_4 buffer, pH 10.55, containing 0.5M NaCl and 0.1% Brij 35 at the flow rate of 1 ml/min at 60°C. Aliquots (25 µl) taken from each fraction (3 ml) were reacted with O-phthalaldehyde reagent (14) and resultant fluorescence was measured at 455 nm (excitation;340 nm). The elution profile was shown in Fig. 1. The fractions correspond to the final peak on the elution chromatogram were collected and passed through a Dowex 50W-X8 column (1 x 9 cm, 100-200 mesh, H^+ form) to remove the salts. The column was washed with H_2O and 2N HCl, and the polyamine was then eluted with 6N HCl. After evaporating HCl under reduced pressure, the residue was dissolved in a minute amount of H_2O and then in a mixture of methanol and ethanol (1:1). The hydrochloride was precipitated from the solution by a dropwise addition of ether. The procedure was repeated until colourless precipitate was obtained. Reprecipitation procedure gave a final yield of 11 mg of canavalmine hydrochloride.

Synthesis of sym-homospermidine and canavalmine- Sym-homospermidine was synthesized according to the method as described in (15) with slight modifications. The synthesis of canavalmine (1,13-diamino-5,9-diazatridecane) was performed by the reaction of monoacetylputrescine with 1,3-dibromopropane as follows. Monoacetylputrescine (68g, 0.53 mol), prepared by the procedure of Aspinall (16), was mixed with 1,3-dibromopropane (48g, 0.24 mol) in 300 ml of iso-propanol and heated under reflux for 18 hr. After removal of the solvent by evaporation, 500g of 12N HCl was added to the residue and refluxed for 3 hr. The hydrolyzate was evaporated to dryness and washed with absolute ethanol. To

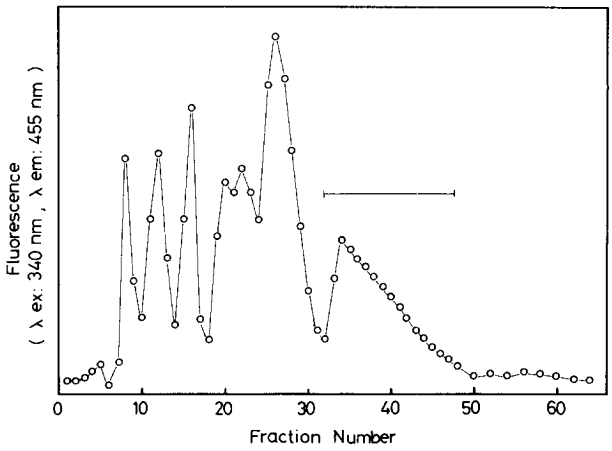

Fig. 1. Separation of unusual tetraamine from *C. gladiata* by cation-exchange chromatography. The extract from the seed of *C. gladiata* was pretreated with an activated Permutit for removal of anionic and neutral compounds and then applied on a Yanaco SCX 1001 cation-exchange column (0.9 x 10 cm). The column conditions and buffer system used are described under MATERIALS AND METHODS. Aliquots (25 µl) taken from each fraction (3 ml) were reacted with O-phthalaldehyde reagent (14) for fluorometric detection of polyamines. The fractions containing unknown tetraamine indicated by the bar were pooled and subjected to further purification procedure. In this buffer system spermidine, homospermidine and spermine overlapped between fraction number 18 to 31.

remove the major by-product putrescine derived from unreacted acetylputrescine, the residue was dissolved in H_2O and applied on a Dowex 50W-X8 column (3 x 10 cm). Putrescine was removed away with 3N HCl and the tetraamine held on the column was eluted with 6N HCl. The tetraamine was further purified by reprecipitation of its hydrochloride salt in ethanol. The purest 1,13-diamino-5,9-diazatridecane hydrochloride was obtained in ca. 15% yield.

Instrument- Hitachi 163 gas chromatograph fitted with flame ionization detector was employed in the quantitative assay of polyamines. The pyrex glass column (150 cm x 3 mm I.D.) was packed with 3% OV-17 on 80-100 mesh Chromosorb W(HP). The N_2 carrier gas flow rate was set at 30 ml/min and the column temperature was programmed 120-280°C at 15°C/min. Mass spectra of PFP derivatives of polyamines were obtained with a double-focusing Hitachi M-80 gas chromatograph mass spectrometer, using an ionizing voltage of 20eV and a He carrier gas flow rate of 50 ml/min. Hitachi 650-10S fluorescence spectrophotometer was used for a fluorometric detection of polyamines. Infrared (IR) spectra and ^{1}H-NMR spectra were recorded on Jasco IRA-2 and JEOL JNM-III, respectively.

RESULTS

Sword bean *C. gladiata* contained two unusual polyamines besides widely occurring polyamines, spermidine and spermine. The gas chromatogram of the PFP derivatives of polyamines extracted from the seeds of *C. gladiata* was shown in Fig. 2. The contents of spermidine and spermine, which are the major polyamines in the seed, were 250 and 184 nmoles/g fresh wt, respectively. Putrescine was also present, but in very low level.

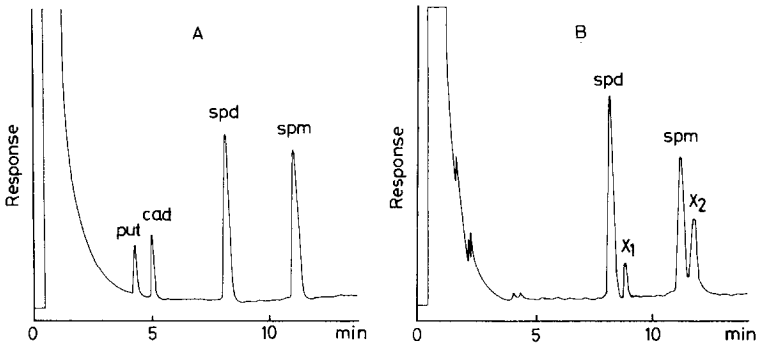

Fig. 2. Gas chromatograms of PFP derivatives of polyamines extracted from the seed of *C. gladiata* and a standard mixture of polyamines. A: standard mixture, B: extract from *C. gladiata*. Put: putrescine, Cad: cadaverine, Spd: spermidine, Spm: spermine.

The unknown peak X_1 eluted after spermidine had the same retention time as PFP derivative of authentic sym-homospermidine. The evidence of identity was established from their mass spectra exhibiting the same fragmentation patterns (Fig. 3-A,B). Very weak molecular ion (M^+) is shown at m/e 597. Two aminobutyl moieties in sym-homospermidine resulted in very intense fragment ions at m/e 216 ($C_2F_5CONHCH_2CH_2CH{=}CH$) and m/e 218 ($C_2F_5CONHCH_2CH_2CH_2CH_2$).

For the identification of the unknown compound X_2 eluted after spermine, several spermine analogues which differ in the length of methylene chain in the molecules were synthesized. Of these, the tetraamines which showed a similar retention time on a gas chromatogram to that of the unknown peak X_2 were $NH_2(CH_2)_4NH(CH_2)_3NH(CH_2)_4NH_2$ and $NH_2(CH_2)_4NH(CH_2)_4NH(CH_2)_3NH_2$. Under electron impact the mass fragmentation of the unknown peak X_2 was identical with that of PFP derivative of $NH_2(CH_2)_4NH(CH_2)_3NH(CH_2)_4NH_2$ (1,13-diamino-5,9-diazatridecane) as shown in Fig. 3-C,D. The loss of C_2F_5 from the parent molecule gave the most intense fragment ion at m/e 681. No visible fragment ions at m/e 435 (= $C_2F_5CONH(CH_2)_4\overset{\overset{COC_2F_5}{|}}{N}(CH_2)_4$) and m/e 379 (= $CH_2\overset{\overset{COC_2F_5}{|}}{N}(CH_2)_3NHCOC_2F_5$) characteristic to the PFP derivative of $NH_2(CH_2)_4NH(CH_2)_4NH(CH_2)_3NH_2$ was obtained.

The identification was also performed by comparing IR and ^{1}H-NMR spectra of the isolated natural tetraamine with those of a synthetic reference compound. The IR spectra of the hydrochlorides (KBr pellets) of synthetic 1,13-diamino-5,9-diazatridecane and of isolated tetraamine are given in Fig. 4.

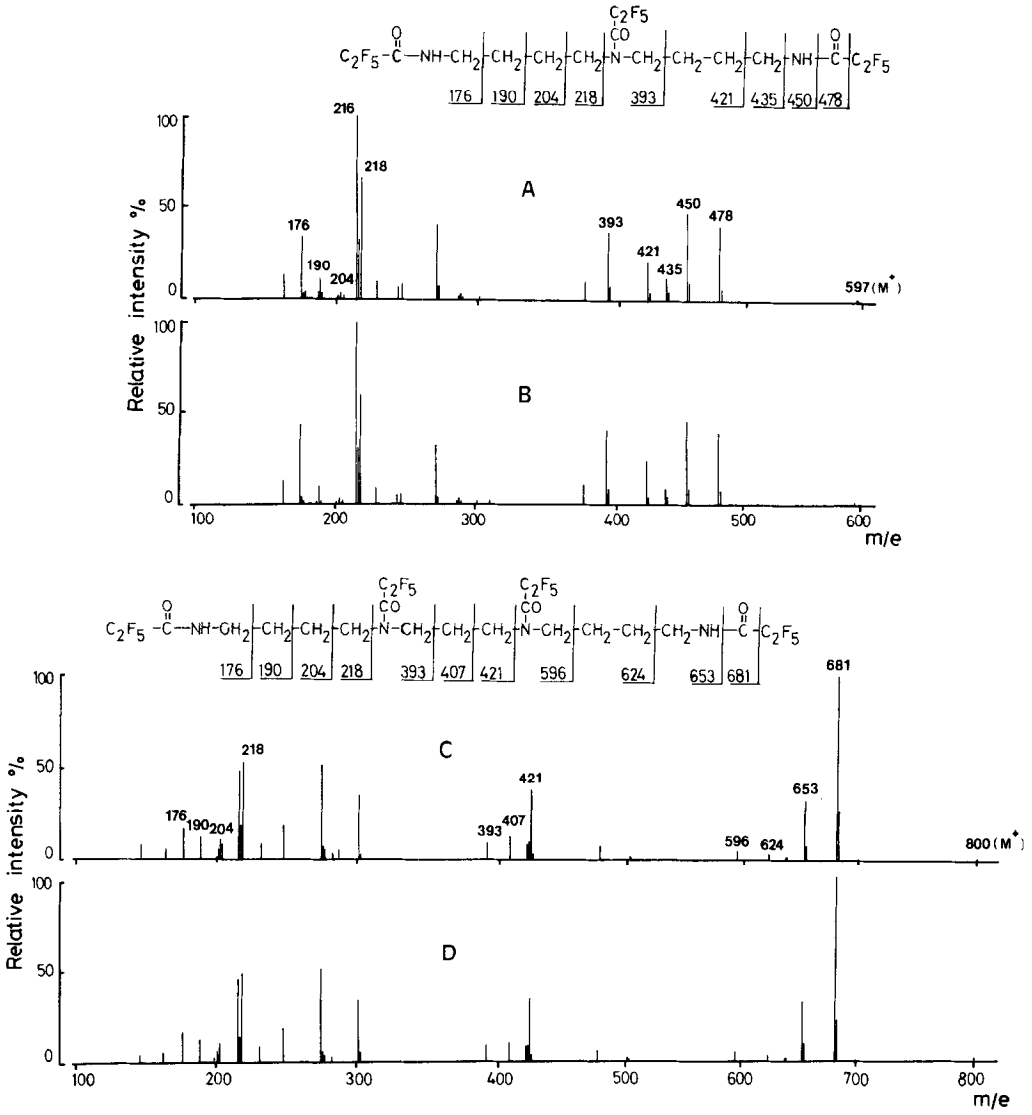

Fig. 3. Mass spectra of PFP derivatives of X_1, X_2 and synthetic reference compounds. A: synthetic 1,9-diamino-5-azanonane (sym-homospermidine), B: X_1 eluted after spermidine, C: synthetic 1,13-diamino-5,9-diazatridecane, D: X_2 eluted after spermine.

All absorption peaks appear in both spectra. Their ^{1}H-NMR spectra in D_2O using trimethylsilylpropanesulfonate (TSP) as an internal standard were also identical (Fig. 5).

These spectral data including GC-MS, IR and ^{1}H-NMR together with its chromatographic behavior on IEC and GLC provide strong evidence that the tetra-amine isolated from seeds of *C. gladiata* is spermine analogue 1,13-diamino-5,9-diazatridecane (= N,N'-bis(4-aminobutyl)1,3-diaminopropane).

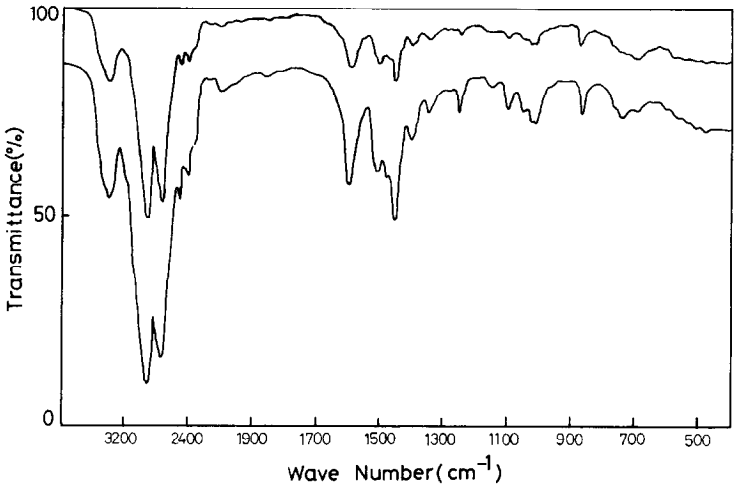

Fig. 4. Infrared spectra of the unusual tetraamine isolated from *C. gladiata* and synthetic reference compound. Upper: isolated tetraamine from *C. gladiata*, Lower: synthetic 1,13-diamino-5,9-diazatridecane. KBr-disks contained 1 mg of the tetraamine hydrochloride.

DISCUSSION

In the present investigation, a new polyamine, named canavalmine, was found as one of the major constituents of polyamines in the seed of *C. gladiata* besides spermidine, spermine and sym-homospermidine. The occurrence of this

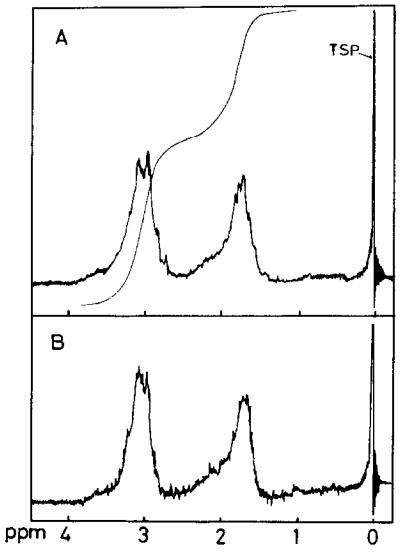

Fig. 5. ^{1}H-NMR spectra of the unusual tetraamine isolated from *C. gladiata* and synthetic reference compound. A: synthetic 1,13-diamino-5,9-diazatridecane, B: isolated tetraamine from *C. gladiata*. Samples (5-8 mg, hydrochloride salts) were dissolved in 0.3 ml of D_2O. TSP (trimethylsilylpropanesulfonate) was used as an internal standard.

new tetraamine in natural sources has been presented for the first time. Among the seeds of 14 species of leguminous plants so far surveyed, canavalmine was detected only in *C. gladiata*. Other unusual polyamines such as sym-norspermidine, sym-norspermine (thermine) and thermospermine reported in various procaryotic and eucaryotic organisms (2-4,7,8,11,12) were not detected in this plant. Distribution of canavalmine in other species of *Canavalia* is currently under investigation.

The presence of two aminobutyl terminal groups in the structure of canavalmine is indicative of the existence of enzyme system(s) transfering aminobutyl moiety to spermidine. Assuming that this is true, the enzyme involved in the transfer of aminobutyl group should specifically act on the aminopropyl terminal of spermidine unlike the aminopropyl transfer to the aminobutyl terminal of spermidine in spermine biosynthesis (1), since the transfer of this group to the aminobutyl terminal of spermidine invariably leads to the formation of $NH_2(CH_2)_4NH(CH_2)_4NH(CH_2)_3NH_2$ which could not be detected in *C. gladiata*.

As to the origin of sym-homospermidine in the seed of *C. gladiata*, the report of Smith (10), that legume root nodules and nitrogen-fixing bacterium *Rhizobium* contained sym-homospermidine, is of special interest, since *C. gladiata* also forms root nodules by symbiotic association with *Rhizobium*.

The pathways and site(s) of biosynthesis of canavalmine and sym-homospermidine together with their physiological functions in *C. gladiata* are the subjects to be elucidated in future.

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