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NOTES

Discovery of New Homologous Pamamycins by Mass Spectrometry and *post mortem* Inhibitory Action on Autolysis of Chicken Embryo Chorioallantoic Membrane Blood Vessels

ALBERT HÄRTL, AXEL STELZNER, ROLF SCHLEGEL, STEPHAN HEINZE, HEIKE HÜLSMANN, WERNER FLECK and UDO GRÄFE*

Hans-Knöll-Institute of Natural Products Research, Beutenbergstraße 11, D-07745 Jena, Germany

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Pamamycins possessing molecular weights of 593, 607, 621, 635 and 649 Da have previously been reported as inducers of aerial mycelium formation by *Streptomyces alboniger*[1~4], as inhibitors of myosin-light-chain kinase[5], vasodilators[6], ionophores[6], mediators of hydrophilic ion transport through lipophilic phases[7~9] and protonophoric agents[10].

The structural diversity of most of the published pamamycins has been ascribed to the substitution of protons or methyl groups at both parts of the macrodiolide ring by ethyl groups (see *c.f.* R1, R2, R3, R4 and R5 in Fig. 1)[1,2,11]. However, variations of the length of the nitrogen-containing side chain at the left side of the pamamycin skeleton (see *c.f.* R6 in Fig 1) have been reported for pamamycins-621 and -635[4,5] harbouring the same ring structure as was shown for pamamycin-607 ($R1=R2=R3=R4=R5=CH_3$)[3]. The homologous mixtures of pamamycins are difficult to separate owing to their high degree of chemical similarity. Structural assignment of the known individual components has employed chemical derivatizations[4,10] in addition to spectroscopic methods.

In the course of screening for embryotoxic compounds, an extract of *Streptomyces* sp. HKI-0118 was found to prevent *post mortem* the autolysis of the chorioallantoic membrane blood vessel tissues of 15-day-old embryonated chicken eggs. This effect was also observed in presence of the above extract when the mortalization of the embryo was induced by ergotamine tartrate.

Here we report the occurence of new pamamycins with molecular weights of 663, 677, 691 and 705 Da in extracts of *Streptomyces* sp. HKI-0118 as constitutive parts of a mixture of homologous pamamycins containing also pamamycins-607, -621, -635 and -649.

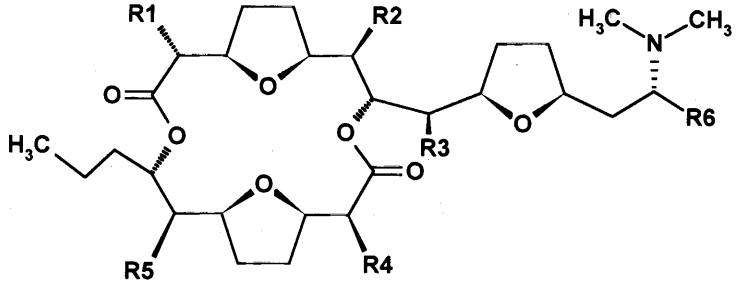
Due to the possibility of daughter-ion generation from the single $[M+H]^+$ ions of a mixture, triple-quadrupole mass spectrometry (CID-MS/MS) appeared as a promising tool for the analysis of the new pamamycin complex.

Experimental

Microorganism and Cultivation

Streptomyces sp. HKI-0118 was obtained from the

Fig. 1. General structure of pamamycins-607, -621, -635, -649, -663, -677 and -691 as part of a mixture isolated from *Streptomyces* sp. HKI-0118.



R1, R2, R3, R4, R5 = CH_3, C_2H_5 or H
R6 = $(CH_2)_{2\sim4}CH_3$

culture collection of the Hans-Knöll-Institute of Natural Products Research Jena (Germany). Twelve days agar plate cultures (25°C) were prepared to inoculate a seed medium (each 80 ml in 500 ml shake flasks) composed as follows (g/liter): glucose 15; soya flour 15; $CaCO_3$ 1; NaCl 5; KH_2PO_4 0.1; pH 6.3. Cultivation was carried out for 48 hours at 28°C on rotary shakers (180 r.p.m.). Three ml of this inoculum culture were used to inoculate the main culture medium (80 ml in 500 ml shake flasks) composed of (g/liter): glucose 20; oat meal 20; yeast extract 3; NaCl 3; $CaCO_3$ 3; $FeSO_4 \cdot 7H_2O$ 0.54 and $MnCl_2 \cdot 4H_2O$ 0.6; pH 6.8 prior to sterilization. After four days of fermentation at 28°C on rotary shaker (180 r.p.m.) the fermentation broth was pooled[8].

Instruments and Analytical Methods

High-resolution fast atom bombardment mass spectra (HRFAB-MS; 3-nitrobenzylalcohol as matrix) and high-resolution electron impact mass spectra (HREI-MS) were recorded on an AMD-402 double-focusing mass spectrometer with direct inlet system and BE geometry (AMD Intectra, Harpstedt, Germany). Positive ion electrospray ionization (ESI-MS) and collision-induced mass spectra of selected pseudomolecular ions (CID-MS/MS) were recorded on a triple quadrupole instrument Quattro (VG Biotech, Altrincham, England). Samples dissolved in methanol/water (1 : 1; 0.05 mg/liter) were directly applied to the nebulizer-assisted electrospray ion source. ^1H and ^{13}C NMR spectra were recorded at 300 K in $CDCl_3$ on an Avance DRX 500 spectrometer (Bruker, Rheinstetten, Germany).

Determination of *post mortem* Activities of Pamamycins from *Streptomyces* sp. HKI-0118 and *Streptomyces aurantiacus* IMET 43917 Using 15 Day-old Chicken Embryos

The preparation of the embryonated chicken embryos occured in the same manner as was described in the literature for the assay of acute toxicity testing[12].

55±5 g fertile White Leghorn eggs (Lohmann weiß) were breeded at 37.5°C and 50 to 60 % relative humidity in a breeder type BSS 160/8103 (Ehret, Emmendingen, Germany), equipped with a ventilation system and an automatic egg-turn mechanism. Beginning from the third up to the fifteenth day the eggs were rotated by 180° for every four hours. At the fifteenth day the sharp pole of the egg was desinfected with Braunoderm® (Braun, Melsungen, Germany) and the eggshell was perforated with a high-speed dental drill. Solutions of the test substances (ergotamine, pamamycin samples) were injected in volumes of 0.1 to 0.2 ml into the albumin. Subsequently the hole in the eggshell was closed with tesafilm and paraffin, and the breeding was continued for 72 hours under the same condition as before but without turning.

Table 1. Inhibitory action of purified pamamycin from *Streptomyces* sp. IMET 43917[1] (see Fig. 3c) on *post mortem* autolysis of chorioallantoic blood vessels of 15 day-old chicken embryos after lethal intoxication by 1 mg ergotamine-tartrate/kg egg (percentage of autolysis = 100-percentage of non-lyzed CAM vessels).

Number of eggs	Pamamycin[a] added (mg/kg egg)	Percentage of autolysis (%) post application		
		24 hours	48 hours	72 hours
26	0	87	95	100
19	1.0	41	50	49
29	2.5	37	39	49
29	5.0	16	27	36

[a] Pamamycin and ergotamine tartrate were simultaneously applied to 15 days chicken embryos.

Ergotamine tartrate was singly applied in a dose of 1 mg/kg egg weight (e.w.); simultaneously pamamycin samples (from *Streptomyces* strains HKI-0118 and IMET 43917) were also singly applied in doses of 1, 2 or 5 mg/kg e.w. The dose of 1 mg/kg e.w. ergotamine tartrate, was sufficient to kill 100% of treated chicken embryos within 24 hours. The viability of the embryos was checked using a candle lamp. The death of the embryo became visible owing to the loss of spontaneous motility. In general, a few hours later the visible structures of blood vessels of the chorioallantoic membrane (CAM) disappeared totally due to the rapid onset of *post mortem* autolysis.

At time points 24, 48 and 72 hours after dosing the degree of autolysis was scored as follows: no visible vessels = 0, weakly visible vessels = 1 and well visible vessels = 2.

The inhibitory effect on autolysis was evaluated coarsely according to the following scheme: for every dose group and time point after administration the single numerical scores were added to give a single numerical value for the pertinent egg group. This value was divided by the number of treated embryos, multiplied by a factor of 100, and the resulting value was divided again by the factor of 2 to calculate the percentage (N) of the embryos with non-lyzed CAM vessels (Table 1; percentage of

autolysis: 100-N)

Results and Discussion

The mycelial extract of a 50 ml shake flask culture of this strain was found to kill the embryo and to concomitantly preserve the *post mortem* autolysis of the chorioallantoic (CAM) blood vessels of 15-day-old chicken embryos in a dose-dependent manner. The effect was also observed when the extract samples were co-administrated with another embryotoxic agent. Thus, 100% mortality of the chicken embryos was induced within 24 hours by administration of ergotamine tartrate, and autolysis of CAM blood vessels was nearly 100% at this time. However, if extract samples of *Streptomyces* sp. HKI-0118 were administrated in addition to ergotamine tartrate the CAM vessels remained well recognizable at least for further 24 hours *post mortem*, even during storage at incubator temperature of 37.5°C (Table 1). This effect was dose dependent and was noted with pamamycins from strain HKI-0018 and *Streptomyces* sp. IMET 43917[1)] in the same manner (Table 1).

To isolate the active compound(s) the mycelia from 5 liters of shake cultures of *Streptomyces* sp. HKI-0118 were twice extracted with 1 liter methanol for 24 hours. The residue of the evaporated extract was subjected to column chromatography on silica gel 60 (0.063 ~ 0.1 mm; $CHCl_3$; acetone; ethyl acetate - triethylamine (95 : 5)) as was described previously[1,7)].

Evaporated samples of fractions containing the mixture of pamamycins (Fig. 2a) were tested for the prevention of autolysis of CAM blood vessels of 15 day-old chicken embryos as was described above. The active fractions eluted by ethyl acetate were pooled and subjected to column chromatography on Sephadex LH-20 (4 × 120 cm, MeOH). The active fractions (see above) were evaporated, again, to yield 185 mg of a colourless waxy mass.

Comparison of the 500 MHz ^1H and 125 MHz ^{13}C NMR chemical shift data with those of a mixture of pamamycins-607 and -621 from *Streptomyces aurantiacus* IMET 43917[1)] suggested that the mixture of compounds from *Streptomyces* sp. HKI-0118 was composed of homologous structures differing by methyl or ethyl substitutions.

High-resolution fast atom bombardment mass spectrometry (HRFAB-MS) showed not less than eight $[M+H]^+$ ions with *m/z* 608.4538 ($C_{35}H_{62}NO_7$; calcd. 608.4526), *m/z* 622.4696 ($C_{36}H_{64}NO_7$; calcd. 622.4683), *m/z* 636.4863 ($C_{37}H_{66}NO_7$; calcd. 636.4839), *m/z* 650.4993 ($C_{38}H_{68}NO_7$; calcd. 650.4996), *m/z* 664.5160 ($C_{39}H_{70}NO_7$; calcd. 664.5152), *m/z* 678.5318 ($C_{40}H_{72}NO_7$; calcd. 678.5309), *m/z* 692.5459 ($C_{41}H_{74}NO_7$; calcd. 692.5465) and *m/z* 706.5623 ($C_{42}H_{76}NO_7$; calcd. 706.5622). As depicted in Fig. 2a the relative intensity of the $[M+H]^+$ ions with *m/z* 608, *m/z* 622, *m/z* 636, *m/z* 650, *m/z* 664, *m/z* 678, *m/z* 692, and 706 was approximately 8 : 16 : 40 : 69 : 91 : 100 : 40 : 12 suggesting the relative amounts of the components in the mixture. For the sake of comparison the FAB mass spectrum of the pamamycin mixture of *Streptomyces aurantiacus* IMET 43917[7)] is shown in Fig. 2b.

Comparable results were obtained by high-resolution electron impact mass spectrometry (HREI-MS).

The FAB-MS EI-MS and electrospray mass spectra (ESI-MS) of the reported pamamycins (R6 = $(CH_2)_{2\sim4}CH_3$) displayed all the diagnostic α-fragmentation of the nitrogen-containing side chain yielding *m/z* 100[1,4,5,10,11)] (see *c.f.* Fig. 2b). However, in the HRFAB and HREI mass spectra of the pamamycins from *Streptomyces* sp. HKI-0118, two additional nitrogen-containing fragments were visible with *m/z* 114.1281 ($C_7H_{16}N$; calcd. 114.1282) and *m/z* 128.1436 ($C_8H_{18}N$; calcd. 128.1433) accompanying *m/z* 100.1114 ($C_6H_{14}N$; calcd. 100.1101; Fig. 2a).

This result suggested the occurrence of pamamycins in the mixture possessing the same side chains as was described for single pamamycins-621 and -635 as inhibitors of myosine-light-chain kinase[5)] (see Fig. 1; R6: n = 3 and 4).

Conclusive support for this contention was obtained from the electrospray ionization mass spectra (ESI-MS) and collision-induced decomposition (CID-MS/MS) of each of the eight single pseudomolecular ($[M+H]^+$) ions in the presence of argon gas (Figs. 3a, 3b). Collision-induced dissociation (CID-MS/MS) of the pseudomolecular ion ($[M+H]^+$) with *m/z* 608 yielded *m/z* 100.1 as the most prominent diagnostic fragment ion but no fragments at *m/z* 114 and *m/z* 128. However, the latter were present in the CID-MS/MS daughter-ion spectra of the $[M+H]^+$ ions at *m/z* 622, 636, 650, 664, 678, 692 and 706 thus confirming the presence of R6 = $(CH_2)_3CH_3$ and $(CH_2)_4CH_3$ residues (Fig. 1), respectively. For comparison in Fig. 3c the CID-MS/MS of pamamycin-635 from *Streptomyces aurantiacus* IMET 43917[7)] is given (R6 = $(CH_2)_2CH_3$) showing *m/z* 99.8 as a single prominent daughter ion. With the exception of *m/z* 608 and *m/z* 706 each of these pseudomolecular ions displayed more than one of these diagnostic fragments (Figs. 3a, 3b) suggesting that they each represent a mixture of

Fig. 2. HRFAB-MS of the mixture of homologous pamamycins from *Streptomyces* sp. HKI-0118 (A) and *Streptomyces aurantiacus* IMET 43917[1)] (B).

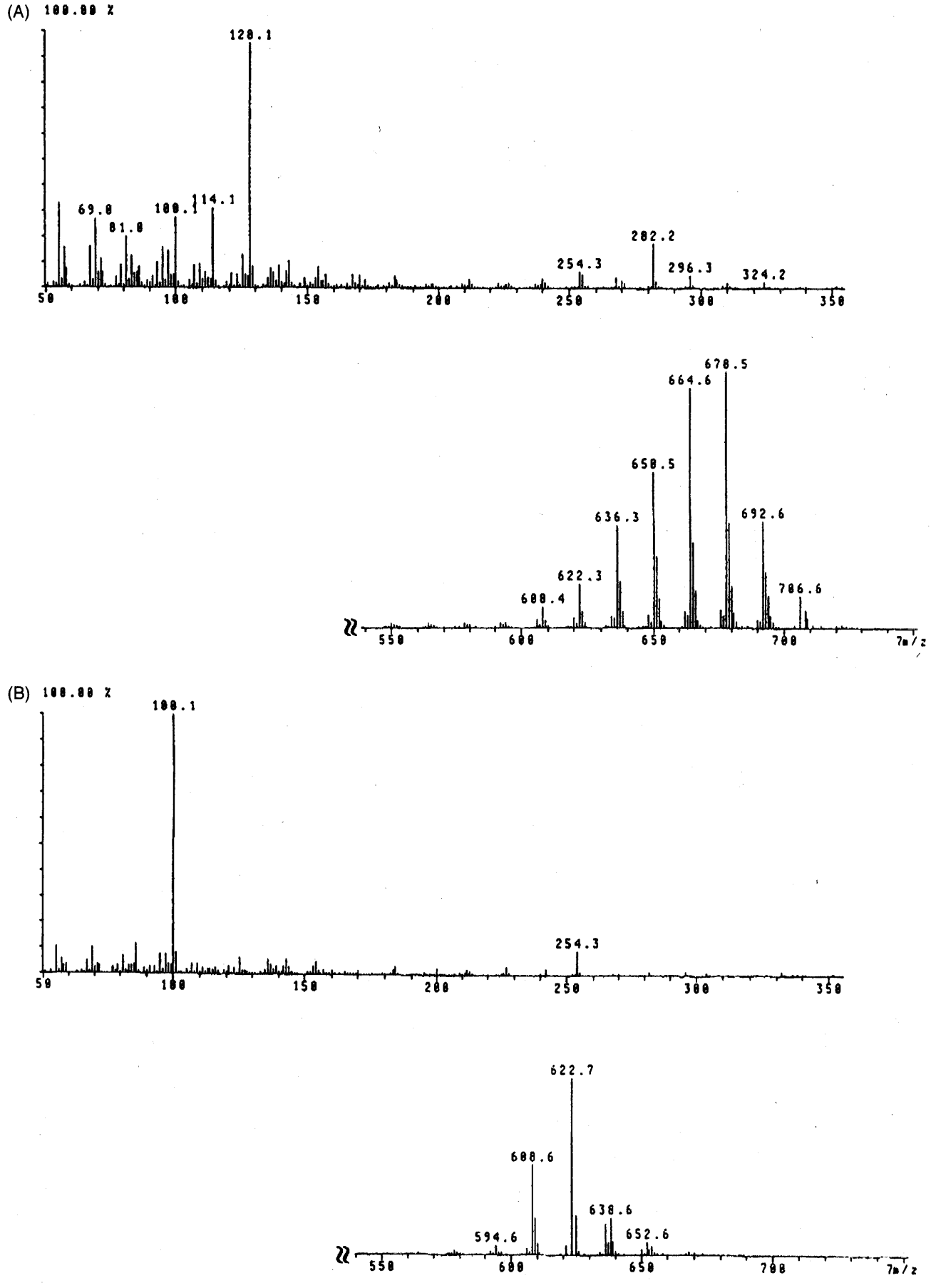
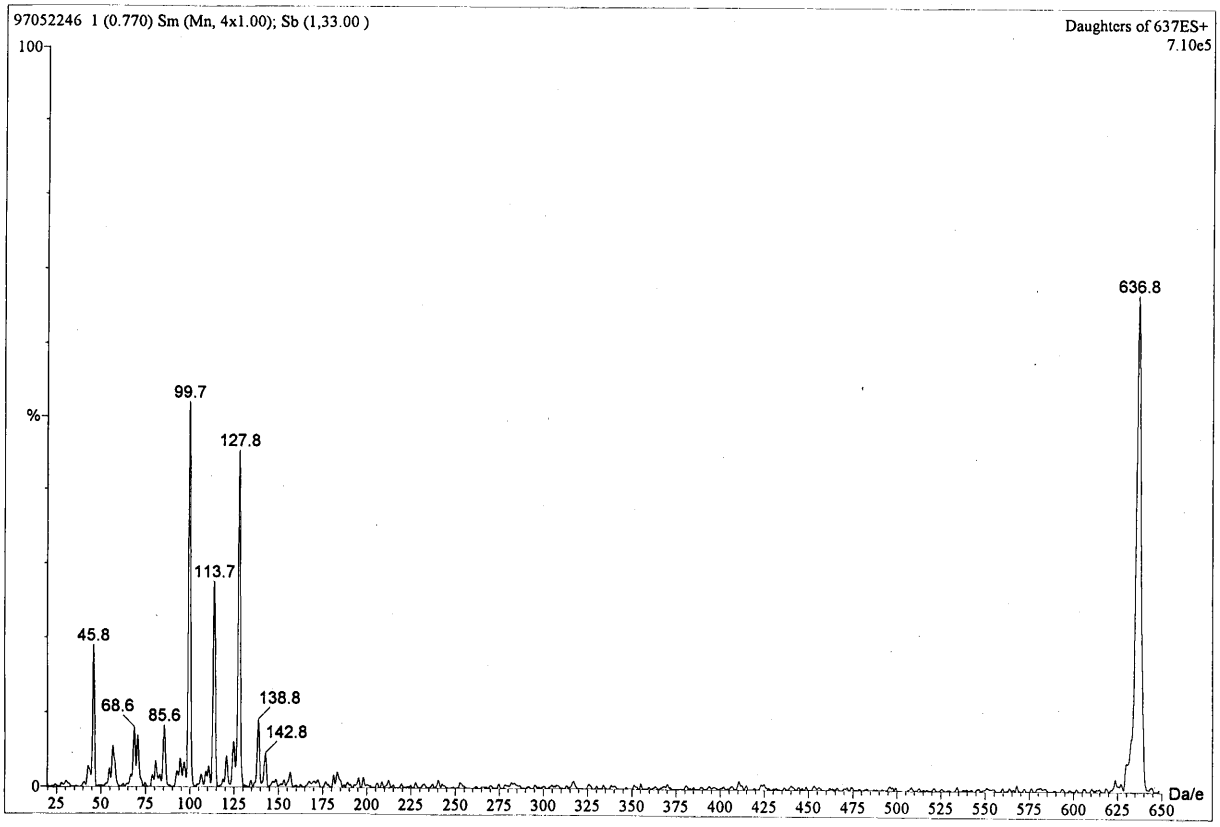
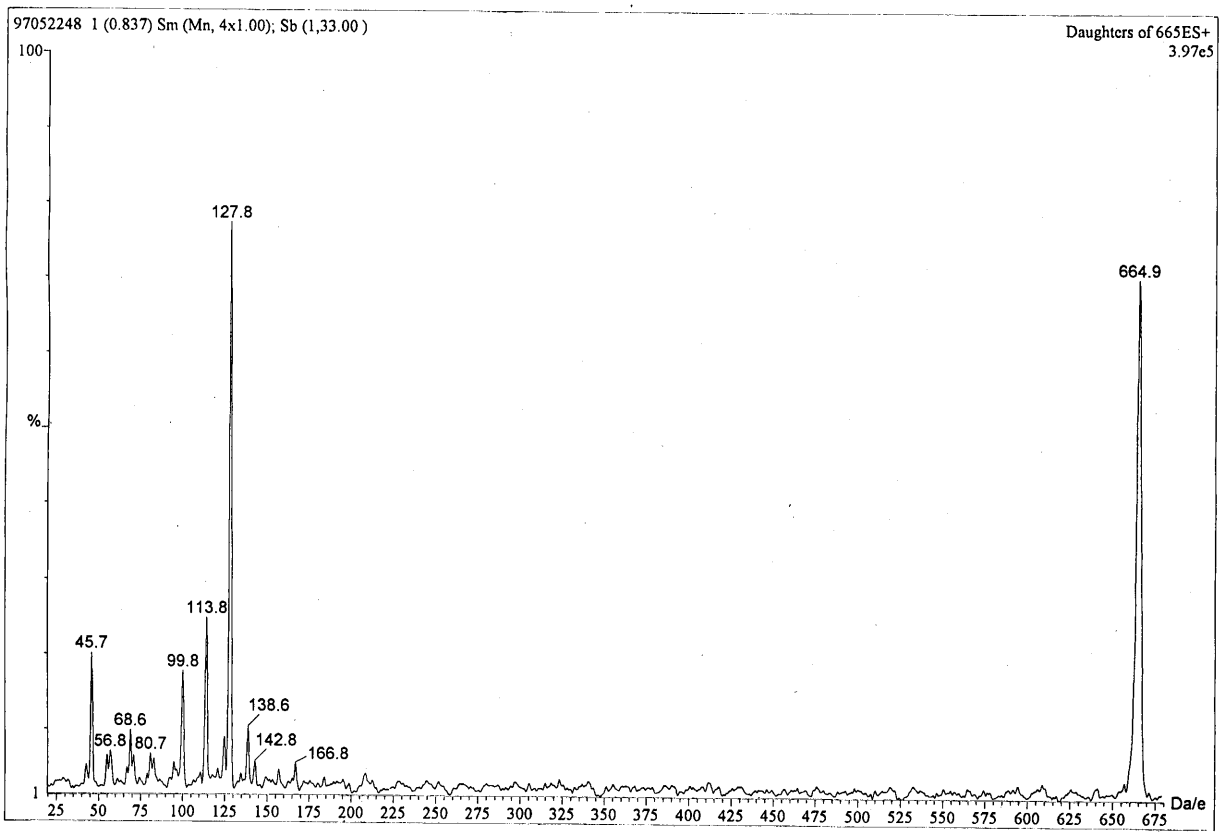


Fig. 3. ESI-CID-MS/MS of selected $[M+H]^+$ ions of pamamycin mixtures.

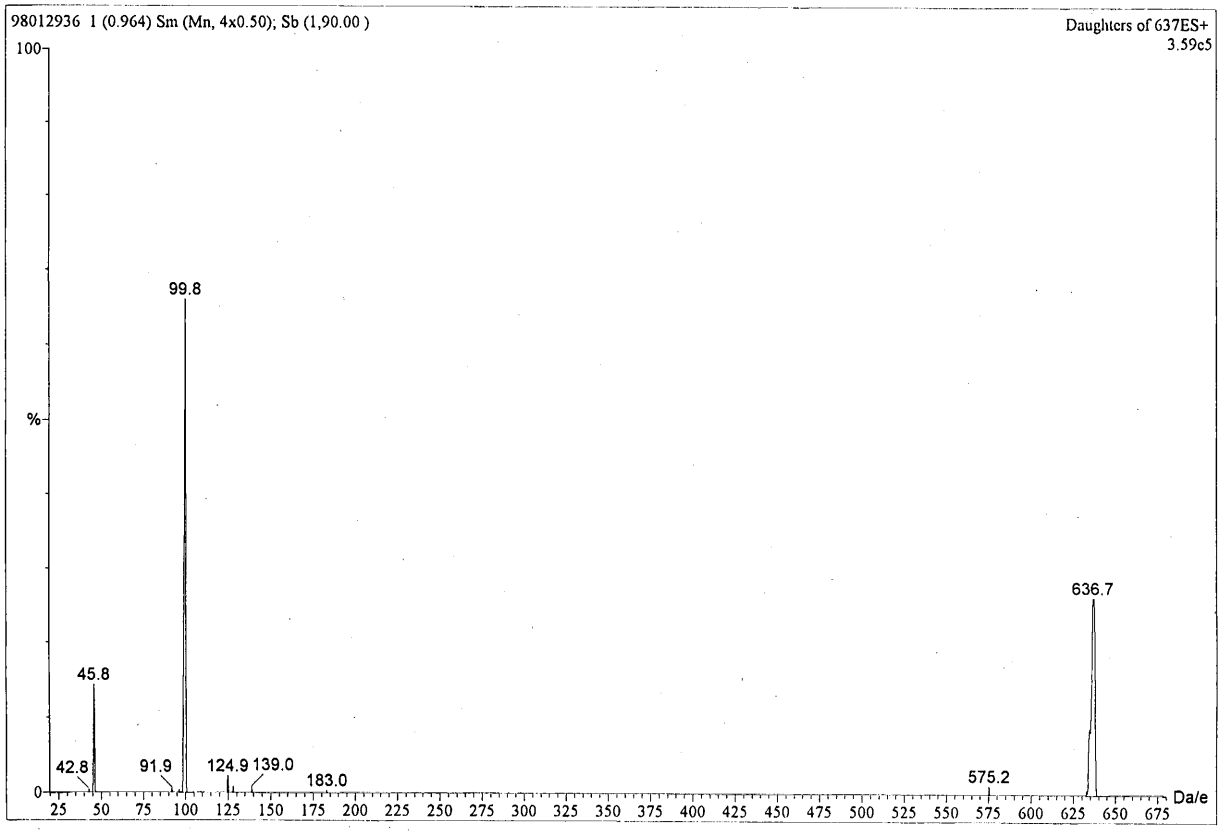
a) CID-MS/MS of *m/z* 636.8 (*Streptomyces* sp. HKI-0118).



b) CID-MS/MS of *m/z* 664.9 (*Streptomyces* sp. HKI-0118).



c) CID-MS/MS of *m/z* 636.7 from the pamamycin mixture of *Streptomyces aurantiacus* IMET 43917[1].



two or three different pamamycins. For instance, the simultaneous occurence of *m/z* 100.1, *m/z* 114 and *m/z* 128.2 in the daughter ion spectra of the $[M+H]^+$ ions with *m/z* 636.8 and *m/z* 664.2 could be ascribed to a mixture of three pamamycins possessing the same molecular weight but differing in the length of the nitrogen-containing side chains. Despite of the same molecular weight ($[M+H]^+$) and occurence of the same side chain, the individual components thus identified could be distinguishable by varying substitutions at the macrolide ring according to the general substitution pattern of the pamamycins[4]. According to the mass spectrometric data the pamamycin complex from *Streptomyces* sp. HKI-0118 thus may be composed of at least 18 to 20 different chemical individuals. The relative intensity of the diagnostic fragment ions with *m/z* 100; *m/z* 114 and *m/z* 128 (Fig. 2, Figs. 3a and 3b) could also be taken as a measure of the ratios of pamamycins in the mixture yielding $C_6H_{14}N$ (R6=$(CH_2)_2CH_3$), $C_7H_{16}N$ (R6=$(CH_2)_3CH_3$ and $C_8H_{18}N$ (R6=$(CH_2)_4CH_3$) daughter-ions and, respectively, diagnostic fragement ions during electrospray-ion-source-assisted CID-MS/MS, EI-MS and FAB-MS. (Fig. 1, Table 2). The separation of the above complex into single components remains a challenge for future work.

Table 2. Relative amounts pamamycins (for the general formula see Fig. 1) as detected by the relative intensity of diagnostic fragment ions in the ESI-CID-MS/MS of single $[M+H]^+$ ions.

$[M+H]^+$	Relative intensity of the diagnostic fragment ions (%)		
	m/z 100	*m/z* 114	*m/z* 128
608	100	0	0
622	73	27	0
636	34	20	46
650	35	19	46
664	18	17	65
678	6	11	83
692	0	7	93

The mixture of pamamycins from *Streptomyces* sp. HKI-0118 and, in the same manner and concentration, the 3:7 mixture of pamamycins-607 and -621 from *Streptomyces aurantiacus* IMET 43917[7], prevented the

post mortem autolysis of the chorioallantoic membrane (CAM) blood vessels of 15-day-old-chicken embryos. In any case the lethality was induced at 15th day of the embryonal development by administration of 1 mg ergotamine tartrate/kg egg weight. Normally 24 hours later all treated chicken embryos were dead and the blood vessels of the chorioallantoic membrane were lysed. Single dosages of pamamycin (0.5 to 5 mg/kg e.w.) preserved the chorioallantoic blood vessels from autolysis for several days after death if they were applied simultaneously with a lethal dose of ergotamine tartrate (Table 1).

This finding suggests that pamamycins are able to protect vascular tissues from an autolytical *post mortem* process. The reason for this phenomenon needs to be elucidated in future. However the observed anti-autolytical potency of pamamycins on 15-day-old chicken embryos supplies an additional facet to the spectrum of interesting biological activities of the pamamycins[1~7,9~11].

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