Production and Isolation of Two Novel Esperamicins in a Chemically Defined Medium

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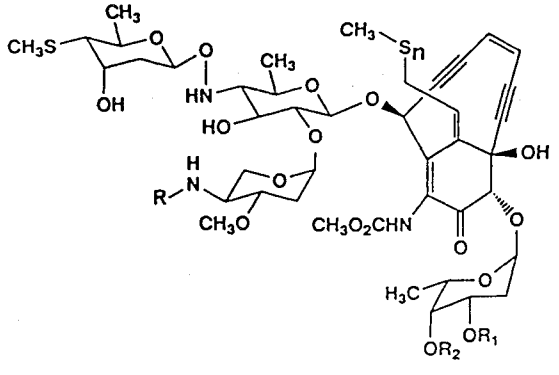
Esperamicins, a family of extremely potent compounds, showing a broad spectrum of antimicrobial and antitumor activities in murine models has been identified in cultures of *Actinomadura verrucosospora* ATCC 39334[1,2]. The isolation and structure elucidation of esperamicins A_1 (esp A_1), A_{1b} (esp A_{1b}) and A_2 (esp A_2) have been reported[1,3,4]. The esperamicins consist of a bicyclic core to which attached a trisaccharide and a substituted 2-deoxy-L-fucose. The bicyclic core contains an enediyne, an allylic trisulfide and a bridgehead eneone.

Esp A_1 (Fig. 1), the major component of the esperamicin family isolated from the fermentation of *A. verrucosospora* ATCC 39334 grown in the complex medium H946[5], is one of the most potent antitumor agents yet discovered[2]. Three minor esperamicins, esp A_2, esp A_{1b} and esp P (Fig. 1) were also isolated from the large scale fermentation of *A. verrucosospora* ATCC 39334 grown in the complex medium[5,6]. While developing a defined medium for more efficient incorporation of labelled precursors into esp A_1 during the biosynthetic studies[7], we detected the formation of two new esperamicin analogs in a defined medium. We report here the production, isolation, structure determination and antitumor activity of these analogs.

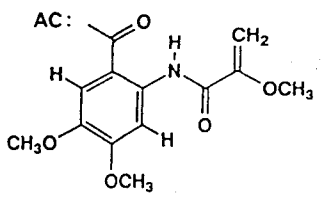
A vegetative culture of *A. verrucosospora* ATCC 39334 was prepared by transferring 4 ml of the frozen stock culture to a 500-ml Erlenmeyer flask containing 100 ml of a vegetative medium consisting of starch 2%, glucose 0.5%, Pharmamedia 1%, yeast extract 1% and $CaCO_3$ 0.2%. The vegetative culture was incubated at 28°C for 96 hours on a rotary shaker operating at 250 rpm. An 8-ml aliquot of incubated seed culture was transferred to a 500-ml Erlenmeyer flask containing 100 ml of complex medium H946. Medium H946 was prepared using cane molasses 6%, starch 2%, fish meal 2%, $CuSO_4 \cdot 5H_2O$ 0.01%, $CaCO_3$ 0.2% and NaI 0.00005%. The metabolite production profile of an extract from a 10-day old culture of *A. verrucosospora* ATCC 39334 grown in the complex medium H946 at 28°C on a rotary shaker operating at 250 rpm analyzed by HPLC is shown in Fig. 2. HPLC analysis was carried out by using a C-18 reverse-phase column (Novapak, 3.9 × 150 mm, Waters Associates), the solvent system was 0.05 M ammonium acetate (pH 4.5)-CH_3OH-CH_3CN (1:1:1) at a flow rate of 1 ml/minute and detected at 254 nm. The major product of fermentation was esp A_1 (Rt. 8.6 minutes) which comprised 75% of the esperamicin complex. The other major component of the fermentation, esp A_2 (Rt. 16.3 minutes) constituted approximately 15% of the complex.

Using the same medium and conditions described above for the preparation of the seed culture, an 8-ml aliquot of incubated seed culture was transferred to a

Fig. 1. Naturally occuring esperamicins.



Esperamicin	n	R	R_1	R_2
A_1	3	$CH(CH_3)_2$	AC	H
A_{1b}	3	CH_2CH_3	AC	H
A_{1c}	3	CH_3	AC	H
P	4	$CH(CH_3)_2$	AC	H
A_2	3	$CH(CH_3)_2$	H	AC
A_{2c}	3	CH_3	H	AC



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500-ml Erlenmeyer flask containing 100 ml of a chemically defined medium DF-15. Shifting the fermentation of *A. verrucosospora* ATCC 39334 from the complex medium to the defined medium DF-15 using sucrose as the sole carbon source and ammonium sulfate as the sole nitrogen source yielded a significantly different production profile for the esperamicin complex. Defined medium DF-15 consisted of sucrose 4%, NH_4Cl 0.2%, Na_2SO_4 0.2%, K_2HPO_4 0.1%, $MgCl_2 \cdot 6H_2O$ 0.1%, NaCl 0.1%, $CaCO_3$ 0.2%, $MnCl_2 \cdot 4H_2O$ 0.0001%, $ZnCl_2$ 0.0001%, $FeCl_2 \cdot 4H_2O$ 0.0001% and NaI 0.00005%. The metabolite production profile of an extract from a 6-day old culture of *A. verrucosospora* ATCC 39334 grown in medium DF-15 analyzed by HPLC is shown in Fig. 3. Esp A_1 and esp A_2 were not detected in the extract of *A. verrucosospora* ATCC 39334 grown in medium DF-15, however, two new esperamicin metabolites (Rts. 6.5 minutes and 10.3 minutes) that had not been previously isolated in our laboratory were identified by HPLC analysis.

Fig. 2. Chromatogram of HPLC analysis of an extract from a 10-day old culture of *Actinomadura verrucosospora* ATCC 39334 grown in complex medium H946.

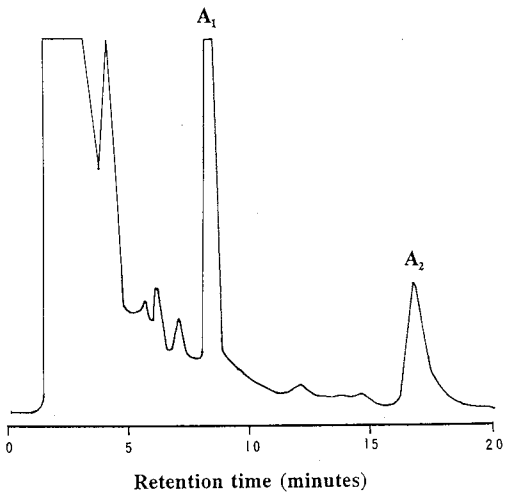
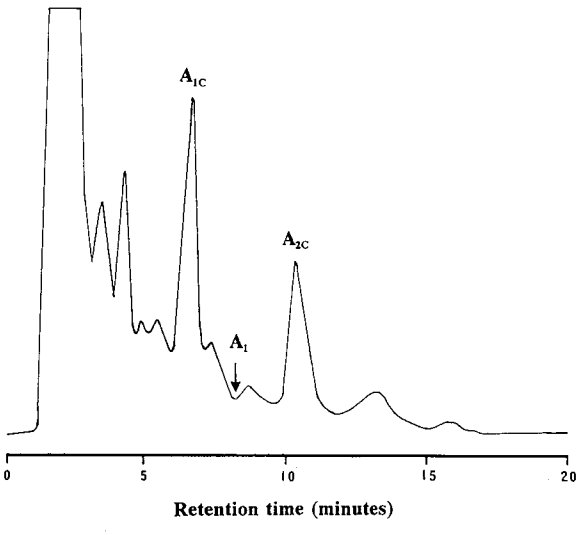
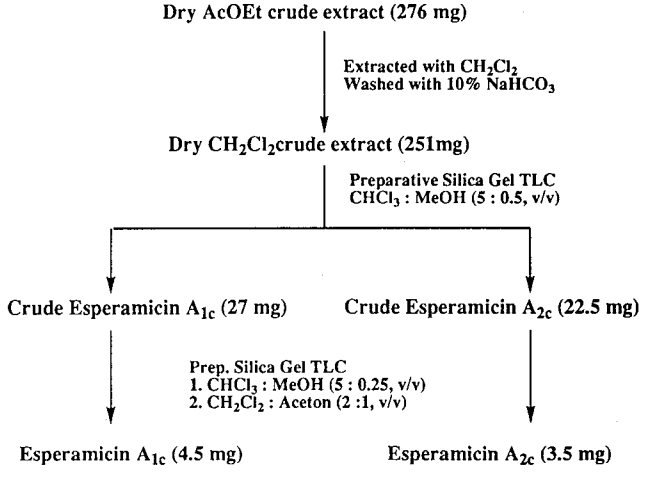


Fig. 3. Chromatogram of HPLC analysis of an extract from a 6-day old culture of *Actinomadura verrucosospora* ATCC 39334 grown in defined medium DF-15.



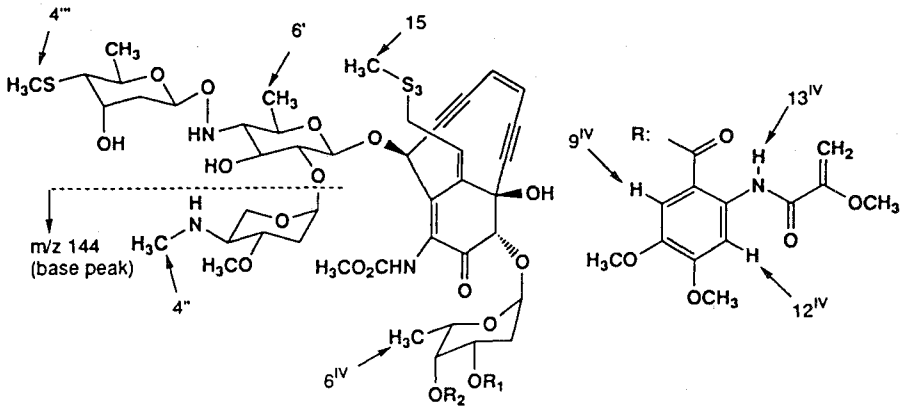
The purification of the two new analogs from a 10-liter fermentation broth is summarized in Fig. 4. The structure determination of these two new analogs is summarized in Fig. 5. Mass spectrometry analysis shows that both analogs have identical molecular ions 28 mass unit less than that of esp A_1 indicating a difference of two methylene groups. In addition, fragmentation of the glycosidic bond of the aminosugar residue results in formation of base peaks at *m/z* 144 that clearly identifies the alkylation pattern of the analyzed congeners (Fig. 5). Lack of availability in sufficient quantities, their instability, and the complexity of their NMR spectra precluded a full assignment of the chemical shifts for the minor components, yet the close structural relations among them allowed us to classify them based on a few readily identifiable diagnostic peaks. In the ^1H spectra the lowest three singlets at 11.77 ppm (br, NH), 8.58 ppm (H-12^{iv}), and 7.47 ppm (H-9^{iv}) are indicative of the A_1 series, while the equivalent peaks for the A_2 series are found at 11.90, 8.61, and 7.57 ppm, respectively (Fig. 5). In the high field region of the esp A_{1c} spectrum a doublet at 1.30 ppm indicates the presence of 6^{iv} methyl group. The equivalent doublet for the A_{2c} congener was shifted to 1.24 ppm (Fig. 5). Additional diagnostic information can be obtained from ^{13}C NMR data of the methyl region of the spectra. The signals for the N-isopropyl groups in esp A_1 and esp A_2 found at 22.2, 22.3 and 47.2 ppm were replaced by the N-methyl signals at 34.1 and 33.6 ppm for esp A_{1c} and esp A_{2c}, respectively (Fig. 5). The above data clearly indicate that the two new esperamicins were esp A_{1c} and esp A_{2c}. Esp A_{1c} was later determined to be the same as FR-900406 reported by Fujisawa Pharm. Co. Ltd.[8,9]. Esp A_{2c} is a novel esperamicin analog that has not been previously reported.

Fig. 4. Scheme for isolation of esperamicins A_{1c} and A_{2c} from a 10 liter shake flask batch.



The extracts and fractions from purification were evaporated to dryness *in vacuo* using a rotary evaporator at 25°C.

Fig. 5. Structure determination of esperamicins A_{1c} and A_{2c}. HPLC separation conditions used in LC-MS study: C-18 Novapak column (3.9 × 150 mm, Waters Associates); CH_3CN-CH_3OH-H_2O (33:33:34) solvent system; 1 ml/minute flowrate; temperature; 25°C.



Esperamicin		A_{1c} (R_1=R, R_2=H)	A_{2c} (R_1=H, R_2=R)
HPLC			
Rt, (min):		6.50	16.10
MS			
LC-TSP (*m/z*):	$(M+H)^+$	1297	1297
	$(M+Na)^+$	1319	1319
	Base peak	144	144
NMR			
^1H, $CDCl_3$ (ppm):	13^{IV}-NH	11.77	11.90
	12^{IV}-H	8.58	8.61
	9^{IV}-H	7.47	7.56
	6^{IV}-H	1.30	1.24
^{13}C, $CDCl_3$ (ppm):	C-4'''-S*Me*	13.7	13.7
	C-6^{IV}	16.7	16.6
	C-6'	17.6	17.5
	C-15-S_3*Me*	22.7	22.7
	C-4''-NH*Me*	34.1	33.6

Table 1. Effect of esperamicins A_1, A_{1c} and A_{2c} on P388 leukemia.

Compound	Dose (mg/kg/inj, ip)	Effect (% T/C)	Compound	Dose (mg/kg/inj, ip)	Effect (% T/C)
Esp A_1	0.016	180		0.001	135
	0.008	155		0.0005	130
	0.004	160	Esp A_{2c}	0.016	145
	0.002	135		0.008	135
	0.001	135		0.004	125
	0.0005	130		0.002	110
Esp A_{1c}	0.016	175		0.001	110
	0.008	160		0.0005	105
	0.004	150	Control	0	100
	0.002	155			

Tumor inoculum: 10^6 ascites cells, ip.
Host: CDF_1 mice.
Schedule: Q1D × 1; 2.
Evaluation: Medium survival time (MST).
Effect: % T/C = (MST treated/MST control) × 100.
Criteria: % T/C > 125 considered significant antitumor activity.
Control mice were given saline injection.

Table 2. Effect of esperamicins A_1 and A_2 on P388 leukemia.

Compound	Dose (mg/kg/inj, ip)	Effect (% T/C)	Compound	Dose (mg/kg/inj, ip)	Effect (% T/C)
Esp A_1	0.01	56		0.01	144
	0.003	104		0.003	144
	0.001	152		0.001	144
	0.0003	152		0.0003	136
	0.0001	144		0.0001	128
	0.00003	128		0.00003	120
	0.00001	128		0.00001	120
Esp A_2	0.1	48	Control	0	100
	0.03	104			

Tumor inoculum: 10^6 ascites cells, ip.
Host: CDF_1 mice.
Schedule: Q1D × 9; 1.
Evaluation: Medium survival time (MST).
Effect: % T/C = (MST treated/MST control) × 100.
Criteria: % T/C > 125 considered significant antitumor activity.
Control mice were given saline injection.

The *in vivo* antitumor activity of esp A_1, esp A_{1c} and esp A_{2c} against P388 leukemia implanted ip in mice is shown in Table 1. The activity and potency of esp A_{1c} against P388 leukemia are similar to those of esp A_1 and both of the compounds were active at the lowest dosage tested, 0.0005 mg/kg/inj. Esp A_{2c} was active against P388 leukemia at 0.004 mg/kg/inj but was inactive at 0.002 mg/kg/inj, indicating that it is at least 8 fold less potent than esp A_1 and esp A_{1c} based on minimal effective dose comparisons. Table 2 compares the effect of esp A_1 and esp A_2 against P388 leukemia implanted ip in mice. Esp A_1 was again active at the lowest dosage tested, 0.00001 mg/kg/inj. Esp A_2 was active against P388 leukemia at 0.0001 mg/kg/inj but was inactive at 0.00003 mg/kg/inj, indicating that it is at least 10 fold less potent than esp A_1 based on minimium effective dose comparisons. The above finding indicates that the position of attachment of the anthranilate chromophore to the 2-deoxy-L-fucose moiety is an important factor in determining the antitumor potency of esperamicins. Mechanism of action studies[10] demonstrated that the anthranilate-deoxyfucose moiety affects the uptake of the drug into the cells and after activation may also sterically hinder the resulting phenylene diradical so as to lead to inefficient double stranded breakage of DNA. Furthermore, results from molecular dynamics simulations and spectroscopic studies[11~13] showed that the anthranilate-deoxyfucose moiety contributes significantly to the biochemistry of esp A_1-induced DNA damage.

In this paper, we have demonstrated that new esperamicin analogs can be generated by changing the composition of the fermentation medium. The comparison of antitumor activity between esp A_{1c} and esp A_{2c} also confirms that the A_1 series are more potent than the A_2 series of esperamicin in the *in vivo* antitumor tests.

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