APHE-1 AND APHE-2, TWO NEW ANTIMICROBIAL AND CYTOTOXIC ANTIBIOTICS

I. TAXONOMY, FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITY

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(Received for publication June 18, 1992)

APHE-1 and APHE-2 are two new antibiotics produced by *Streptoverticillium griseocarneum* NCIMB 40447. They exhibited a remarkable cytotoxic activity against several tumor cell lines from different origin. Furthermore, they showed weak antimicrobial activity against Gram-positive bacteria, filamentous fungi and yeasts.

Over the course of a screening for novel antibiotics, an actinomycete strain 43/16 identified as *Streptoverticillium griseocarneum* was found to produce two new cytotoxic and antimicrobial antibiotics, APHE-1 and APHE-2. The producing organism was isolated from a soil sample taken at Perales de Tajuña (Madrid, Spain).

APHE-1 and APHE-2 exhibited *in vitro* antitumor activity against tumor cell lines of mice and human origin. Moreover, these antibiotics showed activity against Gram-positive bacteria, filamentous fungi and yeasts.

This paper deals with the taxonomy of the producing strain as well as the fermentation, isolation and biological activities of APHE-1 and APHE-2. Physico-chemical properties and structure determination of both compounds will be described in a subsequent paper[1].

Materials and Methods

Microorganism

Streptoverticillium sp. 43/16 was obtained from the culture collection of our Department. The origin of this strain, its maintenance and cultivation media have already been described[2].

Taxonomic studies

Preliminary morphological and physiological chracterizations of the producing strain 43/16 were reported in a previous paper[2].

To determine the definite taxonomic position of the strain, further assays required for numerical taxonomy as recommended by WILLIAMS *et al.*[3] for identification of *Streptoverticillium* species were conducted. In all cases inoculum consisted of dense spores and/or mycelial suspensions obtained by adding sterile water to sporulating cultures on BENNET's agar (BA)[4].

Identification scores were obtained using the Matiden[5] program. The strain was compared with the numbered clusters defined by LOCCI and SCHOFIELD[6].

Fermentation

The producing strain was maintained on BA medium or lyophilized for long periods of time according to HOPWOOD *et al.*[7]. The selected medium for antibiotic production was Tryptic Soy Broth (Difco), pH 7.2.

A standarized lyophilized inoculum of the producing strain was transferred to 500-ml Erlenmeyer flasks containing 100 ml of the production medium. Flasks were incubated at 28°C for 48 hours at 200 rpm on a rotatory shaker. 200 ml of the resulting culture were then transferred to a 15-liter fermenter containing 10 liters of the same medium. The fermentation was carried out at 28°C for 100 hours, aerated at a rate of 7 liters/minute and agitated at 150 rpm. Antibiotic production was monitored during the fermentation by HPLC.

HPLC of APHE-1 and APHE-2

HPLC analysis was performed on a Waters model 510, using a Waters μBondapak C-18 reverse-phase column (3.9 × 150 mm) eluted at 1 ml/minute flow rate with the mobile phase acetonitrile - water (64 : 36). The semipreparative HPLC separations were carried out on a μBondapak C_{18} column (7.8 × 300 mm) with the same mobile phase at 4 ml/minute. After injection the sample solutions elution was followed by UV detector at 254 nm.

Antimicrobial Activity

The antibacterial activity was monitored during the isolation and purification process checking the growth of *Micrococcus luteus* CECT 247 in liquid cultures in presence of the different fractions after 24 hours of incubation at 37°C.

The *in vitro* antimicrobial activities of APHE-1 and APHE-2 were determined by serial broth dilution method in nutrient broth media for bacteria and in Sabouraud media for filamentous fungi and yeasts. Antimicrobial activity was observed after overnight incubation at 37°C for bacteria and after 48 ~ 72 hours incubation at 28°C for filamentous fungi and yeasts.

Cytotoxic Activity on Tumor Cells

In vitro cytotoxicity of APHE-1 and APHE-2 was studied on a diverse group of murine and human tumor cell lines.

Cells were grown in monolayers or suspension cultures at 37°C in a humidified 5% CO_2 atmosphere. They were maintained in DULBECCO's modified essential medium or RPMI-1640 medium supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 10% heat-inactivated fetal bovine serum.

Cytolysis was roughly distinguished from the cytostatic activity by culturing a higher number of cells (8×10^4 vs. $2 \sim 5 \times 10^3$) under low-serum conditions (0.5 vs. 10%) and shorter antibiotic treatments (18 vs. 72 hours). These activities were essentially measured according to an automated colorimetric assay based on the production of dark blue formazan crystals by living cells incubated with the tetrazolium salt MTT[8]. Other standard procedures such as crystal violet staining of living cells[9] or [^{3}H]thymidine uptake for proliferation assays were also employed in several experiments.

Results and Discussion

Taxonomy of the Producing Strain

Based on taxonomic previous studies it was concluded that the producing strain 43/16 belongs to the genus *Streptoverticillium*.

Results of the assays for numerical taxonomy recommended by WILLIAMS *et al.* for identification of *Streptoverticillium* species are reported in Table 1.

Comparison of the strain with the numbered clusters described by LOCCI and SCHOFIELD resulted in a positive identification with *Streptoverticillium griseocarneum*. In Table 2 differences between the strain 43/16 and the type strain *Streptoverticillium griseocarneum* CECT 3250 are shown.

Table 1. Characteristics of the producing strain *Streptoverticillium griseocarneum* NCIMB 40447.

Character	Result	Character	Result
Aerial mycelium cottony	+	Hypoxanthine	+
Spores yellow	−	L-Tyrosine	−
Spores white	+	Tween 20	+
Melanin	−	NO_3^- reduction	−
Utilization of:		H_2S production	+
Mannitol	−	Growth at 12°C	−
D-Melibiose	−	Growth with:	
Raffinose	−	NaCl (5%, w/v)	+
Sorbitol	−	1-Phenylethanol (0.3%, w/v)	+
Coumarin	−	Potassium tellurite (0.01%, w/v)	−
L-Methionine	−	Crystal violet (0.01%, w/v)	−
L-Proline	+	Malachite green (0.01%, w/v)	−
Shikimic acid	−	Resistance to:	
DL-α-Aminobutyric acid	+	Azlocillin	+
Acid production from:		Carbenicillin	−
D-Galactose	−	Cephaloridine	+
meso-Inositol	+	Cephalotin	−
D-Fructose	−	Cefamandole	+
D-Ribose	−	Colistin	+
D-Trehalose	+	Antibiosis to:	
Degradation of:		*Aspergillus niger*	+
Esculin	+	*Bacillus subtilis*	+
Citrate	−	*Candida albicans*	+
DNA	+		

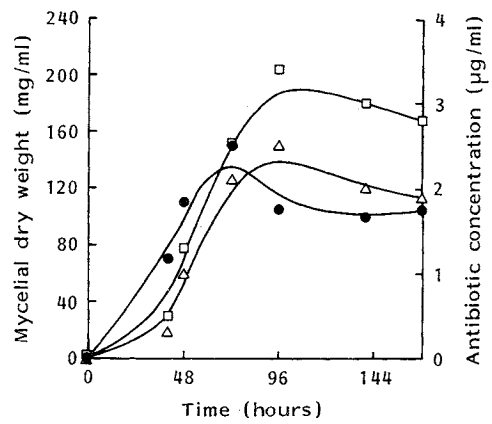
Table 2. Differential characteristics between *Streptoverticillium griseocarneum* NCIMB 40447 strain and the type culture CECT 3250.

	Streptoverticillium griseocarneum	
	CECT 3250	Strain 43/16
Degradation of:		
Esculin	+	−
Citrate	−	+
Tween 20	+	−
Acid production from:		
D-Ribose	−	+

The strain was deposited at the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland, U.K., with the name of *Streptoverticillium griseocarneum* under the accession number NCIMB 40447.

Fig. 1. Time courses of cell growth and antibacterial activity production in a 15-liter jar fermenter.

● Mycelial cell growth, △ APHE-1, □ APHE-2.



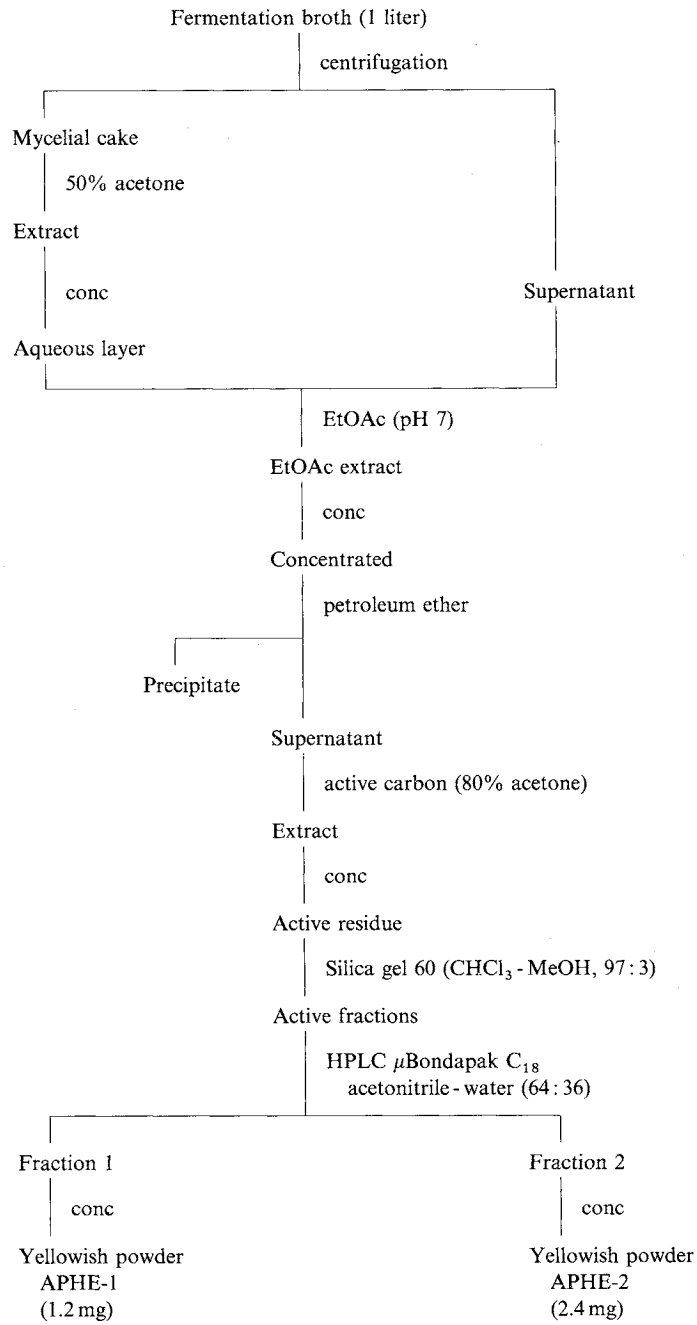
Fermentation

A typical time course for the production of the antimicrobial activity is shown in Fig. 1. Production started at approximately 36 hours after incubation and reached maximum titer at 72 hours.

Isolation of APHE-1 and APHE-2

The procedure for isolation of APHE-1 and APHE-2 is shown in Fig. 2. The antibiotic activity was detected in both mycelium and extracellular medium. The culture filtrate (5 liters) obtained after 72 hours of fermentation was adjusted to pH 7.0 and extracted with ethyl acetate. The mycelial cake was extracted with aqueous acetone and the acetone extract was concentrated *in vacuo* to give an aqueous

Fig. 2. Isolation procedure for APHE-1 and APHE-2.



solution. After adjusting to pH 7.0, the active component was extracted with ethyl acetate. Both extracts were combined and concentrated under reduced pressure, and then petroleum ether was added to eliminate impurities. The organic layer was concentrated to give a brown oily residue that was adsorbed on active carbon and then recovered with 80% aqueous acetone and concentrated under vacuum. This residue was chromatographed on a silica gel column (Kieselgel 60, 230~400 mesh ASTM, Merck; 32 × 2 cm) using a chloroform - methanol mixture as developing solvent. The active fractions were collected and the antibiotics were further purified by semipreparative HPLC (see above) to obtain yellowish powders, after removing the mobile phase under vacuum. By HPLC, both compounds showed a very close retention time, 4.4 and 5.2 minutes for APHE-1 and APHE-2, respectively. The contents of APHE-1 and APHE-2 after 72 hours of fermentation were 1.2 mg/liter and 2.4 mg/liter, respectively.

Antimicrobial Activity

Table 3 shows the antimicrobial activities of APHE-1 and APHE-2 against bacteria, filamentous fungi and yeasts. Both antibiotics showed a low activity against the most of assayed microorganisms. They were inactive against Gram-negative bacteria. The antimicrobial activity of APHE-2 was greater than that of APHE-1.

Table 3. Antimicrobial activity of APHE-1 and APHE-2.

Test organism	MIC (μg/ml)	
	APHE-1	APHE-2
Bacillus subtilis CECT 35	125	50
Staphylococcus epidermidis CECT 231	125	50
Micrococcus luteus CECT 247	62.5	37.5
Escherichia coli CECT 471	>125	>125
Enterobacter aerogenes CECT 684	>125	>125
Shigella flexnerii CECT 585	>125	>125
Klebsiella pneumoniae CECT 142	>125	>125
Penicillium oxalicum IJFM 1331	>125	>125
Fusarium culmorun CECT 2148	37.5	37.5
Cunninghamella echinulata CECT 2120	25	25
Aspergillus niger CECT 2545	>125	>125
Candida utilis CECT 1061	37.5	37.5

Cytotoxic Activity

In vitro cytotoxicity of APHE-1 and APHE-2 against a diverse group of murine and human tumor cell lines is summarized in Table 4. When asynchronous exponentially growing cultures were treated with APHE-1 and APHE-2, proliferation was markedly inhibited in all of the cell lines tested, and the inhibition rate was cell line-dependent (Table 4). [^{3}H]thymidine incorporation assessing DNA synthesis was also significantly reduced during prolonged antibiotic treatment over 72 hours (data not shown). The cytotoxic potency of APHE-1

Table 4. Cytotoxic activity of APHE-1 and APHE-2.

Cell line	Type	APHE-1		APHE-2	
		CS-ID_{50}[a]	CL-ID_{50}[a]	CS-ID_{50}	CL-ID_{50}
EL4	Murine lymphoma	6.5±2.1	20.0±5.1	14.0±5.1	34±11.1
A-1	Murine B hybridoma	2.6±0.7	20.0±4.8	9.4±2.3	25± 6.6
P388	Murine leukemia	11.5±3.8	>50.0	19.0±7.1	>50.0
L929	Murine fibrosarcoma	19.9±5.7	>50.0	14.5±4.3	>50.0
1591-RE-S	Murine fibrosarcoma	8.7±3.1	>50.0	10.5±4.2	>50.0
B16	Murine melanoma	19.0±6.3	>50.0	22.9±8.1	>50.0
L1210	Murine leukemia	25.0±6.5	>50.0	22.9±9.5	>50.0
HL-60	Human histiocytoma	30.1±7.4	>50.0	—	>50.0
HeLa	Human cervix carcinoma	7.4±3.0	36.0±9.2	18.2±6.8	40±12.3

[a] CS-ID_{50} and CL-ID_{50} are concentrations (μg/ml±SD of three experiments) that respectively inhibit growth and viability by 50%. The exposure time in cytostatic and cytolytic assays was 72 and 18 hours, respectively.

and APHE-2 was nearly the same.

Thus, both growth inhibition (through blockade of DNA synthesis or different mechanisms) and disruption of cellular integrity (either necrosis or apoptosis) might account for the overall cytotoxic effect but further in-depth experiments are required to determine its precise mode of action.

Subsequent *in vivo* experiments using grafted tumors will contribute to determine the potential of APHE-1 and APHE-2 as chemotherapeutic agents for antitumor therapy.

Acknowledgments

This investigation was aided by a fellowship to M. L. FIDALGO from the Ministerio de Educación y Ciencia, Spain. The authors wish to thank Prof F. URUBURU, Head of Spanish Type Culture Collection (CECT) for generously providing us the strains used in this work.

References

1) FIDALGO, M. L.; M. S. ARIAS, J. SOLIVERI & M. E. ARIAS: APHE-1 and APHE-2, two new antimicrobial and cytotoxic antibiotics. II. Physico-chemical properties and structure elucidation. J. Antibiotics 45: 1759~1762, 1992
2) SOLIVERI, J.; M. E. ARIAS & F. LABORDA: PA-5 and PA-7, pentaene and heptaene macrolide antibiotics produced by a new isolate of *Streptoverticillium* from Spanish soil. Appl. Microbiol. Biotechnol. 25: 366~371, 1987
3) WILLIAMS, S. T.; R. LOCCI, J. VICKERS, G. M. SCHOFIELD, P. H. A. SNEATH & A. M. MORTIMER: Probabilistic identification of *Streptoverticillium* species. J. Gen. Microbiol. 131: 1681~1689, 1985
4) LOCCI, R.; E. BALDACCI & B. PETROLINI: The genus *Streptoverticillium*. A taxonomic study. Giorn. Microbiol. 17: 1~60, 1969
5) SNEATH, P. H. A.: BASIC program for identification of an unknown with presence-absence data against an identification matrix of percent positive characters. Comput. Geosci. 5: 195~213, 1979
6) LOCCI, R. & M. SCHOFIELD: Genus *Streptoverticillium*. *In* BERGEY's Manual of Systematic Bacteriology. Volume 4. *Ed.*, S. T. WILLIAMS *et al.*, pp. 2492~2504, Williams & Wilkins, 1989
7) HOPWOOD, D. A.; M. J. BIBB, F. CHATER, T. KIESER, C. J. BRUTON, H. M. KIESER, D. J. LYDIATE, C. P. SMITH, J. M. WARD & H. SCHREMPF: Genetic manipulation of *Streptomyces*. A laboratory manual. The John Innes Foundation, 1985
8) MOSMANN, T.: Rapid colorimetric assay for cellular growth and survival. Application to proliferation and cytotoxicity assays. J. Immunol. Methods 65: 55~63, 1983
9) RUFF, M. R. & G. E. GIFFORD: Tumor necrosis factors. *In* Lymphokines II. *Ed.*, E. PICK, pp. 235~272, Academic Press, 1981