APHE-3 and APHE-4, Two New Pyrazoloisoquinolinone Antibiotics Produced by *Streptoverticillium griseocarneum* NCIMB 40447

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In previous studies, APHE-1 and APHE-2, two antibiotics produced by *Streptoverticillium griseocarneum* NCIMB 40447, exhibited *in vitro* antitumor activity against tumor cell lines of mouse and human origin. Furthermore, these antibiotics showed activity against Gram-positive bacteria, filamentous fungi and yeasts[1]. A b-fused 1(2*H*)-isoquinolinone system was proposed as the most probable structure for these antibiotics and thus, they were identified as 3-substituted 1*H*-pyrazolo [2,3-*b*]isoquinolin-9-ones (pyrazoloisoquinolinone antibiotics)[1,2], which only differ in the nature of the aliphatic chain at the C-3 of the pyrazole ring, being an ethyl and a *n*-propyl group for APHE-1 and APHE-2, respectively.

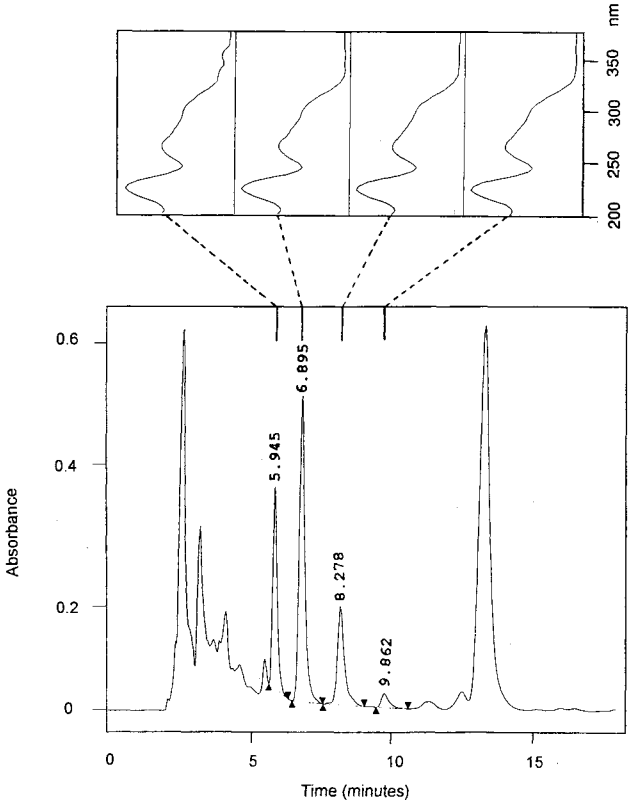
During the fermentation of the producing strain in BENNETT's agar medium[3], other active compounds were also detected by HPLC. This study is focused on the isolation and characterization of new pyrazoloisoquinolinone antibiotics and the comparison of their biological activities.

For antibiotic production in solid cultures, Petri dishes containing 20 ml of BENNETT's agar medium were overlayed with sterile cellophane discs (75 mm of diameter 325P Cello Discs, Cannings Packaging Ltd, Bristol, UK) and inoculated with 100 μl of a standarized spore suspension (1×10^7 viables/ml) in sterile distilled water. These cultures were incubated at 28°C for 9 days. The cellophane discs were used to facilitate the harvest of mycelium. The mycelium was extracted several times with methanol. All extracts were concentrated *in vacuo* to dryness and the residues were dissolved in an appropriate volume of methanol. The antibiotics were further purified by semipreparative HPLC (Shimadzu LC-9A Liquid Chromatograph, Shimadzu Corporation, Kyoto, Japan). HPLC was performed using a reverse phase Nucleosil 120-5C_{18} column (8 × 250 mm, Scharlau S.A., Barcelona, Spain). The mobile phase was acetonitrile - water (64 : 36) at a flow rate of 4 ml/minute. The chromatograms were analyzed with a photodiode array UV-VIS detector Waters 996 set at 254 nm.

The methanol extracts were resolved as several absorption maxima. The maxima corresponding to the retention times of 6.8 and 8.2 minutes were identified as the pyrazoloisoquinolinone antibiotics APHE-1 and APHE-2, respectively. The UV spectra of the maxima corresponding to 5.9 and 9.8 minutes of retention time (APHE-3 and APHE-4, respectively) were identical to those showed by APHE-1 and APHE-2 and exhibited characteristic absorption maxima at 265, 280 and 296 nm indicating a similar aromatic chromophore (Fig. 1).

Mass spectra of the antibiotics were determined with a Hewlett Packard HP5988A mass spectrometer (Hewlett Packard Company, Palo Alto, U.S.A.) interfaced with an HP Series 1050 chromatograph by an HP Particle Beam 59980A LC/MS Interface. The MS source was held at 250°C. The electron impact mass spectra were obtained at 70 eV from *m/z* 40 to 350. In the mass spectra the ions with major molecular magnitude correspond to the fragments with a *m/z* relation of 198 and 240 for APHE-3 and APHE-4, respectively. The ion *m/z* = 197 present in the MS spectrum of APHE-4 indicates the loss of a fragment of 43 (C_3H_7) mass units. The fragmentation sequence of these compounds showed the same essential fragment ions to those obtained for APHE-1 and APHE-2, suggesting the presence of the same or a similar ($C_{11}H_7N_2O$) structural unit[2]. Thus, the aliphatic substituents must correspond to a methyl and a *n*-butyl or *iso*-butyl group for APHE-3 and APHE-4, respectively.

Fig. 1. HPLC profile of the methanol extract from mycelium.



The upper part of the figure shows the UV spectra of the pyrazoloisoquinolinone antibiotics.

The proposed molecular formula for APHE-3 as deduced from LC-MS and elemental analysis is $C_{12}H_{10}N_2O$. The IR spectrum of APHE-3 was, too, very similar to those described for APHE-1 and APHE-2, showing amide carbonyl absorptions at 1640 and 1583 cm^{-1} as well as a NH band at 3200 cm^{-1}.

Unfortunately, it has not been possible to obtain enough quantities of APHE-4 to carry out more assays for its physico-chemical characterization.

The ^1H and ^{13}C NMR spectra of APHE-3 in $CDCl_3$ solution only exhibited a singlet in the high-field region due to a methyl group. The other signals came up in the low-field region, between 7.1 and 8.4 ppm and 106 and 159 ppm, respectively. These proton and carbon resonances were very similar to those previously described for the antibiotics APHE-1 and APHE-2.

The detailed analysis of the spectra was carried out using 2D NMR techniques-homonuclear ^1H-^1H (COSY-45)[4] and heteronuclear ^1H-^{13}C (XHCORD)[5,6] correlated spectra and double resonance (DR) experiments. As in the case of the reported compounds, the NMR data agreed with the presence of one NH, one amide carbonyl and six methine groups, as well as four quaternary carbon atoms.

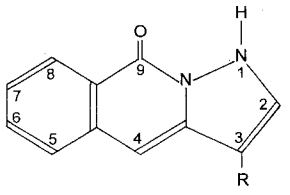
The assignment of the respective proton and carbon signals was achieved on the basis of the spectroscopic data—2D correlations and DR modifications—, from our previous study on APHE-1 and APHE-2[2], as well as substituent steric and electronic effects on proton[7,8] and ^{13}C chemical shifts[8,9] and the magnitude of the ^1H-^1H coupling constants[7,8]. Owing to the similarity of the proton and carbon parameters for the antibiotics APHE-1, APHE-2[2] and APHE-3, it seems to be very clear that these compounds should have the same basic structure which corresponds to a 3-substituted 1*H*-pyrazolo [2,3-*b*]isoquinolin-9-one, and only differ in the nature of the aliphatic chain in position 3 (Fig. 2).

On the basis of these observations, it might be proposed that these condensed heterotricycles have a common origin and are produced by *Streptoverticillium griseocarneum* NCIMB 40447[1] by the same biosynthetic pathway.

The *in vitro* antimicrobial activities of the pyrazoloisoquinolinone antibiotics were determined by serial broth dilution method. The results seem to indicate a relation between the length of the aliphatic chains of these compounds and their antimicrobial activity. Thus, antimicrobial activity was enhanced by the antibiotics with longer aliphatic chains, except in the case of assays against *Staphylococcus epidermidis* and *Candida utilis* (Table 1). Although these antibiotics did not show growth inhibitory activity against *Penicillium oxalicum* and *Aspergillus niger*, the growth rates of these organisms were lower than in the control assays. They were inactive against Gram-negative bacteria.

The effect of APHE antibiotics on the proliferative response of human lymphocytes from peripheral blood or synovial fluid in presence or absence of phytohemagglutinin was carried out using the method of WANG

Fig. 2. Structure of the pyrazoloisoquinolinone antibiotics.



	R
APHE-3	CH_3
APHE-1	CH_2–CH_3
APHE-2	CH_2–CH_2–CH_3
APHE-4	CH_2–CH_2–CH_2–CH_3

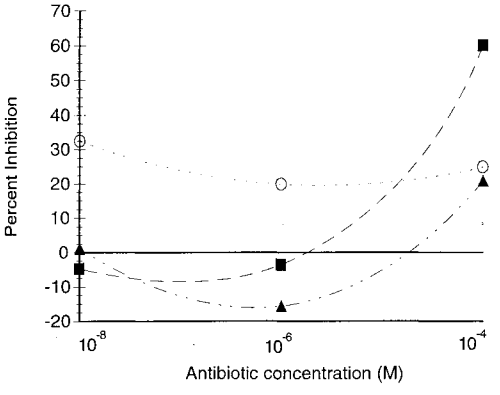
Table 1. Antimicrobial activity of the pyrazoloisoquinolinone antibiotics.

Test organism	MIC (μg/μl)			
	APHE-3	APHE-1	APHE-2	APHE-4
Bacillus subtilis CECT 39	>125	125	62.5	NT
Staphylococcus epidermidis CECT 231	50	125	50	NT
Staphylococcus aureus CECT 59	>125	125	125	NT
Micrococcus luteus CECT 247	>125	62.5	37.5	37.5
Mycobacterium phlei CECT 3009	125	62.5	37.5	NT
Enterococcus faecalis CECT 795	>125	>125	>125	NT
Escherichia coli CECT 405	>125	>125	>125	NT
Enterobacter cloacae CECT 194	>125	>125	>125	>125
Salmonella sp. CECT 545	>125	>125	>125	NT
Aspergillus niger CECT 2545	>125	>125	>125	NT
Penicillium oxalicum IJFM 1331	>125	>125	125	NT
Cunninghamella echinulata CECT 2120	125	25	37.5	37.5
Candida utilis CECT 1061	<12.5	87.5	87.5	125
Candida albicans CECT 1354	>125	>125	125	NT
Nocardia asteroides CECT 3042	87.5	37.5	NT	NT

NT: no tested.

Fig. 3. Comparison of the effects of APHE-3, APHE-1 and APHE-2 on lymphocyte proliferation.

■ APHE-3, ○ APHE-1, ▲ APHE-2.



Lymphocytes were stimulated with phytohemagglutinin in the absence or presence of various concentrations of each drug.

et al.[10]. According to the data showed in Figure 3, only APHE-3 inhibited lymphocyte proliferation in the presence of phytohemagglutinin by more than 50 percent at a concentration of 10^{-4} M. With APHE-1 and APHE-2 the effect was less. This result could be due to the smaller size of APHE-3 being better able to bypass the membrane barrier of the cells.

APHE-3 and APHE-4 showed the same basic structure as the previously described APHE-1 and APHE-2. Antimicrobial and immunomodulatory activity assays denote a relationship between the biological activity of these compounds and the length of their aliphatic chains. Thus, antibiotics with longer aliphatic chains exhibited enhanced antimicrobial activity, while their immunomodulatory activity was decreased.

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