PM-94128, a New Isocoumarin Antitumor Agent Produced by a Marine Bacterium

LIBRADA M. CAÑEDO*, JOSÉ L. FERNÁNDEZ PUENTES and JULIA PÉREZ BAZ

Pharma Mar S.A. Research laboratory, 24196 Carbajal de la Legua, León. Spain

CRISTINA ACEBAL, FERNANDO DE LA CALLE, DOLORES GARCÍA GRÁVALOS and TERESA GARCÍA DE QUESADA

Pharma Mar S.A. Research Laboratory, 28046 Tres Cantos, Madrid. Spain

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In general, isocoumarins have been reported to exhibit antibacterial, antitumor and gastroprotective activity and some have been isolated from culture broths of the genus *Bacillus*. In our screening program for new antitumor compounds produced by marine bacteria, we have isolated a new isocoumarin named PM-94128 from the culture broth of *Bacillus* sp. PhM-PHD-090 isolated from a marine sediment. PM-94128 is a basic substituted isocoumarin antibiotic with cytotoxic activity against several tumor cell lines.

Experimental

A seed medium composed of 0.5% soybean, 0.2% yeast extract, 0.2% dextrose, 0.2% MOPS (3-(*N*-morpholino)propanesulfonic acid) and 2.7% Instant Ocean Salts (Aquarium Systems, Inc.) was inoculated with a slant culture of strain PhM-00-PHD-090 and incubated at 28°C on a reciprocal shaker for 22 hours. The resultant seed culture was transferred to a 16-liter stainless steel jar-fermenter containing 10 liters of the production medium consisting of 0.4% corn steep liquor, 0.5% soybean, 0.2% yeast extract, 1% dextrose, 0.4% $CaCO_3$ and 1.5% Instant Ocean Salts. The fermentation was carried out at 28°C for 24 hours.

The following extraction and purification processes were performed by monitoring the activity against P-388 cell lines.

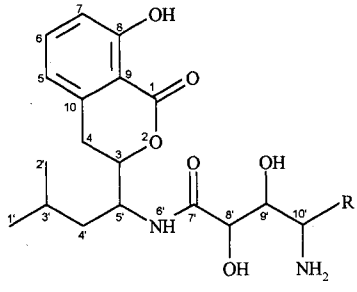
The fermentation broth (10 liters) was extracted with 1:1 volumes of EtOAc and the organic extract was concentrated under vacuum to syrup (2.16 g). The extract was dissolved in 300 ml of a mixture 10% aqueous NaCl-MeOH 1:1 and defatted by partitioning twice with 300 ml of *n*-hexane. The water-alcohol fraction was extracted twice with 300 ml of CH_2Cl_2 and the active CH_2Cl_2 extracts concentrated to yield 600 mg. The extract was chromatographed on a silica gel column using hexane-EtOAc as eluting solvent. Fractions with antitumor activity (280 mg) were eluted with *n*-hexane-EtOAc 75:25. Column chromatography on silica gel was repeated and the activity (90 mg) eluted with $CHCl_3$-MeOH 98:2. The final purification step was carried out by column chromatography on C18 reversed phase using H_2O-MeOH 15:85 as the eluting solvent. Pure fractions on TLC were combined and evaporated to yield 52 mg of pure PM-94128.

Results and Discussion

PM-94128 gave positive color reaction with ninhydrin reagent. The mp was 172~173°C and $[\alpha]_D^{25}$ −88.9° (*c* 2.0, $CHCl_3$). The ultraviolet spectrum, λ_{max} nm (ε): 208 (27,000), 246 (6,400), 314 (4,380), and the infrared spectrum, ν_{max} (KBr) cm^{-1}: 3365, 2954, 1664, 1523 indicated the presence of a benzoic acid moiety with a hydroxyl group on the benzene ring, and an amide group. Comparison of the ultraviolet spectrum of PM-94128 with those of isocoumarin compounds, such as baciphelacin[1], amicoumacin[2,3], AI-77-B[4,5], xenocoumacins[6] and Y-05460M-A[7], suggested that PM-94128 contains a chromophore similar to 3,4-dihydro-8-hydroxyisocoumarin in its structure. The molecular formula $C_{22}H_{34}N_2O_6$ was determined by HRFAB-MS experiment, which gave a $(M+H)^+$ ion at *m/z* 423.2482 (calcd for $C_{22}H_{35}N_2O_6$ 423.2495) and by the carbon number in the ^{13}C NMR spectrum.

The assignments of ^1H and ^{13}C NMR are shown in Table 1. A ^1H-^{13}C correlation spectrum allowed the specific ^{13}C assignments shown in Table 1 and multiplicities were determined by DEPT experiments. The connectivities in the ^1H NMR spectrum of PM-94128 were confirmed completely by decoupling experiments and by the ^1H-^1H COSY spectrum. The ^1H and ^{13}C spectral data of PM-94128 (**1**) are essentially in agreement with the corresponding values reported for the related $C_{21}H_{32}N_2O_6$ isocoumarin Y-05460M-A (**2**)[7], but our compound contains an additional methylene group at δ_C 44.16 (11′) and δ_H 1.18 (11′-H_a), δ_H 1.80 (11′-H_b). In the ^1H-^1H COSY spectrum the methylene signal at δ 1.18 (11′-H_a) connected to a methine at δ 2.90 (10′-H) which

Fig. 1. Structures of PM-94128 (**1**) and related isocoumarin Y-05460M-A (**2**).



PM-94128 (**1**): R = CH_2–CH(CH_3)–CH_3

Y-05460M-A (**2**): R = CH(CH_3)–CH_3

Table 1. NMR spectral data for PM-94128 ($CDCl_3$, δ in ppm).

^{13}C shift	^1H shift	Assignment	^{13}C shift	^1H shift	Assignment
175.20		7′	54.99	2.90	10′
169.50		1	48.54	4.30	5′
162.10		8	44.16	1.18	11′
				1.80	
139.38		10	40.43	1.50	4′
				1.85	
136.41	7.40	6	30.22	3.08	4
				2.80	
118.18	6.68	5	24.77	1.65	3′
116.11	6.88	7	23.53	1.75	12′
108.03		9	24.00	0.97	13′
80.98	4.60	3	20.94	0.90	14′
74.78	4.08	8′	22.99	0.95	1′
73.60	3.34	9′	21.81	0.92	2′

^{13}C NMR (75 MHz) and ^1H NMR (300 MHz) were recorded on a Varian Unity 300 spectrometer.

was coupled to a methine signal (9′-H) at δ 3.34.

The ^1H-^1H COSY spectrum showed an isopropyl group at δ 0.97 and 0.90 (13′ and 14′-H_3) coupled with a methine group at δ 1.75 (12′-H). In the spin decoupling experiment, irradiation at δ 1.75 (12′-H) converted two methyl doublets at δ 0.97 and 0.90 (13′ and 14′-H_3) into singlets and caused substantial change in the shape of the multiplets at δ 1.18 and 1.80 (11′-H_2). These findings suggested that the methylene 11′ was attached to the methines at δ 2.90 (10′-H) and δ 1.75 (12′-H). From these data, we propose that the isocoumarin PM-94128 isolated from PhM-PHD-090 has the structure (**1**).

Biological Activity

In vitro antitumor activity was studied, using an adaptation of the method described by BERGERON *et al.*[8]. The antitumor activity of isocoumarin PM-94128 against tumor cell lines is shown in Table 2.

The effect of the compound PM-94128 on the apparent rates of DNA, RNA and protein synthesis was assessed by measuring cellular incorporation of tritiated thymidine, uridine and leucine (NEN Research Products) from P-388 culture fluids, following the method of TOMITA[9], with small modifications to adapt it to 96 well microplates. Isocoumarin PM-94128 inhibited protein synthesis with an IC_{50} of 0.1 μM and DNA synthesis with an IC_{50} of 2.5 μM.

Table 2. Antitumor activity of PM-94128 (IC_{50} μM).

Compound	P-388	A-549	HT-29	MEL-28
PM-94128	0.05	0.05	0.05	0.05

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