Tartrolon D, a Cytotoxic Macrodiolide from the Marine-Derived Actinomycete *Streptomyces* sp. MDG-04-17-069

Marta Pérez,[†] Cristina Crespo,[†] Carmen Schleissner,[‡] Pilar Rodríguez,[‡] Paz Zúñiga,[‡] and Fernando Reyes*[,†]

Medicinal Chemistry and Microbiology Departments, PharmaMar S.A., Pol. Ind. La Mina Norte, Avenida de los Reyes 1, 28770-Colmenar Viejo (Madrid), Spain

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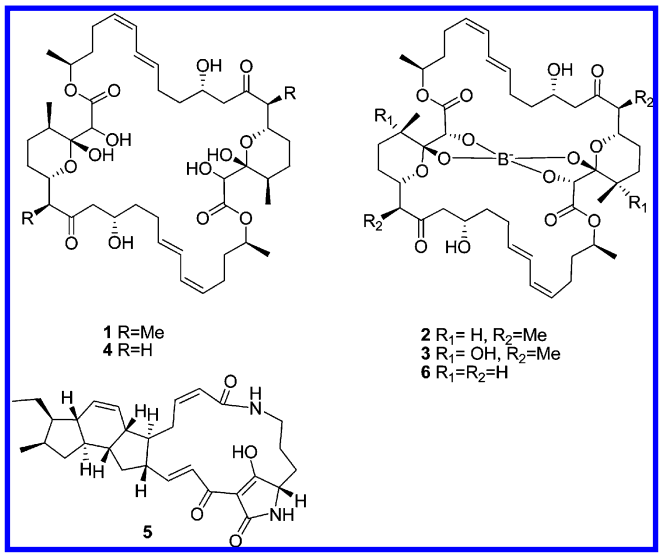
Exploration of marine-derived actinomycetes as a source of antitumor compounds has led to the isolation of a new member of the tartrolon series, tartrolon D (**4**). This new compound was obtained from *Streptomyces* sp. MDG-04-17-069 fermentation broths and displayed strong cytotoxic activity against three human tumor cell lines. Additionally, the known compound ikarugamycin (**5**) was also found in the culture broths of the same microorganism. The structure of this new tartrolon was established by a combination of spectroscopic techniques (1D and 2D NMR, HRMS, and UV) as well as by comparison with published data for similar compounds.

The tartrolons are a class of compounds that have attracted a great deal of attention owing to their interesting biological properties. These compounds, which are symmetric macrodiolides, were first isolated in 1994 by Höfle and Reichenbach from cultures of the myxobacterium *Sorangium cellulosum*.[1] The fermentation broths of this microorganism produced either tartrolon A (**1**) or B (**2**), where the incorporation of boron in the structure to give tartrolon B depended on the use of glass-lined fermentation vessels instead of stainless steel tanks. In addition to the moderate antimicrobial activities exhibited by all the members of the family,[1b] tartrolon A showed cytotoxic activity against the rat fibroblast line L929 with a MIC value of 0.07 mg/mL.[2] In 2003 a new compound of the family, tartrolone C (**3**), with an additional hydroxy group in each monomeric unit, was isolated from a *Streptomyces* species on the basis of its insecticidal activity.[3] This compound also exhibited inhibition of HIF-1 transcriptional activity under hypoxic conditions (IC_{50} = 0.17 μg/mL).[4]



The absolute configuration of tartrolon B was established by X-ray structure analysis,[5] and the substructures and configurations of the boron-binding site were found to be identical to that of the related macrodiolides boromycin,[6] aplasmomycin,[7] and borophycin.[8] The amount of interest in this family of compounds is evidenced by the large number of attempts by different groups to complete their total synthesis. The first total synthesis of tartrolon B was described by the group of Mulzer using a stereoselective boron-mediated aldol reaction as the key step.[9] More recently, another procedure employing a silicon-tethered ring-closing metathesis strategy has been reported by Lee and co-workers.[10]

As part of our ongoing screening for new antitumor compounds from marine microorganisms, we have isolated a new member of the tartrolon family, namely, tartrolon D (**4**), produced by culture broths of the actinomycete *Streptomyces* sp. MDG-04-17-069, isolated from a 30 m deep marine sediment collected off the east coast of Madagascar. The compound was obtained by reversed-phase C-18 column chromatography and repeated preparative and semipreparative reversed-phase HPLC of EtOAc extracts of the supernatant of culture broths of the actinomycete. Additionally, the known compound ikarugamycin (**5**) was also found in these crude extracts.[11]

Tartrolon D was obtained as a white powder with a molecular formula of $C_{44}H_{68}O_{14}$ according to its (+)-HRESIMS (*m/z* 843.4488 $[M + Na]^+$) and ^{13}C NMR data (Table 1), indicating 11 degrees of unsaturation. The dimeric nature of the compound was evidenced by the presence of only 22 signals in its ^{13}C NMR spectrum. As in the case of other tartrolons, several losses of 18 amu appeared in the mass spectrum, indicating multiple hydroxylation sites. The planar structure of **4** was established on the basis of the analysis of its NMR data (Table 1) and comparison with data reported for the related tartrolons A−C.[1,3] Taking into account the dimeric nature of the compound, signals for four methyl groups, 16 methylenes, 10 aliphatic/oxygenated methines, four 1,2-disubstituted double bonds, and six quaternary carbons were assigned by HSQC experiments. Three spin systems (A−C) were identified by careful analysis of COSY and 1D-TOCSY correlations (Figure 1). Connectivities between these spin systems were established by analysis of the HMBC spectrum (Figure 1). Thus, HMBC cross-peaks from both H-8, both H-10, and H-11 protons to carbon C-9 linked fragments B and C through a ketone functionality. On the other hand, correlations from H-2 to C-3 and C-7 (four-bond distance) and from H-4 and the methyl group H-22 to C-3 attached fragment A to B through carbon C-3, whose chemical shift was indicative of the presence of a ketal ring in the molecule, similar to that found in the structures of tartrolons A−C. Finally, to complete the planar structure of tartrolon D, HMBC cross-peaks from H-2 and H-20′ to the carbonyl C-1 and the low-field chemical shift of proton H-20′ (4.98 ppm) closed the ring and proved the symmetric bis-lactone nature of tartrolon D.

* To whom correspondence should be addressed. Tel: +34 91 823 4527. Fax: +34 91 846 6001. E-mail: jfreyes@pharmamar.com.
[†] Medicinal Chemistry Department.
[‡] Microbiology Department.

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Table 1. NMR Spectroscopic Data (500 MHz, CD_3OD) for Compounds **4** and **6**

	4		**6**	
position	δ_C, mult.	δ_H, mult. (*J* in Hz)	δ_C, mult.	δ_H, mult. (*J* in Hz)
1	172.2, qC		175.1, qC	
2	74.6, CH	4.15, s	79.5, CH	4.48, s
3	99.4, qC		105.6, qC	
4	35.3, CH	2.03, m	36.4, CH	1.89, m
5	28.8, CH_2	1.69, m; 1.53, m	29.2, CH_2	1.72, m; 1.60, m
6	32.9, CH_2	1.62, m; 1.23, m	33.7, CH_2	1.67, m; 1.21, m
7	68.3, CH	4.27, m	67.2, CH	4.41, m
8	50.1, CH_2	2.52, dd (15.0, 8.9)	51.9, CH_2	2.62, dd (14.0, 3.5)
		2.39, dd (15.0, 4.3)		2.13, dd (14.0, 12.5)
9	211.3, qC		212.0, qC	
10	52.2, CH_2	2.62, dd (15.8, 3.8)	48.3, CH_2	3.19, dd (18.3, 10.2)
		2.52, dd (15.8, 8.9)		2.48, brd (18.3)
11	67.8, CH	4.07, m	68.3, CH	3.95, m
12	37.8, CH_2	1.55, m, 2H	33.7, CH_2	1.62, m; 1.32, m
13	29.8, CH_2	2.20, m, 2H	29.3, CH_2	2.35, m; 2.00, m
14	135.6, CH	5.70, dt (14.2, 7.1)	135.6, CH	5.84, dt (14.3, 4.9)
15	126.9, CH	6.35, ddd (14.2, 11.4, 0.7)	124.7, CH	6.08, m
16	130.8, CH	5.99, dd (11.4, 10.8)	132.0, CH	6.04, m
17	129.4, CH	5.28, m	128.7, CH	5.26, m
18	24.7, CH_2	2.26, m, 2H	23.8, CH_2	2.44, m ; 1.93, m
19	36.8, CH_2	1.72, m; 1.62, m	37.0, CH_2	1.82, m; 1.40, m
20	72.8, CH	4.98, m	71.2, CH	4.68, qd (6.2, 6.0)
21	20.5, CH_3	1.28, d (6.2)	21.2, CH_3	1.20, d (6.2)
22	16.7, CH_3	0.98, d (6.9)	17.1, CH_3	1.04, d (6.7)

The presence of two diene functionalities in the molecule was confirmed by an UV absorption at $\lambda = 231$ nm, similar to that observed in the spectra of other tartrolons[1] and consistent with the presence in the ^1H and ^{13}C NMR spectra of signals accounting for eight sp^2 methines. The *E* geometry of the Δ^{14} double bond was inferred from the J_{14-15} coupling constant of 14.2 Hz. In a similar manner, a J_{16-17} coupling constant of 10.8 Hz indicated a *Z* geometry for the Δ^{16} double bond.

Similarly to that observed for the structures of tartrolons A and B, and tartrolone C, the relative configuration of the six-membered ketal ring in **4** had the alkyl substituents at C-3, C-4, and C-7 in an equatorial disposition, as could be deduced from correlations observed in a NOESY experiment (Figure 2). The configurations at positions C-11 and C-20 of **4** were assumed to be the same as in the rest of the tartrolons on the basis of biogenetic grounds and also due to the almost identical chemical shifts and multiplicities found in their NMR spectra.[1,3] In fact, the only major differences found in the spectra of tartrolon D with respect to that of tartrolon A were due to the absence of the methyl group at C-8 present in the latter compound, corroborated by the presence of two H-8 methylene protons in the ^1H spectrum of **4** (δ 2.52, dd, $J = 15.0$, 8.9; 2.39, dd, $J = 15.0$, 4.3). Finally, the configuration at C-2 remained undetermined.

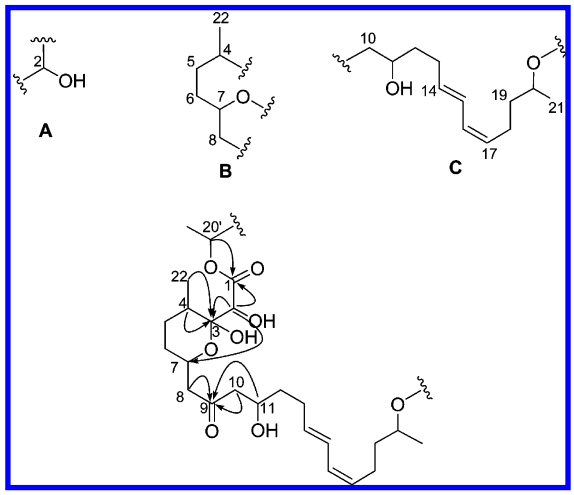

Figure 1. Spin systems (A−C) and key HMBC correlations observed in the structure of **4**.

In addition to the reported data for tartrolon D a second set of signals accounting for the presence of a minor compound was evident in the NMR spectra of samples of **4** (Table 1). This compound was identified as the boron-containing equivalent of tartrolon D (**6**) by comparison of its NMR chemical shifts with those of tartrolon B and was probably formed during the storage of the sample in glass vials, as its proportion increased with time.

Cytotoxicity assays were performed for tartrolon D against a panel of three human tumor cell lines, including lung (A549), colon (HT29), and breast (MDA-MB-231). The compound displayed GI_{50} values in the submicromolar range against all the lines tested (Table 2), making it a promising candidate to pursue further studies to assess its potential as an anticancer agent.

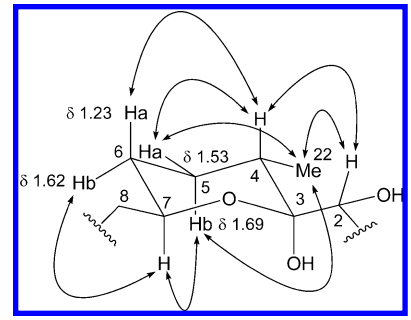

Figure 2. NOESY correlations observed in the six-membered ketal ring of **4**.

Table 2. Cytotoxic Activity (μM) for Tartrolon D (**4**) and Doxorubicin against Three Human Tumor Cell Lines

		cell lines		
		MDA-MB-231	A549	HT29
4	GI_{50}	0.79	0.16	0.31
	TGI	3.41	1.46	1.71
	LC_{50}	11.0	8.05	8.41
doxorubicin	GI_{50}	0.02	0.08	0.07
	TGI	0.12	0.17	0.33
	LC_{50}	0.86	0.35	>17.2

Experimental Section

General Experimental Procedures. Optical rotations were determined using a Jasco P-1020 polarimeter. UV spectra were obtained with an Agilent 1100 DAD. IR spectra were measured on a Perkin-Elmer Spectrum 100 FT-IR spectrometer. NMR spectra were recorded on a Varian "Unity 500" spectrometer at 500/125 MHz (^{1}H/^{13}C). Chemical shifts were reported in ppm using residual CD_3OD (δ 3.31 for ^{1}H and 49.0 for ^{13}C) as internal reference. HMBC experiments were optimized for a $^3J_{CH}$ of 8 Hz. ROESY spectra were measured with a mixing time of 350 ms. (+)-HRMALDITOFMS was performed on a QSTAR Applied Biosystems spectrometer. ESIMS were recorded using an Agilent 1100 Series LC/MSD spectrometer.

Bacterial Strain. The microbial producer *Streptomyces* sp. MDG-04-17-069 was isolated by spreading a marine sediment collected near the east coast of Madagascar on 172B modified agar medium[12] plates supplemented with nalidixic acid (1%). Plates were incubated at 28 °C for 30 days. The strain was subjected to phylogenetic analysis based on 16S rRNA sequences analyzed by BLAST (Basic Local Alignment Search Tool) against the National Centre for Biotechnology Information (NCBI) database,[13] showing 99% identity with that of *Streptomyces* sp. A404-Ydz-Q (596/598 bp). The GenBank accession number for the 16S rRNA sequence of our strain is GU211218.

Fermentation Processes. The seed culture was developed in two scale-up steps, first in 100 mL Erlenmeyer flasks containing 20 mL of seed medium and then in 250 mL Erlenmeryer flasks with 50 mL of the same medium. The seed culture was grown on a medium containing dextrose (0.1%), soluble starch (2.4%), soy peptone (0.3%), yeast extract (0.5%), Tryptone (0.5%), soya flour (0.5%), NaCl (0.54%), KCl (0.02%), MgCl (0.24%), Na_2SO_4 (0.75%), and $CaCO_3$ (0.4%) in tap water and cultured at 28 °C on orbital shakers for 72 h. For production, 12.5 mL of the seed medium was transferred into 2 L Erlenmeyer flasks containing 250 mL of fermentation medium containing yeast extract (0.5%), soy peptone (0.1%), dextrose (0.5%), soya flour (0.3%), Glucidex (Roquette, France) (2%), NaCl (0.53%), KCl (0.02%), $MgCl_2 \cdot 6H_2O$ (0.24%), Na_2SO_4 (0.75%), $MnSO_4 \cdot 4H_2O$ (0.00076%), $CoCl_2 \cdot 6H_2O$ (0.0001%), K_2HPO_4 (0.05%), and $CaCO_3$ (0.4%). The culture was grown at 28 °C using an orbital shaker (5 cm eccentricity, 220 rpm) for 5 days.

Extraction and Isolation. Bioassay-guided isolation of **4** from the fermentation broth was performed as follows: The fermentation broth (2 L) was subjected to centrifugation, and the clarified broth was extracted with EtOAc (1:1, v/v). The solvent was concentrated to give a crude extract (178 mg), which was subjected to preparative HPLC (C-18 Symmetry column (19 × 150 mm, 7 μm, Waters), gradient CH_3CN/H_2O (20−100% in 25 min), flow rate 15 mL/min, UV detection at 215 nm). Under these conditions 10 fractions were separated and the cytotoxic activity was detected in the fraction eluting at 18 min. Further purification of this active fraction by semipreparative HPLC was carried out at room temperature (C-18 Symmetry column (7.8 × 150 mm, 7 μm, Waters), gradient CH_3CN/H_2O (45−90% in 20 min), flow rate 3 mL/min, UV detection at 215 nm). Under these conditions, compound **4** (2 mg, t_R = 13 min) and traces of ikarugamycin were isolated.

A second extract from a 12 L fermentation broth (1150 mg of supernatant extract obtained), fractionated by C-18 column chromatography in a H_2O/MeOH gradient and HPLC under the same conditions described above, yielded 9.2 mg of **4** and 30 mg of ikarugamycin.

Tartrolon D (4): white solid; $[\alpha]^{25}_D$ +11 (*c* 0.2, MeOH); UV λ_{max} 231 nm (MeOH); IR (neat) ν_{max} 3460, 2927, 1737, 1366, 1229, 1216 cm^{-1}; ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz), see Table 1; (+)-ESIMS *m/z* 843 $[M + Na]^+$; (+)-HRESIMS *m/z* 843.4488 $[M + Na]^+$ (calcd for $C_{44}H_{68}O_{14}Na$, 843.4501), 838.4944 $[M + NH_4]^+$ (calcd for $C_{44}H_{72}NO_{14}$, 838.4947).

Biological Activity. A549 (ATCC CCL-185, lung carcinoma), HT29 (ATCC HTB-38, colorectal carcinoma), and MDA-MB-231 (ATCC HTB-26, breast adenocarcinoma) cell lines were obtained from the ATCC. Cell lines were maintained in RPMI medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 100 U/mL penicillin and streptomycin, at 37 °C and 5% CO_2. Triplicate cultures were incubated for 72 h in the presence or absence of test compounds (at 10 concentrations ranging from 10 to 0.0026 μg/mL). For quantitative estimation of cytotoxicity, the colorimetric sulforhodamine B (SRB) method was used.[14] Briefly, cells were washed twice with PBS, fixed for 15 min in 1% glutaraldehyde solution, rinsed twice in PBS, and stained in 0.4% SRB solution for 30 min at room temperature. Cells were then rinsed several times with 1% acetic acid solution and air-dried. Sulforhodamine B was then extracted in 10 mM trizma base solution and the absorbance measured at 490 nm. Results are expressed as GI_{50}, the concentration that causes 50% inhibition in cell growth after correction for cell count at the start of the experiment (NCI algorithm). Doxorubicin and DMSO (solvent) were used as the positive and negative controls in this assay. Prism 3.03 from GraphPad was used for the statistical analysis of the cell growth inhibition results.

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Supporting Information Available: NMR spectra for compound **4** are available free of charge via the Internet at http://pubs.acs.org.

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