Studies on the Secondary Metabolites of a *Pseudoalteromonas* sp. Isolated from Sediments Collected at the Northeastern Coast of Brazil

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Continuing search for anticancer compounds from the marine environment, we have studied microorganisms that inhabit intertidal sediments of the northeastern Brazilian coast. Of the 32 strains isolated, 13 were selected for biological evaluation of their crude extracts. The acetate extract obtained from a *Gram*-negative bacterium was strongly active against cancer cell lines with IC_{50} values that ranged from 0.04 (HL60 leukemia cells) to 0.26 µg/ml (MDA MB-435 melanoma cells). The bacterium was identified as a *Pseudoalteromonas* sp. based on 16S rRNA gene sequencing. A bioassay-guided fractionation of the active extract led to the isolation of prodigiosin, a well-known tripyrrole red pigment with immunosuppressive and anticancer activities. Further experiments with ErbB-2 overexpressing cell lines, including HB4a-C3.6 (moderate overexpression), HB4a-C5.2 (high overexpression), and the parental HB4a cell line, were performed. Prodigiosin was moderately active toward HB4a cells with an IC_{50} of 4.6 µg/ml, while it was 115 and 18 times more active toward HB4a-C3.6 cells (IC_{50} of 0.04 µg/ml) and HB4a-C5.2 (IC_{50} of 0.26 µg/ml) cells, respectively. These data suggest that, in spite of its previously described apoptosis-inducing properties, prodigiosin can selectively recognize cells overexpressing ErbB-2, which could be highly appealing in human breast cancer therapy.

Introduction. – The oceans cover *ca.* 70% of the Earth's surface and represent a highly complex microbiological environment where abundances can reach values as high as 10^6 microorganisms/ml of seawater and 10^9/cm^3 of ocean-bottom sediment [1][2]. This microbiota has recently been realized to be a prolific source of secondary metabolites [1][3]. Two compounds therein originated, salinosporamide A and NPI-2358, are examples of successful drug candidates [4–7].

Due to their great chemical and biological diversity and the perceived sustainability associated with the possibility of fermentation as a resource to rescue a large amount of bioactive compounds for preclinical and clinical studies, marine microorganisms may have revolutionary implications on the natural-product sciences [3]. In addition, marine natural products present an extraordinary diversity of molecular targets and a

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high degree of selectivity, which highlight their therapeutic and pharmacological potential [8][9].

In this context, this study is a pioneering assessment of the biodiversity and biotechnological potential of microorganisms associated with sediments from the northeastern coast of Brazil. This region of the Brazilian coast has already demonstrated a high degree of endemism for marine invertebrates [10][11]. However, until now, there have been no studies with its marine microorganism communities. Thus, here we report the isolation and cultivation of microorganisms from sediments collected in the intertidal zone of Taíba Beach (São Gonçalo do Amarante, Ceará, Brazil), followed by an anticancer screening. The bioassay-guided fractionation of the extract of the most active bacterium led to the isolation of prodigiosin as the active principle along with non-active compounds, the bile acid derivatives deoxycholic acid and cholic acid. Several fatty acids were also identified in the nonpolar fractions of the AcOEt extract. Additional experiments were performed to assess the activity of prodigiosin, which is already known for its anticancer properties [12][13], against ErbB-2-over-expressing breast cells.

Results. – *Strain Isolation, Cultivation, Extraction, and Screening*. Thirty-two strains were isolated from sediment samples collected at Taíba beach, but only 13 were selected for extraction and cytotoxic screening. Among these, four demonstrated some biological activity and inhibited tumor cell proliferation at 50 μg/ml (*Fig. 1*). The most active extract was obtained by AcOEt extraction from the spent media of strain BRA-007, which inhibited almost 100% of the growth of all tested cell lines at 50 μg/ml.

Preliminary morphological characterization of this organism indicated that the isolate was a red marine, obligate, *Gram*-negative bacterium. Based on alignment of its 16S rRNA sequence from the *GenBank* database, the gene sequence of the isolate showed highest similarity to that of *Pseudoalteromonas rubra* (99% similarity). Based on this information, the *EzTaxon* server 2.1 was used to align the isolate's sequence with *Pseudoalteromonas* type strains. Similarity values with *P. rubra* reached 99.68%. The phylogenetic analysis warrants inclusion within the *Pseudoalteromonas* clade and also indicates that *P. rubra* is a sister taxon with a bootstrap value of 97 (*Fig. 2*).

Bioassay-Guided Fractionation of Prodigiosin. The AcOEt extract obtained from BRA-007 was cytotoxic toward the four tumor cell lines tested with IC_{50} values ranging from 0.51 (HL-60 cells) to 3.05 μg/ml (MDA-MB-435 cells). This extract was submitted to successive replicates of preparative silica gel high-performance thin layer chromatography to yield prodigiosin (**1**; *Fig. 3*). All the chemical procedures were guided by the cytotoxic activities of the obtained fractions. The results are compiled in the *Table*. As expected, prodigiosin was strongly active toward tumor cell lines with IC_{50} values ranging from 0.05 to 0.19 μg/ml.

A second set of experiments was performed using ErbB-2 overexpressing cell lines, HB4a-C3.6 (moderate overexpression), HB4a-C5.2 (high overexpression), and the parental cell line HB4a, to access the cytotoxic activity of prodigiosin and to determine whether this compound could differentially recognize these cells. Indeed, prodigiosin was moderately active toward HB4a cells (IC_{50} of 4.6 μg/ml), while it was 115 and 18 times more active toward HB4a-C3.6 (IC_{50} of 0.04 μg/ml) and HB4a-C5.2 (IC_{50} of 0.26 μg/ml) cells, respectively.

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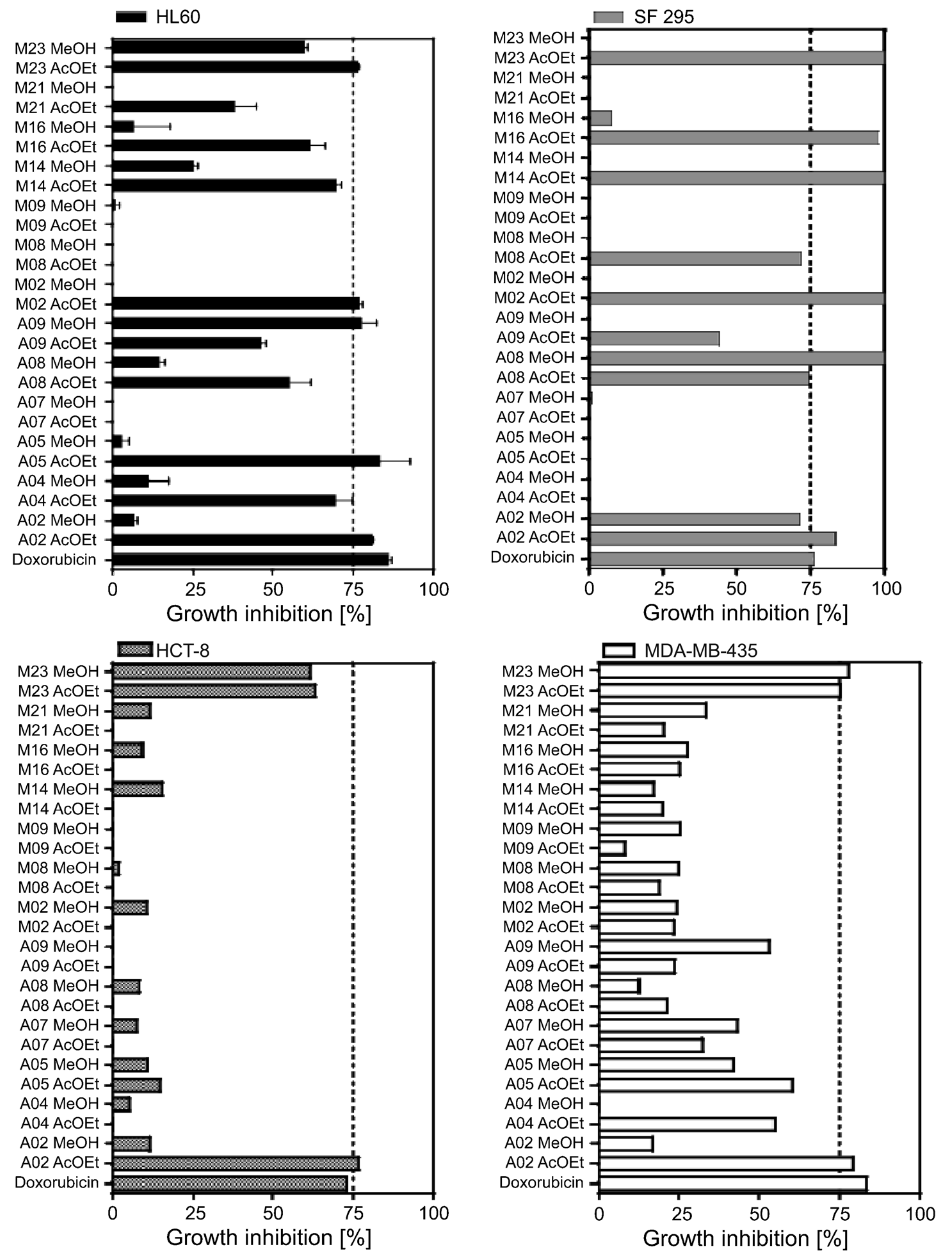

Fig. 1. *Single-dose cytotoxic activity of the microorganism extracts evaluated against SF-295* (glioblastoma)*, HCT-8* (colon cancer)*, MDA-MB-435* (melanoma)*, and HL-60* (promyelocytic leukemia) *cell lines* (at 50 μg/ml) *using the* Alamar *assay after 72 h of incubation.* Data are represented as a percentage of growth inhibition relative to untreated cells. Doxorubicin was used as a positive control.

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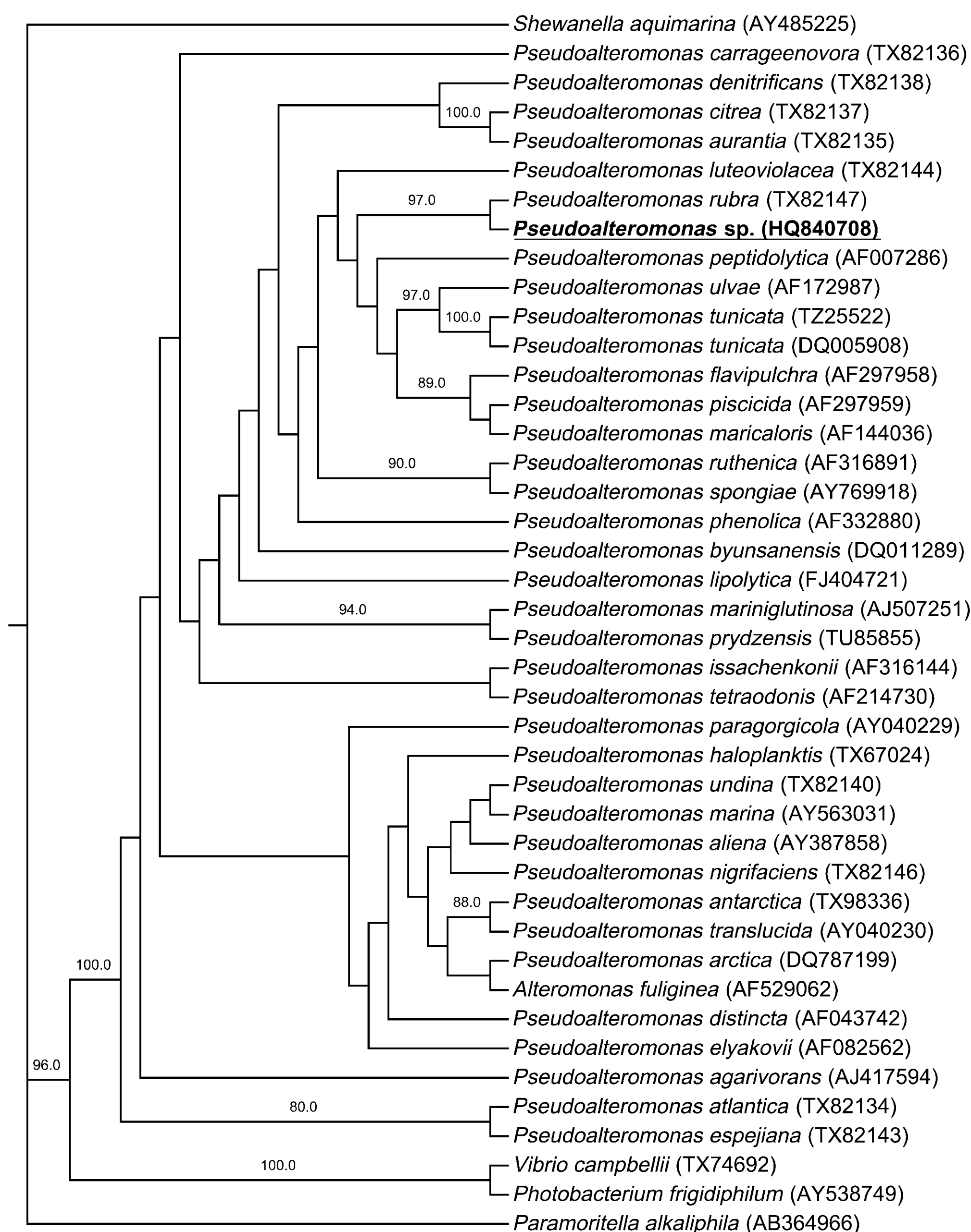

Fig. 2. *Phylogenetic tree showing the location of the new isolate* (bold) *among different* Pseudoalteromonas *and related species.* The numbers at the branches represent bootstrap values (100 bootstrap resamplings, only values larger than 80 are shown). The *GenBank* accession No. for each reference species is shown in parentheses.

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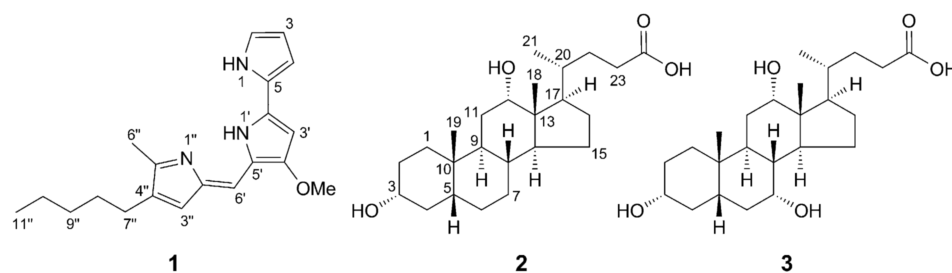

Fig. 3. *Structures of compounds isolated from* Pseudoalteromonas *sp.*

Table. *Cytotoxicity of the* Pseudoalteromonas *sp. Crude Extract, Fraction* (obtained by elution with hexane/AcOEt 3 : 2)*, and Prodigiosin on Tumor Cell Lines*[a]). Doxorubicin was used as positive control.

Sample	IC_{50} [μg/ml] CI 95%			
	HCT-8	HL-60	MDA-MB-435	SF-295
Crude extract	0.97	0.52	0.63	3.05
	0.77 – 1.22	0.36 – 0.75	0.54 – 0.74	1.92 – 4.85
Fr. 3	0.08	0.01	0.05	0.01
	0.07 – 0.09	0.01 – 0.02	0.03 – 0.09	0.00 – 0.04
Prodigiosin	0.05	0.06	0.19	0.06
	0.04 – 0.06	0.04 – 0.08	0.12 – 0.31	0.04 – 0.08
Doxorubicin	0.04	0.06	0.48	0.17
	0.03 – 0.05	0.04 – 0.09	0.35 – 0.65	0.13 – 0.23

[a]) IC_{50} Values and their 95% confidence intervals were obtained by nonlinear regression using the GraphPad program (*Intuitive Software for Science*, San Diego, CA).

Chemical Studies on the Pseudoalteromonas *sp. Extract*. In addition to the isolation of prodigiosin as the active fraction of the *Pseudoalteromonas* sp. extract, the remaining fractions were studied. GC/MS Analysis of the fractions obtained by elution with hexane and hexane/AcOEt 4 : 1 led to the identification of the following fatty acids: (*Z*)-hexadec-9-enoic acid (t_R 23.61 min; 16.6%), (*E*)-hexadec-9-enoic acid (t_R 23.67 min; 12.9%), hexadecanoic acid (t_R 23.93 min; 29.3%), heptadec-9-enoic acid (t_R 24.93 min; 7.5%), heptadecanoic acid (t_R 25.22 min; 5.4%), octadec-9-enoic acid (t_R 26.18 min; 25.1%), and octadecanoic acid (t_R 26.4 min; 3.2%). Additionally, the known bile acids, deoxycholic acid **2** and cholic acid **3**, were isolated from fractions *Fr. 6*, obtained by elution with AcOEt, and *Fr. 7* (128 mg), obtained by elution with AcOEt/ MeOH 4 : 1 (*Fig. 3*).

Discussion. – This study demonstrates the isolation of prodigiosin produced by a *Pseudoalteromonas* strain associated with marine sediments from the northeastern coast of Brazil. The genus *Pseudoalteromonas* was formally described in [14] based on rRNA gene sequence data to accommodate eleven species previously described as *Alteromonas* species. According to [14], this genus includes *Gram*-negative, non-spore-forming, obligatory aerobic, marine bacteria with promising secondary metabolism and

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high ecological significance, especially when considering the clades of pigmented species [14–16].

The species isolated in the present study matched all the characteristics described above and have a high similarity to *P. rubra* (ATCC 29570), which was first isolated by [17] from Mediterranean waters near Nice, France. The chemical study of the obtained AcOEt extract led to the isolation of prodigiosin as the active compound, and two common bile acids, deoxycholic acid and cholic acid [18][19].

Prodigiosin is a tripyrrole red pigment first isolated from *Serratia* species [20]. This pigment and its related compounds have been further isolated from different bacterial species, including *Pseudoalteromonas* spp. [21–25]. Although the literature on this class of compounds is vast, and knowledge on biosynthetic pathways is rapidly increasing, the physiological role of prodigiosin in its producers remains unclear [25]. Nonetheless, the biological activities described for prodigiosin account for most of the published research, and they include antibacterial, antimalarial, antitumor, and immunosuppressive properties [13][26][27]. In this context, prodigiosin is considered a promising molecule in cancer treatment, and, until now, four possible mechanisms for its anticancer activity have been considered: intracellular acidification, DNA damage, modulation of signal transduction pathways, and cell cycle arrest [13][27]. However, it seems that the mechanism may depend on the cell type, and molecular targets have not been conclusively identified. Our present findings corroborate previous data on the cytotoxicity of prodigiosin toward tumor cell lines with IC_{50} values in the nM range. In the *NCI* (*National Cancer Institute*) database, prodigiosin demonstrates an average IC_{50} value of 13.7 nM toward the 60-cell line panel, with an average LC_{50} value of 2.1 μM (www.dtp.nci.nih.gov under NSC 47147-F).

Furthermore, prodigiosin was also assayed for cytotoxicity against cells transfected with ErbB-2 cDNA, and it selectively recognized cells with moderate and high over-expression of ErbB-2 receptors. The tyrosine kinase receptor ErbB-2 is already a validated target for cancer therapy, being over-expressed in 25–30% of all breast tumors and associated with a poor prognosis [28–30]. The mechanisms involved in the selective recognition of ErbB-2 overexpressing cells by prodigiosin are not known; however, there are evidences that tyrosine kinase receptors play an important role. JAK3, a tyrosine kinase associated with cytokine signaling, is considered the target for the immunosuppressive effects of prodigiosin, which may also contribute to its anticancer properties [27]. Further studies are in progress to better understand the effects of prodigiosin on ErbB-2 overexpressing cells and its potential for human breast cancer therapy.

This work was supported by the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (*CNPq*), *Financiadora de Estudos e Projetos* (*FINEP*), and the *International Foundation for Science* (*IFS*). We also acknowledge Dr. *Anamaria Camargo Aranha* (Instituto Ludwig de Pesquisa Sobre o Câncer, Brasil) for donating the transfected cell lines. The manuscript was edited linguistically by *American Journal Experts* (key # 5B9B-CF21-6071-7CA2-3C38).

Experimental Part

Sampling and Bacterial-Strain Isolations. Sediment samples were collected at Taiba beach (03°30′21.23′S; 038°53′40.16′W), which is located on the northeastern coast of Brazil. Collections were

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carried out using aseptic techniques, the material was gathered and stored using sterile utensils. For the isolation of microorganisms from the sediment, samples were suspended in sterile seawater in a proportion of 1:5 (*m/v*). Samples were diluted in a serial manner from 10^{-1} to 10^{-5}. Aliquots of 0.1 ml of each dilution were plated on *Petri* dishes containing starch casein agar or marine agar. Purification of each strain was secured by sequentially restreaking onto new agar plates, followed by *Gram* staining and light microscopy to confirm purity. Pure strains were inoculated into liquid media for scaled-up growth. Cultures of each isolated strain were sampled into cryotubes and supplemented with 20% glycerol (*v/v*) for storage at $-70°$.

Scaled-up Growth and Extraction. First, pure cultures were inoculated into test tubes containing 10 ml of liquid media. Tubes were kept under a constant temp. and agitation for 96 h. Next, the grown cultures were diluted in *Erlenmeyer* flasks containing 250 ml of fresh media and agitated at 200 rpm (28°) for 5–7 d.

The resulting culture was filtered to separate the bacterial biomass from the spent medium, and the biomass was extracted with MeOH, while the medium was extracted overnight in AcOEt. The extracts were evaporated under a vacuum, washed with MeOH to remove excess salt, and fully dried under compressed air to yield the crude extracts. At this point, the extracts were ready to be evaluated for biological activity.

Extraction and Isolation of Pseudoalteromonas *sp. Compounds.* The AcOEt fraction (5 l) was dried (Na_2SO_4) and concentrated under reduced pressure to give 1.42 g of a crude pigment. The resulting material was fractioned by column chromatography (CC; silica gel; hexane, hexane/AcOEt 4:1, 3:2, 2:3, and 1:4, AcOEt, AcOEt/MeOH 4:1, 1:1, and MeOH) to afford 74 fractions of 8 ml each. After TLC, the fractions were grouped into eight main fractions, *Frs. 1–8*. The cytotoxic potential of each fraction was evaluated; however, only *Fr. 3* (obtained by elution with hexane/AcOEt 3:2) was active. An aliquot of *Fr. 3* (83.7 mg) was subjected to prep. silica-gel high-performance thin layer chromatography (HPTLC; *Merck*) and eluted with hexane/CH_2Cl_2/MeOH 5:4.5:0.5. The red pigment (33 mg) obtained was submitted to chromatography for a second time using this same procedure to afford 16.4 mg of a pure compound. On the basis of its NMR data, it was identified as *prodigiosin* (**1**) [31]. An aliquot of *Fr. 1* (5 mg), obtained by elution with hexane and hexane/AcOEt 4:1, was subjected to an acid-catalyzed methanolysis with MeOH/HCl (5%). The methyl esters, after the usual workup, were analyzed by GC/MS. *Fr. 6* (198 mg), obtained by elution with AcOEt, was subjected to CC (silica gel; increasing amounts of CH_2Cl_2/AcOEt 9:1, 4:1, 7:3, 3:2, and 1:1, CH_2Cl_2/MeOH 4:1, and MeOH). The CH_2Cl_2/AcOEt 1:1 fraction (18.1 mg) was subjected to flash-CC (CH_2Cl_2/MeOH 9.5:0.5) to yield a pure compound (14.8 mg) that was identified by means of HR-EI-MS and ^1H- and ^{13}C-NMR data as *deoxycholic acid* (**2**) [18]. *Fr. 7* (128 mg), obtained by elution with AcOEt/MeOH 4:1, was repeatedly subjected to CC (*Sephadex LH-20*; MeOH) to afford a pure compound (13 mg) that was characterized by HR-EI-MS and ^1H- and ^{13}C-NMR data as *cholic acid* (**3**) [18]. The structures of these three compounds were elucidated based on HR-EI-MS, and ^1H- and ^{13}C-NMR data, which included 2D-NMR experiments, in addition to comparisons with published data.

Prodigiosin (=*4-Methoxy-5-[(*Z*)-(5-methyl-4-pentyl-2*H*-pyrrol-2-ylidene)methyl]-1*H*,1′*H*-2,2′-bi-pyrrole*; **1**). Dark red solid. ^1H-NMR (500 MHz, $CDCl_3$): 12.7 (br. *s*, H–C(1′)); 12.5 (br. *s*, H–C(1)); 7.23 (br. *s*, H–C(2)); 6.94 (*s*, H–C(6′)); 6.92 (br. *s*, H–C(4)); 6.67 (*s*, H–C(3″)); 6.35 (br. *s*, H–C(3)); 6.07 (*s*, H–C(3′)); 4.00 (*s*, MeO); 2.54 (br. *s*, Me(6″)); 2.39 (*t*, $J=10.0$, CH_2(7″)); 1.57–1.53 (*m*, CH_2(8″)); 1.32–1.31 (*m*, CH_2(10″)); 1.29–1.26 (*m*, CH_2(9″)); 0.90 (*t*, $J=6.5$, Me(11″)). ^{13}C-NMR (125 MHz, $CDCl_3$): 166.0 (C(4′)); 147.9 (C(2′)); 147.2 (C(5″)); 128.6 (C(3″,4″)); 127.1 (C(2)); 125.4 (C(2″)); 122.4 (C(5)); 120.9 (C(5′)); 117.2 (C(4)); 116.2 (C(6′)); 111.9 (C(3)); 93.0 (C(3′)); 58.9 (MeO); 31.6 (C(9″)); 30.0 (C(8″)); 25.5 (C(7″)); 22.7 (C(10″)); 14.2 (C(1″)); 12.6 (C(6″)). HR-ESI-MS: 324.2059 ($[M+H]^+$, $C_{20}H_{26}N_3O^+$; calc. 324.2075).

Deoxycholic Acid (=*(3α,5β,12α)-3,12-Dihydroxycholan-24-oic Acid*; **2**). White solid. ^1H-NMR ((D_5)pyridine, 500 MHz): 4.21 (*s*, H–C(12)); 3.89–3.85 (*m*, H–C(3)); 2.69–2.64 (*m*, H–C(23a)); 2.56–2.50 (*m*, H–C(23b)); 2.32–2.27 (*m*, H–C(17)); 2.23–2.21 (*m*, H–C(9)); 2.21–2.19 (*m*, H–C(4a)); 2.16–2.14 (*m*, H–C(22a)); 1.99–1.96 (*m*, H–C(2a)); 1.95–1.93 (*m*, H–C(14)); 1.87–1.86 (*m*, CH_2(16)); 1.85–1.84 (*m*, H–C(6a)); 1.83–1.81 (*m*, H–C(1a)); 1.78–1.76 (*m*, H–C(4b)); 1.75–1.72 (*m*, H–C(11a)); 1.65–1.58 (*m*, H–C(11b,15a)); 1.55–1.52 (*m*, H–C(20)); 1.46–1.41 (*m*, H–C(22b,8)); 1.40–1.38 (*m*,

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H–C(5,2b,7a)); 1.24–1.23 (*m*, H–C(6b)); 1.22 (*d*, *J*=6.2, Me(21)); 1.16–1.11 (*m*, H–C(7b,15b)); 1.10–1.09 (*m*, H–C(1b)); 0.96 (*s*, Me(19)); 0.75 (*s*, Me(18)). ^{13}C-NMR ((D_5)pyridine, 125 MHz): 178.0 (C=O); 72.6 (C(12)); 71.6 (C(3)); 48.8 (C(14)); 47.3 (C(17)); 47.7 (C(13)); 42.9 (C(5)); 37.7 (C(4)); 36.8 (C(8)); 36.4 (C(20)); 35.9 (C(22)); 34.8 (C(10)); 34.3 (C(9)); 32.4 (C(23)); 31.7 (C(16)); 30.0 (C(11)); 28.4 (C(2)); 28.0 (C(6)); 27.0 (C(7)); 24.5 (C(15)); 23.9 (C(19)); 17.8 (C(21)); 13.4 (C(18)). HR-ESI-MS: 415.2901 ([*M*+Na]$^+$, $C_{24}H_{40}O_4^+$; calc. 415.2926).

Cholic Acid (=*(3α,5β,7α,12α)-3,7,12-Trihydroxycholan-24-oic Acid*; **3**). White solid. ^{1}H-NMR ((D_5)pyridine, 500 MHz): 4.25 (*s*, H–C(12)); 4.09 (*s*, H–C(7)); 3.78–3.73 (*m*, H–C(3)); 3.11 (*q*, *J*=13.0, H–C(8)); 2.92 (*dt*, *J*=12.0, 4.2, H–C(9)); 2.79–2.73 (*m*, H–C(14)); 2.67–2.61 (*m*, H–C(23a)); 2.56–2.50 (*m*, H–C(23b)); 2.39–2.34 (*m*, H–C(17)); 2.15–2.13 (*m*, CH_2(22)); 2.13–2.11 (*m*, H–C(4a)); 2.07–2.00 (*m*, H–C(15a)); 1.94–1.91 (*m*, H–C(1a,2a)); 1.88–1.86 (*m*, H–C(11a)); 1.68–1.67 (*m*, CH_2(6)); 1.67–1.62 (*m*, H–C(20,4b,2b,11b)); 1.51–1.49 (*m*, H–C(5)); 1.44–1.37 (*m*, CH_2(16)); 1.23 (*d*, *J*=5.5, Me(21)); 1.21–1.19 (*m*, H–C(15b)); 1.09–1.03 (*m*, H–C(1b)); 1.00 (*s*, Me(19)); 0.81 (*s*, Me(18)). ^{13}C-NMR ((D_5)pyridine, 125 MHz): 178.0 (C=O); 72.2 (C(3)); 72.7 (C(12)); 68.0 (C(7)); 47.6 (C(17)); 47.3 (C(13)); 43.0 (C(5)); 42.9 (C(14)); 41.4 (C(8)); 41.0 (C(4)); 36.6 (C(20)); 36.4 (C(1)); 36.2 (C(6)); 35.7 (C(10)); 32.4 (C(22)); 32.3 (C(23)); 32.0 (C(2)); 30.0 (C(11)); 28.5 (C(16)); 27.7 (C(9)); 24.2 (C(15)); 23.6 (C(19)); 17.9 (C(21)); 13.4 (C(18)). HR-ESI-MS: 431.2729 ([*M*+Na]$^+$, $C_{24}H_{40}NaO_5^+$; calc. 431.2773).

Molecular Identification: Nucleic Acid Extraction, 16S rRNA Gene Amplification, and Sequencing. Genomic DNA extraction from selected microorganisms was carried out using a CTAB (cetyl(trimethyl)ammonium bromide)-based protocol [32]. The concentration and quality of the DNA extractions were determined with a *Nanodrop* spectrophotometer *ND-1000* (*NanoDrop*, Wilmington, DE, USA).

The 16S rRNA gene was amplified from genomic DNA by PCR (polymerase chain reaction) using the primers 63F (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 1389R (5′-GGG CGG WGT GTA CAA GGC-3′) [33]. Amplification reactions were performed in a final volume of 25 μl containing 100 ng of genomic DNA (template), 20 mM *Tris*·HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each (dATP, dCTP, dGTP, and dTTP), 0.5 μM of each primer and 1.0 units of Taq DNA Polymerase (*MBI Fermentas Inc.*, USA). PCRs were carried out in a thermocycler (*Eppendorf AG 22331*; Eppendorf, D-Hamburg) programmed for an initial denaturation step (4 min at 94°), followed by 35 cycles of 1 min 94°, 1 min at 55°, and 2 min at 72°. The last cycle was followed by a final extension of 10 min at 72°. The specificity of the amplifications was confirmed by 1.0% agarose gel electrophoresis stained with SYBR Safe DNA Gel Stain (*Invitrogen*, USA). PCR Products were purified using *Wizard SV Gel* and PCR Clean-up System (*Promega Corporation*, Madison, WI, USA).

DNA Sequencing was performed by *Macrogen Inc.* (Seoul, Korea) using the *ABI PRISM BigDye*™ Terminator Cycle Sequencing kit (*Applied Biosystems*, Foster City, CA, USA) following the protocols supplied by the manufacturer. The sequencing reactions were performed with the primers 63F, 1389R, 518F (5′-CCA GCA GCC GCG GTA ATA CG-3′) and 800R (5′-TAC CAG GGT ATC TAA TCC-3′). The fluorescent-labeled fragments were purified according to an EtOH precipitation protocol, resuspended in dist. H_2O, and subjected to electrophoresis in an *ABI 3730* sequencer (*Applied Biosystems*, Foster City, CA, USA).

The nucleotide sequences were assembled, analyzed, and manually edited using the Sequencher software package (Version 4.5; *Gene Codes Co.*, Ann Arbor, MI) and compared to sequences within the NCBI database (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST). The obtained sequence was compared with all partial 16S rRNA gene sequences from *Pseudoalteromonas* type strains using the *EzTaxon* server 2.1 [34]. The nucleotide sequence was submitted to *NCBI GenBank* and is available under the accession No. HQ840708.

Phylogenetic Analysis. The identity of the strain was confirmed after an evaluation of the phylogenetic relationships with the most similar sequences obtained from the *EzTaxon* server. The sequences were aligned using Muscle ver. 3.6, and the reconstruction was performed using a combined bootstrapping and maximum likelihood approach through RAxML [35] on the CIPRES portal [36]. The tree was produced using TreeGraph 2.0 [37].

Anticancer Screening. Cytotoxic activity of the extracts and fractions was evaluated against four human tumor cell lines obtained from the National Cancer Institute (NSI; Bethesda, MD, USA): HL-60 (promyelocytic leukemia), MDA-MB-435 (melanoma), SF-295 (CNS glioblastoma), and HCT-8 (colon

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carcinoma). Cells were grown in RPMI-1640 medium supplemented with 2 mM glutamine, 10% fetal calf serum, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37° in a 5% CO_2 atmosphere. The cell cultures were regularly split to keep them in logarithmic growth phase.

The effect of 50 μg/ml concentrations of the extracts and 25 μg/ml fractions on tumor cell growth following 72 h of incubation was evaluated *in vitro* using the *Alamar* assay. Cells were plated in 96-well plates (2×10^4 cells/well in 100 μl of medium). After 24 h, prodigiosin (0.01 to 25 μg/ml) dissolved in 1% DMSO was added to each well using the HTS (high-throughput screening), and cells were incubated for 72 h. Control groups received the same amount of DMSO. Twenty-four h before the end of the incubation, 10 μl of a stock soln. (0.436 mg/ml) of *Alamar Blue* (resazurin, *Sigma–Aldrich Co.*, St. Louis, MO, USA) was added to each well. Absorbance was measured with a multiplate reader (*DTX 880* Multimode Detector, *Beckman Coulter, Inc.*, Fullerton, California, USA). The drug effect was quantified as the percentage of the control absorbance at 570 and 600 nm [38].

Extracts or fractions that caused more than 90% cell-growth inhibition were tested again at concentrations varying from 0.01 to 50 μg/ml to determine the 50% inhibitory concentration (IC_{50}), which was calculated by non-linear regression using the software GraphPad Prism 5.0.

Testing ErB-2 Overexpressing Cells. To investigate the selectivity of prodigiosin toward ErbB-2 overexpressing cell lines, *Alamar Blue* assays were performed with two transfected cell lines, HB4a-C3.6 (moderate overexpression) and HB4a-C5.2 (high overexpression), which were obtained by transfecting an immortalized human breast luminal epithelial cell line HB4a with ErbB-2 cDNA [28] and the parental cell line HB4a [39]. These cells were kindly donated by Dr. *Anamaria Camargo Aranha* from the *Ludwig Institute* (São Paulo, Brazil). Cells were grown in RPMI-1640 medium supplemented with 2 mM glutamine, 10% fetal calf serum, 100 μg/ml streptomycin, 100 U/ml penicillin, 1 μg/ml hydrocortisone, and 5 μg/ml insulin at 37° in a 5% CO_2 atmosphere. The assays were conducted essentially as previously described for the tumor cell lines.

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