The Journal of Antibiotics (2010) 63, 297–301

www.nature.com/ja

ORIGINAL ARTICLE

Aqabamycins A-G: novel nitro maleimides from a marine *Vibrio* species: I. Taxonomy, fermentation, isolation and biological activities

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In a screening of marine bacteria, a *Vibrio* species isolated from the surface of the soft coral *Sinularia polydactyla* collected in the Red Sea was found to be a prolific producer of secondary metabolites with antibacterial and cytotoxic activities. Seven novel maleimide derivatives named aqabamycin A (1a), aqabamycin B (1b), aqabamycin C (1c), aqabamycin D (1d), aqabamycin E (1e and 1e′), aqabamycin F (1f) and aqabamycin G (2) were isolated together with several known metabolites such as 3-nitro-1*H*-indazole (3), indazole-3-carbaldehyde (4), phenyl-2-bis-indolylmethane (5a), turbomycin B (5b), vibrindole A (6), 1,4-dithiane (7), 3-(3-nitro-4-hydroxyphenyl)-2-propenoic acid (8), 3-nitro-4-hydroxybenzaldehyde (9), phenylacetic acid, benzoic acid, 3-hydroxybenzoic acid and 4-hydroxycinnamic acid. The aqabamycins, except aqabamycin A, bear a nitro group. Compounds 3, 4, 7 are described here for the first time from a natural source and vibrindole A was found to have cytotoxic activity.
The Journal of Antibiotics (2010) **63,** 297–301; doi:10.1038/ja.2010.34; published online 30 April 2010

Keywords: antibacterial activity; aqabamycins; aromatic nitro compounds; cytotoxic activity; marine bacteria; *Vibrio* species

INTRODUCTION

Marine bacteria, in recent years, were the source of many new bioactive metabolites with interesting properties.[1–3] Especially interesting are bacteria from biofilms. These bacteria colonize a large number of plants and sedentary animals and produce diverse chemical compounds that have a role in the protection of the host against pathogenic and fouling microorganisms.[4] Species of the genus *Vibrio* comprise the majority of culturable bacteria in marine environments.[5] Several *Vibrio* species were reported to produce tetrodotoxin and derivatives thereof;[6–8] 1,1,3-tris(3-indol)-butane, 3,3-bis(3-indolyl) butane-2-one and other indoles have been recently reported from *V. parahaemolyticus*.[9]

In a screening of bacterial strains isolated from living marine surfaces in the Red Sea, extracts obtained from submerged cultures of strain WMBA exhibited antimicrobial and cytotoxic activities. Bioassay-guided isolation yielded 19 metabolites, many of which were novel compounds. In this paper we report the taxonomy of strain WMBA, its fermentation, the isolation and purification of the compounds, as well as their biological activities. The physico-chemical properties and the elucidation of the structures will be reported in a separate paper.[10]

RESULTS AND DISCUSSION

Strain WMBA forms light beige colonies on M1-agar. The cells are Gram-negative, mobile rods, 2.3–2.6 μm (3.4) long and 1.0–1.7 μm wide, nonsporogenic and facultative anaerobic. The strain does not accumulate poly-β-hydroxybutyrate and has no arginine dihydrolase system. It is oxidase and catalase positive but lacks a β-galactosidase activity, produces indole and reduces nitrate. It is a psychrotrophic strain unable to grow below 10 °C. Growth in media without marine salts is weak. The results of the biochemical and physiological characterization are summarized in Table 1.

16S rRNA gene sequence analysis revealed that strain WMBA is very closely related to *Vibrio* species (99% similarity level). The nearest identified phylogenetic relatives were *V. splindidus* biovar II (Access. No. AB038030) isolated from North-western Pacific Ocean and Otsuch Bay, Japan,[11] and *V. shilonii* (Access. No. AY911395), a pathogen that causes bleaching of the Mediterranean coral *Oculina patagonica*.[12] Our strain differed from *V. splindidus* in the ability to use D-glucuronic acid and L-leucine and from *V. shilonii* in the ability to grow at salt concentrations higher than 6%. Therefore, this strain represents a distinct species within the genus *Vibrio*.

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Received 16 December 2009; revised 28 January 2010; accepted 26 March 2010; published online 30 April 2010

Table 1 Biochemical and physiological characteristics of *Vibrio* sp. WMBA

Characteristic		*Characteristic*	
NaCl requirement	–	Compound as C- and N-source	
Salinity tolerance	7.5–10%	L-alanine	+
Oxidation/fermentation		L-leucine	+
D-glucose	+/+	L-proline	+
D-lactose	–/–	D-aspartic acid	+
Sucrose	–/–	L-lysine	–
L-arabinose	–/–	L-histidine	+
D-xylose	–/–	L-cysteine	+
Fructose	+/+	L-asparagine	–
Maltose	+/+	L-phenylalanine	–
Rhamnose	–/–	L-glutamine	+
Mannitol	+/+	L-tyrosine	+
Glycerol	+/+	Hydrolysis of	
Raffinose	–/–	Tween 40, 80	+
Sorbitol	–/–	Starch	–
D-galactose	+/+	Gelatine	+
Organic compound as C-source		Esculin	+
		Esterase activity	+
Sodium acetate	+	Arginine decarboxylase	–
D-glucuronic acid	+	Lysine decarboxylase	–
Succinic acid	+	β-Galactosidase	–
Glycine	+	H_2S production	–
DL-lactic acid	+	Indole production	+
Oxalic acid	+	Nitrate reduction	+
Sodium glutamate	+	Motility	+
D-aspartic acid	+	Spore formation	–
Sodium pyruvate	+	Poly-β-hydroxybutyrate	–

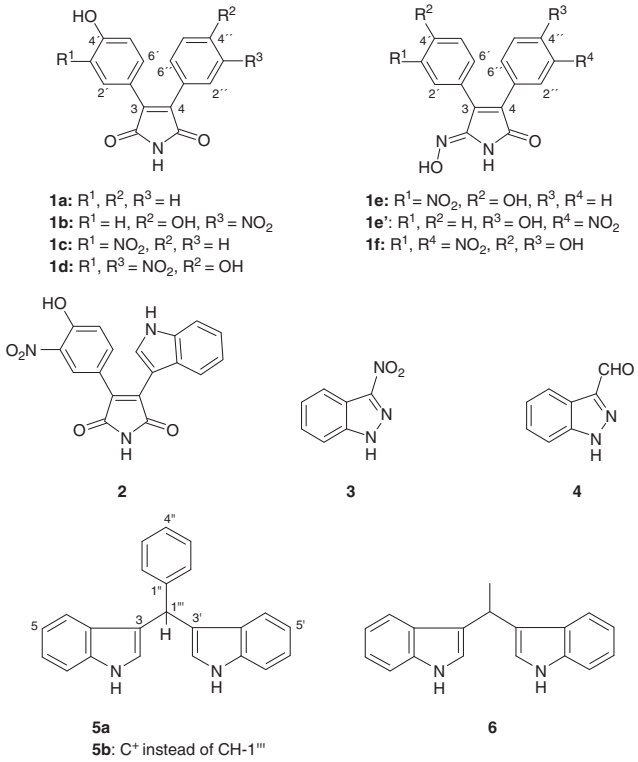

Figure 1 Structures of compounds **1a**, **1b**, **1c**, **1d**, **1e** and **e′**, **1f** and **2**–**6**.

During a typical fermentation of strain WMBA in media with marine salts, the antifungal activity against *Nematospora coryli* reached its maximum after 36–48 h and the cultures were collected at this point. Bioactivity-guided fractionation resulted in the isolation of more than 15 compounds. The structures of the novel compounds named aqabamycins are given in Figure 1. The isolation procedure is described in 'Materials and methods' section. The isolation schemes for the crude extracts of cultures grown in M1 and B1 medium are given in Figures 2 and 3.

More common compounds that were obtained during the isolation such as phenylacetic acid, benzoic acid, 3-hydroxybenzoic acid and 3-(4-hydroxyphenyl)-2-propenoic acid are not included in the description; 3-nitro-1*H*-indazole (**3**), indazole-3-carbaldehyde (**4**) and 1,4-dithiane (**7**) are reported from a natural source for the first time. Vibrindole A (**6**) has been isolated from *V. parahaemolyticus,* but no biological activities have been reported so far.[13]

The total yields of compounds isolated from *Vibrio* sp. WMBA and the retention times measured by analytical HPLC are summarized in Table 2.

All aqabamycins are novel compounds. They exhibited antibacterial and cytotoxic activities as shown in Tables 3 and 4. Not all of the compounds were obtained in amounts to allow biological testing. The mixture of aqabamycin E (**1e** and **1e′**) and aqabamycin F (**1f**) were the most active compounds. The MIC values toward bacteria varied between 3.15 and 25 μg ml^{-1}. Fungi with the exception of *N. coryli* were only affected at concentrations of 50 μg ml^{-1} or higher. Vibrindole A was not active up to 100 μg ml^{-1} (data not shown). As can be depicted from Table 4, L1210 cells were the most sensitive among the tested cell lines. Vibrindole A (**6**) and aqabamycin E (**1e** and **1e′**) were weakly but broadly cytotoxic. None of the compounds had nematicidal activity toward *Caenorhabditis elegans* and *Meloidogyne incognita* up to 100 μg ml^{-1} or phytotoxic activity. The latter was tested with *Setaria italica* and *Lepidium sativum*.

Together with a *Salegentibacter* species,[14,15] *Vibrio* sp. WMBA is the second marine bacterium in our hands that abundantly produces aromatic metabolites with nitro substitutions. Except aqabamycin A, all aqabamycins possess a nitro substitution. Comparison of the activities of aqabamycins A and C shows that the nitro substitution increases the antibacterial activity whereas the cytotoxic activity is decreased.

Maleimides are a growing group of natural products with a variety of biological activities such as fungicidal, antibacterial, cytotoxic and even protein kinase C inhibitory activities.[16] Not only bacteria are found among the producing organisms, also fungi have the capability to produce maleimides like the himanimides A, B and C, diarylmaleimide derivatives from the basidiomycete *Serpula himantoides* with weak antibacterial and antifungal activities.[17]

METHODS

Producing organism

The strain WMBA was isolated from the surface of the soft coral *Sinularia polydactyla* collected at a depth of 15 m in the Red Sea at Aqaba, Jordan. It has been deposited as WMBA-1 in the strain collection of the Institute of Biotechnology and Drug Research (IBWF), Germany.

Taxonomy

Morphological, biochemical and physiological tests. Morphological studies were carried out with a light microscope and a phase contrast microscope using

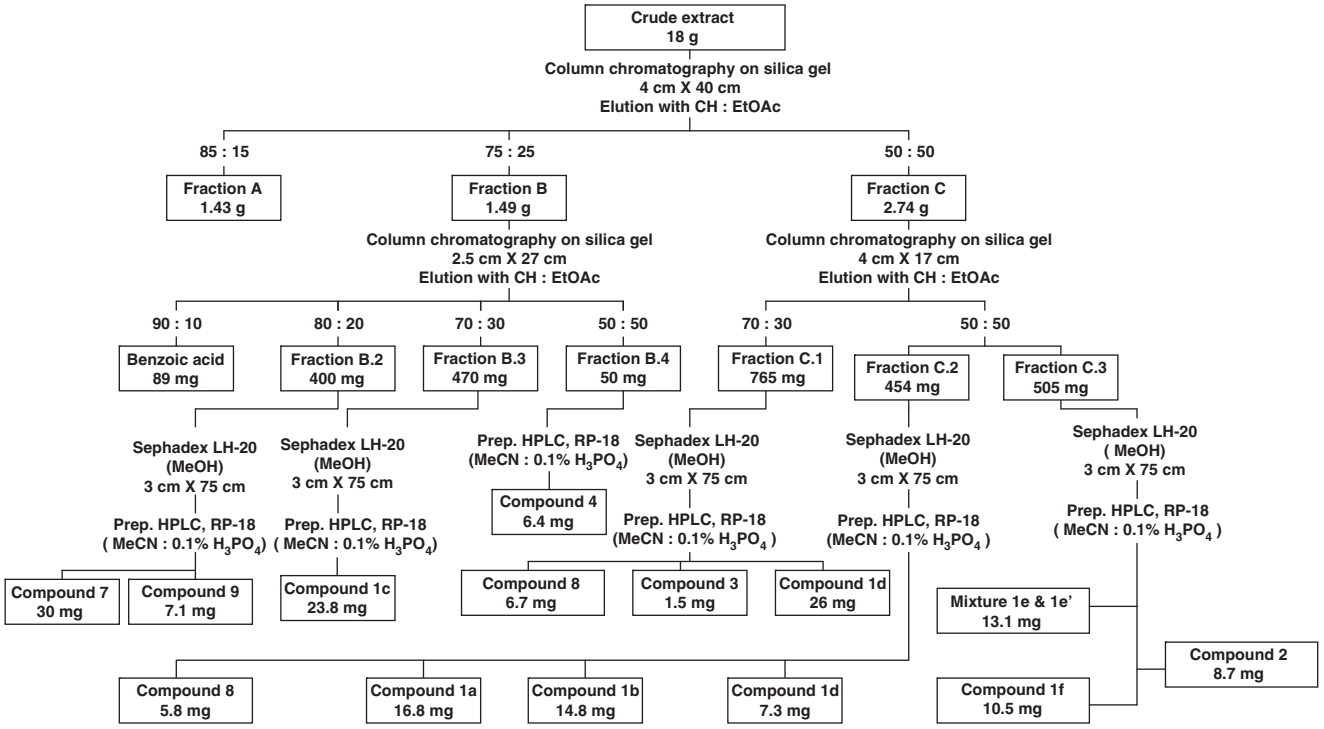

Figure 2 Isolation of compounds from fermentation of *Vibrio* sp. WMBA in medium B1.

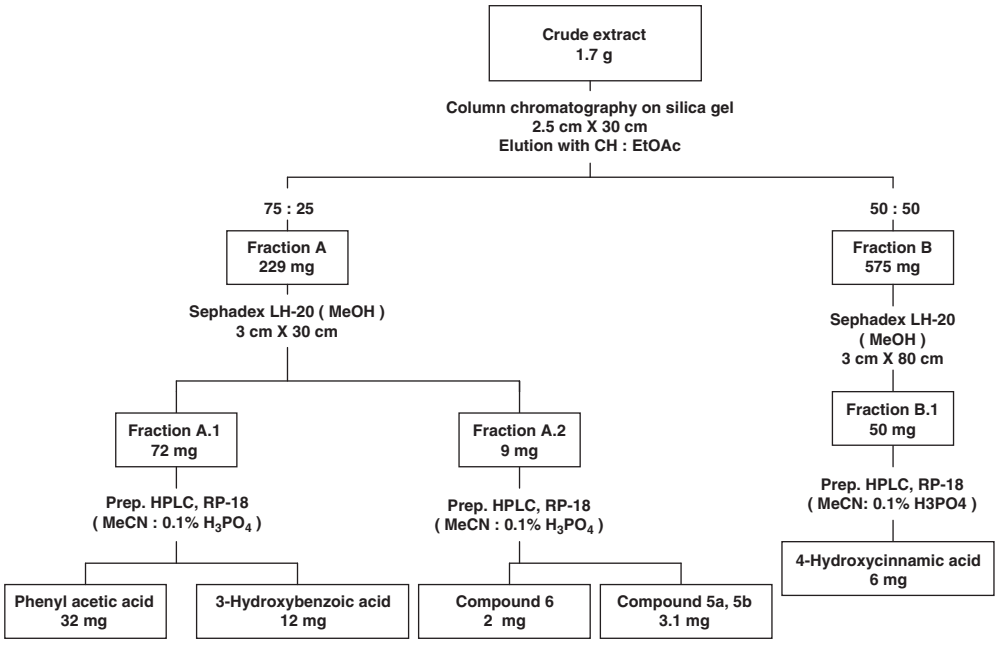

Figure 3 Isolation of compounds from fermentation of *Vibrio* sp. WMBA in medium M1.

cultures grown for 3–4 days at 25 °C on modified M1-agar medium composed of yeast extract 0.5% (Difco, Detroit, MI, USA), tryptone 0.5% (Difco), NaCl 1%, agar 2% in half strength artificial sea water, pH 7.2. Half strength artificial sea water was prepared by dissolving 16.7 g of marine salt mixture, purchased from Tropic marine (Dr Biener, Wartenberg, Germany), in 1 l distilled water.

Biochemical and physiological characteristics were determined using standard procedures as reported before.[14]

Fermentation

The strain was cultured in 500 ml Erlenmeyer flasks containing 250 ml of M1 medium on a rotary shaker (120 r.p.m.) at 25 °C for 36 h. This culture was used to inoculate a Biolafitte C6 fermentor containing 20 l of M1 medium or B1 medium composed of corn steep solids 0.5% (Sigma-Aldrich, Steinheim, Germany), A-Z amine 0.25% (Sigma-Aldrich), beef extract 3.8% (Difco), soy meal 0.1% (sojamin 50 T, Lucas Meyer, Hamburg, Germany), yeast extract

0.25% (Hartge Ingredients, Hamburg, Germany), seaweed extract 0.25% (v/v) (Manufactum, Waltrop, Germany), marine salts mixture 3.33%, pH value adjusted to 8.0. The fermentations were carried out at 25 °C with aeration of 4 l min^{-1} and agitation of 150 r.p.m.

During the fermentation process, samples (150 ml) were taken every 12 h. The culture fluid was separated from the bacterial cells by centrifugation (16 000 *g*, 10 min). The supernatant was adjusted to pH 4 and extracted with an equal volume of EtOAc. The organic phase was dried over Na_2SO_4, concentrated *in vacuo* at 40 °C and the resulting residue was dissolved in MeOH to a final concentration of 10 mg ml^{-1}. Aliquots corresponding to 300 µg of the concentrated residue were used for the evaluation of the antifungal and antibacterial activities in the agar diffusion assay using *N. coryli* and *Bacillus subtilis* as test organisms.

Isolation and purification of the compounds

After 36–48 h of fermentation, when the OD started to decrease and/or the antimicrobial activity had reached its maximum, the cultures were collected. The biomass was separated by centrifugation and discarded. The supernatant (16 l) was adjusted to pH 4 and extracted with EtOAc (10 l). After drying with Na_2SO_4, the organic phase was concentrated to dryness.

The combined crude extracts (18 g) from five fermentations (80 l) in B1 medium were applied onto a column with silica gel (Merck 60, 0.063–0.2 µm; column 4 cm×40 cm, Merck, Darmstadt, Germany). Elution with cyclohexane (CH)-EtOAc (85:15) yielded an inactive fraction A (agar diffusion assay with *B. subtilis* and *N. coryli*), which was discarded. Elution with CH-EtOAc (75:25) yielded fraction B (1.49 g) and CH-EtOAc (50:50) fraction C (2.74 g), both exhibited antimicrobial activity and were further processed. Fraction B on fractionation on silica gel as above (column 2.5 cm×27 cm) yielded 89 mg of benzoic acid (elution with CH-EtOAc, 90:10), 400 mg of fraction B.2 (CH-EtOAc, 80:20), 470 mg of fraction B.3 (CH-EtOAc, 70:30) and 50 mg of fraction B.4 (CH-EtOAc, 50:50); 3-nitro-4-hydroxybenzaldehyde (**9**) and 1,4-dithiane (**7**) were obtained from B.2 by chromatography on Sephadex LH-20 (GE Healthcare, Munich, Germany) in MeOH (column size 3 cm×75 cm) followed by HPLC on RP-18 (7 µm, 2.5 cm×25 cm, elution with MeCN (A):0.1% H_3PO_4 (B), linear gradient in 40 min, 10% A–100% A, flow rate 15 ml min^{-1}), yield 7.1 mg of compound **9** and 30 mg of compound **7**. Aqabamycin C (**1c,** 23.8 mg) was obtained from fraction B.3 by chromatography on Sephadex LH-20 (yield 98 mg enriched product) and HPLC as above. Fraction B.4 yielded after HPLC (conditions as above) indazole-3-carbaldehyde (**4**, 6.4 mg). Fraction C afforded after silica gel chromatography (column 4 cm×17 cm) and elution with CH-EtOAc three fractions, C.1–C.3. These were each subjected to LH20 chromatography (see above) and the resulting active fractions were further

Table 2 Retention times[a] and yields of the aqabamycins and some of the other compounds isolated from *Vibrio* sp. WMBA

Compound	*Yield (µg l^{-1})*	*Retention time (min)*
1a	210	10.95
1b	195	10.65
1c	300	12.43
1d	415	11.70
1e, **1e′**	165	11.68
1f	135	10.97
2	110	11.87
3	20	9.90
4	80	8.75
5a, **5b**	155	15.67
6	100	14.54
7	375	ND
8	100	8.30
9	88.5	8.68

[a]HPLC conditions: RP-18, LiChroCart, 5 µ, 125 mm×4 mm, Merck, Darmstadt, Germany and elution with 0.1% HCOOH (A) and MeCN (B) linear gradient 0–20 min 1–100% B; flow rate 0.45 ml min^{-1}.

Table 4 Cyctotoxic activities (IC_{50}, µg ml^{-1}) of some of the compounds from *Vibrio* sp. WMBA

	Cell line				
Compound	*L1210*	*Jurkat*	*MDA-MB321*	*MCF-7*	*Colo-320*
1a	30	>100	>100	100	40
1b	100	>100	>100	>100	>100
1c	50	>100	>100	>100	>100
1d	15	60	>100	>100	>100
1e, e′	15	20	25	20	50
1f	15	25	>100	>100	100
2	15	50	>100	>100	100
3	50	100	>100	>100	100
4	40	20	>100	>100	100
6	15	25	30	50	30
8	100	>100	>100	>100	>100
9	100	>100	>100	>100	>100

Table 3 Antimicrobial activities (MIC, µg ml^{-1}) of some of the compounds from *Vibrio* sp. WMBA in the serial dilution assay

Organism	***1a***	***1b***	***1c***	***1d***	***1e,e′***	***1f***	***2***	***3***	***4***	***8***	***9***
Bacteria											
Bacillus subtilis	50	100	25	50	6.25	12.5	25	>100	100	100	25
Micrococcus luteus	50	100	25	100	6.25	12.5	25	>100	>100	>100	25
Escherichia coli	100	100	50	100	12.5	12.5	50	>100	>100	>100	50
Proteus vulgaris	50	100	12.5	50	3.15	25	25	>100	>100	>100	25
Fungi											
Candida albicans	>100	>100	100	>100	>100	>100	>100	>100	>100	>100	>100
Magnaporthe grisea	>100	>100	50	>100	50	>100	>100	>100	>100	25	5
Mucor miehei	>100	>100	50	>100	50	>100	100	>100	>100	>100	50
Nematospora coryli	10	100	50	100	50	100	50	>100	50	50	50
Paecilomyces variotii	50	>100	50	>100	50	>100	>100	>100	>100	>100	100
Phytophthora infestans	>100	>100	100	>100	>100	>100	>100	>100	>100	>100	50
Saccharomyces cerevisiae	>100	>100	100	>100	>100	>100	>100	>100	>100	>100	100
Ustilago nuda	>100	>100	50	>100	>100	>100	>100	>100	50	>100	50

processed by HPLC (see above). From fraction C.1 aqabamycin D (**1d**, 26 mg), 3-nitro-1*H*-indazole (**3**, 1.5 mg) and 3-nitro-4-hydroxycinnamic acid (**8**, 6.7 mg) were obtained. Fractions C.2 and C.3 yielded compound **8** (5.8 mg), aqabamycins A (**1a**, 16.8 mg), B (**1b**, 14.8 mg), D (**1d**, 7.3 mg), E (**1e** and **e′**, mixture of isomers, 13.1 mg), F (**1f**, 10.5 mg) and G (**2**, 8.7 mg).

The crude extract from a 20-l fermentation in medium M1 (1.7 g) was applied onto a column (2.5 cm×30 cm) with silica gel (see above). Elution with CH-EtOAc 75:25 and 50:50 yielded fractions A (229 mg) and B (575 mg). Further bioassay-guided fractionation (with *B. subtilis* and *N. coryli* as test organisms) of fractions A and B by chromatography on Sephadex LH-20 (3 cm×30 cm and 3 cm×80 cm, respectively) and elution with MeOH yielded 72 mg of A.1 and 9 mg of A.2, and 50 mg of fraction B.1. All three fractions were further processed by HPLC on RP-18 (2.5 cm×25 cm, elution with MeCN-0.1%H_3PO_4). Phenylacetic acid (32 mg) and 3-hydroxybenzoic acid (12 mg) were obtained from fraction A.1, vibrindole A (**6,** 2 mg), and a mixture (3.1 mg) of phenyl-2-bis-indolylmethane (**5a**) and turbomycin B (**5b**) from A.2. Finally, 4-hydroxycinnamic acid (6 mg) was obtained from B.1.

The purity of all isolated compounds as checked by HPLC using a HP 1100 series instrument (Hewlett Packard, Waldbronn, Germany) equipped with a diode array detector (G1315B) fitted with an RP-18 column (LiChroCart, 5 µ, 125 mm×4 mm, Merck; elution with 0.1% HCOOH (A) and MeCN (B) linear gradient in 20 min 1–100% B; flow rate 0.45 ml min^{-1}) was >98%.

Biological activities

Antimicrobial activities, determined in the serial dilution assay, and cytotoxic activities were assayed as described earlier.[18] Owing to low yields, not all of the compounds could be tested. L1210 (mouse lymphocytic leukemia), Jurkat (human acute T-cell leukemia) and Colo-320 (human colorectal adenocarcinoma) cells were grown in RPMI 1640 medium. MDA-MB-231 (human breast adenocarcinoma), and MCF-7 (human breast adenocarcinoma) cells were grown in DMEM medium. All media contained 10% fetal calf serum, 65 µg ml^{-1} of penicillin G and 100 µg ml^{-1} of streptomycin sulfate. The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

ACKNOWLEDGEMENTS

We thank the DAAD, Bonn (German Academic Exchange Service) and the Federal Minister for Science and Technology (BMBF, Berlin-Bonn) for financial support. We especially thank the members of the Marine Science Station in Aqaba, Jordan, for the generous hospitality and support. The expert technical assistance of W Schuck and A Meffert is gratefully acknowledged.

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