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Essramycin: A First Triazolopyrimidine Antibiotic Isolated from Nature[†]

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Abstract In the course of our screening program for new bio-active compounds, a novel triazolopyrimidine antibiotic, essramycin (**1**), was obtained from the culture broth of the marine *Streptomyces* sp., isolate Merv8102. Structure **1** was established by intensive NMR studies and by mass spectra. The compound is antibacterially active with MIC of 2 to 8 μg/ml against Gram-positive and Gram-negative bacteria, while it showed no antifungal activity. The fermentation and isolation, as well as the structure elucidation and biological activity of **1** are described.

Keywords essramycin, triazolopyrimidine, marine *Streptomyces* sp.

Introduction

In spite of some technical specialities on handling [1], microorganisms derived from marine environments are widely recognized as a rising source of novel natural products [2～4]. In recent years, numerous metabolites possessing uncommon structures and potent bioactivity have been isolated from strains of bacteria and fungi collected from diverse marine environments, such as animals, plants and sediments [5]. Triazolopyrimidines are synthetic heterocycles with valuable bioactivity [6～9]: They are useful therapeutics, especially for the treatment and prevention of cardiovascular diseases and in particular for the treatment of hypertension, cardiac insufficiency and diseases of the arterial wall, especially atherosclerosis [10]. They are known also as smooth muscle cell growth inhibitors [11] and are efficient analgesic and anti-inflammatory agents [12]. However, compounds of this type have never been described as natural products so far.

In our program to investigate secondary metabolites from the marine environment, microbial strains collected from the sediments of the Mediterranean Sea at the Egyptian coast were investigated. The crude extracts obtained from the culture media of *Streptomyces* sp. isolate Merv8102 showed a potent activity against the bacteria *Escherichia coli* (ATCC 10536), *Pseudomonas aeruginosa* (ATCC 10145), *Bacillus subtilis* (ATCC 6051), *Staphylococcus aureus* (ATCC 6538), and *Micrococcus luteus* (ATCC 9341). Additionally, a moderate antifungal

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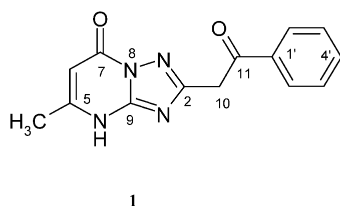
activity against *Aspergillus flavus*, *Trichoderma ressei*, and *Alternaria alternata* was observed.

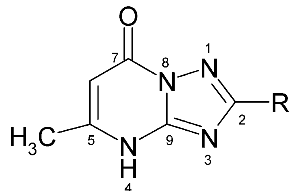
The strain was found to produce a triazolopyrimidine derivative **1**, which has an unprecedented skeleton amongst natural products and was named essramycin. It was obtained as an UV absorbing middle polar substance, which turned pale yellow on TLC by spraying with anisaldehyde/sulfuric acid.

Isolation, Maintenance and Identification of the Producing Strain

The *Streptomyces* sp. Merv8102 has been derived from sediment samples of Paltium coast at the Mediterranean Sea of Egypt, and was isolated on a medium containing 75% natural seawater with incubation at 28°C. The pure culture was maintained on yeast extract-malt extract agar.



1



4: R = H; **5**: R = CH_2OH; **6:** R = $CH_2(CH_2)_5CH_3$
7: R = CH_2OCH_2COOH; **8:** R = CH_2SH

Morphological characterisation of the producing strain *Streptomyces* sp. Merv 8102 was performed using light and scanning electron microscopy. The strain showed spiral or hooked hyphae. No special organs were observed. A mature spore chain comprised more than 30 spores (0.7∼0.9× 0.9∼1.3 μm) with a smooth surface. The cultural characteristics of strain Merv8102 grown on various media at 28°C for 21 days are summarized in Table 1. The strain forms a yellowish white to brownish gray aerial mycelium. Pale to deep brown soluble pigment is observed on all media used except nutrient agar medium. Melanin pigment is produced neither on peptone–yeast extract–iron agar nor on tyrosine agar. The temperature optimum is at approximately 28°C. The strain does not grow at 45°C. Gelatine and starch are degraded, casein and cellulose are hydrolyzed and hydrogen sulfide is produced, the strain is nitrate reductase positive. The peptidoglycan cell wall of the strain contains major amounts of L,L-diaminopimelic acid (L,L-DAP) but no diagnostic sugars (cell wall chemotype I, Table 2). Based on the described morphological, physiological and chemotaxonomic properties, the strain most probably belongs to the genus *Streptomyces*. Details of taxonomy will be discussed in a following article. The strain is deposited in the Chemistry of Natural Compounds Department, National Research Centre, Cairo, Egypt.

Fermentation and Isolation

The marine strain *Streptomyces* sp. isolate Mer8102 was inoculated from well grown agar plates with yellow air mycelia and yellowish-white colonies into 5 of 0.5 liters-Erlenmeyer flasks, each containing 100 ml of production medium: galactose (2.0%), dextrin (2.0%), Bacto-soytone (1.0%), corn steep liquor (0.5%), seawater (75%) and

Table 1 Cultural characteristics of marine *Streptomyces* sp. Merv8102

Medium	Growth	Color		Diffusible soluble pigment
		Aerial mycelium	Substrate mycelium	
Sucrose nitrate agar	Good	Pale olive	Yellowish brown	Brown
Glucose asparagines agar	Good	Yellowish white	Yellowish brown	Pale brown
Glycerol asparagines agar	Good	Brownish grey	Yellowish brown	Deep brown
Inorganic salt starch agar	Good	Whitish grey	Yellow	Yellowish brown
Tyrosine agar	Good	Brownish grey	Yellowish brown	Deep brown
Nutrient agar	Good	Brownish white	Pale pink	None
Yeast extract malt extract agar	Good	Moderate white	Light brown	Deep brown
Oat meal agar	Good	Brownish white	Pale yellowish brown	Deep brown
Peptone yeast extract iron agar	Good	Brownish grey	Pale yellow	Brown
Tryptone yeast extract broth	Good	Pale brownish white	Yellowish brown	Deep brown

Table 2 Physiological properties of *Streptomyces* sp. Merv8102

Melanin formation on	Response	Utilization of carbon source	Response
Tyrosine agar	−	L-Arabinose	+
Peptone yeast extract iron agar	−	D-Xylose	+
Tryptone yeast extract broth	−	D-Glucose	+
H_2S production	+	D-Fructose	+
Nitrate reduction	+	D-Galactose	+
Hydrolysis of starch	+	L-Rhamnose	+
Hydrolysis of casein	+	Sucrose	+
Cellulolytic activity	+	Inositol	+
Liquefaction of gelatine	+	Raffinose	+
Coagulation of milk	+	D-Manitol	+
Peptonization of milk	+	Utilization of amino acids	
Growth at 45°C	−	L-Valine	−
Sodium chloride tolerance	+ (18%)	L-Phenylalanine	−
L,L-diaminopimelic acid (L,L-DAP)	+	L-Histidine	+
G+C content	78%	L-Cysteine	−
		L-Hydroxyproline	−

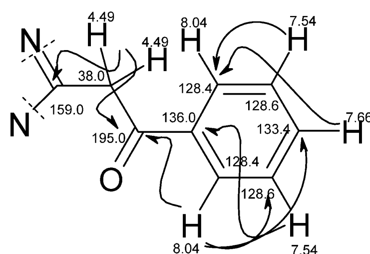


Fig. 1 HMBC correlations in partial structure **A** of essramycin (**1**).

demineralized water (25%). The pH was adjusted to 7.2 using 2 N NaOH before sterilization. Fermentation was carried out at 180 rpm on a rotary shaker for 3 days at 28°C. After cultivation, the seed culture was used to incubate 50 of 1.0 liter-Erlenmeyer flasks, each containing 200 ml of the previous medium. The fermentation was carried out for 5 days under identical conditions. After cultivation, the culture broth was filtered over Celite under pressure. The filtrate was extracted with EtOAc at pH 3, while the mycelium was extracted with MeOH. The MeOH extract was evaporated *in vacuo* and the residual water was extracted with EtOAc. Both organic extracts were combined, as TLC showed identity, and concentrated *in vacuo*. The brown extract (2.50 g) was dissolved in MeOH and defatted by hexane. After evaporation of both fractions (residue from MeOH 1.88 g, from hexane 0.28 g), the MeOH extract was applied to column chromatography on Sephadex LH-20. The column was eluted with MeOH and fractions were analyzed by TLC; spots were visualized by UV and anisaldehyde/sulfuric acid affording a polar fraction, which by application to further column chromatography on Sephadex LH-20 led to compound **1** (25 mg) as a crude product. Further purification by reversed phase column chromatography (RP-18) using a MeOH gradient, afforded 15 mg of **1** as colourless UV absorbing solid (Fig. 2). The physico-chemical properties of **1** are summarised in Table 3.

Results and Discussion

The UV spectra (MeOH) of **1** displayed two strong bands at λ_{max}=244 and 277 nm in neutral solution. Under acidic conditions, the latter band showed a hypsochromic shift to λ_{max}=271 nm, while a bathochromic shift to λ_{max}=282 nm was observed in basic MeOH.

The IR spectra (KBr) of **1** displayed a signal at ν=3350 cm^{-1} of NH or OH groups. Between ν=3080∼2950, two absorption bands were indicative of aromatic and aliphatic C–H bonds, two strong bands at ν=1695 and 1645 cm^{-1} indicated conjugated carbonyl groups and an aromatic skeleton or double bonds.

The molecular weight was determined by ESI-MS: Two and three *quasi*-molecular ion peaks in positive and negative ESI-MS mode, respectively, confirmed the molecular weight of **1** as 268 Dalton. (+)-HRESI-MS of **1** delivered the molecular formula $C_{14}H_{12}N_4O_2$.

Table 3 Physico-chemical properties of essramycin (**1**)

Appearance	Colorless solid
Mp (°C)	219~221
Rf	0.34[a], 0.52[b]
Solubility	Soluble in DMSO, MeOH, EtOH and EtOAc. Insoluble in hexane, benzene and H_2O
Molecular formula	$C_{14}H_{12}N_4O_2$
(+)-ESI MS: *m/z* (%)	291 $[M+Na]^+$ (24); 539 $[2M+Na]^+$ (100)
(−)-ESI MS: *m/z* (%)	267 $[M-H]^-$ (100); 535 $[2M-H]^-$ (72); 557 $[2M-2H+Na]^-$ (54)
(+)-HRESI MS (*m/z*)	
Found	269.103367 (M+H); 291.085319 (M+Na)
Calcd.	269.103306 for $C_{14}H_{13}N_4O_2$; 291.085246 for $C_{14}H_{12}N_4O_2Na$
IR (KBr) ν cm^{-1}	3350, 3080, 2950, 1695, 1645, 1610, 1520, 1456, 1382, 1270
UV/VIS: λ_{max} (log ε)	(MeOH): 244 (4.16), 277 (3.96); (MeOH/HCl): 243 (4.16), 271 (3.95); (MeOH/NaOH): 244 (4.17), 282 (4.05) nm.

[a] CH_2Cl_2/5% MeOH; [b] CH_2Cl_2/10% MeOH

The ^{1}H-NMR spectrum of **1** showed the pattern of a monosubstituted aromatic system. The coupling constants (7.5~8.0, 1.0~1.3 Hz) were indicative of a benzene derivative, and the dd signal at δ=8.04 (2H), and two td signals at δ=7.66 (1H) and 7.54 (2H) pointed to an electron-withdrawing substituent. The spectra displayed only three additional singlets, an olefinic methine signal at δ=5.76, a singlet of a methylene group at δ=4.49, and at δ=2.28 the signal of a methyl group bound to an aromatic system, an acetyl group, or a double bond.

The ^{13}C/HMQC spectra of compound **1** exhibited 12 carbon signals, among them a carbonyl signal at 195.0, and signals of four quaternary carbons at δ=155.6, 151.9, 151.4 and 136.0, the first three being due to O or N connected sp^2 carbon atoms. Further on, three sp^2 methine signals, two of them with double intensity, were due to the phenyl moiety. A fourth methine signal at δ=97.9 gave an HMQC correlation with the olefinic proton at δ=5.76. In the aliphatic region, at δ=38.0 and 18.9, respectively, the expected carbon signals of a methylene and a methyl group were visible; both shifts excluded an attachment to hetero atoms.

In the HMBC spectrum, the *ortho*-coupled aromatic protons at δ=8.04 (H-2′/6′) and 7.54 (H-3′/5′) exhibited ^2J and ^3J couplings with the carbon signals at δ=136.0 (C_q-1′) and 133.4 (CH-4′), confirming the monosubstituted phenyl moiety. Additionally, the methine protons H-2′/6′ (δ=8.04) displayed a significant ^3J coupling with the carbonyl carbon at δ=195.0, which in turn coupled (^2J) with the methylene protons (H_2-10, δ 4.49). The latter protons displayed an additional cross signal with the quaternary carbon at δ=159.0. As no further couplings of the methylene group were visible, and due to the downfield shift of the quaternary C atom, a guanidine fragment was assumed, resulting in partial structure **A** (Fig. 1).

On subtracting the acetophenone substructure of **A**, 6 double bond equivalents were left for the remaining fragment $C_6H_5N_4O$. This pointed firstly to a one of the four *N*-methylhypoxanthins; these were, however, easily excluded, as their *N*-methyl signals appear between δ=3~4 and not at δ=2.28 as in **1**.

According to the HSQC data, the olefinic proton (δ 5.76) is attached to the upfield carbon at δ=97.9. HMBC cross signals of this CH with the methyl group (Fig. 3) and *vice versa* confirmed a propene fragment in that way, that the methyl group is connected with the carbon at δ=151.9. The alkene shifts required a carbonyl group attached to the upfield carbon, whereby the former must be present as an amide to explain its shift. The methyl group coupled weakly with this carbonyl group and with an additional quaternary carbon at δ=151.4, however, due to overlapping with δ=151.9, it was not possible to distinguish between a strong ^4J or a weak ^3J coupling. This latter carbon showed no coupling with the methine proton at δ=5.76, so that a ^3J distance was excluded with respect to the CH and the CH_3 group.

A calculation of all possible isomers containing substructure **A** and fulfilling the conditions listed above delivered 41 hits [13]. When the highly strained or chemically unstable structures were sorted out, only compounds **1**~**3** and further 5 prototrop-isomers with higher ground state energies [14] were left. Two of them

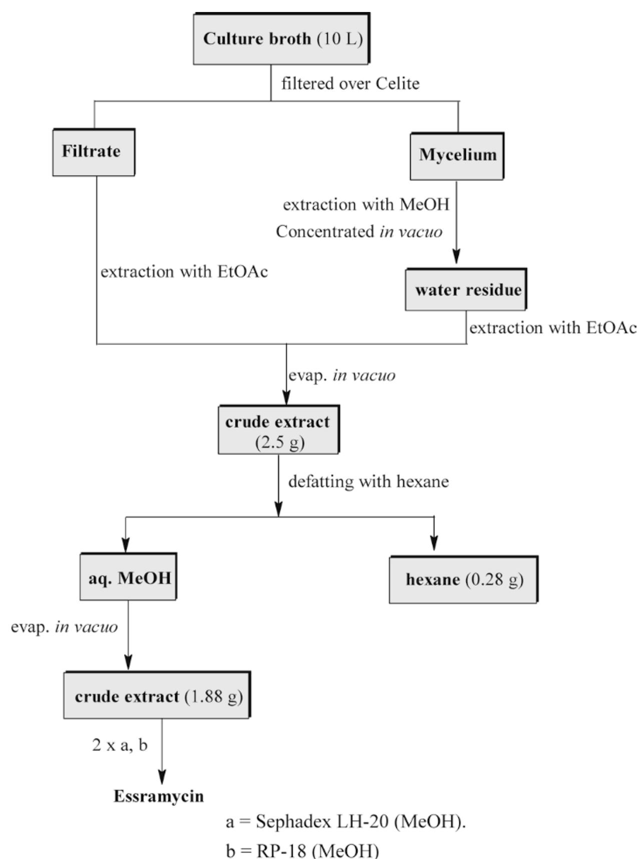


Fig. 2 Working up scheme for extracts of *Streptomyces* sp. Merv8201.

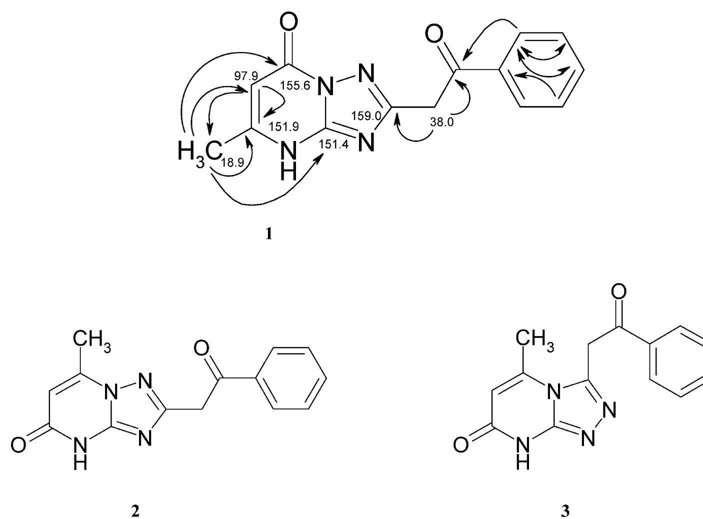


Fig. 3 The three alternative skeletons **1**~**3** for essramycin; for **1**, HMBC (→) correlations are indicated.

were [1,2,4]triazolo[4,3-a]pyrimidines (*e.g.* **3**), with the methyl group and the acetophenone substituent in a *syn*-periplanar position. As essramycin did not show any nuclear Overhauser effect between the methyl group and the phenone moiety (solely an interaction between Me and 6-H is visible), these isomers were excluded.

It was not possible to distinguish between **1** and **2** on the basis of 2D CH correlations, and further measurements were hindered by the low yield. Comparison of the ^{13}C-NMR data of essramycin with values of related synthetic compounds of type **1** delivered, however, a much better agreement with the experimental values than with compounds of type **2**, confirming the skeleton of **1** [15, 16]: A C-2 substituent affects the *ipso* C atom of 1,2,4-triazolo[1,5-a]pyrimidines, however, the influence on the ^{13}C shifts of the other ring atoms is negligible (Table 4).

[1,2,4]Triazolo[1,5-a]pyrimidines have found a broad interest as fungicides [17], herbicide safener [18, 19], kinase inhibitors [20, 21], antiparasitic [22] and plant protecting agents [23]. Thousands of compounds of this type have been described, however, to the best of our knowledge, not a single natural product is amongst them [6, 24, 25].

Table 4 ^{13}C Chemical shifts (δ values in [D$_6$]DMSO) of **1** and further 2-substituted [1,2,4]triazolo[1,5-a]pyrimidine derivatives **4**~**8** [16]

No	C-2	C-5	C-6	C-7	C-9	5-CH$_3$
1	159.0	151.9	97.9	155.6	151.4	18.9
4	151.9	151.5	98.2	155.9	150.6	18.7
5	163.8	151.4	98.1	155.7	150.8	18.6
6	164.1	151.0	98.0	155.0	150.6	18.6
7	160.4	151.5	98.2	155.7	151.0	18.6
8	160.8	151.3	98.3	155.5	151.0	18.6

Table 5 ^{13}C- and ^{1}H-NMR spectral data for **1** ([D$_6$]DMSO, shifts as δ values, *J* in [Hz]).

Position	δ_C	δ_H	HMBC
2	159.0	—	
5	151.9	—	
5-CH$_3$	18.9	2.28 (s)	9, 5, 6, 7
6	97.9	5.76 (s, br)	5, CH$_3$-5
7	155.6	—	
9	151.4	—	
10	38.0	4.49 (s)	2, 11
11	195.0	–	
1′	136.0	—	
2′	128.4	8.04 (dd, 8.1, 1.0)	11, 3′, 4′
3′	128.6	7.54 (td, 7.5, 1.2)	1′, 2′
4′	133.4	7.66 (td, 8.4, 1.3)	
5′	128.6	7.54 (td, 7.5, 1.2)	
6′	128.4	8.04 (dd, 8.1, 1.0)	

Biological Activities

Essramycin (**1**) showed potent antibacterial activities with MIC ranging between 1.0~8.0 μg/ml against Gram-positive and Gram-negative bacteria, using *Escherichia coli* (ATCC 10536), *Pseudomonas aeruginosa* (ATCC 10145), *Bacillus subtilis* (ATCC 6051), *Staphylococcus aureus* (ATCC 6538) and *Micrococcus luteus* (ATCC 9341) as test organisms (Table 6). Especially the high activity against *Pseudomonas aeruginosa* (ATCC 10145) is encouraging for further studies. In spite of the moderate antifungal activity of the crude extract, **1** displayed no activity against *Aspergillus flavus*, *Trichoderma ressei*, and *Alternaria alternata*.

Experimental Section

NMR spectra were measured on Varian Unity 300 and Varian Inova 600 spectrometers. Electron spray ionization mass spectrometry (ESI HR-MS): Finnigan LCQ ion trap mass spectrometer coupled with a Flux Instruments (Basel, Switzerland) quaternary pump Rheos 4000 and a HP 1100 HPLC (nucleosil column EC 125/2, 100-5, C 18) with autosampler (Jasco 851-AS, Jasco Inc., Easton, MD, USA) and a Diode Array Detector (Finnigan Surveyor LC System). High-resolution mass spectra (HR-MS) were recorded by ESI-MS on an Apex IV 7 Tesla Fourier-Transform Ion Cyclotron Resonance Mass Spectrometer (Bruker Daltonics, Billerica, MA, USA). IR spectra were recorded on a Perkin-Elmer 1600 Series FT-IR spectrometer from KBr pellets. UV-VIS spectra were recorded on a Perkin-Elmer Lambda 15 UV/VIS spectrometer. Rf values were measured on Polygram SIL F/UV$_{254}$ (Merck, pre-coated sheets). Size exclusion chromatography was done on Sephadex LH-20 (Pharmacia).

Table 6 Antibacterial activities of **1** (MIC values, conc. in μg/ ml).

Test microorganism	MIC (μg/ml)
Escherichia coli (ATCC 10536)	8
Pseudomonas aeruginosa (ATCC 10145)	3.5
Bacillus subtilis (ATCC 6051)	1
Staphylococcus aureus (ATCC 6538)	1
Micrococcus luteus (ATCC 9341)	1.5

Collection, Isolation and Growth of *Streptomyces* sp. Merv8102

Streptomyces sp. Merv8102 was isolated from sediment collected at a depth of 2 m in the Mediterranean Sea, Paltium coast of Egypt. The samples were immediately processed at the Chemistry of Natural Compounds Department, National Research Center, Cairo, Egypt.

Aliquots of sediments were inoculated in Petri dishes containing different culture media. Marine streptomycetes were selected using standard growth conditions (g/liter): soluble starch (10.0), peptone (2.0), yeast extract (4.0), agar (15.0); the pH was adjusted to 7.0∼7.5 [26]. Spread plates were incubated at 28°C for two weeks. Single colonies were picked and checked for purity. *Streptomyces* sp. Merv8102 was identified by morphological and physiological analyses according to the method of ISP [27∼29] and by chemotaxonomic analyses (cellular wall amino acids and fatty acids composition) using the method of Yamaguchi and Yan *et al.* [30, 31]. Detailed observations of mycelium and spore morphologies were performed with a light microscope and scanning electron microscope according to Williams and Davis [32]. Color determinations were carried out according to ISCC–NBS color charts [33].

Production

A loop-full sporulating mycelium of the strain *Streptomyces* sp. Merv8102 was inoculated into 5 of 0.5 liters-Erlenmeyer flasks, each containing 100 ml of a production medium: galactose (2.0%), dextrin (2.0%), Bacto-soytone (1.0%), corn steep liquor (0.5%), seawater (75%) and demineralized water (25%), and the pH was adjusted at 7.2 before sterilization. The culture was incubated at 28°C for 3 days on a rotary shaker (180 rpm). This seed culture was used to incubate 50 of 1.0 liter-Erlenmeyer flasks, each containing 200 ml of the previous medium. The fermentation was carried out for 5 days at the same conditions. After cultivation, the culture broth was mixed with Celite (1.2 kg) and applied to filtration under pressure. The filtrate was acidified to pH 3 and exhaustively extracted with EtOAc (three times, each with 3.5 liters). The mycelium was extracted with MeOH (1.5 liters), and the MeOH extract was evaporated *in vacuo* to the aqueous residue, which was re-extracted with EtOAc (1.2 liters). Both EtOAc fractions were combined and evaporated to dryness. The obtained brown crude extract (2.5 g) was dissolved in MeOH (250 ml) and defatted by hexane (200 ml). The residual methanol extract (1.88 g) was twice chromatographed on Sephadex LH-20 (75×2.5 cm, MeOH) to deliver 25 mg of crude **1**. The substance was further purified by reversed phase chromatography (RP-18 column, 35×0.5 cm, MeOH) yielding **1** as a colorless solid (15 mg).

Evaluation of Biological Activity

The antimicrobial activities of **1** were determined by serial dilution techniques. The cell growth was measured after 24 hours of incubating the target strains: *Escherichia coli* (ATCC 10536), *Pseudomonas aeruginosa* (ATCC 10145), *Bacillus subtilis* (ATCC 6051), *Staphylococcus aureus* (ATCC 6538) and *Micrococcus luteus* (ATCC 9341) in Luria-Bertani medium [(g/liter): NaCl (10.0), peptone (10.0) and yeast extract (5.0)] at 37°C.

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