J. Antibiot. 61(6): 379–386, 2008

ORIGINAL ARTICLE

THE JOURNAL OF ANTIBIOTICS

Novel Bioactive Metabolites from a Marine Derived Bacterium *Nocardia* sp. ALAA 2000

Mervat M. A. El-Gendy, Usama W. Hawas, Marcel Jaspars

Received: November 4, 2007 / Accepted: June 10, 2008

Abstract Extracts of the Egyptian marine actinomycete, *Nocardia* sp. ALAA 2000, were found to be highly bioactive. It was isolated from the marine red alga *Laurenica spectabilis* collected off the Ras-Gharib coast of the Red Sea, Egypt. According to detailed identification studies, the strain was classified as a member of the genus *Nocardia*. The cultivation and chemical analysis of this species yielded four structurally related compounds namely, chrysophanol 8-methyl ether (**1**), asphodelin; 4,7′-bichrysophanol (**2**) and justicidin B (**3**), in addition to a novel bioactive compound ayamycin; 1,1-dichloro-4-ethyl-5-(4-nitro-phenyl)-hexan-2-one (**4**) which is unique in contain both chlorination and a rarely observed nitro group. The compounds were isolated by a series of chromatographic steps and their structures of **1**∼**3** secured by detailed spectroscopic analysis of the MS and NMR data whereas that of **4** was elucidated by single crystal X-ray diffraction studies. These compounds displayed different potent antimicrobial activity against both Gram-positive and Gram-negative bacteria as well as fungi with MIC ranging from 0.1 to 10 μg/ml.

Keywords *Nocardia* sp. ALAA 2000, ayamycin, justicidin B, NMR, X-ray, antimicrobial

Introduction

The oceans cover more than 70% of the earth's surface and represent an enormous resource for the discovery of chemotherapeutic agents. Given the diversity of marine organisms and habitats, marine natural products encompass a wide variety of chemical classes such as terpenes, polyketides, acetogenins, peptides and alkaloids of varying structures, representing biosynthetic schemes of stunning variety. Over the past 30 to 40 years, marine organisms have been the focus of a worldwide effort for the discovery of novel natural products [1]. Marine microorganisms (actinobacteria) are sources of novel compounds with often unique structures and potential therapeutic applications. The *Actinomycetes* are widely distributed in natural and manmade environments and are also well known as a rich source of antibiotics and bioactive molecules, which are of considerable importance in chemotherapy [2, 3].

Nocardia species, which was reported as an antibiotic producer, is one of the most promising sources of bioactive compounds because among sixteen *Actinomycete* strains that were tested for their potentiality to produce bioactive compounds that inhibit the growth of the pathogen *Vibrio damsela*, *Nocardia brasiliensis* showed the largest inhibition zone and highest activity [4].

Nocardia brasiliensis has revealed such new bioactive metabolites as a new antifungal and antibacterial compound, indole alkaloid named brasilidine A and new cytotoxic antibiotics named brasiliquinones (A, B and C29). A new Transvalencin A, a thiazolidine zinc complex antibiotic was isolated from *Nocardia transvalensis* IFM 10065 while, the remaining strains produced a chelating

M. M. A El-Gendy: Chemistry of Natural and Microbial Products Department, National Research Centre, Dokki, Cairo, Egypt

M. Jaspars (Corresponding author)**, U. W. Hawas:** Department Chemistry, University of Aberdeen, AB 24 3UE Aberdeen, Scotland, UK, E-mail: m.jaspars@abdn.ac.uk

compound *N*,*N*-ethylenediaminedisuccinic acid (EDDS) and nocaracins [5, 6].

We have recently started a program aimed at investigating the bioactive secondary metabolites of the Egyptian marine derived microbial strains as a part of our continued efforts to exploit marine *Actinomycetes* natural products for drug discovery and development. We have been isolating strains of *Actinomycetes* from marine algae of the Red Sea, Egypt. Based on different chemical and biological screening of the crude extracts of these different marine *Actinomycetes*, we have found one marine strain with high bioactivity against different human, animal or plant pathogens. It was found to belonging to the genus *Nocardia*, *Nocardia* sp. ALAA 2000, and it was selected for the further investigations.

According to physical and chemical analysis data, the strain was found to produce chrysophanol 8-methyl ether (**1**) [7], asphodelin; 4,7′-bichrysophanol (**2**) [8] identified as two known anthraquinone metabolites; justicidin B (**3**) as arylnaphthalene lignanolide [9], reported here to be isolated for the first time from microorganisms; and a novel bioactive water soluble compound, ayamycin, 1,1-dichloro-4-ethyl-5-(4-nitro-phenyl)-hexan-2-one (**4**). The absolute stereochemistry of a novel compound **4** has been assigned through X-ray crystallographic analysis. The compounds were potentially active against Gram-positive and Gram-negative bacteria as well as fungi.

The taxonomy of the producing strain ALAA 2000 as well as the extraction, purification, structure elucidation and biological activity of four antimicrobial molecules (**1**∼**4**) from ALAA 2000 strain are described.

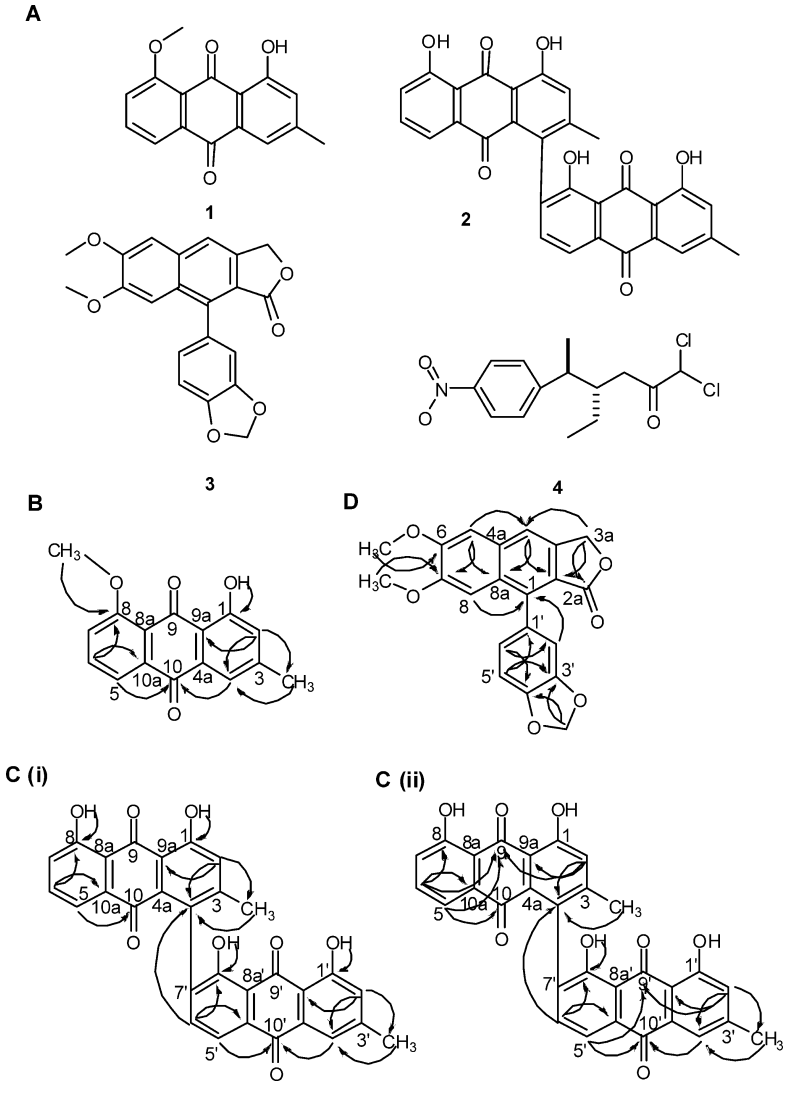

Fig. 1 A; Structures of compounds **1**∼**4**. B; Selected HMBC correlations for compound **1**. C; Selected HMBC correlations for compound **2** (i) at Δ=50 ms and (ii) at Δ=200 ms. D; elected HMBC correlations for compound **3**.

Materials and Methods

Collection of Samples

Samples of the marine red alga *Laurenica spectabilis* from a depth of 1～10 m were collected at Ras-Gharib coast of Red Sea, Egypt. The algal samples were placed inside sterile polyethylene bags underwater and transferred to the lab aseptically in ice boxes. The samples of algae were immediately processed at the Chemistry of Natural and microbial products Department of the National Research Center, Egypt.

Isolation of Marine Actinobacteria

Algae associated actinobacteria were isolated by following the method outlined by Ananda *et al.* [1]. Initially, the algal samples were washed with jets of filtered and autoclaved seawater until they were visibly free of debris. Subsequently the algal surface was sterilized by a rapid wash of 70% EtOH and immediately immersed in autoclaved and filtered seawater and then aspirated. One gram of algal tissue was removed with a sterile scalpel and the tissue was immediately transferred to 99 ml sponge dissociation medium (2.7% NaCl, 0.008% KCl, 0.01% Na_2SO_4, pH 8). The samples were soaked for 20 minutes and then the tissue and diluents were macerated and the homogenate was plated on starch nitrate agar medium of the following composition (g/liter): Starch 20; KNO_3 1; K_2HPO_4 0.5; $MgSO_4 \cdot 7H_2O$ 0.5; NaCl 0.5; $FeSO_4 \cdot 7H_2O$ 0.01; agar 15 [10]. The isolation medium was prepared by using 100% seawater and contained 25 and 75 μg/ml of filter-sterilized cycloheximide and nystatin, respectively, as antifungal agent [11], using a serial dilution plate technique. The plates were incubated at room temperature for 14 days. *Actinomycetes* were recognized by their tough dry colonies, branched vegetative mycelium, aerial mycelium when present and spore formation, and only colonies with these characteristics were continued from the third day onwards up to the fourteenth day and included in this study.

Organisms and Maintenance

Biomass of the ALAA 2000 strain was prepared in shake flasks of tryptic soy broth at 35°C for 7 days. At maximum growth, the broth cultures were checked for purity, harvested by centrifugation, washed twice in TE buffer (pH 7) and kept at 5°C. The strain was maintained on tryptic soy agar plates and as spore suspensions (20%, v/v, glycerol).

Taxonomy of the Producing Strain

Cell morphology of *Nocardia* sp. ALAA 2000 was observed under a Zeiss light microscope at ×1,000, with cells grown on inorganic salts-starch agar (ISP medium 4) after incubating the strain at 35°C for 3 days. The strain stained Gram-positive and examined for a balanced set of phenotypic properties, using procedures described in previous study [12～19]. Color determinations were carried out according to ISCC-NBS color charts [20].

Chemotaxonomically: The presence of the isomeric form of diaminopimelic acid, and the composition of cell-wall sugars was analyzed by the previous method [21]. Polar lipids extracted, examined by two dimensional thin layer chromatography, the frist dimension with chloroform/methanol/water (65 : 24 : 4) and in the second dimension with chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4). Polar lipids were identified using published procedures [22]. Menaquinones were extracted, purified and identified [23]. Mycolic acids were extracted and analyzed by one-dimensional TLC and developed in petroleum benzene/acetone (95 : 5, v/v) as described previously [24] and fatty acids analysisas described before [25].

Molecular characteristics, in order to characterize strain Merv21695 by molecular methods, G+C content of the DNA and 16S rDNA sequencing were carried out. The G+C contents were determined using the thermal denaturation (*Tm*) method in standard saline citrate (SSC) by the method of Mandel & Marmur [26].

Chromosomal DNA was isolated from the test strain using a procedure of Chun and Goodfellow [27], slightly modified from that of Pitcher *et al.* [28].

16S rRNA gene amplification and sequencing from the test strain were carried out using a procedure of Chun *et al*. [29], and Kim *et al*. [30].

Biological Assays

The antimicrobial spectrum and activity of *Nocardia* sp. ALAA 2000 secondary metabolites were determined by the agar plate diffusion assay [31]. Ten μl of the filtrate broth was then applied to sterile paper disc (Whatman) of 6 mm diameter, placed on the surface of indicator test plate and incubated at the temperature that permitted optimal growth of the test organisms. For determination of antibacterial and antifungal activities, indicator bacteria were grown overnight in LB medium, yeasts were grown in YPG medium (10 g/liter yeast extract, 10 g/liter peptone, 100 g/liter glucose) for 24 hours, and fungi were grown in potato dextrose agar (PDA) for 3～4 days. The MIC of the pure compounds was expressed as μg/ml by the dilution method.

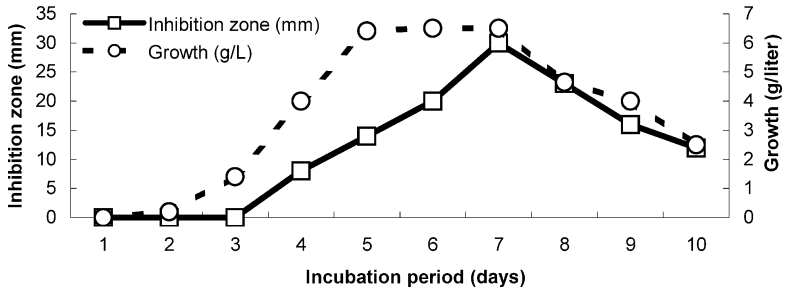

Fig. 2 Typical time course of bioactive secondary metabolites produced by *Nocardia* sp. ALAA 2000.

Instrumental Analysis

NMR experiments were measured on Bruker (250 MHz) and Varian Unity INOVA (400 MHz). The chemical shifts were expressed in δ (ppm) using $CDCl_3$ as solvent and TMS as internal reference. ESI-MS were recorded on a Waters-Micromass Quattro Premier Triple Quadrupole mass spectrometer. UV-VIS spectra were recorded on a Perkin-Elmer Lambda 25 UV/VIS spectrometer. HPLC was carried out on Agilent 1200 HPLC using C-18 column. X-ray was carried out using an Enraf-Nonius 590 Kappa CCD using maXus (Bruker-Nonius, Delft & MacScience, Japan). Rf-values were measured on Polygram SIL F/UV_{254} (Merck-pre-coated sheets). Size exclusion chromatography was done on Sephadex LH-20 (Pharmacia).

Fermentation and Isolation of Bioactive Compounds

The medium containing starch 1.0%, glucose 1.0%, glycerol 1.0%, corn steep powder 0.25%, peptone 0.5%, yeast extract 0.2%, $NaNO_3$ 0.1% and $CaCO_3$ 0.3% in 100% sea water, pH 7.2 was used as seed and fermentation medium [32]. The fermentation 1-liter Erlenmeyer flasks 50, each containing 200 ml of the cultivation medium were inoculated with 5.0% of the seed cultures, grown in 500 ml for 48 hours on a rotary shaker at 120 rpm and 35°C. The fermentation was carried out for 7 days at 35°C with an agitation of 200 rpm. After the time course of fermentation, the culture broth was filtered on celite under pressure. The filtrate was acidified to a pH of 3 and extracted with EtOAc using liquid-liquid partition for several times, at which the solvent and aqueous layer were mixed and stirred magnetically. The EtOAc extract of the filtrate was defatted with *n*-hexane and subjected to flash silica gel column chromatography to give four fractions. Working up of the fractions resulted in the isolation of three bioactive compounds, chrysophanol 8-methyl ether (**1**) (8.4 mg), asphodelin (**2**) (20 mg) and justicidin B (**3**) (20 mg). The mycelium was extracted with Me_2CO followed by MeOH. Both organic extracts were combined and evaporated under pressure. The obtained brown crude extract was defatted by *n*-hexane and applied to column chromatography on Sephadex LH-20 (75×0.5 cm, MeOH/H_2O) affording two fractions. Further purification of the polar fraction with RP-18 column chromatography give a 5.0 mg as white crystals of a new compound ayamycin (**4**).

Table 1 Culture characteristics of marine *Nocardia* sp. ALAA 2000

Characteristic	ALAA 2000
Aerial mass color	Deep greenish red
Substrate mycelium color	Whitish red
Hyphae	Fragmented into rod-shaped to coccid elements at a later stage
Melanin production	Positive
Diffusible pigment	Light olive green

Ayamycin; 1,1-Dichloro-4-ethyl-5-(4-nitro-phenyl)-hexan-2-one (**4**)

Colorless crystals, mp 115~117°C. Rf=0.5 (10% MeOH/CH_2Cl_2), X-ray: crystal data collections and structure refinement (Table 3), CCDC number is 664911.

Results and Discussion

Taxonomy of the Producing Strain

Strain ALAA 2000 exhibited a range of phenotypic properties typical of members of the genus *Nocardia* [19]. Aerobic non motile Gram-positive and slightly acid-alcohol fast *Actinomycetes* which forms an extensively branched whitish red substrate mycelium gives rise to deep greenish red aerial mycelium. Pale olive green diffusible pigments are formed (Table 1). At a late stage of growth the hyphae fragment into rod-shaped to coccid elements as the characteristic of *Nocardia* Strains.

Chemotaxonomically: ALAA 2000 contained chemical

markers that support assignment of this actinobacterium to the genus *Nocardia*. The cell wall was rich in *meso*-diaminopimelic acid as well as arabinose and galactose (*i.e.* cell wall chemotype IV sensu Lechevalier & Lechevalier [32]). The quinone system with the predominant compound (99%) MK-8 (H4, v-cycl) supports affiliation of strain ALAA 2000 to the genus *Nocardia*, where all species have MK-8 (H4, v-cycl) as the major quinone [19]. Polar lipid analysis showed that strain contain phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside and diphosphatidylglycerol as the characteristic phospholipids (*i.e.* phospholipid type PII sensu Lechevalier *et al.* [33]). *i.e.*, the polar lipid profile of strain ALAA 2000 was similar to those reported for other *Nocardia* with the presence of significant amounts of phosphatidylglycerol. The mycolic acid of strain ALAA 2000 showed a chromatographic behavior was very similar to that of the closely related *Nocardia* species, TLC revealed that the strains contained methyl mycolates, its mycolic acids are cleaved upon pyrolysis, releasing fatty acids C14 : 0, C16 : 0, C18 : 1v9c and 10-methyl C16 : 0 as the major cleavage product.

Physiological characteristics of ALAA 2000 showed liquefaction of gelatin; nitrates weakly reduced to nitrites; melanoid pigments are formed; no peptonization or curd formation in purple milk; NaCl tolerance up to 15% in yeast extract agar. Optimal growth temperature 35°C and the strain can grow at 45°C. Carbon source utilization [17] were as follows: Good utilization of glycerol, salicin, and dextrose, L-inositol, D-fructose, maltose, adonitol, L-arabinose, lactose, D-mannitol, L-rhamnose, sucrose and D-xylose. Starch, casein, gelatin, cellulose, xanthine, hypoxanthin, and chitin are degraded but not uric acid and urea. The strain is highly fermentative actinobacteria, acid is formed from, adonitol, L-arabinose, cellobiose, dextrin, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannitol, D-mannose, L-rhamnose, salicin, and sucrose. The organism is resistant to bacitracin, penicillin, gentamicin, and streptomycin (Table 2).

Molecular taxonomy, the G+C content of the DNA is 69.9 mol%. An almost complete gene sequence of the 16S rDNA of the strain was determined following cloning and sequencing of the amplified gene. The sequence was aligned with those available for *Nocardiae* species. It is, therefore, proposed that the organism can be classified in the genus *Nocardia*. The type strain is *Nocardia* sp. ALAA 2000. The strain is deposited in the Chemistry of Natural and microbial products Department, National Research Centre, Cairo, Egypt.

Table 2 Phenotypic characteristics of marine *Nocardia* sp. ALAA 2000

Characteristic	ALAA 2000
Acid production from	
Adonitol	+
Arabinose	+
Glucose	+
Mannitol	+
Rhamnose	+
Sorbitol	−
Decomposition of	
Adenine	+
Elastin	+
Hypoxanthin	+
Xanthin	+
Tyrosine	+
Uric acid	−
Growth at 45°C	+
Utilization of citrate	−
Growth in the presence of (%)	
NaCl (15)	+
Sodium azide (0.01)	−
Phenol (0.1)	+
Resistance to antibiotic (μg/ml)	
Bacitracin	+
Penicillin	+
Gentamycin	+
Streptomycin	+

Optimization of Culture Condition for Maximum Antibiotic Production by ALAA 2000

Productivity of *Actinomycetes* is an important criterion for assessment of potency of *Actinomycetes* producing antibiotics. In order to determine the factor that intensifies the biosynthesis of antibiotics in culture, it is necessary to determine antibiotic productivity during the growth of the microorganism with different culture conditions. Data in (Table 3) and (Fig. 2) showed a profound effect of these conditions on antimicrobial activities of bioactive marine *Nocardia* strain ALAA 2000. The strain established its complete biosynthesis of antimicrobial substances at the 7th day of growth (*i.e.* through the stationary growth phase). Antibiotic production was successively increased with increasing the pH up to 7.5 and the temperature up to 35°C as well. Different carbon and nitrogen sources had a significant effect on the production of antibiotics and it is clear that glucose, starch or glycerol as carbon source ensured a relatively high yield of antibiotic formation by marine ALAA 2000. The highest antagonistic activities

Table 3 The effect of different carbon sources and nitrogen sources on the bioactive metabolites productivity by ALAA 2000

Carbon source (1.0% w/v)	Inhibition zone (mm)	Nitrogen source	Inhibition zone (mm)
Fructose	16	Yeast extract	37
Glucose	38	Meat extract	22
Galactose	10	Peptone	20
Mannose	13	Malt extract	36
Mannitol	22	Soybean	25
Maltose	29	Corn step liquor	40
Lactose	12	$NaNO_3$	36
Sucrose	10	$(NH_4)_2SO_4$	15
Raffinose	0		
Starch	42		
Glycerol	40		

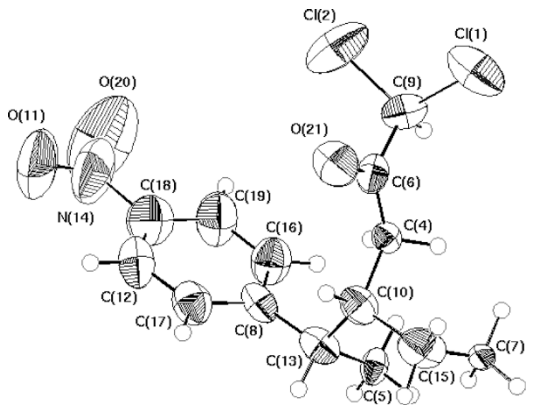

Fig. 3 X-ray crystal structure of compound **4**.

were observed in cultures containing corn step liquor, malt extract, yeast extract or sodium nitrate as nitrogen source.

Purification and Structure Elucidation of the Active Compounds

Bulk fermentation was performed on 10 liters scale of the *Nocardia* strain ALAA 2000. The production of bioactive compounds in the fermentation broth began at day 3 and reached maximum at day 7. The antibiotic formation was monitored by an antibiotic bioassay. The fermentation broth was separated into filtrate (10 liters) and mycelia by celite filtration under vacuum. The filtrate was extracted with acetone and ethyl acetate respectively. Different chromatographic techniques were used on the extract to deliver three compounds. ^{1}H, ^{13}C-NMR and 2D-NMR data (HSQC and HMBC) and ESI-MS spectra were measured and compared with literature data.

Table 4 Summary of crystal data collections and structure refinement of compound **4** (Ayamycin)

Molecular formula	$C_{14}H_{16}Cl_2NO_3$
Molecular weight	317.92
Shape	cube
Temperature	298 K
Wavelength	0.71073
Crystal system	Orthorhombic
Space group	$C2_12_12_1$
Unite cell dimensions	a=7.3408 (3) Å,
	b=17.5498 (6) Å,
	c=22.2939 (12) Å
	$\alpha=\beta=\gamma=90.00°$
Volume	2872.1 (2) Å^3
F(000)	2440
Z, molecules/unit cell	8
Density (calculated)	1.467 mg m^{-3}
Density (measured)	not measured
Index ranges	$h=-8\rightarrow8$, $k=-20\rightarrow21$,
	$l=26\rightarrow26$
θ Range for data collection	2.910~25.350
Measured reflections	2410
Independent reflections	1519
Observed reflections	1076
Final R indices [I>3.0 sigma(I)]	R_{int}=0.023
Refinement method	Full matrix least squares on F^2
No. of variables refined	181
R indices (all data)	R=0.0106 wR=0.174
Largest difference peak and hole	0.67 and −0.53 eÅ
Restrains	0

The presence of *peri*-hydroxy-anthraquinones in compounds **1** and **2** was detected by TLC on silica gel by spraying with 1.0% vanillin-H_2SO_4 and by their red colour with 5% KOH solution, respectively.

Compound **1** with chelated OH and aromartic methyl and methoxy groups was identified as chrysophanol 8-methyl ether. One dimensional NMR data were measured for compound **2** which showed similar ^{1}H- and ^{13}C-NMR signals to three bichrysophanol compounds, cassiamin C, microcarpin; 2,7′-bichrysophanol and asphodelin. 2D-NMR data (HSQC and HMBC) were used to differentiate between these compounds. The normal HMBC experiment at 50 and 200 ms to reveal the ^{3}J and ^{4}J correlations preferentially was performed to identify the compound **2** as asphodelin; 4,7′-bichrysophanol.

The NMR spectroscopic and spectrometric analyses were measured for compound **3** and all data taken together determined the structure of **3** as the arylnaphthalene lignanolide justicidin B, previously known from plants

Table 5 Antimicrobial activities of the isolated pure compounds (**1**~**4**) (conc. in μg/ml)

Test micro-organism	1	2	3	4
Indicator bacteria				
Escherichia coli (ATCC 10536)	10.0	8.0	0.5	0.1
Pseudomonas aeruginosa (ATCC10145)	6.0	6.5	0.2	0.1
Bacillus subtilis (ATCC 6051)	2.0	0.5	2.0	0.1
Bacillus cereus (ATCC 9634)	1.5	2.5	2.5	0.1
Staphylococcus aureus (ATCC 6538)	3.0	1.6	1.0	0.1
Micrococcus luteus (ATCC 9341)	4.5	1.2	0.5	0.1
Mycobacterium smegmatis (ATCC 607)	9.5	6.5	5.5	0.1
Corynebacterium xerosis (NRRL B-1397)	8.0	3.0	7.0	0.1
Indicator fungi				
Rhodotorula acuta	3.0	10.0	3.0	0.5
Pichia angusta	0.8	8.8	0.8	0.5
Candida albicans	4.5	5.5	4.5	0.2
Cryptococcus neoformans	10.2	5.5	0.5	0.1
Aspergillus niger	1.0	7.4	0.2	0.2
Botrytis fabae	1.5	6.0	0.4	0.1

Justicia (Acanthaceae) [9, 34] and *Hoplophyllum* (Rutaceae) [35] species and reported here for the first time from a microorganism.

Water soluble compound **4** was isolated from the methanol extract of the mycelium, and the purification was achieved by column chromatography. Single crystals of the compound were grown from methanol solution as colourless plates by slow evaporation at room temperature. In order to prevent powderization, the crystal was sealed into a glass capillary with some mother liquor. A summary of crystallographic data is given in (Table 4). A single crystal X-ray diffraction study of **4** was carried out, from which its absolute stereochemistry was determined unambiguously and is as shown (Fig. 3). The solution of the X-ray diffraction data indicated a novel compound **4**, ayamycin; 1,1-Dichloro-4-ethyl-5-(4-nitro-phenyl)-hexan-2-one which contains the rare combination of a 1,1-dichloro moiety together with a nitro aromate. The compound bears a superficial resemblance to the well known tyrosine-derived antibiotic chloramphenicol produced by *Streptomyces venezuelae*. Compound **4** is distinctly different and must derive from an unusual biosynthesis.

Biological Activities

The four bioactive secondary metabolites (**1**~**4**) of *Nocardia* sp. ALAA 2000 showed different potent antimicrobial activities with MIC ranging between 0.1~10 μg/ml against Gram-positive and Gram-negative bacteria as well as fungi (Table 5). The results were obtained using *Escherichia coli* (ATCC 10536), *Pseudomonas aeruginosa* (ATCC 10145), *Bacillus subtilis* (ATCC 6051), *Bacillus cereus* (ATCC 9634), *Staphylococcus aureus* (ATCC 6538), *Micrococcus luteus* (ATCC 9341), *Mycobacterium smegmatis* (ATCC 607) and *Corynebacterium xerosis* (NRRL B-1397), as test bacterial strains. The fungal indicators were *Rhodotorula acuta*, *Pichia angusta*, *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus niger* and *Botrytis fabae*. These results indicated that *Nocardia* sp. ALAA 2000 was fruitful as an antibiotic producer. The isolated compounds, especially the novel compound ayamycin (**4**), have powerful antimicrobial activity. The MIC of the pure compounds was expressed as μg/ml by the dilution method.

References

1. Anada TP, Abdul WB, Yogesh SS, Upal R, Jay S, Siddhartha PS. Antimicrobial activity of marine bacteria from the waters off the coast of South East India. Microbiol Res 161: 252–262 (2006)
2. Mirabelli CK, Bartus H, Bartus JL, Johnson R, Mong SM, Sung SP, Crooke ST. Application of tissue culture microtitre test for the detection of cytotoxic agents from natural products. J Antibiot 38: 758–766 (1985)
3. Kanzaki H, Yanagisawa S, Nitoda T. Biosynthetic intermediates of the tetradehydro cyclic dipeptide albonoursin produced by *Streptomyces* albulus KO-23. J Antibiot 53: 1257–1264 (2000)
4. El-Sersy NA, Abou-Elela MG. Antagonistic effect of marine

Nocardia brasiliensis against the fish pathogen *Vibrio damsela*: Application of Plackett-Burman experimental design to evaluate factors affecting the production of the antibacterial agent. International Journal of Oceans and Oceanography 1: 141–150 (2006)
5. Mikami Y. Biological Work on Medically Important *Nocardia* Species. Actinomycetologica 21: 46–51 (2007)
6. Hoshino Y, Mukaia A, Yazawa K, Uno J, Ando A, Mikami Y, Fukai T, Ishikawa J, Yamaguchi K. Transvalencin A, a thiazolidine zinc complex antibiotic produced by a clinical isolate of *Nocardia* transvalensis. J Antibiot 57: 803–807 (2004)
7. Guo H, Chang Z, Yang R, Guo D, Zheng J. Anthraquinone from hairy root cultures of *Cassia obtusifolia*. Phytochemistry 49: 1623–1625 (1998)
8. Adinolfi M, Lanzeita R, Marciano CE, Parrilli M. A new class of anthraquinone-anthrone-C-glycosides from *Asphodelus ramosus* Tubers. Tetrahedron 47: 4435–4440 (1991)
9. Abullaev ND, Yagudaev MR, Batirov EKH, Malikov VM. ^{13}C NMR spectra of arylnaphthalene lignans. Chem Nat Compd 23: 63–74 (1987)
10. Waksman SA. The *Actinomycetes*, Vol. II, The Williams and Wilkins Co., Baltimore (1961)
11. Jensen PR, Dwight R, Fenical W. Distribution of *Actinomycetes* in near-shore tropical marine sediments. Appl Environ Microbiol 57: 1102–1108 (1991)
12. Gerhardt P, Murray RGE, Wood WA, Krieg NR. Methods for General and Molecular Bacteriology. American Society for Microbiology, Washington, DC (1994)
13. Williams ST, Davies FL. Use of a scanning electron microscope for the examination of *Actinomycetes*. J Gen Microbiol 48: 171–177 (1967)
14. Shirling EB, Gottlieb D. Methods for characterization of *Streptomyces* species. Int J Syst Bacteriol 16: 313–340 (1966)
15. Williams ST, Goodfellow M, Alderson G, Wellington EMH, Sneath PHA, Sackin MJ. Numerical classification of *Streptomyces* and related genera. J Gen Microbiol 129: 1743–1813 (1983)
16. Williams ST, Goodfellow M, Alderson G. *In* Bergey's Manual of Systematic Bacteriology, Vol. 4. *Ed.*, Williams ST, Sharpe ME and Holt JG. Williams & Wilkins, Baltimore (1989)
17. Pridham TG, Gottlieb D. The utilization of carbon compounds by some *Actinomycetales* as an aid for species determination. J Bacteriol 56: 107–114 (1948)
18. Goodfellow M. *Nocardia* and related genera. *In* Topley and Wilson's Microbiology and Microbial Infections, 9th ed., Vol. 2, Systematic Bacteriology, pp. 463–489. *Ed.*, Balows A and Duerden BI. Arnold, London (1998)
19. Goodfellow M, Isik K, Yates E. *Actinomycete* systematics: an unfinished synthesis. Nova Acta Leopold 312: 47–82 (1999)
20. Kenneth LK. Prepared research paper RP 2911, Central Nations for the revised ISCC-NBS colour name blocks. J Res NBS 16: 427 (1958)
21. Ruan J, Mahmoud A, Zhou Z, Liang H. *Actinopolyspora iraqiensis* sp. nov., a new halophilic actinomycete isolated from soil. Int J Syst Bacteriol 44: 759–763 (1994)
22. Ventosa A, Marquez MC, Kocur M, Tindall BJ. Comparative study of "*Micrococcus* sp." strains CCM 168 and CCM 1405 and members of the genus *Salinicoccus*. Int J Syst Bacteriol 43: 245–248 (1993)
23. Tindall BJ. Lipid composition of Halobacterium lacusprofundi. FEMS Microbiol Lett 66: 199–202 (1990)
24. Minnikin DE, Iwona G, Hutchinson G, Caldicott AB. Thin-layer chromatography of methanolysates of mycolic acid containing bacteria. J Chromatogr 188: 221–233 (1980)
25. Kämpfer P, Kroppenstedt RM. Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. Can J Microbiol 42: 989–1005 (1996)
26. Mandel M, Marmur J. Use of ultraviolet absorbance temperature profile for determining the guanine plus cytosine content of DNA. Methods Enzymol 12B: 195–206 (1968)
27. Chun J, Goodfellow M. A phylogenetic analysis of the genus *Nocardia* with 16S rRNA gene sequences. Int J Syst Bacteriol 45: 240–245 (1995)
28. Pitcher DG, Saunders NA, Owen RJ. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett Appl Microbiol 8: 151–156 (1989)
29. Chun J, Seong C, Bae K, Lee K, Kang S, Goodfellow M, Hah Y. *Nocardia flavorosea* sp. nov. Int J Syst Bacteriol 48: 901–905 (1998)
30. Kim SB, Falconer C, Williams E, Goodfellow M. *Streptomyces thermocarboxy-dovorans* sp. nov. and *Streptomyces thermocarboxydus* sp. nov., two moderately thermophilic carboxydotrophic species from soil. Int J Syst Bacteriol 48: 59–68 (1998).
31. Egorov NS. Antibiotics. A scientific approach. Mirpublishers. Moscow. (translated from the russian by Alexander Poshinkin) (1985)
32. Lechevalier MP Lechevalier H. Chemical composition as a criterion in the classification of aerobic *Actinomycetes*. Int J Syst Bacteriol 20: 435–443 (1970)
33. Lechevalier MP, De Bièvre C, Lechevalier HA. Chemotaxonomy of aerobic *Actinomycetes*: phospholipid composition. Biochem Syst Ecol 5: 249–260 (1977)
34. Okigawa M, Maeda T, Kawano N. The isolation and structure of three new lignans from *Justicia procumbens* Linn. var. *leucantha* Honda. Tetrahedron 26: 4301–4305 (1970)
35. Nukul GS, Abu Zarga MH, Sabri SS, Al-Eisawi DM. Chemical constituents of the flora of Jordan, part III. Mono-*O*-acetyl diphyllin apioside, a new arylnaphthalene lignan from *Haplophyllum buxbaumii*. J Nat Prod 50: 748–750 (1987)