NPC Natural Product Communications

2009
Vol. 4
No. 3
389 - 394

Aureobasidin, New Antifouling Metabolite from Marine-Derived Fungus *Aureobasidium* sp.

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Received: Received: October 4th, 2008; Accepted: January 8th, 2009

Two antifouling compounds, aureobasidin (**1**), a new ester with an unusual 4,6-dihydroxydecanoic acid residue, and (3*R*,5*S*)-3,5-dihydroxydecanoic acid (**2**), were isolated from the marine-derived fungus *Aureobasidium* sp., in addition to (5*R*,3*Z*)-5-hydroxydec-3-enoic acid (**3**) and (*R*)-3-hydroxydecanoic acid (**4**). The structures were unambiguously established by IR, 1D and 2D NMR spectroscopic and mass spectral data. Compounds **1**-**3** were found to be active against *Bacillus subtilis*, *Escherichia coli* and *Staphyllococcus aureus*. Compound **3** showed fungistatic activity against *Candida albicans*.

Keywords: *Aureobasidium* sp., aureobasidin, hydroxydecanoic acid, antifouling activity.

Marine-derived fungi are an eco-physiologically (Mycophyta) classified not a taxonomically identified group of fungi [1]. The increasing number of secondary metabolites isolated from marine-derived fungi, prove that they are a rich source of bioactive metabolites with therapeutic potential [2,3]. Predominantly these compounds show antibacterial, antifungal [4,5], cytotoxic [6-8] and antitumor [5] activities. The antifouling activity is one of the interesting and potentially economic activities, which has been repeatedly reported for marine fungi derived from seaweeds, sponges, soft corals, and ascidians [9-12]. Biofouling on ship hulls, offshore structures or aquaculture equipment is a major global economic and technical problem. Therefore, the development of environmentally safe and efficient antifouling substances is urgently needed [13].

In our search for new natural products from marine-derived fungi, particularly those having eco-environmental activity such as anti-fouling, we investigated the *Aureobasidium* sp. isolated from *Poseidonia oceanica* collected from Moraira (Mediterranean Sea, Spain).

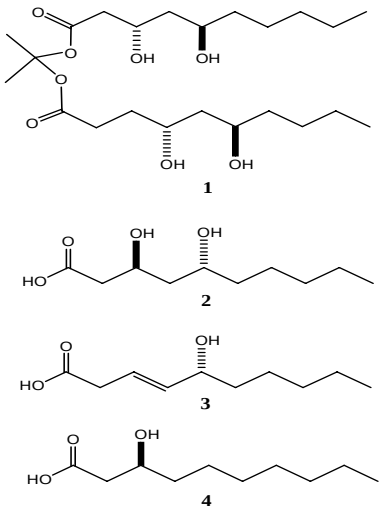

Figure 1: Structures of the isolated compounds.

The bioassay guided isolation afforded a new antifouling didecanoyl-isopropane ester named aureobasidin (**1**), in addition to three hydroxyldecanoic acid derivatives (**2**-**4**). A literature survey showed that only a few compounds have been isolated from fungi belonging to the genus *Aureobasidium*, as for example orcinotriol and exopolysaccharide compounds [14,15].

Compound **1** was isolated as a white amorphous powder. The IR spectrum showed absorptions due to hydroxyl groups (broad band at 3550 cm^{-1}) and ester carbonyls (1730 cm^{-1}). The HREIMS showed a molecular ion peak at m/z 448 $[M]^+$, indicating a molecular formula of $C_{23}H_{44}O_8$, which requires two degrees of unsaturation, which were ascribable to two ester carbonyls with carbon resonances at δ 169.8 and 168.6. The two ester carbonyls and a dioxygenated quaternary carbon at δ_C 98.6 [16], together with the two methyls at δ_H 1.34 and 1.27 (3H, s), were indicative of an isopropane-diester backbone. The HMBC (Figure 2) experiment confirmed the isopropyl moiety because of the cross peaks of the two methyls at δ_H 1.34 and 1.27 (Me-1 and 3) with the dioxygenated quaternary carbon (C-2). Other HMBC cross peaks were observed for CH_2-2' with the carbonyl ester C-1' (169.8) and the hydroxymethine CH-3', and the CH_2-4' with the two hydroxymethines CH-3' and CH-5'. In addition, there were cross peaks of CH_2-6' with CH-5' and CH_2-7' identifying a 3,5-dihydroxy fatty acid residue attached to C-2. In the same way, a 4,6-dihydroxy fatty acid residue attached to C-2 was identified through the cross peaks of CH_2-2" with the second ester carbonyl C-1" (168.6) and CH_2-3". Cross peaks were also detected of the hydroxy-methine CH-4" with CH-3" and CH_2-5", and of the hydroxymethine CH-6" with CH_2-5" and CH_2-7", in addition to cross peaks of CH_2-7" with CH-6" and CH_2-8". The H-H COSY correlations (Figure 2) identified two spin systems. The first confirmed the presence of a 3,5-dihydroxy fatty acid residue, as it showed the segment C-2' to C-7' (CH_2-CHOH-CH_2-CHOH-CH_2-CH_2), and the second spin confirmed the presence of a 4,6-dihydroxy fatty acid residue, as it showed the segment C-2' to C-8" (CH_2-CH_2-CHOH-CH_2-CHOH-CH_2-CH_2). The proton signals at δ_H 1.26 [br., $(CH_2)_n$] and 0.81 (6H, t, *J* = 7.2 Hz, me-10' and 10") showed the presence of an unbranched aliphatic chain [17].

The lengths of the fatty acid chains were determined by alkaline hydrolysis with KOH in methanol (experimental). The reaction residue was purified by Sephadex LH-20 column chromatography followed by LC-MS analysis of the residue, which exposed the presence of two components. One gave a fragmentation pattern identical to that of methyl-3,5-dihydroxydecanoate (compound **2** methyl ester derivative), while the other component (218 $[M]^+$) showed a fragment at m/z 161 (69%) due to cleavage of the bond between C-6 and C-7 [18], and another fragment at m/z 57 (12%) due to C_4H_9, which

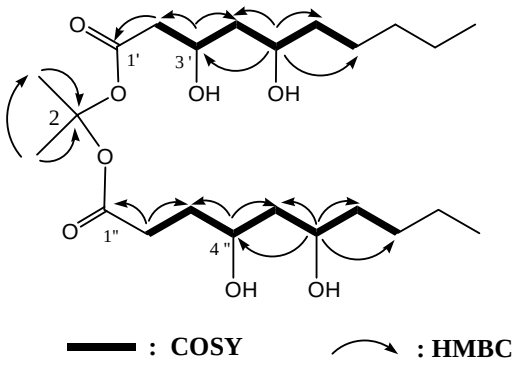

Figure 2: 2D correlations of compound **1**.

confirmed the presence of methyl-4,6-dihydroxy-decanoate, as shown by the 2D experiments. Comparing the chemical shifts of H-3', H-5', H-4" and H-6" of the fatty acid moieties with similar compounds identified the relative configuration as 3'*S*, 5'*S*, 4"*S* and 6"*R* [19-21].

According to previous findings, compound **1** was identified as (3'*S*,5'*S*,4"*S*,6"*R*)2-[(3,5-dihydroxy-decanoyl]-2-[4,6-dihydroxydecanoyl]-isopropane and was named aureobasidin.

Compound **2** was isolated as a yellowish oily residue. EIMS showed a molecular ion peak at m/z 204 $[M]^+$, and fragments at m/z 187 and 168, which revealed the presence of two hydroxyl substituents. The ^{13}C and DEPT spectra revealed a dihydroxy decanoic acid structure as they showed resonances due to acidic carbonyl (171.3), two hydroxymethines (62.7 and 76.7), six methylenes (39, 32.6, 35.8, 31.9, 25.0 and 22.9) and one methyl (14.4). The proton spectrum showed two hydroxymethine signals at δ_H 4.64 (1H, m) and 4.35 (1H,m), a terminal methyl as a signal at δ_H 0.84 (3H, t, *J* = 6.8 Hz), two methylenes at δ_H 2.54 (1H, m) and 2.63 (1H, m), and 1.93 (1H, m) and 1.66 (1H, m), and a methylene cluster at 1.24-1.26 (8H, m). The shift of the two hydroxymethines identified 3,5 dihydroxydecanoic acid, which was confirmed by the EIMS fragment at m/z 134 (89%) resulting from cleavage of the bond between C5 and C6. Comparing the chemical shifts of the two hydroxymethines with similar compounds afforded further evidence for the structure [21,22]. Comparing the chemical shifts and splitting pattern of H-3 and H-5 with those of similar dihydroxy fatty acid residues identified the relative configuration at C-3 and C-5 to be 3*S* and 5*R* [18-20]. According to these results, compound **2** was identified as (3*R*,5*S*)-3,5-dihydroxydecanoic acid.

Table 1: Zones of inhibition of compounds **1**,**2** and **3** (in mm).

Compound		*S. aureus*	*E. coli*	*B. subtilis*	*C. albicans*
1	5 µg	7.5	7.4	8.5	n.a.*
	10 µg	8.1	9	10.3	n.a*
2	5 µg	8.2	7.9	8	n.a.*
	10 µg	10	11	12	n.a.*
3	5 µg	10	8.3	9	n.a.*
	10 µg	12.5	11.4	12.1	7.7

* Not active.

Compound **3** was isolated as an oily residue. EIMS showed a molecular ion peak at m/z 187 $[M+H]^+$. The proton spectrum showed the presence of two *cis* olefinic protons at δ_H 6.85 (1H, dddd, J = 9.8, 3.2, 2.9, 2.2 Hz) and 5.99 (1H, ddd, J = 9.8, 2.2, 1.5 Hz). The ^{13}C NMR spectrum showed resonances due to hydroxydecanoic acid, with an acidic carbonyl (167.4), hydroxymethine (78.3) and two olifenic carbons (145.1 and 121.6), in addition to five methylenes and a terminal methyl.

The mass fragments at m/z 116 ($C_5H_8O_3$) and 99 ($C_5H_7O_2$) due to cleavage between C-5 and C-6, showed a hydroxyl substituent at C-5. The stereochemistry at C-5 was determined as 5*R* by comparing the chemical shift of H-5 with literature values [23]. Accordingly, compound **3** is (5*R*,3*Z*)-5-hydroxydec-3-enoic acid, which, to the best of our knowledge, is the first isolation of this acid from a natural source.

Compound **4** was identified as (*R*)-3-hydroxy-decanoic acid through ^{1}H and ^{13}C NMR spectroscopic and EIMS analysis and comparison with literature data [24]. Confirmation was achieved through GCMS analysis using commercial 3-hydroxydecanoic acid (Sigma, H3648).

Compounds **1** and **2** inhibited larval settlement of the barnacles of *Balanus amphitrite* larvae by 70% and 85%, respectively. Compounds **1, 2** and **3** showed antibacterial activity against *S. aureus*, *E. coli* and *B. subtilis*, while compound **3** showed fungistatic activity against *C. albicans* at concentration of 10 µg.

The natural occurrence of hydroxydecanoic acid derivatives is quite rare. They may exist as intermediates during the synthesis of γ-decalactones [25] or other valuable chemicals, such as antibiotics, vitamins, aromatics, pheromones, and (*S*)-β-amino acids [26] or as intermediates during the biotransformation of glucose [27], linoleic [28] or ricinoleic acids [29]. Hydroxydecanoic acid derivatives reported from natural sources include 3-hydroxydecanoic acid [24,29,30], 4-Hydroxydecanoic [31], 3,4-dihydroxydecanoic [28], 3,10-dihydroxydecanoic acid [17] and 10-hydroxydec-2-enoic acid [32]. To the best of our knowledge, this is the first isolation of 3,5-dihydroxydecanoic (**2**), (*Z*)-5-hydroxydec-3-enoic acid (**3**) and the 4,6-dihydroxydecanoate incorporated in compound **1**.

Experimental

General experimental procedures: ^{1}H (1D, 2D COSY) and ^{13}C (1D, 2D HMBC) NMR spectra were recorded on a JEOL-JNM-EX spectrometer. MS were measured on a Finningan MAT TSQ-7000 spectrometer, and IR spectra on a Schimadzu 470 infrared spectrometer. HPLC was performed on a semipreparative (ARII Cosmosil, 250 x 10 mm) C-18 column (Waters) with a UV detector at 220 nm and a flow rate of 2 mL/min. Pre-coated silica gel 60 F254 plates (E. Merck) were used for TLC. Vacuum liquid chromatography (VLC) was carried out using silica gel 60, 0.04-0.063 mm mesh size (Merck). All solvents were distilled prior to use. NMR grade solvents (Merck) were used for NMR analysis. Compounds were detected by spraying the plates with anisaldehyde reagent.

Isolation, taxonomy and cultivation of the fungal strain: *Poseidonia oceanica* L. was collected from Moraira (Mediterranean Sea, Spain). Under sterile conditions, sterilized plant material was cut into pieces and placed on agar slants containing 15 g/L agar, 1 L of seawater from the sample-collecting site, benzyl penicillin, and streptomycin sulfate (250 mg/L). Fungal colonies growing out of the plant tissue were transferred onto sporulation medium (1.0 g/L glucose, 0.1 g/L yeast extract, 0.5 g/L peptone from meat, enzymatic digest, 15 g/L agar, 1 L of artificial seawater at pH 8). The strain (S2-10) was identified as *Aureobasidium* sp. by Dr R. A. Samson (Centraalbureau Voor Schimmel Cultures, Baarn, Netherlands) and was assigned registry No. 436 (S2-10). Mass cultivation of the fungus was carried out at room temperature for two months in 5 L (12 Fernbach flasks) of solid biomalt agar containing 50 g of Biomalt (Villa Natura Gesundheitsprodukte GmbH, Germany), 15 g of agar (Fluka Chemie AG), and 1 L of 2.5% SERA artificial seawater. Each flask was inoculated with 10 mL of 10-day-old cultures grown in biomalt media.

Extraction and isolation: The mass cultivated fungus, mycelia and medium were homogenized using an Ika Ultra-Turrax at 8000 rpm. The resulting mixture was exhaustively extracted with EtOAc (3 x 6 L) to yield 1.47 g of a viscous brownish black residue, which was fractionated by VLC using *n*-hexane/$CHCl_3$ gradient, followed by $CHCl_3$/EtOAc (100:0; 50:50; 0:100) to yield six fractions (200 mL each). The afforded fractions were subjected to the brine shrimp lethality assay, where fractions 2 and 4 showed lethality of 85 and 60% against the larvae of *Artemia salina*, respectively.

Accordingly, fractions 2 and 4 were selected for further isolation work. Fraction 2 (365 mg) was fractionated by VLC, eluted with a *n*-hexane/ acetone gradient, to yield six fractions (F02-1 to F02-6, 100 mL each). F02-2 (157 mg) was purified by semi-preparative RP-18 HPLC eluted with 60% MeCN/H_2O to 100% MeCN, at 2 mL/min for 40 mins to afford **1** (71 mg) and **2** (17 mg). Meanwhile, fraction 4 (93 mg) was chromatographed on a silica gel column to afford compounds **2** (11 mg), **3** (14 mg) and **4** (20 mg).

Alkaline hydrolysis of 1 [33]***:*** A solution of compound **1** in 3% KOH/MeOH was left to stand for 15 min at room temperature then neutralized with 1 N HCl/MeOH. The solution was passed through Sephadex LH-20 and eluted with MeOH.

Fatty acid analysis: The fatty acid methylesters were prepared as described by Christie [34]. Compound **4** was analyzed by GCMS using a Hewlett-Packard model 6890 (Plus) series gas chromatograph system equipped with a 0.25-μm-diameter HP-INNOWax capillary column 30 m in length. The standard was a commercial product (3-hydroxydecanoic, H3648-Sigma).

Antifouling assay: The culture of *Balanus amphitrite* has been reported previously [35]. Test samples were dissolved in MeOH; aliquots of solutions were pipetted into wells of 24-well polystyrene microtiter plates and air-dried. To each well, 2 mL of filtered seawater diluted to 80% with deionized water and 8 cyprids of *B. amphitrite* (2-3 days fold) were added to each well. Each experiment was carried out with 4 wells. The plates were incubated in the dark for 48 h at 25˚C.The numbers of larvae which swam, metamorphosed, died or did not move were counted daily up to 4 days under a microscope [36]. Each concentration was replicated 3 times.

Agar plate diffusion assay [37]***:*** Sterile filter paper disks were impregnated with 20 µg of the samples using methanol as the carrier solvent. The impregnated disks were then placed on agar plates previously inoculated with *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*. Solvent controls were run against each organism. After the plates had been incubated at 37°C for 24 h, antimicrobial activity was recorded as clear zones (mm) of inhibition surrounding the disk. The test sample was considered active when the inhibition zone was greater than 7 mm.

Brine shrimp lethality assay [38]***:*** Eggs of *Artemia salina* (Dohse, Aquaristik GmbH, Bonn, Germany) were hatched in small tanks filled with artificial seawater. After 48 h, 20 napulii were transferred to 10 mL vials containing 50 and 100 µg of the tested fraction and 20 µL of DMSO. Artificial sea-water was added to obtain a final volume of 5 mL. The percent mortality at each dose level, including the control was determined after 24 h.

Aureobasidin (**1**)

^{1}H NMR (500 MHz, $CDCl_3$): 1.34 (3H,s, Me-1), 1.27(3H,s, Me-3), 2.41 (1H, dd, *J* = 15, 7.8, CH-2'a), 2.33 (1H, dd, *J* = 15, 4.8, CH-2'b), 4.18 (1H, m, CH-3'), 1.44 (1H, m, CH-4'a), 1.08 (1H, m, CH-4'b), 3.73 (1H, m, CH-5'),1.41 (1H, m, CH-6'a), 1.27 (1H, m, CH-6'b), 1.26 (8H, m), 0.81 (6H, t, *J* = 7.2 Hz, Me-10' and 10''), 2.01 (1H, m, CH-2''a), 1.69 (1H, m, CH-2''b), 2.7 (1H, dd, *J* = 18, 5.4 Hz, CH-3''a), 2.62 (1H, m, CH-3''b), 5.17 (1H, m, CH-4''), 1.62 (1H, m, CH-5''a), 1.51 (1H, m, CH-5''b), 4.48 (1H, m, CH-6''), 1.17 (2H, m, CH-9'').
^{13}C NMR (125 MHz, $CDCl_3$): 19.8 (C-1), 98.6 (C-2), 22.6 (C-3), 169.8 (C-1'), 41.7 (C-2'), 66 (C-3'), 36.5 (C-4'), 68.7 (C-5'), 36.3 (C-6'), 31.8 (C-7') , 30.2 (C-8'), 24.6 (C-9'), 14.2 (C-10'), 168.6 (C-1''), 32.9 (C-2''), 35.4 (C-3''), 65.8 (C-4''), 35.5 (C-5''), 76.0 (C-6''), 31.6 (C-7''), 24.5 (C-8''), 22.6 (C-9''), 14.1 (C-10'').
EIMS *m/z*: 449 $[M+H]^+$ (0.2), 411 (0.3), 395 (11.5), 377 (0.9), 359 (25), 191 (100);
HREIMS *m/z*: 448.3034 (calcd for $C_{23}H_{44}O_8$ 448.3036).

(3*R*, 5*S*)3,5-Dihydroxydecanoic acid (**2**)

^{1}H NMR (400 MHz, $CDCl_3$): 2.54 (1H, m, CH_2-2a), 2.63 (1H, m, CH_2-2b), 4.64 (1H, m, CH-3), 1.93 (1H, m, CH_2-4a), 1.66 (1H, m, CH_2-4b), 4.35 (1H, m,

CH-5), 1.24-1.26 (8H, m, CH_2- 6-9), 0.84 (3H, t, J = 6.8 Hz, CH_3-10).
^{13}C NMR (100 MHz, $CDCl_3$): 171.3 (C-1), 39 (C-2), 62.7 (C-3), 36.2 (C-4), 76.7 (C-5), 35.8 (C-6), 31.9 (C-7), 25.0 (C-8), 22.9 (C-9), 14.4 (C-10).
EIMS *m/z* (%): 204 $[M]^+$ (2), 187 (3.8), 168 (16), 161 (14), 126 (41), 103 (36), 80 (78), 60(100).

(5*R*,3*Z*) 5-Hydroxydec-3-enoic acid (**3**)

^{1}H NMR ($CDCl_3$, 400 MHz); 6.85 (1H, dddd, J = 9.8, 3.2, 2.9, 2.2 Hz), 5.99 (1H, ddd, J = 9.7, 2.2, 1.4 Hz), 4.32(1H, m), 2.31 (1H,m), 1.81 (1H,m), 1.73 (2H, m), 1.6-1.3 (6H,m), 0.85 (3H, t, J = 6.8 Hz).
^{13}C NMR ($CDCl_3$, 100 MHz): 167.4, 145.1, 121.6, 78.3, 35.2, 32, 29.6, 24.9, 22.9, 14.4.
EIMS *m/z* (%): 187 $[M+H]^+$ (4), 168 (6), 116 (92), 111 (52), 99 (34), 72 (24), 66 (79), 57 (48), 45 (87).

(*R*)3-Hydroxydecanoic acid (**4**)

EIMS *m/z* (%): 188 $[M]^+$ (2), 171 (5), 90 (100), 100 (4), 46 (92), 43 (39), 57 (18).

Acknowledgments - The authors wish to express their sincere thanks to Prof. G. M. König, Institute of Pharmaceutical Biology, Bonn, Germany, for the fungal strain and performing the spectral analysis. This project is part of the DAAD project on bioactive metabolites from marine derived fungi, Project code 134.104401.212.

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