



## Metabolites from the soft coral -associated bacterium *Micrococcus* sp. strain A-2-28

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### ABSTRACT

The marine actinomyces *Micrococcus* was the most common bacterium among the isolates corals, sponges, and alga isolates. Only a few investigations of natural compounds from *Micrococcus* sp. were reported previously. This study implicated the soft coral-associated *Micrococcus* sp. strain A-2-28 (following relative *Micrococcus flavus* LW<sup>4T</sup>) for large-scale cultivation, chemical analyses, and biological activities. Whereas crude extract of the strain A-2-28 inhibited only *Staphylococcus epidermidis*, metabolites profiles and pure compounds from strain A-2-28 showed that this strain produced phytohormone (IAA), metabolized some new compounds which were almost inactive for biological tests. This work suggested that it is possible to plan a new strategy for improving coral health and resilience through their associated microbial.

**Keywords:** *Micrococcus* sp., coral-associated, metabolites, compound purification, bioassays.

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## INTRODUCTION

*Anthozoan* orders *Alcyonacea* (soft corals) and *Gorgonacea* (sea fans) were being studied as promising sources of bioactive compounds with many species such as *Klyxum simplex*, *Lobophytum* sp., *Sarcophyton crassocaule*, *Sinularia flexibilis*, *Clauvularia* sp which produced antitumor, anti-HIV, antiviral compounds [1]. For a decade, Rosenberg et al. [2] reported that abundant and diverse microorganisms associated with healthy corals or diseased corals, those microorganisms play an essential role in the nutrient and disease resistance of healthy coral. The biological activity of coral-associated bacteria is not only restricted to activity against pathogenic bacteria, fungi, and viruses. However, it has also been found against tumor cell lines, for example, the cyclic thiodepsipeptide thiocoraline, which showed antitumor activity and was obtained from *Micromonospora* sp. strain derived from a soft coral [3].

Conversely, research on the communication relationship between the host and their living microorganisms has now identified small signal molecules such as hormones [4]. Auxin is one of the most well-studied small signals in the relationship between plants and symbionts. One of the most important natural auxins is indole-3-acetic acid (IAA) produced by plants, bacteria, and fungi [5, 6]. Together with several *Bacillus*, *Micrococcus yunnanensis* and *M. luteus* have been reported as potent microorganisms for soil agriculture as a biofertilizer [7, 8]. A marine *M. luteus* exhibited plant growth-promoting with beneficial attributes of phosphate solubilization, IAA production, and siderophore production. When this potent strain was applied to promote the plant, it significantly increased chickpea growth [9]. Recently, a report showed that cultural microbial played a crucial role in coral health [10]. However, the potent metabolites from those associated still need to fulfill many under-investigation functions.

A soft coral-associated *Micrococcus* sp. strain A-2-28 showed weak inhibition of *Staphylococcus epidermidis* in a medium

without tryptophan and will apply for metabolites investigation in this present study.

## MATERIALS AND METHODS

### Materials

*Origin, cultivation, and identification of the strain A-2-28:* The strain A-2-28 was isolated from soft coral *A. digitatum* from the Baltic Sea in SW medium at 10°C and identified as *Micrococcus* sp. because analysis of 16S rRNA gene sequence showed 99.3% sequence similarity with *Micrococcus flavus* LW4T (GenBank accession number DQ491453), 98.6% similarity with *Micrococcus yunnanensis* YIM65004T (GenBank accession number FJ214355), and 98.5% sequence similarity with *Micrococcus luteus* DSM20030T (GenBank accession number AJ310083). The crude extract of strain A-2-28 showed weak inhibition of the bacterium *Staphylococcus epidermidis* DSM20044 [11].

### Methods

#### *Cultivation and extraction of the strain A-2-28*

Cultivation of strain A-2-28 was scaled up in 1 liter GYM medium. The 100 mL of broth culture was extracted after 2, 3, and 4 days and applied to HPLC-DAD-MS to check the presence of three main compounds identified in the screening media. The identified metabolites in 1 liter culture disappeared after 4 days of cultivation. Therefore, 12 liters of broth was harvested after 72 h cultivations at 25°C, extracted with ethyl acetate, and separated with semi-HPLC.

#### *Compound purification*

The crude extract of strain A-2-28 was analyzed by an HPLC (LaChrom Elite, VWR-Hitachi, VWR International system with Diode Array Detector (DAD) L-2450 and an Esquire 4000 Bruker Daltonics Mass Spectrometer. Herein named HPLC-DAD-MS system. Deionized water was purified by the Milli-Q

system (Millipore, Bedford, USA). Milli-Q water, methanol (grade for HPLC-Merk), and acetonitrile (Sigma Aldrich) were used as solvents for HPLC. The mobile phase used was A: 95% Milli-Q-water plus 0.1% formic acid and B: ACN (Acetonitrile) plus 0.1% formic acid, pumped at a rate of 2.0 mL/min with the following gradient: initial, 95% A; 0–4 min 40% A; 6–6.9 min 0% A; 11.5–12.5 min 0% A, and a column reconditioning phase until 8.5 min at a flow of 2.5 mL/min. All samples were dissolved in methanol and filtered through a 0.2 µm PTFE syringe filter (Graphic Controls-Germany) before applying in HPLC.

### Compound analysis

Analysis of crude extracts was conducted at positive ionization mode. As a result, monomer ions ( $[M + H]^+$ ,  $[M + Na]^+$ ) were primarily formed with dimer ions ( $[2M + H]^+$ ,  $[2M + Na]^+$ ). UV-VIS (200–500 nm) and MS data were evaluated using the programs Data Analysis and HyStar Post Processing (Bruker Daltonics, USA). For the identification of known compounds, datasets of metabolites were compared with entries in both database “Dictionary of Natural Products-DNP” and online published available databanks. Data were reconciled with database entries regarding accurate mass, UV maxima, biological sources, and bioassay activity.

### Bioassay tests of pure compounds

Antimicrobial activity test against *Bacillus subtilis* (DSM347), *Escherichia coli*

(DSM498), *Propionibacterium acnes* (DSM1897), *S. epidermidis*, *Staphylococcus aureus* MRSA (DSM18827), and *Klebsiella pneumoniae* (DSM30104) was performed as described by Schneemann et al. [12]. According to Jansen et al., [13] a phytopathogenic *Septoria tritici* dermatophyte fungus *Trichophyton rubrum* were tested. The cytotoxic activity against NIH-3T3, HepG2, and inhibitory against phosphodiesterase (PDE-4β2) was determined according to Schulz et al. [14]. Inhibitory activity against acetylcholinesterase (AChE) and the human pathogenic yeast *C. albicans* was performed according to Ohlendorf et al. [15]. The enzyme inhibition test for glycogen synthase kinase-3β was performed according to Baki et al. [16], and the protein tyrosine phosphatase test was implemented following to Helaly et al. [17].

The final concentration of the pure compounds used in the assays was 100 µM for antibacterial and antifungal activity, 50 µM for cytotoxic activity, and 10 µM for enzymatic inhibition. All experiments in this study were conducted at GEOMAR Centre for Marine Biotechnology, Kiel, Germany.

## RESULTS AND DISCUSSION

### Metabolic profiles

Chemical analysis of metabolite profiles of the strain A-2-28 are shown in Table 1.

Table 1. Chemical profiles of the strain A-2-28

CNo Peak	Rt (min)	$[M + H]^+$	UV (nm) maxima	CP/yield	CN/comment	References
1*	1.8	129 158 175	229, 273, 289	Purification ( $m/z$ 129) 1.5 mg	Maybe a 3-indolylacetic acid ( $m/z$ 175)	[18]
2	3.5	327	225, 292, 390	Purification 60 mg	Unknown Maybe new	This study
3	3.7	716	226, 326, 405, 536	Purification 1 mg	Unknown Maybe new	This study
4	3.9	334/668	224, 395	No purification	Unknown Maybe new	This study

Notes: Peak 1 consists of 3 compounds ( $m/z$  129,  $m/z$  158 and  $m/z$  175), Rt: retention time; CP: compound.

Peak 1 included 3 compounds with  $m/z$  129 [M + H]<sup>+</sup>,  $m/z$  158 and  $m/z$  175. This peak was found in 100 mL culture in GYM and BM. Those three compounds possessed similar UV<sub>max</sub> 229, 273, 278, and 289. In contrast, compound 2 (UV<sub>max</sub> 225, 292, 390;  $m/z$  327) was found in

large amounts in both media. The compound 3 (UV<sub>max</sub> 226, 326, 405, 536;  $m/z$  716) was present in low amounts in BM (100 mL cultures), and compound 4 ( $m/z$  334, UV<sub>max</sub> 224, 395 nm) was likely found in low amounts in 100 mL cultures in GYM (Table 1, Figure 1).

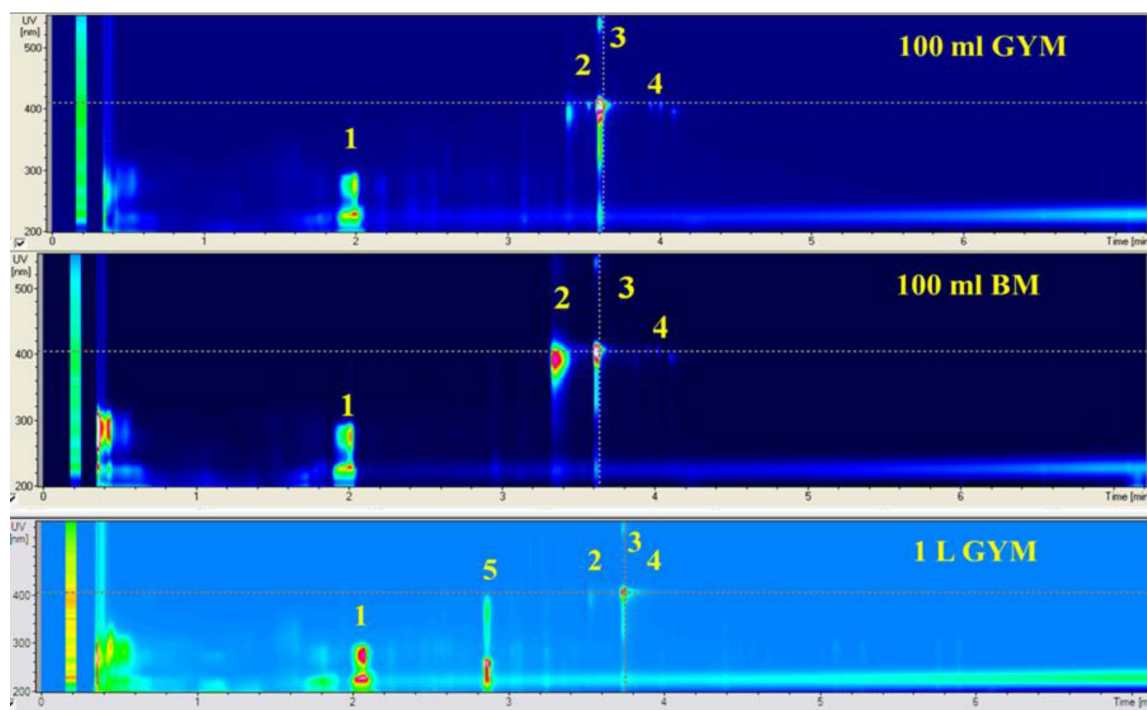


Figure 1. Metabolites of A-2-28 in GYM and BM media

All compounds (compounds in peak 1, compound 2, 3 and 4) were produced in 100 mL cultures and in 1 L cultures in GYM, in addition to compound 5 with unidentified mass. Although the compound 5 showed very good UV signals. It could not be isolated due to the very low amounts in the extract. Compounds 2 ( $m/z$  327) and 3 ( $m/z$  716) were produced in large amounts and had good UV signals, while compounds of peak 1 and the compound 4 ( $m/z$  334) were present in very low amounts, the latter compound showed rather weak UV signals as well (Fig. 1).

### Compound purification

Searching data from DNPs by molecular weight, UV maxima, and biological sources revealed that compounds of peak 1 ( $m/z$  129,

$m/z$  158,  $m/z$  175), compound 2 ( $m/z$  327), compound 3 ( $m/z$  716) extracted from *Micrococcus* sp. A-2-28 in this study were not isolated from this genus so far. Moreover, the compounds from peak 1 with molecular weight  $m/z$  129, compound 2 ( $m/z$  327), and compound 3 ( $m/z$  716) showed good enough UV signals for isolation. Therefore, these compounds were purified.

Semi-Preparative HPLC was used for the separation of the metabolites which A-2-28 produced. Semi-preparation of 1.2 g crude extract obtained from 12 L cultures was carried out with Merck Hitachi Elite LaChrom system with a gradient from 20% B at 0 min increasing to 80% B at 15 min, to 100% B from 27 min to 27.5 min, flow 14 mL/min.

100 mg fraction 1, consisting of 3 compounds  $m/z$  129,  $m/z$  158, and  $m/z$  175, was

eluted at 15 min. 180 mg fraction **2** was eluted at 5 min to 7 min and 60 mg pure compound **2** ( $m/z$  327/654) was obtained.

28 mg fraction 3 [compound **3** ( $m/z$  716), compound **2**, and compound from fraction 1 with  $m/z$  158 (peak 1)] was eluted at 16.5 min, and 7 mg fraction 4 [compound **2** ( $m/z$  327/654) and compound **4** ( $m/z$  334/668)] was eluted at 18 min.

To purify fraction 1 ( $m/z$  129,  $m/z$  158,  $m/z$  175), preparation of 100 mg fraction 1 was carried out with a gradient from 5% B at 0 min increasing to 100% B at 25 min to 27 min, flow 14 mL/min. As a result, 1.5 mg of pure compound  $m/z$  129 (peak 1) was eluted at 19 min, and 20 mg of the mixtures of this compound with compound  $m/z$  158 (peak 1) were eluted at 15 min. The compound with  $m/z$  175 (peak 1) was not harvested due to a low amount after preparative HPLC separation.

The same gradient and column used to purify compound **1** ( $m/z$  129) was applied to

purify the compound **3** ( $m/z$  716). Compound **3** was eluted at 17 min, and 1 mg pure compound was obtained. The mass of this compound was changed during the preparation time, along with the emergence of a new mass previously unavailable in the fraction. Consequently, compound **3** might be unstable and was not further consideration. Compound **4** ( $m/z$  334/668) was found in low amounts in 7 mg of fraction 4, so this compound could not purify.

### Bioactivities of the isolated compounds

The biological activities of three purified compounds are shown in Table 2. Compounds **1** ( $m/z$  129) and compound **2** ( $m/z$  327) were inactive in all tests, while compound **3** ( $m/z$  716) showed weak inhibition of *T. rubrum* with 24% and inhibited the enzyme phosphodiesterase 4 $\beta$ 2 (PDE4) with 43%.

Table 2. Bioactivities assays of pure compounds isolated from A-2-28

Compounds	Antibacterial						Antifungal			Cytotoxic		Enzyme inhibition			
	<i>Bacillus subtilis</i>	<i>Staphylococcus epidermidis</i>	<i>Staphylococcus aureus</i> MRSA	<i>Escherichia coli</i>	<i>Klebsiella pneumoniae</i>	<i>Propionibacterium acnes</i>	<i>Candida albicans</i>	<i>Septoria tritici</i>	<i>Trichophyton rubrum</i>	NIH-3T3 (mouse fibroblasts)	Hep G2 (human liver cancer)	Acetylcholinesterase	Glycogen synthase kinase	Phosphodiesterase 4 $\beta$ 2	Protein-tyrosine-phosphatase
1 ( $m/z$ 129)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2 ( $m/z$ 327)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3 ( $m/z$ 716)	-	-	-	-	-	-	-	-	24	-	-	-	-	43	-

Notes: The numbers indicate the percentage of inhibition; (-): indicate negative results.

## DISCUSSION

Among the compounds of peak 1, there was no hit for compounds with  $m/z$  129 ( $UV_{max}$  229, 275) and  $m/z$  158 ( $UV_{max}$  230, 281). At the same time, the compound with  $m/z$  175 ( $UV_{max}$  229, 273, 278, 289) was assigned as a 3-indolylacetic acid (IAA) which was isolated from marine alga *Undaria pinnatifida* [18].

This substance has  $UV_{max}$  229, 273, 278, 289 in MeOH, which perfectly matched the compound with  $m/z$  175 in peak 1. This compound was isolated from the marine alga and is widely distributed in higher plants, bacteria, algae, yeasts, and fungi. The compound with  $m/z$  129 might be unknown producing from this strain.

There was only one close hit for compound **2** ( $UV_{max}$  225, 292, 360, 375, 390;  $m/z$  327). It

was proviolacein (UV<sub>max</sub> 271, 290, 385, 412, 492; *m/z* 327) produced by *Chromobacterium violaceum* [19]. However, proviolacein has been reported as a red pigment in MeOH (DNPs), while the purified compound **2** was not. In addition, the UV maxima of compound **2** did not match the UV spectra of proviolacein. Consequently, compound **2** was interesting. It could be further investigated for structural and other bioassay as well.

Similarly, compound **3** was unknown. Rubiflavin D (UV<sub>max</sub> 204, 230, 245, 280, 313, 408, 429; *m/z* 716) isolated from *Streptomyces griseus* [20] was found most similar to the compound **3** (UV<sub>max</sub> 226, 326, 405, 536; *m/z* 716). Moreover, rubiflavin D (DNPs) has been reported as an orange substance, whereas the purified compound **3** was a pink pigment. As a result, the compound **3** was regarded as an unknown compound.

There were two closed hits for compound **4** (*m/z* 334). The first closed compound was 6-methyl-dihydrolindbladione (UV<sub>max</sub> 269, 324, 389; *m/z* 334) isolated from myxomycete *Lindbladia tubulina*. It was red pigment and was inactive against P388/S (murine leukemia sensitive P388 cell line) and P388/VCR (vincristine resistant P388 cell line cell) in the absence of VCR, but it was active against P388/VCR cell in the presence of VCR [21]. In addition, the second hit was palmarumycin CP3 (UV<sub>max</sub> 290, 299, 307, 313, 327, 381; *m/z* 334) isolated from the fungi *Coniothyrium palmarum*. Palmarumycin was colorless and showed equal activity against Gram-positive (*B. megaterium*) and Gram-negative bacteria (*E. coli*). It was particularly active against fungi *Ustilago violacea*, *Mycotapha microspore*, *Fusarium oxysporum*, *Eurotium repens*, and in addition against the alga *Chlorella fusca* [22].

In contrast to the crude extract of this strain, all three purified compounds (*m/z* 129, *m/z* 327, and *m/z* 716) were inactive against *S. epidermidis*. It was inferred that the corresponding compound for inhibition of the test organism was not isolated. The compound *m/z* 175 (peak 1) was considered 3-indolylacetic acid or IAA. This phytohormone was not reported to be an antibacterial agent so far. The last two

metabolites, the compounds with *m/z* 158 (peak 1) and the compound 4 (*m/z* 334), were produced in low amounts in GYM and BM and might cause the relatively weak inhibition of the crude extract toward *S. epidermidis*. In other work, this genus showed an excellent candidate for antimicrobial production, such as a sponge-associated *M. yunnanensis* YIM from the Florida Keys was found as a producer of antibiotics when it showed inhibited the growth of methicillin-resistant *S. aureus* [23].

## CONCLUSIONS

Our work provides essential information on the metabolic potential of the coral-associated bacterium. However, these crucial results can be used to refine comprehension of the role of bacteria in coral health, and they could be used to create novel plans for coral resilience through microbiome manipulation.

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