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# New xanthoquinodin-like compounds, JBIR-97, -98 and -99, obtained from marine sponge-derived fungus *Tritirachium* sp. SpB081112MEf2

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Fungi found in marine habitats constitute a rarely used, but promising, resource for discovering novel bioactive substances,[1] such as diketopiperazine alkaloids,[2] trichodermatides[3] and carbonarones.[4] We have recently discovered novel compounds, namely, aspochracin derivative JBIR-15,[5] glycosyl benzenediols JBIR-37 and -38,[6] sorbicillinoid JBIR-59[7] and JBIR-74 and -75[8] obtained from marine sponge-derived fungi. To the best of our knowledge, the isolation of fungi from marine sponge *Pseudoceratina* and their secondary metabolites have been only reported by Boot *et al.*[9] Therefore, we attempted to isolate fungi from a marine sponge, *Pseudoceratina purpurea*, to obtain novel substances from the fungal culture. In this study, we isolated new compounds termed JBIR-97 (**1**), JBIR-98 (**2**) and JBIR-99 (**3**) from the culture of *Tritirachium* sp. SpB081112MEf2 associated with the sponge (Figure 1a). This paper describes the fermentation, isolation and structure elucidation of **1–3**; the biological activity of these compounds is also briefly described.

The fungus, *Tritirachium* sp. SpB081112MEf2 was isolated from the marine sponge, *Pseudoceratina purpurea*, collected from offshore sites in Sakuraguchi, Ishigaki Island, Okinawa Prefecture, Japan, according to a previously reported method that uses Manila clam (*Ruditapes philippinarum*) extract agar plates.[10,11] The sponge was rinsed with sterilized seawater, finely minced using scissors and resuspended in sterilized seawater. A 100-μl aliquot of this suspension was spread on the Manila clam extract agar plates prepared in 50% (v/v) artificial seawater (Marine art SF-1, Tomita Pharmaceutical, Tokyo, Japan) and supplemented with 35 μg ml$^{-1}$ of nalidixic acid and 75 μg ml$^{-1}$ of cycloheximide. Cycloheximide-resistant fungal colonies that grew on the agar plates were transferred to potato dextrose agar slants, on which individual strains were maintained. Strain SpB081112MEf2 was identified by sequence analysis of the ribosomal DNA internal transcribed spacer region. The sequence analysis revealed that the strain was 99% similar to *Tritirachium* sp. IAM 14522 (AB003951). Thus, the strain was identified as *Tritirachium* sp. The strain was cultivated in a 50-ml test tube containing 15 ml of potato dextrose broth (24 g l$^{-1}$ potato dextrose; BD Biosciences, San Jose, CA, USA). The test tube was shaken on a reciprocal shaker (355 r.p.m.) at 27 °C for 3 days. An aliquot (5 ml) of the seed culture was transferred into a 500-ml Erlenmeyer flask containing 15 g of brown rice (Hitomebore, Miyagi, Japan), 30 mg of bacto-yeast extract (BD Biosciences), 15 mg of sodium tartrate, 15 mg of potassium hydrogen phosphate and 45 ml of water, and incubated at 27 °C for 14 days in static culture.

The solid culture (one flask) was extracted with 80% aq. $Me_2CO$ (100 ml). After concentration *in vacuo*, the aqueous concentrate was extracted with EtOAc (50 ml ×3). The collected organic layer was dried over anhydrous $Na_2SO_4$ and evaporated to dryness. The dried residue (62 mg) was subjected to normal-phase medium-pressure liquid chromatography (Purif-Pack SI 60 μm, Moritex, Tokyo, Japan) and eluted with a stepwise solvent system of *n*-hexane–EtOAc (3:1) and $CHCl_3$–MeOH (49:1), successively. The $CHCl_3$–MeOH-eluted fraction (21.9 mg) was rechromatographed on normal-phase TLC ($CHCl_3$:MeOH =20:1) to yield **1** ($R_f$=0.64–0.70, 5.3 mg), together with a band ($R_f$=0.34–0.59) containing **2** and **3**. The band containing **2** and **3** was further purified by preparative reversed-phase HPLC using an L-column2 ODS (5.0 μm, 20 i.d. ×150 mm, Chemicals Evaluation and Research Institute, Tokyo, Japan) developed with 80% MeOH–$H_2O$ (flow rate: 10 ml min$^{-1}$) to yield **2** (0.78 mg) and **3** (1.6 mg).

Compounds **1–3** were obtained as yellow amorphous solids ($[\alpha]_D^{25}$ + 316°, *c* 0.1; UV $\lambda_{max}$ 266 (ε=8800), 340 (ε=21 800) nm for **1**; $[\alpha]_D^{25}$+ 229°, *c* 0.1; UV $\lambda_{max}$ 266 (ε=11 700), 340 (ε=25 400) nm for **2**; $[\alpha]_D^{25}$+ 333°, *c* 0.1; UV $\lambda_{max}$ 267 (8800), 339 (23 300) nm for **3**, in MeOH). The molecular formulas of these compounds were determined to be

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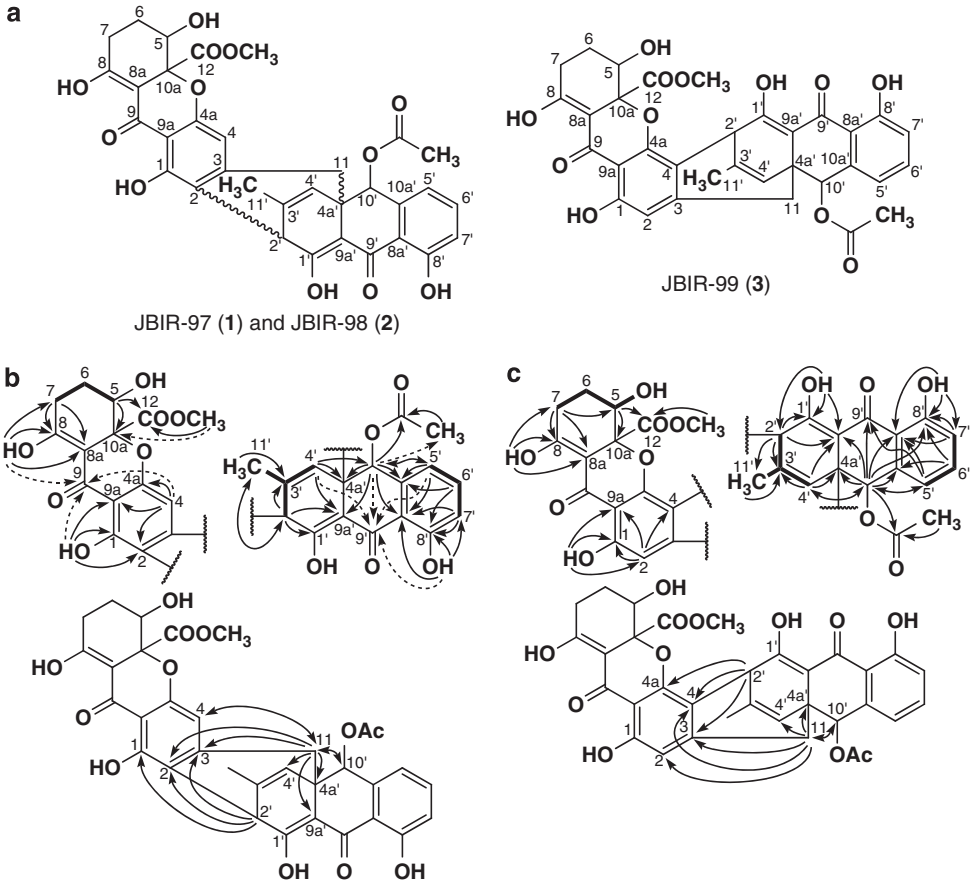


**Figure 1** (**a**) Structures of **1**–**3**. Key correlations of $^{1}$H-$^{1}$H DQF-COSY (bold lines) and HMBC (solid arrows for $J>8$ Hz, dashed arrows for $J>3.5$ Hz, proton to carbon) of xanthone and anthrone units and their bridge site of **1** (**b**) and **3** (**c**).

$C_{33}H_{28}O_{12}$ by HR-electrospray ionization-MS ([M-H]$^-$, 615.1509, 615.1507, and 615.1505, respectively; calculated for $C_{33}H_{27}O_{12}$, 615.1503). Absorptions assignable to ester carbonyl and conjugated carbonyl groups in **1** ($\nu_{max}$ 1740, 1610 cm$^{-1}$), **2** ($\nu_{max}$ 1750, 1620 cm$^{-1}$) and **3** ($\nu_{max}$ 1740, 1620 cm$^{-1}$) were observed in their Infrared spectra. The direct connectivity between each proton and carbon was established from the heteronuclear single quantum coherence spectra. The $^{13}$C and $^{1}$H NMR spectroscopic data for **1**–**3** are listed in Table 1. The planar structures were established by double-quantum filtered (DQF)-COSY and constant time heteronuclear multiple bond correlation[12] spectra as follows.

A sequence from an oximethine proton 5-H ($\delta_H$ 4.24) through methylene protons 6-H ($\delta_H$ 2.15, 2.07) to methylene protons 7-H ($\delta_H$ 2.65) was observed in the DQF-COSY spectrum of **1**, as shown in Figure 1b. In the HMBC spectrum of **1**, the $^{1}$H–$^{13}$C long-range correlations from 5-H to an oxygenated quaternary carbon C-10a ($\delta_C$ 84.3) and an ester carbonyl carbon C-12 ($\delta_C$ 170.0); from a methoxyl proton 12-*O*-Me ($\delta_H$ 3.72) to C-12; from 7-H to two olefinic carbons C-8 ($\delta_C$ 178.1) and C-8a ($\delta_C$ 101.0); and from a hydrogen-bonded hydroxyl proton 8-OH ($\delta_H$ 13.81) to C-7 ($\delta_C$ 27.5), C-8, and C-8a revealed a trioxyheptenoic acid methyl ester moiety (Figure 1b). In the same manner, the correlations from a phenolic hydroxyl proton 1-OH ($\delta_H$ 11.80) to aromatic carbons C-9a ($\delta_C$ 104.7), C-1 ($\delta_C$ 158.8), and C-2 ($\delta_C$ 116.8) and from an aromatic proton 4-H ($\delta_H$ 6.01) to C-9a, C-2 and C-4a ($\delta_C$ 156.8) indicated a phenol moiety. In the long-range selective HMBC experiment, the aromatic proton 4-H and two phenolic hydroxyl protons 1-OH and 8-OH were coupled to a carbonyl carbon C-9 ($\delta_C$ 186.4). These results suggested the existence of a trihydroxyxanthonecarboxylic acid unit.

The interpretation of the DQF-COSY spectrum revealed allylic couplings between a methine proton 2′-H ($\delta_H$ 4.56) and an olefinic proton 4′-H ($\delta_H$ 5.64) and between 4′-H and vinyl methyl protons 11′-H ($\delta_H$ 1.91) and a sequence from an aromatic proton 5′-H ($\delta_H$ 7.07) to 7′-H ($\delta_H$ 6.99) through 6′-H ($\delta_H$ 7.46). $^{1}$H–$^{13}$C long-range couplings from 2′-H to two olefinic carbons C-1′ ($\delta_C$ 187.0) and C-9a′ ($\delta_C$ 105.7); from 4′-H to a quaternary carbon C-4a′ ($\delta_C$ 41.1) and C-9a′; from 5′-H and 7′-H to an aromatic carbon C-8a′ ($\delta_C$ 114.9); from 6′-H to a phenolic carbon C-8′ ($\delta_C$ 161.5) and an aromatic carbon C-10a′ ($\delta_C$ 136.7); and from a phenolic hydroxyl proton 8′-OH ($\delta_H$ 11.54) to three aromatic carbons C-7′ ($\delta_C$ 119.3), C-8′ and C-8a′ indicated a cyclohexadienol and a phenol moiety. An oxymethine proton 10′-H ($\delta_H$ 5.93) was long-range coupled to the acetyl carbonyl carbon ($\delta_C$ 170.5), which in turn was coupled to a singlet methyl proton 10′-*O*-COCH$_3$ ($\delta_H$ 2.00), C-4a′ ($\delta_C$ 41.1), C-10a′, C-8a′, C-9a′ and a carbonyl carbon C-9′ ($\delta_C$ 185.5) in the HMBC spectrum. Thus, these results indicated that a 4,5-dihydroxy-10-oxo-9,10-dihydroanthracen-9-yl acetate unit was established. Finally, the connectivity between the two units was determined by HMBC correlations from 2′-H to C-2 and from methylene protons 11-H ($\delta_H$ 2.73, 2.55) to C-3 and C-4a′ (Figure 1a). The $^{13}$C and $^{1}$H NMR, DQF-COSY and HMBC spectra of **2** were identical to those of **1**. Thus, the structure of **2** was determined to be a diastereomer of **1**, as shown in Figure 1a, left. The similar fungus metabolites,

**Table 1** $^{13}C$ and $^{1}H$ NMR data for 1–3

| | *1* | | *2* | | *3* | |
|---|---|---|---|---|---|---|
| *Position* | $\delta_C$ | $\delta_H$ *(Multiplicity, J in Hz)* | $\delta_C$ | $\delta_H$ *(Multiplicity, J in Hz)* | $\delta_C$ | $\delta_H$ *(Multiplicity, J in Hz)* |
| 1 | 158.8 | | 158.8 | | 160.1 | |
| 1-OH | | 11.80 (br s) | | 11.81 (br s) | | 11.14 (s) |
| 2 | 116.8 | | 116.8 | | 114.2 | 6.12 (s) |
| 3 | 147.9 | | 147.9 | | 147.8 | |
| 4 | 110.9 | 6.01 (br s) | 110.9 | 6.08 (s) | 114.7 | |
| 4a | 156.8 | | 156.8 | | 154.8 | |
| 5 | 71.6 | 4.24 (m) | 71.7 | 4.24 (ddd, 12.5, 5.0, 2.0) | 72.0 | 4.40 (dd, 12.5, 5.0) |
| 5-OH | | 2.84 (br s) | | 2.75 (br s) | | 2.89 (br s) |
| 6 | 23.8 | 2.15 (m); 2.07 (m) | 23.7 | 2.15 (m); 2.06 (m) | 23.9 | 2.21 (m); 2.13 (m) |
| 7 | 27.5 | 2.65 (m) | 27.6 | 2.68 (m); 2.65 (m) | 27.6 | 2.68 (m) |
| 8 | 178.1 | | 178.1 | | 178.5 | |
| 8-OH | | 13.81 (br s) | | 13.82 (br s) | | 13.93 (s) |
| 8a | 101.0 | | 101.0 | | 101.3 | |
| 9 | 186.4 | | 186.2 | | 186.7 | |
| 9a | 104.7 | | 104.5 | | 105.1 | |
| 10a | 84.3 | | 84.3 | | 85.0 | |
| 11 | 35.4 | 2.73 (d, 17.5); 2.55 (d, 17.5) | 35.6 | 2.76 (d, 17.5); 2.58 (d, 17.5) | 35.6 | 2.74 (d, 18.0); 2.67 (d, 18.0) |
| 12 | 170.0 | | 169.8 | | 170.2 | |
| 12-*O*-$CH_3$ | 53.3 | 3.72 (s) | 53.3 | 3.72 (s) | 53.4 | 3.72 (s) |
| 1′ | 187.0 | | 186.5 | | 186.2 | |
| 1′-OH | | 14.11 (br s) | | 14.11 (br s) | | 14.27 (br s) |
| 2′ | 42.4 | 4.56 (d, 1.0) | 42.4 | 4.56 (d, 1.5) | 43.2 | 4.54 (d, 1.5) |
| 3′ | 141.5 | | 141.5 | | 141.7 | |
| 4′ | 125.2 | 5.64 (quintet, 1.0) | 125.2 | 5.65 (quintet, 1.5) | 125.1 | 5.65 (quintet, 1.5) |
| 4a′ | 41.1 | | 41.1 | | 41.1 | |
| 5′ | 122.0 | 7.07 (d, 7.5) | 121.9 | 7.03 (dd, 7.5, 1.0) | 121.9 | 7.07 (dd, 7.5, 1.0) |
| 6′ | 135.8 | 7.46 (t-like, ~8.0) | 135.8 | 7.46 (dd, 8.0, 7.5) | 136.0 | 7.46 (dd, 8.0, 7.5) |
| 7′ | 119.3 | 6.99 (d, 8.5) | 119.2 | 6.99 (dd, 8.0, 1.0) | 119.3 | 6.99 (dd, 8.0, 1.0) |
| 8′ | 161.5 | | 161.5 | | 161.5 | |
| 8′-OH | | 11.54 (br s) | | 11.60 (br s) | | 11.57 (s) |
| 8a′ | 114.9 | | 114.9 | | 114.9 | |
| 9′ | 185.5 | | 185.7 | | 186.0 | |
| 9a′ | 105.7 | | 105.5 | | 106.3 | |
| 10′ | 73.0 | 5.93 (s) | 73.0 | 5.95 (s) | 73.0 | 5.97 (s) |
| 10′-*O*-$COCH_3$ | 170.5 | | 170.5 | | 170.4 | |
| 10′-*O*-$COCH_3$ | 21.2 | 2.00 (s) | 21.2 | 2.00 (s) | 21.2 | 2.01 (s) |
| 10a′ | 136.7 | | 136.3 | | 136.7 | |
| 11′ | 21.2 | 1.91 (d, 1.0) | 21.2 | 1.91 (d, 1.5) | 20.8 | 1.90 (d, 1.5) |

NMR spectra were obtained using an NMR System 500 NB CL (Varian, Palo Alto, CA, USA) in chloroform-*d* with the residual solvent peak as an internal standard ($\delta_C$ 77.0, $\delta_H$ 7.26 p.p.m.).

xanthoquinodin A1 and A2, which are epimer at C-10a each other, showed the difference among their chemical shifts at C-10a ($\Delta\delta_C \sim 5$ p.p.m.).[13–15] However, the difference between **1** and **2** was not observed, whereas the slight differences at 4′-H, 11-H, 5′-H, C-1′ and C-10a′ between **1** and **2** were detected. They suggest that **1** and **2** are diastereomers at C-10′ and/or bridge sites C-2′ and C-4a′.

In the same manner, the analyses of the DQF-COSY and HMBC spectra of **3** revealed that the structure of **3** partially consisted of a xanthone and an oxyanthrone unit. The $^{1}H$–$^{13}C$ NMR long-range couplings from 1-OH ($\delta_H$ 11.14) to an aromatic methine carbon C-2 ($\delta_H$ 6.12, $\delta_C$ 114.2), from 2′-H ($\delta_H$ 4.54) to C-4 ($\delta_C$ 114.7) and from 11-H ($\delta_H$ 2.74, 2.67) to C-3 ($\delta_C$ 147.8) established the connectivity of the partial structures, as shown in Figure 1c. Thus, the planar structure of **3** was characterized as shown in Figure 1a, right.

Cytotoxic activities of **1–3** against human cervical carcinoma HeLa cells and human malignant pleural mesothelioma ACC-MESO-1 cells[16–19] were tested by the WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt) colorimetric assay (Cell Counting Kit, Dojindo, Kumamoto, Japan). Compounds **1–3** exhibited cytotoxic effects against HeLa cells ($IC_{50}$=11, 17 and 17 μM, respectively) and ACC-MESO-1 cells ($IC_{50}$=31, 63 and 59 μM, respectively) for 48 h.

In this study, we isolated three new heterodimeric compounds that consist of tetrahydroxanthone and oxyanthrone from *Tritirachium* sp. SpB081112MEf2 associated with *Pseudoceratina purpurea*. The compounds xanthoquinodins, which are similar to those isolated in this study, consist of a tetrahydroxanthone and an anthraquinone skeleton and have been reported to be isolated from the fermentation broth of *Humicola* sp. as anticoccidial agents.[13–15] The results of this study suggest that fungi isolated from *Pseudoceratina* sp. possess the attractive ability to produce new active compounds.

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