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Marine Bacterial Inhibitors from the Sponge-Derived Fungus *Aspergillus* sp.

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Abstract

Chromatographic separation of a crude extract obtained from the fungus *Aspergillus* sp., isolated from the Mediterranean sponge *Tethya aurantium*, yielded a new tryptophan derived alkaloid, 3-((1-hydroxy-3-(2-methylbut-3-en-2-yl)-2-oxoindolin-3-yl)methyl)-1-methyl-3,4-dihydrobenzo[*e*][1,4]diazepine-2,5-dione (**1**) and a new meroterpenoid, austalide R (**2**), together with three known compounds (**3**-**5**). The structures of the new compounds were unambiguously elucidated on the basis of extensive one and two-dimensional NMR (^{1}H, ^{13}C, COSY, HMBC, and ROESY) and mass spectral analysis. Interestingly, the compounds exhibited antibacterial activity when tested against a panel of marine bacteria, with **1** selectively inhibiting *Vibrio* species and **2** showing a broad spectrum of activity. In contrast, no significant activity was observed against terrestrial bacterial strains and the murine cancer cell line L5178Y.

Keywords *Aspergillus*; Marine natural products; Sponge-derived fungi; *Tethya aurantium*.

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Introduction

Prevention or treatment of bacterial disease outbreaks in aquacultures is a major challenge facing this industry.[1] For instance, destructive infections caused by bacteria of the genus *Vibrio* and those causing necrotizing hepatopancreatitis (NHP) are the main diseases commonly affecting shrimp farms.[2] Hence, there is a great need for new antibiotics to combat such diseases and the resulting stock loss, especially with the development of bacterial resistance to traditionally used antibiotics.[1]

A potential source of novel antibacterial compounds are marine-derived fungi, which have attracted considerable attention in recent years.[3-5] They have been isolated from virtually every possible marine habitat, including inorganic matter, microbial communities, plants, invertebrates and vertebrates. In particular, sponges have yielded numerous fungal strains, which have been reported to produce a variety of pharmacologically active and structurally diverse metabolites.[3,6-11] The need of these organisms to adapt and survive in an environment that is significantly different from that of terrestrial organisms may have shaped their natural product patterns resulting in many cases in the production of unique secondary metabolites.[12-15]

The chemical profiles of both terrestrial and marine *Aspergillus* species have been studied by several research groups, and a vast diversity of secondary metabolites with novel structures and interesting biological activities was already elucidated.[16-25] In continuation of our previous studies on the sponge-derived *Aspergillus* sp. strain, isolated from the Adriatic Sea sponge *Tethya aurantium*,[6,25] two new compounds, 3-((1-hydroxy-3-(2-methylbut-3-en-2-yl)-2-oxoindolin-3-yl)methyl)-1-methyl-3,4-

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dihydrobenzo[*e*][1,4]diazepine-2,5-dione (**1**) and austalide R (**2**), as well as the known compounds 8-*O*-4-dehydrodiferulic acid (**3**), cytochalasin Z17 (**4**) and dihydroisoflavipucine (**5**) (Figure 1), were now isolated and identified. All compounds exhibited antibacterial activity against marine-derived strains, with **1** selectively inhibiting *Vibrio* species and **2** showing a broad spectrum of activity, which may raise the prospect of using such compounds as antifouling agents or to combat epizootics in aquaculture in the future.

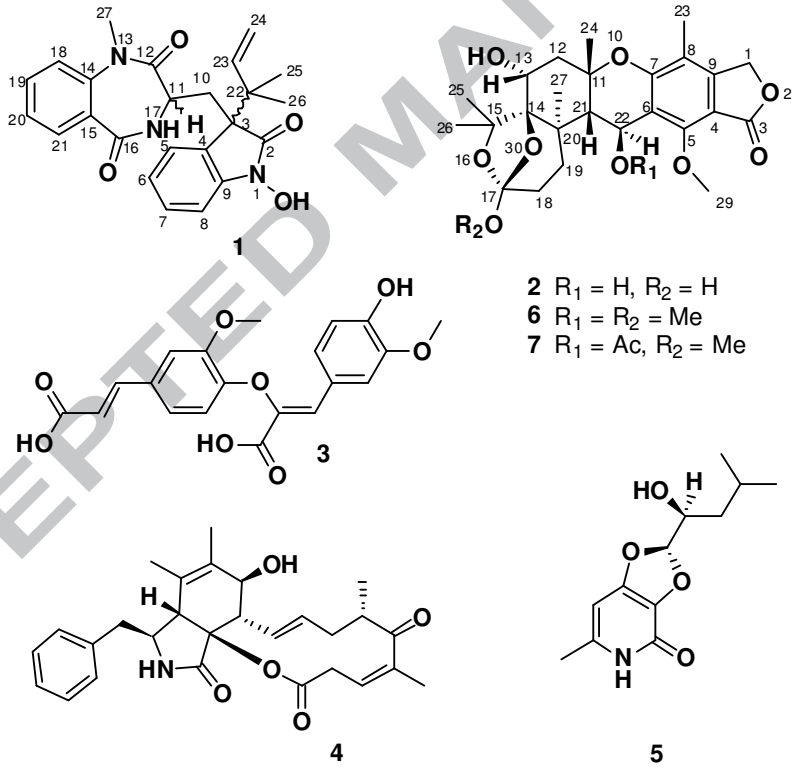

Figure 1. Structures of **1-7**.

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Results and Discussion

The crude ethyl acetate extract of the fungus *Aspergillus* sp. was subjected to repeated column chromatography, followed by semi preparative HPLC separation, to afford two new compounds (**1** and **2**), along with three known compounds (**3**-**5**) (Figure 1). The molecular formula of **1** was established as $C_{24}H_{25}N_3O_4$ on the basis of the $[M+H]^+$ signal at *m/z* 420.1917 in the HRESIMS. The UV absorbance bands observed at λ_{max} (MeOH) 214.1, 250.1 and 290.0 nm suggested the presence of an indoline chromophore.[26] The ^{1}H NMR and COSY spectra of **1** (Table 1) revealed the presence of eight aromatic protons corresponding to two ABCD spin systems resonating at δ_H 7.30, 6.90, 7.21, 6.73 ppm (H-5 to H-8, respectively) and at δ_H 7.24, 7.50, 7.18, 7.27 ppm (H-18 to H-21, respectively), an olefinic ABX spin system at δ_H 4.94/5.02 (H_2-24) and 6.07 (H-23) ppm, an aliphatic ABX spin system at δ_H 2.30/2.70 (H_2-10) and 2.90 (H-11) ppm, two geminal methyl groups at δ_H 0.98 ppm (H_3-25 and H_3-26), a nitrogen bearing methyl group at δ_H 3.15 ppm (H_3-27), and a NH group at δ_H 8.18 ppm (H-17). The ^{13}C NMR (Table 1) and DEPT spectra confirmed the presence of 24 carbon atoms in the structure of **1**, including one aliphatic and nine olefinic methine groups, one aliphatic and one olefinic methylene groups, three methyl groups, as well as two aliphatic and seven olefinic quaternary carbon atoms, the latter including three amide carbonyl carbons resonating at δ_C 172.3, 169.9, and 166.8 ppm (C-2, C-12, and C-16, respectively). Furthermore, analysis of the HMQC spectrum allowed the assignment of proton signals to the corresponding proton bearing carbon atoms.

Table 1. ^{1}H, ^{13}C NMR, COSY and HMBC data of **1** at 300 (^{1}H) and 100 (^{13}C) MHz

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(DMSO-d_6, δ in ppm, J in Hz)

Position	δ_H	δ_C	COSY	HMBC
1				
2		172.3		
3		54.7		
4		124.8		
5	7.30 d (7.8)	126.6	6	3, 7, 9
6	6.90 dt (0.1, 7.6)	120.8	5, 7	4, 7, 8
7	7.21 t (7.7)	128.0	6, 8	5, 8, 9
8	6.73 d (7.6)	106.3	7	4, 6, 9
9		142.7		
10	2.30 dd (7.7, 14.9)	28.9	11	2, 3, 4, 11, 12, 22
	2.70 dd (3.2, 14.9)			
11	2.90 brm	49.6	10, 17	
12		169.9		
13				
14		140.1		
15		128.2		
16		166.8		
17	8.18 d (5.9)		11	10, 11, 15
18	7.24 d (8.3)	121.6	19	15, 20
19	7.50 dt (1.7, 8.5)	132.0	18, 20	14, 18, 21
20	7.18 t (8.4)	125.1	19, 21	15, 18
21	7.27 d (8.3)	128.8	20	14, 16, 19
22		41.8		
23	6.07 dd (10.8, 17.4)	142.9	24	22, 25, 26
24	4.94 dd (0.1, 17.4)	113.4	23	22, 23
	5.02 dd (0.1, 10.9)			
25	0.98 s	21.3		3, 22, 23, 26
26	0.98 s	22.5		3, 22, 23, 25
27	3.15 s	34.9		12, 14

The identified spin systems of **1** were connected based on inspection of the HMBC spectrum (Table 1, Figure 2). Correlations of the tertiary methyl group protons H_3-27 (δ_C 34.9 ppm) to the amide carbonyl C-12 and to C-14 (δ_C 140.1 ppm), of H-18 to C-15 (δ_C 128.2 ppm) and C-20, of H-21 to C-14, C-16 and C-19, of the amide proton H-17 to C-10 (δ_C 28.9 ppm), C-11 (δ_C 49.6 ppm) and C-15, and of H_2-10 to C-11 and

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C-12, established the 1-methyl-1,4-benzodiazepine-2,5-dione moiety of **1**. Further correlations of H_2-10 to C-2 (δ_C 172.3 ppm), C-3 (δ_C 54.7 ppm), C-4 (δ_C 124.8 ppm), and C-22 (δ_C 41.8 ppm), of H-5 to C-3, C-7 and C-9 (δ_C 142.7 ppm), and of H-8 to C-4, C-6 and C-9, corroborated the presence of an indolin-2-one moiety and revealed its connection with the 1,4-diazepine-2,5-dione ring through CH_2-10. The 2-methylbut-3-en-2-yl side chain was verified by correlations of both methyl groups CH_3-25 (δ_C 21.3 ppm) and CH_3-26 (δ_C 22.5 ppm) to each other and to C-3, C-22, and C-23 (δ_C 142.9 ppm), of the olefinic proton H-23 to C-22, C-25 and C-26, and of H_2-24 (δ_C 113.4 ppm) to C-22 and C-23. The hydroxyl group was located at N-1 as the assignment of all other atoms was completed, and based on comparison of observed chemical shift values with those reported for similar 1-hydroxyindolin-2-one substructures.[27] Hence, **1** was determined as a novel metabolite with an unusual structural framework and named 3-((1-hydroxy-3-(2-methylbut-3-en-2-yl)-2-oxoindolin-3-yl)methyl)-1-methyl-3,4-dihydrobenzo[*e*][1,4]diazepine-2,5-dione.

Attempts to determine the relative configuration of **1** by analysis of the ROESY spectrum failed due to free rotation around the methylene bridge CH_2-10.

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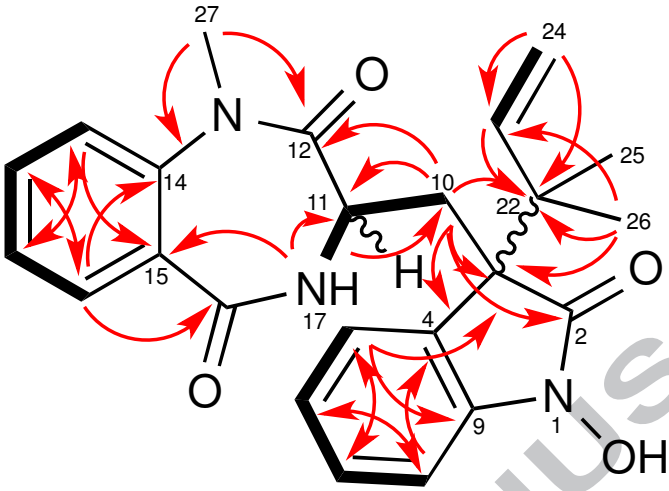

Figure 2. Key COSY (▬) and HMBC (→) correlations observed for **1**.

HRESIMS indicated the molecular formula $C_{25}H_{32}O_9$ for **2** in accordance with the $[M+H]^+$ signal at *m/z* 477.2119. Its UV spectrum showed characteristic maxima of an austalide at λ_{max} (MeOH) 222.6 and 268.5 nm.[28] The ^1H and ^{13}C NMR spectra (Table 2) revealed five methyl groups at δ_H (δ_C) 0.79 (18.2), 1.29 (28.8), 1.32 (29.1), 1.43 (25.6) and 1.99 (10.5) ppm (CH_3-27, -24, -26, -25 and -23, respectively), and one methoxy group at δ_H 4.01 (δ_C 62.5) ppm (OCH_3-29). Additionally, four methylene groups were observed, including the oxygenated benzylic methylene group at C-1 (δ_H 5.25, δ_C 68.1 ppm), and three methine groups, two of which situated on oxygen-bearing carbon atoms based on their chemical shift values at δ_H (δ_C) 3.89 (67.6) and 4.83 (59.5) ppm (CH-13 and CH-22, respectively).

Table 2. ^1H, ^{13}C NMR, COSY, HMBC and ROESY data of **2** (δ in ppm, *J* in Hz)

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Position	δ_H[a]	δ_C[b]	COSY[a]	HMBC[a]	ROESY[c]
1	5.25 s	68.1		3,4,7,8,9	
3		168.6			
4		107.0			
5		156.7			
6		119.4			
7		157.2			
8		113.9			
9		147.3			
11		75.2			
12	2.01 d (4.7)	42.8	13	11, 14	
	2.20 d (14.3)				
13	3.89 brs	67.6	12		26
13-OH	4.62 brs				
14		85.5			
15		83.8			
17		117.1			
17-OH	7.04 brs				
18	1.50 m, 1.74 m	30.6	19	17,20	
19	1.80 m, 1.93 m	31.0	18	17	
20		38.0			
21	2.34 brs	46.3	22	6,11,19,20,22,24,27	24
22	4.83 d (2.8)	59.5	21	6,7,11,20	27
22-OH	5.20 brs			6	
23	1.99 s	10.5		6,7,8,9	
24	1.29 s	28.8		11,12,21	21
25	1.43 s	25.6		14,15,26	27
26	1.32 s	29.1		14,15,25	
27	0.79 s	18.2		14,19,20,21	22,25
29	4.01 s	62.5		5	

[a] 300 MHz (DMSO-d_6)

[b] 75 MHz (DMSO-d_6)

[c] 600 MHz (MeOH-d_4)

Furthermore, three hydroxyl groups were observed at δ_H 4.62, 5.20 and 7.04 ppm (13-, 22-, and 17-OH, respectively). The ^{13}C NMR (Table 2) and DEPT spectra confirmed the presence of 25 carbon atoms in the structure, including 12 quaternary carbon atoms. These data were in accordance with the data reported for austalide O,[6]

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previously isolated from the same fungal strain, suggesting that **2** has the same molecular skeleton as austalide O. Comparison of the NMR spectra of both compounds disclosed the disappearance of the methoxy group located at C-17 of austalide O in **2** and the appearance of a hydroxyl group (δ_H 7.04 ppm) instead. This was also consistent with the 14 amu decrease in the molecular weight of **2** compared to austalide O.[6] The structure of **2** was further confirmed by inspection of COSY, HMQC and HMBC spectra (Table 2). Based on the ROESY spectrum of **2**, comparison of the optical rotation values and the corresponding chirality centers of austalides M and O,[6] **2** was assigned to have (11*S*,13*R*,14*R*,20*R*,21*S*,22*S*) absolute configuration. Accordingly, **2** was characterized as a new natural product named austalide R.

The known compounds (**3**-**5**) were identified as 8-*O*-4-dehydrodiferulic acid,[29,30] cytochalasin Z17[31] and dihydroisoflavipucine,[32] respectively, by comparing their data (^{1}H and ^{13}C NMR, MS and $[\alpha]_D$) with literature values. This is the first report of **4** and **5** from a sponge-derived fungus. Previous studies described the isolation of **4** from *A. terreus* and *A. flavipes* obtained from *Artemisia annua* and the mangrove plant *Acanthus ilicifolius*,[31,33] respectively, and of **5** from *Phoma* sp. isolated from *Salsola oppositifolia*.[32]

Compounds **1**-**5**, in addition to austalides M (**6**) and N (**7**) that were previously isolated from the same fungal strain,[6] were evaluated for their antibacterial activity against a panel of terrestrial and marine-derived bacteria, as well as for their cytotoxic activity against the murine cancer cell line L5178Y. All compounds showed

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antibacterial activity against marine-derived strains (Table 3), at levels sometimes equivalent and/or lower than the positive control Seanine™ for *Vibrio harveyi*, *V. natriegens*, *Roseobacter litoralis*, *Pseudoalteromonas elyakovii*, *Halomonas aquamarina*, *Polaribacter irgensii* and *Shewanella putrefaciens*. Austalides **2** and **6** showed a broad spectrum of activity inhibiting 8 out of 11 tested strains at MIC values equal or inferior to the Seanine™'s ones (for *V. harveyi*, *R. litoralis*, *P. elyakovii*, *H. aquamarina*, *P. irgensii* and *S. putrefaciens*), whereas **7** inhibited only *V. natriegens* and *R. litoralis*. This indicates that a bulky substituent at C-22 may alter the spectrum of antibacterial activity. Furthermore, **1** exclusively inhibited *Vibrio* species, **4** exhibited selective and pronounced activity against *R. litoralis* (with a significantly lower MIC than Seanine™), and **5** displayed strong activity against *S. putrefaciens* and *V. natriegens*. In contrast, only **5** showed considerable activity against terrestrial *Staphylococcus aureus*. Furthermore, all compounds proved inactive against the murine cancer cell line L5178Y in the cytotoxicity assay. These data indicate that compounds **1**-**7** selectively inhibit marine-derived bacterial strains and lack cytotoxicity as judged from the cell line assay. This is of special interest as it may raise the prospect of using such compounds as antifouling agents or to combat epizootics in aquaculture in the future.

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Table 3. Results of antibacterial assay for **1**-**7** expressed as Minimum Inhibitory Concentrations (MIC)

	MIC [μg/mL][a]							
Bacterial strain	Seanine	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Terrestrial								
Escherichia coli	1	>10	>10	>10	>10	>10	>10	>10
Staphylococcus aureus	0.0001	>10	>10	>10	>10	0.001	>10	>10
Marine								
Halomonas aquamarina	0.1	>10	0.1	>10	>10	>10	0.001	>10
Polaribacter irgensii	1	>10	0.1	>10	>10	>10	0.01	>10
Pseudoalteromonas elyakovii	0.1	>10	0.1	>10	>10	>10	0.001	>10
Roseobacter litoralis	1	>10	0.01	1	0.0001	10	0.001	0.01
Shewanella putrefaciens	1	>10	0.1	>10	>10	0.001	0.001	>10
Vibrio harveyi	1	1	0.1	>10	>10	>10	0.001	>10
V. natriegens	1	1	>10	>10	10	0.001	10	0.01
V. proteolyticus	0.01	0.1	0.1	0.1	>10	>10	>10	>10
V. carchariae	0.0001	0.1	0.01	1	>10	>10	0.01	>10

[a] MIC values indicating the same or higher bioactivity than Seanine are highlighted.

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Acknowledgments

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Supplementary data

Supplementary data (experimental section and compound characterization) associated with this article can be found in the online version, at http://XXXXXXXXXXXXXX

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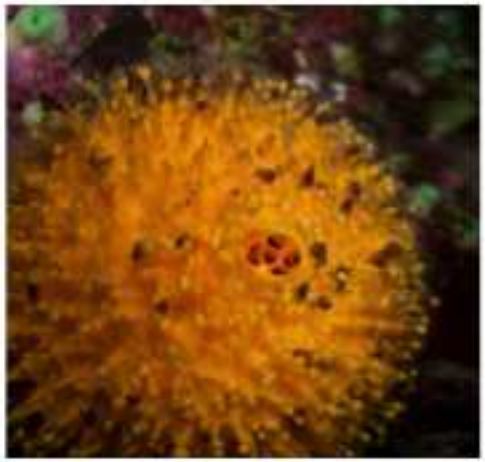

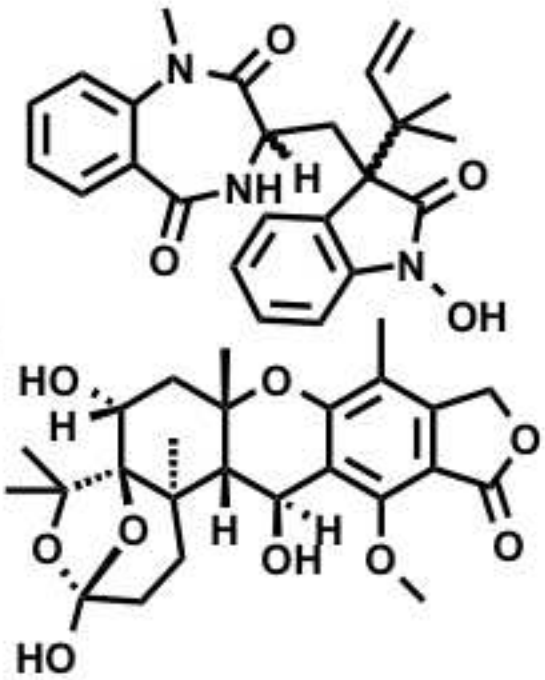

ACCEPTED