Triazoles and Other N-Containing Metabolites from the Marine-Derived Endophytic Fungus *Penicillium chrysogenum* EN-118

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Two new triazoles, chrysotriazoles A and B (**1** and **2**, resp.), along with two known quinazolinones, **3** and **4**, two known 2-(4-hydroxyphenyl)acetamides, **5** and **6**, and two known *N*-(4-hydroxystyryl)formamides, **7** and **8**, were isolated and identified from the culture extract of *Penicillium chrysogenum* EN-118, an endophytic fungus obtained from the marine brown alga *Sargassum pallidium*. The structures of the isolated compounds were elucidated by NMR-spectroscopic analysis, and that of compound **1** was confirmed by X-ray crystallographic analysis of its *p*-bromobenzoate derivative. Compounds **4**, **5**, and **7** showed moderate cytotoxicities against Du145, A-549, and HeLa cell lines.

Introduction. – Marine-derived fungi have yielded a variety of novel chemical compounds which exhibited diverse bioactivities such as antitumor, anti-microbial, and insecticidal properties [1]. Among these, marine alga-derived endophytic fungi have been extensively investigated and were found to be rich sources of alkaloids [1]. Triazole alkaloids, some of which have been synthesized, have been proved to possess antitubulin, cytotoxic, and antibacterial activities [2–6].

In our continuing investigation of bioactive secondary metabolites from marine alga-derived endophytic fungi [7–14], a fungal strain, *Penicillium chrysogenum* EN-118, was obtained from the marine brown alga *Sargassum palladium*. The crude extract of this fungus displayed weak cytotoxic activity in the preliminary assays. Further investigation of the fungal strain has resulted in the isolation and identification of two new triazole alkaloids, chrysotriazoles A and B (**1** and **2**, resp.; *Fig. 1*), together with six

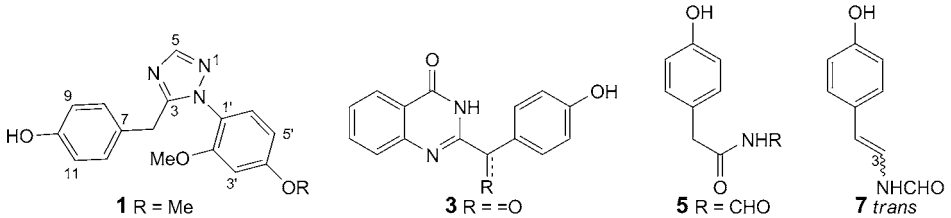

Fig. 1. *The chemical structures for the isolated compounds* **1**–**8**

known alkaloids, **3**–**8**. The structures of these metabolites were established by extensive analyses of spectroscopic data, and the structure of **1** was confirmed by X-ray crystallographic analysis of its 4-bromobenzoate derivative. Compounds **4**, **5**, and **7** exhibited moderate cytotoxicities against Du145, A-549, and HeLa tumor cell lines. Herein, we describe the isolation, structure elucidation, and bioactivities of compounds **1**–**8**.

Results and Discussion. – 1. *Structure Elucidation.* The fungal strain *P. chrysogenum* EN-118 was cultured in MH2 liquid medium at 28° for 30 d (see *Exper. Part*). The culture broth and mycelium were extracted with AcOEt and MeOH, respectively, to yield two crude extracts, which were analyzed by means of TLC and HPLC. The two extracts were combined for further separation, since their TLC and HPLC profiles were similar. As a result, two new triazoles, **1** and **2**, together with six known alkaloids, **3**–**8**, were isolated by using a combination of column chromatography (CC) on SiO_2, *Sephadex LH-20*, reversed-phase (RP) silica gel, preparative TLC, and semi-preparative HPLC.

Compound **1** was obtained as colorless oil. Its molecular formula was determined as $C_{17}H_{17}N_3O_3$ by HR-ESI-MS (*m*/*z* 312.1343 ($[M+H]^+$, $C_{17}H_{18}N_3O_3^+$; calc. 312.1348)), with eleven degrees of unsaturation. The ^{13}C-NMR data (DEPT) indicated the presence of two MeO, one sp^3 CH_2, eight sp^2 CH groups (including two sets of overlapped CH groups), and six sp^2 quaternary C-atoms (four of which were connected to O-atom/N-atom). The ^{1}H- and ^{13}C-NMR spectra of **1** (*Table*) displayed two sets of *doublet*s ($J=8.2$) at δ(H) 6.79 (H–C(8) and H–C(12)) and 6.60 (H–C(9) and H–C(11)), suggesting the presence of a 4-substituted aromatic ring system with an O-atom at C(10) (δ(C) 155.8 (*s*)) (*Table*). The HMBCs from H–C(6) to C(7) and from H–C(8)/H–C(12) to C(6) verified the presence of a 4-hydroxybenzyl moiety in **1** (*Fig. 2*). Further analysis of the coupling constants of the other three aromatic H-atoms at δ(H) 6.74 (*d*, $J=2.3$, H–C(3′)), 6.61 (*dd*, $J=2.3$, 8.6, H–C(5′)), and 7.17 (*d*, $J=8.6$, H–C(6′)) indicated the presence of a 1,2,4-trisubstituted phenyl moiety in **1**. The HMBCs from H–C(3′) to C(1′) and C(5′), from H–C(5′) to C(1′) and C(4′), as well as from H–C(6′) to C(2′) and C(4′) supported the substitution pattern of this phenyl system. Moreover, the observed HMBCs from the two MeO groups at δ(H) 3.67 (MeO–C(2′)) and 3.84 (MeO–C(4′)) to C(2′) and C(4′), respectively, confirmed their connection to C(2′) and C(4′) (*Fig. 2*). Besides the above deduced two aromatic rings, the assignments of the remaining elements ($C_2H_1N_3$) were difficult, because only the correlations from H–C(6) and H–C(5) to C(3) could be observed. Compound **1** was esterified with 4-bromobenzoyl chloride, and a colorless single crystal (needle) of the 4-bromobenzoate derivative of **1** was obtained. The crystal was submitted for X-ray crystallographic analysis (*Fig. 3*). As indicated, a 1,2,4-triazole ring system, which was connected with the phenyl unit at N(2) and the benzyl group at C(3), was eventually established. The structure of compound **1** was, therefore, determined as 2-(2,4-dimethoxylphenyl)-3-(4-hydroxybenzyl)-1,2,4-triazole, named chrysotriazole A.

Compound **2** was obtained as a colorless oil. Its molecular formula was assigned as $C_{16}H_{15}N_3O_3$, 14 units less than that of **1**, on the basis of HR-ESI-MS (*m*/*z* 298.1186 ($[M+H]^+$, $C_{16}H_{16}N_3O_3^+$; calc. 298.1192)). The 1D- and 2D-NMR data of **2** resembled those of **1**. However, only one MeO signal was observed in the NMR spectra of

Table. *^{1}H- and ^{13}C-NMR Data* (500 and 125 MHz, resp.) *of* **1** *and* **2** *in* CD_3OD. δ in ppm, J in Hz. Assignments were corroborated by ^{1}H,^{1}H-COSY, HSQC, and HMBC experiments. For atom numbering, see *Fig. 1*.

Position	**1**		**2**	
	δ(H)	δ(C)	δ(H)	δ(C)
3	–	156.0 (*s*)	–	157.2 (*s*)
5	7.96 (*s*)	150.7 (*d*)	7.86 (*s*)	151.5 (*d*)
6	3.76 (*s*)	30.6 (*t*)	3.83 (*s*)	32.0 (*t*)
7	–	126.2 (*s*)	–	128.2 (*s*)
8/12	6.79 (*d*, J = 8.2)	129.4 (*d*)	6.87 (*d*, J = 8.5)	130.6 (*d*)
9/11	6.60 (*d*, J = 8.2)	114.9 (*d*)	6.67 (*d*, J = 8.5)	115.9 (*d*)
10	–	155.8 (*s*)	–	157.0 (*s*)
1′	–	118.5 (*s*)	–	119.3 (*s*)
2′	–	155.1 (*s*)	–	156.6 (*s*)
3′	6.74 (*d*, J = 2.3)	99.2 (*d*)	6.62 (*d*, J = 2.5)	100.8 (*d*)
4′	–	161.4 (*s*)	–	160.9 (*s*)
5′	6.61 (*dd*, J = 2.3, 8.6)	105.1 (*d*)	6.51 (*dd*, J = 2.5, 8.5)	108.0 (*d*)
6′	7.17 (*d*, J = 8.6)	129.2 (*d*)	7.03 (*d*, J = 8.5)	130.3 (*d*)
*Me*O–C(2′)	3.67 (*s*)	55.6 (*q*)	3.67 (*s*)	56.0 (*s*)
*Me*O–C(4′)/HO–C(4′)	3.84 (*s*)	55.7 (*q*)	8.30 (br. *s*)	–
HO–C(10)	9.25 (br. *s*)	–	9.09 (br. *s*)	–

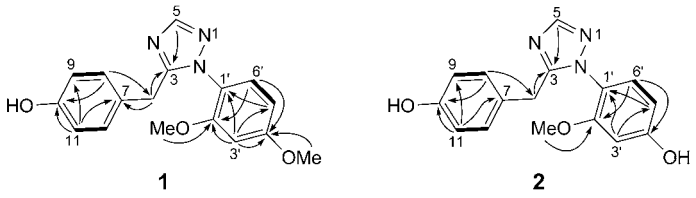

Fig. 2. *Key COSY and HMB correlations of compounds* **1** *and* **2**

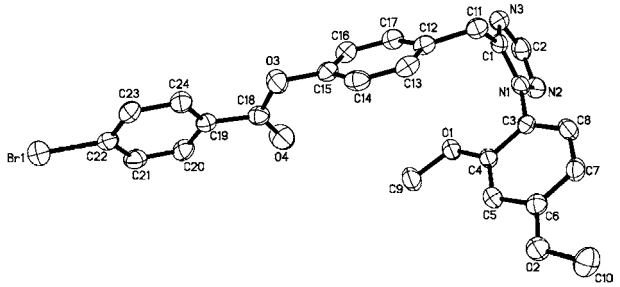

Fig. 3. *Crystal structure of the* p-*bromobenzoyl ester of* **1** (numbering system different from that given in *Fig. 1*)

2 (at δ(H) 3.67 (*s*) and δ(C) 56.0 (*s*)). Based on the obvious HMBC from the MeO group to C(2′) (δ(C) 156.6), this MeO group was determined to be at C(2′), as shown in

Fig. 2. The structure of compound **2** was eventually determined as 3-(4-hydroxybenzyl)-2-(4-hydroxy-2-methoxylphenyl))-1,2,4-triazole, named chrysotriazole B.

In addition to the new compounds **1** and **2**, six known compounds, *i.e.*, 2-(4-hydroxybenzoyl)-4(3*H*)-quinazolinone (**3**) [15], 2-(4-hydroxybenzyl)quinazolin-4(3*H*)-one (**4**) [16], *N*-[2-(4-hydroxyphenyl)acetyl]formamide (**5**) [17], 2-(4-hydroxyphenyl)acetylamide (**6**) [18], *N*-[(2*E*)-(4-hydroxyphenyl)ethenyl]formamide (**7**) [19], and *N*-[(2*Z*)-(4-hydroxyphenyl)ethenyl]formamide (**8**) [19], were also isolated and identified.

2. *Biological Activity.* Compounds **1**–**8** were tested for their cytotoxic activities against eight tumor cell lines. The results indicated that compounds **4**, **5**, and **7** were active against Du145, A-549, and HeLa cell lines, with the IC_{50} values of 8, 20, and 20 μg/ml, respectively.

Compounds **1** and **2** were also evaluated for radical-scavenging activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method and for antibacterial activity against two bacteria, *Escherichia coli* and *Staphylococcus aureus*, but none of them displayed inhibitory activity.

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Experimental Part

General. TLC: precoated silica-gel plates (*GF-254*, *Qingdao Haiyang Chemical Factory*). Column chromatography (CC): commercial silica gel (SiO_2; 100–200 and 200–300 mesh; *Qingdao Haiyang Chemical Factory*), *Lobar LiChroprep RP-18* gel (40–63 μm; *Merck*), and *Sephadex LH-20* gel (*Pharmacia*). Anal. HPLC: *Dionex* HPLC system equipped with a *P680* pump, an *ASI-100* automated sample injector, a *TCC-100* column oven, a *UV-DAD 340U* detector, and a *Dionex Acclaim ODS* column (4.6 × 250 mm, 5 μm). Prep. HPLC: *Dionex UltiMate U3000* system using an *Agilent Prep RP-18* column (21.2 × 250 mm, 10 μm) with UV detection. UV Spectra: *Gold Spectrumlab 54* UV/VIS spectrophotometer (*Shanghai Lengguang Tech. Co.*); λ_{max} (log ε) in nm. NMR Spectra: *Bruker Avance 500* spectrometer (^{1}H: 500 and ^{13}C: 125 MHz); δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. Low- and high-resolution (LR and HR, resp.). ESI-MS: *VG Autospec-3000* mass spectrometer; in *m*/*z*.

Fungal Material. The endophytic fungus *P. chrysogenum* EN-118 was isolated from the marine fresh alga *Sargassum palladium* that was collected from Fujian Province of China in May 2009. The fungal strain grew fast on potato dextrose agar plate, and the green mycelium with plenty spores could be observed in *ca.* 3 d at 28°. Fungal identification was performed by the method as described in [7], and the sequence data obtained from the fungal strain were deposited with Genbank, NIH, with accession No. JQ712997. A BLAST search result showed that the sequence was identical (100%) to the sequence of *Penicillium chrysogenum* (compared to accession No. JQ422624). The stain was deposited with the Institute of Oceanology, Chinese Academy of Sciences.

Fermentation, Extraction, and Isolation. After 5 d growth on potato dextrose agar plate, a piece of the fresh mycelium (*ca.* 4 cm^2) was incubated into a 1000-ml flask containing 300 ml of the MH2 culture medium (6.0 g of mannite, 0.9 g of yeast extract, 3.0 g of monosodium glutamate, 3.0 g of glucose, 0.15 g of KH_2PO_4, 6.0 g of maltose, 0.09 g of $MgSO_4 \cdot 7\,H_2O$, 0.3 g of corn steep liquor, 0.15 g of tryptophan, and 300 ml of seawater; pH 6.5). The fungus was fermented in 100 flasks at 28° for 30 d.

The fermented culture broth and mycelium were exhaustively extracted with AcOEt and MeOH by ultrasonic processor, resp. Since the TLC and HPLC profiles of both extracts were nearly identical, they were combined for further separation. The combined extract (41 g) was subjected to CC (SiO_2; petroleum ether/AcOEt 20 : 1 to 1 : 1; and then $CHCl_3$/MeOH 30 : 1 to 1 : 1) to give 13 fractions, *Frs. 1–13*. *Fr. 5* (1.0 g) was separated by CC (*Sephadex LH-20*; $CHCl_3$/MeOH 1 : 1) to give five subfractions,

Frs. 5.1–5.5. *Fr. 5.4* (0.88 g) was further purified by CC (SiO_2; petroleum/AcOEt 5:1 to 1:1; and then *Sephadex LH-20*; MeOH) to yield compounds **3** (6.9 mg), **5** (99.2 mg), and **6** (91.9 mg). *Fr. 6* (1.5 g) was separated by CC (*Sephadex LH-20*; $CHCl_3$/MeOH, 1:1) to give five subfractions, *Frs. 6.1–6.5*. *Fr. 6.3* (1.0 g) was then subjected to CC (SiO_2; petroleum/AcOEt 10:1 to 1:1; and then $CHCl_3$/MeOH 20:1 to 5:1; *Lobar LiChroprep C18*; MeOH/H_2O 3:7 to 0:1) to afford a semipurified fraction, which was purified by semi-prep. HPLC (60% MeOH; 3.0 ml/min) to yield **7** (t_R 11.19 min, 10.4 mg) and **8** (t_R 13.74 min, 25.7 mg). *Fr. 8* (1.2 g) was fractionated by CC (*Lobar LiChroprep C18*; MeOH/H_2O 3:7 to 1:0) to obtain *Frs. 8.1–8.5*. *Fr. 8.3* was further purified by CC (*Sephadex LH-20*; MeOH; and SiO_2; $CHCl_3$/MeOH 30:1 to 5:1) to afford **4** (12.3 mg). *Fr. 9* (0.65 g) was purified by CC (*Sephadex LH-20*; MeOH, and SiO_2; petroleum ether/actone 2:1 to 1:1, then $CHCl_3$/MeOH 30:1 to 15:1), and semi-prep. HPLC (65% MeOH, 3.0 ml/min) to yield **1** (t_R 15.17 min, 25.5 mg). *Fr. 10* (0.6 g) was subjected to CC (SiO_2; $CHCl_3$/MeOH 50:1 to 5:1; and *Sephadex LH-20*; MeOH) to afford **2** (11.1 mg).

Chrysotriazole A (=*2-(2,4-Dimethoxyphenyl)-3-(4-hydroxybenzyl)-1,2,4-triazole*; **1**). Colorless oil. UV (MeOH): 203 (4.55), 229 (4.03), 279 (3.29). ^{1}H- and ^{13}C-NMR: see the *Table*. HR-ESI-MS: 312.1343 ($[M+H]^+$, $C_{17}H_{18}N_3O_3^+$; calc. 312.1348).

Chrysotriazole B (=*3-(4-Hydroxybenzyl)-2-(4-hydroxy-2-methoxyphenyl)-1,2,4-triazole*; **2**). Colorless oil. UV (MeOH): 203 (4.60), 229 (4.19), 279 (3.56). ^{1}H- and ^{13}C-NMR: see the *Table*. HR-ESI-MS: 298.1186 ($[M+H]^+$, $C_{16}H_{16}N_3O_3^+$; calc. 298.1192).

Esterification of Compound **1**. Compound **1** (9.8 mg) was dissolved in pyridine (1.0 ml) and mixed with excess 4-bromobenzoyl chloride (13.5 mg) and with 4-(dimethylamino)pyridine (DMAP; 0.5 mg) as catalyst. The mixture was stirred at 25° for 24 h, followed by addition of 500 µl of distilled H_2O to terminate the reaction. The mixture was separated by prep. TLC to afford the corresponding 4-bromobenzoate derivative of **1** [20], which was crystallized to yield a suitable crystal for X-ray analysis.

X-Ray Crystallographic Data for 4-Bromobenzoate Derivative of **1**[1]). $C_{24}H_{20}BrN_3O_4$, M_r 494.34, orthorhombic space group *Pbca*, $a = 5.9280(4)$ Å, $b = 17.5761(16)$ Å, $c = 43.390(3)$ Å, $V = 4520.9(6)$ Å^3, $Z = 8$, $d = 1.453$ Mg/m^3. $F(000) = 2016$, $\mu = 1.854$ mm^{-1}. A single crystal of dimensions $0.43 \times 0.13 \times 0.08$ mm was used for X-ray measurements. The intensity data of all unique reflections within the θ range 2.36–25.02° were collected on a *Bruker Smart-1000 CCD* diffractometer equipped with a graphite-monochromatic MoK_α radiation ($\lambda = 0.71073$ Å) at 298(2) K. A total of 21,790 independent reflections were collected, and 3,969 were considered to be observed ($|F|^2 \geq 2\sigma|F|^2$) after equivalent data were averaged, and *Lorentz* and polarization corrections were applied. The data with 291 parameters were corrected for absorption by using the program SADABS [21]. The structure was solved by direct methods with the SHELXTL software package [22]. All non-H-atoms were refined anisotropically. The H-atoms were located by geometrical calculations, and their positions and thermal parameters were fixed during the structure refinement. The structure was refined by full-matrix least-squares techniques [23]. The final refinement gave $R_1 = 0.0587$, w$R_2 = 0.1288[I > 2\sigma(I)]$, $(\Delta/\sigma)_{max} = 0.000$, S = 1.037, $(\Delta\rho)_{max} = 0.450$ and $(\Delta\rho)_{min} = -0.527$ e/Å^3.

Cytotoxicity Assay. The cytotoxicity evaluation was carried out by MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) method [24]. The tested cell lines were MCF-7 (human breast adenocarcinoma), SW1990 (human pancreatic cancer), HepG2 (human hepatocellular liver carcinoma), NCI-H460 (human non-small cell lung cancer), A-549 (human lung cancer), HeLa (human epithelial carcinoma), and Du145 (human prostate carcinoma).

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[1]) CCDC-894326 contains the supplementary crystallographic data of compound **1**. These data can be obtained free of charge *via* http://www.ccdc.cam.ac.uk/data_request/cif (or from the *Cambridge Crystallographic Data Centre*, 12 Union Road, Cambridge CB21EZ, U.K.; fax: +441223336033; e-mail: deposit@ccdc.cam.ac.uk).

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