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RESEARCH ARTICLE

Induced production of 6,9-dibromoflavasperone, a new radical scavenging naphthopyranone in the marine-mudflat-derived fungus *Aspergillus niger*

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Abstract The addition of metal bromides (NaBr and $CaBr_2$) during fermentation of the marine-mudflat-derived fungus *Aspergillus niger* induced production of a new radical scavenging brominated naphthopyranone, 6,9-dibromoflavasperone (**1**); and three known naphtho-γ-pyranone monomers, flavasperone (**2**), TMC-256A1 (**3**), and fonsecin (**4**); and one naphtho-γ-pyranone dimer, aurasperone B (**5**). The structure of 6,9-dibromoflavasperone (**1**) was assigned through the combination of spectroscopic data analyses and comparison with the spectral data of flavasperone (**2**). Compounds **1**–**5** displayed potent radical scavenging activity against 2,2-diphenyl-1-picrylhydrazyl, with IC_{50} values of 21, 25, 0.3, 0.02, and 0.01 μM, respectively, and **3**–**5** were more potent than the positive control, ascorbic acid (IC_{50}, 20.0 μM).

Keywords 6,9-Dibromoflavasperone · Flavasperone · TMC-256A1 · Fonsecin · Aurasperone B · Radical scavenging activity

Introduction

When marine-derived microorganisms are cultured under saline conditions, they rarely produce interesting biological halogenated metabolites [e.g., salinosporamide A (Feling et al. 2003), a highly potent inhibitor of the 20S proteasome, and its halogenated derivatives (Lam et al. 2007); methyl bromodihydroxyphenyl acetates (Leutou et al. 2013)]. Encouraged by the detection of bioactive halogenated marine analogs, and in an effort to gain access to a wider cross-section of halogenated secondary metabolites, we added metal bromides, NaBr and $CaBr_2$, to the culture medium of marine-mudflat-derived fungus *Aspergillus niger* to induce the production of halogenated secondary metabolites (Stadler et al. 1995a, b), and we discovered a new brominated radical-scavenger naphtho-γ-pyranone, 6,9-dibromoflavasperone (**1**).

In this paper, we report the production, isolation, identification, and radical scavenging activity of a new 6,9-dibromoflavasperone (**1**); and three known naphtho-γ-pyranone monomers, flavasperone (**2**) (Sakurai et al. 2002, 2008; Huang et al. 2011; Siriwardane et al. 2015), TMC-256A1 (**3**) (Sakurai et al. 2002; Huang et al. 2011), and fonsecin (**4**) (Sakurai et al. 2002; Huang et al. 2011); and one naphtho-γ-pyranone dimer, aurasperone B (**5**) (Siriwardane et al. 2015).

Materials and methods

General experimental procedures

UV/visible spectra were measured on a Hitachi U-2001 UV/Vis spectrometer. IR spectra were recorded on a Bruker FT-IR model IFS-88 spectrometer. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were obtained on a JEOL JNM-ECP 400 NMR spectrometer, using TMS or solvent peaks [DMSO-d_6: ^{1}H (δ 2.50) and ^{13}C (δ 39.5)] as reference standard. MS spectra were recorded with a JMS-700 spectrometer (JEOL, Japan)

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using a MS-Mp9020D data system and an LCMS-IT-TOF (Shimadzu, Japan) mass spectrometers. HPLC was performed on a YOUNG LIN-ACME HPLC system using a reversed-phase analytical column (Gemini C18, 4.6 × 250 mm, 5 μM) with UV detection.

Fungal isolation and culture

The fungal strain, *Aspergillus niger*, was isolated from the marine mudflat collected at Suncheon Bay, Jeonnam Province, Korea, and identified based on 16S rRNA analyses (SolGent Co., Ltd., Daejeon, Korea), identity of 98 %. A voucher specimen is deposited at Pukyong National University (code MSA773). The fungus was cultured (1 L × 10) in SWS medium consisting of soytone (0.1 %), soluble starch (1.0 %), and seawater (100 %). The cultures were incubated at 29 °C for 10 days on a rotary shaker (120 rpm), and NaBr and $CaBr_2$ (each 50 mM) were subsequently added (Stadler et al. 1995a, b). Incubation was continued again for 10 days by the same method. The culture control was incubated in the absence of metal halides by the same method. TLC analysis showed that the composition of the extract differed from the extract derived from bromide-free SWS medium.

Extraction and isolation

The mycelium and broth were separated by filtration through cheesecloth, and the filtered mycelium was extracted with CH_2Cl_2–MeOH (1:1) to afford the mycelium extract (940 mg). A portion of this extract (920 mg) was subjected to silica gel flash chromatography with CH_2Cl_2–EtOAc (stepwise, 0–100 % EtOAc) to yield seven fractions. Fractions 3–6 exhibited radical-scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) and were purified further by medium-pressure liquid chromatography (ODS column), with a step gradient from 1:10 H_2O/MeOH to MeOH to yield the crude compounds **1** (9 mg) and **2** (15 mg) from fraction 3, **3** (12 mg) from fraction 4, **4** (14 mg) from fraction 5, and **5** (35 mg) from fraction 6. Crude compounds **1**–**5** displayed radical-scavenging activity and were purified by HPLC (Gemini C18, 4.6 × 250 mm, 1 mL/min) with a 30 min gradient program of 50–100 % MeOH in H_2O to obtain compounds **1** (6 mg), **2** (11 mg), **3** (9 mg), **4** (8 mg), and **5** (23 mg), respectively.

6,9-Dibromoflavasperone (**1**): yellowish solid; UV (MeOH) λ_{max} (log ε) 288 (4.2), 317(*sh*) (3.7), 370 (3.0) nm; IR (KBr) ν_{max} 3445, 1734, 1668, 1595, 1421, 1309, 1230, 824, 653 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz) (see Table 1; Figs. S3, S4 in the Supplementary Material); LR-EI-MS *m/z* 438 [M (^{79}Br)]$^+$ (rel. int. 50), 440 [M (^{79}Br^{81}Br)]$^+$ (100), 442 [M (^{81}Br)]$^+$ (50), 399 (19), 401 (37), 403 (21), 384 (10), 386 (14), 388 (8), 358 (12), 350 (14), 348 (10) (see Fig. S1 in the Supplementary Material); HR-EI-MS *m/z* 441.9051 [M (^{79}Br)]$^+$ (calcd for $C_{16}H_{12}O_5^{79}Br_2$, 441.9051) (−0.1 ppm) *m/z* 443.9011 [M (^{79}Br, ^{81}Br)]$^+$ (calcd for $C_{16}H_{12}O_5^{79}Br^{81}Br$, 443.9032) (−4.6 ppm), *m/z* 445.9014 [M (^{81}Br)]$^+$ (calcd for $C_{16}H_{12}O_5^{81}Br_2$, 445.9014) (+0.8 ppm) (see Fig. S2 in the Supplementary Material).

Table 1 NMR spectral data for 6,9-dibromoflavasperone (**1**)

Carbon position	6,9-Dibromoflavasperone (**1**)		
	δ_H (mult., *J* in Hz)	δ_C (mult.)	HMBC (H to C)
2		168.6 (s)	
3	6.60 (s)	110.3 (d)	2, 4, 12, 2-Me
4		182.4 (s)	
5		153.6 (s)	
6		108.3 (s)	
7	7.43 (s)	102.6 (d)	6, 8, 9, 13
8		158.6 (s)	
9		101.0 (s)	
10		155.4 (s)	
11		152.6 (s)	
12		108.7 (s)	
13		137.0 (s)	
14		102.6 (s)	
2-Me	2.63 (s)	20.7 (q)	2, 3
8-OMe	4.09 (s)	56.7 (q)	7, 8
10-OMe	3.98 (s)	61.2 (q)	10

Recorded in $CDCl_3$ at 400 MHz (^{1}H) and 100 MHz (^{13}C)

Flavasperone (**2**) (Sakurai et al. 2002; Sakai et al. 2008; Huang et al. 2011; Siriwardane et al. 2015), TMC-256A1 (**3**) (Sakurai et al. 2002; Huang et al. 2011), fonsecin (**4**) (Sakurai et al. 2002; Huang et al. 2011), and aurasperone B (**5**) (Siriwardane et al. 2015): Spectroscopic data were virtually identical to those reported in the literature.

Radical scavenging assay

Samples to be tested were dissolved in MeOH and the solution (160 μL) was dispensed into wells of a 96-well microtiter tray. In all, 40 μL of the DPPH solution in MeOH (1.5×10^{-4} M) was added to each well. The mixture was shaken and left to stand for 30 min, and the absorbance of the resulting solution was measured at 520 nm with microplate reader (Molecular Devices, VERSAmax). The DPPH radical was treated in the triplicate with the same concentrations of the tested compounds, respectively. The scavenging activity on DPPH radical was expressed as IC_{50}, which is the concentration of the tested compound required to give a 50 % decrease of the absorbance from that of the

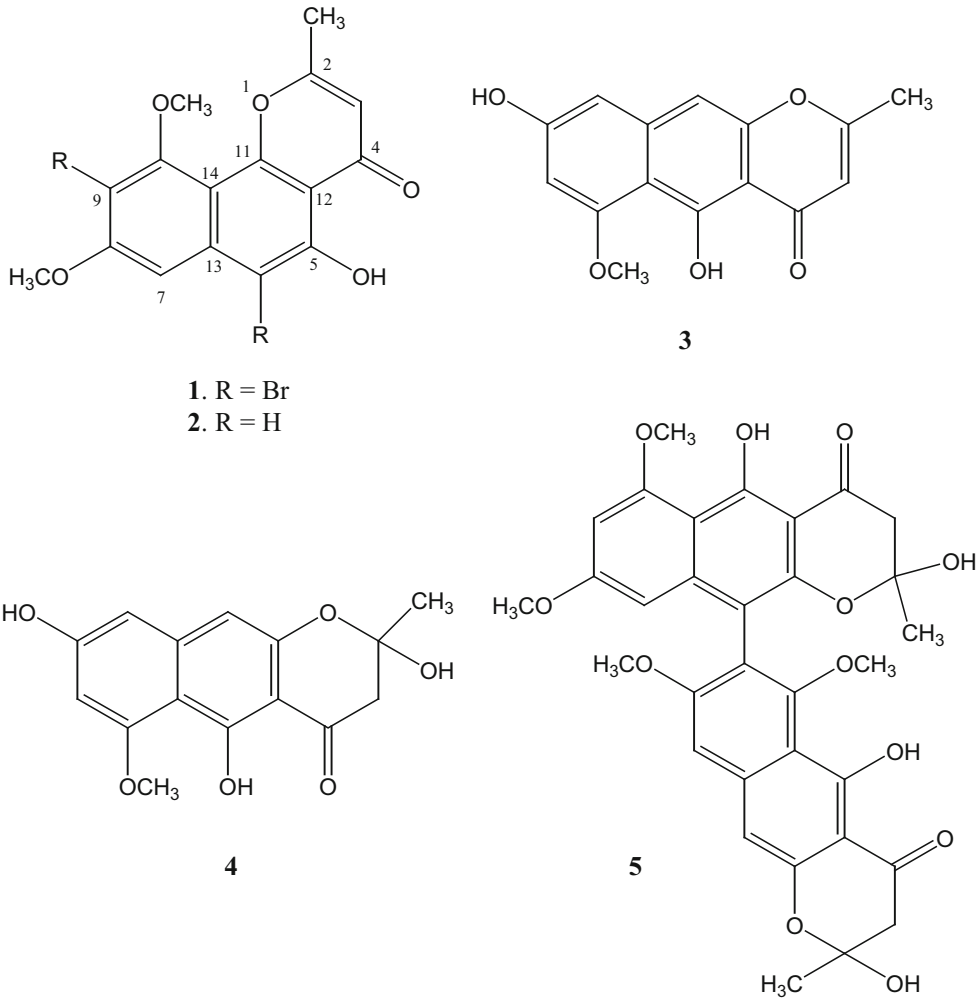
blank solution [consisting of MeOH (160 µL) and DPPH solution (40 µL)] (Leutou et al. 2014).

Results

Compound **1** was isolated as a yellowish needles, identified as $C_{16}H_{12}Br_2O_5$ by HR-EI-MS and ^{13}C-NMR (Figs. S2, S4 in the Supplementary Material). The LR-EI-MS spectrum contained an isotopic cluster at *m/z* 438 [M (^{79}Br)]$^+$ (50), 440 [M (^{79}Br^{81}Br)]$^+$ (100), and 442 [M (^{81}Br)]$^+$ (50) with a ratio of 1:2:1 (Fig. S1 in the Supplementary Material), suggesting the presence of two bromine atoms. The IR spectrum showed bands characteristic of hydroxyl (3445 cm^{-1}), conjugated carbonyl (1734 cm^{-1}), and aromatic (1668, 1595 cm^{-1}) functionalities. The UV absorption at 317(*sh*) nm (log ε = 3.7) indicates the presence of polyconjugated enone chromophore.

1D-NMR (^{1}H- and ^{13}C-NMR, DEPT) data of **1** revealed the presence of one olefinic methyl [δ_H 2.63 (3H, s, 2-Me), δ_C 20.7 (q)], two methoxyls [δ_H 4.09 (3H, s, 8-OMe), δ_C 56.7 (q) and δ_H 3.98 (3H, s, 10-OMe), δ_C 61.2 (q)], five oxygenated sp^2 quaternary carbons [δ_C 168.6 (s, C-2), 153.6 (s, C-5), 158.6 (s, C-8), 155.4 (s, C-10), and 152.6 (s, C-11)], five sp^2 quaternary carbons [δ_C 108.3 (s, C-6), 101.0 (s, C-9), 108.7 (s, C-12), 137.0 (s, C-13), and 102.6 (s, C-14)], two sp^2 methines [δ_H 6.60 (1H, s, H-3), δ_C 110.3 (d, C-3) and δ_H 7.43 (1H, s, H-7), 102.6 (d, C-7)], and one conjugated-carbonyl carbon [δ_C 182.4 (s, C-4)] (Table 1; Fig. 1). The HMBC correlations, from 2-CH_3 to C-2 and C-3, and from H-3 to C-2, C-4, C-12, and 2-CH_3, indicated a 2-methyl γ-pyranone moiety. The HMBC correlations from H-7 to C-6, C-8, C-9, and C-13, from 8-OCH_3 to C-7 and C-8, and from 10-OCH_3 to C-10 indicated a 6,9-dibromo-8,10-dimethoxynaphthalene moiety (Table 1; Fig. 2).

The known naphtho-γ-pyranone derivatives, flavasperone (**2**) (Sakurai et al. 2002; Sakai et al. 2008; Huang et al. 2011; Siriwardane et al. 2015), TMC-256A1 (4*H*-5,8-dihydroxy-6-methoxy-2-methylnaphtho[2,3-*b*]pyran-4-one) (**3**) (Sakurai et al. 2002; Huang et al. 2011), fonsecin (**4**) (Sakurai et al. 2002; Huang et al. 2011), and aurasperone B (**5**) (Siriwardane et al. 2015), were also isolated in this study (Fig. 1).



1. R = Br
2. R = H

3

4

5

Fig. 1 The structure of 6,9-dibromoflavasperone (**1**), flavasperone (**2**), TMC-256A1 (**3**), fonsecin (**4**), and aurasperone B (**5**) isolated from *Aspergillus niger*

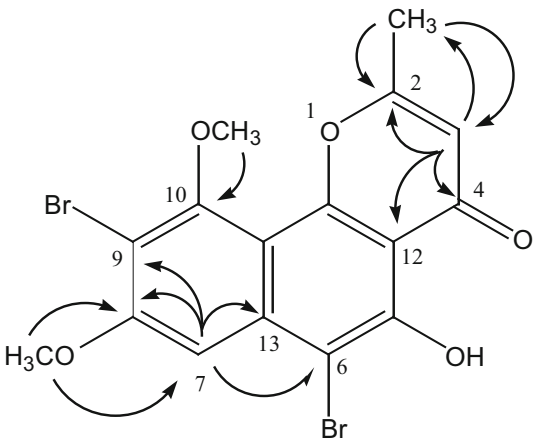


Fig. 2 Key HMBC correlations of 6,9-dibromoflavasperone (**1**)

We examined compounds **1**–**5** for radical scavenging activity against DPPH. Compounds **1**–**5** displayed potent radical scavenging activity, with IC_{50} values of 21, 25, 0.3, 0.02, and 0.01 μM, respectively.

Discussion

The physicochemical data of compound **2** suggested the molecular composition of $C_{16}H_{12}Br_2O_5$ (see Figs. S1, S2, S4 in the Supplementary Material) and the presence of partial structures consisting of 2,3,6-trisubstituted γ-pyranone and 1,2,3,4,6,7,8-heptasubstituted naphthalene moieties bearing two bromines, one hydroxyl, two methoxyl, and one methyl groups (Table 1; Fig. 1). The presence of a γ-pyranone was further supported by the IR (1668 cm^{-1}) (Huang et al. 2011; Sakai et al. 2008; Sakurai et al. 2002), and UV [317(*sh*) nm (log ε = 3.7)] (Scott 1964) data.

The connectivities of the partial structures and the positions of the two bromines, one methyl, and two methoxyl substituents, were determined based on the HMBC spectral data (Table 1; Fig. 2). The position of the hydroxyl group remained to be determined. The point of attachment could be either on the γ-pyranone moiety at C-11 or to the naphthalene moiety at C-5. Based on the comparison of the NMR spectral data with those of compounds **2** and **3**, the hydroxyl group was concluded to be attached to the naphthalene moiety at C-5.

These spectroscopic features reveal that compound **1** has the general structural features of flavasperone (**2**). The NMR data for both compounds show similar patterns, except that two sp^2 methines [δ_H 6.86 (1H, s, H-6), δ_C 105.8 (d, C-6) and δ_H 6.39 (1H, d, J = 2.3 Hz, H-9), δ_C 97.0 (d, C-9)] in **2** were replaced with two sp^2 quaternary carbons [δ_C 108.3 (s, C-6) and δ_C 101.0 (s, C-9)] in **1** (Tables 1, S1 in the Supplementary Material). Based on these results, compound **1** was assigned as 6,9-dibromo-5-hydroxy-8,10-dimethoxynaptho[1,2-*b*]pyran-4-one (Fig. 1).

The known naphtho-γ-pyranone derivatives, **2**–**5** were identified by comparing their $[\alpha]_D$, LR-EI-MS, NMR spectra with literature data (Fig. 1).

Compounds **1**–**5** displayed potent radical scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH), with IC_{50} values of 21, 25, 0.3, 0.02, and 0.01 μM, respectively, and **3**–**5** were more potent than the positive control, ascorbic acid (IC_{50}, 20.0 μM). The different radical scavenging activity for compounds **1**–**5** indicates that the number of aromatic hydroxyl group is important for such activity.

The naphto-γ-pyranones inhibit IL-4 signaling transduction (Sakurai et al. 2002), selectively inhibit Acyl-CoA (Sakai et al. 2008), and display brine shrimp toxicity (Siriwardane et al. 2015) and cytotoxicity against cancer lines (Huang et al. 2011). Therefore, the biological activity of compound **1** should be investigated in future work.

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Compliance with ethical standards

Conflict of interest All the authors have no conflict of interest to declare.

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