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NOTE

Benzomalvin E, an indoleamine 2,3-dioxygenase inhibitor isolated from *Penicillium* sp. FN070315

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Indoleamine 2,3-dioxygenase (IDO) is an extrahepatic heme-containing dioxygenase that catalyzes the addition of oxygen across the C-2/C-3 bond of indole ring of tryptophan (Trp).[1,2] This is the initial and rate-limiting step in the catabolism of the essential amino acid Trp to *N*-formylkynurenine along the kynurenine pathway, the *de novo* biosynthetic route leading to NAD.[3,4] T-cell lymphocytes are extremely sensitive to Trp shortage, which cause them to undergo cell cycle arrest in G1, and leads to apoptosis and immunosuppression.[5] Degradation of Trp by the placenta inhibits T-cell proliferation and, as a result, prevents immunological rejection of tumor or fetus. IDO is expressed ubiquitously but predominately in cells within the immune system, where it is specifically induced in dendritic cells and macrophages at the site of inflammation by cytokines.[1] It is known that IDO is overexpressed in a variety of diseases, including cancer,[6] alzheimer's disease,[7] age-related cataract[8] and HIV encephalitis.[9] Recent studies have shown that IDO inhibition might enhance the efficacy of cancer treatment. Indeed result from *in vitro* and *in vivo* experiment have suggested an improvement of the efficacy of therapeutic vaccination or chemotherapy by concomitant administration of an IDO inhibitor thus highlighting IDO as an attractive target.[6,10–12] Although there have been a number of reports on the development of IDO inhibitors,[13–16] new types of IDO inhibitors having improved pharmacological properties remain to be discovered.

In the course of our screening of the extracts of fungus for IDO inhibitors, we found activity in the culture broth of the soil fungus FN070315. Bioassay-guided fractionation of the extract led us to isolate a new benzodiazepine alkaloid, named benzomalvin E (**1**), together with two known benzomalvins B (**2**) and C (**3**). In this paper, we describe the fermentation, isolation, structure determination and biological activity of benzomalvins. Strain of a fungus, FN070315 was isolated from the soil sample collected from Daejeon in the Korea and was identified on the basis of the ribosomal RNA (rRNA) sequences and morphological evaluation. A GenBank search with the 26S rRNA gene of FN070315 indicated *Penicillium jensenii* (AY443470), *Penicillium canescens* (AY484896) as the closest matches, showing sequence identities of 100% and 99.98%, respectively. Therefore, the fungal strain FN070315 was identified and named as a *Penicillium* sp. FN070315 (deposited as KCTC1818P at the Korean Collection for Type Culture). *Penicillium* sp. FN070315 was grown on the PD agar medium for 7 days and was then inoculated into a 500-ml Erlenmeyer flask containing 75 ml of seed culture medium PD broth (24 g l^{-1} potato dextrose; BD Bioscience, San Jose, CA, USA). Incubation was carried out at 28 °C for 3 days on a rotary shaker operating at 135 r.p.m. This seed medium (150 ml) was transferred to 8 l of the same production medium in a two 14-l jar fermentor. The fermentation was carried out at 28 °C for 6 days with agitation at 165 r.p.m. and an air flow of 10 l min^{-1}. The culture broth (16 l) was filtered and extracted three times with an equal volume of EtOAc and the EtOAc layer was concentrated *in vacuo*. The EtOAc extract (1.3 g) was subjected to reversed-phase C_{18} flash column chromatography using a stepwise gradient of MeOH/H_2O (from 20/80, 40/60, 60/40, 80/20 to 100/0; 700 ml for each step), to yield five fractions (fractions 1–5). The active fraction 3 (68.0 mg) eluted with MeOH/H_2O (60/40) was

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Table 1 ^{1}H and ^{13}C NMR data for benzomalvin E (1) in $CDCl_3$

Position	δ_C	δ_H *mult (J in Hz)*	*HMBC*
2	165.4		
3	131.8		
4	130.9	8.04, d (8.1)	2, 3, 8
5	129.3	7.62, t (8.1)	3
6	131.1	7.72, t (8.1)	4, 8
7	128.5	7.61, d (8.1)	8
8	132.8		
10	161.3		
11	121.0		
12	127.3	8.23, d (8.1)	10, 11, 16
13	127.7	7.47, t (8.1)	11
14	134.8	7.70, t (8.1)	12, 16
15	127.5	7.51, d (8.1)	11
16	146.2		
18	152.0		
19	75.6	4.82, d (9.9)	2, 18, 20, 27
20	73.1	4.15, d (9.9)	19, 22, 26
20-OH[a]		5.99, d (4.5)	19, 20, 21
21	138.7		
22/26	126.4	7.06, br d (7.2)	21, 24
23/25	128.7	7.23, br t (7.2)	21, 25
24	129.1	7.20, t (7.2)	26
27	39.0	3.48, s	2

[a]Spectra were measured in dimethyl sulfoxide (DMSO)-d_6.

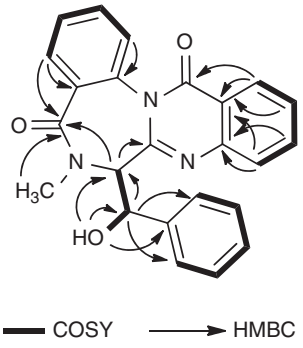


Figure 1 Key 2D NMR correlations of **1**.

purified by semi-preparative reverse phase HPLC using an isocratic solvent system of MeCN/H_2O (50/50), to yield compound **1** (t_R 16.3 min, 3.5 mg), **2** (t_R 23.5 min, 5.5 mg) and **3** (t_R 26.8 min, 5.2 mg). Compounds **2** and **3** were identified as benzomalvins B and C by the comparison of NMR and MS data (Supplementary Figures S8–S11) with those in the literature, respectively.[17]

Compound **1** was assigned the molecular formula $C_{24}H_{19}N_3O_3$ on the basis of HRESIMS data (*m/z* 398.1499 $[M+H]^+$; calculated for $C_{24}H_{20}N_3O_3$, 398.1498) (Supplementary Figure S12) in combination with ^{1}H and ^{13}C NMR data (Table 1). The IR spectrum (Supplementary Figure S13) displayed absorptions due to hydroxyl groups at 3428 cm^{-1} and carbonyl groups at 1646 and 1697 cm^{-1}. The ^{1}H, ^{13}C, DEPT and HMQC data (Supplementary Figures S1–S3, S5) of **1** displayed signals assignable to 24 carbons, which were classified into 1 *N*-methyl carbon at δ_C 39.0, 2 sp^3 methine carbons (δ_C 75.6 and 73.1), 13 sp^2 protonated carbons, 6 sp^2 quaternary carbons and 2 carbonyl carbons (δ_C 165.4 and 161.3). Additionally, one exchangeable proton signal was observed in dimethyl sulfoxide (DMSO)-d_6, at δ 5.99 (d, 4.5), and was assigned as hydroxyl proton. The ^{1}H-^{1}H COSY and HMQC data (Supplementary Figures S4, S5) suggested the presence of one *N*-methyl ($\delta_{H/C}$ 3.48/39.0), two sets of 1,2-disubstituted benzenes (($\delta_{H/C}$ 8.04/130.9, 7.72/131.1, 7.62/129.3 and 7.61/128.5) and ($\delta_{H/C}$ 8.23/127.3, 7.70/134.8, 7.51/127.5, and 7.47/127.7)), one mono-substituted benzene ($\delta_{H/C}$ 7.06/126.4 (×2), 7.23/128.7 (×2), and 7.20/129.1), one oxygenated methine ($\delta_{H/C}$ 4.15/73.1) and one nitrogenous methine ($\delta_{H/C}$ 4.82/75.6). These physicochemical properties and NMR data suggested that **1** was related to benzomalvins.[17] Interpretation of the 2D NMR data, including COSY, HMQC, HMBC and NOESY spectra (Supplementary Figures S1–S7), enabled the structure of **1** to be deduced (Figure 1). The HMBC correlation peaks (H_3-27/C-2, H-4/C-2, C-3 and C-8, H-12/C-10, C-11 and C-16, H-19/C-2, C-18, C-20 and C-27, H-20/C-19, C-22 and C-26) and the remaining ^{1}H-^{1}H COSY cross-peak (H-19/H-20), thus, the structure of **1** was determined to be 7-(hydroxy(phenyl)methyl)-6-methyl-6,7-dihydrobenzo(6,7)(1,4)diazepino(2,1-b)quinazoline-5,13-dione, as a new benzodiazepine alkaloid, designated as benzomalvin E (**1**) as shown in Figure 2. Furthermore, the location of a hydroxyl group was confirmed by ^{1}H-^{1}H COSY and HMBC correlations in DMSO-d_6 (COSY, H-20 (δ_H 3.87)/20-OH (δ_H 5.99); HMBC, 20-OH (δ_H 5.99)/C-19 (δ_C 75.8), C-20 (δ_C 71.6) and C-21 (δ_C 141.4)). The absolute configuration of C-20 was determined by applying the modified Mosher's method. Treatment of **1** with (*S*)- and (*R*)-methoxy(trifluoromethyl)phenylacetic acid(MTPA) chloride, yielding the (*R*)- and (*S*)-MTPA esters (**1a** and **1b**), respectively. The difference in chemical shift values ($\Delta\delta=\delta_S-\delta_R$) for the two diastereomeric esters **1a** and **1b** were calculated in order to assign the absolute configurations at C-20 (Figure 3 Supplementary Figures

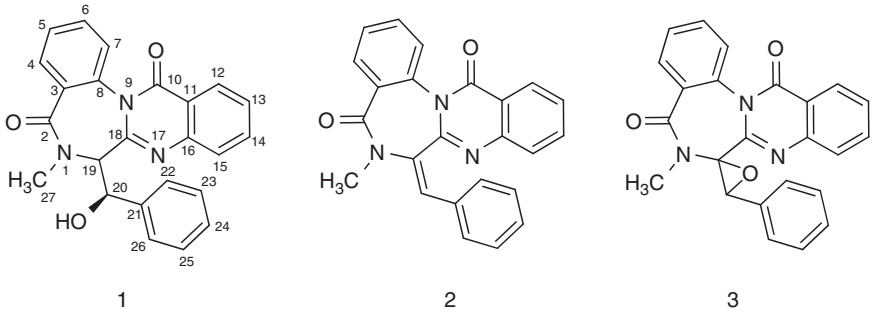
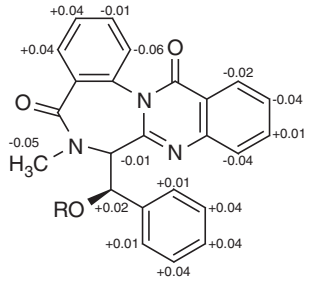


Figure 2 Chemical structures of **1**, **2** and **3**.



1a: R = (*R*)-MTPA ester

1b: R = (*S*)-MTPA ester

Figure 3 $\Delta\delta$ values ($\Delta\delta=\delta_S-\delta_R$ in ppm) obtained for MTPA esters (**1a** and **1b**) of **1**.

S14, S15). These data allowed assignment of the absolute configuration of C-20 as *R* (Figure 3). However, at present, the absolute configuration at C-19 is not determined due to the lack of relevant data. Though **1** was listed in the CAS Registry file (registry No. 1246066-81-8), there are no published data or reports on its stereochemistry.

Compounds **1**, **2** and **3** were evaluated for inhibitory activity against IDO. Benzomalvin E (**1**) showed that the activity of IDO in a dose-dependent manner, and its IC_{50} values were determined as $21.4 \pm 1.2\,\mu M$. A known IDO inhibitor, menadione ($IC_{50} = 3.7 \pm 0.5\,\mu M$), was employed as a positive control in the assay. On the other hand, compounds **2** and **3** showed weakly inhibitory activity against IDO with IC_{50} values of 126 and 130 μM, respectively.

Benzomalvin derivatives have been reported to function as inhibitor against neuropeptide substance-P at the guinea pig, rat and human neurokinin-1 receptor.[17] Several IDO inhibitors have been reported to date.[18] 1-Methyltrytophan is the most frequently used inhibitor with a weak K_i of 34 μM and is in clinical development at the National Cancer Institute.[19,20] To our knowledge, the IDO inhibitory activity of the benzomalvin derivatives are now being reported for the first time in this study. Further investigation and optimization of benzomalvins might enable the preparation of new IDO inhibitors potentially useful in the treatment of cancer.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)