Isolation and Structure Determination of Two New Carbazoles from *Streptomyces ehimensis* JB201

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Carbazole and its derivatives have been of special pharmaceutical interest because of their versatile biological aspects, such as antiinflammation,[1] antibacterial,[2] antifungal,[3] antiviral,[4] anticancer,[5] and protein-kinase inhibition activities[6] for the last decades. A recent study reported that new derivatives of carbazole could be obtained through biotransformation by biphenyl (BP)-utilizing bacteria.[7] Among them, carbazomycins have been known as the first antibiotics possessing a carbazole framework isolated from *Streptomyces* or *Streptoverticillium* species.[8] In our attempt of finding antibiotics from the secondary metabolites produced by microorganisms, two new carbazole derivatives were obtained from the cultured cells of *Streptomyces ehimensis* JB201 (KCTC18166P), along with four known carbazomycins; carbazomycin A (**3**), B (**5**), G (**6**), and *N*-methyl carbazomycin A (**4**).[8-10] However, all compounds except for compound **1** showed no antifungal activity against *Candida albicans* and *Cryptococcus neoformans* by paper disc diffusion method. Consecutive research revealed that the strong antifungal activity for the extract of the cultured cells came from other skeletal compounds mixed with the isolated carbazomycins. In this paper we describe the isolation and complete structure determination of two new compounds by the NMR spectroscopic analysis and chemical reaction.

Compound **1** was isolated as a white amorphous solid which determined for $C_{14}H_{15}NO_2$ by the measured pseudomolecular ion peak at m/z 230.1181 $[M + H]^+$ in the high resolution FAB-MS, consistent with eight unsaturation degrees. The IR spectrum displayed absorption bands at 3368, 1739, and 1260 cm^{-1}. The ^1H NMR spectrum showed the existence of two methyl doublets, four successive aromatic protons, one hydroxy proton (δ 5.21, $J = 3.4$ Hz), and one amide proton (δ 11.95). Furthermore, from a combination of the ^{13}C and the edited HSQC spectra, **1** was revealed to have one oxymethine, two aliphatic methines, one keto group, and four quaternary carbons. The values of eight sp^2 carbon chemical shifts (δ 108.6, 117.8, 120.1, 121.6, 122.5, 124.6, 136.8, and 154.6) and the amide proton were reminiscent of an indole moiety.[11] This suggestion was supported the COSY and HMBC correlations shown in Fig. 1. Collectively,
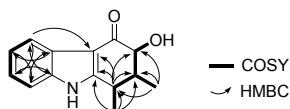
all information accounted for seven of eight unsaturation degrees, supposing a new alkylated ring. This suggested a structure which is somewhat different from biarylic compounds like carbazomycins.

The COSY spectrum exhibited that the methyl protons H_3-10 at δ 1.45 coupled to H-1 at δ 3.05, which subsequently coupled with the proton H-2 at δ 2.19. In succession, the latter methine proton further showed the couplings with both the methyl signal at δ 1.02 and the oxymethine proton H-3 at δ 4.20, constructing an aliphatic chain, as shown in Fig. 1. One terminus of this chain was connected to C-9a (δ 154.6) in the indole unit, which was determined by the HMBC correlations of H-1 and H_3-10 with the carbon at δ 154.6. On the other hands, it could be deduced that the unassigned keto group was bonded to C-4a position of the indole unit and designated as an α,β-unsaturated keto. This suggestion was supported by an UV absorption band at 238 nm (log $\varepsilon \sim 3.6$) and more downfield-shifted carbon chemical shift of C-9a in the indole moiety. The overall structure of **1** could be completed by the linkage of the oxymethine carbon and the keto group on the basis of the molecular weight and the remaining one unsaturation degree. Accordingly, **1** was assigned as 3-hydroxy-1,2-dimethyl-1,2,3,9-tetrahydrocarbazol-4-one.
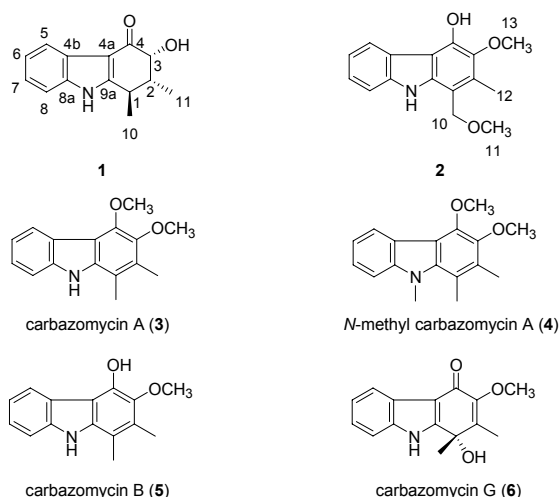
Compared with the reported carbazole compounds, the feature of compound **1** possessed three stereocenters in the cyclohexenone ring. For the purpose of determining the configuration of the ring obviously, first of all, the absolute configuration of the 3-hydroxy group was performed by the modified Mosher method. Esterification of **1** with *R*-(-)- and *S*-(+)-MTPA chlo-

1a (*S*)-MTPA
1b (*R*)-MTPA
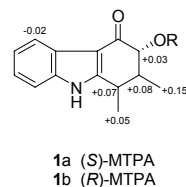
Figure 2. ^1H NMR chemical shift differences ($\Delta\delta^{S-R}$) in ppm for (*S/R*)-MTPA ester of **1** in CDCl₃.

Figure 1. COSY and HMBC correlations of compound **1**.
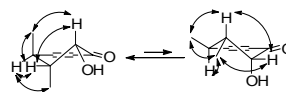
Figure 3. NOE correlations in the aliphatic ring portion of compound **1**.

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Scheme 1. The molecular structures of carbazole alkaloids from *S. ehimens* JB201

rides yielded an *S*-MTPA ester and *R*-MTPA, respectively,[12] and the difference in proton chemical shifts of their ester derivatives was shown in Fig. 2, indicating that the stereochemistry on C-3 is *R*-form. On the basis of the stereochemistry of C-3, the relative orientations of two methyl groups on C-1 and C-2 could be determined by the ROESY spectrum and coupling constants. The NOE correlations of H-3/H-2, H-3/H$_3$-10, and H-3/H$_3$-11 suggested the conformational motion as given in Fig. 3. Especially the NOE observation between H-3 and H$_3$-10 allowed the stereochemistry of C-1 to be *R*-form. In a similar way, along with the NOE signal between H$_3$-10 and H$_3$-11, the NOE correlations of H-2 with H-1, H-3, and H$_3$-10 were observed due to ring interconversion, disabling to determine the explicit configuration of C-2. However, a careful analysis of NOE signals showed that the intensity of NOE signal between H-2 and H-3 was relatively stronger than that between H-3 and the methyl protons H$_3$-11. This could be explained that H-2 is always placed in a *gauche* state with H-3, irrespective of ring motion, indicating that C-2 was assigned as *R*-form. The configuration of C-2 was also supported by the coupling constant ($^3J_{H1,H2}$ = 5.1 Hz) between H-1 and H-2 which contributed by a partial *trans* state, compared to that ($^3J_{H2,H3}$ = 3.4 Hz) between H-2 and H-3.

Therefore, compound **1** was established as (1*R*, 2*R*, 3*R*)-3-hydroxy-1,2-dimethyl-1,2,3,9-tetrahydrocarbazol-4-one, an unusual skeleton oxidized from carbazole.

Compound **2** was isolated as a pale yellow oil and its molecular formula was determined for $C_{16}H_{17}NO_3$ by the high resolution FAB-MS, consistent with nine unsaturation degrees. 1D and 2D NMR interpretation showed that the structure of **2** were very closely similar to that of carbazomycin B, but possessed one methylene group which is not common in carbazole derivatives. The HMBC experiment revealed that the methoxy protons at δ 3.31 were correlated to the methylene carbon C-10 at δ 67.1 to form an ether linkage. The methylene group was in turn attached to C-1, which is confirmed by HMBC correlations of H$_2$-10 with C-1, -2, and 9a. Therefore, **2** was determined as 1-(methoxymethyl)-carbazomycin B, derived from the oxidation of carbazomycin B.

The extract of the cultured cells showed significant antifungal activity against *Candida albicans* and *Cryptococcus neoformans* and the isolated compounds were tested by a paper disc diffusion method. However, five compounds except for compound **1** showed no inhibition zone. Compound **1** had an inhibition zone of 4 mm in diameter for *C. albicans* after 24 hr, compared with that (5 mm in diameter) of amphotericin B used as a positive control. We conclude that other sources in the extract might exhibit the activity, and are under study in finding out them.

Experimental Section

Experimental procedures. Optical rotations were measured on a JASCO P-1010 digital polarimeter in a 5 cm cell. UV spectra were obtained in MeOH using a Varian Cary 50, and IR spectra were measured on a JASCO FT/IR 4100 spectrometer. All NMR spectra were recorded on a Varian VNMRS 500 spectrometer in DMSO-d_6. Chemical shifts of proton and carbon spectra were reported in reference to residual solvent peaks at 2.50 ppm and 39.5 ppm, respectively. Positive HRFAB mass spectra were obtained on a JEOL JMS-700 spectrometer at Korea Basic Science Research Institute, Daegu, South Korea. HPLC was carried out on a Varian system (Prostar 210 pump and Prostar 355 refractive index detector).

Bacterial strain and cultivation. The bacterial strain isolated from the Korean seawater was taxonomically recognized as *Streptomyces ehimensis* JB201 and deposited as KCTC18166P in the biological resource center, Korea Research Institute of Bioscience & Biotechnology (KRIBB). Strain *S. ehimensis* JB201 was cultured in a plate with a solid production medium composed of 0.4% glucose, 0.4% yeast extract, 1% malt extract, 0.2% $CaCO_3$ and 1.2% agar at 25 °C for 5 days. The plate culture was used to inoculate 10 mL of liquid medium of the same composition and cultured at a condition of 25 °C, pH 6.5, and 180 rpm. After cultivation for 3 days, seed culture was inoculated to 100 mL of liquid medium under the same environment and grown for 5 days for harvest.

Extraction and isolation. After cultivation, the cells were harvested by centrifugation with a speed of 12,000 rpm for 5 min to remove aqueous supernatants of culture broth. Cell pallet was extracted with acetone solvent at room temperature for 12 hr. The filtrate was evaporated to dryness in vacuo and then the crude extract was subjected to reversed phase flash chromatography using solvent systems of H_2O and MeOH with 10% increase of MeOH to yield six fractions (MeOH/H_2O = 50/50, 60/40, 70/30, 80/20, 90/10, and 100/0). Three subfractions (60% ~ 80% MeOH) showed antifungal activity. The 60% MeOH fraction was separated by reversed phase HPLC (Varian Pursuit XRS 25 × 4.6 mm, 5 μm, RI detector, 2 mL/min) by eluting 35% H_2O and 65% MeOH solvents to afford a mixture at a retention time of 17 min. For purification, the mixture was rechromatographed by using 40% ACN and 60% H_2O solvents to give compound **1** and carbazomycin G (**6**). Similarly, the 70% MeOH fraction was separated with 30% H_2O and 70% MeOH solvents to yield compound **2**, carbazomycin B (**5**). Finally, cabazomycins A (**3**) and *N*-methyl carbazomycin B (**4**) was isolated from 80% MeOH subfraction by using 25% H_2O

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Table 1. Spectral data for compounds **1** and **2** in DMSO-d_6

position	1		2	
	^{1}H	^{13}C	^{1}H	^{13}C
1	3.05 (1H, dq, 5.1, 7.2)	33.2, d		109.4, s
2	2.19 (1H, m)	42.5, d		129.0, s
3	4.20 (1H, t, 3.4)	73.3, d		138.1, s
4		192.6, s		144.8, s
4a		108.6, s		109.7, s
4b		124.6, s		122.4, s
5	7.92 (1H, d, 7.2)	120.1, d	8.09 (1H, d, 7.6)	121.8, d
6	7.15 (1H, dd, 7.2, 7.6)	121.6, d	7.08 (1H, dd, 7.6, 7.1)	118.2, s
7	7.18 (1H, dd, 7.6, 7.6)	122.5, d	7.28 (1H, dd, 8.1, 7.1)	124.1, d
8	7.41 (1H, d, 7.6)	117.8, d	7.42(1H, d, 8.1)	110.3, d
8a		136.8, s		139.5, s
9a		154.6, s		137.5, s
10	1.45 (3H, d, 7.2)	17.7, q	4.78 (2H, s)	67.1, t
11	1.02 (3H, d, 6.9)	14.2, q	3.31 (3H, s)	57.0, q
12			2.36 (3H, s)	12.1, q
13			3.67 (3H, s)	60.6, q
9-NH	11.95 (1H, br s)		10.92 (1H, s)	
3-OH	5.21 (1H, br d, 3.4)		9.43 (1H, br s)	

Data were recorded at 500 MHz for ^{1}H and 125 MHz for ^{13}C. Assignments were made by COSY, TOCSY, HSQC, and HMBC experiments. s = singlet, d = doublet, t = triplet, q = quartet

and 75% MeOH solvents.

Compound 1: $[\alpha]_D^{25}$ +29.7° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 204 nm (3.7), 238 nm (3.6); IR (film) 3368, 2924, 1739, 1636, 1260, 802 cm^{-1}; ^{1}H and ^{13}C NMR data were given in Table 1; HRFAB-MS [M + H]$^+$ *m/z* 230.1181 (Δ −0.5 mmu).

Compound 2: UV (MeOH) λ_{max} (log ε) 205 nm (3.9), 228 nm (4.0), 266 nm (3.3), 279 nm (3.5); IR (film) 3365, 2929, 1612, 1419. 1146 cm^{-1}; ^{1}H and ^{13}C NMR data were given in Table 1; HRFAB-MS [M + H]$^+$ *m/z* 272.1280 (Δ −0.1 mmu).

Mosher reaction of compound 1. To a stirred solution of compound **1** (2 mg) and dried pyridine (20 μL) in dry CH$_2$Cl$_2$ (0.5 mL) at room temperature, *R*-(-)-MTPA-Cl (150 μL) was added. The reaction progress was monitored by TLC chromatography on silica gel (Hex : EtOAc = 4 : 1). After ~ 10 hr, the reaction mixture was quenched by the addition of H$_2$O and dimethyl ether. The organic layer was concentrated in vacuo. The crude product mixture was eluted by silica-gel SPE with hexane/ ethyl acetate (5 : 1) to give the (*S*)-MTPA ester (**1a**) as a pale yellow gum. ^{1}H NMR (CDCl$_3$, 500 MHz) δ 8.16 (1H, d, *J* = 6.1 Hz, H-5), 6.06 (1H, d, *J* = 4.4 Hz, H-3), 3.16 (1H, dq, *J* = 7.1, 4.4 Hz, H-2), 2.57 (1H, m, H-1), 1.60 (1H, d, *J* = 7.1 Hz, H-10), 1.21 (1H, d, *J* = 7.1 Hz, H-11). In an entirely analogous way, the (*R*)-MTPA ester (**1b**) was obtained using *S*-(+)-MTPA-Cl. ^{1}H NMR (CDCl$_3$, 500 MHz) δ 8.18 (1H, d, *J* = 6.3 Hz, H-5), 6.03 (1H, d, *J* = 4.2 Hz, H-3), 3.08 (1H, dq, *J* = 7.1, 3.7 Hz, H-2), 2.44 (1H, m, H-1), 1.55 (1H, d, *J* = 7.3 Hz, H-10), 1.06 (1H, d, *J* = 7.1 Hz, H-11).

Antifungal activity test. The antifungal activity of all isolated compounds was tested against the fungi *Candida albicans* (ATCC10231) and *Cryptococcus neoformans* (ATCC2334). These fungi were grown in ATCC790 media (0.5% D-glucose, 0.1% yeast extract, 0.1% peptone, and 3% sea salt) at 25 °C for 2 days. After incubation, each 100 μL fungus was smeared on an ATCC790 agar plate and on it paper discs were placed to spot compounds. The plates with compounds were incubated 25 °C for 24 hr. The activity degree of agar-diffusible compounds was estimated by measuring the diameter of the inhibition area.

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