Hygrolansamycins A-D, O-Heterocyclic Macrolides from Streptomyces sp. KCB17JA11

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Six ansamycin derivatives were isolated from the culture broth of Streptomyces sp. KCB17JA11, including four new hygrolansamycins A-D (1-4) and known congeners divergolide O (5) and hygrocin C (6). Compounds 1-5 featured an unusual six-membered O-heterocyclic moiety. The isolation workflow was guided by a Molecular Networking-based dereplication strategy. The structures of 1-4 were elucidated using NMR and HRESIMS experiments, and the absolute configuration was established by the Mosher’s method. Compound 2 exhibited mild cytotoxicity against five cancer cell lines with IC50 values ranging from 24.60 ± 3.37 μM to 49.93 ± 4.52 μM.

Keywords: Molecular networking, Streptomyces, ansamycin family, hygrolansamycins

Introduction

Actinomycetes are the primary producers of ansamycins, including rifamycin, ansamitocin, and geldanamycin, which have potent antibiotic and anticancer properties [1, 2]. In general, 3-amino-5-hydroxybenzoic acid (AHBA) is utilized for the polyketides assembly of ansamycins containing a benzene- or naphthalene-based mC7N chromophore [1]. It has recently been reported that modified ansamycin scaffolds can be generated through divergent biosynthetic pathways involving divergolides and hygrocins [3-7]. Baeyer-Villiger oxygenation of a macrolactam intermediate has also been proposed as a biosynthetic route to the divergolides and hygrocins [6, 8, 9]. Furthermore, several divergolides undergo optional acyl migration following oxidative β-hydroxy formation adjacent to the ester bond [4, 6].

Tandem mass-based dereplication is a common technique for screening known bioactive compounds, such as ansamycins, in complex microbial samples [10-16]. Global Natural Products Social Molecular Networking (GNPS), an open-access data-driven tandem mass spectral platform, is particularly well-suited for this purpose and is widely used. Previously, during our tandem-mass-based dereplication study of Streptomyces species, we discovered geldanamycin [10] and streptimidine [11] derivatives. In this study, we investigated the fermentation of Streptomyces sp. KCB17JA11, a geldanamycin producer, and using the GNPS system, we discovered new nodes, corresponding to divergolide O (5) [7] and hygrocin C (6) [5] with molecular weights of 513 and 509, respectively. Four other compounds were found to have the same molecular formula (C31H39NO8) as divergolide O (5). Herein, we present four new stereochemical derivatives of 5, namely hygrolansamycins A-D (1-4).

Materials and Methods

General Experimental Procedures.

Optical rotation was obtained using a JASCO P-1020 polarimeter. UV spectra were recorded on an Optizen 2120 UV spectrophotometer. NMR experiments were operated on a Bruker AVANCE HD 800 MHz NMR spectrometer (Bruker, Germany) at the Korea Basic Science Institute (KBSI) in Ochang, Korea. NMR spectra were recorded in DMSO-d_6 as an internal standard (δ_H 2.50/δ_C 39.51). High resolution electrospray ionization mass spectra (HR-ESIMS) were recorded on a Waters Vion IM-QTOF mass spectrometer (Waters, USA) at the KRRBB in Ochang, Korea. Column chromatography was performed on reversed phase silica gel (0.075 mm; Cosmosal, Japan). Analytical C18 (Waters Sunfire, 5 μm, 4.6 × 150 mm) and semi-preparative C18 (Waters Atlantis T3, 5μm, 10 × 250 mm) columns were used for reverse phase HPLC on a 515 pump HPLC system (Waters) equipped with a 2996 PDA detector (Waters) using HPLC grade solvents (Honeywell). Liquid chromatography-mass spectrometry (LC-MS) was operated using an LTQ XL linear ion trap (Thermo Scientific, USA) equipped with an electrospray...
ionization (ESI) source that was coupled to a rapid separation LC (RSLC, ultimate 3000, Thermo Scientific) system (ESI-MS).

Strain Identification
A soil sample was collected at Ochang, Cheongju, Republic of Korea. Analysis of 16S rRNA gene sequences showed that strain KCB17JA11 was most closely related to the Streptomyces rapamycinicus gene (99.79% identity, GenBank Accession No. KP209440.1). Therefore, strain KCB17JA11 was named Streptomyces sp. KCB17JA11 and used in the subsequent culture experiments.

Culture Conditions
Streptomyces sp. KCB17JA11 was cultured for 3 days at 28°C on a rotary shaker with agitation at 125 rpm in a 250 ml Erlenmeyer flask containing 50 ml of seed culture medium (soluble starch 1%, yeast extract 0.1%, and tryptone 0.1%). For a large culture, 1% of the pre-culture broth was inoculated into 40 x 1,000 ml baffled Erlenmeyer flasks containing 250 ml of YM broth (glucose 1%, soluble starch 2%, meat extract 0.3%, yeast extract 0.5%, malt extract 0.5%, and CaCO3 0.05%), and cultured for 8 days at 28°C on a rotary shaker with agitation at 125 rpm.

LC-MS Analysis Conditions and Dereplication through Molecular Networking
The samples from the EtOAc extract were dissolved in a methanol and analyzed using a Thermo U3000-LTQ XL ion trap mass spectrometer (Thermo Scientific) equipped with an electrospray ionization (ESI) mass source. Chromatographic separation of the compounds was achieved using a Waters HSS T3 C18 column (2.1 x 150 mm; 2.5 μm) at a flow rate of 0.3 ml/min. The mobile phases A and B were water and acetonitrile, respectively, both containing 0.1% formic acid. Gradient elution was conducted as follows: 5-100% B for 0-15 min with a linear gradient, followed by 5 min of 100% B. The MS/MS system was operated in ESI mode. Typical operating parameters were as follows: spray needle voltage, +5 kV; ion transfer capillary temperature, 275°C; nitrogen sheath gas, 35; and auxiliary gas, 5 (arbitrary units). The ion trap contained helium damping gas which was introduced in accordance with the manufacturer's recommendations. Mass spectra were acquired in an m/z range of 100-2,000, applying three microscans and a maximum ion injection time of 100 ms. Data-dependent mass spectrometry experiments were controlled using the menu-driven software provided in the Xcalibur system (version 4.0; Thermo Scientific). The acquired data file was exported in *.mzXML format using the application of MS-convert. The parameters were set as follows: product ion tolerance of 0.5 Da and a precursor ion mass tolerance of 2.0 Da. The results were visualized using Cytoscape 3.7.2 software.

Extraction and Isolation
To remove EtOAc, the residue was partitioned three times with EtOAc and evaporated. The crude extract was fractionated employing reversed-phase C18 vacuum column chromatography eluting with a stepwise MeOH:H2O solvent system of (20:80 to 100:0, each x 1 L). The 70% (0.143 mg) fraction was further fractionated using a CombiFlash RF (Teledyne ISCO) medium-pressure chromatography system (MPLC) on a Redesip RF C18 reverse-phase column under stepwise gradient elution with MeOH:H2O (from 20:80, 40:60, 60:80, 80:100:0; 1 L for each step). To obtain compound 6, fraction 3 (314 mg) was subjected to semi-preparative HPLC [Waters Atlantis T3 C18 column (10 x 250 mm, 5 μm, 3 ml/min)] under isocratic elution using 65% MeOH-H2O (0.05% TFA) over 45 min to (6.0 mg, RT:16.1). The same MPLC fraction 3 was subjected to semipreparative HPLC [Waters Atlantis T3 C18 column (2.1 x 150 mm; 2.5 μm) at a flow rate of 0.3 ml/min. The mobile phases A and B were water and acetonitrile, respectively, both containing 0.1% formic acid. Gradient elution was conducted as follows: 5-100% B for 0-15 min with a linear gradient, followed by 5 min of 100% B. The MS/MS system was operated in ESI mode. Typical operating parameters were as follows: spray needle voltage, +5 kV; ion transfer capillary temperature, 275°C; nitrogen sheath gas, 35; and auxiliary gas, 5 (arbitrary units). The ion trap contained helium damping gas which was introduced in accordance with the manufacturer's recommendations. Mass spectra were acquired in an m/z range of 100-2,000, applying three microscans and a maximum ion injection time of 100 ms. Data-dependent mass spectrometry experiments were controlled using the menu-driven software provided in the Xcalibur system (version 4.0; Thermo Scientific). The acquired data file was exported in *.mzXML format using the application of MS-convert. The parameters were set as follows: product ion tolerance of 0.5 Da and a precursor ion mass tolerance of 2.0 Da. The results were visualized using Cytoscape 3.7.2 software.

Modified Mosher's Method
Compounds 1 and 4 (0.5 mg) was dissolved in anhydrous pyridine (1 ml), and a catalytic amount of dimethylaminopyridine (DMAP) was added. After 5 min of stirring, 25 μl of (R)-MPA-Cl was added, and the mixture was stirred at room temperature for 16 h. Repeat treatment of same method with (S)-MPA-Cl instead of (R)-MPA-Cl. Each mixture was subjected to semi-preparative reversed phase HPLC (column as above; flow rate 3 ml/min; 50-100% CH3CN-H2O containing 0.05% TFA over 25 min) to yield (S)-MPA ester 1A (0.3 mg, tR 16.5 min) and 4A (0.3 mg, tR 16.8 min) and (R)-MPA ester 1B (0.3 mg, tR 17.0 min) and 4B (0.3 mg, tR 17.3 min). The 1H NMR chemical shifts of MPA esters 1A, 1B, 4A, and 4B were assigned on the basis of interpretation of 1H, COSY, and HSQC-DEPT NMR data (see Supporting Information for NMR data).

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Table 1. $^1$H (800 MHz) and $^{13}$C (200 MHz) NMR data for hygrolansamycins A-D (1–4) in DMSO-$d_6$.

| No. | $^1$H (800 MHz) | $^{13}$C (200 MHz) |
|-----|----------------|------------------|
|     | $\delta$ (mult., in Hz) | $\delta$ (mult., in Hz) |
| 1   | 74.9 | 5.04 (m$^\alpha$) | 74.8 | 5.02 (m$^\alpha$) |
| 2   | 51.5 | 2.45 (dd, 14.5, 7.1) | 51.5 | 2.45 (dd, 14.5, 7.2) |
| 3   | 208.3 | 208.4 |
| 4   | 48.4 | 3.08 (d, 16.7), 2.12 (d, 16.7) | 48.6 | 3.08 (d, 16.7), 2.30 (d, 16.7) |
| 5   | 102.1 | 102.0 |
| 6   | 38.1 | 1.81 (m$^\alpha$), 1.58 (t, 12.6) | 37.9 | 1.94 (t, 12.3), 1.87 (m$^\alpha$) |
| 7   | 27.9 | 1.67 (m$^\alpha$), 1.22 (m$^\alpha$) | 28.5 | 1.68 (td, 12.0, 6.5), 1.08 (d, 6.3) |
| 8   | 43.5 | 1.77 (m$^\alpha$) | 43.7 | 1.75 (dt, 14.2, 9.3) |
| 9   | 136.3 | 134.9 | 4.94 (d, 15.7, 9.1, 1.3) |
| 10  | 128.0 | 124.7 | 5.51 (dd, 15.7, 3.8) |
| 11  | 77.3 | 5.02 (dd, 6.8, 4.8, 1.0) | 78.2 | 5.04 (m$^\alpha$) |
| 12  | 68.5 | 3.77 (qd, 6.8, 6.5) | 67.5 | 3.72 (dq, 6.8, 7.0) |
| 13  | 19.2 | 1.08 (d, 6.4) | 18.8 | 1.08 (d, 6.3) |
| 14  | 15.5 | 1.28 (d, 7.2) | 15.3 | 1.29 (d, 7.2) |
| 15  | 28.8 | 1.37 (m$^\alpha$), 1.22 (m$^\alpha$) | 27.6 | 1.37 (qd, 12.6, 7.2), 1.29 (d, 6.3) |
| 16  | 12.4 | 0.83 (t, 7.7) | 12.5 | 0.83 (t, 7.4) |
| 1’  | 106.6 | 6.22 (d, 2.7) | 105.9 | 6.16 (d, 2.7) |
| 2’  | 151.2 | 150.0 |
| 3’  | 107.7 | 7.51 (d, 2.7) | 106.9 | 7.68 (d, 2.7) |
| 4’  | 126.4 | 127.2 |
| 5’  | 132.6 | 131.7 | 132.0 |
| 6’  | 122.5 | 122.4 | 119.3 |
| 1’’ | 166.6 | 168.2 | 168.2 |
| 2’’ | 37.5 | 3.37 (dd, 16.6, 8.1), 3.29 (dd, 16.6, 8.0) | 39.9 | 4.06 (t, 11.6), 2.85 (dd, 11.7, 7.2) |
| 3’’ | 133.4 | 7.00 (td, 7.9, 1.1) | 136.7 | 6.29 (dd, 11.4, 7.0) |
| 4’’ | 133.9 | 134.1 |
| 5’’ | 166.4 | 166.5 |
| 6’’ | 13.1 | 1.86 (s) | 20.4 | 1.99 (s) |
| NH  | 7.97 (s) | 8.82 (s) |
| 2’-OH | 9.15 (s) | 9.08 (s) | 9.02 (s) | 9.05 (s) |

*Resonances overlapped or multiplet.

Cell Viability Assay

B16F10, HeLa, MDA-MB-231, and PC12 cell lines were grown in Dulbecco’s modified Eagle’s medium (DMEM; Welgene, LM 001-05) supplemented with 10% fetal bovine serum (FBS; Welgene, S001-07), 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco, 15140-122) in a humidified atmosphere at 37°C with 5% CO$_2$. The AGS cells were grown in Roswell Park Memorial Institute 1640 (RPMI 1640; Welgene, LM 011-01) medium supplemented with 10% FBS, penicillin, and streptomycin. Cells were seeded in 96-well cell culture plates (0.7 × 10$^3$ cells/well) overnight. Varying concentrations of compounds were treated for 24 h, and 10 μl of EZ-Cytox cell viability assay solution (Dael Lab Service, Korea) was directly added. After 2 h of incubation at 37°C, the absorbance was measured at 450 nm using a microplate reader (Molecular Devices, USA).

Results and Discussion

**Streptomyces** sp. KCB17J11 was cultured in YMG media at 28°C for 7 days, before the broth and mycelia extracts were partitioned using EtOAc. The EtOAc extracts were examined using ESI-ion trap-MS/MS and the data were processed into molecular networks using the GNPS platform (http://gnps.ucsd.edu) [13]. Among the nodes, the node shown in Fig. 1 was annotated through library searching, corresponding to diergolide O (5) and hygrocin C (6). The precursor ion at this node had an $m/z$ value of 514.3 [M+H]+. The high-performance liquid chromatography (HPLC) data and total ion current of the crude extract revealed the presence of six peaks eluting at 11.52, 11.71, 12.23, 12.65, 12.78, and 13.58 min, all of which had the same $m/z$ values (514.3 [M+H]+) as the standard 5, which eluted at 12.65 min, as confirmed by retention time and MS/MS spectra comparison (Fig. 1). To obtain compounds 1-5, purification was performed using silica open-column liquid chromatography, reverse-phase MPLC, and semi-preparative HPLC. Thus, five compounds had $m/z$ values of 514.3. Furthermore, 6 was isolated and identified from the same crude fractions (Fig. 2).
Hygrolansamycin A (1) was obtained as a yellow powder. Based on high resolution electrospray ionisation mass spectroscopy (HRESIMS) data, the molecular formula of 1 was assigned as $C_{28}H_{35}NO_{8}$, having 12 degrees of
unsaturation. The $^1$H NMR spectrum of data of 1 contained signals for five sp$^2$ protons ($\delta_1$ 7.51, 7.00, 6.22, 5.63, and 5.30), three sp$^3$ oxymethine protons ($\delta_5$ 5.04, 5.02, and 3.72), one methyl singlet ($\delta_6$ 1.86), two methyl doublets ($\delta_1$ 1.08 and 1.28), and one methyl triplet ($\delta_6$ 0.83). The $^{13}$C NMR was combined with HSQC-DEPT data, which indicated resonances for one ketone carbonyl ($\delta_4$ 208.3), two ester/amide carbonyls ($\delta_4$ 166.6, 166.4), ten methines including five olefinic methines ($\delta_4$ 136.3, 133.4, 128.0, 106.6, and 107.7) and three oxygenated methines ($\delta_4$ 77.3, 75.4, and 68.5), five methylenes ($\delta_4$ 14.5 Hz) between H-1 and H-2 showed the configuration. Thus, the 2D NMR correlations from H-1 to C-2, C-14, C-3, C-5, and C-5′′ indicated the presence of two fragments linked via the ester moiety and the methylated position at C-6′′. Furthermore, six typical aromatic carbon signals at $\delta_4$ 106.6 (C-1′′), 151.2 (C-2′′), 107.7 (C-3′′), 126.4 (C-4′′), 132.6 (C-5′′) and 122.5 (C-6′′), as well as HMBC correlations of NH/C-4′′ and 1″′, H-3′′/C-1′′ and C-2′′, H-1′′/C-5′′ and C-6′′, suggest the presence of the 1-amino-3-hydroxybenzene moiety. Further analysis of HMBC correlations from H-1 to C-2, C-14, C-3, C-5, and C-5′′, from H-4 to C-2, C-3, C-5, and C-6 and from H-6 to C-3, C-4 and C-5 suggested that compound 1 is an unusual six-membered O-heterocyclic ring (Fig. 3). Comparison of the planar structural data with those reported for divergolide O (5) revealed that 1 was closely related to 5, except for only relative configurations from H-2 to H-4 rotating-frame nuclear Overhauser effect correlation spectroscopy (ROESY) correlation (Fig. 2). In the previous report, the stereochemical study of O-heterocyclic ring moiety has been performed by NMR spectral analysis and X-ray crystallography in divergolide A [4].

The $^{1}$H-$^{1}$H correlation spectroscopy (COSY) spectrum, combined with Heteronuclear multiple bond correlation (HMBC) correlation analysis, established the presence of two spin systems; thus, fragments C-6/C-7/C-8(C-15/15.5) (Table 1). Further analysis of HMBC correlations from H-1 to C-2, C-14, C-3, C-5, and C-5′′ indicated the presence of two fragments linked via the ester moiety and the methylated position at C-6′′. Furthermore, six typical aromatic carbon signals at $\delta_4$ 106.6 (C-1′′), 151.2 (C-2′′), 107.7 (C-3′′), 126.4 (C-4′′), 132.6 (C-5′′) and 122.5 (C-6′′), as well as HMBC correlations of NH/C-4′′ and 1″′, H-3′′/C-1′′ and C-2′′, H-1′′/C-5′′ and C-6′′, suggest the presence of the 1-amino-3-hydroxybenzene moiety. Further analysis of HMBC correlations from H-1 to C-2, C-14, C-3, C-5, and C-5′′, from H-4 to C-2, C-3, C-5, and C-6 and from H-6 to C-3, C-4 and C-5 suggested that compound 1 is an unusual six-membered O-heterocyclic ring (Fig. 3). Comparison of the planar structural data with those reported for divergolide O (5) revealed that 1 was closely related to 5, except for only relative configurations from H-2 to H-4 rotating-frame nuclear Overhauser effect correlation spectroscopy (ROESY) correlation (Fig. 2). In the previous report, the stereochemical study of O-heterocyclic ring moiety has been performed by NMR spectral analysis and X-ray crystallography in divergolide A [4]. In order to establish the relative configuration of O-heterocyclic ring moiety of compound 1, detailed analyses of $^1$H NMR and ROESY data were performed. A large coupling constant ($\text{J} = 14.5$ Hz) between H-1 and H-2 showed the anti orientation for the two protons, whereas H-2 and H-14 was showed simply three bond coupling ($\text{J} = 7.2$ Hz). The ROESY correlation between H-2 ($\delta_6$ 2.45) and H-4$_{1}$ ($\delta_6$ 2.12) of 1 indicate that equatorial preferred for both H-2 and H-4$_{1}$ (Fig. 4A). On the other hand, the relative stereochemistry of the O-heterocyclic ring moiety in reported divergolide O (5) was described through NOE correlation between H-2 and H-4$_{1}$ [7]. In addition, the configuration of the C-3′′/4′′ double bond was assigned as E configuration because of the chemical shift of the allylic methyl group C-6′′ ($\delta_6$ 13.1) [4]. The absolute configuration of C-11 was determine using a modified Mosher’s method. Methoxyphenylacetic (MPA) esters were prepared by treating 1 with (R)- and (S)-MPA-Cl in anhydrous pyridine, yielding the corresponding (S)- and (R)-MPA esters 1a and 1b. C-11S configuration was established for 1 based on the $\Delta\delta$ ($\delta(S) - \delta(R)$) values of the MPA esters (Fig. 4D). Furthermore, the coupling constant of $\text{J}_{\text{H11H2}} = 6.8$ Hz in 1 indicate that the dihedral angle of these two protons was approximately 60° (Fig. 4C). In addition, the strong ROESY correlation between H-11 and H-12, but not between H-11 and the H-13 methyl protons of 1, indicated that H-11 was oriented anti to this methyl group (Fig. 4C). Thus, the relative configuration of C-1/C-2/C-5/C-11/C-12 was determined as $\Delta^5\beta^5\Delta^{11\prime\prime}\Delta^{12\prime\prime}$. However, the relative configuration C-8 bearing the ethylated branch remained unknown because of the lack of relevant ROESY correlations, which was attributed to the flexibility of that portion of the ansa macro lactam. To confirm the configuration of C-8 stereocenter in 1, using chiroptical analysis involving circular dichroism (CD) spectroscopy. The previously reported CD spectra of 5 and divergolides were elucidated as 1S/2R/5S/8R/11S/12S by contrasting with the mirror image and confirming a closely similar Cotton effect to divergolide A [4, 6, 7]. Further, the experimental CD spectra of 1 show strikingly similar Cotton effect from 5 over the entire wavelength range (Fig. 5). Therefore, the absolute configuration of 1 was established 1S/2S/5S/8R/11S/12S. Thus, compound 1 was named as hygroansamycin A.

Hygroansamycin B (2) was isolated as a yellow powder. According to HRESIMS data the molecular formula of 2 was determined to be $\text{C}_{18}\text{H}_{14}$NO$_3$, which is the same as that of 1. The orientation of the C-3′′/4′′ double bond was the only difference between the structures of 1 and 2. The relative downfield shift of the C-6′′ allylic methyl group ($\delta_6$ 13.1, $\delta_6$ 1.86 in 1 and $\delta_6$ 20.4, $\delta_6$ 1.99 in 2) was used to deduce that the C-3′′/4′′ double bond was in Z configuration. Thus, 1 is a geometric isomer of 2 [4].

Fig. 3. Key 2D NMR correlations of compound 1.
Hygrolansamycin C (3) was isolated as a yellow powder. The molecular formula was determined to be C_{28}H_{35}NO_{8} on the HRESIMS data, which is the same as that of 2. The high similarity between the 1 and 2D NMR spectra of 2 and 3 indicated that they shared the same planar structure. The ROESY spectrum correlation between H-2 and H-4_{ax} (\(\delta_{H} 2.98\)) indicates that O-heterocyclic ring is axial preferred for both H-2 and H-4_{ax} (Fig. 4B) [6, 7]. Furthermore, the 1D NMR chemical shifts of C-14 (\(\delta_{C} 15.3\), \(\delta_{H} 1.29\) in 2 and \(\delta_{C} 9.9\), \(\delta_{H} 0.89\) in 3) were shown relative configuration of C-2, which is opposite in between 2 and 3 [17]. Thus, 3 is a stereoisomer of 1.

Hygrolansamycin D (4) was isolated as a yellow powder. The HRESIMS data indicated that the molecular formula of 4 was C_{28}H_{35}NO_{8}. The high similarity between the 1 and 2D NMR spectra of 1 and 4 indicated that they shared the same planar structure. The ROESY spectrum (Fig 4B) and chemical shifts of C-14 (\(\delta_{C} 15.5\), \(\delta_{H} 1.28\) in 1

Fig. 4. Determination of stereochemistry of compounds. The key coupling constants and ROESY correlations from H-2 to H-4_{eq} or H-4_{ax} in O-heterocyclic ring moiety of 1 (A) and 3 (B). The key ROESY correlations and dihedral angle model for determining the relative configurations of C-11 and C-12 in 1 (C). Modified Mosher’s ester analysis of 1 (D) and 4 (E). \(\Delta\delta_{\text{ax}}\) value of \(1^H\) for (S)- and (R)-MPA esters.

Fig. 5. Comparison of the experimental CD spectra of 1-5 in MeOH.
and δ4 9.9, δ6 0.92 in 4 were shown configuration of C-2, which is opposite in between 1 and 4. Furthermore the spectroscopic data of 4 were different the upfield shift of H-11 (δ6 4.06) and downfield shift of H-12 (δ6 4.87). The different absolute configuration of 4 was established by the same method as that of 1. The Δ4 [δ(S) - δ(R)] values of the MPA esters indicate the R-configuration at C-11 in 4 (Figs. 4C and 4E). Therefore, the absolute configuration of 4 was established 1S/2S/3S/8R/11R/12R.

The O-heterocyclic ring moiety of 5 [7] was present in the four hygrolansamycin congeners 1-4. However, closer examination of the NMR data revealed some variations at position C-2 in the O-heterocyclic ring. The H-1 signals appeared as doublets in the 1H NMR spectra of 3 and 4, but not in those of 1 and 2 [7, 17]. Therefore, H-1 and H-2 are anti oriented in 1 and 4, while in 3 and 4, they are syn. Furthermore, the C-3’/C-4’ double bond shifted to the β, γ-position, rather than the typical α, β-position, which results from traditional syn elimination of water from a β-hydroxy function. From the relatively shielded chemical shifts of C-6’ (δ4 13.1 in 1 and δ6 13.2 in 4) of compounds 1, 4, and 5, C-3’/4’ was E configuration, whereas the C-6’ (δ4 20.4) of 2 and 3, C-3’/4’ was determined to be Z configuration [6]. Except for the C-3’/4’ double bond, other chromophores have little effect on the Cotton effects. Compounds 1, 4, and 5 with E forms show different Cotton effects from compounds 2 and 3 in the 200-280 nm wavelength range, but all spectra are very similar at wavelengths above 290 nm. In addition, reported divergolides E, G, H, and O (5) show a positive Cotton effects at 260 nm, while 2 and 3 with the Z form show a lower negative Cotton effects at 230-260 nm, similar to those of reported divergolides A and F (Fig. 5) [4, 6, 9]. Although the O-heterocyclic moiety, C-3’/4’ double bond and isobutyl moiety of divergolides may serve as additional chromophores, the hygrolansamycins found in this study showed similar CD spectral patterns. These results show that the differences in chromophore of O-heterocyclic ring moiety does not affect the CD spectrum patterns of compounds.

Many divergolides and hygrocins are cytotoxic against several cancer cell lines [3-5, 7]. The cytotoxicity of 1-6 was evaluated against five cancer cell lines, namely human gastric adenocarcinoma (AGS), murine melanoma (B16F10), human cervical carcinoma (HeLa), human breast cancer (MDA-MB-231), and human pheochromocytoma (PC12) cell line.

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### Conflicts of Interest

The authors have no financial conflicts of interest to declare.

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