NOTES

New Radical Scavenging and Ultraviolet-A Protecting Prenylated Dioxopiperazine Alkaloid Related to Isoechinulin A from a Marine Isolate of the Fungus *Aspergillus*

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A number of isoechinulin-type metabolites, characterized by a dehydrotryptophan unit, all containing isoprenic and reversed isoprenic chains in the 2- and 5-positions of the indole nucleus, respectively, have been isolated from molds of the genus *Aspergillus*.[1)]

Their common biosynthetic origin from cyclo-L-alanyl-L-tryptophanyl has been established.[2)]

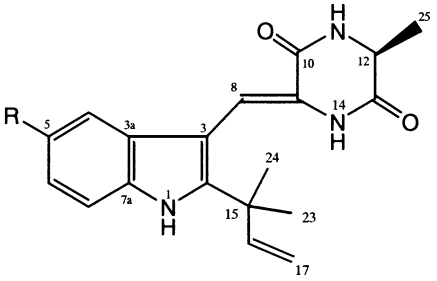
During a search for bioactive constituents from marine microorganisms,[3)] we have previously isolated diketopiperazines (**2**, **3**, **4**) from a marine-derived *Aspergillus* sp.[4)] In a continuing study of the more polar fractions from the same fungus, we have isolated a new metabolite, dihydroxyisoechinulin A (**1**), and related echinulin (**5**).[5)]

Materials and Methods

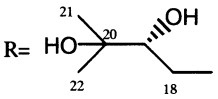
General

Melting points were determined on an Electrothermal model IA 9100 micro-melting point apparatus and are uncorrected. Optical rotations were determined on a Perkin Elmer model 341 polarimeter. IR spectra were recorded on a Bruker FT-IR model IFS-88 spectrometer. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectra were obtained on a JEOL JNM-ECP 400 NMR spectrometer, using TMS or solvent

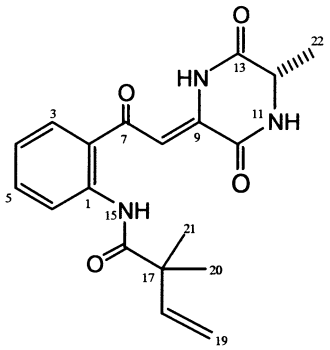
Fig. 1. Structures of dihydroxyisoechinulin A (**1**) and its analogs (**2**~**5**).



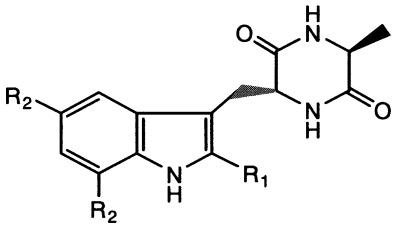
dihydroxyisoechinulin A (**1**) :



neoechinulin A (**3**) : R= H

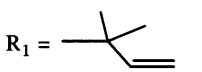


golmaenone (**2**)



(**4**) R_1=R_2= H

echinulin (**5**) R_1 =



R_2 =

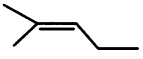


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Table 1. Physico-chemical properties of dihydroxyisoechinulin A (**1**).

Appearance	Colorless oil
$[\alpha]_D$ (*c* 0.4, $CHCl_3$)	-47°
Molecular formula	$C_{24}H_{31}N_3O_4$
LREI-MS (*m/z*)	425 $[M]^+$ (72), 407 $[M-H_2O]^+$ (1), 382 $[M-H_2O-C_3H_7]^+$ (1), 367 $[M-C_3H_6O]^+$ (13), 356 $[M-C_5H_9]^+$ (42), 336 $[M-C_4H_9O_2]^+$ (47), 298 $[356-C_3H_6O]^+$ (100), 268 $[336-C_5H_8]^+$ (81), 194 (41), 69 $[C_5H_9]^+$ (17), 59 $[C_3H_7O]^+$ (51)
HREI-MS (*m/z*)	
Found	425.2320 $[M]^+$
Calcd for $C_{24}H_{31}N_3O_4$	425.2315
IR ν_{max} (neat) cm^{-1}	3358, 3262, 3085, 1673, 1629, 1425, 1381, 1323, 1242, 1160, 1024, 1000, 902, 756
UV λ_{max} (MeOH) nm (log ϵ)	209 (3.9), 226 (3.9), 289 (3.4), 340 (3.5)
CD (MeOH) nm ($\Delta\varepsilon$)	212 (-6.6), 239 (+3.2), 266 (+1.7), 284 (+1.6), 341 (-1.1)

Table 2. ^1H (δ, mult, *J*) and ^{13}C (δ, mult) NMR data for dihydroxyisoechinulin A (**1**)[a].

Position	δ_H	δ_C	HMBC (H to C)
1	10.91 (s)		2, 3, 3a, 7a
2		144.0 (s)	
3		103.1 (s)	
3a		126.2 (s)	
4	7.02 (br. s)	119.2 (d)	3, 6, 7a, 18
5		132.3 (s)	
6	6.98 (dd, 8.2, 1.3)	123.0 (d)	4, 7a, 18
7	7.29 (d, 8.2)	111.1 (d)	3a, 5
7a		133.9 (s)	
8	6.87 (s)	110.8 (d)	2, 3a, 10
9		124.8 (s)	
10		160.0 (s)	
11	8.36 (d, 1.9)		9, 13
12	4.10 (qd, 6.5, 1.9)	51.0 (d)	10, 13, 25
13		166.6 (s)	
14	8.51 (s)		10, 12, 13
15		39.2 (s)	
16	6.06 (dd, 17.0, 10.5)	145.4 (d)	2, 15, 23, 24
17	5.01 (d, 17.0) 5.03 (d, 10.5)	111.6 (t)	15, 16
18	2.36 (dd, 13.5, 10.0) 2.93 (d, 13.5)	38.0 (t)	4, 5, 6, 19
19	3.27 (m)	80.0 (d)	5, 20, 21, 22
20		72.0 (s)	
21	1.09 (s)[b]	26.5 (q)[c]	19, 20, 22
22	1.06 (s)[b]	24.7 (q)[c]	19, 20, 21
23	1.46 (s)[c]	27.6 (q)[c]	2, 15, 16, 24
24	1.45 (s)[c]	27.7 (q)[c]	2, 15, 16, 23
25	1.39 (d, 6.5)	20.3 (q)	12, 13
19-OH	4.11 (s)[d]		18, 19, 20
20-OH	4.16 (s)[d]		20, 21, 22

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

[b-e] Exchangable.

peaks as reference standard. MS spectra were obtained on a JEOL JMS-700 spectrometer. UV/visible spectra were measured on an Hitachi U-2001 UV/Vis spectrometer. CD spectra were taken on a JASCO J-715 spectropolarimeter.

Fungal Isolation and Culture

The fungal strain (stock # MFA 212) was isolated from the surface of the marine red alga *Lomentaria catenata* collected in the Golmae village, Ulsan City, Korea in 2002 and identified as an *Aspergillus* sp. based on fatty acid methyl ester analysis (Korean Culture Center of Microorganisms, Seoul, Korea), similarity index 0.62. The fungus was cultured (20 liters) for 30 days (static) at 29°C in SWS medium: soytone (0.1%), soluble starch (1.0%), and seawater (100%).

Extraction and Isolation

The culture broth and mycelium were separated and the broth was extracted with ethyl acetate to provide a crude extract (1.5 g) that was fractionated by silica gel flash chromatography (*n*-hexane/EtOAc) to generate five fractions containing diketopiperazine alkaloids **2** (20 mg), **3** (120 mg), **4** (35 mg), **1** (15 mg), and **5** (10 mg). Final purification of the fractions containing **1** and **5** by ODS column chromatography (H_2O in MeOH), followed by HPLC (YMC ODS-A, MeOH), yielded the new compound dihydroxyisoechnulin A (**1**, 10 mg), as well as the known echinulin (**5**, 6 mg).

Compound (**1**): See Tables 1 and 2 for physicochemical and NMR data.

Compound (**5**) was isolated as a colorless solid that

showed spectral data virtually identical to those reported in the literature.[5)]

Acid Hydrolysis and Marfey Analysis

Samples (0.5 mg) of compounds **1** and **5** were subjected to acid hydrolysis with 6 N HCl (1 ml) at 110°C for 12 hours. The hydrolyzates were dried, resuspended in H_2O (100 μl), and derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide. The derivatives were compared with similarly derivatized L- and D-alanine by HPLC [HiQ sil C18W (4.6×250 mm), 5 μm, flow rate 1 ml/minute, UV detection at 340 nm], using a linear gradient of MeCN in 0.1% (v/v) aqueous TFA (30~70% MeCN over 50 minutes). The retention times of the derivatives of L- and D-alanine were 14.4 and 17.1 minutes, respectively, and the retention time of the derivative from both hydrolyzates was 14.4 minutes.

Absolute Stereochemistry at C-19 of Compound (**1**)

(±)-α-Phenylbutyric anhydride (15 mg) was added to a solution of **1** (3.5 mg) and dimethylaminopyridine (1.0 mg) in pyridine (0.5 ml), and the mixture was stirred under N_2 atmosphere for 48 hours at r.t. The reaction mixture was partitioned into an EtOAc - sat. aq. $NaHCO_3$ mixture. The organic phase was dried under vacuum and the residue was chromatographed on silica gel (EtOAc), followed by HPLC (Applo-C18, MeOH - H_2O=5 : 1) to afford the ester (1.0 mg). The aq. $NaHCO_3$ phase was acidified with aq. 2 N HCl and extracted with EtOAc. Work-up of the EtOAc extract in the usual manner afforded the recovered acid, which was purified by HPLC (ODS-A, MeOH - H_2O=10 : 1) to furnish α-phenylbutric acid (5 mg) of $[\alpha]_D$ +4.9° (*c* 0.6, benzene). The following data were recorded for the ester: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14 (1H, br.s, H-1), 7.00 (1H, br.s, H-4), 6.82 (1H, dd, *J*=8.3, 1.5 Hz, H-6), 7.03 (1H, d, *J*=8.3 Hz, H-7), 7.21 (1H, s, H-8), 5.88 (1H, br.s, H-11), 4.32 (1H, q, *J*=7.0 Hz, H-12), 7.37 (1H, br.s, H-14), 6.08 (1H, dd, *J*=17.5, 10.5 Hz, H-16), 5.21, 5.25 (each 1H, d, *J*=17.5, 10.5 Hz, respectively, H_2-17), 2.78 (1H, dd, *J*=14.5, 9.0 Hz, H_a-18), 3.06 (1H, dd, *J*=14.5, 4.5 Hz, H_b-18), 5.09 (1H, dd, *J*=9.0, 4.5 Hz, H-19), 1.19 (3H, s, H_3-21), 1.17 (3H, s, H_3-22), 1.54 (6H, s, H_3-23/24), 1.60 (3H, d, *J*=7.0 Hz, H_3-25), 7.09 (2H, m, ph-α), 7.15 (3H, m, ph-α), 3.39 (1H, t, *J*=7.6 Hz, H-α), 1.69 (2H, m, H_2-β), 0.78 (3H, t, *J*=7.5 Hz, H_3-γ); HREI-MS *m/z* 571.3092 $[M]^+$ (calcd for $C_{34}H_{41}N_3O_5$, 571.3046); LREI-MS *m/z* 571 $[M]^+$ (rel. int. 24), 407 [M-(2-phenylbutyric acid)]$^+$ (22), 338 (19), 336 (18), 320 (8), 194 (12), 164 [2-phenylbutyric acid]$^+$ (22), 119 (35), 91 (100).

Results and Discussion

19,20-Dihydroxyisoechinulin A (**1**) was isolated as a colorless oil with a molecular composition of $C_{24}H_{31}N_3O_4$ from the HREI-MS and ^{13}C NMR data. The eleven unsaturations by HREI-MS implied that **1** contained two carbonyl groups, six double bonds and three rings.

The IR spectrum of **1** showed broad absorptions for multiple hydroxyl and amine (3358, 3262 cm^{-1}), and amide (1673, 1629 cm^{-1}) functionality.

The UV spectrum of **1** showed the presence of amide [209 nm (log ε 3.9), 226 (3.9)] and conjugated indole [289 nm (3.4), 340 (3.5)] chromophores.[4)]

In the ^{1}H NMR spectrum, five protons were exchanged by D_2O, suggesting that **1** has one aromatic amine proton [δ 10.91 (H-1)], two amide protons [δ 8.36 (H-11), 8.51 (H-14)] and two hydroxyl protons [δ 4.11 (19-OH), 4.16 (20-OH)].

The ^{1}H and ^{13}C NMR spectra of **1** showed signals ascribable to methyl substituted diketopiperazine [δ 8.36 (H-11), 4.10 (H-12), 8.51 (H-14), 1.39 (H_3-25), 124.8 (C-9), 160.0 (C-10), 51.0 (C-12), 166.6 (C-13), 20.3 (C-25)], a trisubstituted indole [δ 10.91 (H-1), 7.02 (H-4), 6.98 (H-6), 7.29 (H-7), 144.0 (C-2), 103.1 (C-3), 126.2 (C-3a), 119.2 (C-4), 132.3 (C-5), 123.0 (C-6), 111.1 (C-7), 133.9 (C-7a)], an isopentenyl [δ 6.06 (H-16), 5.01, 5.03 (H_2-17), 1.45, 1.46 (CH_3-23/24), 39.2 (C-15), 145.4 (C-16), 111.6 (C-17), 27.6 (C-23), 27.7 (C-24)], a dihydroxyisopentanyl [δ 2.36 (H_a-18), 2.93 (H_b-18), 3.27 (H-19), 1.09, 1.06 (H_3-21/22), 4.11 (19-OH), 4.16 (20-OH), 38.0 (C-18), 80.0 (C-19), 72.0 (C-20), 26.5 (C-21), 24.7 (C-22)], and a trisubstitited double bond [δ 6.87 (H-8), 110.8 (C-8), 124.8 (C-9)] (Table 2).

The connection of the functional groups in **1**, which led to the planar structure, was achieved on the basis of COSY, HMQC, HMBC and NOESY correlations. Key HMBC correlations between H-4 and C-18; between H-6 and C-18; between H-8 and C-2, C-3a, and C-10; between H-16 and C-2; between H-18 and C-4, C-6 and C-19; and between H-19 and C-5, clearly established planar structure of **1**.

The 1.9 Hz coupling constant for H-11, H-12 indicates a pseudoequatorial orientation at C-12.[6)] The configuration at C-12 was established using Marfey's method.[7)]

For this analysis alanine enantiomers were derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide and analyzed by reversed-phase HPLC. The retention times of the corresponding enantiomers (2*S* and 2*R*) were observed at 14.4 and 17.1 minutes, respectively. Analogous derivatization of the acid hydrolyzate of compound **1** followed by HPLC analysis and comparison with the

standard derivatives enabled us to deduce the (*S*) configuration at C-12.

Two factors enabled us to identify the geometry of C-8/C-9 double bond as (*Z*) configuration. The first is the NOE correlations between H-8 and H_3-23 and H_3-24, and the second is the chemical shift of H-8 (δ 6.87), which is shifted to low field by the deshielding effect of the carbonyl group on β-vinyl proton.[6)]

The absolute configuration at C-19 in compound **1** was determined to be (*R*) by the application of HOREAU'S method[8)] to **1**, where the recovered α-phenylbutyric acid showed $[\alpha]_D$ +4.9° (*c* 0.6, benzene).

Compound **1** exhibited significant radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (IC_{50}, 20 μM), the same as was observed for ascorbic acid, which was used as the positive control. Compound **1** also showed ultraviolet-A protecting activity with ED_{50} value of 130 μM, which is more active than oxybenzone (ED_{50}, 350 μM), a currently used sunscreen agent.

Further biological evaluation of dihydroxyisoechinulin A (**1**) is in progress.

Acknowledgements

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