Bionectins A−C, Epidithiodioxopiperazines with Anti-MRSA Activity, from *Bionectra byssicola* F120

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Three new epidithiodioxopiperazine compounds, bionectins A (**1**), B (**2**), and C (**3**), along with a known compound, verticillin D (**4**), have been isolated from the mycelium of liquid fermentation cultures of the fungus *Bionectra byssicola* F120. The structures of compounds **1**−**3** were assigned on the basis of MS and NMR data. Compounds **1** and **2** incorporate a dioxopiperazine moiety with a disulfide bridge in their molecules, while **3** contains a dioxopiperazine ring with two methylsulfanyl groups. Compounds **1** and **2** exhibited antibacterial activity against *S. aureus* including methicillin-resistant *S. aureus* (MRSA) and quinolone-resistant *S. aureus* (QRSA), with MIC values of 10−30 μg/mL, while **3** showed no antibacterial activity even at 100 μg/mL.

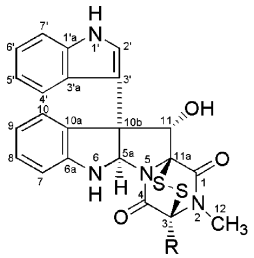
Gram-positive eubacteria are representative of pathogenic microorganisms. In particular, *Staphylococcus aureus* is the most important clinically of these pathogens because of its exceptional virulence, stress tolerance, and capacity to induce antimicrobial resistance.[1] Methicillin-resistant *S. aureus* (MRSA) is known as a major nosocomial pathogen that has also developed resistance to many other antibiotics. Moreover, MRSA has been reported to acquire resistance to the antibiotic of last resort, vancomycin.[2] It has been suggested that *S. aureus* will acquire full resistance to vancomycin in the near future. Therefore, it is increasingly important and necessary to find new classes of antibacterials.

In the course of our screening for new antibacterials from microbial metabolites, three new epidithiodioxopiperazine compounds named bionectins A−C (**1**−**3**) were isolated from the mycelium of liquid fermentation cultures of a fungal strain F120. Compounds **1**−**3** are monomeric epidithiodioxopiperazines with an indole ring like leptosins D−F[3] and bliocladines C−E.[4] These compounds are rare microbial metabolites since most of the known epidithiodioxopiperazines have been known to be dimeric, such as verticillins A−F,[5,6] leptosins A−C,[3] K, K_1, K_2,[7] and M−N_1,[8] Sch52900,[9] chaetosin and chetracin A,[10] and gliocladines A and B.[4] In this paper, we report the fermentation, isolation, structure determination, and anti-MRSA activity of **1**−**3**.

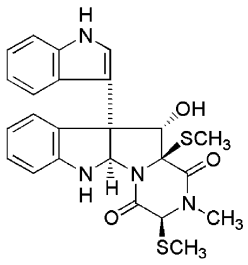
The fungal strain F120 was isolated from soil collected in Chungnam Province, South Korea. The EtOAc extract of the mycelium of liquid fermentation cultures of strain F120 was fractionated by silica gel chromatography. Final separation of the active fraction by reversed-phase HPLC afforded three new compounds (**1**−**3**), along with a known compound, verticillin D (**4**).[6]

The ^{1}H and ^{13}C NMR spectroscopic data of **4** together with its molecular weight suggested that this compound is a member of the dimeric epidithiodioxopiperazine class.[5,6] Interpretation of the ^{1}H and ^{13}C NMR data led to the identification of **4** as verticillin D.[6] The [α] value (+225, *c* 0.1, MeOH) of **4** was almost identical with the literature value for this compound (+220, *c* 0.1, MeOH).

The molecular formula of **1** was determined as $C_{22}H_{18}N_4O_3S_2$ on the basis of the HRESIMS [$(M + H)^+$, 451.0885 *m/z* (−0.8 mmu error)] in combination with the ^{1}H and ^{13}C NMR data. Compound **1** gave characteristic UV maxima at 281 and 290 nm, which were different from that of **4** (301 nm). IR absorptions of **1** at 1671 and 3403 cm^{-1} suggested the presence of carboxyl and



1 R = H
2 R = -^{13}CH(OH)^{14}CH$_3$



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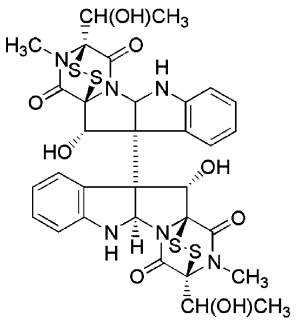

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hydroxyl moieties, respectively. The ^{1}H and ^{13}C NMR data (Table 1) supported by the ^{1}H−^{1}H COSY, DEPT, and HMQC NMR data suggested the presence of two 1,2-disubstituted benzenes, an olefinic methine (δ 7.11, d; δ 123.3), an isolated methine (δ 6.54; 83.1) attached to two nitrogens, two more isolated methines (δ 5.28; 80.1 and δ 5.19; 70.6), one *N*-methyl group, two exchangeable protons (δ 5.63 and 8.08), an sp^2 quaternary carbon (δ 113.6), two sp^3 quaternary carbons (δ 59.1 and 79.1), and two amide carbonyls (δ 163.1 and 167.0). These spectroscopic data of **1** were substantially different from those of **4**. The major differences were that one more 1,2-disubstituted benzene ring, an olefinic methine (δ 7.11, d; δ 123.3), and a quaternary sp^2 carbon (δ 113.6) appeared in **1**, instead of signals for a CH(OH)CH$_3$ group in **4**. This suggested the presence of a dithiodioxopiperazine moiety without a side chain at C-3, and an indole ring in **1**. The structure of the dithiodioxopiperazine moiety was determined by the HMBC spectrum (Figure 1). The methine proton at δ 5.19 (3-H) was long-range coupled to the two amide carbonyl carbons at δ 167.0 (C-1) and 163.1 (C-4) and the *N*-methyl carbon at δ 33.1 (C-12). The *N*-methyl protons at δ 3.02 (H_3-12) were in turn long-range coupled with the methine carbon at δ 70.6 (C-3) and the C-1 carbon. These data revealed the presence

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Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Spectroscopic Data for Compounds **1**−**3** in $CDCl_3$[a]

	1		**2**		**3**	
position	δ_H (mult., J_{HH})	δ_C	δ_H (mult., J_{HH})	δ_C	δ_H (mult., J_{HH})	δ_C
1		167.0 C		167.1 C		164.9 C
3	5.19 (s)	70.6 CH		77.2 C	4.60 (s)	60.8 CH
4		163.1 C		163.0 C		165.0 C
5a	6.54 (s)	83.1 CH	6.35 (s)	82.5 CH	6.35 (s)	81.8 CH
6	5.63 (s)		5.29 (s)		5.12 (s)	
6a		147.0 C		147.1 C		147.6 C
7	6.68 (dd, 7.5, 1.0)	110.5 CH	6.74 (dd, 7.5, 1.0)	111.1 CH	6.65 (dd, 7.5, 1.0)	110.1 CH
8	7.07 (td, 7.5, 1.0)	129.3 CH	7.16 (td, 7.5, 1.0)	129.5 CH	7.09 (td, 7.5, 1.0)	129.0 CH
9	6.75 (td, 7.5, 1.0)	120.3 CH	6.86 (td, 7.5, 1.0)	120.1 CH	6.73 (td, 7.5, 1.0)	119.3 CH
10	7.32 (dd, 7.5, 1.0)	125.3 CH	7.46 (dd, 7.5, 1.0)	124.9 CH	7.42 (dd, 7.5, 1.0)	123.5 CH
10a		131.5 C		130.9 C		131.9 C
10b		59.1 C		61.2 C		59.1 C
11	5.28 (s)	80.1 CH	5.37 (s)	81.3 CH	5.32 (s)	80.6 CH
11a		79.1 C		75.7 C		73.3 C
12	3.02 (s)	33.1 CH_3	3.23 (s)	27.8 CH_3	3.11 (s)	32.2 CH_3
13			4.57 (m)	68.1 CH		
14			1.74 (d, 6.9)	20.0 CH_3		
SCH_3-3					2.47 (s)	18.2 CH_3
SCH_3-11a					2.08 (s)	15.7 CH_3
1′	8.08 (br s)		8.05 (br s)		8.06 (br s)	
1a′		137.1 C		137.0 C		137.1 C
2′	7.11 (d, 2.4)	123.3 CH	7.06 (d, 2.4)	123.7 CH	7.11 (d, 2.5)	123.3 CH
3′		113.6 C		113.5 C		115.0 C
3a′		126.1 C		126.0 C		125.9 C
4′	7.93 (dd, 7.8, 1.0)	121.4 CH	7.95 (dd, 7.5, 1.0)	121.7 CH	7.87 (dd, 7.8, 1.0)	121.3 CH
5′	7.15 (td, 7.8, 1.0)	120.1 CH	7.18 (td, 7.5, 1.0)	120.1 CH	7.15 (td, 7.8, 1.0)	120.2 CH
6′	7.20 (td, 7.8, 1.0)	122.4 CH	7.20 (td, 7.5, 1.0)	122.6 CH	7.19 (td, 7.8, 1.0)	122.6 CH
7′	7.33 (dd, 7.8, 1.0)	111.7 CH	7.33 (dd, 7.5, 1.0)	111.6 CH	7.32 (dd, 7.8, 1.0)	111.8 CH

[a] The assignments were aided by the ^{1}H−^{1}H COSY, DEPT, HMQC, and HMBC NMR spectra.

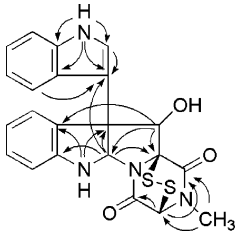

Figure 1. Key HMBC correlations of compound **1**.

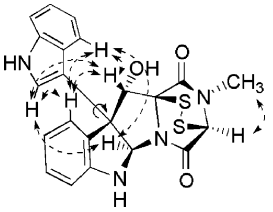

Figure 2. Key NOEs of compound **1**.

of an epidithiodioxopiperazine moiety without a side chain at C-3. Also, the indole ring was determined by the HMBC spectrum. HMBC correlations were observed from the olefinic proton at δ 7.11 (H-2′) to the quaternary sp^2 carbon at δ 115.0 (C-3′) and two quaternary sp^2 carbons at δ 137.1 (C-1′a) and 125.9 (C-3′a) of one of the 1,2-disubstituted benzene rings. The exchangeable proton at δ 8.08 (H-1′) was also long-range coupled with the carbons C-3′ and C-3′a. This indicated the presence of a 3′-substituted indole ring. The connectivity of the 3′-substituted indole ring with the epidithiodioxopiperazine-bearing part was determined by the HMBC spectroscopic data. The methine at δ 6.54 (H-5a) attached to two nitrogens was long-range coupled with C-3′ of the indole ring, indicating that the C-3′ carbon is connected to the sp^3 quaternary carbons at δ 59.1 (C-10b). The remaining structure was also confirmed by the HMBC spectroscopic data (Figure 1). Thus, the planar structure of **1** was assigned as a new derivative of leptosin D,[3] in which the isopropyl group at C-3 of leptosin D was absent. The relative configuration of **1** was determined from the NOESY NMR data (Figure 2). An NOE between H-10 and H-11 was observed, while no NOE between H-11 and H-5a was detected. The H-5a proton signal showed NOEs with H-2′ and H-4′ of the indole ring. These data indicated that H-11 and H-5a have a *trans* configuration, and also H-11 and the C-10b−C-10b′ bond have a *trans* configuration. H-10 and H-11 showed NOEs with both H-2′ and H-4′ of the indole ring, suggesting that the C-10b−C-10b′ bond freely rotates. Considering the absolute configuration of **4**, these data indicated the absolute configuration of **1** to be the same as that of leptosin D.[3]

The molecular formula of **2** was determined to be $C_{24}H_{22}N_4O_4S_2$ on the basis of the HRESIMS [(M + H)$^+$, 495.11801 *m*/*z* (+2.5 mmu error)], in combination with ^{1}H and ^{13}C NMR data. Compound **2** exhibited characteristic UV maxima at 282 and 291 nm similar to those of **1**. The IR data suggested the presence of carboxyl (1672 cm^{-1}) and hydroxyl (3407 cm^{-1}) groups. The ^{1}H and ^{13}C NMR data (Table 1) revealed signals similar to those of **1**. The major differences between those compounds were that a sp^3 quaternary carbon (δ 77.2) and signals (δ 4.57, m; δ 68.1 and 1.74, d; δ 20.0) attributable to a 1-hydroxyethyl group appeared in **2** instead of the methine (δ 5.19, s; δ 70.6) of C-3 in **1**. These spectroscopic data suggested that the 1-hydroxyethyl group is attached to C-3 in **2**. This structure was further corroborated from the HMBC spectrum. The methine proton at δ 4.57 (H-13) of the 1-hydroxylethyl group was long-range coupled with one amide carbonyl carbon at δ 163.0 (C-4), the sp^3 quaternary carbon at δ 77.2 (C-3), and the methyl carbon at δ 20.0 (C-14) of the 1-hydroxyethyl group. In addition, HMBC correlations were observed from the *N*-methyl protons at δ 3.23 (H_3-12) to the carbon of C-3 and the other amide carbonyl carbon at δ 167.1 (C-1). These data indicated the presence of the 1-hydroxyethyl group at C-3. The relative configuration of **2** was examined using the NOESY NMR spectrum. NOEs between H-10, H-11, H-5a, H-4′, and H-2′ were the same as those observed for **1**.

The molecular formula of **3** was determined to be $C_{24}H_{24}N_4O_3S_2$ on the basis of HRESIMS [(M + H)$^+$, 503.12009 *m*/*z* (+1.8 mmu error)] in combination with the ^{1}H and ^{13}C NMR data. The IR spectrum suggested the presence of carboxyl (1660 cm^{-1}) and hydroxyl (3404 cm^{-1}) groups. The ^{1}H and ^{13}C NMR data (Table

Table 2. Antibacterial Activity of Compounds **1**, **2**, and **4** (MIC, μg/mL)

organism	**1**	**2**	**4**	vancomycin
S. aureus RN4220	10	30	3	0.5
S. aureus 503	10	30	3	0.25
S. aureus CCARM 3167 (MRSA)	30	10	3	1
S. aureus CCARM 3506 (MRSA)	30	10	3	0.5
S. aureus CCARM 3505 (QRSA)	10	30	10	1
S. aureus CCARM 3519 (QRSA)	10	30	10	0.5

1) were similar to those of **1**. The differences between **1** and **3** were that two singlet methyls (δ 2.08, s; δ 15.7 and 2.47, s; δ 18.2) appeared in **3**. In addition, the methine of C-3 and the sp^3 quaternary carbon of C-11a were downfield-shifted in **3**. These data suggested that two *S*-methyl groups were formed at both C-3 and C-11a. The structure was further elucidated by the HMBC spectrum. The *S*-methyl protons at δ 2.47 (SCH_3-3) were long-range coupled to the methine carbon at δ 60.8 (C-3), which was, in turn, long-range coupled with the *N*-methyl protons at δ 3.11 (CH_3-12). HMBC correlations were also observed from the methine proton at δ 4.60 (H-3) to two amide carbonyl carbons at δ 164.9 (C-1) and 165.0 (C-4). The other *S*-methyl protons at δ 2.08 (SCH_3-11a) were long-range coupled to the sp^3 quaternary carbon at δ 73.3 (C-11a). These data demonstrated the presence of *S*-methyl groups at both C-3 and C-11a. The remaining structure was confirmed using the HMBC NMR spectrum. The relative configuration of **3** was determined using the NOESY NMR spectrum. NOEs between H-10, H-11, H-5a, H-4′, and H-2′ were also the same as those of **1**. In particular, the *S*-methyl protons of SCH_3-11a showed NOEs with H-11 and H-10, while no NOE was observed with H-5a. This confirmed that H-11 and H-5a have a *trans* configuration. Also, these data indicated that H-11 and SCH_3-11a have the *cis* configuration as expected from the stereochemistry of the disulfide bond in **1** and **2**.

Compounds **1** and **2**, monomeric epidithiodioxopiperazines with an indole moiety, exhibited antibacterial activity against various strains of *S. aureus* including methicillin-resistant *S. aureus* (MRSA) and quinolone-resistant *S. aureus* (QRSA) with MIC values of 10−30 μg/mL, as shown in Table 2. Compound **4**, a dimeric epidithiodioxopiperazine, showed stronger antibacterial activity, with MIC values of 3−10 μg /mL, than **1** and **2**. Compound **3**, however, showed no antibacterial activity even at 100 μg /mL. Vancomycin as a positive control exhibited antibacterial activity with MIC values of 0.25−1 μg/mL.

Structurally, **1**, **2**, and **4** incorporate a disulfide bridge in their dioxopiperazine ring, while **3** does not contain this functionality. The epidithiodioxopiperazine compounds such as the verticillins, the leptosins, and Sch52900 are known to have antibacterial, nematocidial, antifungal, and antitumor activities.[3,4,6,8] Leptosins A−C, dimeric epidithiodioxopiperazines, have been reported to show more potent antitumor activity than leptosin D, a monomeric epidithiodioxopiperazine. Leptosins A and C also show in vivo antitumor effects.[3] Sch52900 exerts antitumor activity by inhibiting the induction of *c-fos* proto-oncogene, a early gene involved in cell proliferation.[9] Leptosin M is known to exhibit cytotoxicity against human cancer cell lines and inhibit specifically two protein kinases, PTK and CaMKII, and human topoisomerase II.[8] The active moiety of the epidithiodioxopiperazine compounds, however, has not been reported. Our present results indicate clearly that the antibacterial activity of the epidithiodioxopiperazine class of compounds is mediated in part due to the disulfide bridge in the epidithiodioxopiperazine moiety.

Experimental Section

General Experimental Procedures. Optical rotations were determined on a JASCO P-1020 polarimeter. UV spectra were measured on a Shimadzu UV-1601 UV−visible spectrophotometer. IR spectra were obtained using a Bruker EQUINOX 55 spectrophotometer. NMR spectra were recorded on a Bruker Biospin Avance 500 spectrometer. HRESIMS data were recorded on a JEOL JMS-HX110/110A mass spectrometer.

Fungal Material. The fungal strain F120 was isolated from a soil sample collected in May 2002 near Gongju-city, Chungnam Province, Korea. The strain was identified as *Bionectria byssicola* (Berk. & Broome) Schroers & Samuels 1997 on the basis of standard biological and physiological tests and taxonomic determination by staff at the Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea. The strain was deposited in the Korean Collection for Type Cultures, Daejeon, with accession number KCTC 10881BP.

Fermentation and Isolation. Fermentation was carried out in a liquid culture medium containing YPS medium (2% glucose, 0.2% yeast extract, 0.5% peptone, 0.05% $MgSO_4$, and 0.1% KH_2PO_4, pH 5.7 before sterilization). A piece of the strain F120 from a mature plate culture was inoculated into a 500 mL Erlenmeyer flask containing 80 mL of the above sterile liquid medium and cultured on a rotary shaker (150 rpm) at 28 °C for 3 days. For the production of active compounds, 15 mL of the seed culture was transferred into 1000 mL Erlenmeyer flasks containing 300 mL of the YPS medium and cultivated on a rotary shaker (150 rpm) for 7 days under 28 °C. After incubation, the fermented liquid cultures (73 L) were centrifuged at 6000 rpm for 20 min. Then only the mycelial parts were extracted with 80% acetone since compounds **1**−**4** were not detected in the supernatants. The acetone extracts were concentrated in vacuo to an aqueous solution, which was then extracted with an equal volume of EtOAc three times.

The EtOAc extract (5 g) was subjected to silica gel (Merck Art. No. 7734.9025) column chromatography, by stepwise elution with $CHCl_3$−MeOH (100:1, 50:1). Active fractions were combined on the basis of their TLC profiles and antibacterial activity. The active fractions eluted with $CHCl_3$−MeOH (100:1) were pooled and concentrated in vacuo. The residue dissolved in MeOH was further purified by reversed-phase HPLC column (YMC C_{18}, 10 × 250 mm) chromatography. The column was eluted with MeOH−H_2O (75:25) at a flow rate of 5 mL/min to afford **1** (3 mg), with a retention time of 18.7 min.

The active fractions eluted with $CHCl_3$−MeOH (50:1) were pooled and concentrated in vacuo. Next, the residue was purified by reversed-phase HPLC column chromatography with the above conditions to afford **4** (35 mg), **2** (2.8 mg), and **3** (3 mg), with retention times of 24.3, 20.1, and 22.5 min, respectively.

Bionectin A (1): white powder; $[\alpha]_D$ +584 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 220 (4.54), 245 (sh 4.06), 281 (3.81), 290 (3.79) nm; IR (KBr) ν_{max} 3403 (OH), 2921, 1671 (CO), 745 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS $[M + H]^+$ *m/z* 451.0885 (calcd for $C_{22}H_{18}N_4O_3S_2$ + H, 451.0893).

Bionectin B (2): white powder; $[\alpha]_D$ +493 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 219 (4.59), 246 (sh 3.99), 282 (3.81), 291 (3.79) nm; IR (KBr) ν_{max} 3407 (OH), 2920, 1672 (CO), 745 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS $[M + H]^+$ *m/z* 495.1180 (calcd for $C_{24}H_{22}N_4O_4S_2$ + H, 495.1155).

Bionectin C (3): white powder; $[\alpha]_D$ +244 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 207 (4.56), 248 (sh 3.86), 282 (3.68), 290 nm (3.68); IR (KBr) ν_{max} 3404 (OH), 2921, 1660 (CO), 1425, 877 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS $[M + Na]^+$ *m/z* 503.1200 (calcd for $C_{24}H_{24}N_4O_4S_3$ + Na, 503.1182).

Determination of Antibacterial Susceptibility.[11] Two MRSA strains (CCARM3167, CCARM3506) and two QRSA strains (CCARM 3505, CCARM 3519) were obtained from the Culture Collection of Antimicrobial Resistant Microbes of Korea. *Staphylococcus aureus* was grown to mid-log phase in Mueller−Hinton broth and diluted 1000-fold in the same medium. Cells (10^5/mL) were inoculated into Mueller−Hinton broth and dispensed at 0.2 mL/well in a 96-well microtiter plate. MICs were determined in triplicate by serial dilution of test compounds. The MIC was defined as the concentration of a test compound that completely inhibited cell growth during a 24 h incubation at 30 °C. Bacterial growth was determined by measuring the absorption at 650 nm using a microtiter ELISA reader.

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