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Epicorazine C, an Antimicrobial Metabolite from *Stereum hirsutum* HKI 0195

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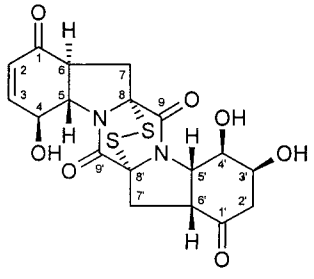
In the course of screening for new antibiotics active against methicillin-resistant *Staphylococcus aureus*, we isolated an antibacterial metabolite from the culture broth of a basidiomycete, which was named epicorazine C (**1**).

Spectroscopic analysis revealed that **1** was a member of the epidithiopiperazinedione group, represented by epicorazine A (**2**), B (**3**)[1~3] and 3822-A (**4**)[4], which were isolated also from fermentations of the same culture (Fig. 1). In this case, it was the first isolation of epicorazines from a basidiomycete. Antibiotic **1** has already been mentioned in a previous paper as derivative of **4**[5], but it was not named. No details of biological and physicochemical data of **1** have so far been published although the congeners **2** and **3** were well known in early times. Antibiotic **1** inhibited growth of Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecalis* (VRE) as well as *Candida albicans*. The isolation, structure determination and biological properties of **1** will be described in the following.

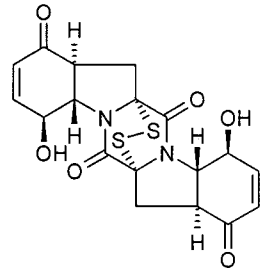
The producing strain HKI 0195 from the strain collection of the Hans-Knöll-Institut was conserved as *Stereum hirsutum* HKI 0195. Mycelial cultures of the strain HKI 0195 were derived from tissue plugs of fruit bodies of *Stereum hirsutum* collected from a forestal district of Thuringia, Germany.

A small piece of a mature slant culture of the strain HKI 0195 grown on malt extract agar (malt extract 4%, yeast extract 0.4%, agar 1.5%, deionized water at pH 6, 1 liter)

Fig. 1. Structures of epicorazines A~C (**1**~**3**) and 3822-A (**4**).



1



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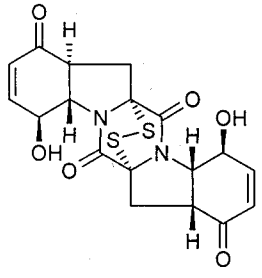

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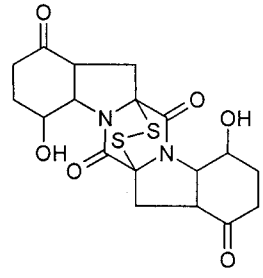

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was used to inoculate 500 ml-Erlenmeyer bottles containing the medium consisting of glucose 1%, sucrose 1%, corn starch 1%, yeast extract 0.1%, casein peptone 0.1%, soybean meal 0.5%, $(NH_4)_2SO_4$ 0.5%, $(NH_4)_2HPO_4$ 0.05%, and $CaCO_3$ 0.03%. The surface cultures were incubated for 28 days at 23°C.

For the isolation of metabolites **1**~**4**, the culture broth (10 liters) was extracted at pH 6 with equal volumes of EtOAc, and the metabolites were obtained by several subsequent chromatography steps. At first the residue (6.95 g) was purified by flash chromatography on silica gel (Merck, 0.063~0.1 mm) prepared with $CHCl_3$. The fractions were eluted stepwise with $CHCl_3$/MeOH (9 : 1 and 1 : 1) and MeOH. The eluates were analyzed by HPLC and tested for antimicrobial activity. The active fractions were combined and finally subjected to preparative HPLC in 20 mg batches (20 runs) using a binary gradient of 0.1% TFA to 83% CH_3CN (99.5 : 0.5 to 0.5 : 99.5, 22 minutes, column 10×250 mm, Nucleosil 100-5, C18, flow rate 5 ml/minute, detection at 230 nm). The fractions were concentrated and lyophilized. Retention time (Rt) of the fraction containing **1**: 10.3 minutes (yield 12 mg), Rt of **2**: 12.7 minutes (yield 10 mg), Rt of **3**: 11.1 minutes (yield 9 mg) and Rt of **4**: 10.0 minutes (trace).

Table 1. ^1H and ^{13}C NMR shifts of epicorazine C (**1**) (in DMSO-d_6 and $CHCl_3$, δ in ppm).

Carbon No.	δ_C[a] (DEPT) (DMSO-d_6)	δ_C[a] ($CDCl_3$)	δ_H[b] (mult, *J* Hz) (DMSO-d_6)	δ_H[b] (mult, *J* Hz) ($CDCl_3$)
1	194.87 (C)	193.72		
2	128.64 (CH)	129.23	6.06 (dd, 10.0, 2.3)	6.13 (dd, 10.2, 2.4)
3	151.16 (CH)	150.65	6.89 (dd, 10.0, 1.8)	6.93 (dd, 10.2, 1.8)
4	70.50 (CH)	71.05	4.67 (ddd, 8.6, 2.3, 1.8)	4.77 (ddd, 8.2, 2.4, 1.8)
5	68.18 (CH)	69.29	3.98 (dd, 13.0, 8.6)	3.77 (dd, 13.1, 8.2)
6	48.11 (CH)	48.95	3.34 (ddd, 13.0, 12.3, 5.6)	3.23 (ddd, 13.1, 12.4, 5.5)
7	29.67 (CH_2)	30.65	2.84 (dd, 14.2, 12.3)	3.00 (dd, 14.9, 12.4)
			2.54 (dd, 14.2, 5.6)	2.57 (dd, 14.9, 5.5)
8	75.28 (C)	75.65		
9	162.31 (C)	163.93		
1'	208.28 (C)	204.65		
2'	43.64 (CH_2)	42.83	2.69 (dd, 16.3, 10.3)	2.76 (dd, 19.2, 2.6)
			2.52 (m)	2.59 (dd, 19.2, 4.3)
3'	65.87 (CH)	68.98	3.71 (ddd, 10.3, 4.5, 2.0)	4.33 (ddd, 4.3, 2.6, 1.8)
4'	65.79 (CH)	72.79	4.78 (m)	3.89 (dd, 6.5, 1.8)
5'	63.68 (CH)	66.68	4.40 (dd, 7.6, 4.4)	4.69 (dd, 9.9, 6.5)
6'	46.31 (CH)	46.93	3.17 (m)	3.61 (ddd, 9.9, 9.1, 1.8)
7'	32.42 (CH_2)	30.65	2.97 (dd, 14.8, 8.2)	3.14 (dd, 15.4, 1.8)
			2.76 (dd, 14.8, 2.4)	3.01 (dd, 15.4, 9.1)
8'	76.09 (C)	76.35		
9'	164.05 (C)	164.55		

[a] 125 MHz, [b] 500 MHz

The UV and IR spectra of **1** resemble those of **2**~**4**[1~3]. The UV spectra of **1**~**4** displayed only a λ_{max} at 220 nm. Further support was inferred from the IR spectrum in KBr due to λ_{max} 1672 cm^{-1} (α,β-unsaturated carbonyl), 1695 cm^{-1} (amide carbonyl of a *N*-substituted diketopiperazine) and 3400 cm^{-1} (OH). **1** has optical rotation properties: $[\alpha]_D^{20}$ −350.5° (*c* 0.19, MeOH).

Structure elucidation of **1** was based on mass spectrometric and NMR spectroscopic measurements. The electrospray mass spectrum (ESI-MS, triple quadrupole mass spectrometer Quattro 400, VG Biotech, Altrincham, U.K.) of **1** showed *m/z* 439 ($[M+H]^+$), 456 ($[M+NH_4]^+$), and 461 ($[M+Na]^+$). The elemental composition of **1** was suggested by 439.0624 ($[M+H]^+$; calcd. 439.0634 for $C_{18}H_{19}N_2O_7S_2$) in HRFAB-MS (double-focusing mass spectrometer AMD 402, Intectra, Harpstedt, Germany). Besides the pseudomolecular ions a strong fragment was visible at *m/z* 375 ($[M+H-S_2]^+$), comparable to that reported for **3**[3]. Similarly, the formulas of **2** ($C_{18}H_{17}N_2O_6S_2$; $[M+H]^+$ *m/z* 421.0546; calcd. 421.0528), **3** ($C_{18}H_{17}N_2O_6S_2$; $[M+H]^+$ *m/z* 421.0549; calcd. 421.0528), and **4** ($C_{18}H_{21}N_2O_6S_2$; $[M+H]^+$ *m/z* 425.0883; calcd. 425.0841) were determined by HRFAB-MS.

The chemical structure of **1** as an epidithiopiperazinedione was unambiguously settled by detailed homo- and heteronuclear 2D-NMR experiments (COSY, NOESY, HSQC, HMBC). The multiplicities of the carbon atoms were assigned using DEPT spectra. The assignment of the ^1H and ^{13}C NMR signals is shown in Table 1.

By comparison of the physical-chemical data of **1**, **2** and **3** the close structural relationship of these three metabolites is apparent.

The absolute stereochemistry of **2** and **3** was derived by X-ray diffraction analysis of crystalline **2** and **3** and their CD and NMR data[2,3]. Although the absolute configuration of **1** was not determined by X-ray analysis, the absolute stereochemistry of **1** could be derived from its CD and NMR measurements.

The sign of the charge transfer transition CD bands can be used to predict the absolute configuration of epidithiopiperazinedione systems[6]. The CD spectrum of **1** was similar to that of **3**. It showed a strong negative band at 236 nm and a positive at 265 nm which are consistent with a diketopiperazine ring bridged by a disulfide bond. The

Table 2. Antimicrobial activity of epicorazines A~C (**1**~**3**).

Microorganisms	MIC (μg/ml)		
	1	**2**	**3**
1. *Bacillus subtilis* ATCC 6633	25	25	25
2. *Staphylococcus aureus* SG 511	25	25	25
3. *S. aureus* 134/94 MRSA	25	25	25
4. *Enterococcus faecalis* 1528 VRE	12.5	12.5	12.5
5. *Escherichia coli* SG 458	>100	>100	>100
6. *Pseudomonas aeruginosa* SG 137	>100	>100	>100
7. *Mycobacterium smegmatis* SG 987	>100	>100	>100
8. *M. aureum* SB 66	50	50	50
9. *Sporobolomyces salmonicolor* SBUG 549	>100	>100	>100
10. *Penicillium notatum* JP 36	>100	>100	>100
11. *Candida albicans* BMSY 212	12.5	12.5	12.5

1~8 MIC obtained from microtiter test[10]

9~10 MIC obtained from agar well diffusion test[11]

11 MIC obtained from microtiter test[12]

Table 3. Antiproliferative and cytotoxic effects of epicorazines A~C (**1**~**3**).

Effect	Cell line	IC_{50} (μg/ml)		
		1	**2**	**3**
Antiproliferative effect	L 929 (mouse fibroblast cells)	2.60	0.10	4.00
	K 562 (human leukemia)	0.14	0.04	1.10
Cytotoxic effect	HeLa (human cervical carcinom)	1.40	1.90	3.60

similarity of both CD spectra indicates that **1** possesses the same absolute configuration as **3**.

The stereochemistry of the hexagonal ringsystems was determined by the different intensities of NOE cross peaks observed in the NOESY spectra.

Moreover, the signals at δ 4.33 and 3.89 ($CDCl_3$) could be assigned to the two protons H-3′ and H-4′. They are in *cis* position, as it could be expected from the proton coupling constant $J_{3',4'}=1.8$ Hz. According to this assignment, all these data are consistent with the absolute configuration of C-4′ and C-3′ as shown in Fig. 1. Since the absolute configuration of C-4′ is known for **2** and **3**, it is assumed that the stereochemistry of C-4′ is preserved through this series of metabolites.

Consequently, since **1**, **2** and **3** are co-produced in the same fermentation, it can be concluded that the absolute configuration at the asymmetric centers in all three metabolites is identical and represented by structures **1**, **2** and **3** (Fig.1). Also, on a biogenetic basis, such an assignment is reasonable.

The interest in diketopiperazine-type metabolites is due to their activity in various pharmacological assay systems[7~9]. The antimicrobial activities of **1** were compared with those of the other epidithioketopiperazinediones **2** and **3**. Table 2 summarizes the antibacterial activities, which were determined by the broth dilution susceptibility tests. The metabolites were dissolved in methanol. The epicorazines A~C exhibited comparable results. Gram-positive bacteria were more sensible than Gram-negative bacteria and also *Candida albicans* was inhibited. An interesting fact was the sensitivity of resistant strains such as *Staphylococcus aureus* (MRSA) and *Enterococcus faecalis* (VRE).

The epicorazines A~C also exhibited antiproliferative effects against several mouse fibroblast and cancer cell lines. They were highly cytotoxic against HeLa cells (Table 3), where epicoracine B was most potent amongst the congeners.

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