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Full Papers

Microbial Metabolism Studies of Cyanthiwigin B and Synergetic Antibiotic Effects

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Microbial transformation studies of the marine diterpene cyanthiwigin B (**1**), isolated from the Jamaican sponge *Myrmekioderma styx*, were accomplished. Two actinomycete cultures, *Streptomyces* NRRL 5690 and *Streptomyces spheroides*, significantly metabolized cyanthiwigin B to new metabolites. *Streptomyces* NRRL 5690 transformed cyanthiwigin B to three new compounds, cyanthiwigins AE (**2**), AF (**3**), and AG (**4**), and the known cyanthiwigin R (**5**). *S. spheroides* transformed cyanthiwigin B to cyanthiwigins S (**6**), E (**7**), and AE (**2**). All microbial-metabolized derivatives (**2–7**) of cyanthiwigin B exhibited the ability to increase the antimicrobial activity of curcuphenol, the major antimicrobial sesquiterpene isolated from *M. styx*.

Cyanthiwigins are a class of 5,6,7-tricarbocyclic diterpenes, which were first isolated from the Jamaican sponge *Epipolasis reiswigi* in 1992.¹ Cyanthiwigin C and two analogous epoxides were reported in the same year from the Venezuelan sponge *Myrmekioderma styx*.² Since this diterpene class has the same tricarbocyclic skeleton as the cyanthins, metabolites from the bird's nest fungus *Cyanthus* sp.,³ they were named cyanthiwigins. In the interest of identifying anticancer and anti-infective leads from Jamaican deep reef invertebrates, we isolated thirty diterpenes, cyanthiwigins A–AD, from the sponge *Myrmekioderma styx*,^{4,5} collected using closed-circuit rebreathers and of which cyanthiwigin B (**1**) is produced in the highest yield. Additionally, cyanthiwigin B has the unique characteristic of increasing the antimicrobial activity of curcuphenol (**8**), a sesquiterpene isolated from the same sponge.⁶ Recently, a total synthesis of cyanthiwigin U was achieved.⁷ Microbial transformations have been shown to be a productive method for the modification of natural products⁸ and represents a useful in vitro system to evaluate animal metabolism. To optimize the synergetic antimicrobial effect, cyanthiwigin B (**1**) was subjected to a biotransformation study.

Biotransformations of terpenoids have been reported as having particular significance due to their application as fragrances or flavoring agents, their antibacterial, antiviral, or cytotoxic activities,

and their increasing importance as specific pharmacological tools. The ability of microbial transformations to introduce functional groups with regio- and stereoselectivity remains unrivaled by existing chemical methodologies.⁹ In most cases biotransformation leads to the inactivation of drugs. However the biotransformation can also lead to the generation of more active or less toxic metabolites. In some instances, biotransformations can produce reactive or toxic intermediates with potential toxicological implications. Hence, an understanding of the metabolism of new chemical entities is a valuable component of the drug discovery process.¹⁰

Results and Discussion

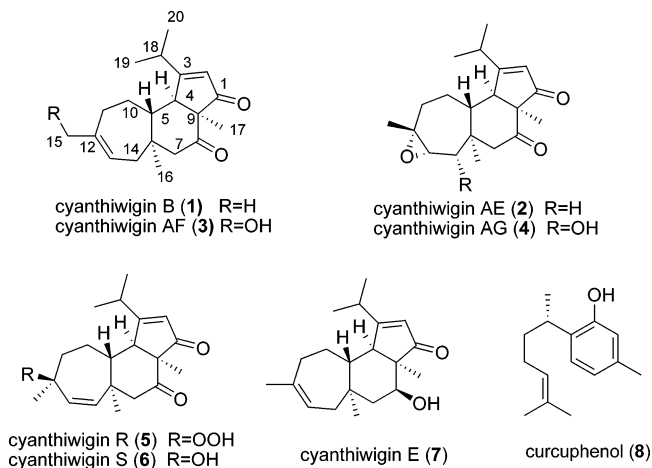
Ten microorganisms were screened for their ability to transform cyanthiwigin B (**1**) to more active metabolites. Two actinomycete cultures, *Streptomyces* NRRL 5690 and *Streptomyces spheroides*, showed significant biocatalysis of cyanthiwigin B and were selected for preparative-scale fermentation. *Streptomyces* NRRL 5690 was able to transform cyanthiwigin B to cyanthiwigins AE (**2**, 13.5%), AF (**3**, 3%), AG (**4**, 3%), and R (**5**, 2%). *S. spheroides* transformed cyanthiwigin B to cyanthiwigins S (**6**, 2%), E (**7**, 1%), and AE (**2**, 1.5%).

Cyanthiwigin AE (**2**) was obtained as a white powder. Its molecular formula was deduced as C₂₀H₂₈O₃ by high-resolution ESIMS [M + Na] 339.1918 (calcd 339.1936). The ¹H NMR provided characteristic signals of cyanthiwigin-type diterpenes, including an olefinic proton at δ 5.81 (H-2), a methine proton at δ 2.73 (d, *J* = 8.9 Hz, H-4), and five methyl protons at δ 0.94 (s),

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1.16 (s), 1.22 (s), 1.19 (d, $J = 7.0$ Hz), and 1.29 (d, $J = 6.5$ Hz). In comparison to cyanthiwigin B (1), 2 revealed the absence of carbon signals of the double bond between C-12 and C-13 and new carbon signals at δ 60.0 and 62.3, which indicated the double bond was converted to an epoxy functionality. The structure was verified by long-range heteronuclear correlations (HMBC) of δ 2.03 (H-11), 1.88 (H-14), and 1.22 (H-15) to δ 60.0 (C-12) and 62.3 (C-13), as well as δ 2.84 (H-13) to δ 41.3 (C-6,14), 60.0 (C-12), and 24.5 (C-15). The 1D and 2D NMR data for the remainder of the structure were superimposable with that of cyanthiwigin B (1). The relative configuration of the epoxide group was assigned as α by comparison of the coupling constant and NOE correlations of H-13 with those of cyanthiwigins H and J.⁴ The signal of H-13 was shown to be a triplet with a small 3J coupling constant of 3.6 Hz for cyanthiwigin AE, which is significantly different compared to the large 3J values of 8.0 and 7.2 Hz for cyanthiwigins H and J. In the NOESY spectrum, H-13 (δ 2.84) did not show correlations to H-16 (δ 0.94), while the NOE correlations between H-13 and H-16 were observed in cyanthiwigins H and J.⁴ These indicated the α orientation of the epoxide group of cyanthiwigin AE rather than the β orientation for cyanthiwigins H and J. To confirm the stereochemistry, crystals of 2 were grown in acetone. X-ray

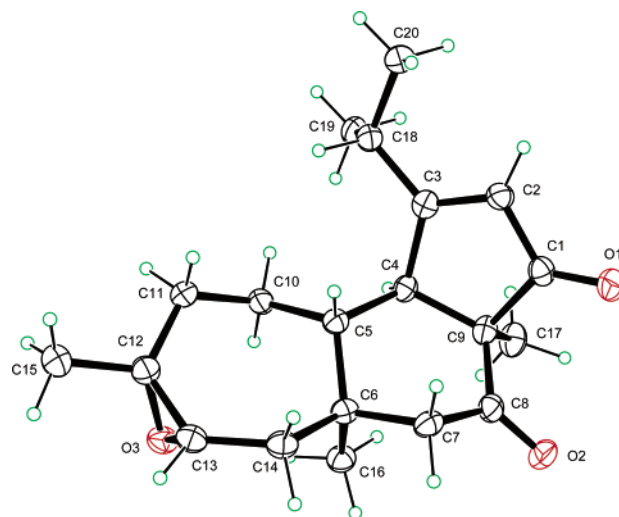


Figure 1. X-ray structure of 2.

diffraction analysis was performed, and the crystal structure with relative configuration is shown in Figure 1.

Cyanthiwigin AF (3) was obtained as a white powder. The molecular formula $C_{20}H_{28}O_3$ was shown by high-resolution ESIMS [$M + Na$] 339.1927 (calcd 339.1936) to have one more oxygen than that of cyanthiwigin B (1). The 1H NMR spectral data of 3 were comparable with that of cyanthiwigin B with the exception of the loss of a methyl proton signal at δ 1.74 (H-15) and the addition of an oxygenated methylene proton at δ 3.94, which suggested a hydroxyl group at C-15. The ^{13}C NMR spectrum revealed the hydroxymethylene carbon at δ 66.8, which was confirmed to be at C-15 by long-range heteronuclear correlation with H-11 (δ 2.19).

Cyanthiwigin AG (4) was isolated as a white powder, and its molecular formula $C_{20}H_{28}O_4$ was obtained from the high-resolution ESIMS [$M + Na$] 355.1854 (calcd 355.1885), which has the same degree of unsaturation but two more oxygen atoms than cyanthiwigin B (1). The 1H and ^{13}C NMR data (see Table 1) showed that cyanthiwigin AG is both a hydroxylated and epoxidized derivative

Table 1. 1H and ^{13}C NMR Data of Cyanthiwigins AE, AF, and AG (2–4)^a

	2		3		4	
	δ_H (J in Hz)	δ_C , mult.	δ_H (J in Hz)	δ_C , mult.	δ_H (J in Hz)	δ_C , mult.
1		204.3, qC		204.2, qC		203.7, qC
2	5.81, s	122.4, CH	5.84, s	122.7, CH	5.81, s	122.2, CH
3		192.9, qC		192.9, qC		192.6, qC
4 α	2.73, d (8.9)	58.9, CH	2.71, d (9.2)	58.7, CH	2.76, d (8.4)	58.9, CH
5 β	1.50, t (8.8)	52.5, CH	1.77, t (9.6)	56.5, CH	1.45, t (9.2)	49.0, CH
6		41.3, qC		38.8, qC		46.1, qC
7 α	1.91, d (13.0)	56.1, CH ₂	1.91, d (13.6)	56.3, CH ₂	1.85, d (12.4)	51.5, CH ₂
7 β	2.10, d (13.0)		2.15, d (13.6)		2.67, d (12.4)	
8		204.0, qC		204.7, qC		204.2, qC
9		63.1, qC		63.4, qC		62.8, qC
10 α	1.81, m	27.3, CH ₂	1.51, m	28.7, CH ₂	1.89, m	27.6, CH ₂
10 β	1.73, m		2.03, m		2.04, m	
11 α	2.03, m	33.7, CH ₂	2.01, m	38.5, CH ₂	2.05, m	33.9, CH ₂
11 β			2.19, m		1.61, dt (1.2, 15.2)	
12		60.0, qC		145.5, qC		58.8, qC
13	2.85, t (3.6)	62.3, CH	5.57, m	121.2, CH	3.06, br. s	69.7, CH
14 α	1.88, br. s	41.3, CH ₂	1.86, dd (8.8, 14.4)	42.0, CH ₂	3.66, d (5.6)	74.4, CH
14 β			2.22, m			
15	1.22, s	24.5, CH ₃	3.94, s	66.8, CH ₂	1.24, s	24.9, CH ₃
16	0.94, s	19.6, CH ₃	0.72, s	17.5, CH ₃	0.95, s	14.2, CH ₃
17	1.16, s	23.9, CH ₃	1.19, s	24.0, CH ₃	1.16, s	23.6, CH ₃
18	2.87, m	30.5, CH	2.91, m	31.2, CH	2.85, m	29.9, CH
19	1.29, d (6.5)	21.0, CH ₃	1.29, d (6.8)	20.7, CH ₃	1.30, d (6.8)	20.8, CH ₃
20	1.19, d (6.5)	21.3, CH ₃	1.18, d (6.8)	21.3, CH ₃	1.20, d (6.8)	21.2, CH ₃
OH			3.79, br. s		4.45, d (5.6)	

^a 1H NMR at 400 MHz, ^{13}C NMR at 100 MHz, referenced to residual solvent acetone-*d*₆.

Table 2. Antimicrobial Activity^a

	antifungal IC ₅₀ (μg/mL)		antibacterial IC ₅₀ (μg/mL)	
	<i>Candida albicans</i>	<i>Cryptococcus neoformans</i>	<i>Staphylococcus aureus</i>	MRS ^b
curcuphenol	>15	15	>15	>15
cyanthiwigin B	NA	NA	NA	NA
cyanthiwigin B + curcuphenol, 1.1:1.8	>15	6.0	6.5	7.0
cyanthiwigin E	NA	NA	NA	NA
cyanthiwigin E + curcuphenol, 0.7:1.7	>15	6.0	6.5	6.5
cyanthiwigin R	NA	NA	NA	NA
cyanthiwigin R + curcuphenol 1.0:1.8	>15	10	>15	>15
cyanthiwigin S	NA	NA	NA	NA
cyanthiwigin S + curcuphenol, 0.7:1.8	15	6.5	8.0	7.5
cyanthiwigin AE	NA	NA	NA	NA
cyanthiwigin AE + curcuphenol, 0.6:1.8	15	6.0	6.5	6.5
cyanthiwigin AF + curcuphenol, 0.5:1.8	15	5.0	6.5	6.0
cyanthiwigin AG + curcuphenol, 0.5:1.8	>15	7.5	8.5	9.5

^a IC₅₀ values for curcuphenol and cyanthiwigin mixtures were calculated on the basis of the mass of curcuphenol. ^b MRS = methicillin-resistant *Staphylococcus aureus*.

of cyanthiwigin B. The epoxide functionality, δ_{H} 3.06, δ_{C} 58.8 and 69.7, was located between C-12 and C-13 on the basis of the HMBC correlations between δ 1.24 (H-15), 2.05 (H-11 α), 1.61 (H-11 β) and C-12 and C-13. The hydroxyl group was assigned to C-14 by the HMBC correlations from δ 0.95 (H-16) to δ 74.4 (C-14) and from δ 3.66 (H-14) to δ 46.1 (C-6) and 14.2 (C-16). The rest of the molecule remained unmetabolized. A correlation between H-14 and H-5 was observed in the NOESY experiment, indicating that H-14 has the same β orientation with H-5. The broad singlet of the H-13 signal suggested a *cis* axial–equatorial relationship of H-13 and H-14. H-13 has NOE correlations with H-14 and H-15, indicating the β orientation of H-13 and CH₃-15 and the α orientation of the epoxy group.

Compounds **5–7** were identified as cyanthiwigins R, S, and E by comparison of the TLC and NMR data with the authentic samples isolated from the same sample of sponge from which cyanthiwigin B was isolated.⁴ These data suggest that cyanthiwigins R, S, and E, originally isolated from the sponge *M. styx*, may be metabolites of sponge-associated microbes.

All microbial metabolites (**2–7**) of cyanthiwigin B were tested for their ability to increase the antimicrobial activity of curcuphenol (**8**), and the results are shown in Table 2. Cyanthiwigins were first tested for antimicrobial activity and then mixed with curcuphenol, followed by repeat testing for antimicrobial activity. Due to the limited amount of cyanthiwigins AF (**3**) and AG (**4**), they were directly mixed with curcuphenol. Curcuphenol exhibited antimicrobial activity with an IC₅₀ value of 15 μg/mL against *Cryptococcus neoformans* and an IC₅₀ > 15 μg/mL against *Candida albicans*, *Staphylococcus aureus*, and methicillin-resistant *S. aureus* (MRS). Cyanthiwigins B (**1**), AE (**2**), R (**5**), S (**6**), and E (**7**) were not active against all the tested microbes when assayed alone. All mixtures exhibited better activity than curcuphenol, indicating the ability of cyanthiwigins to increase the antimicrobial activity. Cyanthiwigins AE (**2**) and AF (**3**) are more potent than cyanthiwigins B (**1**), E (**7**), and S (**6**), and cyanthiwigin R (**5**) has the least potency.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. IR and UV spectra were obtained using an AATI Mattson Genesis Series FTIR and a Hewlett-Packard 8452A diode array spectrometer. NMR spectra were measured on a Bruker Avance DRX-400. ¹H and ¹³C NMR spectra were measured and reported in ppm by using the residual solvent peak as an internal standard (acetone-*d*₆, δ 2.05 for proton, δ 206.7 for carbon). ESI-FTMS analyses were measured on a Bruker-Magnex BioAPEX 30es ion cyclotron HR-FT spectrometer by direct injection into an electrospray interface. HPLC was carried out on a Waters 510 model system and a dual-wavelength detector.

X-ray Analysis. The crystal was colorless orthorhombic, space group *P*₂₁₂₁ with cell dimensions *a* = 8.474(3) Å, *b* = 10.392(3) Å, *c* = 19.749(8) Å, *V* = 1739.1(11) Å³, *Z* = 4, *D*_x = 1.208 Mg m⁻³. Intensity data for cyanthiwigin AE (**2**)¹¹ were collected at 102 K on a Nonius KappaCCD (with Oxford Cryostream) diffractometer equipped with Mo K α (λ = 0.71073 Å) radiation using ω - 2θ scans, 2θ range 2.5–27.5°. A total of 2266 independent reflections were obtained with 1871 reflections having *I* > 2 σ (*I*). Data reduction was by DENZOSMN¹¹ and SCALEPACK.¹¹ The structure was solved by direct methods and refined by full-matrix least-squares using SHELXL.¹³ All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were constrained in calculated positions. Atomic coordinates, crystal data, final *R* values, and other details are included in Tables 3, 4, and 5, Supporting Information.¹³

Material. Cyanthiwigin B (**1**) was isolated from *Myrmekioderma styx*, and its structure was determined by spectral analysis as previously reported.⁴

Microorganisms. *Streptomyces* NRRL 5690, *S. argenteolus*, *S. seokies*, *S. spheroides*, *Saccharomyces cerevisiae*, *Penicillium* sp., *Aspergillus* sp., *Geotrichum* sp., *Fusarium solani*, and *F. oxysporium* were obtained from the collection at the Department of Pharmacognosy, The University of Mississippi.

Microbial Metabolism. All microorganisms were subjected to screening for their ability to transform cyanthiwigin B by inoculating them in 25 mL of media that contained 3 mg of cyanthiwigin B. The cultures were shaken in an incubator shaker at 200 rpm, 28 °C. The biotransformation process was monitored at 3, 5, 10, 14, and 18 days using TLC. *Streptomyces* NRRL 5690 was able to metabolize the compound in 5 days, while *S. spheroides* metabolized the compounds from 5 to 10 days. The rest of the microorganisms did not metabolize the compound until the 18th day of fermentation.

For scaling-up *Streptomyces* NRRL 5690 and *S. spheroides* were grown in 100 mL of media containing 20 mg of cyanthiwigin B. The cultures were shaken in an incubator shaker at 200 rpm, 28 °C. The fermentation was stopped after 5 days for *Streptomyces* NRRL 5690 and 10 days for *S. spheroides*.

Isolation. The whole culture of *Streptomyces* NRRL 5690 was extracted with EtOAc. The extract (15.9 mg) was subjected to HPLC using a Phenomenex Luna C8 column, 5 μm, 250 × 21.5 mm, eluted with a gradient of 50% acetonitrile in H₂O to 100% acetonitrile over 50 min at a flow rate of 6 mL/min and a detector wavelength of 254 nm. Fractions 9 and 13 gave pure cyanthiwigins AE (**2**, 2.7 mg) and R (**5**, 0.4 mg), respectively. Fraction 5 was separated by silica gel preparative thin-layer chromatography using hexane–EtOAc (1:1) as a developing solvent to yield cyanthiwigins AF (**3**, 0.6 mg) and AG (**4**, 0.6 mg).

The whole extract of *S. spheroides* was extracted with EtOAc. The extract (13.2 mg) was chromatographed using an HPLC column with the same conditions as above. Fractions 9 and 17 gave cyanthiwigins S (**6**, 0.4 mg) and E (**7**, 0.2 mg). Fraction 13 yielded cyanthiwigin AE (**2**, 0.3 mg) after purification by PTLC (silica gel, hexanes–EtOAc, 1:1).

Cyanthiwigin AE (2): colorless crystal; $[\alpha]_D^{26} -285$ (*c* 0.09, MeOH); UV (MeOH) λ_{\max} (log ϵ) 236 (4.15) nm; IR (film) ν_{\max} 2971, 1725, 1689, 1605, 1433, 1383, 1261, 1171, 870 cm^{-1} ; HRESIMS *m/z* 339.1918 [M + Na]⁺ (calcd for C₂₀H₂₈O₃Na 339.1936), 655.3964 [2M + Na]⁺ (calcd for C₄₀H₅₆O₆Na 655.3975).

Cyanthiwigin AF (3): white powder; $[\alpha]_D^{26} -284$ (*c* 0.06, MeOH); UV (MeOH) λ_{\max} (log ϵ) 236 (4.00) nm; IR (film) ν_{\max} 3448, 2963, 1724, 1687, 1603, 1455, 1384, 1260, 1086, 868 cm^{-1} ; HRESIMS *m/z* 339.1927 [M + Na]⁺ (calcd for C₂₀H₂₈O₃Na 339.1936), 655.3981 [2M + Na]⁺ (calcd for C₄₀H₅₆O₆Na 655.3975).

Cyanthiwigin AG (4): white powder; $[\alpha]_D^{26} -145$ (*c* 0.06, MeOH); UV (MeOH) λ_{\max} (log ϵ) 236 (3.86) nm; IR (film) ν_{\max} 3436, 2964, 1725, 1691, 1605, 1454, 1259, 1006, 826 cm^{-1} ; HRESIMS *m/z* 355.1854 [M + Na]⁺ (calcd for C₂₀H₂₈O₄Na 355.1885), 687.3824 [2M + Na]⁺ (calcd for C₄₀H₅₆O₈Na 687.3872).

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Supporting Information Available: X-ray analysis and crystal data for cyanthiwigin AE (2). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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