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Microbial Metabolism Studies of Cyanthiwigin B and the Synergetic Antibiotic effects

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Abstract

Microbial transformation studies of the marine diterpene cyanthiwigin B (**1**), isolated from the Jamaican sponge *Myrmekioderma styx*, were accomplished. Two actinomycetes 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**), 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 a Jamaican sponge *Epipolasis reiswigi* in 1992.[1] Cyanthiwigin C and two analogous epoxides were reported in the same year from a 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] of which cyanthiwigin B (**1**) is presented 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, total synthesis of cyanthiwigin U was achieved.[7] Microbial transformations have been shown to be a productive method for the modification of natural products.[8] In order to improve 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 agents. The ability of microbial transformations to introduce functional groups with regio- and stereo-selectivity 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 a more active or less toxic metabolites. In some instances, biotransformations can produce reactive or toxic intermediates with potential toxicological implications. Hence, an

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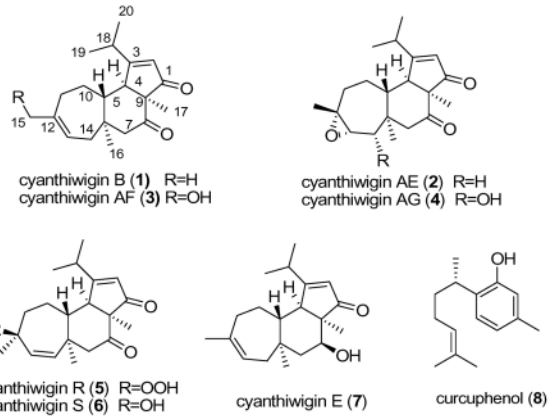
understanding of the metabolism of new chemical entities is a valuable component of the drug discovery process.[10]

Results and Discussion

Ten microorganisms were screened for their ability to transform cyanthiwigin B (**1**) to more active metabolites. Two actinomycetes 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 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 $C_{20}H_{28}O_3$ by high-resolution ESIMS [M+Na] 339.1918 (calcd. 339.1936). The ^{1}H 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), 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 bound between C-12 and C-13 and new carbon signals at δ 60.0 and 62.3, which indicated the double bound 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), 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 stereochemistry 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 as 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 diffraction analysis was performed and the crystal structure with relative configuration is shown in Fig. 1.

Cyanthiwigin AF (**3**) was also 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 ^{1}H 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 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 based on the HMBC correlations between δ 1.24 (H-15), 2.05 (H-11α), 1.61 (H-11β) 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 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 CH_3-15 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] This data suggested that cyanthiwigins R, S, and E, originally isolated from the sponge *M. styx*, maybe metabolites of sponge associated microbes.

All microbial metabolites (**2**–**7**) of cyanthiwigin B were tested for their ability to increase the anti-microbial activity of curcuphenol (**8**) and the results are shown in Table 2. Cyanthiwigins were first tested for the antimicrobial activity, and then mixed with curcuphenol followed by the 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 anti-microbial activity with IC_{50} 15μg/mL against *Cryptococcus neoformans* and IC_{50} > 15μg/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. Cyanthiwigin AE (**2**) and AF (**3**) are more potent than cyanthiwigin B (**1**), E (**7**), and S (**6**), and cyanthiwigin R (**5**) has the least potency.

Experimental Section

General Experiment 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 Bruker Avance DRX-400. ^1H and ^{13}C-NMR spectra were measured and reported in ppm by using the residual solvent peak as an internal standard (acetone-d6, 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 spectrometry 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 $P2_12_12_1$ with cell dimensions a = 8.474 (3) Å, b = 10.392 (3) Å, c = 19.749 (8) Å, V = 1739.1 (11) $Å^3$, Z = 1, D_x = 1.208 Mgm^{-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 scan, 2θ range 2.5–27.5°. 2266 independent reflections were obtained with 1871 reflections having $I > 2\sigma(I)$. Data reduction was by DENZOSMN[11] and SCALEPAK.[12] 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.

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, Saccahromyces cerevisiae, Penicillium sp, Aspergillus sp, Geotrichum sp, *Fusarium solani* and *F. oxysporium.* All microorganisms 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 media that contain 3 mg cyanthiwigin B. The cultures were shaken in an incubator shaker at 200 rpm, 28°. 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 18 days fermentation. For scaling-up *Streptomyces* NRRL 5690 and *S. spheroides* were grown in 100 mL media containing 20 mg of cyanthiwigin B. The cultures were shaken in an incubator shaker at 200 rpm, 28°. 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 H_2O to 100% acetonitrile over 50 min at a flow rate of 6 mL/min and a detector wavelength of 254 nm. Fraction 9 and 13 gave pure cyanthiwigin 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 cyanthiwigin AF (**3**, 0.6 mg) and AG (**4**, 0.6 mg).

The whole extract of *Streptomyces spheroides* was extracted with EtOAc. The extract (13.2 mg) was chromatographed using an HPLC column using the same condition as above. Fraction 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; $[\alpha]^{26}_{D}$ –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 $C_{20}H_{28}O_3Na$ 339.1936), 655.3964 $[2M+Na]^+$ (calcd for $C_{40}H_{56}O_6Na$ 655.3975).

Cyanthiwigin AF (3)

white powder; $[\alpha]^{26}_{D}$ -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 $C_{20}H_{28}O_3Na$ 339.1936), 655.3981 $[2M+Na]^+$ (calcd for $C_{40}H_{56}O_6Na$ 655.3975).

Cyanthiwigin AG (4)

white powder; $[\alpha]^{26}_{D}$ –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 $C_{20}H_{28}O_4Na$ 355.1885), 687.3824 $[2M+Na]^+$ (calcd for $C_{40}H_{56}O_8Na$ 687.3872).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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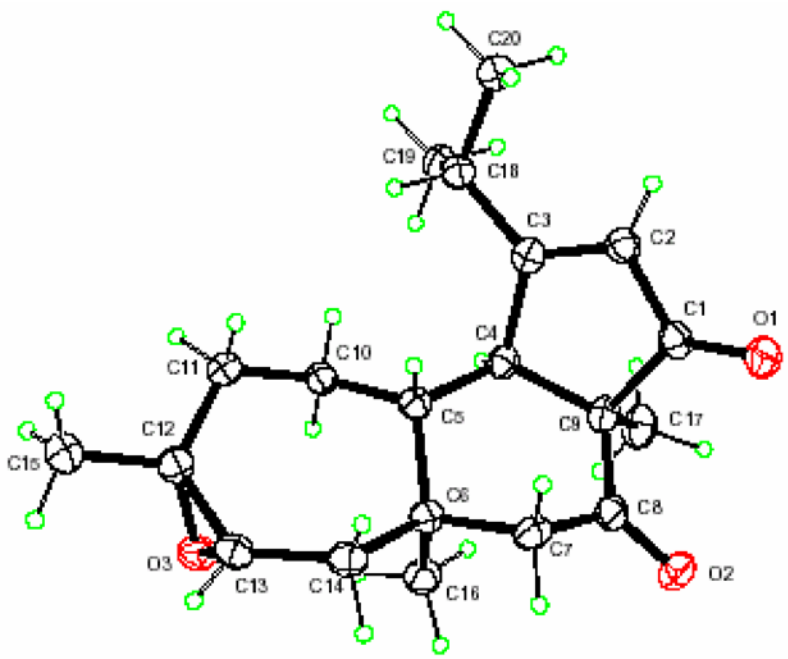

Figure 1.
X-ray structure of **2**

Table 1

^{1}H and ^{13}C NMR Data of cyanthiwigins 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
4α	2.73, d (8.9)	58.9, CH	2.71, d (9.2)	58.7, CH	2.76, d (8.4)	58.9, CH
5β	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
7α	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
7β	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
10α	1.81, m	27.3, CH_2	1.51, m	28.7, CH_2	1.89, m	27.6, CH_2
10β	1.73, m		2.03, m		2.04, m	
11α	2.03, m	33.7, CH_2	2.01, m	38.5, CH_2	2.05, m	33.9, CH_2
11β			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
14α	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
14β			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	14.2, CH_3
17	1.16, s	23.9, CH_3	1.19, s	24., 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] ^{1}H NMR at 400 MHz, ^{13}C NMR at 100MHz, referenced to residual solvent acetone-d_6.

Table 2

Antimicrobial Activity

	Antifungal IC_{50} (μg/mL)		Antibacterial IC_{50} (μg/mL)	
	Candida albicans	***Cryptococcus neoformans***	***Staphylococcus aureus***	**MRS[a]**
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

MRS= methicillin-resistant *Staphylococcus aureus*.

IC_{50} values for curcuphenol and cyanthiwigin mixtures were calculated based on the mass of curcuphenol.