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Cytoskyrins A and B, New BIA Active Bisanthraquinones Isolated from an Endophytic Fungus

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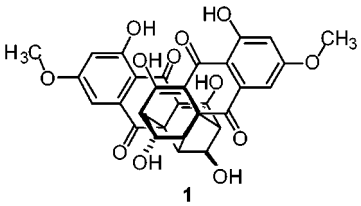
ABSTRACT



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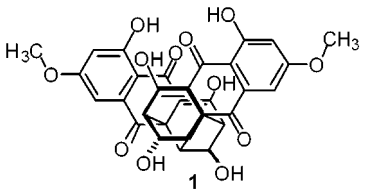
The biochemical induction assay (BIA) is a rapid (colorimetric) bacterial assay used to identify compounds that damage DNA or inhibit DNA synthesis and thereby identify potential natural product anticancer agents. Bisanthraquinones based on a 1,3,6,8-tetrahydroxyanthraquinone-type carbon skeleton were isolated from an endophytic fungus and characterized by NMR and X-ray crystallography. Cytoskyrin A (1) is highly active in the biochemical induction assay, while the closely related cytoskyrin B (2) has no detectable activity in this assay.

The biochemical induction assay (BIA), which measures the induction of the SOS response in bacteria, is used to identify compounds that inhibit DNA synthesis either directly by inhibiting the DNA replication machinery or more often indirectly by modifying DNA.[1] Because small molecules that interact with DNA have been successful anticancer agents,[2] the BIA has been used as an initial screen to identify potential small molecule anticancer agents.[3] We recently reported the characterization of the cytosporones, antibiotics produced in large-scale cultures of an endophytic fungus, CR200 (*Cytospora* sp.), isolated from the branch of a *Conocarpus erecta* tree in the Guanacaste Conservation Area of Costa Rica.[4]

We now report the isolation, from small-scale cultures of the same fungus, and characterization by X-ray crystallography and NMR of the BIA active bisanthraquinone cytoskyrin A (**1**) and the related but inactive bisanthraquinone cytoskyrin B (**2**).



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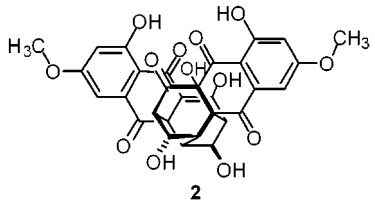

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Using a modified Kupchan scheme, the ethyl acetate extract of small-scale CR200 cultures grown in potato

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(1) (a) Elespuru, R. K.; Yarmolinsky, M. B. *Environ. Mutagen.* **1979**, *1*, 65−78. (b) Elespuru, R. K. A biochemical phage induction assay for carcinogens. In *Environmental Physiology and Medicine, Short-Term Tests for Chemical Carcinogens*; Stich, H., San, R. H. C., Eds.; Springer: New York, 1981; pp 1−11. (c) Elespuru, R. K.; Moor, S. G. *Mutat. Res.* **1986**, *164*, 31−40.

(2) Mountzouris, J. A.; Hurley, L. H. Small Molecule-DNA Interaction. In *Bioorganic Chemistry: Nucleic Acids*; Hecht, S., Ed.; Oxford University Press: New York, 1996; pp 288−479.

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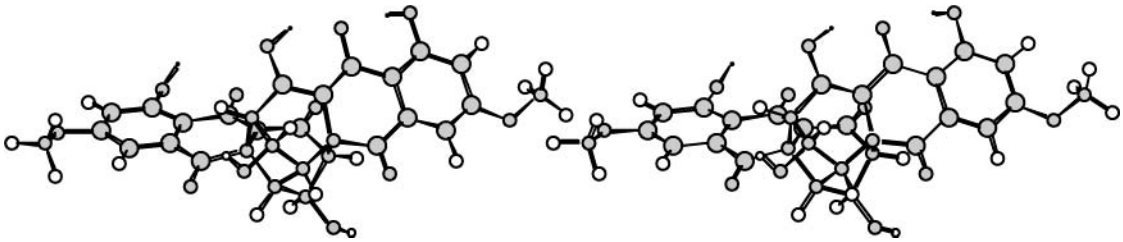


Figure 1. Stereoview of the computer-generated perspective drawing for cytoskyrin A. No absolute stereochemistry is implied.

dextrose broth was partitioned into three fractions (hexanes, CH_2Cl_2, MeOH:H_2O).[5] The CH_2Cl_2 fraction was further partitioned by reversed phase flash chromatography using a THF:H_2O step gradient modified with 0.1% HOAc. The BIA active material that eluted from the flash column with 100% THF was extracted three times with methanol. The remaining methanol insoluble-material was then dissolved in basic methanol (0.1% TEA), and cytoskyrin A was collected as a precipitate upon the addition of acidic methanol (0.1% HOAc). Cytoskyrin A was obtained as a homogeneous yellow powder following three rounds of precipitation (Figure 1). Cytoskyrin B was partitioned, using reversed phase flash chromatography (MeOH:H_2O step gradient modified with 500 mM NH_4OAc, pH 4.5), from the ethyl acetate extract obtained from cultures that showed significantly reduced BIA activity. Cytoskyrin B was purified by reversed phase C-18 HPLC (40:60 THF:H_2O) from the material that eluted from the flash column with 60% MeOH.

The ^{1}H NMR spectrum of cytoskyrin A (**1**) contains 11 protons (1 oxygen-substituted methyl, 3 methine protons, 3 exchangeable protons, and 2 aromatic protons), while the ^{13}C NMR spectrum shows 15 unique carbon chemical shifts (Table 1). HRFABMS predicts $C_{30}H_{22}O_{12}$ as the molecular formula, indicating that cytoskyrin A must be a dimer of 15 carbon monomers (HRMS−FAB (*m*/*z*): $[M + H]^+$ calcd for $C_{30}H_{23}O_{12}$ 575.1190, found 575.1188; $[\alpha]^{20}_D$ +328 (*c* 1.0 in acetone + 0.2% TEA).

C-4, -4a, and -10 show characteristic 1,3-diketo ^{13}C chemical shifts (δ 188.5, 106.2, and 183.5) and are linked to the C-3 methine by HMBC correlations from both C-4 and -4a to H-3. H-3 also shows a very weak long-range ^{1}H−^{1}H RelayH correlation to H-1 which is indicative of a rigid 4-bond W correlation. The position of the oxygen-substituted C-2 methine between C-1 and -3 is defined by HMBC correlations from C-1, -2, and -3 to the hydroxyl proton at C-2. The aromatic ring is connected to the left portion of the monomer (**3**) through HMBC correlations from the C-9 ketone to both the H-8 aromatic proton and the H-1 methine proton. The highly deshielded aromatic carbons at δ 165.4 and 167.6 and the weak (2.5 Hz) coupling observed between H-6 and H-8 suggested the placement of the two protons meta to each other on the dioxygenated aromatic ring seen in cytoskyrin A. This partial structure is confirmed by HMBC correlations from C-10a, -8, -5, and -7 to H-6 and from C-8a, -10a, and -6 to H-8.

The C(9a)−C(3′) and C(9a′)−C(3) bonds between monomers are defined by HMBC correlations from C-9/C-9′ to H-3′/H-3. The dimerization of the 15 carbon monomers (**3**) is completed by a third bond, C(1)−C(1′), between monomers, which is the only way to create the two additional rings that are required to satisfy the remaining two unsaturations of the molecular formula.

Similar 1- and 2-D NMR arguments can be used to show that the carbon skeleton of cytoskyrin B[6] (**2**) is identical to

Table 1. NMR Data for Cytoskyrin A (**1**)

		^{13}C[a]	^{1}H[b,c]
1	1′	51.8	4.04 (2H, s)
2	2′	74.8	4.00 (2H, d, 4)
		(OH)	4.40 (2H, d, 4)
3	3′	61.9	2.85 (2H, s)
4	4′	183.5	
		(OH)	14.38 (2H, s)
4a	4a′	106.2	
5	5′	165.4	
		(OH)	11.88 (2H, s)
6	6′	107.1	6.75 (2H, d, 2.5)
7	7′	167.6	
8	8′	108.0	7.07 (2H, d, 2.5)
8a	8a′	137.1	
9	9′	194.4	
9a	9a′	61.0	
10	10′	188.5	
10a	10a′	111.7	
11	11′	56.7	3.92 (6H, s)

[a] ^{13}C NMR spectra were recorded at 100 MHz in tetrahydrofuran-d_8 and referenced at 67.6 ppm. [b] ^{1}H NMR spectra were recorded at 500 MHz in tetrahydrofuran-d_8 and referenced at 3.58. [c] Assignments are based on ^{1}H−^{13}C HMQC.

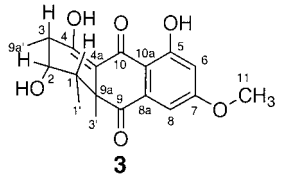
(3) (a) Endo, H.; Ishizawa, M.; Kamiya, T.; Sonoda, S. *Nature* **1963**, *198*, 258−260. (b) Elespuru, R. K.; White, R. J. *Cancer Res.* **1983**, *43*, 2819−2830. (c) Heinemann, B. Prophage induction in lysogenic bacteria as a method of detecting potential mutagenic, carcinogenic, carcinostatic and teratogenic agents. In *Chemical Mutagens: Principles and Methods for Their Detection, vol. 1*; Hollaender, A., Ed.; Plenum: New York, 1971; pp 235−266.

(4) Clardy, J.; Brady, S. F.; Wagenaar, M. M.; Singh, M. P.; Janso, J. E. *Org. Lett.* **2000**, *2*, 4043.

(5) The crude extract was resuspended at 1 mg/mL in 90:10 (MeOH:H_2O) and extracted three times with hexanes. The aqueous phase, following the addition of 20% H_2O, was then re-extracted (3×) with CH_2Cl_2. Kupchan, S. M.; Britton, R. W.; Ziegler, M. F.; Sigel, C. W. *J. Org. Chem.* **1973**, *38*, 178−179.

that of cytoskyrin A (**1**). However, HRFABMS analysis of cytoskyrin B (HRMS−FAB (*m*/*z*): $[M + H]^+$ calcd for $C_{30}H_{23}O_{13}$ 591.1139, found 591.1141) indicates the presence of an additional oxygen, suggesting that cytoskyrin B is a bisanthraquinone heterodimer. The heterodimer is observed in the ^1H and ^{13}C spectra as a slightly offset doubling of each NMR signal. The significant shielding observed at C-4a and deshielding at C-10 and -4 in one of the cytoskyrin B monomers relative to the other establishes cytoskyrin B as the C-4a hydroxyl substituted derivative of cytoskyrin A.

The relative positions of the methoxy and the hydroxy on the aromatic ring in addition to the relative stereochemistry at C-2 and C-2′ could not be assigned with absolute certainty from the NMR data alone. We therefore chose to continue the structural studies of cytoskyrin A using single-crystal X-ray diffraction analysis. Cytoskyrin A crystallizes by slow evaporation from acetone modified with 2.0% TEA. Cytoskyrin A crystals are triclinic in the *P*1 space group and have lattice parameters $a = 7.374(2)$, $b = 10.246(4)$, and $c = 11.359(4)$ Å, $\alpha = 2.97(2)°$, $\beta = 78.71(2)°$, and $\gamma = 74.04(3)°$. The core of the dimer is a tetracyclic cage with two cyclopentane rings that are roughly perpendicular to the plane of each monomer. The cytoskyrin A crystal structure establishes the position of the hydroxy as C-5 and the methoxy as C-7. In addition, the configuration of the C-2 hydroxy is defined such that the H-1, -2 and H-2, -3 methine protons are cis,cis within a monomer (**3**).[7]



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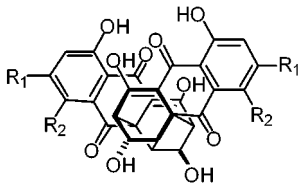
The rigid W confirmation in which H-1, C-2, and H-3 are held in the central cage structure allows for the determination, based on the Karplus equation, of the relative stereochemistry of C-1, -2, and -3 in related bisanthraquinones.[8] In NMR experiments with cytoskyrins A and B, we observed weak long-range couplings between H-1 and -3; however, we did not see a significant coupling between H-1and -2 or H-2 and -3. Molecular modeling[9] experiments predict that the experimentally observed coupling pattern will only occur if the H-1, -2 and H-2, -3 proton pairs are cis,cis within monomers of both cytoskyrins A and B. The cis,cis conformation for cytoskyrin A is confirmed by the X-ray crystallography structure and therefore by analogy assumed to be true for cytoskyrin B.

The cytoskyrins likely arise from the dimerization of 1,3,6,8-tetrahydroxyanthraquinone monomers by a one-electron oxidation and condensation process while previously described bisanthraquinones[10] likely arise from the dimerization of emodin (6 methyl-1,3,8-trihydroxyanthraquinones) or oxidized emodin-type anthraquinones. BIA activity is highly dependent on the three-dimensional structure and not a general property of these polyphenolic compounds. The closely related bisanthraquinones luteoskyrin (**4**) and rugulosin (**5**) are known to interact with DNA.[11] Luteoskyrin did



(**1**) R_1=OCH_3, R_2=H Cytoskyrin A
(**4**) R_1=CH_3, R_2=OH Luteoskyrin
(**5**) R_1=CH_3, R_2=H Rugulosin

not induce a BIA response in the standard BIA; however, it has been reported to produce a BIA response at 500 ng after extended periods of incubation.[3b] Pure cytoskyrin A shows strong BIA activity down to 12.5 ng in the standard assay while cytoskyrin B shows no significant BIA response at any of the concentrations tested (<50 μg). Preliminary studies (data not shown) suggest that cytoskyrin A inhibits both in vitro DNA synthesis and transcription to a greater extent than either luteoskyrin or cytoskyrin B. The structural basis of these differences is not yet clear.

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Supporting Information Available: Representative ^1H and ^{13}C NMR spectra for cytoskyrins A and B. Archival X-ray data for cytoskyrin A. This material is available free of charge via the Internet at http://pubs.acs.org.

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(6) **Cytoskyrin B**: HRMS−FAB (*m*/*z*): $[M + H]^+$ calcd for $C_{30}H_{23}O_{13}$ 591.1139, found 591.1141; ^1H (methanol-d_4:tetrahydrofuran-d_8 (1:1) referenced at 3.31 ppm) 7.01 (d, 2.5, H8), 7.01 (d, 2.5, H8′), 6.81 (d, 2.0, H6), 6.79 (d, 2.5, H6′), 4.92 (m, H1), 4.89 (s, H2), 4.01 (m, H1′), 3.97 (6H, s, H11, H11′), 3.94 (s, H2′), 2.93 (d, 2.0, H3), 2.72 (d, 1.5, H3′); ^{13}C (methanol-d_4:tetrahydrofuran-d_8 (1:1) referenced at 49.2 ppm) C1−C11′: 51.5, 71.2, 68.2, 199.7, 77.0, 165.8, 107.1, 168.2, 108.2*, 138.6*, 194.3, 59.1, 195.6, 112.4, 57.0, 51.2, 77.7, 59.6, 176.9, 106.8, 166.7, 106.6, 167.7, 108.0*, 138.1*, 192.6, 66.6, 189.7, 110.7, 57.0 * indicates that assignment between monomers is interchangeable.

(7) Crystallographic data for cytoskyrin A have been deposited with the Cambridge Crystallographic Data Center. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, U.K. (fax +44-(0) 1223 336033 or e-mail deposit@ccdc.cam.ac.uk).

(8) Ejiri, H.; Sankawa, U.; Shibatat, S. *Phytochemistry* **1975**, *14*, 277−279.

(9) *CS Chem3D Pro*, version 2.5; CambridgeSoft Corporation: Cambridge, MA, 1997. The MM2 force field was used.

(10) (a) Takeda, N.; Seo, S.; Ogihara, Y.; Sankawa, U.; Iitaka, I.; Kitagaw, I.; Shibata, S. *Tetrahedron* **1973**, *29*, 3703−3719. (b) Thomson, R. H. *Naturally Occurring Quinones III: Recent Advances*; Chapman and Hall: New York, 1987. (c) Thomson, R. H. *Naturally Occurring Quinones*; Chapman and Hall: New York, 1971.

(11) (a) Ueno, Y.; Platel, A.; Fromageot, P. *Biochim. Biophys. Acta* **1967**, *134*, 27−36. (b) Ueno Y.; Ueno, I.; Mizumoto, K. *Japn. J. Exp. Med.* **1968**, *38*, 47−64. (c) Bouchet, J. C.; Chuong, P. P. V. *Ann. Nutr. Alim.* **1977**, *31*, 811−830. (d) Umeda, M.; Saito, M.; Shibata, S. *Japan J. Exp. Med.* **1974**, *44*, 249−255.