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Plectosphaeroic Acids A, B, and C, Indoleamine 2,3-Dioxygenase Inhibitors Produced in Culture by a Marine Isolate of the Fungus *Plectosphaerella cucumerina*

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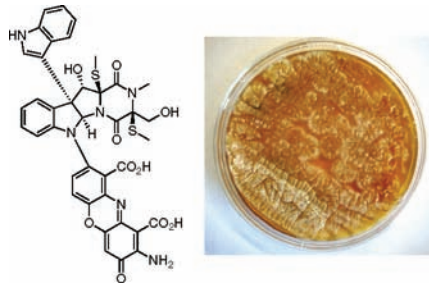
ABSTRACT



Laboratory cultures of the fungus *Plectosphaerella cucumerina* obtained from marine sediments collected in Barkley Sound, British Columbia, yielded the novel alkaloids plectosphaeroic acids A (1) to C (3). The alkaloids 1−3 are inhibitors of indoleamine 2,3-dioxygenase (IDO).

Indoleamine 2,3-dioxygenase (IDO) has emerged as a promising molecular target for the development of a new class of therapeutic agents for treating cancer that work by modulating an extrinsic property of tumor cells.[1] The interest in IDO arises from its putative role in the ability of tumors to escape the T-lymphocyte-based immune response, a characteristic trait of human cancer that plays a central role in the progesssion of the disease from small dormant localized tumors to the often lethal state of metastasis. IDO catalyzes the cleavage of the 2,3-indole bond of L-tryptophan, converting it to *N*-formylkynurenine in the first and rate-limiting step of the kynurenine catabolic pathway. IDO-expressing cancer cells use this transformation to reduce tryptophan concentrations in their microenvironments to levels that prevent T-lymphocyte activation and proliferation. Evidence also suggests that other catabolites in the kynurenine pathway are toxic to T-cells and further inhibit their action. A growing body of clinical data shows that many primary tumor cell lines obtained from patients overexpress IDO, and this strongly correlates with a poor prognosis for survival.[2]

Muller, Prendergast, and co-workers have provided compelling evidence from murine cancer models for the promise

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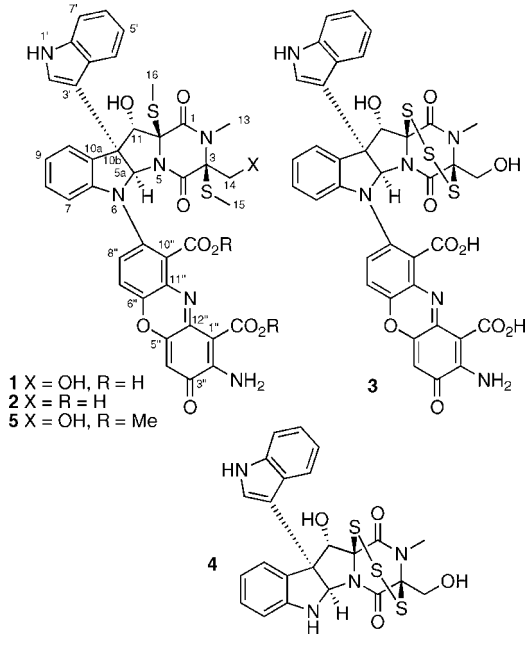
of IDO inhibitors as anticancer drugs that can marshal the immune system to help regress established tumors. Their experiments have been conducted with the three modestly active IDO inhibitors *N*-methyltryptophan,[3] 5-bromobrassinin,[4] and menadione.[5] Each of these compounds failed to regress murine tumors when used as a monotherapy, but they all showed impressive regression of established tumors when used in combination with the antimitotic drug paclitaxel, that was also ineffective when used alone. Careful control experiments confirmed that in each case the target IDO was required for the positive in vivo results.

Although *N*-methyltryptophan, 5-bromobrassinin, and menadione have provided important proof-of-principle demonstrations for the use of IDO inhibitors in cancer chemotherapy, none of them are potent enough to be realistic drug development candidates. In an attempt to discover more potent IDO inhibitors belonging to new structural classes, we have screened a library of crude extracts of marine invertebrates and laboratory cultures of microorganisms obtained from marine habitats with an in vitro assay for IDO inhibition that uses purified recombinant human IDO. This screening program identified the marine natural products annulins A and B[6] ($K_i = 120$ nM) and exiguamines A ($K_i = 40$ nM)[7] and B[8] as potent IDO inhibitors. Annulin B inspired a medicinal chemistry program that generated a series of synthetic IDO inhibitory pyranonaphthoquinones with low nanomolar potency,[5] and it stimulated the discovery in our laboratories that menadione was an IDO inhibitor.[9] Similarly, exiguamine A was the starting point for the preparation of synthetic tryptaminequinone analogues that are also submicromolar inhibitors of IDO.[8,10]



A third crude extract that showed promising IDO inhibition in the in vitro screen came from laboratory cultures of the fungus *Plectosphaerella cucumerina*[11] obtained from marine sediments collected at −100 m depth in Barkley Sound, British Columbia. Bioassay guided fractionation of the crude extract identified the novel alkaloids plectosphaeroic acids A (**1**), B (**2**), and C (**3**) as in vitro inhibitors of IDO. Details of the isolation, structure elucidation, and biological activities of **1**−**3** are presented below.

Production cultures of *P. cucumerina* were grown as lawns on solid agar (medium: ISP4) at rt for 14 days. The mature cultures were sliced into small squares containing both fungal biomass and media, and these were soaked in EtOAc. Concentration of the EtOAc extracts in vacuo gave a residue that was dissolved in MeOH and then fractionated using Sephadex LH20 chromatography eluting with MeOH. The IDO inhibitory fractions were pooled and purified further using reversed-phase HPLC (Supporting Information) to give pure samples of plectosphaeroic acids A (**1**), B (**2**), and C (**3**) along with the known alkaloid T988 A (**4**), which was identified by comparison of its spectroscopic data with literature values.[12]

Plectosphaeroic acid A (**1**) was obtained as an optically active red/orange solid that gave an $[M - H]^-$ peak at *m*/*z* 807.1534 in the negative ion HRESIMS appropriate for a molecular formula of $C_{39}H_{32}N_6O_{10}S_2$ (calcd for $C_{39}H_{31}N_6O_{10}S_2$ 807.1543) requiring 27 sites of unsaturation. Thirty nine well-resolved carbon resonances could be identified in the ^{13}C NMR spectrum of **1**, and the HSQC spectrum showed that there were 25 hydrogen atoms attached to carbon ($3 \times CH_3$; $1 \times CH_2$; $14 \times CH$). The LRESIMS recorded in CD_3OD gave an $[M - D]^-$ peak at *m*/*z* 813 and an $[M - D + 2Na]^+$ ion at *m*/*z* 859 confirming that there were seven exchangeable hydrogen atoms in **1**, which together with the 25 hydrogens attached to carbon accounted for the 32 hydrogen atoms present in the molecular formula indicated by the HRESIMS data.

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Analysis of the COSY, HSQC, and HMBC data recorded for plectosphaeroic acid A (**1**) identified two fragments **A** and **B** (Figure 1) that are also present in the co-occurring

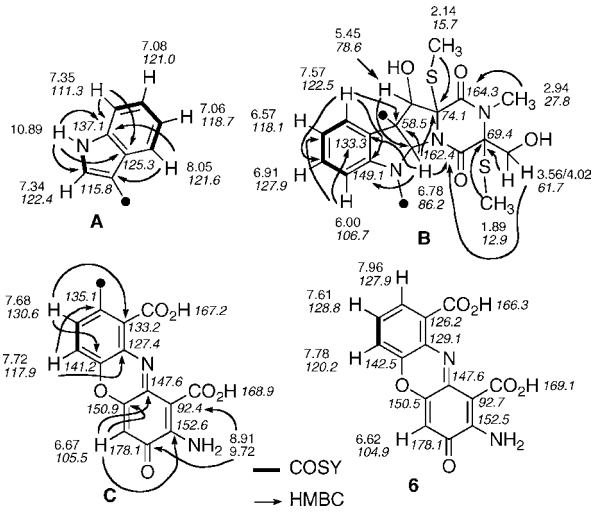

Figure 1. 2D NMR correlations for plectosphaeroic acid A (**1**).

metabolite T988 A (**4**).[12] COSY correlations revealed the two isolated ^{1}H spin systems (H-4′ to H-7′ and H-2′/NH-1′) of the C-3′ substituted indole fragment **A**, and HSQC/HMBC correlations confirmed the carbon assignments. The carbon chemical shifts assigned to fragment **A** in **1** were nearly identical to the corresponding carbon chemical shifts assigned to this fragment in T988 A (**4**) (Figure 1 and Supporting Information).[12]

The presence of fragment B in **1** was indicated by the existence of ^{1}H and ^{13}C NMR resonances in the 1D spectra recorded for **1** that showed chemical shifts and scalar coupling patterns in the COSY and HMBC data nearly identical with those observed for the corresponding resonances assigned to the same fragment in **4**.[12] A series of COSY correlations identified the resonances in the H-7 to H-10 spin system at δ 6.00 (d, J = 8.0 Hz, H-7), 6.91 (t, J = 7.6 Hz, H-8), 6.57 (t, J= 7.6 Hz, H-9), and 7.57 (d, J = 7.5 Hz, H-10). A singlet resonance at δ 5.45 that showed an HSQC correlation to a carbon resonance at δ 78.6 was assigned to the H-11 carbinol methine proton. A second ^{1}H singlet at δ 6.78, correlated in the HSQC to a carbon resonance at δ 86.2, was assigned to the H-5a proton. Methyl singlets at δ 1.89 (^{13}C δ 12.9), 2.14 (^{13}C δ 15.7), and 2.94 (^{13}C δ 27.8) in the ^{1}H NMR spectrum could be assigned to two thiomethyls (Me-15 and Me-16) and an *N*-methyl (Me-13), respectively.

Three bond HMBC correlations observed between H-10 (δ 7.57) and a carbon resonance at δ 149.1 (C-6a) and between H-7 (δ 6.00) and a carbon resonance at δ 133.3 (H-10a), along with the HSQC correlations from the aromatic protons H-7 to H-10 to their attached carbons, assigned all of the carbon resonances in the C-6a to C10a aromatic ring. There was very close agreement between these assignments in **1** and the assignments for the corresponding carbons in **4** (Table 1, Supporting Information). An HMBC correlation between the *N*-methyl resonance at δ 2.94 and a carbon resonance at δ 164.3 revealed the C-1 diketopiperazine carbonyl, and the C-4 carbonyl resonance at δ 162.4 was identified by an HMBC correlation from the H-14 resonance at δ 4.02. The Me-16 thiomethyl resonance at δ 2.14 showed an HMBC correlation to the C-12 carbon resonance at δ 74.1, and both of the Me-15 thiomethyl and H-14 resonances at δ 1.89 and 3.56, respectively, showed HMBC correlations to the C-3 resonance at δ 69.4.

A set of HMBC correlations from the H-5a methine resonance at δ 6.78 to the aromatic carbon resonances at δ 149.1 (C-6a) and 133.3 (C-10a), to the C-4 carbonyl resonance at δ 162.4, and to the C-10b and C-12 quaternary carbon resonances at δ 58.5 and 74.1, respectively, and additional HMBC correlations from the H-10 resonance at δ 7.57 to the C-10b resonanace at δ 58.5, and from the H-11 resonanance at δ 5.45 to the C-5a resonance at δ 86.2, were all in agreement with the proposed tetracyclic substructure **B** containing phenyl, carbinol methine, and diketopiperazine moieties. The ^{13}C NMR assignments for the nonaromatic carbons in substructure **B** in **1** (Figure 1) were again in very good agreement with the literature assignments to their counterparts in T988 A (**4**) and B.[12]

Subtracting the atoms accounted for by fragments **A** and **B** ($C_{25}H_{25}N_4O_4S_2$) from the molecular formula of **1** showed that the remaining fragment had to account for $C_{14}H_7N_2O_6$, 12 sites of unsaturation, and four exchangeable hydrogens. Resonances at δ 167.2 (C-14″) and 168.9 (C-13″) in the ^{13}C NMR spectrum were tentatively assigned to carboxylic acid functionalities. This assignment was confirmed by reacting **1** with methyl iodide and base to produce the dimethyl ester **5**. A carbon resonance at δ 178.1 (C-3″) had a chemcial shift appropriate for a quinone carbonyl, but since there was not a second carbon with a similar shift, we assumed this was part of an iminoquinone. The COSY spectrum identified a pair of ortho protons (δ 7.68, d, J = 8.8 Hz, H-8″; 7.72, d, J = 8.6 Hz, H-7″), and the HSQC data showed that there was one additional proton (δ 6.67, s, H-4″) attached to carbon (δ 105.5, C-4″).

Plectosphaeroic acid A (**1**) had a UV λ_{max} at 428 nm, which is similar to that reported for phenoxazinone dyes.[13] Thus, it appeared that the final fragment of plectosphaeroic acid A (**1**) might be a substituted cinnabarinic acid moiety, which would account for all of the remaining atoms in the structure, the functionality identified from the NMR data, and the UV λ_{max}. To confirm this hypothesis, cinnabarinic acid (**6**) was synthesized from 3-hydroxy anthranilic acid following literature procedures,[14] and its carbon and proton NMR spectra were assigned as shown in Figure 1. There was excellent agreement between the ^{1}H and ^{13}C NMR assigments for fragment **C** in plectosphaeroic acid A (**1**) and the synthetic cinnabarinic acid (Figure 1). It was also apparent from these assignments that fragment **C** had to be linked to

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fragments **A** or **B** via C-9″ since the resonance corresponding to H-9″ in the ^{1}H NMR spectrum of cinnabarinic acid (**6**) (δ 7.96), which is the most deshielded of the proton resonances and shows an HMBC correlation to the carbon of the carboxylic acid attached to C-10″, was missing in the ^{1}H NMR spectrum of **1**. Synthetic cinnabarinic acid had UV λ_{maxs} at 231 and 429 nm.

The remaining unsatisfied valences in fragments **A**, **B**, and **C** were at N-6, C-10b, C-3′ and C-9″. ROESY correlations between H-4′ (δ 8.05) and both H-10 (δ 7.57) and H-11 (δ 5.45) were consistent with a bond between C-10b and C-3′ as in T988 A (**4**), and a ROESY correlation between H-7 (δ 6.00) and H-8″ (δ 7.68) identified a bond between N-6 and C-9″, completing the consitution of **1** (Figure 2). The

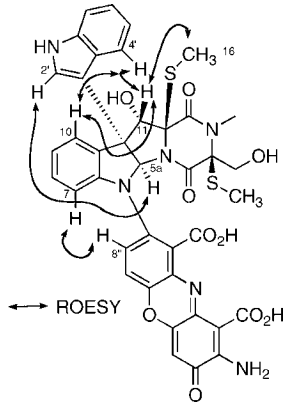

Figure 2. ROESY correlations for plectosphaeroic acid A (**1**).

observation of a ROESY correlation between H-5a (δ 6.78) and H-2′ (δ 7.34) showed that H-5a and the indole ring were *cis* to each other. ROESY correlations between H-11 (δ 5.45) and both Me-16 (δ 2.14) and H-10 (δ 7.57) required that H-11 and Me-16 were *cis* to each other and *trans* to H-5a as shown (Figure 2).

Plectosphaeroic acid B (**2**) was isolated as an optically active red/orange solid that gave an $[M - H]^-$ peak at *m*/*z* 791.1599 in the negative ion HRESIMS appropriate for a molecular formula of $C_{39}H_{32}N_6O_9S_2$ (calcd for $C_{39}H_{31}N_6O_9S_2$ 791.1594) that differed from the formula for **1** by loss of an oxygen atom. Routine analysis of the NMR data for **2** (Supporting Information) showed that it was missing the alcohol functionality found at C-14 in **1**.

Plectosphaeroic acid C (**3**) was isolated as an optically active red/orange solid that gave an $[M - H]^-$ peak at *m*/*z* 809.0786 in the negative ion HRESIMS appropriate for a molecular formula of $C_{37}H_{26}N_6O_{10}S_3$ (calcd for $C_{37}H_{25}N_6O_{10}S_3$ 809.0794) that differed from the formula for **1** by loss of C_2H_6 and addition of one sulfur atom. The NMR data obtained for **3** were missing the ^{1}H and ^{13}C resonances assigned to the C-15 and C-16 thiomethyl groups in the NMR data for **1** but were in all other ways nearly identical with the data for **1**. Therefore, plectosphaeroic acid C was assigned the structure **3**, containing a trisulfide bridge across the diketopiperazine ring. Comparison of the CD spectra recorded for plectosphaeroic acids A (**1**), B (**2**), C (**3**), and T988 A (**4**) (Supporting Information) with the literature values for the structurally related leptosins[15] revealed that compounds **1**−**4** have the 3*S*, 5a*R*, 10b*R*, 11*S*, and 12*S* absolute configurations shown.

The plectospaeroic acids represent a new family of complex fungal alkaloids. Biogenetically they appear to be derived from 4 equiv of tryptophan and 1 equiv of either serine or alanine. Remarkably, the tryptophan residues have been processed in three distinctly different ways to produce the final structure. The indole ring in fragment **A** presumably arises from a tryptophan residue where the side chain has been removed, possibly via a Grob fragmentation of a diketopiperazine similar to substructure **B**.[16] Fragment **B** contains an intact tryptophan residue that has formed normal peptide bonds with either serine or alanine to produce the core of the modified diketopiperazine. The phenoxazinone heterocycle found in fragment **C** is known to arise from oxidative coupling of two 3-hydroxyanthranilic acid units[17] that can in turn arise from catabolic degradation of tryptophan via the kynurenine pathway.[18]

Plectosphaeroic acids A (**1**), B (**2**), and C (**3**) all inhibited purified, recombinant human IDO assayed in vitro by the method of Takikawa et al.[19] and exhibited identical IC_{50}s of $\approx$2 μM, while T988 A (**4**) was completely inactive. Synthetic cinnabarinic acid (**6**) was also active in the assay with an IC_{50} of $\approx$2 μM indicating that some portion of the phenoxazinone fragment **C** represents a new IDO inhibitory pharmacophore. The discovery of the plectosphaeroic acids further highlights the ability of marine isolates of apparently well-known microorganisms to produce novel bioactive secondary metabolites that are potential drug leads.[20]

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Supporting Information Available: Experimental details, tables of NMR assignments, 1D and 2D NMR spectra, and CD spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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