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Our goal is to find new antimitotic drugs for the treatment of breast cancer using a novel cell-based assay to screen natural product libraries and guide the purification of their active components.

In year 1, we carried out a screen of over 30,000 extracts of terrestrial plants, algae and marine organisms, obtained 223 positive extracts, and isolated and identified active compounds from 5 of the positive extracts.

In year 2, we isolated several new analogs of the microtubule-stabilizing compound eleutherobin and produced several more by synthetic transformation. We used the analogs to carry out an extended structure-activity relationship study of eleutherobin and identified unanticipated structural requirements that will be important for future pharmacophore models. We have also isolated sufficient amounts of eleutherobin and desmethyleleutherobin for in vivo testing. We isolated active compounds from 5 additional extracts, 3 of which have novel mechanisms of action that are currently under investigation.

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## Table of Contents

## INTRODUCTION

Antimitotics are drugs that kill cancer cells by causing them to arrest in mitosis. Antimitotics currently used in breast cancer therapy include paclitaxel, vincristine and vinblastine. Although these drugs are extremely valuable, they are not ideal because they have serious side effects, and, most importantly, many breast cancers are resistant to them. Our goal is to find new antimitotic drugs for the treatment of breast cancer using a novel cell-based assay to screen natural product libraries and guide the purification of their active components.

## BODY

### TASK 1

Screen the natural product collection (5,000 extracts) for antimitotic activity (months 1-4)

This task was completed in Year 1. We obtained 233 positive extracts out of about 30,000 extracts screened.

### TASK 2

Isolate and identify the chemical structure of active compounds (months 5-30)

In Year 1 we completed the process of fractionation, dereplication, purification and chemical structure elucidation of active compounds for five positive extracts, yielding a new rhizoxin analog, six new eleutherobin analogs, and two paclitaxel analogs.

Our discovery in Year 1 of a new source of eleutherobin and elucidation of the structures of several active analogs created considerable interest. We have pursued this further, obtaining 12 positive extracts from corals related to *Erythropodium caribaeorum* that we suspected might contain other eleutherobin analogs. In Year 2 we have completed the isolation and identification of four additional eleutherobin analogs (Britton et al., 2001a; Appendix 1) and produced several analogs by synthetic transformation (Britton et al., 2001b; Appendix 1), shown below in Figure 1. We have also demonstrated that the eleutherobin initially described by Fenical and coworkers (Lindel et al., 1997) is an isolation artifact and that desmethyleleutherobin (first described by us in Year 1) is the natural product (Britton et al, 2001a; Appendix 1).

In Year 2 we also carried out this process for 17 positive extracts from species unrelated to *E. caribaeorum*. There was insufficient material to complete the purification of 7 extracts. For one of these, the National Cancer Institute is arranging for the collection of additional material. 5 purifications have been completed. The structural formulae of the purified antimitotics are shown in Figure 2. From a plant we isolated 3 active flavonoids (3'-methoxycalycopterin, 4',5,7-trihydroxy-3,3',6,8-

tetramethoxyflavone, 3,3'dimethoxyquercaetin) and one inactive analog. We isolated 2 okadaic acid analogs, 27-O-acyl-dinophysistoxin and 27-O-acyl-okadaic acid, from a marine invertebrate. 3'acetoxychavicol acetate, vernonataloide, and 13-hydroxy-15-oxozoapatlin were isolated from different plants. 5 other purifications are ongoing.

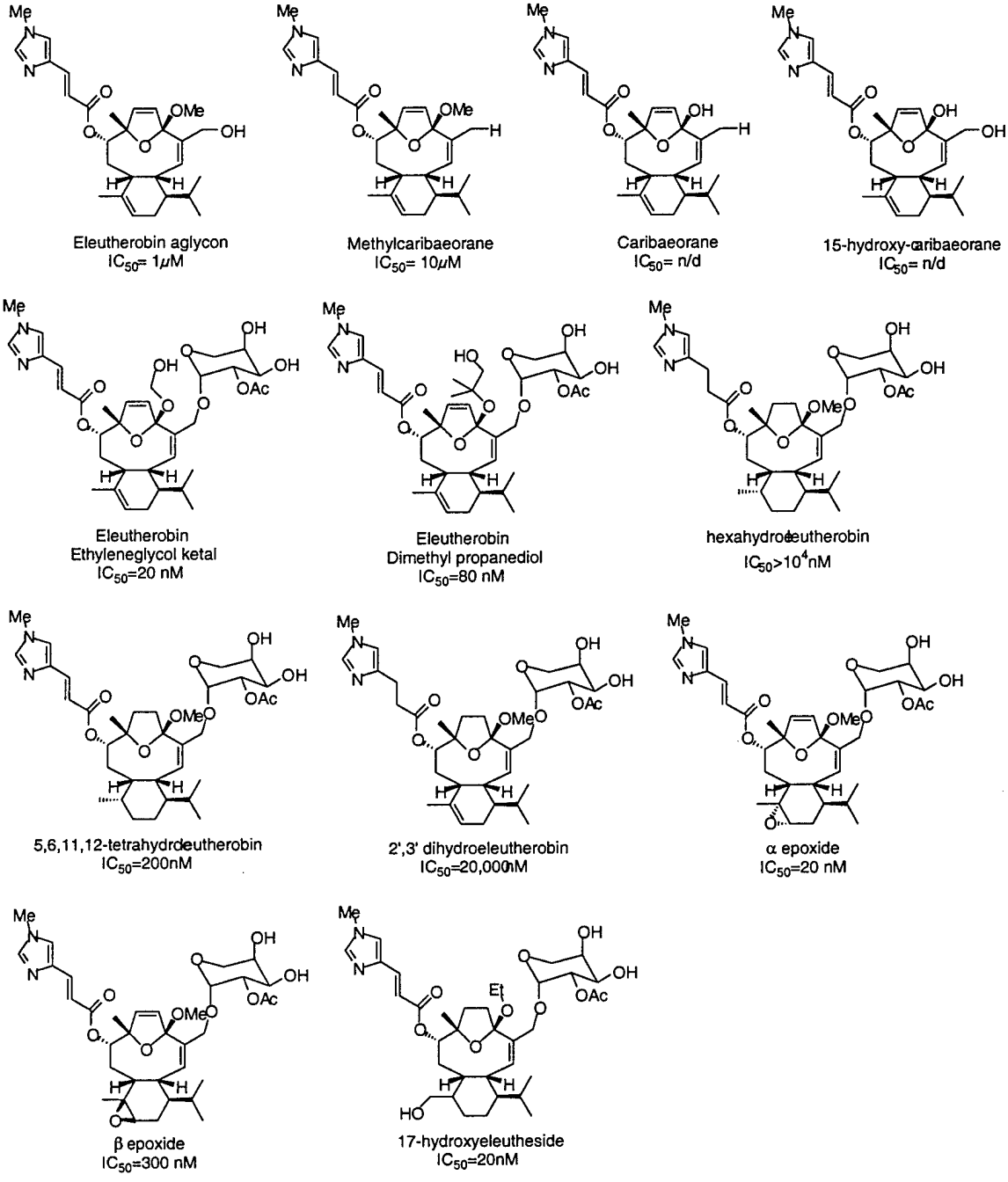


Figure 1. Structural formulae of eleutherobin analogs purified or synthesized in year 2 and their $IC_{50}$ for mitotic arrest.

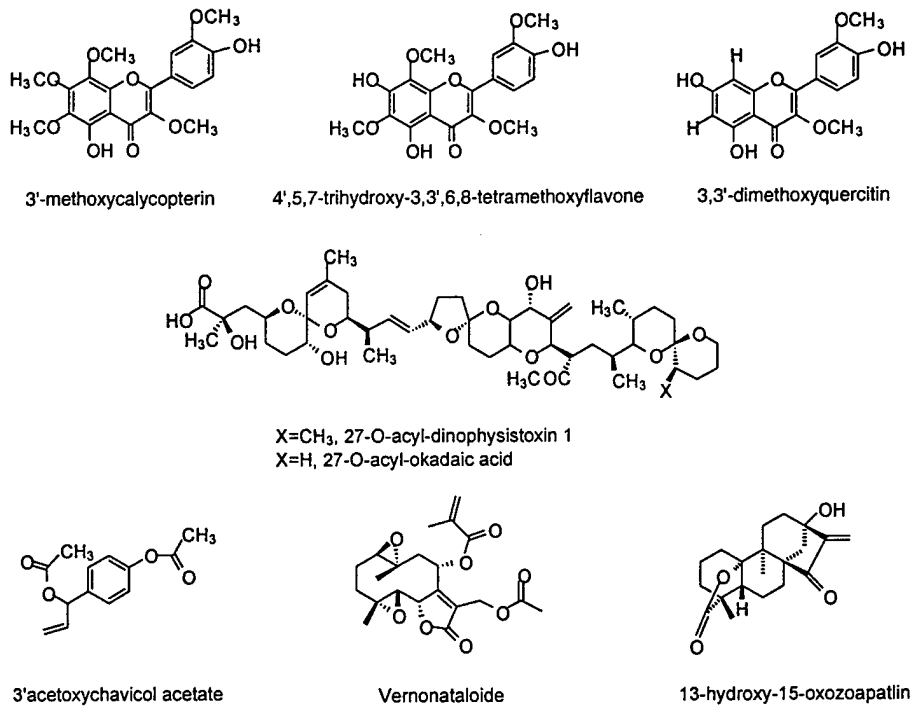


Figure 2. Structural formulae of non-eleutherobin antimitotics purified in year 2.

## TASK 3

Mechanism of action of new antimitotic compounds (months 13-32)

We have used several assays in Year 2 to investigate the mechanism of action of our purified antimitotics. The first assay examines the effect of the compounds on cells. Cells are grown on coverslips, treated with the compounds, and examined by immunofluorescence microscopy using the DNA dye bisbenzimide to visualize the chromosomes and a monoclonal antibody to β-tubulin to examine the microtubules of interphase cells and the spindle of mitotic cells. A second assay examines whether the compounds can interact directly with purified tubulin to cause either its depolymerization or its polymerization. A third assay examines whether the antimitotic effect of the compounds is due to inhibition of Ser/Thr protein phosphatases. In a 96-well format phosphatases, either in crude extract form or as purified protein phosphatase 1A or 2A, and a phosphopeptide substrate are incubated with the antimitotic compound and inhibition of release of phosphate is detected by decreased malachite green signal.

The new eleutherobin analogs all arrest cells in mitosis with bundled microtubules. Using the in vitro tubulin assay confirmed that the bundling of microtubules observed in cells is caused by a direct interaction between the eleutherobins and tubulin. We carried out a structure-activity study for the different analogs. Their $IC_{50}$

for mitotic arrest is shown in Figure 1. The interpretation and significance of these results for the development of a tubulin-binding pharmacophore for compounds that stabilize microtubules is presented in (Britton et al., 2001b; Appendix 1). A refined solution structure of eleutherobin has also been obtained (Cornett et al., submitted).

The flavonoids caused the cells to arrest in mitosis with depolymerized microtubules. This is the most common mechanism of action for antimitotics. The $IC_{50}$ of the most active analog was about 10 μg/ml. These compounds belong to a family of flavones related to centaureidin whose antimitotic properties have been extensively studied (Beutler et al., 1998).

The okadaic acid analogs did not cause microtubule bundling or depolymerization in the cell-based tubulin immunofluorescence assay. However they were potent inhibitors of Serine/Threonine protein phosphatases ($IC_{50}$ 0.3 ng/ml).

3'acetoxychavicol acetate and vernonataloide did not cause microtubule bundling or depolymerization. In fact treated cells, although rounded up, had none of the other standard morphological features of mitotic cells i.e. the chromosomes were not condensed, there was no mitotic spindle, and the nuclear lamina remained intact. However, further study showed that at the biochemical level treated cells had undergone many proline-directed phosphorylation events strictly associated with mitosis, such as phosphorylation of histone H3 at Ser10, phosphorylation of nucleolin at the TG3-immunoreactive site, phosphorylation of GF-7 antigen, and phosphorylation of MPM2 antigens. This strongly suggests that these two compounds are activating a proline-directed kinase. They did not act as Serine/Threonine phosphatase inhibitors. We are now carrying out biochemical studies to determine the kinase involved and its mechanism of activation.

13-hydroxy-15-oxozoapatlin caused cells to arrest in mitosis ($IC_{50}$ 10 μM), but with an unusual morphology not produced by other antimitotics: their chromosomes were condensed and a mitotic spindle was formed, but the chromosomes failed to align properly on the mitotic spindle. It was also able to act as a G2 checkpoint inhibitor, i.e. it released cells from arrest in G2 phase of the cell cycle following DNA damage and caused them to enter mitosis. This interesting combination of effects suggests a novel mechanism of action and we are now pursuing the identification of the relevant targets.

## TASK 4

Initial studies on therapeutic potential (months 14-36)

The purified compounds were tested for their ability to inhibit the proliferation of MCF-7mp53 breast cancer cells. The eleutherobin analogs were particularly promising. Their effectiveness as antimitotics was closely correlated with their ability to inhibit cell

proliferation, desmethyleleutherobin and eleutherobin being the most active, with an $IC_{50}$ for inhibition of proliferation of about 18 nM.

The flavonoids were not studied further since they are members of a family of compounds extensively studied by others [Beutler, 1998].

The okadaic acid analogs were found to be very potent inhibitors of proliferation ($IC_{50}$ 1 ng/ml) and were considered likely to be too toxic for therapeutic applications.

3'acetoxychavicol acetate had an $IC_{50}$ for inhibition of proliferation of 4 μg/ml.

13-hydroxy-15-oxozoapatlin had an $IC_{50}$ for inhibition of proliferation of 0.8 μM.

## KEY RESEARCH ACCOMPLISHMENTS

- We have completed the purification and structural elucidation of a further 4 active eleutherobin analogs and produced several analogs by synthetic transformation. This has allowed us to carry out a more extensive structure-activity study than previously possible and revealed some striking and unanticipated features of its antimitotic properties. A detailed understanding of the structural requirements for binding of microtubule-stabilizing agents to tubulin is an important tool for the rational design of new microtubule-stabilizing drugs.

- We have completed the purification and structural elucidation of antimitotics from a further 5 positive extracts. The flavonoids belong to a recently described family of antimitotics [Beutler, 1998] that act by depolymerizing microtubules. The okadaic acid analogs act through inhibition of Ser/Thr phosphatases but were deemed potentially too toxic for therapeutic use. 3'acetoxychavicol acetate, vernonataloide and 13-hydroxy-15-oxozoapatlin had novel mechanisms of action currently under further study. This success rate for novelty is in part attributable to our efforts at secondary screening of crude extracts to optimize for novelty, implemented in Year 1.

- Synthetic schemes have been published for eleutherobin, but they are not practical and sufficient amounts for administration to even a few mice were not previously available. We have been able to isolate from natural sources over 20 mg of eleutherobin and desmethyleleutherobin, the members of this family with the most potent antimitotic and antiproliferative activity. This makes the testing of their anticancer activity in experimental tumors in mice possible in the immediate future.

## REPORTABLE OUTCOMES

Manuscripts published in year 2 (copies provided in Appendix 1):

1. Roberge, M., Cinel, B., Anderson, H. J., Lim, L., Jiang, X., Xu, L., Kelly, M. T. and Andersen, R. J. "Cell-based screen for antimitotic agents and identification of new analogs of rhizoxin and eleutherobin" Cancer Res. 60: 5052-5058 (2000)

2. Britton R., Roberge M., Berisch H. and Andersen R.J. (2001a). Antimitotic diterpenoids from Erythropodium caribaeorum: isolation artifacts and putative biosynthetic intermediates. Tetrahedron Letters 42, 2953-2956.

3. Britton R., de Silva E.D., Bigg C.M., McHardy L.M., Roberge M and Andersen, R.J. (2001b). Synthetic transformations of eleutherobin reveal new features of its microtubule-stabilizing pharmacophore. J. Am. Chem Soc. In press.

Manuscript submitted:

4. Cornett, B., Monteagudo, E., Cicero, D. O., Cinel, B., Andersen, R. J., Roberge, M., Liotta, D. C. and Snyder, J. P. Conformational analysis of the microtubule-stabilizing agent eleutherobin in $CDCl_3$. Submitted (2001).

## CONCLUSIONS

We have accomplished all our goals for Year 2 of the grant. We have continued to pursue our study of analogs of eleutherobin, the class of antimitotics of greatest therapeutic interest to us at the present time. We have produced sufficient compound for extensive structural studies and in vivo evaluation. We have succeeded in isolating structurally novel antimitotics.

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Lindel T., Jensen P.R., Fenical W., Long B.H., Casazza A.M., Carboni J and Fairchild C.R. (1997). Eleutherobin, a new cytotoxin that mimics paclitaxel (Taxol) by stabilizing microtubules. J. Am. Chem. Soc. 119, 8744-8745.

**APPENDIX 1**

Papers published during Year 2 of this grant.

[CANCER RESEARCH 60, 5052–5058, September 15, 2000]

# Cell-based Screen for Antimitotic Agents and Identification of Analogues of Rhizoxin, Eleutherobin, and Paclitaxel in Natural Extracts[1]

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## ABSTRACT

**We describe a cell-based assay for antimitotic compounds that is suitable for drug discovery and for quantitative determination of antimitotic activity. In the assay, cells arrested in mitosis as a result of exposure to antimitotic agents in pure form or in crude natural extracts are detected by ELISA using the monoclonal antibody TG-3. The assay was used to screen >24,000 extracts of marine microorganisms and invertebrates and terrestrial plants and to guide the purification of active compounds from 5 of 119 positive extracts. A new rhizoxin analogue was found in a *Pseudomonas* species, six new eleutherobin analogues were identified from the octocoral *Erythropodium caribaeorum*, and two paclitaxel analogues were found in the stem bark of the tree *Ilex macrophylla*. The assay was also used for quantitative comparison of the antimitotic activity of different analogues. It revealed the importance of the C-11 to C-13 segment of the diterpene core of eleutherobin for its antimitotic activity. The identification of antimitotic compounds in very low abundance and their high (0.5%) occurrence in natural extracts indicates that drug discovery efforts using this cell-based assay may lead to the identification of structurally novel antimitotic agents.**

## INTRODUCTION

Antimitotic agents are compounds that arrest cells in mitosis. Several are clinically important anticancer drugs, including the *Vinca* alkaloids vinblastine, vincristine, and vinorelbine (1) and the taxanes paclitaxel and docetaxel (2). They cause mitotic arrest by interfering with the assembly or disassembly of $\alpha$- and $\beta$-tubulin into microtubules. At high concentrations, the *Vinca* alkaloids and most other antimitotics cause complete microtubule depolymerization, whereas the taxanes cause bundling of microtubules by stabilizing them against depolymerization. At low concentrations, neither depolymerization nor bundling is observed, but there is sufficient alteration in the dynamics of tubulin loss or addition at the ends of mitotic spindle microtubules to prevent the spindle from carrying out its function of attaching to and segregating the chromosomes, and cells arrest in mitosis (3, 4). Prolonged arrest eventually leads to cell death, either in mitosis or after an eventual escape from mitotic arrest (5, 6). Another class of antimitotic agents, represented by estramustine, does bind tubulin (7) but may also bind microtubule-associated proteins and prevent them from regulating interactions between tubulin polymers (8). Agents that are not known to interact with microtubules, such as inhibitors of protein phosphatases 1 and 2A and mitotic kinesin inhibitors, can also arrest cells in mitosis (9–11).

The *Vinca* alkaloids were isolated from the periwinkle plant, which originally attracted attention because of reported hypoglycemic properties. However, periwinkle extracts showed no antidiabetic action but were found to prolong the life of mice bearing a transplantable lymphocytic leukemia (1). This led to the identification of vincristine and vinblastine. Paclitaxel was isolated from the bark of the Pacific yew tree, an extract of which showed antineoplastic activity in the NCI[3] large-scale screen (2). Vinorelbine and docetaxel are semisynthetic analogues.

These drugs, although extremely valuable, are not ideal. They have numerous toxicities, principally myelosuppression and neurotoxicity. More importantly, many cancers are inherently resistant to these drugs or become so during prolonged treatment (1, 2). This is often the result of multidrug resistance caused by overexpression of P-glycoprotein, which functions as a drug efflux pump. Other sources of resistance include increased expression of tubulin isotypes to which a particular drug binds less effectively and alterations in $\alpha$- and $\beta$-tubulin structure, by mutation or posttranslational modification, that reduce binding.

Antimitotics with different chemical structures might show increased specificity to mitotic microtubules rather than neuronal microtubules and reduce unwanted side effects and might be effective against resistant cancers. Many other antimitotics have been discovered, some of which show promise in preclinical studies or have entered clinical trials (12). However, they were discovered either by serendipity or by cytotoxicity screening, or because they showed patterns of cytotoxic activity against panels of cancer cell lines similar to patterns shown by other antimitotic agents (13). The search for better antimitotics would be greatly aided by rational assays for use in drug screens.

We have developed a rapid and reliable cell-based screen for antimitotic agents. In this report, we describe the assay, its application to a screen of >24,000 natural extracts, and the purification and characterization of paclitaxel analogues and new rhizoxin and eleutherobin analogues.

## MATERIALS AND METHODS

**Cell Culture and Treatment.** Human breast carcinoma MCF-7 cells were cultured as monolayers (14). The cells were seeded at 10,000/well of 96-well polystyrene tissue culture plates (Falcon) in 100 $\mu$l of medium and were allowed to grow overnight. Crude extracts were then added at about 10 or 1 $\mu$g/ml from 1000-fold stocks in DMSO. Untreated samples received an equivalent amount of DMSO and served as negative controls. Cells treated with 100 ng/ml nocodazole (Sigma), from a 1000-fold stock in DMSO, served as positive controls. Cells were incubated for 16–20 h. The relative number of cells in mitosis was then determined by microscopy (14), by ELISA, or by ELICA (see below).

**ELISA of Mitotic Cells.** After incubation with extracts, the cell culture medium was withdrawn carefully using a pipettor. This did not result in any loss of the rounded-up mitotic cells, which remained attached to the plates. The cells were lysed by adding 100 $\mu$l of ice-cold lysis buffer (1 mM EGTA, pH

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[3] The abbreviations used are: NCI, National Cancer Institute; ELICA, enzyme-linked immunocytochemical assay; HRP, horseradish peroxidase.

7.4, 0.5 mM phenylmethylsulfonyl fluoride) and by pipetting up-and-down 10 times. The cell lysates were transferred to 96-well PolySorp plates (Nunc) and dried completely in a stream of air at about 37°C from a hair dryer. Vacant protein binding sites were blocked by adding 200 μl/well of antibody buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, and 3% (w/v) dried nonfat milk (Carnation)] for 1 h at room temperature. This was removed and replaced with 100 μl of antibody buffer containing 0.1–0.15 μg/ml TG-3 monoclonal antibody (15, 16). This antibody recognizes a phosphoepitope on nucleolin that is present only at mitosis and was provided by Dr. Peter Davies (Albert Einstein College of Medicine, Bronx, NY). After 16–20 h incubation at 4°C, the antibody solution was removed, and the wells were rinsed twice with 200 μl of 10 mM Tris-HCl (pH 7.4), 0.02% Tween 20. HRP-conjugated goat antimouse IgM secondary antibody (Southern Biotechnology Associates) was added at a dilution of 1:500. After overnight incubation at 4°C, the antibody solution was removed, and the wells were rinsed three times with 200 μl of 10 mM Tris-HCl (pH 7.4), 0.02% Tween 20. One hundred μl of 120 mM $Na_2HPO_4$, 100 mM citric acid (pH 4.0) containing 0.5 μg/ml 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and 0.01% hydrogen peroxide was added for 1 h at room temperature, and absorbance at 405 nm was determined using a Dynex MRX plate reader.

**ELICA of Mitotic Cells.** After incubation with extracts, the medium was withdrawn carefully using a pipettor, and 100 μl of 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, containing 3.7% formaldehyde, were added to fix the cells for 30 min at 4°C. The fixative was removed and replaced with 100 μl of cold (−20°C) methanol for 5 min to permeabilize the fixed cells. The methanol was removed, and the wells were rinsed briefly with 200 μl of antibody buffer. Then, 100 μl of antibody buffer containing 0.1–0.15 μg/ml TG-3 monoclonal antibody and HRP-conjugated goat antimouse IgM secondary antibody at a dilution of 1:500 was added for 16–20 h at 4°C. The plates were washed twice with 200 μl of 10 mM Tris-HCl (pH 7.4), 0.02% Tween 20. One hundred μl of 120 mM $Na_2HPO_4$, 100 mM citric acid (pH 4.0) containing 0.5 μg/ml 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and 0.01% hydrogen peroxide were added for 1 h at room temperature, and absorbance at 405 nm was measured. Additional information about this assay is provided in "Results."

**Sample Collection and Extract Preparation.** Approximately 250 g each of marine invertebrates were collected by hand, using scuba, from the cold temperate waters of the Pacific Ocean along the coast of British Columbia, from tropical Pacific Ocean reefs off Motupore and Madang in Papua New Guinea, and from tropical waters off the island of Dominica in the Caribbean. Samples were deep frozen on site and transported to Vancouver over dry ice. Voucher samples of each invertebrate are stored in methanol at −20°C at the University of British Columbia for taxonomic identification. Marine microorganisms were isolated from the invertebrates on site using various marine culture media. and pure cultures were grown as lawns on solid agar marine media in 10-cm Petri plates for several days and then freeze-dried.

Extracts of invertebrates were prepared by homogenizing in methanol ~200 g of each sample. The homogenates were filtered and concentrated to dryness *in vacuo* to give a gummy residue. Extracts of microorganisms were prepared by extracting the freeze-dried culture (cells and agar) multiple times with dry methanol:acetone, followed by lyophilization. A small amount of each extract was dissolved in DMSO for the antimitotic screen. Extracts of terrestrial plants were obtained from the Open Repository Program of the Natural Products Repository of the NCI Developmental Therapeutics Program as 500-μg samples that were dissolved in 100 μl of DMSO. All diluted extracts were stored at −20°C.

## RESULTS

**Screen for Antimitotic Agents.** The TG-3 monoclonal antibody, originally described as a marker of Alzheimer's disease (15), is highly specific for mitotic cells. Flow cytometry shows that TG-3 immunofluorescence is >50-fold more intense in mitotic cells than in interphase cells (16). In Western blots, the antibody reacts with a $M_r$ 105,000 protein that is present in abundance in extracts of cells treated for 20 h with the antimitotic agent nocodazole but present at only low levels in extracts from cycling MCF-7 cells (Fig. 1). This protein has been identified as a mitotically phosphorylated form of nucleolin (17). Densitometric scanning of the bands showed a 27-fold difference in intensity between nocodazole-treated and untreated cells, corresponding well to the difference in the number of mitotic cells in the two samples: 80% for the nocodazole-treated sample and 3% for the untreated sample, as measured by microscopy.

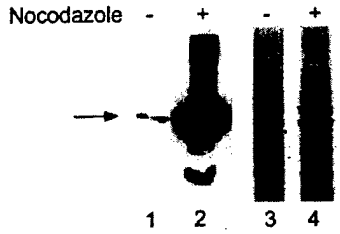


Fig. 1. Western blot, using the TG-3 antibody, of total protein extracts from cycling cells and nocodazole-treated cells (*Lanes 1* and *2*). *Arrow*, mitotically phosphorylated form of nucleolin at $M_r$ 105,000. The film was overexposed to illustrate the quantitative difference between cells treated or not with the antimitotic agent. Coomassie blue-stained lanes used as gel loading controls (*Lanes 3* and *4*).

TG-3 also recognizes mitotic cells in ELISA using microtiter plates (18). In this standard assay (19), cells grown in 96-well plates are lysed, and the lysates are transferred to protein-binding ELISA plates for adsorption to the plastic surface. The antigen is detected by incubating with TG-3 antibody, followed by an HRP-conjugated secondary antibody and colorimetric determination of HRP activity.

We first tested the suitability of the ELISA for quantifying the activity of antimitotic agents. MCF-7 cells were incubated for 20 h with different concentrations of the antimitotic drug paclitaxel, and the proportion of cells arrested in mitosis was measured by counting mitotic figures in the microscope and by ELISA. Paclitaxel induced mitotic arrest in a concentration-dependent manner with half-maximal activity at 10 nM measured by microscopy (Fig. 2*A*) and at 4 nM measured by ELISA (Fig. 2*B*).

ELISA is a lengthy and labor-intensive procedure requiring the preparation of cell lysates, their transfer to protein-binding plates, and many solution changes. We subsequently simplified it, reducing the time of the procedure and the number of steps by half and avoiding transfer of samples to ELISA plates. In this procedure, the cells are fixed with formaldehyde in their microtiter culture plate and permeabilized with methanol and detergents, and the TG-3 primary antibody and HRP-conjugated secondary antibody are added simultaneously. Colorimetric detection of HRP activity remains unchanged. Because cell fixation and permeabilization *in situ* are steps commonly used in immunocytochemistry, we termed the assay ELICA.

The ELICA was tested as above. Dose-dependent arrest of cells in mitosis by paclitaxel was detected by ELICA with half-maximal activity at 1.5 nM (Fig. 2). The ELICA showed a higher signal at low paclitaxel concentrations and a lower signal at high concentrations than did the ELISA (Fig. 2*B*). These differences probably resulted from higher nonspecific staining of interphase cells because of reduced washing and from lower specific staining of mitotic cells because of fixation and reduced antibody incubation times. Nevertheless, the ELICA consistently showed sufficient difference in absorbance between cells treated or not with antimitotic agents to allow unambiguous detection of mitotic cells. Measurements obtained by ELICA consistently showed smaller SDs than obtained by ELISA, probably because the reduced number of manipulations reduced experimental variation.

**Screening of Natural Extracts.** We first tested the suitability of the ELISA for drug screening using a small selection of crude extracts from marine microorganisms (Table 1). Of the 264 extracts tested, 261 showed no activity, giving absorbance readings not statistically different from those of untreated cells. Three extracts clearly showed

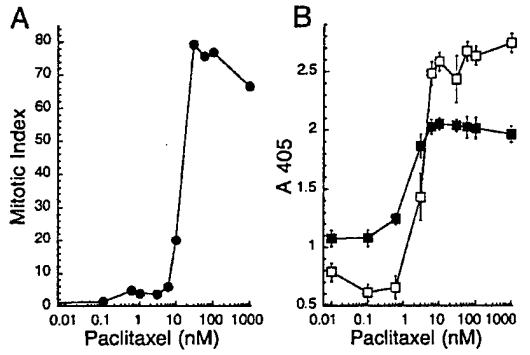


Fig. 2. Evaluation of the ELISA and ELICA using paclitaxel. *A* and *B*, cells were treated with different concentrations of paclitaxel for 20 h, and antimitotic activity was determined using mitotic spreads (●), ELISA (□), or ELICA (■). Experiments were carried out in triplicate, and values indicate means; *bars*, SD.

activity, with absorbance readings of 1.135, 1.437, and 1.245, close to the values obtained with nocodazole as a positive control.

We then screened over 2000 crude extracts of marine sponges, tunicates, soft corals, starfish, and nudibranchs. This screen identified 16 additional extracts with antimitotic activity. The positive extracts were retested by counting mitotic cells in the microscope, and all were confirmed to arrest cells in mitosis.

Finally, we screened crude extracts of terrestrial plants from the NCI Natural Products Repository by ELICA. The suitability of the ELICA for drug screening is illustrated in Table 2, which displays a screen of 264 plant extracts from three randomly selected 96-well plates. Five extracts showed activity, with absorbance readings close to or higher than those obtained with nocodazole. These positive readings were well above those obtained with negative controls or extracts showing no activity. Of 21,600 plant extracts tested in this manner, 100 showed activity, all of which were confirmed to be positive by microscopy.

All positive extracts from marine organisms and most positive extracts from plants were then rescreened using tubulin immunofluorescence microscopy (14) to examine their effects on microtubule structure. We then purified and identified the active agents in the three microbial extracts, in the single marine invertebrate extract that produced paclitaxel-like bundling of microtubules, and in the terrestrial plant extract that showed clearest evidence of microtubule bundling. The other extracts remain to be studied.

**Identification of New Rhizoxin Analogues.** Marine bacterial isolate MK7020 collected off the coast of British Columbia was identified as a *Pseudomonas* sp. by gas chromatographic analysis of cellular fatty acids. The active compounds **1** and **2** (Fig. 3) were purified by chromatographic procedures using the ELISA to guide fractionation. The two other microbial extracts were found to be independent isolates of the same *Pseudomonas* species and contained the same active compounds as MK7020.

Compound **1** is identical to WF-1360C (20, 21), a previously reported analogue of the antimitotic agent rhizoxin (Fig. 3). Compound **1** showed half-maximal antimitotic activity ($IC_{50}$) at 52 nM, as determined by ELISA (data not shown). Compound **2** is a new δ-lactone seco-hydroxy acid and had an $IC_{50}$ of 8 nM (data not shown).

**Identification of New Eleutherobin Analogues.** An extract of octocoral *Erythropodium cf. caribaeorum* collected from shallow reefs near Dominica showed antimitotic activity and bundling of microtubules. The active compounds **3–10** (Fig. 4) were isolated, and their chemical structure was elucidated as described in detail elsewhere (22).

Compound **3** was identified as eleutherobin, a recently discovered antimitotic agent that acts like paclitaxel by stabilizing microtubules (23, 24). Compound **4** was identified as sarcodictyin A (25) and differs from eleutherobin by replacement of the C-15 β-linked 2′-*O*-acetyl-D-arabinopyranose side chain of **3** with a methyl ester and replacement of the C-4 methoxyl with a hydroxyl group. Compounds **5–10** have not been reported previously. Desacetyleleutherobin (**5**) retains the arabinose, but not the 2′ acetyl substituent. Isoeleutherobin A (**6**) has an acetyl group at the 3′ position instead of the 2′ position. *Z*-Eleutherobin (**7**) is a geometric isomer of eleutherobin at the C-2′ to C-3′ double bond of the C-8 *N*-(6′)-methylurocanic acid ester side chain. Desmethyleleutherobin (**8**) differs from eleutherobin by the presence of a hydroxyl instead of a methoxyl at C-4. Caribaeoside (**9**) differs from eleutherobin by the presence of a hydroxyl at C-11 of the tricyclic core, and a double bond at C-12 to C-13 instead of C-11 to C-12, significantly altering the cyclohexene ring. Caribaeolin (**10**) differs from caribaeoside only by the presence of a $-CH_2OCO-CH_3$ substituent in the C-3 side chain.

The antimitotic activity profile of these compounds determined by

Table 1 *Pilot ELISA screen of microbial extracts*

| | $A_{405}$[a] | | | |
|---|---|---|---|---|
| 96-well plate | Positive extracts | Negative extracts | Negative control (no extract added) | Positive control (nocodazole) |
| A | 1.135<br>1.437 | 0.270 ± 0.051 ($n$ = 86) | 0.294 ± 0.098 ($n$ = 4) | 1.615 ± 0.068 ($n$ = 4) |
| B | — | 0.280 ± 0.040 ($n$ = 88) | 0.267 ± 0.033 ($n$ = 4) | 1.298 ± 0.136 ($n$ = 4) |
| C | 1.245 | 0.276 ± 0.040 ($n$ = 87) | 0.305 ± 0.035 ($n$ = 4) | 1.448 ± 0.059 ($n$ = 4) |

[a] Values shown are mean and SD of the number of measurements shown in parentheses. The absorbance readings are raw data, not corrected for background caused by the microtiter plate and reagents.

Table 2 *ELICA screen of plant extracts*

| | $A_{405}$[a] | | | |
|---|---|---|---|---|
| 96-well plate | Positive extracts | Negative extracts | Negative control (no extract added) | Positive control (nocodazole) |
| 97040140 | 2.141<br>2.366<br>2.181 | 0.731 ± 0.346 ($n$ = 85) | 0.752 ± 0.047 ($n$ = 4) | 2.379 ± 0.057 ($n$ = 4) |
| 97040141 | 2.313 | 0.651 ± 0.198 ($n$ = 88) | 0.712 ± 0.048 ($n$ = 4) | 1.555 ± 0.113 ($n$ = 4) |
| 97040143 | 1.421 | 0.558 ± 0.240 ($n$ = 86) | 0.558 ± 0.046 ($n$ = 4) | 1.681 ± 0.030 ($n$ = 4) |

[a] Values shown are mean and SD of the number of measurements shown in parentheses. The absorbance readings are raw data, not corrected for background caused by the microtiter plate and reagents.

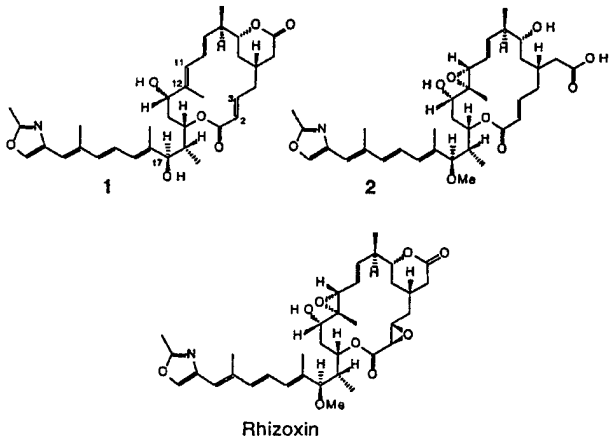


Fig. 3. Structural formulae of rhizoxin and analogues.

ELICA is shown in Fig. 5. Eleutherobin (**3**) had an $IC_{50}$ of 100 nM. The activity of *Z*-eleutherobin (**7**) was close, with an $IC_{50}$ of 250 nM. Desmethyleleutherobin (**8**) and isoeleutherobin A (**6**) were slightly more potent than eleutherobin, with $IC_{50}$ of 20 and 50 nM, respectively. Desacetyleleutherobin (**5**) was slightly less potent, with an $IC_{50}$ of 400 nM. Sarcodictyin A (**4**) showed lower activity, with an $IC_{50}$ of 2 $\mu$M. Caribaeoside (**9**) and caribaeolin (**10**) were considerably less potent, with an $IC_{50}$ of 20 $\mu$M for both compounds.

**Identification of Paclitaxel Analogues in a Non-*Taxus* Species.** NCI Natural Products Repository extract N29701 was obtained from the stem bark of the tree *Ilex macrophylla* in Kalimantan, Indonesia. It showed antimitotic activity and caused bundling of microtubules. The active compounds were isolated and analyzed using ELICA and identified as the known paclitaxel analogues 10-deacetylaxuyunnanine A (**11**) and 7- ($\beta$-xylosyl)-10-deacetyltaxol C (**12**) (Fig. 6) by analysis of their nuclear magnetic resonance data and comparison with published values (26, 27). Compounds **11** ($IC_{50}$, 0.3 $\mu$M) and **12** ($IC_{50}$, 10 $\mu$M) were much less potent than paclitaxel ($IC_{50}$, 1.5 nM).

## DISCUSSION

**Cell-based Assay.** We have described a cell-based assay for antimitotic compounds. When searching for therapeutic agents, cell-based assays are particularly valuable compared with cell-free assays because they select not only for activity against a particular target but also for other desirable properties, such as the ability to permeate cells and to retain activity in tissue culture medium and in cells. In one study, >90% of compounds found on the basis of *in vitro* target-based assays showed no cytotoxic activity because they did not cross the plasma membrane or were degraded rapidly (28). In addition, assays based on measuring arrest of cells in mitosis have the potential to identify not only agents that interact with microtubules but also agents that cause mitotic arrest by other mechanisms, such as protein phosphatase inhibitors and mitotic kinesin inhibitors (9–11).

The ELISA and the ELICA procedures both allow unambiguous detection of antimitotic activity in crude natural extracts. The ELICA was used for most of the screening described here because it is faster, less labor-intensive, and less costly than the ELISA.

Our screen of over 24,000 crude extracts from different natural sources identified unambiguously 119 with antimitotic activity. The absence of false-positive results was confirmed by microscopy, and all five positive crude extracts that were subjected to further study yielded known or novel antimitotic agents; three extracts from the pilot screen contained members of the rhizoxin family, one marine invertebrate extract contained compounds related to eleutherobin, and a tree extract contained paclitaxel analogues.

**Structure-Antimitotic Activity Relationships.** The assay is useful not only for identifying and purifying antimitotics but also for providing a quantitative measure of their antimitotic activity. This is a helpful indicator of a compound's pharmacological potential because it measures not simply the interaction of the compound with its target, as an *in vitro* assay would do, but its ability to interact with its target within a cell. We used it to compare the antimitotic activity of different analogues of rhizoxin, eleutherobin, and paclitaxel.

Rhizoxin is a 16-membered macrolide isolated in 1984 (29) and later found to cause the accumulation of cells in mitosis (30, 31) and to inhibit microtubule assembly (31, 32). Rhizoxin is very cytotoxic to cancer cells *in vitro* or in mice (20, 30), including cell lines resistant to the *Vinca* alkaloids (30). It has been the subject of several Phase I and II clinical trials, but results have been disappointing (reviewed in Ref. 33). To the best of our knowledge, the seco-hydroxy acid **2** was not known previously as a natural product, having been reported in the patent literature only as a semisynthetic derivative of the correspond-

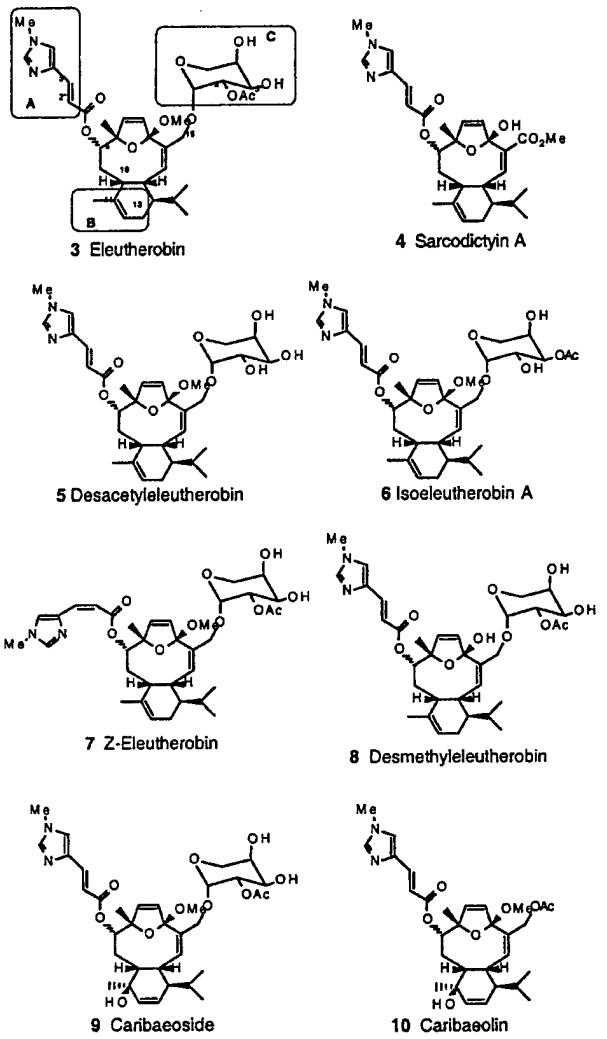


Fig. 4. Structural formulae of eleutherobin and analogues. The *boxed regions A, B,* and *C* of eleutherobin are those considered important for activity in the pharmacophore proposed in (44).

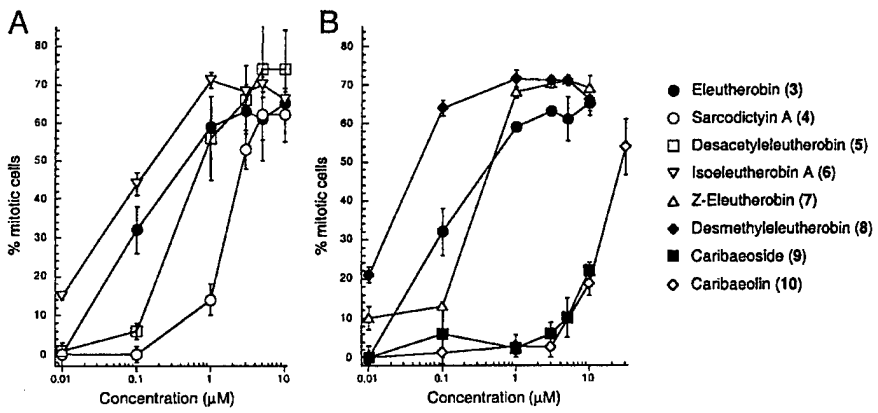


Fig. 5. Antimitotic activity of eleutherobin and analogues. Cells were treated with different concentrations of the compounds for 20 h, and mitotic arrest was determined by ELICA. The absorbance values were transformed into the percentage of mitotic cells using a standard curve constructed by measuring the absorbance values of cell populations containing defined percentages of mitotic cells (18). *Bars*, SD.

ing δ-lactone. WF-1360C (**1**) was 15-fold less toxic to P388 cells than rhizoxin (20). It differs from rhizoxin by the presence of a hydroxyl group instead of a methoxyl at C-17 and the absence of the two epoxides at C-2 to C-3 and C-11 to C-12. Compound **2** retains the methoxyl and one epoxide but has an open lactone ring. Comparison of the antimitotic activity of WF-1360C ($IC_{50}$, 52 nM) to that of compound **2** ($IC_{50}$, 8 nM) and to published cytotoxicity data for other analogues (20, 32) indicates that a closed lactone ring is not required for antimitotic activity and that the presence of a methoxyl substituent at C-17 contributes to the high potency of rhizoxin.

Eleutherobin was identified as a compound with paclitaxel-like properties in 1997 (23), but sarcodictyins A-D were the first members of the eleutherobin class of compounds to be identified (25, 34), their paclitaxel-like properties were recognized only later (35). Sarcodictyin A (**4**) was 20-fold less active than eleutherobin (**3**), indicating that the C-15 β-linked 2′-*O*-acetyl-D-arabinopyranose side chain or the C-4 methoxyl group is important for antimitotic activity. Desmethyleleutherobin (**8**) was active, showing that it is the C-15 side chain and not the C-4 methoxyl that is required. Desacetyleleutherobin (**5**) and isoeleutherobin A (**6**) showed activity similar to eleutherobin, indicating that the acetyl group does not contribute importantly to activity. Therefore, although the sugar moiety is not absolutely required for antimitotic activity, it contributes to the high potency of eleutherobin.

Isomerization of the C-2′ to C-3′ double bond of the C-8 side chain of *Z*-eleutherobin (**7**) had little effect on the antimitotic activity of the compound, showing that the *E* configuration in eleutherobin is not required for antimitotic activity. Desmethyleleutherobin (**8**) was the most active of the compounds tested, suggesting that the C-4 hydroxyl might enhance activity through additional hydrogen bonding, or that the C-4 methoxyl somehow hinders the activity of eleutherobin. Caribaeoside (**9**) was 200-fold less active than eleutherobin, revealing the importance of the C-11 to C-13 segment for antimitotic activity. Caribaeolin (**10**) differs from caribaeoside (**9**) only in the C-3 side chain, and the activities of these compounds are similar. Likewise, sarcodictyin A differs from desmethyleleutherobin only in the C-3 side chain, but its activity is lower than that of desmethyleleutherobin. These data indicate that the C-15 acetyl-D-arabinopyranose can be replaced with an acetoxy functionality without significant loss of activity, confirming earlier data with synthetic analogues (36, 37), but not with a methyl ester.

Thirteen synthetic eleutherobin analogues have recently been described and tested in tubulin polymerization and cytotoxicity assays (36–38). Overall, these studies underlined the importance of the C-8 and C-3 side chains for activity, the C-8 side chain being essential and the sugar or another bulky substituent being needed at C-3 for optimal activity. All of the synthetic analogues retained the original eleutherobin core and therefore provided no information about the importance of segments of the tricyclic core.

Eleutherobin represents one of five chemical structural types known to arrest cells in mitosis by stabilizing microtubules. The other four are paclitaxel, discodermolide, the epothilones, and the laulimalides (39–41). Several pharmacophores have been proposed for members of this group (42–44). The latter (44) included eleutherobin and proposed three regions of common overlap between the chemotypes, shown as boxes A, B and C in Fig. 4. Region A of eleutherobin consists of the C-8 side chain, region B encompasses the C-11 to C-13 segment of the tricyclic skeleton, and region C consists of the C-15 substituent. The importance of regions A and C is supported by the published structure-activity data for eleutherobin analogues mentioned above (36–38). Our demonstration that caribaeoside (**9**), which differs from eleutherobin only in region B, shows a 200-fold lower

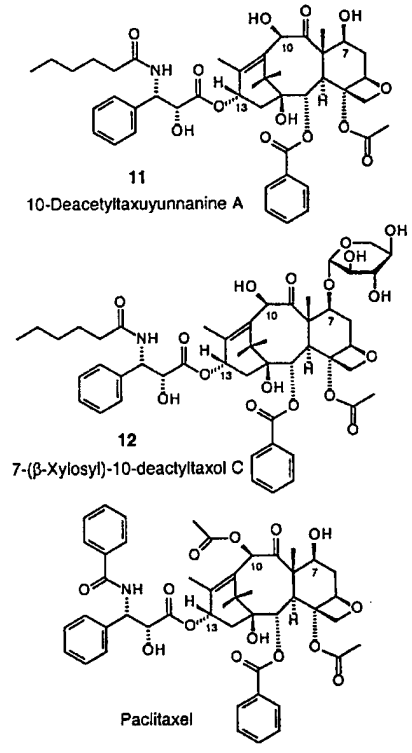


Fig. 6. Structural formulae of paclitaxel and analogues.

activity demonstrates an important role for this region in antimitotic activity. Further studies will be required to determine whether the reduced antimitotic activity of caribaeoside is attributable to reduced affinity for tubulin and microtubules or to factors such as drug uptake, extrusion, or metabolism.

Paclitaxel is an approved drug for the treatment of advanced ovarian cancer and metastatic breast cancer. It was originally isolated from *Taxus brevifolia* in 1971 (45). Since then, over 350 related diterpenoids have been isolated from different species of the genus *Taxus* (46), including compounds **11** and **12** described here. Compound **11** differs from paclitaxel in the nature of the *N*-acyl substituent on the C-13 phenylisoserine side chain and in the absence of the acetyl substituent at C-10. It was less active than paclitaxel, showing that the C-13 and C-10 substituents, although not essential for activity, contribute to the high potency of paclitaxel. Compound **12** further differs from paclitaxel by the presence of a β-xylosyl substituent at C-7. Compound **12** was less active than compound **11,** indicating that the C-7 substituent also contributes to the potency of paclitaxel.

**Unexpected Outcomes.** An unexpected outcome of this study is that although the active compounds we isolated belong to known antimitotic chemotypes, they were found in organisms not known or suspected to produce them. To our knowledge, rhizoxin compounds have previously only been isolated from the rice seedling blight fungus *Rhizopus chinensis* and unidentified species of the same genus (29). We have now identified rhizoxin analogues in marine bacterial isolates of the genus *Pseudomonas*, which is common in Pacific Northwest waters. Eleutherobin was originally isolated from the soft coral *Eleutherobia* sp. (possibly *E. albiflora*) collected in Western Australia (23). We now identify eleutherobin in the Caribbean octocoral *Erythropodium caribaeorum*. This is of practical significance because it has not been possible to obtain sufficient amounts of natural or synthetic eleutherobin for preclinical development (47). The taxonomic classification of this source was confirmed by the identification of large quantities of the erythrolide diterpenoids characteristic of this species (48). In contrast to *Eleutherobia*, *E. caribaeorum* is widespread in the Caribbean and Florida (49–51), abundant in certain areas, and has been grown in aquaria. It may thus constitute a suitable source of eleutherobin for preclinical and early phase clinical trials. Paclitaxel and analogues have all been isolated from the bark of yew trees (46), from endophytic fungi isolated from the *Taxus* species or *Taxodium distichum* (52, 53), and recently from an epiphytic fungus on the rubiaceous plant *Maguireothamnus speciosus* (54). It was surprising to find paclitaxel analogues in the bark of a non-*Taxus* tree. The taxonomic classification of our extract was confirmed by the presence of the triterpenoid glycosides characteristic of the genus *Ilex* (55). It is possible that an endophytic fungus is responsible for their production in *Ilex macrophylla*.

Perhaps the most important outcome of this study is that the assay permitted us to detect antimitotic agents in extracts that were not found to contain them using other methods. *E. caribaeorum* has been subjected to extensive chemical characterization (48), but eleutherobin compounds were not detected because they are very minor components. The COMPARE algorithm, which is able to detect similar differential patterns of growth inhibition for the 60 human cell lines in the NCI anticancer drug screen, has been used successfully to identify new antimitotic agents within the NCI chemical repository of pure compounds (13). Extracts in the NCI Natural Products Repository have also been tested against the NCI cell line panel. A COMPARE analysis using paclitaxel as the probe compound identified 47 plant extracts with a Pearson correlation coefficient above 0.6 (not shown). Three of these extracts were positive in our antimitotic screen, and all three were from *Catharanthus roseus*, the plant from which the *Vinca* alkaloids were originally isolated (1). The analysis did not identify the extract from *Ilex macrophylla,* the growth inhibition pattern of which does not resemble that of paclitaxel and other antimitotic compounds. This illustrates the usefulness of the cell-based assay for the identification of active compounds present in very low abundance in crude natural extracts. The assay should greatly facilitate the discovery and development of novel antimitotic agents and their characterization in the context of living cells.

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# Antimitotic diterpenoids from *Erythropodium caribaeorum*: isolation artifacts and putative biosynthetic intermediates

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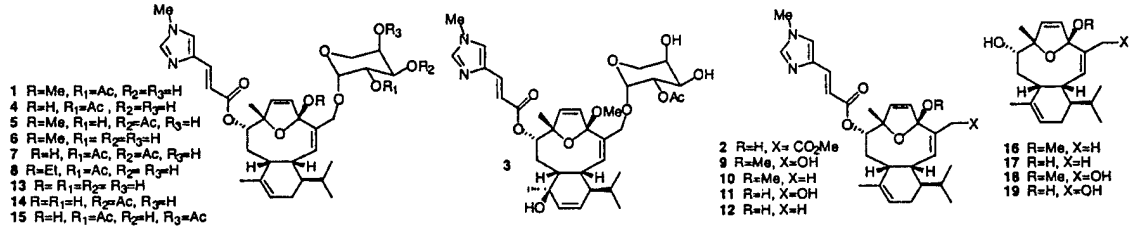
**Abstract**—Two new natural products, caribaeorane (**12**) and 15-hydroxycaribaeorane (**11**), have been identified in *Erythropodium caribaeorum* extracts by isolation of their C-4 methylketals **10** and **9**. It has been demonstrated that eleutherobin (**1**) is an isolation artifact. A proposal for the late stages of the biosynthetic pathway to the *E. caribaeorum* antimitotic diterpenoids is presented.


Eleutherobin (**1**), isolated by Fenical and coworkers from the Western Australian soft coral *Eleutherobia* sp., belongs to a small family of soft coral diterpenoids that are microtubule-stabilizing antimitotic agents.[1] A large amount of effort has been directed towards the total synthesis of eleutherobin and other members of this family.[2] Although these efforts have culminated in the total synthesis of eleutherobin (**1**), its preclinical evaluation as an anticancer drug has been stalled by the limited supply of material available for testing.[3] Recently, we reported that the relatively abundant Caribbean soft coral *Erythropodium caribaeorum* is a good source of eleutherobin (**1**) and a number of analogs including sarcodictyin A (**2**), caribaeoside (**3**), *Z*-eleutherobin, desmethyleleutherobin (**4**), isoeleutherobin A (**5**), and desacetyleleutherobin (**6**).[4,5] *E. caribaeorum* could provide adequate quantities of material for preclinical evaluation of this promising family of anticancer drug leads and even clinical trials should the compounds progress that far.

Initial collections of *E. caribaeorum* examined by our laboratory were extracted with MeOH, resulting in the isolation of highly variable ratios of eleutherobin (**1**) and desmethyleleutherobin (**4**). Ketzinel et al. have reported isolating the eleuthosides (e.g. **7**), which are all hemiketals at C-4, from *Eleutherobia aurea* by using non-alcohol solvents such EtOAc.[6] These observations raised the possibility that eleutherobin (**1**) was actually an isolation artifact. To resolve this issue, fresh specimens of *E. caribaeorum* were collected in Dominica and one portion of the sample was extracted with EtOH and a second portion was extracted with MeOH. The MeOH extracted sample yielded eleutherobin (**1**), desmethyleleutherobin (**4**), and sarcodictyin A (**2**) as the major components along with minor amounts of **3**, **5**, and **6** as before,[5] while the EtOH extracted sample yielded the C-4 ethylketal **8** along with **2** and **4** as the major components. Eleutherobin (**1**) could not be detected by analytical HPLC or NMR analysis of the chromatography fractions from the EtOH extract.

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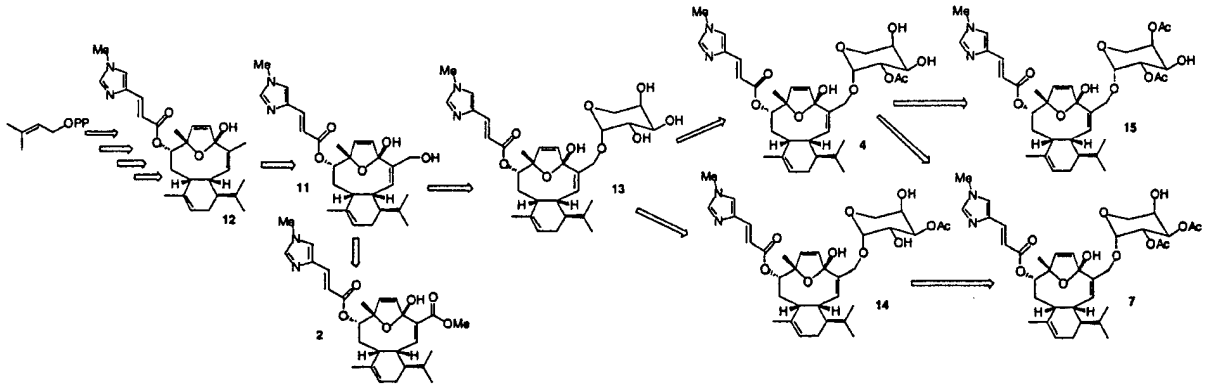
These results show that the C-4 methyl ketal in eleutherobin (**1**) isolated from *E. caribaeorum* is indeed an artifact.

During the course of fractionating a large scale *E. caribaeorum* MeOH extract in order to obtain sufficient eleutherobin (**1**) for biological testing, the eleutherobin aglycon **9**[7] and methylcaribaeorane (**10**) were isolated as very minor components along with sarcodictyin A (**2**) from the least polar fractions eluting from silica gel with EtOAc:MeOH (85:15). The eleutherobin aglycon **9** has previously been reported as a synthetic intermediate.[2a] Comparison of the spectroscopic data collected on the material isolated from *E. caribaeorum*[7] with the literature data for the synthetic compound confirmed that they were identical. The eleutherobin aglycon **9** is presumed to be an artifact formed from the corresponding hemiketal natural product 15-hydroxycaribaeorane (**11**) during the MeOH extraction.

Methylcaribaeorane (**10**),[8] isolated as a white amorphous solid, gave an $[M+H]^+$ ion at $m/z$ 467.2912 in the HRFABMS appropriate for a molecular formula of $C_{28}H_{38}N_2O_4$ that differed from the molecular formula of the eleutherobin aglycon **9** simply by the loss of one oxygen atom. Examination of the $^1H$ and $^{13}C$ NMR data obtained for methylcaribaeorane (**10**) revealed the presence of two olefinic methyl groups ($^1H$ NMR, $C_6D_6$: $\delta$ 1.65, s, 3H; 1.80, s, 3H) and the absence of resonances that could be assigned to an allylic hydroxymethyl fragment. These observations indicated that methylcaribaeorane (**10**) was simply missing the C-15 allylic alcohol found in the eleutherobin aglycon **9**. HMBC correlations observed between a methyl resonance at $\delta$ 3.18 and a carbon resonance at $\delta$ 117.4 assigned the carbon resonance to the C-4 ketal. The olefinic methyl resonance at $\delta$ 1.80 showed HMBC correlations to the C-4 ketal carbon resonance at $\delta$ 117.4 and to the C-2 and C-3 olefinic carbon resonances at $\delta$ 131.3 and 134.0, respectively, confirming that there was an allylic methyl at C-15. The remaining NMR data for methylcaribaeorane was completely consistent with the structure **10**.[8] Methylcaribaeorane (**10**) is also presumed to be an isolation artifact formed from the corresponding hemiketal natural product caribaeorane (**12**). The eleutherobin aglycon **9** and methylcaribaeorane (**10**) were active in a cell-based assay for antimitotic activity at 1 and 10 μM, respectively.[5]

Most of the compounds isolated from the *E. caribaeorum* MeOH extract had C-4 methylketals, which are artifacts. Interestingly, sarcodictyin A (**2**) was only isolated as the C-4 hemiketal. Since methylketal transformations clearly take place during the extraction process, it seemed possible that the eleutherobin aglycon **9** might also be an artifact formed from eleutherobin (**1**) by hydrolysis/methanolysis of the glycosidic linkage during extraction. An investigation of the ketal transformations was undertaken in order to confirm that glycoside hydrolysis was not occurring during extraction and to gain insight into the lack of ketal formation in sarcodictyin A (**2**). First, it was found that desmethyleleutherobin (**4**) can be converted quantitatively to eleutherobin (**1**) by treatment with a catalytic amount of pyridinium *p*-tolunesulfonate (PPTS) in methanol at room temperature.[2] The reverse transformation, **1** to **4**, can be quantitatively effected using PPTS and water/$CH_2Cl_2$. Neither of these transformations result in glycoside hydrolysis. Saracodictyin A (**2**) could also be converted quantitatively to its methylketal using PPTS and MeOH at rt. The combination of the extraction and the laboratory observations indicate that the MeOH extraction conditions are able to readily convert the C-4 hemiketal to the methylketal when there is a methyl, hydroxymethyl, or glycosidic functionality at C-15, but that they cannot effect methylketal formation when there is a ester at C-15. The PPTS in MeOH or PPTS in water/$CH_2Cl_2$ quantitatively interconverts all C-4 hemiketals and methylketals, but still does not bring about glycoside hydrolysis. Therefore, it is apparent that the milder MeOH extraction conditions will not cleave the glycoside linkage, confirming that 15-hydroxycaribaeorane (**11**) is indeed a natural product.

The discovery of methylcaribaeorane (**10**) and the eleutherobin aglycon **9** as minor constituents in the MeOH extracts of *E. caribaeorum* along with the previously reported compounds **1** to **6**, and the demonstration that the C-4 methyl ketal functionalities in these



**Scheme 1.**

compounds are isolation artifacts, led to the biosynthetic proposal presented in Scheme 1. This proposal suggests that geranylgeranylpyrophosphate undergoes cyclization and oxidative functionalization to give the diterpenoid core which is esterified at the C-8 hydroxyl with the urocanic acid residue to give caribaeorane (**12**), the first known intermediate in the pathway. Caribaeorane (**12**) would then be oxidized to 15-hydroxycaribaeorane (**11**) and the arabinose residue would be added to give desacetyldesmethyleleutherobin (**13**), which if monoacetylated at C-2′ would give desmethyleleutherobin (**4**) or at C-3′ would give desmethylisoeleutherobin A (**14**). Further acetylation of either **4** or **14** would give eleuthoside A (**7**), while acetylation of desmethyleleutherobin (**4**) at C-4′ would give eleuthoside B (**15**). In this proposal, sarcodictyin A (**2**) represents a shunt metabolite formed by further oxidation of 15-hydroxycaribaeorane (**11**) to the 15-carboxylic acid followed by SAM methylation to give the methyl ester. It should be noted that there is no evidence for the occurrence of either **7** or **15** in the *E. caribaeorum* extract, however, their biosynthesis in *Eleutherobia aurea* would presumably follow the same pathway.

One significant aspect of the hypothesis presented in Scheme 1 is the proposal that the urocanic ester residue is added to the diterpenoid core before the C-15 alcohol required for glycoside formation is introduced. In an attempt to provide further evidence for this suggestion, the urocanic acid residue was removed from methylcaribaeorane (**10**) by hydrolysis with a few drops of 5N NaOH in MeOH overnight at rt to give **16**,[9] the methyl ketal analog of one of the potential biosynthetic precursors **17** of caribaeorane (**12**). Careful examination of the *E. caribaeorum* MeOH extract chromatography fractions by TLC and GC analysis using **16** as a reference failed to show any evidence for its presence. The corresponding hydrolysis product **18**[10] of the eleutherobin aglycon **9** was also prepared and once again TLC and GC analysis failed to show any evidence for its presence in the *E. caribaeorum* MeOH extract. This negative evidence does not eliminate **17** as the immediate biosynthetic precursor to caribaeorane (**12**) or rule out the possibility that **19** is the direct precursor to **11**, but it does raise the interesting possibility that the urocanic ester is formed before all the diterpenoid functionality, such as the C-7 tertiary alcohol or the C-4 ketone, are in place.

## Acknowledgements

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7. Eleutherobin aglycon **9**: $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ 7.52, d, $J$=15.4 Hz (H-3′), 7.45, s (H-7′), 7.08, s (H-5′), 6.54, d, $J$=15.4 Hz (H-2′), 6.21, d, $J$=6.0 Hz (H-6), 6.02, d, $J$=6.0 Hz (H-5), 5.56, d, $J$=9.4 Hz (H-2), 5.25, m (H-12), 4.80, d, $J$=7.4 Hz (H-8), 4.16, d, $J$=12.0 Hz (H-15), 3.85–3.95, m (H-15, H-1), 3.69, s (H-9′), 3.23, s (H-21), 2.67, m (OH), 2.59, m (H-10), 2.31, m (H-13), 1.98, m (H-13), 1.50–1.65, m (H-9, H-18), 1.50, s (H-17), 1.45, s (H-16), 1.25–1.40, m (H-9, H-14), 0.97, d, $J$=6.5 Hz (H-19), 0.91, d, $J$=6.5 Hz (H-20); HRFABMS [M+H]$^+$ calcd for $C_{28}H_{39}N_2O_5$: 483.2859, found: 483.2860; $[\alpha]_D$ −24.2°.
8. Methylcaribaeorane (**10**): $^1$H NMR ($C_6D_6$, 500 MHz) $\delta$ 7.95, d, $J$=15.4 Hz (H-3′), 7.26, d, $J$=15.4 Hz (H-2′), 6.72, s (H-7′), 6.00, s (H-5′), 5.95, d, $J$=5.8 Hz (H-6), 5.78, d, $J$=5.8 Hz (H-5), 5.49, d, $J$=9.7 Hz (H-2), 5.33, m (H-12), 5.25, d, $J$=7.3 Hz (H-8), 4.30, m (H-1), 3.18, s (H-21), 2.95, m (H-10), 2.37, m (H-13), 2.25, s (H-9′), 2.00, m (H-9), 1.98, m (H-13), 1.81, m (H-9), 1.80, s (H-15), 1.65, s (H-17), 1.57, m (H-18), 1.46, s (H-16), 1.28, m (H-14), 1.06, d, $J$=6.5 Hz (H-19), 0.89, d, $J$=6.5 Hz (H-20); $^{13}$C NMR ($C_6D_6$, 100 MHz) $\delta$ 167.1 (C-1′), 139.2 (C-4′), 139.2 (C-7′), 137.3 (C-3′), 134.9 (C-11), 134.8 (C-6), 134.0 (C-3), 131.3 (C-2), 130.1 (C-5), 122.6 (C-5′), 121.6 (C-12), 117.4 (C-4), 116.5 (C-2′), 90.5 (C-7), 82.2 (C-8), 49.5 (C-21), 43.3 (C-14), 39.6 (C-10), 34.6 (C-1), 32.3 (C-9), 31.9 (C-9′), 29.4 (C-18), 24.9 (C-16), 24.8 (C-13), 22.4 (C-15), 22.3 (C-17), 22.2 (C-20), 20.8 (C-19); HRFABMS [M+H]$^+$ calcd for $C_{28}H_{39}N_2O_4$: 467.2910, found: 467.2912.
9. Compound **16**: $^1$H NMR ($C_6D_6$, 400 MHz) $\delta$ 5.99, d, $J$=5.9 Hz (H-6), 5.74, d, $J$=5.9 Hz (H-5), 5.41, dd, 1H, $J$=9.3, 1.3 Hz (H-2), 5.36, m (H-12), 4.15, m (H-1), 3.47, m (H-8), 3.19, s (H-21), 2.35, m (H-10), 2.28, m (H-13), 1.97, m (H-13), 1.80, s (H-15), 1.62, s (H-17), 1.50, s (H-16), 1.50–1.72, m (H-9, H-18, H-14, OH), 1.27, m (H-9), 0.98, d, $J$=6.5 Hz (H-19), 0.93, d, $J$=6.5 Hz (H-20); HRDCI$^+$MS calcd for $C_{21}H_{32}O_3$: 332.2351, found: 332.2351.

10. Compound **18**: $^1$H NMR ($C_6D_6$, 400 MHz) $\delta$ 5.94, d, $J$=5.9 Hz (H-6), 5.68, d, $J$=5.9 Hz (H-5), 5.67, d, $J$=9.5 Hz (H-2), 5.31, m (H-12), 4.00–4.20, m (H-1, H-15, H-15), 3.44, bd, $J$=6.1 Hz (H-8), 3.00, s (H-21), 2.23–2.38, m (OH, H-10, H-13), 1.93, m (H-13), 1.59, s (H-17), 1.43, s (H-16), 1.42–1.62, m (H-9, H-18, OH), 1.30, m (H-14), 1.25, m (H-9), 0.93, d, $J$=6.9 Hz (H-19), 0.90, d, $J$=6.7 Hz (H-20); HRDCI$^+$MS calcd for $C_{21}H_{32}O_4$: 348.2301, found: 348.2303.

# Synthetic Transformations of Eleutherobin Reveal New Features of its Microtubule-Stabilizing Pharmacophore

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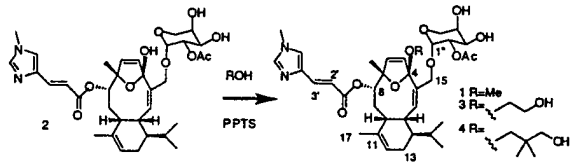
The clinical usefulness of paclitaxel for treating cancer has generated significant interest in finding other compound classes that stabilize microtubules.[1] Discodermolide, epothilones, laulimalide, rhizinilam, WS9885B, xanthochymol, and the eleuthesides also have this important biological property. Several pharmacophore models have attempted to reconcile the SAR data for the taxoids and other compound types in order to generate a sufficiently detailed understanding of the tubulin binding requirements to allow rational design of new classes of microtubule-stabilizing drugs.[2] The predictive power of the pharmacophore models relies on accurate knowledge of the structural features required for activity in each compound class.

Eleutherobin (**1**), isolated by Fenical et al. from the rare alcyonacean *Eleutherobia* sp., belongs to the 'eleutheside' family of microtubule-stabilizing diterpenoids.[3] Total synthesis of eleutherobin, eleuthosides A and B, sarcodictyins A and B, and a combinatorial library of unnatural sarcodictyin analogs[4] has provided information about the nature of the C-8 ester, the C-4 ketal, and the C-15 functionality required for strong interaction of eleuthesides with tubulin, which has facilitated the incorporation of this diterpenoid template into pharmacophore models.[2] However, neither synthesis nor the original natural source has provided sufficient quantities of eleutherobin to permit full *in vivo* evaluation and this has thwarted its further development.

The Caribbean soft coral *Erythropodium caribaeorum* was recently found to be a good source of eleuthesides.[5] Eleutherobin (**1**) can be obtained directly as an isolation artifact from MeOH extracts of *E. caribaeorum* or by chemical transformation of desmethyleleutherobin (**2**), the major eleutheside in the soft coral.[5b] The ready availability of **1** has led to the determination of its solid-state and solution conformations,[6] paved the way for *in vivo* testing, and created an opportunity to investigate chemical transformations of the molecule. In this communication, we report the first detailed investigation of the reactivity of intact eleutherobin, which has uncovered some striking and unanticipated features of its antimitotic pharmacophore.

The total syntheses of eleuthesides have generated very limited diversity in the diterpenoid core, with major variations reported only in the C-15 functionality.[4] Nevertheless, all of the pharmacophore models suggest, without supporting data other than structural overlays, that the cyclohexene ring and its appended substituents (i.e. the isopropyl residue) are important determinants of antimitotic activity. There is also general agreement that the urocanic ester is crucial and the Giannakakou model[2a] suggests that the C-4/C-7 ether bridge in **1** is a hydrogen bond acceptor corresponding to the oxetane oxygen in the taxoids and to the 12,13 epoxide in the epothilones. The importance of the cyclohexene ring is supported by the observation that caribaeoside, which has a β-hydroxyl at C-11 and a $\Delta^{12,13}$ olefin, is ≈ 1,000-fold less active than eleutherobin.[5a] Prompted by the potential importance of the C-4/C-7 ether bridge, the urocanic ester, and the cyclohexene ring for antimitotic activity in the eleuthesides, our chemical transformations focussed on the masked C-4 ketone and olefin oxidation/reduction reactions.




Scheme 1

The first objective was to trap the C-4 ketone in **1** as a cyclic ketal in order to liberate the C-4/C-7 ether oxygen as a C-7 alcohol and to change the oxygen atom's spatial relationship with the C-14 isopropyl group. In order to test the reactivity of the C-4 hemiketal, desmethyleleutherobin (**2**) was treated with various neat aliphatic alcohols (ROH: R = Et, $^n$Pr, $^n$Bu, $^i$Pr) and excess PPTS at rt, which gave the corresponding C-4 ketal analogs in excellent yield (Scheme 1). Attempts to make the C-4 cyclic ketals of **2** with ethylene glycol or 2,2-dimethyl-1,3-propanediol under a variety of conditions using PPTS as a catalyst gave only **3** and **4**. The x-ray structure of eleutherobin[6a] shows significant distortion of the C-1/C-2/C-3 (132°) and C-2/C-3/C-4 (127°) bond angles. This angle strain may act like a clamp to keep the dihydrofuran ring from opening during the transketalization reactions.

Next we turned our attention to oxidation reactions involving the $\Delta^{11,12}$ olefin. Reaction of **1** with MCPBA in $CH_2Cl_2$ at rt for 8 h gave a mixture of two epoxides, **5** and **6** (Scheme 2). The $^1$H NMR data obtained for both **5** and **6** showed the absence of a resonance that could be assigned to H-12 and in both spectra the Me-17 resonance had undergone a significant upfield shift. The observation of a 1D NOESY

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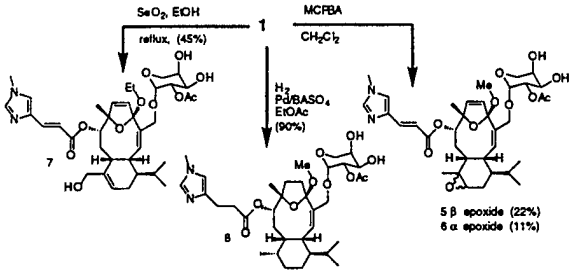
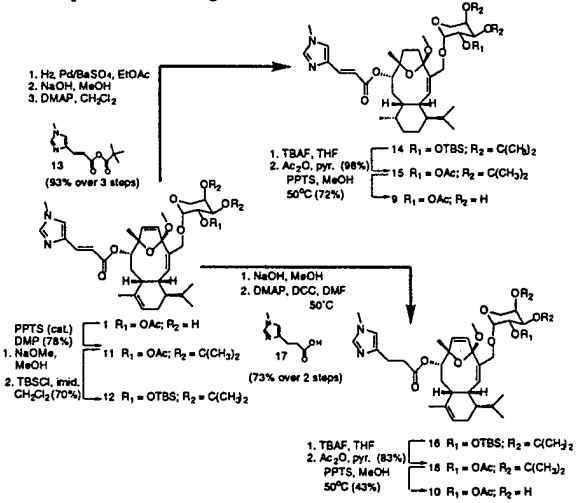
correlation between the Me-17 resonance at δ 1.06 and the H-2 resonance at δ 5.47 showed that the major epoxide was the β isomer **5**. Treatment of **1** with $SeO_2$ (5 equiv.) in refluxing EtOH gave a single product **7** in modest yield.




Scheme 2

Finally, we examined reduction of the olefins. A solution of **1** in EtOAc containing catalytic Pd on $BaSO_4$ was stirred at rt for 1 h under 1 atm of $H_2$ resulting in the formation of hexahydroeleutherobin **8** (Scheme 2). The $^1H$ NMR of **8** contained only a single olefinic proton resonance at δ 5.78 (d, J = 9.3 Hz), assigned to H-2, indicating that the $\Delta^{5,6}$, $\Delta^{11,12}$, and $\Delta^{2',3'}$ double bonds had been reduced. A ROESY correlation observed between the Me-17 resonance (δ 0.76 (d, J = 7.0 Hz)) and the H-2 resonance demonstrated that hydrogen had added to the β face of the $\Delta^{11,12}$ olefin. Hexahydroeleutherobin **8** ($IC_{50}$ $>10^4$ nM) was found to be more than five thousand-fold less active than eleutherobin (**1**) ($IC_{50}$ 20 nM) in a cell-based antimitotic assay,[5a] indicating that the presence of one or more of the reduced double bonds is important for tubulin binding. Biological evaluation of the synthetic sarcodictyin library had suggested that reduction of the $\Delta^{5,6}$ olefin had minimal effect on the potency of tubulin polymerization.[4d] Therefore, 5,6,11,12-tetrahydroeleutherobin (**9**) and 2',3'-dihydroeleutherobin (**10**) were selected as logical targets to further probe the biological effects of olefin reduction.




Scheme 3

The synthesis of **9** started from eleutherobin (**1**), by first forming the 3",4"-acetonide **11**, which was subsequently deacetylated to the 2" alcohol and converted directly to the 2" TBS ether **12**. Hydrogenation of **12**, using a catalytic amount of Pd on $BaSO_4$, gave the 5,6,11,12,2',3'-hexahydro derivative *vide supra* (Scheme 3). Hydrolysis of the crude hydrogenation product cleanly cleaved the C-8 ester side chain, which was replaced with a N-methylurocanic ester residue[4c] to afford **14**. Removal of the TBS protecting group, followed by acetylation provided **15**, which was subsequently deprotected under mildly acidic conditions to give 5,6,11,12-tetrahydroeleutherobin (**9**). Similarily, 2'3'-dihydroeleutherobin (**10**) was prepared from the 2" TBS ether **12**, by hydrolysis of the N-methylurocanic ester residue (NaOH, MeOH) to provide a secondary alcohol at C-8, which was directly coupled with 2,3-dihydro-N-methylurocanic acid (**17**) using DCC and DMAP in warm (50°C) DMF to afford **16**. A deprotection/acetylation sequence similar to that employed in the synthesis of the tetrahydro derivative **9**, provided **10** in good overall yield.

The C-4 ketals **3** and **4** had antimitotic activity ($IC_{50}$ 20 and 80 nM) comparable to eleutherobin ($IC_{50}$ 20 nM) demonstrating that a bulky group can be tolerated at this position. Figure 1A shows dose response curves for antimitotic activity in a cell-based assay[5a] for taxol, eleutherobin (**1**), and the synthetic derivatives **5**, **6**, **7**, **8**, and **10**. The α-epoxide **6** and the 17-hydroxyeleutheside **7** had antimitotic potencies comparable to **1**, while the β-epoxide **5** was ten-fold less active. ($IC_{50}$ **5**: 300, **6**: 30, **7**: 20 nM). This was unexpected in light of the dramatic decrease in activity previously shown by caribaeoside,[5] attributed to the presence of a polar alcohol functionality in a binding region that was thought to require lipophilic character.[2b] Since hydroxylation at Me-17 and α-epoxidation at C-11/C-12 doesn't affect activity, the major negative effect of the C-11 OH in caribaeoside must be more subtle than originally thought.[5a]

Tetrahydroeleutherobin **9** ($IC_{50}$ 200 nM) was only ten-fold less active than eleutherobin, suggesting that reduction of the $\Delta^{2',3'}$ olefin was primarily responsible for the dramatically reduced activity of **8** ($IC_{50}$ $>10^4$ nM). This was confirmed by the observation that 2',3'-dihydroeleutherobin (**10**) ($IC_{50}$ ≈ 20,000 nM) is a thousand-fold less active than eleutherobin (**1**). The lack of activity of 2',3'-dihydroeleutherobin (**10**) in the cell-based antimitotic assay is mirrored in its complete lack of ability to promote the polymerization of purified bovine tubulin in a standard *in vitro* assay (Figure 1B).

The pharmacophore models put forward to date[2a-c] have all suggested that the C-8 urocanic ester is important for tubulin binding in the eleuthesides, however, the remarkably stringent requirement for the 2',3' double bond demonstrated here was completely unanticipated. This important feature of eleutheside binding, along with the observed tolerance for certain types of oxygenated functionality in the cyclohexane ring, will have to be accommodated in future iterations of the models.

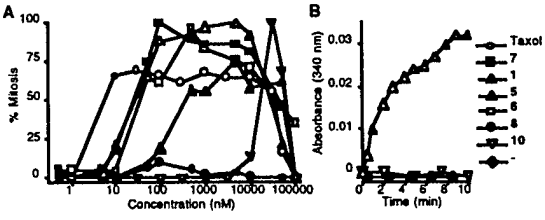



Figure 1. A, antimitotic activity of different compounds; B, microtubule-stabilizing activity of eleutherobin and 2',3'-dihydroeleutherobin.

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Supporting Information. Experimental procedures for the synthetic transformations (14 pages).