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Isolation, structure elucidation and cytotoxic evaluation of endiandrin B from the Australian rainforest plant *Endiandra anthropophagorum*

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ABSTRACT

Chemical investigations of the DCM extract from the roots of *Endiandra anthropophagorum* resulted in the isolation of a new cyclobutane lignan endiandrin B (**1**), together with the known natural products, endiandrin A (**2**), and (−)-dihydroguaiaretic acid (**3**). The structure of **1** was determined by extensive spectroscopic analyses, and confirmed by single crystal X-ray crystallography. Methylation of **1** using diazomethane afforded the previously reported natural product, cinbalansan (**4**). All compounds were evaluated for their cytotoxicity towards human lung carcinoma cells (A549) using high-content screening. (−)-Dihydroguaiaretic acid (**3**) was found to be the most potent cytotoxin against the A549 lung carcinoma cell line, with an IC_{50} value of 7.49 μM.

1. Introduction

The plant family Lauraceae has been the source of over 200 natural products, however only a small number of these secondary metabolites have had any bioactivity reported.[1] Examples of bioactive Lauraceae compounds include the antibacterial agent launobine,[2,3] the antiviral cryptopleurine,[4,5] and the bisbenzylisoquinoline antimalarial dehatrine.[6] The plant genus *Endiandra*, which belongs to the family Lauraceae, has rarely been studied for its chemical composition and prior to 2007 only five compounds had been isolated from this genus.[1] During 2007 we reported the bioassay-guided fractionation of the DCM extract from the roots of *Endiandra anthropophagorum* that resulted in the isolation of a new cyclobutane lignan, endiandrin A (**2**), along with previously reported natural products nectandrin B (**5**) and (−)-dihydroguaiaretic acid (**3**) (Fig. 1).[7] These 3 lignans were shown to display glucocorticoid receptor (GR) binding activity with IC_{50} values of 0.9 (**2**), 27 (**5**) and 35 μM (**3**), respectively.[7] Recollection of this plant sample has enabled us to further investigate the chemistry of this rainforest species, as well as obtain several lignan natural products in sufficient quantities for cytotoxicity evaluations. Herein we report the isolation and structure elucidation of a new cyclobutane lignan that we have named endiandrin B (**1**). The cytotoxicity of endiandrins A (**2**) and B (**1**), (−)-dihydroguaiaretic acid (**3**), and cinbalansan (**4**) against human lung carcinoma cells (A549) determined using high-content screening, is also reported.

2. Results and discussion

The air-dried and ground roots of *E. anthropophagorum* were extracted with DCM and the resulting lipophilic material was partitioned between aq MeOH and hexanes. The polar material was fractionated on a diol bonded silica flash column using a hexanes/EtOAc gradient. Further purification of a lignan enriched fraction using diol bonded silica HPLC (*i*-PrOH/hexanes) yielded (−)-dihydroguaiaretic acid (**3**, 25.7 mg, 0.010% dry wt), endiandrin A (**2**, 75.8 mg, 0.030% dry wt), and endiandrin B (**1**, 7.1 mg, 0.0028% dry wt).

Endiandrin B (**1**) was isolated as clear needles and was assigned the molecular formula $C_{20}H_{24}O_4$ on the basis of HRESIMS and NMR data. The small number of resonances in both the ^1H and ^{13}C NMR spectra (Table 1) coupled with the MS data indicated that **1** was a symmetrical molecule containing nine degrees of unsaturation. The ^1H NMR spectrum of **1** displayed one exchangeable resonance (δ_H 5.34), three aromatic resonances indicative of a trisubstituted

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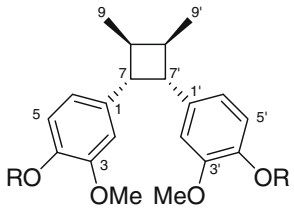
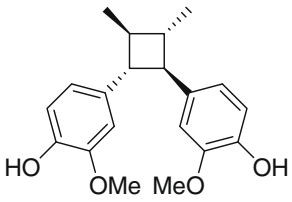
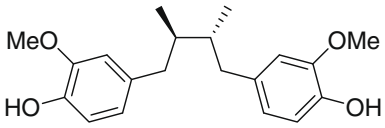


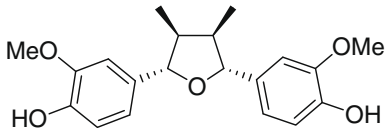
R = H Endiandrin B (**1**)
R = Me Cinbalansan (**4**)



Endiandrin A (**2**)



(-)-Dihydroguaiaretic acid (**3**)



Nectandrin B (**5**)

Figure 1. Chemical structures for compounds **1–5**.

Table 1
NMR data for endiandrin B (**1**)[a]

Position	^{13}C	^{1}H (mult., *J*, int.)	gCOSY	gHMBC	ROESY
1	133.3				
2	111.4	6.23 (d, 1.8, 1H)	3-OMe, 6	1, 3, 4, 6, 7	3-OMe, 7, 8
3	146.0				
3-OMe	55.8	3.60 (s, 3H)	2	3	2
4	143.5				
4-OH		5.34 (s, 1H)		3, 4, 5	
5	113.4	6.71 (d, 7.8, 1H)	6	1, 3, 4	6
6	120.7	6.58 (dd, 7.8, 1.8, 1H)	2, 5	2, 4, 5, 7	5, 7, 8
7	49.9	3.40 (d, 4.8, 1H)	8	1, 2, 6, 7′, 8, 9	2, 6, 9
8	34.4	2.77 (br m, 1H)	7, 9	1, 7, 8′, 9′, 9	2, 6, 9
9	15.0	1.18 (d, 6.6, 3H)	8	1, 7, 8, 8′, 9′	7, 8, 8′
1′	133.3				
2′	111.4	6.23 (d, 1.8, 1H)	3′-OMe, 6′	1′, 3′, 4′, 6′, 7′	3′-OMe, 7′, 8′
3′	146.0				
3′-OMe	55.8	3.60 (s, 3H)	2′	3′	2′
4′	143.5				
4′-OH		5.34 (s, 1H)		3′, 4′, 5′	
5′	113.4	6.71 (d, 7.8, 1H)	6′	1′, 3′, 4′	6′
6′	120.7	6.58 (dd, 7.8, 1.8, 1H)	2′, 5′	2′, 4′, 5′, 7′	5′, 7′, 8′
7′	49.9	3.40 (d, 4.8, 1H)	8′	1′, 2′, 6′, 7, 8′, 9′	2′, 6′, 9′
8′	34.4	2.77 (br m, 1H)	7′, 9′	1′, 7′, 8, 9, 9′	2′, 6′, 9′
9′	15.0	1.18 (d, 6.6, 3H)	8′	1′, 7′, 8′, 8, 9	7′, 8′, 8

[a] Spectra were recorded in $CDCl_3$ at 30 °C.

benzene system [δ_H 6.23 (d, J = 1.8 Hz), 6.71 (d, J = 7.8 Hz) and 6.58 (dd, J = 7.8, 1.8 Hz)], one methoxy group (δ_H 3.60), two aliphatic resonances (δ_H 3.40 and 2.77) and a methyl resonance (δ_H 1.18). The ^{13}C NMR spectrum of **1** contained only 10 signals, six of which resonated between δ_C 110 and 147. The ^{1}H/^{13}C NMR and MS data readily identified **1** as an isomer of endiandrin A (**2**) and HMBC data analysis (Table 1) confirmed that the planar structures of **1** and **2** were identical.[7] The major NMR chemical shift differences between **1** and **2** were noted about the cyclobutane ring moiety, suggesting that endiandrin B had a different relative configuration about the four-membered ring. The optical rotation of endiandrin B (**1**), $[\alpha]_D^{27}$ 0 (*c* 0.12, $CHCl_3$), identified **1** as a *meso*-isomer. With two *meso* compounds possible, ROESY data analysis was undertaken in order to determine the relative configuration of **1**. Strong ROESY correlations between the benzylic protons at δ_H 3.40 (H-7/H-7′) and the methyl doublets at δ_H 1.18 (H-9/H-9′), as well as ROESY cross-peaks between the aliphatic multiplets at δ_H 2.77 (H-8/H-8′) and the aryl signals at δ_H 6.58 (H-6/H-6′) and 6.73 (H-2/H-2′), indicated that the methyl and aryl components of **1** had a *trans* orientation about the cyclobutane ring. On the basis of these data we assigned the relative configuration of **1** as 8β,8′β-dimethyl-7α,7′α-bis(3-methoxy-4-hydroxyphenyl)cyclobutane. Crystals of **1** suitable for X-ray diffraction analysis were obtained by slow evaporation of a *i*-PrOH/hexanes (1:9) mix. An ORTEP[8] representation of the structure of **1** is shown in Figure 2 and confirmed the *trans* arrangement of the methyl and phenyl groups on the cyclobutyl ring. The C9–C8–C18–C19 and C1–C7–C17–C11 torsion angles were 26.8(4)° and 26.5(4)°, respectively, whilst C6–C1–C7–C17 and C16–C11–C17–C7 were 73.7(3)° and −78.0(3)°, respectively. Intramolecular O–H···O bonding was observed between the hydroxy and methoxy groups. In the crystal lattice, strong inter-molecular O–H···O hydrogen bonds between the O4 and O14 hydroxy groups link clusters of four molecules to form R_4^4(**8**) rings[9] disposed about crystallographic centres of symmetry.

Methylation of **1** using diazomethane in a MeOH/diethyl ether mixture afforded a compound that was determined to be cinba-

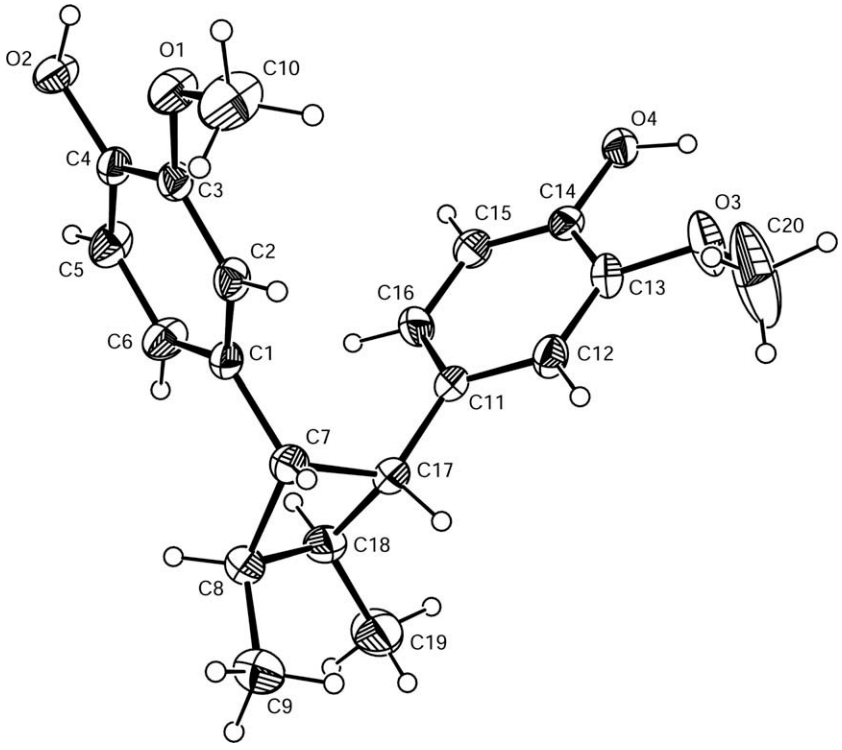


Figure 2. ORTEP plot for endiandrin B (**1**).

lansan (**4**), following spectroscopic data comparison with literature values.[10] In a similar manner, endiandrin A and (−)-dihydroguaiaretic acid were assigned to compounds **2** and **3**, respectively, after NMR and $[\alpha]_D$ data comparison with literature values.[7,11–13]

Due to our interest in natural products that exhibit anticancer activity,[14] detailed profiling of the cytotoxic properties of compounds **1–4** against the lung carcinoma cell line, A549, was determined using a high-content imaging system [Opera™ (PerkinElmer)]. The Opera™ was utilised due to its ability to simultaneously capture and quantify confocal images to measure an array of cellular changes. Several fluorescent markers were employed to investigate changes to nuclear morphology, and membrane permeability, which both undergo significant changes during cell death. Nuclear morphology was visualized using the DNA/RNA binding dyes DRAQ5, YO-PRO-1, propidium iodide and Hoechst 34580. DRAQ5 and Hoechst are both cell permeable dyes and therefore stain all cells, however YO-PRO-1 and propidium iodide are membrane impermeable. Thus, only during the late stages of apoptosis, or programmed cell death, as the permeability of the cell membrane increases, does nuclear staining with YO-PRO-1 occur. Propidium iodide, however, remains cell impermeable until cell death has occurred. Cells were also labelled with Mitotracker Red and phalloidin, which label the mitochondria[15] or actin cytoskeleton,[16] respectively. The mitotracker dye is sequestered by functioning mitochondria with the level of staining dependant on mitochondrial membrane potential.[15] Mitochondria have been shown to undergo major changes in their structure and function early on in the apoptotic process.[17,18] Thus, using these markers in correlation with the Opera™ high-throughput imaging system we are able to measure the effect of compounds on multiple stages within the cytotoxicity pathway. In the HCS assays (−)-dihydroguaiaretic acid (**3**) was found to be the most potent, displaying cytotoxicity towards the A549 cell line with an IC_{50} value of 7.49 μM after 24 h incubation in both the propidium iodide and YO-PRO-1 assays (Fig. 3). This effect was less pronounced in the mitotracker assay with (−)-dihydroguaiaretic acid found to have an IC_{50} of 31.2 μM. Endiandrins A (**2**) and B (**1**) were found to have a moderate effect on all three assays giving an inhibition of 76% and 75% at 100 μM, respectively. Cinbalansan (**4**) was found to have a very minor effect in the three assays, giving a maximum inhibition of 34%, however, as can be seen from the qualitative data, cinbalasan did have an effect on the uptake of the mitotracker dye (Fig. 4). This may indicate early stages of apoptosis, however further work would be required to determine the exact mechanism of action.

3. Conclusion

In conclusion this paper describes the isolation and structure elucidation of the new cyclobutane lignan, endiandrin B (**1**), along with the cytotoxicity data for endiandrins A (**2**) and B (**1**), (−)-dihydroguaiaretic acid (**3**), and cinbalansan (**4**) against the human lung carcinoma cell line, A549. Whilst over 2000 compounds belonging to the lignan structure class have been isolated, lignans containing a cyclobutane moiety are rare with less than 30 reported in the literature.[1]

4. Experimental

4.1. General methods

Optical rotations were recorded on a Jasco P-1020 polarimeter. UV and IR spectra were recorded on a Camspec M501 spectrophotometer and a Bruker Tensor 27 spectrometer, respectively. NMR spectra were recorded at 30 °C on either a Varian 500 MHz or 600 MHz Unity INOVA spectrometer. The latter spectrometer was equipped with a triple resonance cold probe. The ^1H and ^{13}C chemical shifts were referenced to the proto-deutero solvent peaks for DMSO-d_6 at δ_H 2.49 and δ_C 39.5 or for $CDCl_3$ at δ_H 7.26 and δ_C

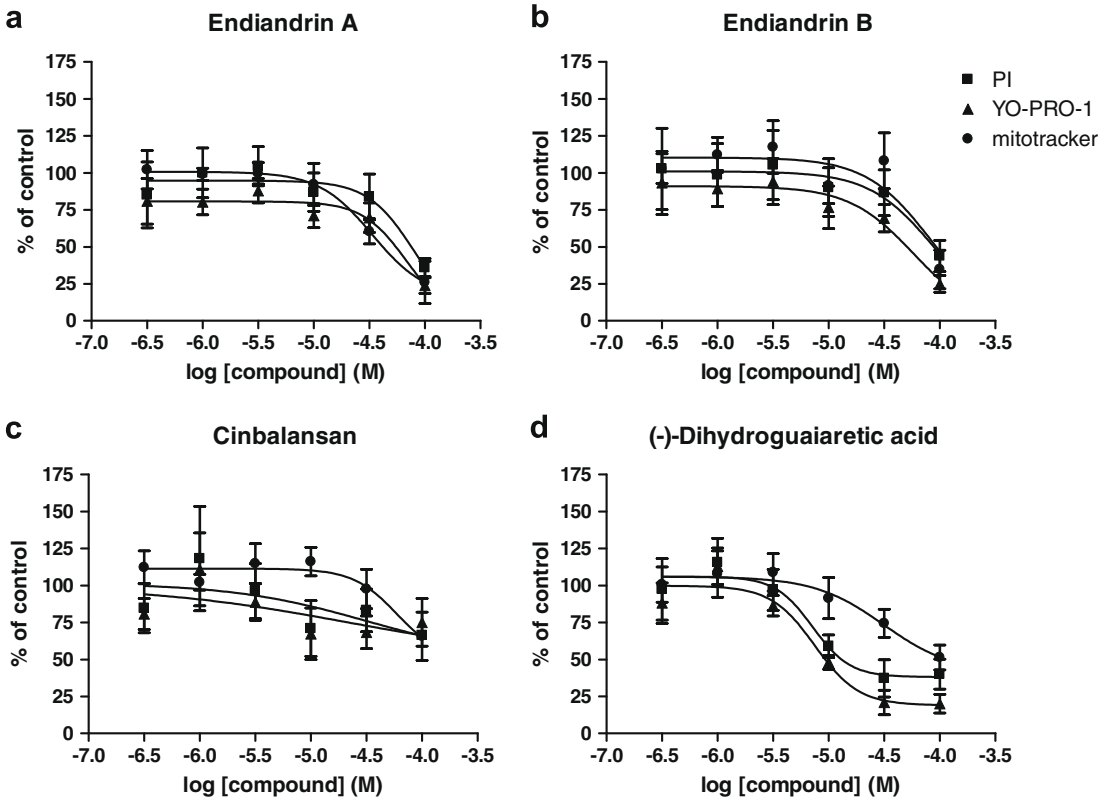


Figure 3. The effect of the compounds **1–4** on three indicators of cytotoxicity. Data is expressed as a percentage of the negative control i.e. untreated and is mean ± standard error of the mean from three separate experiments.

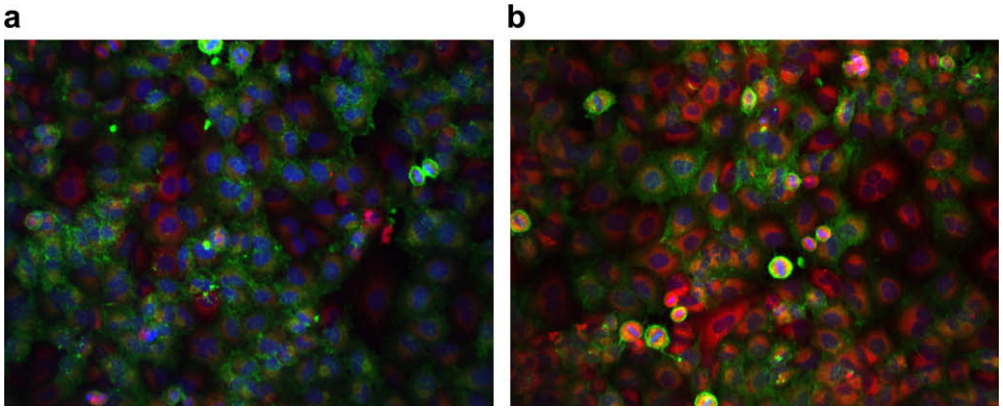


Figure 4. The effect of cinbalansan (**4**) on the uptake of mitotracker by A549 cells. Untreated cells are shown in (a) whilst cells treated with 30 μM cinbalansan for 24 h are shown in (b). Mitotracker staining is shown in red, Hoechst 34580 staining in blue and phalloidin staining in green. Images were captured using a ×20 objective on the Evotec Opera (PerkinElmer).

77.0. LRESIMS were recorded on a Waters ZQ mass spectrometer. HRESIMS were recorded on a Bruker Daltonics Apex III 4.7e Fourier-transform mass spectrometer. An open glass column (40 mm × 110 mm) packed with Alltech Davisil diol-bonded silica 30–40 μm 60 Å was used for flash chromatography. A Waters 600 pump equipped with a Waters 996 PDA detector and a Waters 717 autosampler were used for HPLC. A ThermoElectron C_{18} Betasil 5 μm 143 Å column (21.2 mm × 150 mm) and a YMC diol 5 μm 120 Å column (20 mm × 150 mm) were used for semi-preparative HPLC separations. All solvents used for chromatography, UV, optical rotations and MS were Lab-Scan HPLC grade, and the H_2O was Millipore Milli-Q PF filtered. All synthetic reagents were purchased from Sigma–Aldrich.

4.2. Biological material

The roots of *E. anthropophagorum* were collected at State Forest 143, Mt Lewis, Queensland, Australia during 2005. A voucher sample (AQ603481) has been lodged at the Queensland Herbarium, Brisbane, Australia.

4.3. Extraction and isolation

The air-dried and ground roots of *E. anthropophagorum* (250 g) were exhaustively extracted with DCM (3 × 1 L). The solvent was evaporated to yield a dark brown residue (1.23 g) that was dissolved in 10% aq MeOH (250 mL) then extracted with hexanes

(3 × 250 mL). The aq MeOH layer was evaporated to dryness (0.7 g) then pre-adsorbed to diol-bonded silica (~7 g). The pre-adsorbed material was loaded onto a diol-bonded silica flash column and a 10% stepwise gradient from 100% hexanes to 100% EtOAc was performed (100 mL washes). The 40% EtOAc/60% hexanes flush contained a complex mixture of lignans (387 mg) that was further separated by diol semi-preparative HPLC chromatography. A linear gradient from 100% hexanes to 15% *i*-PrOH/85% hexanes was run at a flowrate of 9 mL/min over 60 min, 120 (0.5 min each) fractions were collected. Fractions 62–65 contained (−)-dihydroguaiaretic acid (**3**, 25.7 mg, 0.010% dry wt), fractions 68–70 yielded endiandrin A (**2**, 75.8 mg, 0.030% yield), and fraction 74 afforded endiandrin B (**1**, 7.1 mg, 0.0028% dry wt).

4.3.1. Endiandrin B (1)

Colourless needles; mp 145–147 °C; $[\alpha]_D^{27}$ 0 (*c* 0.12, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 233 (3.78), 284 (3.44) nm; IR ν_{max} (KBr) 3600–3100, 1601, 1515, 1462, 1430, 1376, 1262, 1234, 1155, 1124, 1033, 861, 809 cm^{-1}; ^1H and ^{13}C NMR data ($CDCl_3$) see Table 1; (+)-LRESIMS *m*/*z* (rel. int.) 351 (100) $[M+Na]^+$; (+)-HRESIMS *m*/*z* 351.15,787 ($C_{20}H_{24}O_4Na$ $[M+Na]^+$ requires 351.15669).

4.4. X-ray data for endiandrin B (1)

Diffraction data were collected on a crystal of **1** at 295 K on a Rigaku AFC7R rotating anode four circle diffractometer using monochromated Mo Kα radiation (λ = 0.71069 Å) in the monoclinic space group $P2_1/n$ with a = 19.501(5), b = 6.676 (5), c = 14.786(3) Å. The structure was solved by direct methods using the program SIR-97[19] with atom positions and displacement parameters refined using SHELXL97[20] within the TEXSAN program package.[21] Final refinement to convergence was against F^2 with 1835 unique reflections (2θ = 50°) to give a final conventional *R*-factor ($I > 2\sigma(I)$) of 0.047 and wR^2(all data) = 0.152. Non-hydrogen atoms were refined anisotropically and the H-atoms placed in idealised geometries. Full crystallographic data for the structure reported in this paper have been deposited at the Cambridge Crystallographic Data Centre (CCDC No. 697474). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (Fax: +44 1223 336033; email: deposit@ccdc.cam.ac.uk).

4.5. Methylation of endiandrin B (1)

Endiandrin B (3.0 mg, 9.1 μmol) was dissolved in dry MeOH (1.5 mL) and Et_2O (1.5 mL) then treated with excess CH_2N_2-Et_2O at 0 °C for 1 h. The reaction was allowed to warm to rt overnight and the resulting residue was pre-adsorbed to C18 (~1 g), packed into stainless steel cartridge (10 × 30 mm) then attached to a C_{18} semi-preparative HPLC column. A linear gradient from 100% H_2O to 100% MeOH over 50 min was run at a flowrate of 9 mL/min, isocratic conditions of 100% MeOH were maintained for a further 10 min at a flowrate of 9 mL/min. Sixty (1 min each) fractions were collected from time = 0 min. Fraction 49 contained a compound that was spectroscopically identical to the known cyclobutane, cinbalansan (**4**, 2.4 mg, 74% yield).[10]

4.6. Biology methods

A549 lung carcinoma cells were routinely cultured in Dulbecco's modified Eagles medium (Invitrogen) supplemented with 10% foetal bovine serum (Invitrogen). Cells were split from flasks using Accutase (Sigma) and plated into 96-well Packard Viewplates (PerkinElmer) at a density of 10,000 cells per well in 99 μL of medium. Compounds were diluted, along with four reference compounds (colchicine, staurosporin, taxol and wortmannin), in 12 point half log dose response curves in 100% DMSO before 1 μL of each dilution was transferred into the cell plate to give a final concentration of 1% DMSO. Cells were incubated with compound for 24 h at 37 °C, 95% humidity, 5% CO_2 before being fixed and stained. Cells were fixed by the addition of 100 μL of 4% paraformaldehyde into each well and incubated for 20 min at room temperature before being washed with phosphate buffered saline (PBS) and stained. Cells were stained with either a mixture of Hoechst 34580, Alexa Fluor 488 phalloidin and mitotracker red, all of which were diluted in PBS to the required concentration (1/1000, 1/100 and 1/5000, respectively). Alternatively, cells were stained with DRAQ5, YO-PRO-1 and propidium iodide (1/1000 in PBS). Cells were incubated with dyes for 20 min in the dark before being washed with PBS and imaged.

Microscopy was performed on an Opera™ (PerkinElmer), using a ×20 objective with a 405 nm excitation line and 420–490 nm band pass filter for Hoechst 34580 detection, a 488 nm excitation line and a 510–535 nm band pass emission filter for YO-PRO-1 and phalloidin detection, a 535 nm excitation line and a 580–620 nm band pass emission filter for propidium iodide detection and a 635 nm excitation line and a 665–715 nm band pass emission filter for DRAQ5 and Mitotracker detection.

Cytotoxicity was quantified using the Acapella™ analysis software. Firstly, cell nuclei were detected using the Opera Nuclear Detection Library. This algorithm was used to detect nuclei (S4a—Supplementary data) stained with DRAQ5 or Hoechst using the parameters of minimum nuclear area, nuclear intensity and minimum nuclear distance (the minimum distance between two nuclear centers). Based on these identified nuclei the fluorescence was detected within the nuclei itself (S4b—Supplementary data), for YO-PRO-1 and propidium iodide staining, or in a 2-pixel wide band around the nucleus (S4c—Supplementary data) for mitochondrial staining. Fluorescence intensity readings were captured for each of these three dyes (Mitotracker, YO-PRO-1 and propidium iodide) in each cell, and then averaged for all the cells in each field of view (430 × 345 μm^2). One field was captured in each experiment with the experiments repeated three times. The positive controls were colchicine, wortmannin, staurosporine and taxol, and were found to be cytotoxic with IC_{50} values of 10.78 ng/mL, 4.28 ng/mL, 139.95 ng/mL and 19.64 ng/mL, respectively.[22,23]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.12.030.

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