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Cytotoxic C_{20} Diterpenoid Alkaloids from the Australian Endemic Rainforest Plant *Anopterus macleayanus*

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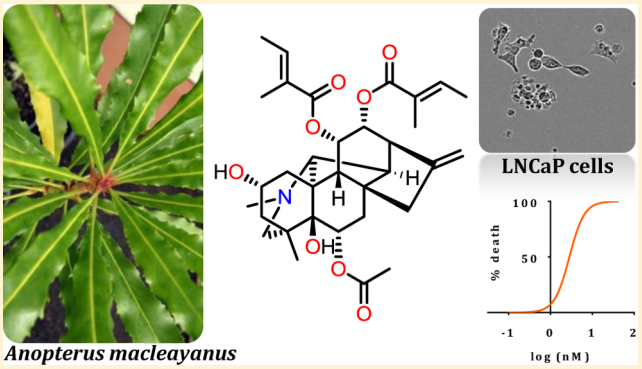
Supporting Information

ABSTRACT: In order to identify new anticancer compounds from nature, a prefractionated library derived from Australian endemic plants was generated and screened against the prostate cancer cell line LNCaP using a metabolic assay. Fractions from the seeds, leaves, and wood of *Anopterus macleayanus* showed cytotoxic activity and were subsequently investigated using a combination of bioassay-guided fractionation and mass-directed isolation. This led to the identification of four new diterpenoid alkaloids, 6α-acetoxyanopterine (**1**), 4′-hydroxy-6α-acetoxyanopterine (**2**), 4′-hydroxyanopterine (**3**), and 11α-benzoylanopterine (**4**), along with four known compounds, anopterine (**5**), 7β-hydroxyanopterine (**6**), 7β,4′-dihydroxyanopterine (**7**), and 7β-hydroxy-11α-benzoylanopterine (**8**); all compounds were purified as their trifluoroacetate salt. The chemical structures of **1**−**8** were elucidated after analysis of 1D/2D NMR and MS data. Compounds **1**−**8** were evaluated for cytotoxic activity against a panel of human prostate cancer cells (LNCaP, C4-2B, and DuCaP) and nonmalignant cell lines (BPH-1 and WPMY-1), using a live-cell imaging system and a metabolic assay. All compounds showed potent cytotoxicity with IC_{50} values of <400 nM; compound **1** was the most active natural product from this series, with an IC_{50} value of 3.1 nM toward the LNCaP cell line. The live-cell imaging assay on **1**−**8** showed a concentration- and time-dependent effect on the cell morphology and proliferation of LNCaP cells.



The genus *Anopterus* belongs to the plant family Escalloniaceae and consists of only two species, *Anopterus macleayanus* and *A. glandulosus*, which are both found only in Australia. *A. macleayanus* F. Muell., also known as Queensland Laurel or Macleay Laurel, is a tree growing to a height of up to 16 m and is found in the subtropical rainforests of Southern Queensland and Northern New South Wales. *A. glandulosus* Labill., also known as Native Laurel or Tasmanian Laurel, is a small tree (2−4 m) that grows in the mountain regions of Tasmania.[1]

Only three chemical investigations of *A. glandulosus* and *A. macleayanus* have been reported; these include Denne et al. in 1972,[2] Hart et al. in 1976,[3,4] and Johns et al. in 1985.[5] Those studies yielded several novel *ent*-kaurenoid alkaloids, belonging to the C_{20} diterpenoid alkaloid class. This structure class is currently divided into three groupings that include the atisane, kaurane, and bisditerpene alkaloid classes, which can all be further characterized according to the position and number of additional C−C or N−C bonds. For example, the kaurane class includes secondary metabolites that contain veatchine, napelline, and anopterine scaffolds.[6] The distribution of C_{20} diterpenoid alkaloids in nature is limited, with all examples of this chemotype having been isolated from only eight plant genera and five plant families.[6] The anopterine structure class has only been isolated from *Anopterus* species and is unique due to its C-14/C-20 linkage,[6] which was first identified by X-ray crystallography.[2] Currently, there are a total of nine natural products based on the anopterine scaffold described.[5,7] Biological investigations of *Anopterus* plants have been previously reported, with extracts of different samples of both *A. macleayanus* and *A. glandulosus* tested for cytotoxicity and subsequently pursued by the National Cancer Institute (NCI), USA (1961−1981).[8] These two species have also been investigated by the Commonwealth Scientific and Industrial Research Organization (CSIRO) as part of a drug discovery screening campaign in collaboration with the NCI; this work was published in 1990.[9] In the CSIRO study, *A. macleayanus* extracts exhibited cardiovascular, diuretic, and antitumor [L-1210 lymphoid leukemia in mice and human epidermoid carcinoma of nasopharynx cells (KB)] activities, but showed no antispasmodic or anticonvulsing effects.[9] *A. glandulosus* extracts showed cardiovascular activity and antitumor effects against Dunning ascites leukemia and P388 lymphocytic leukemia

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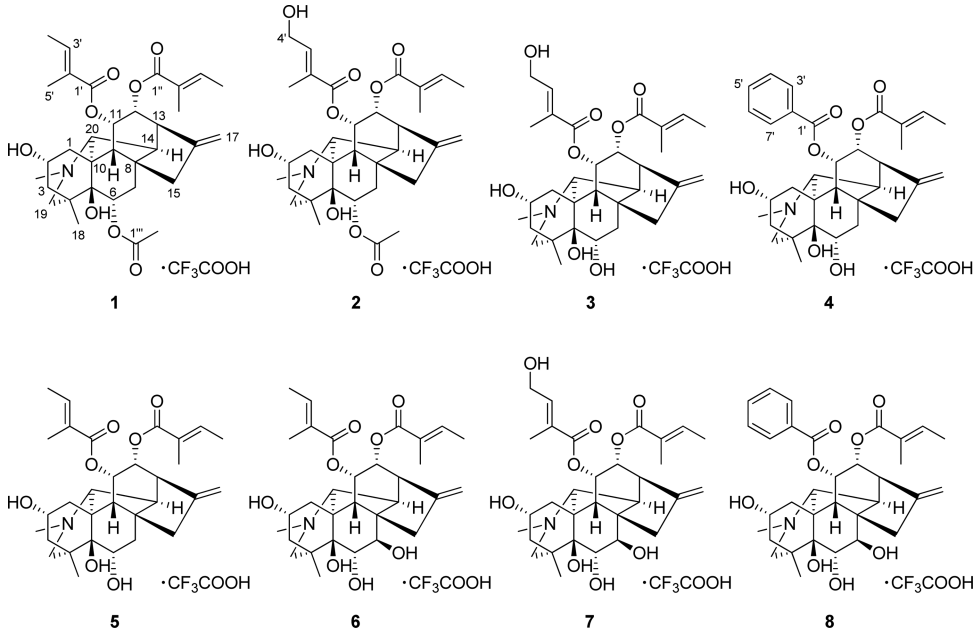
Chart 1



mouse models as well as cytotoxicity against the KB cell line.[9] In 1987, Wall et al., through NCI-funded research, evaluated the antitumor activity of *A. glandulosus* leaves and twig extracts (in vitro using the KB cell line and in vivo using an iv-implanted murine P388 leukemia model).[10] With the exception of one compound (11α-destigloylanopterine), which was considered inactive, all other anopterine analogues exhibited considerable in vitro cytotoxicity (ED_{50} values ranging from 1 to 50 ng/mL).[10] The in vivo bioactivity study of the plant extracts by Wall et al. in the P388 leukemia model showed promising dose−response data; however, the activity was lost when pure compounds were tested.[10] It was suggested by the authors that minor alkaloids might be responsible for the activity of the plant extracts.[10]

Owing to a continuing interest in the discovery and development of new anticancer compounds from nature,[11−13] we have created a prefractionated library derived from Australian endemic plants and screened this unique library for cytotoxic activity against the LNCaP cell line (human prostate carcinoma) using a metabolic assay. Three fractions derived from the seeds, leaves, and wood extracts of *A. macleayanus* were found to display potent activity and were selected for further investigations.

This paper describes the bioassay-guided fractionation, mass-directed isolation, and structure elucidation of four new and four known anopterine analogues from *A. macleayanus*. Furthermore, the in vitro cytotoxicity evaluation of all compounds toward prostate cancer cell lines (LNCaP, C4-2B, and DuCaP) and nonmalignant cell lines (BPH-1 and WPMY-1) is also reported.

RESULTS AND DISCUSSION

Thirty-eight Australian endemic plant samples were selected for the creation of the plant-derived prefractionated library, based on published phytochemical studies from the CSIRO.[9] The MeOH extract for each plant specimen was prepared using a small quantity of air-dried and ground material. These 38 MeOH extracts were fractionated using reversed-phase analytical HPLC and a reported lead-like enhanced fractionation process,[14] which yielded 38 optimized fractions that were tested for their cytotoxic activity toward the LNCaP cell line using a metabolic assay.

The fractions derived from the seeds, leaves, and wood of *A. macleayanus* all showed potent activity (>70% inhibition at 10 μg/mL) and were selected for further investigations. A portion of the MeOH extract derived from the large-scale extraction of the seeds (10 g) was subjected to semipreparative C_{18} HPLC ($MeOH/H_2O$/0.1% TFA) and yielded eight fractions, which were tested for cytotoxicity. LCMS data for the active fractions indicated UV-active compounds that contained prominent ions in the (+)-ESI mode at m/z 542, 558, 564, 574, 580, and 584, suggesting the presence of alkaloids. The active fractions obtained from the semipreparative HPLC did not yield sufficient quantities of pure compounds to allow structure determination. Owing to the identification of alkaloids, the remaining MeOH extract was subjected to an alkaloid extraction and purified using phenyl HPLC ($MeCN/H_2O$/0.1% TFA) to afford the trifluoroacetate salt of the known alkaloid 7β-hydroxy-11α-benzoylanopterine (**8**). In order to isolate more of the bioactive compounds, it was decided to investigate the leaves and wood of *A. macleayanus*, which were more plentiful. Fortuitously, the LCMS data from both the wood and leaf extracts indicated that these samples contained the same ions as the seeds, with an additional ion at m/z 600 detected in the leaves.

The wood (65 g) of *A. macleayanus* was sequentially extracted with CH_2Cl_2 and MeOH. The CH_2Cl_2/MeOH extracts were combined, evaporated, then resuspended in $MeOH/H_2O$, and subjected to an alkaloid extraction followed by a liquid/liquid partition using CH_2Cl_2/H_2O. Subsequent fractionations of the organic layer using semipreparative phenyl HPLC (MeOH or $MeCN/H_2O$/0.1% TFA) afforded the trifluoroacetate salts of the new alkaloids 6α-acetoxyanopterine (**1**), 4′-hydroxyanopterine (**3**), and 11α-benzoylanopterine (**4**), along with the known alkaloids anopterine (**5**), 7β-hydroxyanopterine (**6**), 7β,4′-dihydroxyanopterine (**7**), and 7β-hydroxy-11α-benzoylanopterine (**8**).

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The leaves (270 g) of *A. macleayanus* were extracted in an identical manner to the wood material, and the resulting CH_2Cl_2/MeOH extract was subjected to chlorophyll removal via an *n*-hexane/H_2O partition. The aqueous layer was subsequently extracted with CH_2Cl_2, and the organic phase was fractionated using phenyl HPLC (MeOH or MeCN/H_2O/0.1% TFA) and yielded the trifluoroacetate salts of one additional new alkaloid, 4′-hydroxy-6α-acetoxyanopterine (**2**), along with larger quantities of 6α-acetoxyanopterine (**1**).

Compound **1** was obtained as a stable yellow gum. A molecular formula of $C_{33}H_{45}NO_8$ for the free base of **1** was assigned following analysis of both NMR and (+)-HRESIMS data. The ^{1}H NMR spectrum of **1** displayed signals for 10 methines (δ_H 2.60, 3.05, 3.30, 4.24, 5.12, 5.22, 5.24, 5.54, 6.77, 7.26), six methylenes (δ_H 1.66/2.12, 1.89/2.72, 2.23/2.88, 2.22/2.75, 3.74/4.52, 4.96/5.13), five methyls (δ_H 1.02, 1.75, 1.76, 1.83, 1.90), one *O*-acetyl (δ_H 2.29), and an *N*-methyl (δ_H 3.29). The ^{13}C NMR data that was obtained from the HSQC/HMBC spectra showed signals for 10 methine carbons (δ_C 53.5, 53.6, 54.8, 70.4, 138.4, 139.6 including four oxymethine carbons δ_C 65.6, 69.9, 72.8, 73.5), six methylene carbons (δ_C 36.2, 39.3, 40.7, 43.6, 64.6, 109.4), six methyl carbons (δ_C 11.6, 12.3, 14.4, 14.7, 21.8, 23.2), 10 nonprotonated carbons (δ_C 36.3, 49.9, 53.1, 75.8, 128.1, 128.6, 148.2, 166.2, 167.6, 169.0), and one *N*-methyl group (δ_C 47.3).

Analysis of the ^{1}H−^{1}H COSY spectrum, in combination with the ^{1}H−^{1}H coupling constants, identified two spin systems. The first system included protons at δ_H 2.60 (H-9), 5.54 (H-11), 5.24 (H-12), 3.30 (H-13), and 3.05 (H-14). HMBC correlations between H-11 (δ_H 5.54) and the quaternary carbon at δ_C 49.9 (C-8) and from H-14 (δ_H 3.05) to the carbon at C-9 (δ_C 53.5) allowed construction of ring C (Figure 1). The ^{1}H NMR spectrum of **1** also displayed signals corresponding to two nonequivalent olefinic methine protons (δ_H 6.77 and 7.26) and four nonequivalent deshielded methyl groups (δ_H 1.75, 1.76, 1.83, and 1.90). The ^{1}H−^{1}H COSY data (Figure 1) in conjunction with the multiplicity and coupling constants of the olefinic methine protons at δ_H 6.77 (br q, J = 6.7 Hz) and 7.26 (br q, J = 7.0 Hz) indicated two identical groups that were each associated with one olefinic proton that was geminal to one methyl group (δ_H 1.76, d, J = 6.7 Hz and δ_H 1.83, d, J = 7.0 Hz, respectively). The HMBC data between the second deshielded methyl of each group (δ_H 1.75 and 1.90), the carbonyl (δ_C 166.2 and 167.6), and the carbon bearing both olefinic methine protons and the vicinal methyl were used to unambiguously establish two 2-methyl-2-butenoate moieties.[15] The coupling constants and chemical shifts were typical for a tigloyl group,[15] and this was further supported by comparison of the ^{1}H/^{13}C NMR data of **1** with commercially available tiglic acid (Figures S46 and S47, Supporting Information). The HMBC correlations between the doublet of doublets resonating at δ_H 5.54 (H-11) and the tiglic carbonyl at δ_C 166.2 (C-1′) indicated that a tigloyl group was attached at C-11. As shown in Figure 1, the same process was applied with the proton at δ_H 5.24 (H-12) and the second tigloyl group. The two broad singlets at δ_H 4.96/5.13 (H_2-17) were assigned to an exocyclic methylene group, forming a two-carbon bridge between C-13 and C-8 of ring C, following HMBC and ^{1}H−^{1}H COSY data analysis. Rings B and C were linked together via the correlations of the methylene protons at C-15 (δ_H 2.23/2.88) with C-14 (δ_C 54.8) and with the ^{1}H−^{1}H COSY correlations between the methylene protons H_2-7 (δ_H 1.89/2.72) and the methine proton H-6 (δ_H 5.22). Ring A was identified using the second spin system, which included the protons δ_H 2.22/2.75 (H_2-1), 4.24 (H-2), and 1.66/2.12 (H_2-3), as well as the HMBC correlations from the angular methyl group (δ_H 1.02) to the carbons at δ_C 36.3 (C-4) and 43.6 (C-3). The carbons C-5 (δ_C 75.8) and C-10 (δ_C 53.1) finalized the diterpenoid structure, as protons from rings A and B showed strong $^3J_{CH}$ correlations to these central carbons. A pair of diastereotopic protons (δ_H 3.74/4.52 and δ_C 64.6), a methine (δ_H 5.12 and δ_C 70.4), and a methyl (δ_H 3.29 and δ_C 47.3) were all attached to a nitrogen atom based on HMBC correlations (Figure 1). Furthermore, HMBC correlations from the diastereotopic protons (δ_H 3.74/4.52, H_2-19) to C-3 (δ_C 43.6), C-4 (δ_C 36.3), C-5 (δ_C 75.8), and the C-18 methyl group (δ_C 23.2) positioned the nitrogen-containing bridge at C-4. The HMBC correlations between H-20 (δ_H 5.12) and C-8 (δ_C 49.9) and C-13 (δ_C 53.6) established the C-20/C-14 linkage. The HMBC correlations from H-20 (δ_H 5.12) to C-1 (δ_C 36.2), C-10 (δ_C 53.1), and C-5 (δ_C 75.8) confirmed the C-10/C-20 linkage. These spectroscopic data indicated that **1** belonged to the C_{20} diterpenoid alkaloid class.[6,16] Comparison of the NMR data indicated that compound **1** had a structure similar to anopterine (**5**), the major compound previously reported from *Anopterus* species.[3,5] The relative configuration of **1** was determined on the basis of ROESY experiments in both acetone-d_6 and DMSO-d_6. The data in acetone-d_6 showed correlations between H-9 and H-11, and also between H-11 and H-12 and the methylene proton H-15(β); H-6 showed a strong ROESY correlation to CH_3-18. These connectivities, along with ROESY correlations such as H-3(β)/CH_3-18, H-3(α)/CH_3-18, H-13/H-20, H-20/N-CH_3, and H-19/CH_3-18, indicated the relative configuration of compound **1** was identical to anopterine (**5**); however the OH-5 orientation could not be assigned since this signal was not observed in acetone-d_6. In order to assign the orientation of OH-5, NMR data for compound **1** in DMSO-d_6 were collected. Fortuitously, ROESY correlations were identified for all exchangeable protons in DMSO-d_6. These included ROESY correlations between OH-5 (δ_H 5.38) and CH_3-18, H-1(β), H-3(β), H-6, and H-9 and also between the acetate methyl (δ_H 2.25) and CH_3-18, H-6, H-19, and H-N-CH_3 (δ_H 7.27). These data allowed the orientation of OH-5 to be assigned as β. Thus, the structure of **1** was determined to be 2α,5β-dihydroxy-6α-acetoxy-11α,12α-ditigloyloxy-19,20-methylimino-14,20-cyclo-

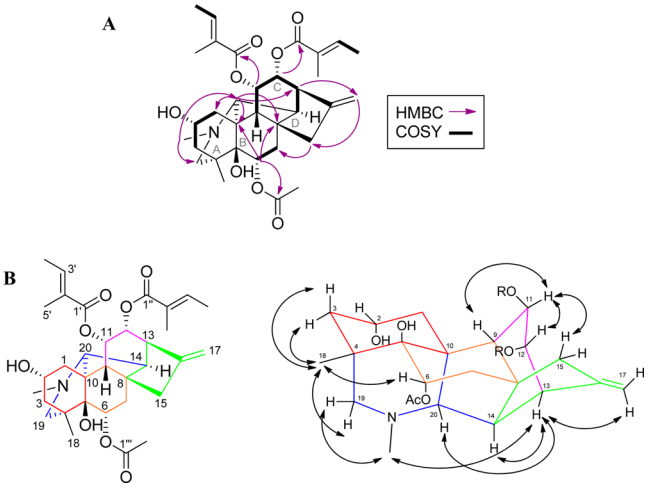

Figure 1. Selected (A) HMBC and ^{1}H−^{1}H COSY and (B) ROESY correlations of 6α-acetoxyanopterine (**1**).

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ent-kaur-16-ene, to which the trivial name 6α-acetoxyanopterine (**1**) was assigned.

Compound **2** was assigned the molecular formula $C_{33}H_{45}NO_9$ (free base) following the analysis of the NMR and (+)-HRESIMS data. The ^{1}H NMR spectrum of **2** displayed a high degree of homology with **1**; however several notable differences were identified. Specifically, one set of tigloyl signals in **1** had been replaced with another small ester unit that contained only one methyl (δ_H 1.73) in **2**. In addition, the vinylic proton resonated as a doublet of doublets of quartets at δ_H 6.76 (ddq, J = 5.6, 5.6, 1.3 Hz, H-3′) in **2** instead of the broad quartet that was observed in compound **1**. Furthermore, compound **2** possessed one more signal for a set of methylene protons (δ_H 4.23/4.27, dd, J = 15.0, 5.6 Hz, H_2-4′). As shown in Figure 2, these methylene protons (H_2-4′) showed ^{1}H−^{1}H

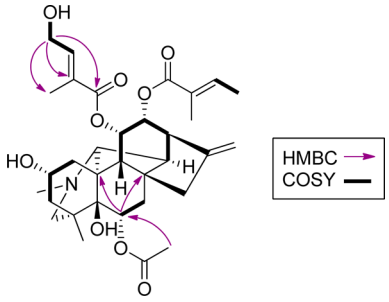

Figure 2. Selected HMBC and ^{1}H−^{1}H COSY correlations of 4′-hydroxy-6α-acetoxyanopterine (**2**).

COSY correlations to a proton at δ_H 6.76 (H-3′) and HMBC correlations to carbons at δ_C 12.4 (C-5′), 127.1 (C-2′), and 166.7 (C-1′). The HMBC correlation between H-11 and C-1′ positioned this small ester unit at C-11, while the tigloyl group was attached to C-12 on account of a strong HMBC cross-peak between H-12 and C-1″. From these data it was deduced that the 11-tigloyl moiety of **1** has been replaced with an (*E*)-4-hydroxy-2-methyl-2-butenoate (hydroxytigloyl[4]) unit in **2**. Thus, the structure of **2** was determined to be 2α,5β-dihydroxy-6α-acetoxy-11α-(4′-hydroxytigloyloxy)-12α-tigloyloxy-19,20-methylimino-14,20-cyclo-*ent*-kaur-16-ene, to which the trivial name 4′-hydroxy-6α-acetoxyanopterine (**2**) was assigned.

Compound **3** was obtained as a stable yellow gum. The molecular formula ($C_{31}H_{43}NO_8$) of the free base of **3** was established following interpretation of the NMR data and the (+)-HRESIMS ion [M − TFA]$^+$ at m/z 558.3067 (calcd 558.3061). Analysis of the 1D and 2D NMR spectra of **3** indicated it was also structurally related to anopterine (**5**), the difference being the presence of four methyl group protons and an additional set of methylene protons (δ_H 4.23/4.27) in **3**, compared to anopterine (**5**), which displayed five methyl signals. The ^{1}H−^{1}H COSY correlations between the extra methylene protons (δ_H 4.23/4.27, dd, J = 15.0, 5.8 Hz, H_2-4′) and H-3′ resonating at δ_H 6.75 (ddq, J = 5.8, 5.8, 1.3 Hz) confirmed the presence of a hydroxytigloyl group in **3** (Figure 3). The HMBC cross-peaks between H-11/C-1′ and H-12/C-1″ allowed the hydroxytigloyl and tigloyl moieties to be positioned at C-11 and C-12, respectively. Thus, the structure of **3** was determined to be 2α,5β,6α-trihydroxy-11α-(4′-hydroxytigloyloxy)-12α-tigloyloxy-19,20-methylimino-14,20-cyclo-*ent*-kaur-16-ene, to which the trivial name 4′-hydroxyanopterine (**3**) was assigned.

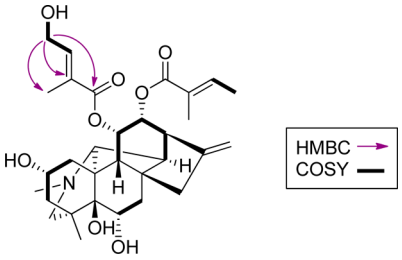

Figure 3. Selected HMBC and ^{1}H−^{1}H COSY correlations of 4′-hydroxyanopterine (**3**).

The molecular formula of compound **4** was determined to be $C_{33}H_{41}NO_7$ (free base) following analysis of both the NMR and MS data. The ^{1}H and ^{13}C NMR data of **4** closely matched those of compound **3**; however **4** contained resonances for a benzoyl group (δ_H 7.49, 7.63, 7.94 and δ_C 129.0, 130.0, 130.7, 134.0) and lacked the signals of the hydroxy-tigloyl group present in **3**. The strong $^3J_{CH}$ correlations from the methine protons at δ_H 7.94 (H-3′ and H-7′) and 5.72 (H-11) to the same carbonyl at δ_C 165.5 indicated that the hydroxytigloyl moiety in **3** had been replaced with a benzoyl group in **4** (Figure 4). Consequently, the structure of compound **4** was assigned as 2α,5β,6α-trihydroxy-11α-benzoyloxy-12α-tigloyloxy-19,20-methylimino-14,20-cyclo-*ent*-kaur-16-ene and was given the trivial name 11α-benzoylanopterine (**4**).

The four known compounds were identified as the TFA salts of anopterine (**5**),[2,3,5] 7β-hydroxyanopterine (**6**),[5] 7β,4′-

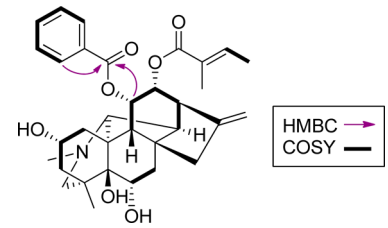

Figure 4. Selected HMBC and ^{1}H−^{1}H COSY correlations of 11α-benzoylanopterine (**4**).

dihydroxyanopterine (**7**),[5] and 7β-hydroxy-11α-benzoylanopterine (**8**)[5] following detailed spectroscopic/spectrometric data analysis. Since compounds **5**−**8** were first purified and reported as their free bases,[2,3,5] we obtained and analyzed 1D and 2D NMR data to confirm the chemical structures. Tables containing the NMR data for compounds **5**−**8** as their free base and TFA salt, as well as COSY, HSQC, and HMBC data, can be accessed in the Supporting Information (Figures S25−S45). Only minor NMR differences were identified between the free base and TFA salt, with the exception of carbon and hydrogen atoms that are in close proximity to the nitrogen atom.

The structures of 7β-hydroxyanopterine (**6**) and 7β,4′-dihydroxyanopterine (**7**) were initially misassigned by Hart et al. in 1976 since only ^{1}H NMR data were recorded for these molecules and subsequently compared with that of anopterine for the structure determination and assignment of both **6** and **7**.[4] The chemical structures of both **6** and **7** were corrected by Johns et al. in 1985,[5] using 1D and 2D NMR data. However, our HMBC data for **6** and **7** showed that C-6, C-7, C9, and C-10 had been misassigned by Johns et al. The full NMR

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Table 1. ^{1}H NMR Spectroscopic Data for Compounds 1−4[a]

	1	2	3	4
position	δ_H (mult. J in Hz)	δ_H (mult. J in Hz)	δ_H (mult. J in Hz)	δ_H (mult. J in Hz)
1	2.22, dd (14.6, 4.7)	2.21, br d (14.6)	2.18, dd (14.8, 4.9)	2.29, dd (14.6, 4.6)
	2.75, br d (14.6)	2.72, br d (14.6)	2.68, br d (14.8)	2.80, ddd (14.6, 2.1, 2.1)
2	4.24, m	4.21, m	4.18, m	4.22, m
3	1.66, br d (14.3)	1.65, br d (14.3)	1.56, ddd (14.3, 2.0, 2.0)	1.59, ddd (14.3, 2.1, 2.1)
	2.12, dd (14.3, 3.8)	2.08, m	2.08, m	2.08, m
6	5.22, br d (6.7)	5.17, br d (6.7)	4.16, d (5.4)	4.20, d (5.5)
7	1.89, br d (16.2)	1.86, br d (16.0)	2.09, m	2.09, m
	2.72, dd (16.2, 6.7)	2.70, m	2.54, dd (14.9, 5.4)	2.62, dd (15.1, 5.5)
9	2.60, m	2.58, m	2.51, m	2.65, m
11	5.54, dd (6.1, 4.4)	5.55, dd (6.1, 4.4)	5.53, dd (6.1, 4.2)	5.72, dd (6.1, 4.4)
12	5.24, dd (6.1, 2.4)	5.24, m	5.23, dd (6.1, 2.1)	5.35, dd (6.1, 2.3)
13	3.30, m	3.29, m	3.22, m	3.29, m
14	3.05, m	3.18, m	2.88, m	2.91, m
15	2.23, ddd (18.6, 2.1, 2.1)	2.22, br d (18.7)	2.22, ddd (18.5, 2.1, 2.1)	2.27, br d (18.8)
	2.88, br d (18.6)	2.83, br d (18.7)	2.83, br d (18.5)	2.92, br d (18.8)
17	4.96, m	4.95, m	4.95, m	4.99, m
	5.13, m	5.13, m	5.11, m	5.15, m
18	1.02, s	1.01, s	1.21, s	1.23, s
19	3.74, m	3.68, br d (12.7)	3.39, br d (12.0)	3.44, br d (12.0)
	4.52, m	4.41, br d (12.7)	4.43, br d (12.0)	4.47, br d (12.0)
20	5.12, m	5.10, m	4.89, m	4.98, m
N−CH_3	3.29, s	3.23, s	3.00, s	3.05, s
3′	6.77, br q (6.7)	6.76, ddq (5.6, 5.6, 1.3)	6.75, ddq (5.8, 5.8, 1.3)	7.94, m
4′	1.76, d (6.7)	4.23, dd (15.0, 5.6)	4.23, dd (15.0, 5.8)	7.49, m
		4.27 dd (15.0, 5.6)	4.27 dd (15.0, 5.8)	
5′	1.75, s	1.73, s	1.73, s	7.63, tt (7.6, 1.3)
6′				7.49, m
7′				7.94, m
3″	7.26, br q (7.0)	7.26, br q (7.0)	7.25, br q (7.0)	7.25, br q (7.1)
4″	1.83, d (7.0)	1.82, d (7.0)	1.83, d (7.0)	1.82, d (7.1)
5″	1.90, s	1.90, s	1.90, s	1.86, s
2‴	2.29, s	2.29, s		

[a]Spectra recorded at 600 MHz in acetone-d_6 at 30 °C.

assignments can be accessed in the Supporting Information (Figures S31−40).

The free base of 7β-hydroxy-11α-benzoylanopterine (**8**) was also only partially characterized (^{1}H NMR and MS data only) in the original publication.[5] We report the full NMR assignment (Figures S41−45, Supporting Information) of the TFA salt of 7β-hydroxy-11α-benzoylanopterine (**8**).

Finally, UV data for the known compounds were not reported in the original publications; hence we have included this data along with additional $[\alpha]_D$ data in the Experimental Section of this paper.

Compounds **1**−**8** were evaluated for their cytotoxicity against a panel of human cell lines, including the prostate cancer cell lines LNCaP (lymph node metastasis, androgen-sensitive), C4-2B (LNCaP-derivative, androgen-insensitive), and DuCaP (metastatic prostate cancer, androgen-sensitive) and the nonmalignant cell lines BPH-1 (human benign prostatic hyperplasia) and WPMY-1 (human prostatic stromal myofibroblast) using a metabolic assay and a live-cell imaging experiment. Prior to screening, all compounds were reanalyzed by ^{1}H NMR spectroscopy and shown to be >95% pure. The metabolic activity of alkaloids **1**−**8** against the panel of cell lines (Table 3) showed that they were highly active toward the prostate cancer cell lines, with IC_{50} values of <400 nM. Compounds **1**, **2**, and **8** were the most active, with IC_{50} values of 3.1, 10.9, and 6.7 nM against LNCaP cells, respectively. Compounds **1**−**8** were also cytotoxic in the nonmalignant cell lines BPH-1 and WPMY-1, which translated to small selectivity indices (SI = IC_{50} cancer cells/IC_{50} noncancer cells). For instance, the SI of compound **1** was 0.3 and 0.5 between the LNCaP cells and the cell lines BPH-1 and WPMY-1, respectively. Notably, the known anticancer drug vinblastine, which inhibits microtubule functions,[17−19] was also cytotoxic in both malignant and nonmalignant cell lines (Table 3), raising the possibility that compounds **1**−**8** specifically target proliferating cells.

The effect of compounds **1**−**8** on cell growth was also evaluated using a live-cell imaging assay. Proliferation of LNCaP cells was monitored in real time as a function of cell confluence, using an IncuCyte system (Figure 5 for compound **1** and Figures S48 and S49, Supporting Information for **2**−**8**). As illustrated in Figure 5A with 6α-acetoxyanopterine (**1**) as an example, the confluence of LNCaP cells decreased with increasing concentrations of this compound, indicating that **1** inhibited cell growth and visibly reduced the cell number when compared to the DMSO control. Morphologically, LNCaP cells treated with **1** showed loss of cell−cell contacts, round cell bodies, and burst cells with membrane blebbing, which are typical signs of apoptosis (Figure 5B).[20,21]

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Table 2. ^{13}C NMR Spectroscopic Data for Compounds 1−4[a]

position	1	2	3	4
1	36.2	36.2	36.5	36.7
2	65.6	65.2	65.4	65.6
3	43.6	43.6	43.1	43.0
4	36.3	36.7	36.6	36.7
5	75.8	76.1	76.4	76.5
6	73.5	73.4	70.3	70.5
7	40.7	41.3	43.9	44.2
8	49.9	49.9	49.8	50.1
9	53.5	53.3	54.4	54.6
10	53.1	52.8	52.3	52.5
11	69.9	70.0	70.4	70.9
12	72.8	72.8	73.0	72.9
13	53.6	53.7	53.7	53.7
14	54.8	54.2	54.8	55.0
15	39.3	39.5	39.8	39.9
16	148.2	148.8	149.1	149.2
17	109.4	109.0	108.8	109.9
18	23.2	23.3	23.8	23.6
19	64.6	64.4	63.4	63.4
20	70.4	70.1	68.9	68.9
N-CH_3	47.3	47.7	44.6	44.6
1′	166.2	166.7	166.4	165.5
2′	128.6	127.1	127.0	130.7
3′	138.4	144.1	144.0	130.0
4′	14.4	59.2	59.4	129.0
5′	11.6	12.4	12.7	134.0
6′				129.0
7′				130.0
1″	167.6	167.5	167.3	167.4
2″	128.1	128.6	128.4	128.5
3″	139.6	139.9	139.7	139.9
4″	14.7	14.3	14.6	14.6
5″	12.3	12.3	12.6	12.6
1‴	169.0	169.7		
2‴	21.8	21.7		

[a]Spectra recorded at 150 MHz in acetone-d_6 at 30 °C; ^{13}C chemical shifts obtained from HSQC and HMBC data.

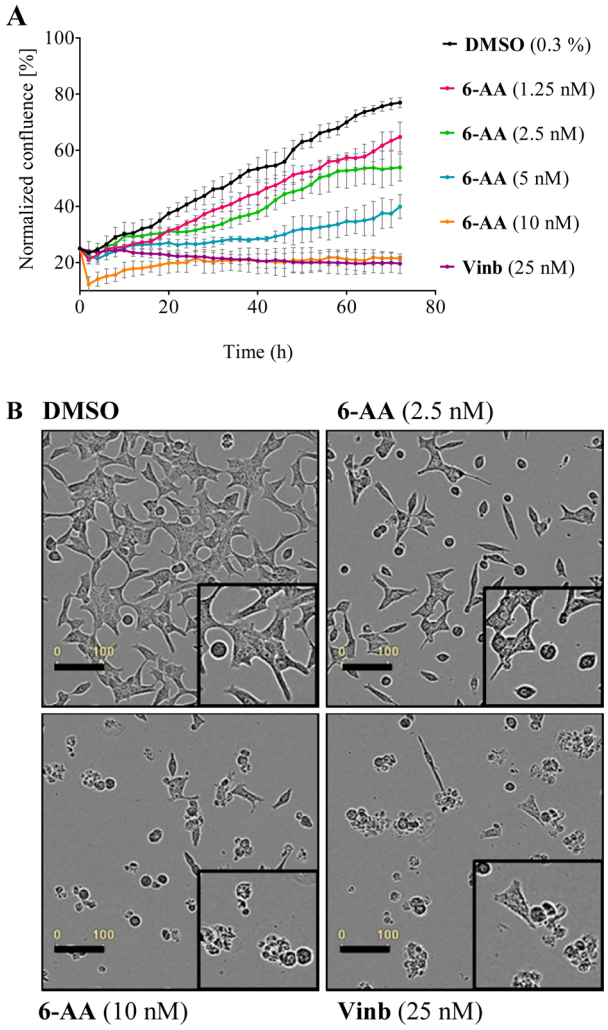

Figure 5. (A) Changes to cell confluence and (B) cell morphology of LNCaP cells after 72 h of treatment with indicated doses of 6α-acetoxyanopterine (6-AA, **1**), DMSO, or vinblastine (Vinb) monitored using an IncuCyte real-time live-cell imaging system (scale bar 100 μm).

Wall et al.[10] have reported the cytotoxity of the known anopterine analogues (**5**−**8**) toward the KB cell line and observed similar levels of activity. No mechanism of action studies have been reported for this rare structure class; however, the potent cytotoxicity warrants further biological investigations.

Table 3. Cytotoxic Activities (IC_{50} ± SD) of Compounds 1−8 against Human Cell Lines[a]

	IC_{50} ± SD (nM)				
compound	LNCaP	C4-2B	DuCaP	BPH-1	WPMY-1
1	3.1 ± 1.8	6.2 ± 0.3	8.7 ± 0.5	11.5 ± 5.7	6.6 ± 0.5
2	10.9 ± 3.2	7.0 ± 2.3	9.9 ± 1.2	11.4 ± 2.8	9.6 ± 2.1
3	378.8 ± 85.8	339.8 ± 71.7	355.0 ± 55.9	177.3 ± 52.8	328.5 ± 18.6
4	190.3 ± 96.2	205.4 ± 63.9	153.5 ± 16.5	236.1 ± 12.9	140.5 ± 34.5
5	27.2 ± 13.2	45.0 ± 10.5	86.9 ± 8.4	29.6 ± 2.0	48.2 ± 5.4
6	50.8 ± 23.9	93.8 ± 6.0	55.7 ± 4.4	64.3 ± 12.3	86.0 ± 13.8
7	72.6 ± 18.3	134.6 ± 30.9	70.7 ± 12.4	133.7 ± 27.3	154.6 ± 35.3
8	6.7 ± 1.8	5.2 ± 0.3	4.1 ± 0.3	9.2 ± 1.9	4.9 ± 1.2
vinblastine	3.2 ± 0.1	2.4 ± 0.2	3.7 ± 0.7	3.8 ± 0.6	4.2 ± 0.6

[a]Cell lines: LNCaP, human prostate cancer (lymph node metastasis, androgen-sensitive); C4-2B, bone metastatic LNCaP-derivative (androgen-insensitive); DuCaP, metastatic prostate cancer (androgen-sensitive); BPH-1, human benign prostatic hyperplasia; WPMY-1, human prostatic stromal myofibroblast.

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EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were recorded on a JASCO P-1020 polarimeter (Easton, MD, USA). UV spectra were recorded on a JASCO V-650 UV/vis spectrophotometer. NMR spectra were recorded at 30 °C on a Varian 600 MHz Unity INOVA spectrometer (Palo Alto, CA, USA). The ^{1}H and ^{13}C chemical shifts were referenced to the solvent peaks for acetone-d_6 at δ_H 2.05 and δ_C 29.9/206.7 and for DMSO-d_6 at δ_H 2.50 and δ_C 39.5. Standard parameters were used for the 2D NMR experiments, which included HSQC ($^{1}J_{CH}$ = 140 Hz) and HMBC ($^{n}J_{CH}$ = 8.0 Hz). Phenomenex solid-phase extraction (SPE) cartridges (10 × 50 mm, nylon frit, Torrance, CA, USA) were used for small-scale plant extraction. LRESIMS data were recorded on a Waters LCMS system (Milford, MA, USA) equipped with a Phenomenex Luna C_{18} column (3 μm, 100 Å, 50 × 4.6 mm), a PDA detector, and a ZQ ESI mass spectrometer. HRESIMS were recorded on a Bruker MicrOTof-Q spectrometer (Billerica, MA, USA) with the Dionex UltiMate 3000 micro LC system, ESI mode (Sunnyvale, CA, USA). All solvents used for chromatography, $[\alpha]_D$, UV, and MS were HPLC grade (Honeywell Burdick & Jackson, Morristown, NJ, USA), and the H_2O was Millipore Milli-Q PF filtered (Billerica, MA, USA). Phenomenex end-capped Sepra C_{18} bonded silica (35−75 μm, 150 Å) was used for extract/fraction preadsorption work, and the resulting material was packed into an Alltech stainless steel guard cartridge (10 × 30 mm, Columbia, MD, USA). A Waters 600 pump and controller fitted with a Waters 996 PDA detector and Gilson 717 Plus autosampler (Middleton, WI, USA) were used for semipreparative HPLC separations. A Phenomenex C_{18} Onyx analytical HPLC column (130 Å, 100 × 4.6 mm) was used for the generation of the plant-derived fraction library. A Thermo Fisher Scientific Betasil C_{18} column (5 μm, 100 Å, 150 × 21.2 mm, Waltham, MA, USA) and a Thermo Fisher Scientific Betasil phenyl column (5 μm, 143 Å, 150 × 21.2 mm) were used for semipreparative HPLC. A Fritsch Universal Cutting Mill Pulverisette 19 (Idar-Oberstein, Germany) was used to grind the air-dried plant material. An Edwards Instrument Company Bioline orbital shaker (Narangba, Australia) was used for the large-scale plant extractions. Tiglic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plant Material. Thirty-six of the 38 Australian endemic plant samples were obtained from the Nature Bank biota library, which is housed at the Eskitis Institute for Drug Discovery, Griffith University, Brisbane, Australia.[22] All samples were taxonomically identified by the Queensland Herbarium, and voucher specimens have been deposited at the Queensland Herbarium, Brisbane, Australia. *Anopterus macleayanus* F.Muell leaf and wood samples were obtained from the Burringbar Rainforest Nursery (Burringbar, Australia) during May of 2013. A voucher specimen (RAD071) has been deposited at the Eskitis Institute for Drug Discovery, Griffith University, Brisbane, Australia. The plant material was air-dried, ground to a fine powder, and stored at room temperature prior to extraction.

Generation of the Plant-Derived Prefractionated Library. A small amount (300 mg) of air-dried and ground plant material from each of the 38 samples was packed into an SPE cartridge, washed with *n*-hexane (8 mL), and successively extracted with CH_2Cl_2 (10 mL) and MeOH (8 mL). The MeOH extracts were dried, weighed, and resuspended in DMSO in order to create a stock solution at 1 mg/100 μL. Liquid injections (100 μL) of each crude MeOH extract were fractionated by HPLC using a C_{18} Onyx analytical column and a gradient solvent method that has previously been reported.[14] The initial and previously published fractionation process resulted in the collection of 11 fractions per extract;[14] however we decided to collect only one fraction (between 2.0 and 7.0 min) in order to simplify the process and minimize cost and time expenditure.

LCMS Analysis. The active fractions were resuspended in MeOH (1 mL), prior to LCMS injection (20 μL). The LCMS was performed using an analytical Phenomenex Luna column and a gradient from 95% H_2O (0.1% formic acid)/5% MeOH (0.1% formic acid) to 100% MeOH (0.1% formic acid) in 10 min, at a flow rate of 1 mL/min.

Large-Scale Extraction and Isolation. The ground seeds (10 g) of *A. macleayanus* were sequentially extracted at room temperature with CH_2Cl_2 (200 mL) and MeOH (200 mL × 3). The resulting extracts were filtered under gravity, dried under reduced pressure, weighed, and stored at room temperature (CH_2Cl_2, 468 mg; MeOH, 1.9 g). A portion of the MeOH extract (1.7 g) was resuspended in MeOH, preadsorbed to C_{18} silica, and packed into four separate stainless steel guard cartridges, and each cartridge was subjected to semipreparative HPLC using a C_{18} Betasil column at a flow rate of 9 mL/min. Isocratic conditions of 10% MeOH (0.1% TFA)/90% H_2O (0.1% TFA) were initially performed for the first 10 min, followed by a linear gradient to 100% MeOH (0.1% TFA) in 40 min; then isocratic conditions of 100% MeOH (0.1% TFA) were run for 10 min. Sixty fractions (60 × 1 min) were collected from the start of the HPLC run. Fractions from the first HPLC run were all tested for activity toward LNCaP cells. In order to minimize the number of fractions tested, aliquots from five consecutive fractions were pooled and tested. Fractions containing biological activity were analyzed by (±)-LRESIMS and ^{1}H NMR spectroscopy. The CH_2Cl_2 extract was also subjected to the same purification process, in an attempt to purify more material. None of these HPLC runs yielded sufficient quantities of pure compounds to allow structure determination or biology testing. The remaining MeOH extract (200 mg) was subjected to a modified separation protocol, which included an alkaloid extraction using EtOAc/H_2O (1:1, 200 mL × 3), with the H_2O basified with ammonia (pH = 10). The enriched alkaloid fraction was subjected to semipreparative Betasil phenyl HPLC. Isocratic conditions of 10% MeCN (0.1% TFA)/90% H_2O (0.1% TFA) were initially performed for the first 5 min, followed by a linear gradient to 100% MeCN (0.1% TFA) in 60 min, at a flow rate of 9 mL/min. Sixty-five fractions (65 × 1 min) were collected from the start of the HPLC run. Fractions were analyzed by (±)-LRESIMS in order to detect the ions belonging to the active fractions previously identified. Fractions 34−36 contained some of the desired ions and were combined (m = 8.3 mg), dried, and further purified by HPLC using a Betasil phenyl column with solvent conditions consisting of a linear gradient from 35% MeCN (0.1% TFA)/65% H_2O (0.1% TFA) to 80% MeCN (0.1% TFA)/20% H_2O (0.1% TFA) in 90 min at a flow rate of 9 mL/min. Manual collection yielded one pure compound as its TFA salt, 7β-hydroxy-11α-benzoylanopterine (**8**, t_R = 19 min, 1.5 mg, M_w 579 (free base), 0.015% dry wt).

The air-dried and ground wood (65 g) of *A. macleayanus* was sequentially extracted at room temperature with CH_2Cl_2 (500 mL) and MeOH (500 mL × 3). The CH_2Cl_2 and MeOH extractions were combined and dried under reduced pressure to yield a crude extract (∼8 g). The MeOH/CH_2Cl_2 extract was subjected to an alkaloid extraction using EtOAc/H_2O (1:1, 300 mL × 3), with the H_2O basified with ammonia (pH = 10). The enriched alkaloid fraction (organic layer ∼1.6 g) was further purified with a liquid/liquid partition using the system CH_2Cl_2/H_2O (1:1, 500 mL × 3). The CH_2Cl_2 extract (775 mg) was resuspended in MeOH, preadsorbed to C_{18} silica, and packed into three separate stainless steel guard cartridges, and each cartridge was subjected to semipreparative Betasil phenyl HPLC. Linear gradient conditions from 20% MeOH (0.1% TFA)/80% H_2O (0.1% TFA) to 40% MeOH (0.1% TFA)/60% H_2O (0.1% TFA) were performed for the first 20 min, followed by a linear gradient to 80% MeOH (0.1% TFA)/20% H_2O (0.1% TFA) for 30 min, then a gradient to 100% MeOH (0.1% TFA) in 10 min, at a flow rate of 9 mL/min. Sixty fractions (60 × 1 min) were collected from the start of the HPLC run, and identical fractions from the three runs were gathered. Fractions were analyzed by (±)-LRESIMS in order to detect the ions belonging to the active fractions previously identified during the investigations of the seed extract. Fractions 31 and 32 were combined (m = 10.5 mg), dried, and further purified by HPLC using a Betasil phenyl column with solvent conditions consisting of a linear gradient from 30% MeCN (0.1% TFA)/70% H_2O (0.1% TFA) to 80% MeCN (0.1% TFA)/20% H_2O (0.1% TFA) in 60 min at a flow rate of 9 mL/min. Manual collection yielded two pure compounds as their TFA salts, 7β,4′-dihydroxyanopterine (**7**, t_R = 13 min, 1.6 mg, M_w 573 (free base), 0.002% dry wt) and 4′-hydroxyanopterine (**3**, t_R = 18 min, 2.1 mg, M_w 557 (free base), 0.003% dry wt). Fraction 38 (m = 6.5 mg) obtained from the first isolation step was further purified by HPLC

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using a Betasil phenyl column with solvent conditions consisting of a linear gradient from 35% MeCN (0.1% TFA)/65% H_2O (0.1% TFA) to 80% MeCN (0.1% TFA)/20% H_2O (0.1% TFA) in 90 min at a flow rate of 9 mL/min to yield the TFA salts of 7β-hydroxyanopterine (**6**, t_R = 18 min, 1.9 mg, M_w 557 (free base), 0.003% dry wt) and 6α-acetoxyanopterine (**1**, t_R = 26 min, 1.1 mg, M_w 583 (free base), 0.002% dry wt). Fraction 39 (m = 7.2 mg) obtained from the first isolation step was further purified by HPLC using a Betasil phenyl column with solvent conditions consisting of a linear gradient from 35% MeCN (0.1% TFA)/65% H_2O (0.1% TFA) to 80% MeCN (0.1% TFA)/20% H_2O (0.1% TFA) in 90 min at a flow rate of 9 mL/min to yield the TFA salt of anopterine (**5**, t_R = 29 min, 3.4 mg, M_w 541 (free base), 0.005% dry wt). Fraction 43 (m = 4.2 mg) from the first isolation step was further purified by HPLC using a Betasil phenyl column with solvent conditions consisting of a linear gradient from 35% MeCN (0.1% TFA)/65% H_2O (0.1% TFA) to 80% MeCN (0.1% TFA)/20% H_2O (0.1% TFA) in 90 min at a flow rate of 9 mL/min. Manual collection yielded one pure compound as its TFA salt, 11α-benzoylanopterine (**4**, t_R = 24 min, 0.7 mg, M_w 563 (free base), 0.001% dry wt). Fraction 44 from the first isolation step (m = 2.8 mg) was further purified by HPLC using a Betasil phenyl column with solvent conditions consisting of a linear gradient from 35% MeCN (0.1% TFA)/65% H_2O (0.1% TFA) to 80% MeCN (0.1% TFA)/20% H_2O (0.1% TFA) in 90 min at a flow rate of 9 mL/min. Manual collection yielded one pure compound as its TFA salt, 7β-hydroxy-11α-benzoylanopterine (**8**, t_R = 27 min, 0.4 mg, M_w 580 (free base), 0.001% dry wt).

The air-dried and ground leaves (270 g) of *A. macleayanus* were sequentially extracted at room temperature with CH_2Cl_2 (1 L) and MeOH (1 L × 3). The CH_2Cl_2 and MeOH extractions were combined and dried under reduced pressure to yield a crude extract (95 g). The chlorophyll of the MeOH/CH_2Cl_2 extract was removed by liquid/liquid partition (*n*-hexane/H_2O, 1:1, 800 mL × 3). The resulting aqueous extract (~60 g) was further purified using the system CH_2Cl_2/H_2O (1:1, 800 mL × 3). The CH_2Cl_2 extract (375 mg) was resuspended in MeOH, preadsorbed to C_{18} silica, and packed into a stainless steel guard cartridge. This cartridge was subsequently attached to a semipreparative Betasil phenyl HPLC column. Linear gradient conditions from 10% MeOH (0.1% TFA)/90% H_2O (0.1% TFA) to 100% MeOH (0.1% TFA) were performed for the first 40 min; then conditions were held at 100% MeOH (0.1% TFA) for 20 min, at a flow rate of 9 mL/min. Sixty fractions (60 × 1 min) were collected from the start of the HPLC run. Fractions were analyzed by (±)-LRESIMS in order to detect the ions belonging to the active fractions previously identified. Fractions 25 and 26 were combined (m = 32.1 mg) and further purified by HPLC using a Betasil phenyl column with solvent conditions consisting of a linear gradient from 30% MeCN (0.1% TFA)/70% H_2O (0.1% TFA) to 80% MeCN (0.1% TFA)/20% H_2O (0.1% TFA) in 60 min at a flow rate of 9 mL/min and afforded the TFA salt of 4′-hydroxy-6α-acetoxyanopterine (**2**, t_R = 15 min, 5.6 mg, M_w 599 (free base), 0.002% dry wt). Fractions 29 to 31 from the first isolation step were combined (m = 44.7 mg) and further purified by HPLC using a Betasil phenyl column with solvent conditions consisting of a linear gradient from 30% MeCN (0.1% TFA)/70% H_2O (0.1% TFA) to 80% MeCN (0.1% TFA)/20% H_2O (0.1% TFA) in 60 min at a flow rate of 9 mL/min and yielded the TFA salt of 6α-acetoxyanopterine (**1**, t_R = 21 min, 10.6 mg, M_w 583 (free base), 0.004% dry wt).

*TFA Salt of 6α-Acetoxyanopterine (**1**):* stable yellow gum; $[\alpha]^{25}_D$ +63 (c 0.1, MeOH); $[\alpha]^{25}_D$ −6 (c 0.2, $CHCl_3$); UV λ_{max} (MeOH) (log ε) 210 (4.26) nm; ^{1}H NMR (600 MHz, acetone-d_6) see Table 1; ^{13}C NMR (150 MHz, acetone-d_6) see Table 2; (+)-LRESIMS m/z 584 (100) [M − CF_3COO^-]$^+$; (+)-HRESIMS m/z 584.3215 ($C_{33}H_{46}NO_8^+$ [M − CF_3COO^-]$^+$ requires 584.3218).

*TFA Salt of 4′-Hydroxy-6α-acetoxyanopterine (**2**):* stable yellow gum; $[\alpha]^{25}_D$ +48 (c 0.04, MeOH); $[\alpha]^{25}_D$ +53 (c 0.03, $CHCl_3$); UV λ_{max} (MeOH) (log ε) 206 (4.17) nm; ^{1}H NMR (600 MHz, acetone-d_6), see Table 1; ^{13}C NMR (150 MHz, acetone-d_6), see Table 2; (+)-LRESIMS m/z 600 (100) [M − CF_3COO^-]$^+$; (+)-HRESIMS m/z 600.3171 ($C_{33}H_{46}NO_9^+$ [M − CF_3COO^-]$^+$ requires 600.3167).

*TFA Salt of 4′-Hydroxyanopterine (**3**):* stable yellow gum; $[\alpha]^{25}_D$ +19 (c 0.05, MeOH); $[\alpha]^{25}_D$ +45 (c 0.01, $CHCl_3$); UV λ_{max} (MeOH) (log ε) 209 (4.21) nm; ^{1}H NMR (600 MHz, acetone-d_6), see Table 1; ^{13}C NMR (150 MHz, acetone-d_6), see Table 2; (+)-LRESIMS m/z 558 (100) [M − CF_3COO^-]$^+$; (+)-HRESIMS m/z 558.3067 ($C_{31}H_{44}NO_8^+$ [M − CF_3COO^-]$^+$ requires 558.3061).

*TFA Salt of 11α-Benzoylanopterine (**4**):* stable yellow gum; $[\alpha]^{25}_D$ +6 (c 0.1, MeOH); $[\alpha]^{25}_D$ −92 (c 0.1, $CHCl_3$); UV λ_{max} (MeOH) (log ε) 222 (4.03) nm; ^{1}H NMR (600 MHz, acetone-d_6), see Table 1; ^{13}C NMR (150 MHz, acetone-d_6), see Table 2; (+)-LRESIMS m/z 564 (100) [M − CF_3COO^-]$^+$; (+)-HRESIMS m/z 564.2959 ($C_{33}H_{42}NO_7^+$ [M − CF_3COO^-]$^+$ requires 564.2956).

*TFA Salt of Anopterine (**5**):* stable yellow gum; $[\alpha]^{25}_D$ +10 (c 0.1, MeOH); $[\alpha]^{25}_D$ −5 (c 0.2, $CHCl_3$); literature value for the free base of **5**, $[\alpha]^{25}_D$ −12 (c 1.5, $CHCl_3$);[2] UV λ_{max} (MeOH) (log ε) 208 (4.13) nm; (+)-LRESIMS m/z 542 (100) [M − CF_3COO^-]$^+$.

*TFA Salt of 7β-Hydroxyanopterine (**6**):* stable yellow gum; $[\alpha]^{25}_D$ +20 (c 0.03, $CHCl_3$); $[\alpha]^{25}_D$ +15 (c 0.03, $CHCl_3$/MeOH); literature value for the free base of **6**, $[\alpha]^{25}_D$ −12 (c 1.0, $CHCl_3$/MeOH);[5] UV λ_{max} (MeOH) (log ε) 209 (4.39) nm; (+)-LRESIMS m/z 558 (100) [M − CF_3COO^-]$^+$.

*TFA Salt of 7β,4′-Dihydroxyanopterine (**7**):* stable yellow gum; $[\alpha]^{25}_D$ +15 (c 0.01, $CHCl_3$); $[\alpha]^{25}_D$ +19 (c 0.1, MeOH); literature value for the free base of **7**, $[\alpha]^{25}_D$ −9 (c 1.2, MeOH);[5] UV λ_{max} (MeOH) (log ε) 209 (4.65) nm; (+)-LRESIMS m/z 574 (100) [M − CF_3COO^-]$^+$.

*TFA Salt of 7β-Hydroxy-11α-benzoylanopterine (**8**):* stable yellow gum; $[\alpha]^{25}_D$ +53 (c 0.01, $CHCl_3$); $[\alpha]^{25}_D$ −50 (c 0.01, MeOH); literature value for the free base of **8**, $[\alpha]^{25}_D$ −10 (c 1.0, MeOH);[10] UV λ_{max} (MeOH) (log ε) 226 (4.13) nm; (+)-LRESIMS m/z 580 (100) [M − CF_3COO^-]$^+$.

AlamarBlue and Live-Cell Imaging Assays. LNCaP and C4-2B cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). BPH-1 and WPMY-1 cells were kind gifts from J. Clements (APCRC-Q, QUT, Australia). DuCaP cells were a generous gift from M. Ness (VTT Technical Research Centre of Finland). LNCaP, C4-2B, BPH-1, and WPMY-1 cells were cultured in phenol-red-free RPMI-1640 medium (Thermo Fisher Scientific) supplemented with 5% FBS (Thermo Fisher Scientific) at 37 °C in an atmosphere containing 5% CO_2 and maintained in log phase growth. DuCaP cells were cultured in phenol-red free RPMI-1640 medium (Thermo Fisher Scientific) supplemented with 10% FBS (Thermo Fisher Scientific) at 37 °C in an atmosphere containing 5% CO_2 and maintained in log phase growth. Cell viability as a function of metabolic activity was measured by an AlamarBlue end point assay (Thermo Fisher Scientific) as previously described.[23] Briefly, LNCaP (4000 cells per well), C4-2B (3000 cells per well), DuCaP (15 000 cells per well), BPH-1 (3000 cells per well), and WPMY-1 (2000 cells per well) were seeded for 24 h into 96-well tissue culture plates (Corning, Corning, NY, USA) and treated with the indicated compounds. Metabolic activity was measured with AlamarBlue according to the manufacturer's instructions (Thermo Fisher Scientific) after 72 h of treatment. Fractions or compounds were dissolved in DMSO and diluted in growth medium (final concentration 0.3%). The prefractionated library was screened at 10 μg/mL. Control cells were treated with the equivalent dose of DMSO (negative control) or vinblastine (25 nM, Sigma-Aldrich) as positive control. For live-cell imaging, cells were seeded and treated as above. As described before,[23] the 96-well plates were loaded into an IncuCyte live-cell imaging system (Essen BioScience, Ann Arbor, MI, USA), cells were imaged every 2 h for 72 h, and growth was measured as a function of increasing confluence. Calculations of half-maximal inhibitory concentration (IC_{50}) after 72 h of treatment were performed with GraphPad Prism (GraphPad Software). Each data point was performed in triplicate and repeated in at least three independent experiments.

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■ ASSOCIATED CONTENT

Supporting Information

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1D and 2D NMR data for compounds **1**−**8** and cytotoxicity evaluation of compounds **2**−**8** using a live-cell imaging assay (PDF)

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Notes

The authors declare no competing financial interest.

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