PLANT ANTITUMOR AGENTS, 26.[1] ISOLATION, STRUCTURE, AND ANTITUMOR ACTIVITY OF ALKALOIDS FROM *ANOPTERUS GLANDULOSUS*

MONROE E. WALL,* M.C. WANI,* BRIAN N. MEYER,[2] and HAROLD TAYLOR

Research Triangle Institute, Research Triangle Park, North Carolina 27709

During our studies of plants with cytotoxic (KB) activity or life-prolongation activity in mouse leukemia assays (P-388 or L-1210), it was found that a crude EtOH extract of *Anopterus glandulosus* Labill. (Escalloniaceae) exhibited high activity in the P-388 (T/C 193 at 50 mg/kg) and KB (ED$_{50}$ 5×10^{-1} μg/ml) systems, and, consequently, fractionation studies were initiated.

There are only two known *Anopterus* species, both restricted in distribution to Australia, *Anopterus macleayanus*, F. Muell. is a tree found in southern Queensland, and *A. glandulosus* is a small Tasmanian tree. The alkaloids of *A. macleayanus* were first examined by Hart *et al.* (1,2) who discovered several novel bases with an *ent*-kaurenoid skeleton. Although the alkaloids were reported to have activity in the KB and P-388 assays, no details were presented (1), and no bases were isolated in crystalline form from *A. glandulosus*, which has a much lower alkaloid content. More recently we have presented a preliminary report of the alkaloids of *A. glandulosus* (3). Subsequently a 2D-nmr study by Johns *et al.* (4) has clarified the uncertain position of one hydroxyl group present in some of the bases. In this report we present a full account of the isolation and structure-activity relationships found in the *ent*-kaurene alkaloids isolated from *A. glandulosus*, including the isolation of two new bases. Details of structural

elucidation will be limited because all of the compounds **1-5** have now been described (1,2,4). The observed biological activity was not due to the non-alkaloidal components as this fraction was inactive in the KB and P-388 test systems.

RESULTS AND DISCUSSION

The crude EtOH extract of the leaves and twigs of *A. glandulosus* was treated with dilute H$_2$SO$_4$. The acid-soluble fraction was rendered alkaline and extracted with CHCl$_3$. After extensive column chromatography on Si gel (see Experimental section) followed in many cases by preparative centrifugal radial thin layer chromatography (cptlc) and/or preparative hplc, five alkaloids were isolated in low overall yield (see Experimental section).

The anopterine alkaloids are related to *ent*-kaurene diterpenes (1) (Figure 1). The alkaloids isolated were anopterine [1] (1,2), 7β-hydroxyanopterine [2] (1-4), 4′,7β-dihydroxyanopterine [3] (1-4), 11α-benzoyl-7β-hydroxy-11α-destigloylanopterine [4] (3,4), and 11α-destigloylanopterine [5] (3,4).

Structure proof of the alkaloids **1-5** was based on the single-crystal X-ray analysis of **1** (5), and our independent ^{1}H-nmr and hrms studies of **2-4**, which confirm structures previously assigned by Hart *et al.* (1,2) and establish the structures of **4** and **5**. We accept the revised location of the hydroxyl groups, previously believed to be at C-1 or C-3, to 7β based on 2D-nmr studies by Johns *et al.* (4).
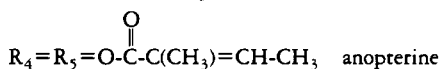
The hrms of **4** (*m/z* 579.282, C$_{33}$H$_{41}$NO$_8$) suggested it to be a derivative of hydroxyanopterine [2] in which a tigloyl group has been replaced by a ben-

1 R=R$_1$=R$_2$=OH, R$_3$=H,

$$\overset{\textstyle O}{\underset{\textstyle \|}{}}$$

R$_4$=R$_5$=O-C-C(CH$_3$)=CH-CH$_3$ anopterine
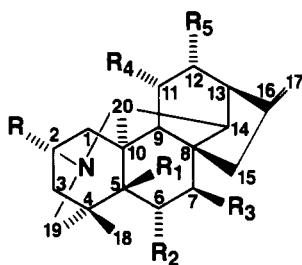
2 R=R$_1$=R$_2$=R$_3$=OH,
R$_4$=R$_5$=tigloyloxy   7β-hydroxyanopterine

3 R=R$_1$=R$_2$=R$_3$=OH,

$$\overset{\textstyle O}{\underset{\textstyle \|}{}}$$

R$_4$=OC-C(CH$_3$)=CHCH$_2$OH,
R$_5$=tigloyloxy   4′,7β-dihydroxyanopterine

4 R=R$_1$=R$_2$=R$_3$=OH,

$$\overset{\textstyle O}{\underset{\textstyle \|}{}}$$

R$_4$=OCC$_6$H$_5$,
R$_5$=tigloyloxy   11α-benzoyl-7β-hydroxy-11α-destigloylanopterine

5 R=R$_1$=R$_2$=R$_4$=OH, R$_3$=H,
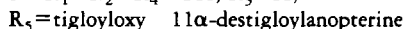R$_5$=tigloyloxy   11α-destigloylanopterine
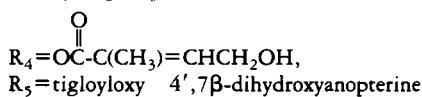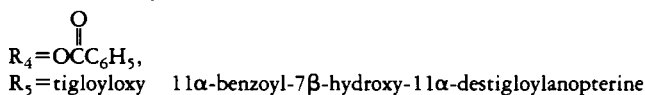
FIGURE 1.   Structure of anopterine alkaloids

zoyl group. This was borne out by the presence of only one set of signals due to the tigloyl moiety and an aromatic pattern characteristic of the benzoyl moiety in the ^{1}H-nmr spectrum of **4**. Further support for the presence of the benzoyl moiety came from an exact mass measurement of the mass spectral fragment at 105 (*m/z* 105.034=C$_7$H$_5$O). Evidence for the location of the benzoyl ester at C-11 came from the ^{1}H nmr of **5** (see below).

The accurate mass of **5** (*m/z* 459.262=C$_{26}$H$_{37}$NO$_6$) suggested it to be a destigloyl derivative of anopterine [**1**], and this was supported by the presence of only one set of signals due to the tigloyl moiety in the ^{1}H-nmr spectrum of **5**. The signal at δ 5.03 was assigned to H-12 based on its multiplicity to H-13 as shown by a double resonance experiment. The signal at δ 4.39 (cf. δ 5.49 in **2** and δ 5.58 in **1**), therefore, represented H-11 on a carbon bearing an unesterified, hydroxyl function.

The vinyl proton signal at δ 7.12 in **5** arises from the tigloyl group at C-12. Similar signals occur in **1** and **2** along with a multiplet around δ 6.8 for the C-11 tigloyl group. The benzoyl ester group must, therefore, be placed on C-11.

Table 1 presents the biological activity data available for the five compounds in the KB cytotoxcity and P-388 mouse leukemia assays. With the exception of compound **5**, which was essentially inactive, all of the other anopterine bases exhibited considerable cytotoxicity of the order ED$_{50}$ 1×10^{-2}-1×10^{-3} μg/ml. We doubt if the differences in cytotoxicity of the active compounds are significant. In view of the high activity of the crude, aqueous, MeOH fraction from *A. glandulosus* in P-388 (see Table 1) with doses from 6.25 mg/kg to 50 mg/kg showing excellent dose response (max T/C 190 at 50 mg/kg), the in vivo P-388 data for the pure compounds **1-5** were both surprising and disappoint-

TABLE 1.   KB and P-388 Activity of Anopterine Alkaloids

Compound	Maximal T/C Dose (mg/kg)	Active Dose Range (mg/kg)	Toxic Dose (mg/kg)	ED_{50} for KB (μg/ml)
90% MeOH fraction from Anopterus glandulosus	190 (50)	6.25-50	100	5×10^{-2}
Anopterine [1]	122 (13)[a]	—[b]	—[c]	5×10^{-2}
7β-Hydroxyanopterine [2] . .	140 (4.4)[a]	2.2-4.4	—[c]	1×10^{-3}
4',7β-Dihydroxy- anopterine [4]	120 (3.5)[a]	—[b]	—[c]	5×10^{-2}
11α-Benzoyl-7β-hydroxy- 11α-destigloyl- anopterine [4]	125 (1.5)[a]	1.5	—[c]	1×10^{-3}
11-Destigloyl- anopterine [5]	127 (6)[a]	6	—[c]	1×10^{0}

[a]Highest dose tested.
[b]Inactive at all doses.
[c]Not toxic at highest dose tested.

ingly low. The most active compound was **2** with a maximal T/C at 140 mg/kg. Of the others, **1** and **3** were inactive and compounds **4** and **5** marginally active at the highest tested dose. The small quantity of alkaloid available in all cases except for **1** restricted the evaluation of P-388 activity, because in no case was a toxic dose level attained. It is evident that for cytotoxic activity the presence of diester moieties at C-11 and C-12 is required as evidenced by the inactivity of the 11-destigloyl ester **5**. Only anopterine [1] was tested at a level high enough to state that it was either inactive or much less potent than the other *ent*-kaurene alkaloids. Hydroxyanopterine [2] was seemingly more active or had higher potency than other *ent*-kaurene alkaloids; however, there were several other alkaloids present in such low quantity, but their isolation was unsuccessful. In view of the high P-388 activity in the crude extracts, it is conceivable that maximal activity may reside in one of these.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.— Melting points were determined on a Kofler hot-stage microscope and are uncorrected. [1]H-nmr spectra were obtained with a Bruker WM250 spectrometer using TMS as internal standard. High resolution mass spectra were obtained with an AEI MS-902 instrument.

Cptlc was performed on a Chromatotron, Model 7924, Harrison Research. Rotors were poured using Kieselgel 60 PF_{254} (E. Merck). R_f measurements were made on analytical, precoated, glass plates, Si gel 60 PF-254 (E. Merck). Solvent systems: A, $CHCl_3$-MeOH-NH_4OH (9:1:0.5, lower layer); B, EtOAc-MeOH-NH_4OH (17:2:1). Semipreparative hplc was performed on a 9.4 mm × 25 cm column packed with Zorbax ODS (DuPont) attached to a Waters 6000A pump with U6K injector and a Schoeffel Instruments SF770 Spectroflow monitor set at 230 nm. The solvent system was pH 3.5 buffer-MeCN-$(iC_3H_7)_2$NH (72:28:0.5). The buffer consisted of 0.1 M aqueous NH_4OAc adjusted to pH 3.5 with glacial HOAc. The flow rate was 6 ml/min, and the load was 6 mg per run.

PLANT MATERIAL.—Twigs and leaves (164 kg) of *A. glandulosus* were collected in Tasmania, April 1982, and assigned PR 57190 by the USDA, Beltsville, Maryland.

EXTRACTION.—The dried, ground plant material (9.1 kg) was subjected to continuous extraction with hot 95% EtOH. This extract was condensed to a thick syrup (1.12 kg) that was triturated with 1% H_2SO_4 (5 liters), centrifuged, and filtered. The filtrate was rendered basic with NH_4OH and extracted with $CHCl_3$ (3 × 2 liters). The combined $CHCl_3$ layers were dried with anhydrous Na_2SO_4 and concentrated to yield a crude alkaloid fraction (10.3 g).

Anopterine [1].—The crude alkaloids were applied to a column of silicAR CC-7 (Mallinckrodt) (95 g) and separated into three fractions (F1, F2, F3) by elution with $CHCl_3$ containing, respectively, 5% and 50% MeOH, and finally with 100% MeOH.

Fraction F1 (6.24 g) was chromatographed in several batches on Si gel using increasing concen-

trations of MeOH in EtOAc (0-100% MeOH). Solvent mixtures were shaken with a 5% volume of NH_4OH. Analogous fractions from separate runs were combined. The pooled fractions giving a Dragendorff-positive spot at R_f 0.69 on tlc (solvent A) were twice chromatographed by cptlc [$CHCl_3$-Me_2CO-(Et)$_2NH$-C_7H_{16}; 5:4:1:6]. The least polar fractions were combined, and after removal of the solvents, the residue was recrystallized from Me_2CO to give anopterine [1] (114 mg); yield 0.001%; mp 220-221° [lit. 222-223° (1)]; ms m/z 541.3036 ($C_{31}H_{43}NO_7$=541.3038) (100%); ^1H-nmr spectra as in the literature (1,2,4).

7β-Hydroxyanopterine [2] *and 11α-benxoyl-7β-hydroxy-11α-destigloyl-anopterine* [4].—The pooled fractions from Si gel chromatography of F1, giving a Dragendorff-positive spot at R_f 0.38 on tlc (solvent B), were chromatographed twice by cptlc ($CHCl_3$-MeOH-NH_4OH; 94:6:10, lower layer) to yield a fraction which crystallized from Me_2CO, mp 246-250°. This material, one spot on silica tlc, was shown to be a mixture by ms and nmr. Two components could be separated by analytical reverse phase hplc. The material was subjected to semipreparative reverse phase hplc on Zorbax ODS (C-18) {mobile phase: pH 3.5 NH_4OAc buffer-MeCN-(iC_3H_7)NH (72:28:0.25); flow rate: 6.0 ml/min; load: 6 mg]. Buffer was prepared by dissolving NH_4OAc (7.7 g) in H_2O (1 liter, 0.1 M) and adjusting to pH 3.5 by adding HOAc. Two components having retention times of 12 and 20 min were obtained. The hplc fractions were adjusted to pH ca. 7 with NH_4OH, and the organic solvent was removed in vacuo. The aqueous fractions were basified to pH 10 and extracted with $CHCl_3$. After removal of the $CHCl_3$, the residues were chromatographed over Si gel (5 g) eluting with $CHCl_3$-MeOH-NH_4OH (98:2:5, lower layer). The Dragendorff-positive eluates were recrystallized from Me_2CO to yield, respectively, 2 (14 mg) and 4 (14 mg), yield, 0.0002% each.

Compound 2.—Mp 246-248° [lit. (2) 248-249°]; ms m/z 557.2980 ($C_{31}H_{43}NO_8$=557.2987) (100%); ^1H-nmr spectra as in Johns *et al.* (4).

Compound 4.—Mp 268-269°; ms m/z 579.2820 ($C_{23}H_{41}NO_8$=579.2829) (100%);

^1H-nmr spectra similar to that in Johns *et al.* (4); $[α]^{25}D$−9.5 (c=1.04, MeOH) [lit. −14° (2)].

11α-Destigloylanopterine [5] *and 4',7β-dihydroxyanopterine* [3].—Fraction F2 was further separated into two fractions (F4 and F5) by chromatography over Si gel (10 g) with EtOAc containing 0-40% MeOH and 60-100% MeOH, respectively. Fraction F4 was chromatographed twice by cptlc (EtOAc-MeOH-NH_4OH, 17:2:1) to yield fractions giving Dragendorff-positive spots at R_f 0.53 (F6) and 0.33 (F7) on tlc (solvent B). Fraction F6 was recrystallized from Me_2CO to yield 5 (22 mg) yield, 0.0002%; mp 184-187°; ms m/z 459.2621 ($C_{26}H_{37}NO_6$=459.2620) (100%); ^1H-nmr spectra similar to that in Johns *et al.* (4).

Fraction F7 resisted crystallization but yielded a white, powdery precipitate 3 (20 mg) yield, 0.0002% on addition of EtOAc to the condensed residue; mp 246-248° [lit. 242-244° (2)]; ms m/z 573.2721 ($C_{31}H_{43}NO_9$=573.2938) (100%), ^1H-nmr spectra as in Johns *et al.* (4); $[α]^{25}D$−6.5° (c=1.2, MeOH) [lit. 9° (2)].

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