Plant Antitumor Agents. 31.[1] The Calycopterones, a New Class of Biflavonoids with Novel Cytotoxicity in a Diverse Panel of Human Tumor Cell Lines

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Three new biflavonoids to which we have accorded the trivial names calycopterone (1), isocalycopterone (2), and 4-demethylcalycopterone (3) and the known flavone 4′,5-dihydroxy-3,3′,6,7-tetramethoxyflavone (4) were isolated as cytotoxic constituents from the flowers of *Calycopteris floribunda* Lamk. (Combretaceae). Compounds 1–3 showed a wide range of activity against a panel of solid tumor cell lines. Among the biflavonoids, calycopterone (1) is the major constituent.

Introduction

As part of our ongoing program for the discovery of cytotoxic/antitumor agents from plants, we have studied the flowers of *Calycopteris floribunda* Lamk. which were collected in Thailand in March 1992. It is a tree that grows to a height of about 10 m and is locally known as "ting tang." The plant also grows in central and southern parts of India, where the leaves are reputed to have medicinal uses as a laxative and an anthelmintic medicine, while the juice derived from the twigs is used for the treatment of malaria.[2,3] Previous chemical studies have reported on the isolation of the flavonoids calycopterin and quercetin from the leaves and flowers of *C. floribunda*.[2-4] In the present investigation, a chloroform-soluble extract of the flowers of this plant was found to exhibit significant cytotoxic activity against a panel of solid tumor cell lines. The crude extract was subjected to numerous silica gel chromatographies. Guided by KB cell line growth inhibition assays, active fractions were selected and further purified by preparative high-performance liquid chromatography (PHPLC). In this manner, three new biflavonoids, calycopterone (1), isocalycopterone (2), and 4-demethylcalycopterone (3), and the known flavonoid 4′,5-dihydroxy-3,3′,6,7-tetramethoxyflavone[5] (4) were obtained. In this communication, we wish to report the isolation, structure elucidation, and cytotoxic activities of these compounds with particular reference to the NCI human tumor, disease-oriented in vitro screen.[6-8]

Chemistry

A crude extract was obtained by extracting dried and ground *C. floribunda* flowers in a Soxhlet apparatus with 50% methanol/chloroform. After removal of the solvent, the residue was partitioned between chloroform and water. The chloroform-soluble residue was then partitioned between 90% methanol/water and hexane. The residue from the 90% methanol phase was then subjected to a series of cytotoxicity-guided silica gel column chromatographies, as well as normal and reversed-phase HPLC, to afford compounds 1–4.

The high-resolution EI-MS of calycopterone (1) indicated a molecular formula of $C_{35}H_{34}O_{10}$ (*m/z* 614.2156), which was confirmed by elemental analysis. In the IR spectrum, absorptions occurred at 3400 cm^{-1} (phenolic OH), 1660 cm^{-1} (α,β-unsaturated C=O), 1620 cm^{-1} (conjugated C=C), and 1595 and 1448 cm^{-1}(aromatic C=C). The EI-MS diagnostic fragments at *m/z* 582 ($M^+ - OCH_3 - H$), 551 ($M^+ - 2 \times OCH_3 - H$), 519 ($M^+ - 3 \times OCH_3 - 2H$), 478 ($M^+ - 4 \times OCH_3 - 2H$), and 419 ($M^+ - 5 \times OCH_3 - CO - 2H$) were consistent with the presence of five methoxyl groups and a carbonyl functionality. The ^{1}H-NMR spectrum revealed aromatic protons in the region δ 7.28–7.49, integrating for 10 protons. In the downfield region of the spectrum, the resonance at δ 8.23 was due to a phenolic hydroxyl group (7-OH), while a vinylic proton appeared at δ 7.13 (H-4″) (cf. Table 1). The connectivities between H-2″ (δ 5.18), H-3″ (δ 2.46 and 2.77), and H-4″ (δ 7.13) and between H-2 (δ 5.08), H-3 (δ 1.93 and 2.20), and H-4 (δ 4.40), respectively, were readily discernible as two disjoint spin systems from the ^{1}H–^{1}H COSY spectrum. Signals at δ 3.26, 3.41, 3.61, 3.67, and 4.00 in the ^{1}H-NMR spectrum and δ 53.1, 55.2, 60.2, 60.4, and 61.3 in the ^{13}C-NMR spectrum indicated the presence of five methoxyl groups. The ^{13}C-NMR assignments were facilitated by DEPT, HMQC, and HMBC NMR experiments (Table 2).

A single-crystal X-ray analysis established the complete structure and relative stereochemistry of calycopterone (1). The asymmetric unit consists of two crystallographically independent molecules. A view of the solid-state conformation of one of the molecules (molecule I) is presented in Figure 1; the conformation of molecule II is overall quite similar, but its methoxy methyl group C-12 is disordered with a 2:1 occupancy over two positions (torsion angles: C-7–C-8–O-8–C-12 = –112.8(3)° and C-9–C-8–O-8–C-12 = 72.7(3)° for molecule I; pairs of angles associated with the two conformations in molecule II are –121.8(3)°, 59.0(4)° for C-12 and 124.0(5)°, –55.3(6)° for

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Table 1. ^{1}H-NMR Spectral Data of Compounds 1–3 in DMSO-d_6[a] (Chemical shifts are in ppm with TMS as internal standard)

	δ (ppm)[b]		
proton no.	1	2	3
7-OH	8.29 (1H,s)		8.13 (1H,s)
Ar-H	7.28–7.49 (10H)	7.31–7.46 (10H)	7.28–7.44 (10H)
4″	7.13 (1H,m)	7.16 (1H,m)	7.13 (1H,m)
2″	5.18 (1H,d,11.9)	5.14 (1H,d,11.4)	5.14 (1H,d,10.8)
2	5.08 (1H,d,12.1)	5.04 (1H,d,12.3)	5.28 (1H,dd,11.2,3.9)
4	4.40 (1H,d,br t)	4.46 (1H, br t)	4.67 (1H,m)
7″-OMe	4.0 (3H,s)	4.07 (3H,s)	3.99 (3H,s)
6″-OMe	3.67 (3H,s)	3.69 (3H,s)	3.71 (3H,s)
7-OMe		3.58 (3H,s)	
8-OMe	3.61 (3H,s)		3.59 (3H,s)
8″-OMe	3.41 (3H,s)	3.37 (3H,s)	3.38 (3H,s)
4-OMe	3.26 (3H,s)	3.33 (3H,s)	
3″β	2.77 (1H,d,20.3)	2.78 (1H,d,19.4)	2.74 (1H,dt,21.0,4.3)
3″α	2.46 (1H,dd,20,11.1)	2.55 (1H,dd,19.4,11.6)	2.50 (1H,qd,28,3)
3β	2.20 (1H,d,14.3)	2.24 (1H,d,14.2)	1.93 1H, overlapping signal)
3α	1.93 (1H,t,11.4)	1.86 (1H,t,10.2)	1.93 (1H, overlapping signal)

[a] Recorded on a Bruker AMX-500 spectrometer (500 MHz). [b] Number of protons, multiplicity, and J (Hz) in parentheses.

Table 2. ^{13}C-NMR Spectral Data of 1–3 (Taken in DMSO-d_6 with TMS as internal standard)[a]

	δ (ppm)		
carbon	1	2	3
2	73.3	73.1	72.9
3	34.0	33.1	38.6
4	66.6	67.0	56.9
5	151.7	149.9	151.6
6	104.2	105.4	104.3
7	148.9	148.3	148.2
8	130.7	128.0	130.5
9	149.8	148.6	149.7
10	98.5	105.0	101.6
1′	140.6	140.7	140.6
2′	126.2	126.1	126.2
3′	128.3	128.4	128.4
4′	127.9	128.0	127.9
5′	128.3	128.7	128.4
6′	126.2	126.1	126.2
2″	70.7	70.6	70.6
3″	32.2	31.7	32.2
4″	138.4	138.8	138.2
5″	180.9	180.8	181.0
6″	131.2	131.1	131.4
7″	158.9	158.2	158.6
8″	87.1	86.1	87.1
9″	103.1	103.3	103.2
10″	139.2	140.1	139.1
1‴	140.8	140.7	141.1
2‴	126.4	126.4	126.2
3‴	128.3	128.4	128.5
4‴	128.0	128.0	128.5
5‴	128.3	128.4	128.5
6‴	126.4	126.4	126.2
4-OMe	55.2	55.9	
7-OMe		61.6	
8-OMe	61.3		61.2
6″-OMe	60.2	60.5	60.2
7″-OMe	60.4	60.5	60.4
8″-OMe	53.1	52.7	53.1

[a] Recorded on a Bruker AMX-500 spectrometer at 125 MHz.

C-12B). Corresponding bond lengths in each of the molecules agree well, and they are in accord with expectations.[9] Ring A-1 is quite flat, as expected, but several of its directly bonded atoms deviate significantly from the least-squares plane through the ring atoms (displacements (Å) for molecule I, with corresponding values for molecule II in parentheses, follow: O-1 −0.062 (0.006), C-4 −0.168 (−0.173), O-5 −0.013 (−0.014), C-8″ 0.074 (0.166), O-7 −0.062 (−0.098), and O-8 −0.098 (0.008)). The conformation of enone ring A-2 (endocyclic torsion angles ($\sigma \pm 0.2$–$0.5°$): $\varpi_{5'',6''}$ −10.0°, $\varpi_{6'',7''}$ 12.6°, $\varpi_{7'',8''}$ 9.0°, $\varpi_{8'',9''}$ −32.4°, $\varpi_{9'',10''}$ 36.3°, and $\varpi_{10'',5''}$ −14.9° in molecule I; corresponding values in molecule II are −7.1°, 13.1°, 5.0°, −28.1°, 35.0°, and −17.4°) is best described as being intermediate between a boat and a half-boat (envelope) form with C-9″ as the out-of-plane atom. Endocyclic torsion angles characterizing the conformations of rings C-1 and C-2 in both molecules are related by approximate C_2 symmetry axes passing through the midpoints of the C-9–C-10 and C-2–C-3 bonds in the former and through the C-4″–C-10″ and C-1″–O-1″ bonds in the latter; both rings are in half-chair forms with their phenyl ring substituent pseudoequatorially oriented. The central five-membered ring has an envelope form in both molecules with C-9″ as the out-of-plane atom. In crystals of 1, molecules related by unit translation along the c direction are linked by O-H···O hydrogen bonds (O-7···O-5″ = 2.807(3) and 2.780(3) Å for molecules I and II, respectively).

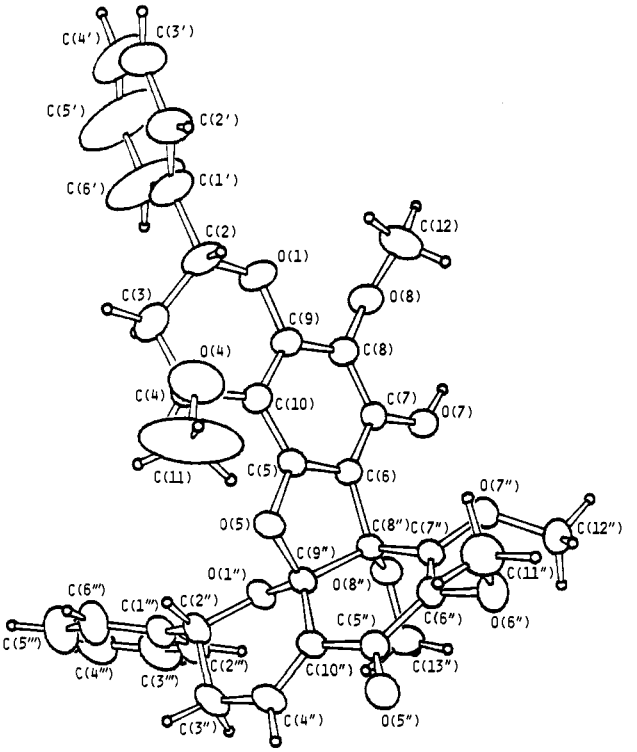


Figure 1. ORTEP diagram (40% probability ellipsoids) showing the crystallographic atom-numbering scheme and solid-state conformation of molecule I. Small circles represent hydrogen atoms.

The positive FAB mass spectrum of isocalycopterone (2) showed a protonated molecular ion at m/z 615 $(M + H)^+$, which clearly suggested that this compound is an isomer of 1. The ^{1}H- and ^{13}C-NMR spectra of 2 are closely comparable with those of 1 (Tables 1 and 2). However, the ^{13}C-NMR spectrum of 2 showed a more downfield resonance at δ 105.0 (C-10) as compared to δ 98.5 for the same carbon in 1. This observation can be accounted for only if the phenolic hydroxyl at C-7 and the methoxyl substituent at C-8 in 1 are interchanged as in 2. The anomalous large combined deshielding effect (6.5 ppm) of the 7-methoxy and 8-phenolic substituents on C-10 might be due to steric effects in ring A-1, as has been observed before for other substituents in ring A of flavonoids.[10-12] The rest of the ^{1}H- and ^{13}C-NMR assignments of 2 were made by comparison with the data obtained for 1 and ^{1}H–^{1}H COSY, DEPT, HMQC, and HMBC experiments.

Compound 3 was isolated as an amorphous off-white powder. The positive high-resolution FAB mass spectrum showed a protonated molecular ion at m/z 601.2074 $(M + H)^+$, which analyzed for $C_{34}H_{32}O_{10}$. The fragment at m/z 583 indicated the loss of H_2O. Comparing the ^{1}H- and ^{13}C-NMR spectral data of 3 with those of 1 and 2 indicated that the former has one methoxy group less than 1 and 2 (Tables 1 and 2). These methoxy signals occurred at δ 3.38, 3.59, 3.71, and 3.99 in the ^{1}H-NMR spectrum and at δ 53.1, 60.2, 60.4, and 61.2 in the ^{13}C-NMR spectrum, and their respective assignments were made by comparison with data obtained for 1 and 2. The downfield chemical shifts of H-2 (δ 5.28) and H-4 (δ 4.67) and the more upfield chemical shifts of H-3β (δ 1.93) in the ^{1}H-NMR spectrum of 3 as compared to δ 5.08, 4.40, and 2.20 of the corresponding protons in 1 indicated that a hydroxy group was affixed at C-4. This was supported by ^{13}C-NMR data of 3 which showed signals at δ 56.9 (C-4) and 38.6 (C-3). Thus, the flavonoid 3 was formulated as a 4-demethyl analog of 1.

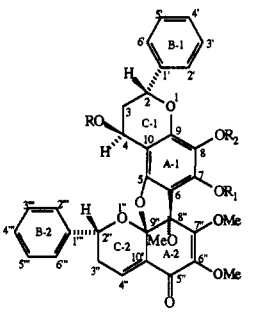
Compound 4 was obtained as crystalline needles from methanol and identified as the known 4′,5-dihydroxy-3,3′,6,7-tetramethoxyflavone by comparison of its physical and spectral parameters (mp, UV, IR, MS, and ^{1}H- and ^{13}C-NMR) with those published in the literature.[5] The structures of compounds 1–4 are shown in Chart 1.

The calycopterones 1–3 have a unique biflavonoid structure completely different from any biflavonoids noted in previous literature.[11-18] Notable features are the linkages of ring A-1, which may be considered the dihydroflavonol moiety of the calycopterones, to ring A-2 by a pyran ring (cf. Chart 1). The A-2,C-2,B-2 moiety has a structure different from any known flavonoid. In particular, ring A-2 is nonaromatic with a keto group conjugated to both exo and endo double bonds. The A-1,B-1,C-1 and A-2,B-2,C-2 components of the calycopterones are also unrelated to any of the flavonoids previously found in *C. floribunda*.[2-4]

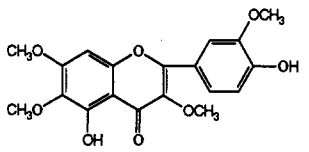
Biology

The calycopterones 1–3 and the flavone 4 were initially screened for in vitro cytotoxicity in a number of cancer cell lines.[19] Compounds 1–3 displayed broad cytotoxic activity of about the same order; with ED_{50} values ranging from 10^{-1} to 10^{0} μg/mL (Table 3). The flavone 4 was inactive in most of the cell lines. Compounds 1–4 were evaluated in the Frederick–NCI human tumor, disease-oriented in vitro screen.[6-8] Calycopterone (1) displayed

Chart 1. Structures of Compounds 1–4



	R	R_1	R_2
Calycopterone (1)	Me	H	Me
Isocalycopterone (2)	Me	Me	H
4-Demethylcalycopterone (3)	H	H	Me



4′,5-Dihydroxy-3,3′,6,7-tetramethoxyflavone (4)

specific sensitivity in the leukemia cell line panels with $\log_{10}$ GI_{50} and TGI values 1–2 orders of magnitude more sensitive than those of most of the other cell lines (cf. Experimental Section). The response parameters GI_{50}, TGI, and LC_{50} are interpolated values at which PG (percent growth) is 50, 0, and −50, respectively.[8] Data for some of the cell lines in which 1 was tested are presented in the Experimental Section. Several measures of subpanel selectivity were noted for 1. D_{GI50}, D_{TGI}, and D_{LC50} are measures of subpanel selectivity; values of $\geq$50 are considered statistically significant.[8] For 1, D_{GI50} = 83.0, D_{TGI} = 81.0, and D_{LC50} = 40.0. D_H values provide a more general measure of selective effect; values of $D_H \geq 75$ are statistically significant. For 1, the value of D_H = 100. Specific sensitivity for 1 was not displayed by the other cell lines (cf. Experimental Section). Calycopterone (1) has been selected for in vivo testing by Frederick–NCI in cell lines in which it has the greatest growth inhibition.[20] 4-Demethylcalycopterone (3), which differs from 1 only at the 4-position of ring C-1, displayed activity similar to that of 1 in regard to selective inhibition of leukemia cell lines at the GI_{50} and TGI response parameters. D_{GI50}, D_{TGI}, and D_{LC50} values for 3 were 83.0, 58.0, and 38.0, respectively, and D_H = 100. In contrast to 1 and 3, isocalycopterone (2) was not sensitive toward the leukemia cell line panel at the GI_{50} and TGI parameters and was inactive at the LC_{50} parameter. It should be noted that 2 differs from 1 and 3 at the 7,8-positions of ring A-1. Compounds 1 and 3 have 7-hydroxy, 8-methoxy substituents, whereas, in the case of 2, the substituents in ring A-1 are 7-methoxy, 8-hydroxy. Further studies are underway in regard to SAR of compounds 1–3.

COMPARE is a program[8] by means of which data for calycopterone (1) was compared with the various levels of growth inhibition, zero growth, or cell killing noted for approximately 150 standard antitumor agents. Poor correlations were found for 1 and the standard antitumor agents, thus indicating that the mechanism of action probably does not correspond to any of the standard agents.

Table 3. In Vitro Cytotoxic Activity of Compounds 1–4

compound[b]	cell lines[a]								
	BC1	HT	Lu1	Col2	KB	KB-V1	P388	A431	U373
calycopterone (1)	5.4	1.8	1.2	0.4	0.8	9.6	0.78	1.0	0.1
isocalycopterone (2)	0.4	0.4	0.7	0.3	0.4		0.2	0.4	0.8
4-demethylcalycopterone (3)	1.4	0.8	1.6	0.3	1.2	8.6	0.42	0.2	0.3
4′,5-dihydroxy-3,3′,6,7-tetramethoxyflavone (4)	>20	4.2	>20	>20	>20	0.8	>20	>20	>20

[a] Key: BC1, breast cancer; HT, fibrosarcoma; Lu1, lung cancer; Col2, colon cancer; KB, oral epidermoid carcinoma; KB-V1, vinblastine-resistant KB; P-388, murine lymphoid neoplasm; A431, epidermoid carcinoma; U373, glioblastoma. [b] Results are expressed as ED_{50} values (μg/mL).

Table 4. Comparison of Cytotoxicity of Calycopterone (1) and Flavone 4 with Doxorubicin

compound	in vitro cytotoxicity—IC_{50} (μM)		
	SKOV3[a]	SKVLB[b]	HT29[c]
flavone 4	79	>133	14.2
calycopterone (1)	0.125		0.24
	1.0	1.90	0.93
doxorubicin	0.043	>10	0.18

[a] SKOV3 (ovarian adenocarcinoma)—provided by Dr. V. Ling, Ontario Cancer Institute. [b] SKVLB (ovarian adenocarcinoma, multidrug resistant)—Dr. V. Ling, Ontario Cancer Institute. [c] HT29 (colon adenocarcinoma)—ATCC catalog #HTB 38.

Therefore, the mechanism may be either a new mechanism for an anticancer drug or a cell toxicity mechanism with no relation to cancer. Inspection of the structure of 1 suggests that the double bond at the 4″-position may be required for activity, and this is currently under investigation.

Table 4 compares the activity of compounds 1 and 4 in several in vitro cytotoxicity tests with the known clinical antitumor drug doxorubicin. The cell lines tested were SKOV3, ovarian adenocarcinoma; SKVLB, multidrug-resistant ovarian adenocarcinoma; and HT29, colon adenocarcinoma. As shown in Table 4, 1 was much more potent than 4 in all three cell lines and was less potent than doxorubicin in SKOV3 and HT29 but considerably more potent than doxorubicin in SKVLB.

Only one in vivo study has been conducted with 1 thus far. This study was conducted with mice in which 1 was administered ip at levels of 10–40 mg/kg. The tumor was human epidermoid carcinoma (KB) implanted sc. Calycopterone (1) was inactive at nontoxic doses of 10–30 mg/kg and toxic at 40 mg/kg. Under the same conditions, doxorubicin inhibited tumor growth at a dose of 10 mg/kg. Other in vivo studies of 1 with additional cancer cell lines are planned.

Experimental Section

General Procedures. Melting points were determined on a Kofler hot-stage microscope and are uncorrected. UV spectra were obtained with a Varian 2290 UV–vis spectrometer and IR spectra with a Perkin-Elmer 467 grating spectrometer. ^1H- and ^{13}C-NMR spectra were recorded with a Bruker AMX 500 spectrometer using TMS as an internal standard. Standard chromatography was carried out on a Si gel (E. Merck), 100–300 mesh, or a Baker flash chromatography Si gel. For TLC determinations, precoated Si gel plates (E. Merck Si gel 60, F254) were used. The plates were visualized under UV after development followed by spraying with phosphomolybdate reagent (5% phosphomolybidic acid in EtOH) and heating the plate at 110 °C for about 10 min in an oven. Preparative HPLC (PHPLC) was performed with a Waters Model Prep-3000 instrument equipped with a Lambda Model 481 LC spectrophotometer and a Waters 740 data module. A Dynamax reversed-phase C_{18} column (2.15- × 25-cm) and a normal phase Si column (2.15- × 25-cm) were used for reversed phase and normal phase, respectively.

Plant Material. The flowers of *C. floribunda* were collected in Thailand in March 1992. A voucher specimen representing this collection has been deposited at the John G. Searle Herbarium of the Field Museum of Natural History, Chicago, IL.

Extraction and Isolation. Dried and ground flowers of *C. floribunda* (1.3 kg) were extracted with 50% MeOH/$CHCl_3$ (2 × 1 L) in a Soxhlet apparatus. Extracts were concentrated in vacuo at 40 °C and partitioned between chloroform and water. The chloroform-soluble portion was concentrated in vacuo and the residue partitioned between 90% MeOH/H_2O–hexane. The weight of the 90% methanol fraction was 25.9 g.

This fraction was taken up in chloroform and chromatographed over Si gel (1.3 kg) using a chloroform–methanol gradient. Cytotoxic fractions were eluted with 1–2% MeOH/$CHCl_3$ (6.6 g). These fractions were combined and further chromatographed over Si gel (0.25 kg), using a hexane–chloroform, chloroform, and methanol–chloroform gradient with cytotoxic fractions being eluted with 10% hexane–chloroform (A, 0.78 g), chloroform (B, 2.6 g, and C, 1.6 g), and with 2% methanol–chloroform (D, 0.23 g).

Calycopterone (1) (250 mg) was obtained by crystallization from a methanol solution of fraction B. A further quantity (165 mg) was obtained by PHPLC. The sample in methanol solution was subjected to reversed-phase PHPLC, C_{18} Dynamax column, eluted isocratically with 75% MeOH–25% H_2O, UV detector 220 nm, t_R = 16 min. The semipurified product was subjected to normal-phase PHPLC, silica column, with a gradient of 8–25% ethanol–isooctane. Pure 1 was obtained; t_R = 20 min. The total yield of calycopterone was 0.415 g (0.032%, w/w) mp 222–223 °C; $[\alpha]_D$ −274° (c 0.3, $CHCl_3$); UV (MeOH) λ_{max} 289 nm (log ε 4.09); IR (KBr) ν_{max} 3485, 3400, 2915, 1660, 1620, 1595, 1448, 1317, 1157 cm^{-1}; EI-MS (70 eV) *m/z* found M^+ 614.2156, calcd for $C_{35}H_{34}O_{10}$ 614.2152, percent rel. intensity M^+ 614 (100), 582 (9), 582 (9), 551 (19), 519 (10), 478 (4), 450 (9), 419 (4), 351 (4), 342 (7), 267 (4), 155 (5), 121 (14); for ^1H and ^{13}C NMR, see Tables 1 and 2. Anal. ($C_{39}H_{34}O_{10}$) C, H.

Isocalycopterone (2) (43 mg) was obtained by normal-phase PHPLC followed by reversed-phase PHPLC of fraction A, using a gradient of 1–5% MeOH/$CHCl_3$ over 40 min and isocratic 75% MeOH/H_2O, respectively. Compound 2 was obtained as an amorphous white powder; $[\alpha]_D$ −274° (c 0.3, $CHCl_3$); UV (MeOH) λ_{max} 277 nm (log ε 4.63); IR (KBr) ν_{max} 3480, 2910, 1661, 1631, 1591, 1444, 1420, 1317, 1096, 1030, 933 cm^{-1}; FAB^+ *m/z* 615 $(M + H)^+$; for ^1H and ^{13}C NMR, cf. Tables 1 and 2.

4-Demethylcalycopterone (3) (56 mg) was obtained by repeated PHPLC of fraction C, using a 5–50% isooctane/EtOH gradient over 45 min. Pure 3 (0.043 g) eluted in 20–25 min as an off-white amorphous powder: $[\alpha]_D$ −327° (c 0.3, $CHCl_3$); UV (MeOH) λ_{max} 288 nm (log ε 4.78); IR (KBr) ν_{max} 3480, 2815, 1660, 1628, 1601, 1492, 1446, 1315, 1156, 1034, 918 cm^{-1}; FAB^+ *m/z* found 601.2049, calcd for M + H, $C_{34}H_{33}O_{10}$, 601.2073 (δ −4.1 ppm); for ^1H and ^{13}C NMR, cf. Tables 1 and 2.

4′,5-Dihydroxy-3,3′,6,7-tetramethoxyflavone (4) (330 mg) was obtained by purification of fraction D by chromatography over silica gel. The fraction containing the flavonoid was eluted with 1% MeOH/$CHCl_3$ and subsequently crystallized from methanol: mp 180–181 °C; UV (MeOH) λ_{max} 350 (log ε 4.64), 270 (log ε 3.53), 256 nm (log ε 3.89). The mp, UV, IR, MS, and NMR data for 4 were identical with data presented in the original literature.[5]

X-Ray Crystal Structure Analysis of Calycopterone (1).[21] Crystal data: $C_{35}H_{34}O_{10}$, MW = 614.66, triclinic, space group $P1(C_1^1)$; a = 10.996(1), b = 15.580(2), c = 9.822(1) Å; α = 106.57(1)°, β = 95.35(1)°, γ = 93.04(1)°; V = 1595.8(7) Å^3; Z =

2; D_c = 1.279 g cm^{-3}; μ(Cu Kα radiation, λ = 1.5418 Å) = 7.4 cm^{-1}. Crystal dimensions: 0.12 × 0.24 × 0.54 mm^3. Intensity data ($+h$, $\pm k$, $\pm l$; θ_{max} = 75°; 6575 nonequivalent reflections) were recorded on an Enraf-Nonius CAD-4 diffractometer (Cu Kα radiation; incident-beam graphite monochromator; $\omega - 2\theta$ scans; scanwidth (0.90 + 0.14 tan θ)°). The space group was determined from the Laue symmetry and the fact that 1 is chiral. Refined unit-cell parameters were derived from the diffractometer setting angles for 25 reflections (36° < θ < 40°) widely separated in reciprocal space. The intensities of four reference reflections, monitored every 2 h during data collection, showed no significant variation (<0.5% overall). In addition to the usual Lorentz and polarization corrections, an empirical absorption correction (T_{max}: T_{min} = 1.00:0.92) was applied to the data, and 5840 reflections with $I > 3.0\sigma(I)$ were retained for the analysis.

The crystal structure was solved by direct methods (RANTAN). Approximate coordinates for 40 non-hydrogen atoms were derived from an E map. The remaining carbon and oxygen atoms were located in a series of weighted F_o Fourier syntheses phased successively by an increasing number of atoms. One of the methoxy methyl groups was found to be disordered over two orientations (2:1). Following several rounds of full-matrix least-squares adjustment of positional and thermal parameters of all non-hydrogen atoms (at first isotropic and then anisotropic), a difference Fourier synthesis yielded hydrogen-atom positions. In the next series of least-squares iterations, hydrogen atoms were incorporated at their calculated positions and latterly an extinction correction (g) was introduced as a variable. The parameter refinement converged (maximum shift: esd = 0.02) at R = 0.039 (R_w = 0.056; GOF = 1.37; g = 2.3(3) × 10^{-6}) ($R = \sum||F_o|-|F_c||/\sum|F_o|$; $R_w = [\sum w(|F_o|-|F_c|)^2/\sum w|F_o|^2]^{1/2}$; GOF = $[\sum w(|F_o| - |F_c|)^2/(N_{observations} - N_{parameters})]^{1/2}$; $\sum w(|F_o| - |F_c|)^2$ [$w = 1/\sigma^2(|F_o|)$] was minimized). A final difference Fourier synthesis contained no unusual features ($\Delta\rho$ (e Å^{-3}) = 0.24 (max) and −0.17 (min)).

Crystallographic calculations were performed on PDP11/44 and MicroVAX computers by use of the Enraf-Nonius Structure Determination Package (SDP). For structure-factor calculations, neutral atom scattering factors and their anomalous dispersion corrections were taken from Ibers and Hamilton.[22]

Biology. The biflavonoids 1–3 and the flavonoid 4 were screened for cytotoxicity in a number of tumor cell lines using previously described procedures.[19] Values of >20 μg/mL were regarded as negative. As shown in Table 3, the biflavonoids 1–3 displayed broad cytotoxicity in a number of cell lines. The flavonoid 4 was generally inactive. Calycopterone (1) was tested in vivo in mice against the tumor human epidermoid carcinoma (KB) implanted sc. The compound was administered ip on days 1, 5, and 9 in 10% DMSO and 1% Tween at doses of 10–40 mg/kg. Doxorubicin at 10 mg/kg was the positive control. A vehicle control was also included. Groups of six to eight animals were used in each study. The percent tumor volume and body weight were noted at days 0, 5, 7, 9, and 12. At doses of 10–30 mg/kg, 1 was inactive but was toxic at 40 mg/kg.

Biological Testing Protocol: Data Display and Analysis and NCI Human Tumor, Disease-Oriented in Vitro Screen.[6] Compounds 1–4 were tested in the NCI in vitro screen. Data calculations and display were performed as described elsewhere.[6-8,23]

Screening Data Summary. The $-\log_{10}$ GI_{50}, TGI, and LC_{50} values, respectively, for a number of panel/cell lines in which 1 was tested were obtained from the standard NCI screening data report[23] and are listed below in sequence following the individual cell line names (cf. ref 24 for an example of this method for reporting data).

Panel: Leukemia: CEM: 7.76, 7.30, 5.69; HL-60: 7.00, 6.53, 6.06; K562: 7.17, 6.48, 74.00; MOLT: 7.40, 6.71, 5.16; RPMI: 7.08, 6.47, 5.39; SR: 7.50, 6.88.

Colon Cancer: COLO-209: 6.52, 5.93, 5.24; HCC-2998: 6.04, 5.60, 5.19; HCT-116: 6.73, 6.03; HCT-15: 6.27, 6.42, 6.07; HT-29: 6.81, 6.50, 6.20; KM12: 5.80, 5.50, 5.19; SW630: 6.56, 5.70, >4.00.

Melanoma: M14: 6.40, 5.74, >4.00; SKMEL-28: 6.74, 6.21, 5.55; SKMEL-5: 6.41, 5.86, 5.28; UACC-257: 6.30, 5.75, 5.34.

Renal Cancer: 786-O: 6.48, 6.00, 5.37; ACHN: 6.25, 6.00, 5.37; SW12C: 6.61, 6.22, 5.65; TK10: 6.37, 5.90, 5.43; UO-31: 6.02, 5.67, 5.34.

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Supplementary Material Available: Tables of fractional atomic coordinates, temperature factor parameters, bond lengths, bond angles, and torsion angles for calycopterone (1) (20 pages); a table of observed and calculated structure factors (39 pages). Ordering information is given on any current masthead page.

References

(1) For preceding paper in this series, see: Wall, M. E.; Wani, M. C.; Nicholas, A. W.; Manikumar, G.; Moore, L.; Truesdale, A.; Leitner, P.; Besterman, J. M. Plant Antitumor Agents. 30. Synthesis and Structure Activity of Novel Camptothecin Analogs. *J. Med. Chem.* **1993**, *36*, 2689–2700.

(2) Chopra, R. N. *Indigenous Drugs of India*; V. N. Dhur and Sons: Calcutta, India, 1958; pp 308, 527, 677, 688.

(3) Casim, S. M.; Neelakantan, S.; Raman, P. V. Chemical Components of Indian Medicinal Plants (*Thespesia populnea* wood and *Calycopteris floribunda* flowers). *Curr. Sci.* **1975**, *44*, 888–889.

(4) Rodriguez, E.; van der Velde, G.; Mabry, T. J.; Subramanian, S. S.; Nair, A. G. R. The Structure of Calycopterin. *Phytochem.* **1972**, *11*, 2311–2312.

(5) Rahman, Atta ur; Ahmed, D.; Choudhary, M. I.; Turkoz, S.; Sener, B. Chemical Constituents of *Buxus sempervirens*. *Planta Med.* **1987**, 173–174.

(6) Boyd, M. R. Status of the NCI Preclinical Antitumor Drug Discovery Screen: Implications for Selection of New Agents for Clinical Trial. In *Cancer: Principles and Practice of Oncology, Update Series*; DeVita, V. T., Jr., Hellman, S., Rosenberg, S. A., Eds.; Lippincott: Philadelphia, 1989; Vol. 3, No. 10, pp 1–12.

(7) Monks, A.; Scudiero, D.; Skehan, P.; Shoemaker, R.; Paull, K.; Vistica, D.; Hose, C.; Langley, J.; Cronise, P.; Vaigro-Wolff, A.; Gray-Goodrich, M.; Campbell, H.; Boyd, M. Feasibility of a High-Flux Anticancer Drug Screen Utilizing a Diverse Panel of Human Tumor Cell Lines in Culture. *J. Natl. Cancer Inst.* **1991**, *83*, 757–766.

(8) Paull, K. D.; Shoemaker, R. H.; Hodes, L.; Monks, A.; Scudiero, D. A.; Rubinstein, L.; Plowman, J.; Boyd, M. R. Display and Analysis of Patterns of Differential Activity of Drugs Against Human Tumor Cell Lines: Development of the Mean Graph and COMPARE Algorithm. *J. Natl. Cancer Inst.* **1989**, *81*, 1088–1092.

(9) Allen, F. H.; Kennard, O.; Watson, D. G.; Bremmer, L.; Orpen, A. G.; Taylor, R. Tables of Bond Lengths Determined by X-ray and Neutron Diffraction. Part 1. Bond Lengths in Organic Compounds. *J. Chem. Soc., Perkin Trans. II* **1987**, S1–S19.

(10) Markham, K. R.; Chari, V. M.; Mabry, T. J. Carbon-13 NMR Spectroscopy of Flavonoids. In *The Flavonoids: Advances in Research*; Harborne, J. B., Mabry, T. J., Eds.; Chapman and Hall: London, 1975; pp 19–134.

(11) Cotterill, P. J.; Scheinmann, F.; Stenhouse, I. A. Extractives from Guttiferae, Part 34. Kolaflavanone, A New Bioflavonone from the Nuts of *Garcinia kola*. Applications of ^{13}C NMR in Elucidation of the Structures of Flavonoids. *J. Chem. Soc., Perkin Trans. I* **1978**, 532.

(12) Chari, V. M.; Ilyas, M.; Wagner, H.; Neszmelyi, A.; Chen, F.; Chen, L.; Lin, Y.-C.; Lin, Y.-M. ^{13}C NMR Spectroscopy of Biflavonoids. *Phytochem.* **1977**, *16*, 1273–1278.

(13) Geiger, H.; Quinn, C. In *Biflavonoids*; Harborne, J. B., Mabry, T. J., Mabry, H., Eds.; Academic Press: New York, 1975; pp 692–742.

(14) El Sohly, M. A.; Craig, J. C.; Waller, C. W.; Turner, C. E. Biflavonoids from Fruits of Poison Ivy *Toxicodendron* radicans. *Phytochem.* **1978**, *17*, 2140–2141.

(15) Duddeck, H.; Snatzke, G.; Yemul, S. S. ^{13}C-NMR and CD of Some 3,8″-Biflavonoids from Garcinia species and of Related Flavanones. *Phytochem.* **1978**, *17*, 1369–1373.

(16) Crichton, E. G.; Waterman, P. G. Manniflavanone: A New 3,8-Linked Flavanone Dimer from the Stern Bark of *Garcinia mannii*. *Phytochem.* **1979**, *18*, 1553–1557.

(17) Hikino, H.; Takahashi, M.; Konno, C. Structure of Ephedrannin: A Hypotensive Principle of Ephedra Roots. *Tetrahedron Lett.* **1982**, *23*, 673–676.

(18) Hikino, H.; Shimoyama, S.; Kasahara, Y.; Takahashi, M. Structures of Mahuannin A and B. Hypotensive Principles of Ephedra Roots. *Heterocycles* **1982**, *19*, 1381–1384.

(19) Likhitwitayawuid, K.; Angerhofer, C. K.; Cordell, G. A.; Pezzuto, J. M.; Ruangrungsi, N. Cytotoxic and Antimalarial Bisbenzyliso-

quinoline Alkaloids from *Stephania erecta*. *J. Nat. Prod.* **1993**, *56*, 30–38.
(20) Private communication from Dr. Anthony Mauger, NCI.
(21) Atomic coordinates for calycopterone (1) have been deposited at the Cambridge Crystallographic Data Centre and can be obtained on request from Dr. Olga Kennard, University Chemical Laboratory, 12 Union Rd., Cambridge CB2 1EW, U.K.
(22) Ibers, J. A., Hamilton, W. C., Eds. *International Tables for X-Ray Crystallography*; The Kynoch Press: Birmingham, U.K., 1974; Vol. IV.
(23) Boyd, M. R.; Paull, K. D.; Rubenstein, L. R. Data Display and Analysis Strategies for the NCI Disease-Oriented In Vitro Antitumor Drug Screen. In *Antitumor Drug Discovery and Development*; Valeriote, F. A., Corbett, T., Baker, L., Eds.; Kluwer Academic Publishers: Amsterdam, 1992; pp 11–34.
(24) Fuller, R. W.; Cardellina, J. H., II; Kato, Y.; Brinen, L. S.; Clardy, J.; Snader, K. M.; Boyd, M. R. A Pentahalogenated Monoterpene from the Red Algae *Portieria hornemamnii* Produces a Novel Cytotoxicity Profile Against a Diverse Panel of Human Tumor Cell Lines. *J. Med. Chem.* **1992**, *35*, 3007–3011.