Bioorganic & Medicinal Chemistry 19 (2011) 186–196

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis of antiproliferative flavones from calycopterin, major flavonoid of *Calycopteris floribunda* Lamk.

Guy Lewin [a,*], Narayan Bhat Shridhar [b], Geneviève Aubert [c], Sylviane Thoret [c], Joëlle Dubois [c], Thierry Cresteil [c]

[a] Laboratoire de Pharmacognosie, (Univ. Paris-Sud 11, BIOCIS, UMR-8076 CNRS), Faculté de Pharmacie, Av. J.B. Clément, 92296 Châtenay-Malabry Cedex, France
[b] Department of Pharmacology and Toxicology, KVAFSU Veterinary College, Hebbal, Bangalore 560 024, India
[c] ICSN, CNRS, UPR 2301, 91190 Gif-sur-Yvette, France

ARTICLE INFO

Article history:
Received 28 September 2010
Revised 13 November 2010
Accepted 16 November 2010
Available online 20 November 2010

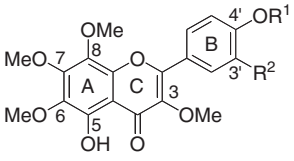
Keywords:
Calycopterin
Calycopteris floribunda
Flavones
Antiproliferative activity
Antimitotic
Tubulin
Combretastatins

ABSTRACT

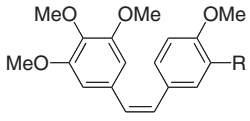
Eighteen new analogues of 5,3′-dihydroxy-3,6,7,8,4′-pentamethoxy-flavone, a potent natural cytotoxic and antimitotic flavone, were synthesized from calycopterin, the major flavonoid of *Calycopteris floribunda* Lamk., a traditional Asian medicinal plant. One of them, the 3′-amino substituted analogue, displayed almost the same activity as the reference compound. Pharmacomodulation at C-3′ on the B-ring, and at C-5,6,7 and 8 on the A-ring allowed to refine structure–activity relationships within the cytotoxic flavones series.

1. Introduction

In 1998, Beutler et al. reported results of a comparative cytotoxicity screening and subsequent tubulin polymerization studies carried out with a series of 79 natural and synthetic flavones.[1] Most of the studied flavones exhibited only hydrogen, hydroxy and methoxy substituents. Maximum potencies for cytotoxicity and tubulin interaction were found only with compounds bearing an OH group at C-5 on the A-ring, 3′-hydroxy-4′-methoxy groups on the B-ring and an OCH_3 at C-3 on the C-ring. The best activity was found with 5,3′-dihydroxy-3,6,7,8,4′-pentamethoxy-flavone **1**, a natural flavone first isolated by Mabry et al. in 1986 from *Gutierrezia microcephala*.[2] In 1994 then 1995, the Sévenet[3] then Lee[4] groups reported, respectively the strong cytotoxic and IPT (Inhibition of Polymerization of Tubulin) properties of **1**, which remains the most antimitotic natural flavone isolated to date. Though the substitution pattern of the A-ring was apparently not critical for activity (except hydroxylation at C-5), a trimethoxylation at C-6, C-7 and C-8 appeared the most favourable, as it has been recently confirmed in the laboratory.[5]



R^1	R^2	
Me	OH	**1**
H	H	**4**
Me	NH_2	**5**



R = OH	**2**
R = NH_2	**3**

We noticed also that the substituents of **1** at C-6, C-7, C-8 on the A-ring and C-3′, C-4′ on the B-ring were the same as in

* Corresponding author. Tel.: +33 146835593; fax: +33 146835399.
E-mail address: guy.lewin@u-psud.fr (G. Lewin).

0968-0896/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2010.11.035

combretastatin A4 **2**, a powerful inhibitor of tubulin assembly now under clinical investigation as its phosphate prodrug. SARs within the combretastatin series reveal that the hydroxy group, though highly favourable to the activity, was not crucial. A slight increase in potency compared to combretastatin A4 was even reported for the amino analogue **3**,[6] an other combretastatin under clinical investigation as its serine prodrug. Therefore, we decided to prepare and to evaluate for activities related to cancer (antiproliferative and proapoptotic activities, inhibition of tubulin assembly) a series of original analogues of **1**, which would differ from the reference compound only in the substituent at C-3′. Access to these analogues could be planned by total synthesis, but this approach was turn down owing to multi-step processes, partly due to the tetramethoxylation pattern of ring A. A semisynthetic method was then envisaged from a natural flavone. The ideal starting compound would be a 3,5,6,7,8,4′-hexa-oxygenated flavone bearing at C-4′ an OH, as strong activating group of the 3′ *ortho* position towards electrophilic reagents. A thorough examination of the literature in the field of natural flavones pointed out calycopterin, 5,4′-dihydroxy-3,6,7,8-tetramethoxy-flavone **4**, as a choice raw material to achieve our purpose. Calycopterin, the main flavonoid of leaves of *Digitalis thapsi* L. (Plantaginaceae) and *Calycopteris floribunda* Lamk. (Combretaceae) was isolated both by Karrer[7] and Ratnagiriswaran et al.[8] in 1934. *Calycopteris floribunda* is a traditional Asian medicinal plant which has been recently suspected to cause morbidity and mortality in grazing cattle in India. In 2006, one of us (N.B. Shridhar) reported on the toxicity of this plant in calf, rabbit and rat,[9] and subsequently provided us with dried leaves of *C. floribunda* for extraction and isolation of calycopterin.

2. Chemistry

2.1. Synthesis of analogues of flavone 1 differing in the 3′-substituent

Owing to the strong activity of the aminocombretastatin **3**, we began the study by the synthesis of flavone **5**, the 3′-amino analogue of the reference compound **1** (Scheme 1).

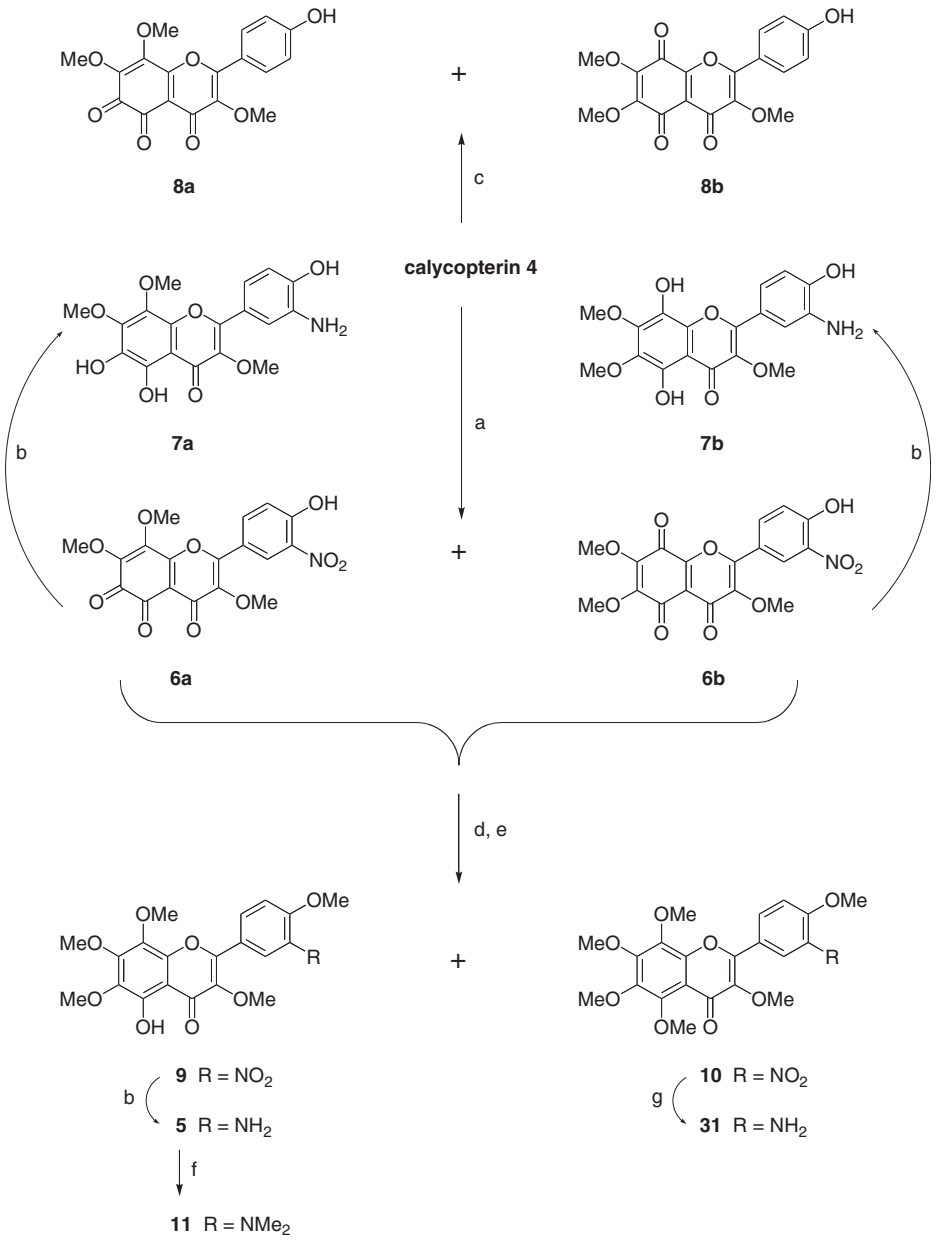
Access to **5** was attempted via nitration of **4** with HNO_3 (1 equiv) in TFA at 0 °C, as previously and successfully used in the laboratory for 3′-nitration of some other 4′-hydroxyflavones.[10,11] Under these conditions, instead of the expected 3′-nitrocalycopterin, a mixture (6–4) of two orange red-coloured compounds **6a** (major) and **6b** (minor) was obtained (yield 77% by crystallization in MeOH). Purification of an aliquot part of these crystals led to pure **6a** and **6b**. EIMS (pseudomolecular ion $[M+Na]^+$ 426) and ^1H NMR spectroscopy (three remaining OCH_3 singlets) indicated that **6a** and **6b** are isomeric 3′-nitro-5,6-*ortho* and 5,8-*para*-flavoquinones. Structures of these two quinones were established unambiguously after reduction (H_2, Pd–C 10%, rt, 5 h in DMF) into the corresponding 3′-aminoflavones **7a** and **7b**, and were inferred from NOESY experiments (for **7a**, significant NOE correlations were observed between both OCH_3 signals at C-3 and C-8 and H-2′ and H-6′; for **7b**, significant NOE correlations were observed between OCH_3 signal at C-3 and H-2′ and H-6′ on one hand, and signals of OCH_3 at C-6 and OH at C-5 and on the other hand). These experiments proved unambiguously **6a** to be 3′-nitro-5,6-*ortho*-flavoquinone, and **6b** 3′-nitro-5,8-*para*-flavoquinone. Changing nitration reagent of **1** for 1 equiv NO_2BF_4 in acetonitrile led to the mixture 45–55 of **8a** and **8b**, an other couple of *ortho* and *para*-flavoquinones, non nitrated on the B-ring. These results demonstrate that oxidation of the A-ring into *ortho* and *para*-quinones occurs before nitration of the B-ring at C-3′ (recovery of **6a** and **6b** can then be explained by oxidation of the A-ring by HNO_3 followed by nitration at the B-ring by resulting nitrite ion in TFA, as already reported in the literature[12]). Next steps of the synthesis were carried out with the crystallized mixture **6a**–**6b** and consisted successively of: a reduction of the isomeric quinones (H_2, Pd–C 10%, rt, 15 min in DMF) to the corresponding *ortho* and *para*-diphenols; a methylation of the crude resulting mixture (iodomethane, K_2CO_3, rt in DMF) leading after purification to 5-hydroxy-3′-nitro-3,6,7,8,4′-pentamethoxyflavone **9** as main product (48% from **6a**–**6b**), and 3′-nitro-3,5,6,7,8,4′-hexamethoxyflavone **10** (4.5% from **6a**–**6b**) as minor one; a reduction of **9** (H_2, Pd–C 10%, rt, 5 h in DMF) to the expected 3′-amino-5-hydroxy-3,6,7,8,4′-pentamethoxyflavone **5**, analogue of **1** (78%). Lastly, with the view of biological evaluation, the dimethylaminoflavone **11** was also prepared from **5** (aq formaldehyde, $NaBH_3CN$, rt, 24 h in acetic acid) in 66% yield.

Since SAR's of combretastatins indicate that analogues of combretastatin A4 with an hydrogen, a fluoride or a boronic acid group in place of the hydroxyl retain a good biological activity,[6] in a second time we undertook the synthesis of the three same corresponding derivatives of **1**. The 3′-deoxy analogue of **1** is the known natural calycopterin 4′*O*-methyl ether **12**,[13,14] and it was prepared from **4** (iodomethane, $KHCO_3$, rt in DMF) in 61% yield. Access to the 3′-boronic acid analogue was attempted via the synthesis of 3′-iodoflavone **15**, and its subsequent palladium-catalyzed cross-coupling reaction with bis(pinacolato)diboron according to Miyaura et al.[15] (Scheme 2).

Iodination of calycopterin **4** was carried out with *N*-iodosuccinimide (NIS), followed by 4′-O-methylation (in the reverse order, oxidation of the A-ring to the mixture of quinones was observed in place of 3′-iodination). Direct monoiodination of **4** at C-3′ occurred in poor yield, for 1 equiv NIS leads to a mixture of remaining **4**, mono and diiodo derivatives. Therefore access to the expected 3′-iodoflavone **15** was achieved according to the following three-step sequence with a 51% overall yield: (a) diodination of **4** (2.2 equiv NIS, rt in CH_2Cl_2–MeOH) to 3′,5′-diodo-calycopterin **13** (71%); (b) 4′-O-methylation of **13** (iodomethane, $KHCO_3$, rt in DMF) to **14** (86%); (c) monodeiodination at C-5′ (Zn in acetic acid, 95 °C)[16] to **15** (85%). Unfortunately, applying Miyaura's conditions[15] [bis(pinacolato)diboron, $PdCl_2$(dppf), potassium acetate, 80 °C in DMSO] to the iodoflavone **15** did not provide 3′-boronic ester, but two compounds resulting from a 3′-3′ dimerization (**16**, 22%) and a 3′-deiodination (**12**, 8%), respectively. The dimeric and symmetrical structure of **16** was unambiguously deduced from mass and NMR spectra. Its isolation as main compound of the reaction is unexpected according to literature data,[17,18] but could be explained by quenching of the boronic ester intermediate by the starting iodoflavone **15**. Synthesis of the 3′-fluoro analogue from calycopterin was no more fruitful: all attempts were carried out with *N*-fluorobenzenesulfonimide (NFSI, Accufluor™) in various solvents (CH_2Cl_2–MeOH, CH_2Cl_2–MeCN, DMF) and under solvent-free fluorination conditions,[19] but they led to complex mixtures of coloured compounds.

The last series of original 3′-substituted analogues of **1** resulted from formylation studies carried out on calycopterin **4**. Indeed, the formyl group appears to us as an excellent starting function to introduce easily chemical diversity at C-3′ (Scheme 3).

Formylation was achieved by a Duff reaction [$(CH_2)_6N_4$ in CF_3COOH at reflux], which afforded 3′-formyl-calycopterin **17** in 55% yield. Despite the presence of two chelated phenol groups at C-5 and C-4′, **17** underwent a surprisingly selective methylation at 4′ (iodomethane, $KHCO_3$, rt in DMF), which led to **18**, the 3′-formyl analogue of **1**, in 89% yield. Flavone **18** was the hub for the access to the following 3′-substituted analogues: 3′-hydroxymethyl **19** ($NaBH_4$, rt in CH_2Cl_2–MeOH, 94% yield), 3′-aminomethyl and 3′-dimethylaminomethyl **20** and **21,** respectively [**20**: NH_4OAc, $NaBH_3CN$, rt in MeOH, 34% yield; **21**: $(CH_3)_2NH$, HCl, $NaBH_3CN$, rt in MeOH, 23% yield] and 3′-cyano **22** (NH_2OH, HCl, reflux in HCOOH, 56% yield).



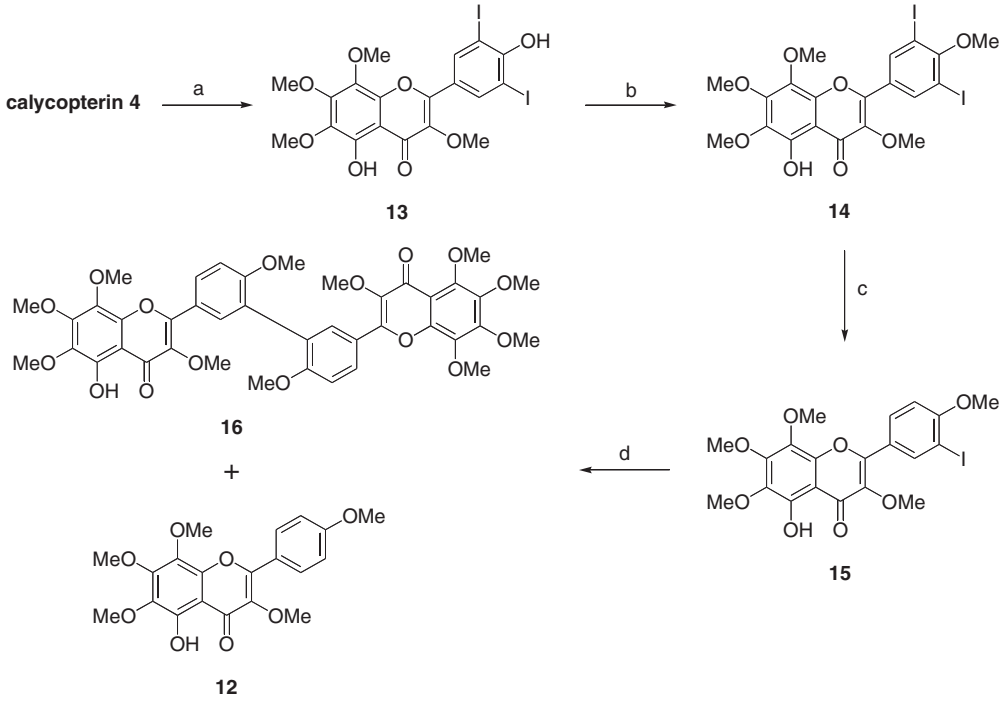
Scheme 1. Reagents and conditions: (a) HNO_3 1 equiv in TFA, 0 °C, 0.5 h, 77% [**6a**, **6b** in mixture (6:4)]; (b) H_2, Pd–C 10% in DMF, rt, 5 h; (c) NO_2BF_4 1.4 equiv in MeCN, 80 °C, 15 min, 45% [**8a**, **8b** in mixture (45:55)]; (d) H_2, Pd–C 10% in DMF, rt, 15 min; (e) iodomethane, K_2CO_3 in DMF, rt, 5.75 h, 48% **9** and 4.5% **10** (from **6a**, **6b**); (f) 37% aq HCHO, $NaBH_3CN$ in AcOH, rt, 24 h, 66%; (g) $HCOONH_4$, Pd–C 10% in MeOH, reflux, 2 h, 86%.

2.2. Synthesis of analogues of aminoflavone 5 differing in the A-ring substitution

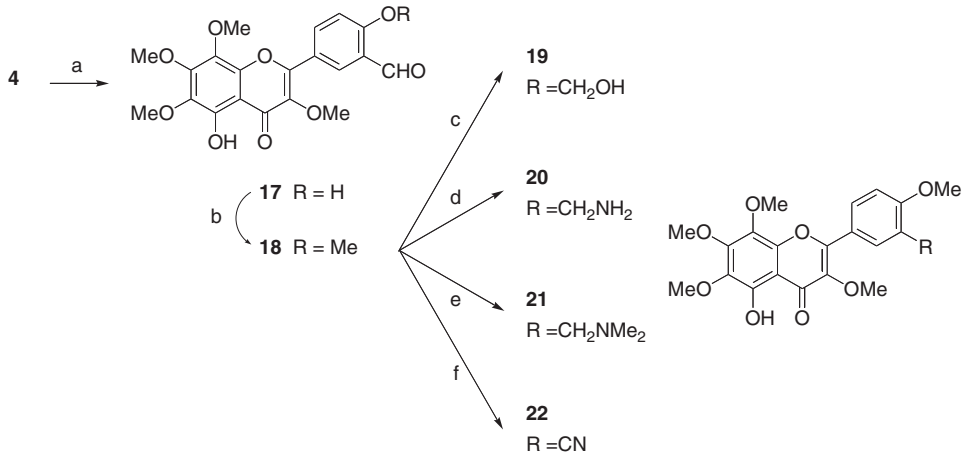
Since the flavone **5** appeared the most potent of the synthesized analogues (cf. biological results), we prepared a second series of flavones which differs from **5** by the A-ring substitution pattern. We successively focused on the 5-hydroxyl known to be critical for the activity,[1] then on the 6,7,8-trimethoxy substitution pattern.

2.2.1. Substituent at C-5

We first studied the influence of the 5-hydroxyl by its substitution for an amino group (such a substituent at C-5 is present in some previously reported very cytotoxic flavones[20]). In the course of the synthesis of this 5-amino analogue **29**, the 5-deoxy analogue **30** was also isolated. Access to **29** and **30** was achieved from calycopterin **4** by multi-step sequences having the following five steps in common (Scheme 4): (a) 4′-O-benzylation (benzyl bromide, $KHCO_3$ in DMF, 115 °C) to **23** (72% yield); (b) 5-O-triflation of **23** ($PhN(Tf)_2$, NaHMDS, rt in THF)[21] to **24** (75% yield); (c) transfer hydrogenolysis (Pd–C 10%, $HCOONH_4$, in MeOH at reflux) of **24** to **25** (80% yield); (d) 3′-nitration of **25** (1 equiv HNO_3 1 equiv in TFA at 0 °C) to **26**; (e) 4′-O-methylation of **26** (iodomethane, K_2CO_3, rt in DMF) to 3′-nitro-3,6,7,8,4′-pentamethoxy-flavone **27** (69% from **25**). A new nitration step carried out with **27** led in a very weak yield (16%) to 5,3′-dinitroflavone **28**, which provided **29**, the expected 5-amino analogue of **1**, by a last hydrogenation step (85% yield). When **27** was directly submitted to hydrogenation, the 5-deoxy analogue **30** was isolated in 86% yield. Lastly, though a 5-methoxy group is known to be detrimental to the cytotoxic activity,[22] **31**, the 5-*O*-methyl ether of **5**, was also prepared from the side compound **10** (cf. Scheme 1) by hydrogenation of the 3′-nitro group (86% yield).



Scheme 2. Reagents and conditions: (a) NIS 2.2 equiv in CH_2Cl_2–MeOH 1–1, rt, 15 h, 71%; (b) iodomethane, $KHCO_3$ in DMF, rt, 8 h, 86%; (c) Zn powder in AcOH, 95 °C, 50 min, 85%; (d) bis(pinacolato)diboron 1.3 equiv, KOAc 4 equiv, $PdCl_2$ (dppf) 5% in anhydrous DMSO, 90 °C, 18 h, 22% **16** and 8% **12**.



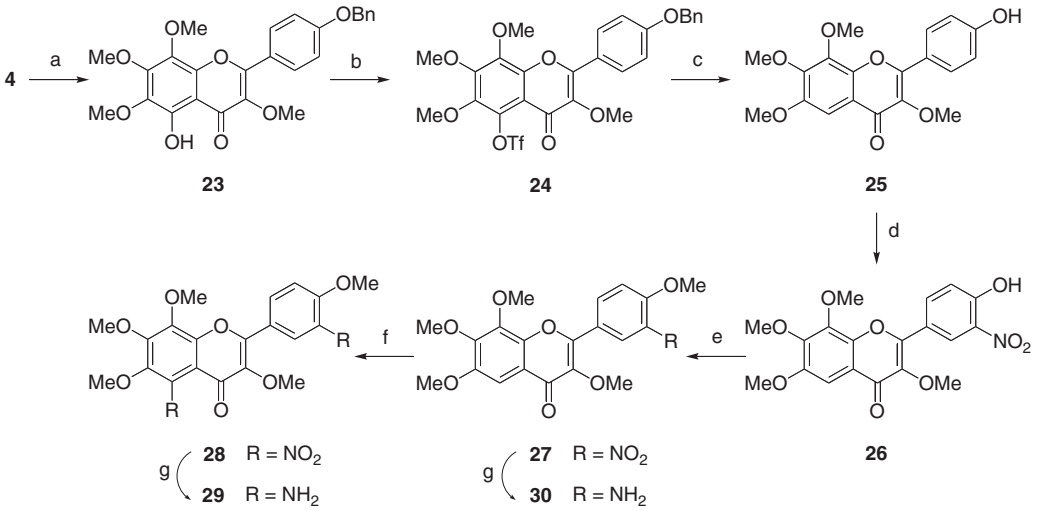
Scheme 3. Reagents and conditions: (a) hexamethylenetetramine 1.06 equiv in TFA, reflux, 5 h, 55%; (b) iodomethane, $KHCO_3$ in DMF, rt, 2.5 h, 89%; (c) $NaBH_4$ in MeOH–CH_2Cl_2 5–1, rt, 30 min, 94%; (d) NH_4OAc 15 equiv, $NaBH_3CN$ in MeOH, rt, 20 h, 34%; (e) Me_2NH, HCl 6 equiv, $NaBH_3CN$ in MeOH, rt, 20 h, 23%; (f) NH_2OH, HCl in HCOOH, reflux, 2 h, 56%.

2.2.2. Substituent at C-6, C-7 and C-8

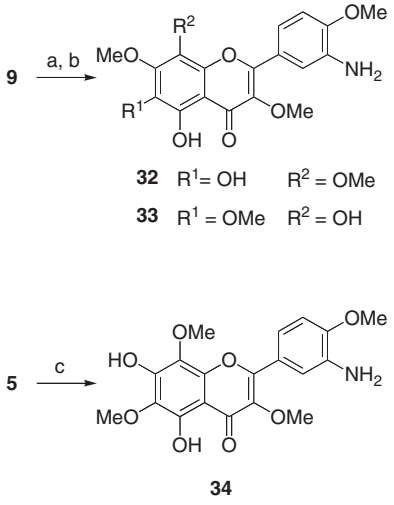
Role of the 6,7,8-trimethoxy substitution was investigated by preparing each of the three mono *O*-demethyl derivatives (Scheme 5). Access to the 6 and 8-*O*-demethyl analogues was achieved from the 3′-nitroflavone **9** by the following two-step sequence: (a) oxidation (1 equiv HNO_3 in TFA at 0 °C) to the couple of isomeric *ortho* and *para*-flavoquinones; (b) reduction of both quinone and nitro groups (H_2, Pd–C 10%, rt, 1.5 h in DMF) to the corresponding 5,6 and 5,8-dihydroxy-3′-aminoflavones **32** and **33**. Structure of each isomer was deduced from NOESY experiments, as mentioned for **7a** and **7b** (two observed NOE correlations between H-2′, 6′ and 3 and 8 methoxy groups in **32**; one only with the 3-methoxyl in **33**). Lastly, reaction of **5** with LiCl/DMF, as demethylating reagent, provided the third analog **34** by an expected selective 7-O-demethylation.[23]

3. Biology

In a first attempt, the antiproliferative effect of flavones assayed on KB human buccal carcinoma cells was associated with the activation of caspases 3/7 in HL60 human leukemia cells and the in vitro inhibition of tubulin polymerization for the most active compounds. The biological evaluation was undertaken with fourteen analogues of **1** having a various B-ring substitution pattern: 11 flavones (**5**, **9**, **11**, **12**, **15**, **16**, **18**–**22**) differ only in the substituent at C-3′, the three others being calycopterin **4**, and two synthetic intermediates, the diiodoflavone **14** and 3′-formyl-calycopterin **17** (Table 1). As expected, the 3′-aminoflavone **5** possesses strong biological activities (antiproliferative, proapoptotic and IPT) very near from **1**, though a little less potent. No interesting responses were noticed with other synthesized flavones, except calycopterin



Scheme 4. Reagents and conditions: (a) benzyl bromide 3 equiv, $KHCO_3$ in DMF, 115 °C, 3 h, 72%; (b) NaHMDS 2.1 equiv, $PhN(Tf)_2$ 2.7 equiv in THF, rt, 22 h, 75%; (c) $HCOONH_4$, Pd–C 10% in MeOH, reflux, 1 h, 80%; (d) HNO_3 1 equiv in TFA, 0 °C, 0.5 h, 90%; (e) iodomethane, K_2CO_3 in DMF, rt, 6 h, 69% from **25**; (f) HNO_3 1 equiv in TFA, 0 °C, 2 h, 16%; (g) $HCOONH_4$, Pd–C 10% in MeOH, reflux, 2 h, 85% **29** and 86% **30**.



Scheme 5. Reagents and conditions: (a) HNO_3 1 equiv in TFA, 0 °C, 0.5 h; (b) H_2, Pd–C 10% in DMF, rt, 1.5 h, 6% **32** and 6% **33** from **9**; (c) LiCl, 3.5 equiv in DMF, 180 °C, 17 h, 26%.

4′*O*-methyl ether **12**, which displays a fair cytotoxicity, but without IPT property. So, it appears clearly that homologation of the 3′-hydroxy or amino substituents (**19**, **20**), such as replacement by some electron donating (**11**) or withdrawing (**18**, **22**) groups of small size are highly unfavourable to the activity. From SAR's point of view, the strong activities observed only with flavones bearing 3′-hydroxy or amino substituents confirmed the analogy with the combretastatins family. Potent activities of **5** led us, in a second time, to synthesize and evaluate analogues of this aminoflavone differing in the substitution pattern on the A-ring (Table 1). Replacement of the 5-hydroxyl by an amino group (**29**), or deoxygenation at C-5 (**30**) resulted in a decrease of antiproliferative and proapoptotic activities, which remain however strong enough. On the contrary, methylation of this phenol group (**31**) is very detrimental to the activity, as previously reported.[1] Lastly, the biological evaluation of **32**–**34**, the three monodemethylated analogues at C-6, C-8 and C-7, respectively, indicated a strong decrease of the antiproliferative activity. This observation, as well as results of our recent study,[5] confirms that A-ring pattern substitution of **1** seems highly favorable for strong antiproliferative and IPT activities within class of flavones. Compound **5** was the most potent newly synthesized flavone, and we compared its biological activity with that of the reference flavone **1** in several human cancer cell lines (Table 2). The antiproliferative activity was comparable for both flavones, except in PC3 which was resistant to the two compounds. One can also observed a striking difference within HT29 cell line, which is sensible to **5** but resistant to **1**. To go further, apoptosis was explored by flow cytometry (Table 3): compounds **1** and **5** similarly induced apoptosis as early as 24 h leading later to the activation of caspases 3/7 reported in Table 1. The inhibition of tubulin polymerization was correlated with the blockade of cell cycle in phase G2/M after 24 h of treatment, prior to a partial DNA degradation (apparent S phase) and finally cell death (sub G1 phase). A similar behavior was reported for combretastatin A4 **2** and, to a lesser extent, compounds **1** and **5** (Table 4).

Table 1
Antiproliferative, proapoptotic and antitubulin activities of synthesized flavones

Compd	Cytotoxicity on KB cells[a] IC_{50} (nM)	Activation of caspases 3/7 in HL60[b]	IPT activity[c]
1	96% IC_{50} = 8	100 nM (×5.7)	8.9 μM (3)[d]
*Analogues of **1** differing in the B-ring substitution*			
4	0%	nd	nd
5	96% IC_{50} = 25	100 nM (×3.2)	13 μM (4.5)[d]
9	13%	nd	nd
11	13%	nd	nd
12	73% IC_{50} = 720	100 μM (×4.5)	2% Inhibition[c]
14–**22**	⩽13%	nd	nd
*Analogues of **5** differing in the A-ring substitution*			
29	78% IC_{50} = 128	100 μM (×3.6)	24 μM (8.3)[d]
30	84% IC_{50} = 78	100 μM (×3.1)	12 μM (4)[d]
31	17%	No activation at 100 μM	5% Inhibition[c]
32	38% $IC_{50} \approx 10{,}000$	nd	nd
33	34% $IC_{50} \gg 10{,}000$	nd	nd
34	IC_{50} = 6200	nd	85 μM (29)[d]

[a] As measured by the MTS assay after 72 h incubation of cells with drug: results are expressed as the percentage of inhibition of cell growth with 10^{-6} M flavone concentration, or as IC_{50} (nM), calculated only for the most active compounds.
[b] Activation of caspases 3/7 activity: optimal concentration of compound and fold activation over controls.
[c] Results are expressed as the percentage of IPT at $\approx 2 \times 10^{-5}$ M, or as IC_{50} (μM).
[d] $IC_{50\ compound}/IC_{50\ deoxypodophyllotoxin}$. nd: not determined.

Table 2
Comparison of antiproliferative activities of **1** and **5**

Cell line	IC_{50}[a] (nM)	
	1	**5**
HCT-116[b]	23	25
HCT-15[b]	14	12
HT29[b]	3140	50
MCF7R[c]	17	34
OVCAR-8[d]	40	105
SK-OV-3[d]	22	37
A549[e]	58	32
PC-3[f]	>1000	>1000
Mia PaCa-2[g]	12	33
HepG2[h]	174	81
SF-268[i]	186	400
HL-60[j]	36	36
HL-60R[j]	5	14
K-562[j]	7	9

[a] As measured by the MTS assay after 72 h incubation of cells with drug.
[b] Colon cancer.
[c] Breast cancer.
[d] Ovarian cancer.
[e] Non-small cell lung cancer.
[f] Prostate cancer.
[g] Pancreas cancer.
[h] Liver cancer.
[i] CNS cancer.
[j] Leukemia.

Table 3
Early and late apoptosis in HL60 cells treated with **1** and **5**[a]

Compd	Viable[b]	% Cells	
		In early apoptosis[c]	In late apoptosis[d]
24 h			
Control	97.2	2.6	0.2
Doxorubicin (200 nM)	52.8	45.9	1.2
1 (50 nM)	72.4	26.7	0.8
5 (50 nM)	77.1	20.7	2.1
48 h			
Control	97.4	2.5	0.1
Doxorubicin (200 nM)	5.2	89.2	5.6
1 (50 nM)	30.8	61.8	7.2
5 (50 nM)	60.1	37.6	2.2

[a] Expressed as the percentage of viable cells, cells in early and late apoptosis, measured by respective fluorescence intensities of 7-AAD (7-Aminoactinomycin D) versus annexin V-PE.
[b] Annexin negative/7-AAD negative.
[c] Annexin positive/7-AAD negative.
[d] Annexin positive/7-AAD.

4. Conclusion

In conclusion, this study reports synthesis and evaluation of the antiproliferative activity of 20 3-methoxy-flavones including 18 new compounds. Nineteen of these flavones were prepared by semisynthesis from calycopterin, a flavonoid isolated from leaves of *Calycopteris floribunda* Lamk., a traditional Asian medicinal plant. One of the prepared products, aminoflavone **5**, displayed a strong antiproliferative activity and proved to possess the same mechanism and about the same potency (though slightly weaker) as 5,3′-dihydroxy-3,6,7,8,4′-pentamethoxy-flavone **1**, a natural flavone, which remains the most antimitotic natural flavone isolated to date. From a pharmacomodulation point of view relating to antiproliferative activity within the flavone series, this study refines the previously predicted structure–activity relationships.[1,24] Lastly, access to these original flavones by semisynthesis confirms the interest of natural products as raw materials for medicinal chemistry.

Table 4
Cell cycle analysis of KB cells treated with **1** and **5**[a]

Compd	% Cells in			
	Sub G1	G0/G1	S	G2/M
24 h				
Control	0.7	72.9	6.9	19.6
C-A4[b] (20 nM)	13.8	9.5	26.7	50.1
1 (50 nM)	14.9	21.3	18.5	45.3
5 (50 nM)	7.3	39.3	19.2	34.5
48 h				
Control	1.2	66.6	18.4	13.8
C-A4 (20 nM)	43.5	27.4	44	0.8
1 (50 nM)	22.2	27.4	38.6	11.8
5 (50 nM)	15.5	38.5	32	14

[a] Expressed as the percentage of KB cells in the various mitotic phases.
[b] Combretastatin A4.

5. Experimental section

5.1. General experimental procedures

Melting points were determined with a micro-Koffler apparatus and are uncorrected. NMR spectra, including NOESY, ^{1}H–^{13}C (HMQC and HMBC) experiments, were recorded on Bruker AC-300 (300 MHz) or Bruker AM-400 (400 MHz) spectrometers. ESIMS were recorded on a Navigator Aqua thermoquest spectrometer or an Agilent HP 1100 MSD spectrometer (ESI source) and APCIMS on a Esquire-LC Bruker 00040 spectrometer. Flash chromatographies were performed with Silica Gel 60 (9385 Merck) or aluminium oxide 90 (1097 Merck). Preparative tlc were performed with 60 F 254 silica gel (5715 Merck) or 60 F 254 aluminium oxide (5713 Merck).

5.2. Plant material

The plant was collected in India from a place named 'Talaguppa' Shimoga District, Karnataka State and its identification confirmed by Dr. Gopalakrishna Bhat, Professor and Head, Department of Botany, Poornaprajna College, Udupi.

5.3. Extraction and isolation of calycopterin 4

Dried and ground leaves of *C. floribunda* (2.5 kg) were extracted with CH_2Cl_2 in a Soxhlet apparatus. Extract was filtered over a Celite pad, then evaporated to dryness. The residue (98 g) was purified by flash chromatography (silica gel, CH_2Cl_2 then CH_2CL_2–MeOH 99–1). The most interesting fractions were combined and evaporated to dryness. Crystallization of the dried residue (36 g) with MeOH afforded pure calycopterin (8.6 g, 0.34%). Bright-yellow crystals: mp: 225–226 °C (lit.[14,25]: 226 °C; 225–226 °C); ^{1}H NMR (DMSO-d_6) δ ppm 3.80, 3.82, 3.90 and 4.02 (4s, 12H, OMe-3, 6, 7 and 8), 6.98 (d, J = 8.7 Hz, 2H, H-3′ and 5′), 7.98 (d, J = 8.7 Hz, 2H, H-2′ and 6′), 12.46 (br s, 1H, 5-OH).

5.4. Synthesis of flavones from calycopterin

5.4.1. 3′-Nitro-5,6-*ortho*-flavoquinone 6a and 3′-nitro-5,8-*para*-flavoquinone 6b

A solution of **1** (1.122 g, 3 mmol) in trifluoroacetic acid (30 mL) at 0 °C was added with 11.19 N HNO_3 (1 equiv) then stirred for 0.5 h. The reaction mixture was taken up in ice water, then extracted with CH_2Cl_2. Standard work-up of the organic layer afforded a red dried residue, which was crystallized in MeOH (0.931 g, 77%). According to ^{1}H NMR spectrum, the crystals consist of a mixture (6–4) of **6a** and **6b**, which were separated from an aliquot part by preparative tlc (silica gel, CH_2CH_2–MeOH 97.5–2.5).

Compound **6a** orange red crystals: mp 195–200 °C (MeOH); ^{1}H NMR (DMSO-d_6) δ ppm 3.90, 3.92 and 4.18 (3s, 9H, OMe-3, 7, 8), 7.32 (d, J = 8.8 Hz, 1H, H-5′), 8.10 (dd, J = 8.8 and 2.3 Hz, 1H, H-6′), 8.48 (d, J = 2.3 Hz, 1H, H-2′). ESIMS (+) m/z 404 $[M+H]^+$, 426 $[M+Na]^+$, $[M+K]^+$ 442. Compound **6b** orange red crystals mp: 217–221 °C (MeOH); ^{1}H NMR (DMSO-d_6) δ ppm 3.88, 3.92 and 4.00 (3s, 9H, OMe-3, 6, 7), 7.32 (d, J = 8.8 Hz, 1H, H-5′), 8.10 (dd, J = 8.8 and 2.3 Hz, 1H, H-6′), 8.46 (d, J = 2.3 Hz, 1H, H-2′). ESIMS (+) m/z 404 $[M+H]^+$, 426 $[M+Na]^+$, $[M+K]^+$ 442.

5.4.2. 3′-Amino-5,6-4′-trihydroxy-3,7,8-trimethoxy-flavone 7a and 3′-amino-5,8-4′-trihydroxy-3,6,7-trimethoxy-flavone 7b

Solutions of **6a** (14 mg, 0.035 mmol) and **6b** (12 mg, 0.03 mmol) in DMF (3 mL) were hydrogenated under 1 atm pressure hydrogen with 10% Pd–C (15 mg) at room temperature for 5 h. The catalyst was separated and the filtrate concentrated to dryness. Dried residues were purified by tlc (silica gel, CH_2CH_2–MeOH 95–5) and afforded pure **7a** (4.5 mg) and **7b** (4 mg). Compound **7a** Amorphe; ^{1}H NMR (DMSO-d_6) δ ppm 3.75, 3.87 and 3.92 (3s, 9H, OMe-3, 7, 8), 6.81 (d, J = 8.8 Hz, 1H, H-5′), 7.26 (dd, J = 8.8 and 2.3 Hz, 1H, H-6′), 7.38 (d, J = 2.3 Hz, 1H, H-2′), 12.2 (br s, 1H, 5-OH). ESIMS (+) m/z 376 $[M+H]^+$, 398 $[M+Na]^+$. Compound **7b** Amorphe; ^{1}H NMR (DMSO-d_6) δ ppm 3.75, 3.80 and 3.90 (3s, 9H, OMe-3, 6, 7), 6.81 (d, J = 8.8 Hz, 1H, H-5′), 7.29 (dd, J = 8.8 and 2.3 Hz, 1H, H-6′), 7.42 (d, J = 2.3 Hz, 1H, H-2′), 12.2 (s, 1H, 5-OH). ESIMS (+) m/z 376 $[M+H]^+$, 398 $[M+Na]^+$.

5.4.3. 5,6-*ortho*-Flavoquinone 8a and 5,8-*para*-flavoquinone 8b

A mixture of **1** (0.187 g, 0.5 mmol) in acetonitrile (20 mL) was heated at 80 °C till dissolution, left at rt for 15 min then added with nitronium tetrafluoroborate (0.092 g, 0.7 mmol). The reaction was stirred for 15 min at rt under nitrogen, then diluted with CH_2Cl_2. Standard work-up of the organic layer afforded an orange reddish dried residue, which was crystallized in MeOH (0.080 g, 45%). According to ^{1}H NMR spectrum, the crystals consist of a mixture (45–55) of **8a** and **8b**. ^{1}H NMR (DMSO-d_6) signals of **8a** at δ ppm 3.82, 3.90 and 4.18 (3s, 9H, OMe-3, 7, 8), 6.95 (d, J = 8.7 Hz, 2H, H-3′ and 5′), 7.86 (d, J = 8.7 Hz, 2H, H-2′ and 6′); signals of **8b** at δ ppm 3.82, 3.92 and 4.00 (3s, 9H, OMe-3, 6, 7), 6.98 (d, J = 8.7 Hz, 2H, H-3′ and 5′), 7.88 (d, J = 8.7 Hz, 2H, H-2′ and 6′).

5.4.4. 5-Hydroxy-3′-nitro-3,6,7,8,4′-pentamethoxy-flavone 9 and 3′-nitro-3,5,6,7,8,4′-hexamethoxy-flavone 10

A solution of the mixture (6–4) **6a–6b** (0.806 g, 2 mmol) in DMF (80 mL) was hydrogenated under 1 atm pressure hydrogen with 10% Pd–C (0.4 g) at room temperature for 15 min. The catalyst was separated, the filtrate was concentrated to 40 mL, added with K_2CO_3 (0.345 g, 2.5 mmol) and iodomethane (0.9 mL, 14 mmol), then the reaction mixture was stirred for 5 h at room temperature. After monitoring of the reaction by tlc, same amounts of K_2CO_3 and iodomethane were added and the medium stirred for further 45 min. The reaction mixture was diluted with iced water, and thoroughly extracted by CH_2Cl_2. Standard work-up of the organic layer afforded a dried residue (0.622 g). Two successive purifications by flash chromatography (silica gel, CH_2CH_2–MeOH 99–1) provided the pure 5-hydroxylated flavone **9**, which was crystallized in MeOH (0.415 g, 48%). Fractions (0.1 g) containing mainly the 5-methoxylated flavone **10** were purified by a third flash chromatography (alumina, CH_2CH_2–cyclohexane 3–1), and led to pure compound **10**, which crystallized by evaporation to dryness (0.045 g, 4.5%). Compound **9** bright-yellow crystals: mp 160–163 °C (MeOH); ^{1}H NMR ($CDCl_3$) δ ppm 3.97, 3.98, 4.10 and 4.15 (4s, 15H, OMe-3, 6, 7, 8, 4′), 7.25 (d, J = 8.8 Hz, 1H, H-5′), 8.39 (dd, J = 8.8 and 2.3 Hz, 1H, H-6′), 8.70 (d, J = 2.3 Hz, 1H, H-2′). APCIMS (+) m/z 434 $[M+H]^+$. Compound **10** light-yellow crystals mp: 151–154 °C; ^{1}H NMR ($CDCl_3$) δ ppm 3.97, 3.98, 4.00, 4.03, 4.10 and 4.15 (6s, 18H, OMe-3, 5, 6, 7, 8, 4′), 7.25 (d, J = 8.8 Hz, 1H, H-5′), 8.37 (dd, J = 8.8 and 2.3 Hz, 1H, H-6′), 8.70 (d, J = 2.3 Hz, 1H, H-2′), 12.21 (s, 1H, 5-OH). APCIMS (+) m/z 448 $[M+H]^+$.

5.4.5. 3′-Amino-5-hydroxy-3,6,7,8,4′-pentamethoxy-flavone 5

A solution of **9** (0.2 g, 0.46 mmol) in DMF (15 mL) was hydrogenated under 1 atm pressure hydrogen with 10% Pd–C (0.2 g) at room temperature for 5 h. The catalyst was separated and the filtrate concentrated to dryness. Dried residue was purified by flash chromatography (silica gel, CH_2CH_2–MeOH 98–2), and provided pure expected flavone **5**, which crystallized by evaporation to dryness (0.146 g, 78%). Compound **5** bright-yellow crystals: mp 116–118 °C; ^{1}H NMR ($CDCl_3$) δ ppm 3.85 (s, 3H, OMe-3), 3.94 (s, 9H, OMe-6, 8 and 4′), 4.10 (s, 3H, OMe-7), 6.91 (d, J = 8.8 Hz, 1H, H-5′), 7.55 (d, J = 2.3 Hz, 1H, H-2′), 7.63 (dd, J = 8.8 and 2.3 Hz, 1H, H-6′), 12.45 (s, 1H, 5-OH). ^{13}C NMR ($CDCl_3$) δ ppm 55.6 (OMe-4′), 60.1 (OMe-3), 61.1, 61.7 and 62.1 (OMe-6, 7, 8), 107.5 (C-10), 110.1 (C-5′), 114.4 (C-2′), 120.1 (C-6′), 123.1 (C-1′), 132.8 (C-8), 136.0 (C-6 and C-3′), 138.7 (C-3), 144.9 (C-9), 149.1 and 149.7 (C-5 and C-4′), 152.8 (C-7), 156.5 (C-2), 179.3 (C-4). HRESIMS (+) m/z $[M+H]^+$ 404.1323 (calcd for $C_{20}H_{22}NO_8$, 404.1339).

5.4.6. 3′-Dimethylamino-5-hydroxy-3,6,7,8,4′-pentamethoxy-flavone 11

A solution of **5** (12 mg, 0.03 mmol) in acetic acid (4 mL) was added with aqueous 37% formaldehyde and $NaBH_3CN$ in excess, then stirred for 24 h at rt. Standard work-up of the reaction, then purification of the residue by preparative tlc (silica gel, CH_2CH_2–MeOH 98.5–1.5) led to pure **11** in 66% yield. Compound **11** Amorphous; ^{1}H NMR ($CDCl_3$) δ ppm 2.86 (s, 6H, $N(CH_3)_2$), 3.87, 3.93, 3.94, 3.97 (s, 12H, OMe-3, 6, 8 and 4′), 4.09 (s, 3H, OMe-7), 6.98 (d, J = 8.8 Hz, 1H, H-5′), 7.82 (d, J = 2.3 Hz, 1H, H-2′), 7.86 (dd, J = 8.8 and 2.3 Hz, 1H, H-6′), 12.40 (s, 1H, 5-OH). ESIMS (+) m/z 432 $[M+H]^+$, 454 $[M+Na]^+$, $[M+K]^+$ 470.

5.4.7. 5-Hydroxy-3,6,7,8,4′-pentamethoxy-flavone (calycopterin 4′*O*-methyl ether) 12

A solution of calycopterin **4** (0.094 g, 0.25 mmol) in DMF (5 mL) was stirred for 24 h at rt, and successively added with $KHCO_3$ (0.027 g, 0.27 mmol) and iodomethane (0.095 mL, 1.5 mmol) at t = 0, 2, 4, 6 and 22 h. Standard work-up of the reaction, then purification of the dried residue by flash chromatography (silica gel, CH_2CH_2–MeOH 99–1) and crystallization with MeOH gave pure compound **12** (0.059 g, 61%). Bright-yellow crystals: mp 119–122 °C (lit.[13,14]: 122–123 °C; 125–127 °C); ^{1}H NMR ($CDCl_3$) δ ppm 3.87, 391 and 3.96 (3s, 12H, OMe-3, 6, 8, 4′), 4.11 (s, 3H, OMe-7), 7.05 (d, J = 8.7 Hz, 2H, H-3′ and 5′), 8.17 (d, J = 8.7 Hz, 2H, H-2′ and 6′), 12.45 (br s, 1H, 5-OH). ^{13}C NMR ($CDCl_3$) δ ppm 55.5 (OMe-4′), 60.1 (OMe-3), 61.2, 61.8, 62.2 (OMe-6, 7, 8), 107.5 (C-10), 114.3 (C-3′ and 5′), 122.9 (C-1′), 130.3 (C-2′ and 6′), 132.9 (C-8), 136.2 (C-6), 138.6 (C-3), 144.9 (C-9), 149.2 (C-5), 152.9 (C-7), 156.1 (C-2), 161.9 (C-4′), 179.3 (C-4).

5.4.8. 3′,5′-Diodo-calycopterin 13

A solution of calycopterin **4** (0.374 g, 1 mmol) in CH_2Cl_2–MeOH 1–1 (80 mL) was added with NIS (0.495 g, 2.2 mmol), and stirred overnight at rt. Standard work-up of the reaction (with washing of the organic phase with 1 N aqueous thiosulfate), then purification of the dried residue by crystallization with MeOH then flash chromatography (silica gel, CH_2CH_2–MeOH 99–1) led to pure diodoflavone **13** (0.444 g) in 71% yield. Bright-yellow crystals: mp 206–209 °C; ^{1}H NMR ($CDCl_3$) δ ppm 3.88, 393 (2s, 9H, OMe-3, 6, 8), 4.09 (s, 3H, OMe-7), 6.10 (s, 1H, OH-4′), 8.49 (s, 2H, H-2′ and 6′), 12.23 (s, 1H, 5-OH). ^{13}C NMR ($CDCl_3$) δ ppm 60.4 (OMe-3), 61.2, 61.8, 62.2 (OMe-6, 7, 8), 80.3 (C-3′and 5′), 107.5 (C-10), 126.5 (C-1′), 132.8 (C-8), 136.4 (C-6), 139.1 (C-3), 139.4 (C-2′ and

6′), 144.7 (C-9), 149.2 (C-5), 152.4 and 153.3 (C-7 and C-4′), 155.8 (C-2), 179.1 (C-4).

5.4.9. 3′,5′-Diodo-5-hydroxy-3,6,7,8,4′-pentamethoxy-flavone 14

A solution of diodoflavone **13** (0.406 g, 0.65 mmol) in DMF (35 mL) was added with 5 equiv $KHCO_3$ (0.325 g, 3.25 mmol) and iodomethane (0.3 mL, 4.8 mmol) and stirred for 5 h at rt. New amounts of $KHCO_3$ (0.16 g, 1.6 mmol) and iodomethane (0.2 mL, 3.2 mmol) were added, and the mixture stirred 3 h more. The reaction was diluted with CH_2Cl_2, filtered, and concentrated to dryness. The dried residue was taken up with CH_2Cl_2, and the organic phase submitted to the standard work-up. Crystallization of the residue with MeOH gave pure compound **12** (0.357 g, 86%). Bright-yellow crystals: mp 170–172 °C; ^{1}H NMR ($CDCl_3$) δ ppm 3.94, 395 and 3.96 (3s, 12H, OMe-3, 6, 8, 4′), 4.12 (s, 3H, OMe-7), 8.54 (s, 2H, H-2′ and 6′), 12.12 (s, 1H, 5-OH). ^{13}C NMR ($CDCl_3$) δ ppm 60.5, 60.9, 61.2, 61.8, 62.2 (OMe-3, 6, 7, 8, 4′), 90.6 (C-3′ and 5′), 107.5 (C-10), 130.0 (C-1′), 132.9 (C-8), 136.4 (C-6), 139.6 (C-3), 139.8 (C-2′ and 6′), 144.9 (C-9), 149.2 (C-5), 152.1 and 153.4 (C-2 and C-7), 161.0 (C-4′), 179.2 (C-4). ESIMS (+) *m/z* 641 $[M+H]^+$, 663 $[M+Na]^+$, $[M+K]^+$ 679.

5.4.10. 5-Hydroxy-3′-iodo-3,6,7,8,4′-pentamethoxy-flavone 15

A mixture of flavone **14** (0.34 g, 0.53 mmol) in acetic acid was stirred at 95 °C till solubilization, then added with Zn powder (0.34 g) and heated to the same temperature 50 min more. The reaction mixture was diluted with H_2O and thoroughly extracted with CH_2Cl_2. Standard work-up of the reaction, then crystallization of the dried residue with MeOH provided pure compound **15** (0.235 g) in 86% yield. Compound **15** bright-yellow crystals: mp 137–139 °C; ^{1}H NMR ($CDCl_3$) δ ppm 3.89 (s, 3H, OMe-3), 3.96 and 3.99 (2s, 9H, OMe-6, 8 and 4′), 4.11 (s, 3H, OMe-7), 6.95 (d, J = 8.8 Hz, 1H, H-5′), 8.19 (dd, J = 8.8 and 2.3 Hz, 1H, H-6′), 8.61 (d, J = 2.3 Hz, 1H, H-2′), 12.25 (s, 1H, 5-OH). ^{13}C NMR ($CDCl_3$) δ ppm 56.6 (OMe-4′), 60.2 (OMe-3), 61.2, 61.8 and 62.2 (OMe-6, 7, 8), 80.1 (C-3′) 107.5 (C-10), 110.6 (C-5′), 124.7 (C-1′), 130.3 (C-2′), 132.9 (C-8), 136.3 (C-6), 138.9 (C-3), 139.6 (C-6′), 144.9 (C-9), 149.2 (C-5), 153.1 (C-7), 154.4 (C-2), 160.2 (C-4′), 179.3 (C-4). ESIMS (+) *m/z* 514 $[M+H]^+$, 537 $[M+Na]^+$, $[M+K]^+$ 553.

5.4.11. 3′,3′-Biflavone 16

A mixture of flavone **15** (0.103, 0.2 mmol), bis(pinacolato)diboron (0.066 g, 0.26 mmol), potassium acetate (0.078, 0.8 mmol) and $PdCl_2$ (dppf) (8 mg, 0.01 mmol) in anhydrous DMSO (5 mL) was stirred at 90 °C for 18 h. The reaction mixture was diluted with H_2O, and thoroughly extracted with Et_2O. Standard work-up of the organic phase gave a rather complex dried residue (0.05 g). Purification by preparative tlc (silica gel, CH_2CH_2–MeOH 99.5–0.5) allowed isolation of pure biflavone **16** (0.017 g, 22%) and calycopterin 4′*O*-methyl ether **12** (0.006 g, 8%). Compound **16** light-yellow crystals (MeOH): mp 148–150 °C; ^{1}H NMR ($CDCl_3$) δ ppm 3.90, 3.93 and 3.96, (3s, 12H, OMe-3, 6, 8 and 4′), 4.10 (s, 3H, OMe-7), 7.19 (d, J = 8.8 Hz, 1H, H-5′), 8.19 (d, J = 2.3 Hz, 1H, H-2′), 8.24 (dd, J = 8.8 and 2.3 Hz, 1H, H-6′), 12.36 (s, 1H, 5-OH). ^{13}C NMR ($CDCl_3$) δ ppm 55.9 (OMe-4′), 60.2 (OMe-3), 61.2, 61.8 and 62.2 (OMe-6, 7, 8), 107.6 (C-10) 111.2 (C-5′), 122.7 (C-1′), 127.0 (C-3′), 130.0 and 132.1 (C-2′ and 6′), 132.9 (C-8), 136.2 (C-6), 138.8 (C-3), 145.0 (C-9), 149.2 (C-5), 152.9 (C-7), 156.1 (C-2), 159.3 (C-4′), 179.3 (C-4). HRESIMS (+) *m/z* $[M+H]^+$ 775.2252 (calcd for $C_{40}H_{39}O_{16}$, 775.2226), $[M+Na]^+$ 797.2063 (calcd for $C_{40}H_{38}O_{16}Na$, 797.2046).

5.4.12. 3′-Formyl-calycopterin 17

A solution of calycopterin **4** (0.497 g, 1.33 mmol) in trifluoroacetic acid (15 mL) was added with 1.06 equiv hexamethylenetetramine (0.2 g, 1.41 mmol) and heated for 5 h at reflux. The mixture was diluted with iced water, stirred for 5 min, then extracted with CH_2Cl_2. Standard work-up of the organic phase, then purification by flash chromatography provide pure **17** which crystallized by evaporation to dryness (0.292 g, 55%). Compound **17** bright-yellow crystals: mp 125–127 °C; ^{1}H NMR ($CDCl_3$) δ ppm 3.92 (s, 3H, OMe-3), 3.94 and 3.95 (2s, 6H, OMe-6 and 8), 4.11 (s, 3H, OMe-7), 7.16 (d, J = 8.8 Hz, 1H, H-5′), 8.34 (dd, J = 8.8 and 2.3 Hz, 1H, H-6′), 8.47 (d, J = 2.3 Hz, 1H, H-2′), 10.01 (s, 1H, CHO), 11.36 (s, 1H, 4-OH′), 12.28 (s, 1H, 5-OH). ^{13}C NMR ($CDCl_3$) δ ppm 60.3 (OMe-3), 61.2, 61.7 and 62.1 (OMe-6, 7, 8), 107.5 (C-10), 118.5 (C-5′), 120.5 (C-3′), 122.6 (C-1′), 132.9 (C-8), 134.6 (C-2′), 136.1 (C-6), 136.4 (C-6′), 138.9 (C-3), 144.8 (C-9), 149.3 (C-5), 153.2 (C-7), 154.1 (C-2), 163.5 (C-4′), 179.2 (C-4), 195.4 (CHO).

5.4.13. 3′-Formyl-5-hydroxy-3,6,7,8,4′-pentamethoxy-flavone 18

A solution of compound **17** (0.241 g, 0.6 mmol) in DMF (25 mL) was added with 5 equiv $KHCO_3$ (0.3 g, 3 mmol) and iodomethane (0.15 mL, 2.4 mmol) and stirred for 2.5 h at rt. The reaction was diluted with H_2O, and extracted with CH_2Cl_2. Standard work-up of the organic phase, then crystallization of the residue with MeOH gave pure compound **18** (0.223 g, 89%). Compound **18** bright-yellow crystals: mp 159–163 °C; ^{1}H NMR ($CDCl_3$) δ ppm 3.92 (s, 3H, OMe-3), 3.95 (s, 3H, OMe-6), 3.97 (s, 3H, OMe-8), 4.05 (s, 3H, OMe-4′), 4.11 (s, 3H, OMe-7), 7.16 (d, J = 8.8 Hz, 1H, H-5′), 8.39 (dd, J = 8.8 and 2.3 Hz, 1H, H-6′), 8.62 (d, J = 2.3 Hz, 1H, H-2′), 10.52 (s, 1H, CHO), 12.30 (s, 1H, 5-OH). ^{13}C NMR ($CDCl_3$) δ ppm 56.1 (OMe-4′), 60.3 (OMe-3), 61.2 and 61.7 (OMe-6 and 8), 62.1 (OMe-7), 107.5 (C-10), 112.1 (C-5′), 123.2 (C-1′), 124.9 (C-3′), 129.2 (C-2′), 132.8 (C-8), 135.8 (C-6′), 136.2 (C-6), 139.0 (C-3), 144.9 (C-9), 149.1 (C-5), 153.1 (C-7), 154.7 (C-2), 163.2 (C-4′), 179.3 (C-4), 188.7 (CHO). HRESIMS (+) *m/z* $[M+H]^+$ 417.1201 (calcd for $C_{21}H_{21}O_9$, 417.1179), $[M+Na]^+$ 439.1015 (calcd for $C_{21}H_{20}O_9Na$, 439.0999).

5.4.14. 3′-Hydroxymethyl-5-hydroxy-3,6,7,8,4′-pentamethoxy-flavone 19

A solution of compound **18** (16 mg, 0.04 mmol) in CH_2Cl_2 (2 mL) was diluted to 10 mL with MeOH, added with $NaBH_4$ in excess, then stirred for 30 min at rt. The mixture was taken up with water at pH 4, then extracted with CH_2Cl_2. Standard work-up of the organic phase, then crystallization of the residue with MeOH gave pure compound **19** (15 mg, 94%). Compound **19** bright-yellow crystals: mp 171–174 °C; ^{1}H NMR ($CDCl_3$) δ ppm 3.87 (s, 3H, OMe-3), 3.95 and 3.96 (2s, 9H, OMe-6, 8 and 4′), 4.10 (s, 3H, OMe-7), 4.77 (s, 2H, CH_2OH), 7.02 (d, J = 8.8 Hz, 1H, H-5′), 8.14 (d, J = 2.3 Hz, 1H, H-2′), 8.15 (dd, J = 8.8 and 2.3 Hz, 1H, H-6′), 12.38 (s, 1H, 5-OH). ^{13}C NMR ($CDCl_3$) δ ppm 55.6 (OMe-4′), 60.1 (OMe-3), 61.1 (OMe-6 and 8), 61.7 (CH_2OH), 62.1 (OMe-7), 107.5 (C-10), 110.4 (C-5′), 122.9 (C-1′), 128.7 (C-2′), 129.6 (C-3′), 129.9 (C-6′), 132.8 (C-8), 136.1 (C-6), 138.7 (C-3), 144.9 (C-9), 149.1 (C-5), 152.9 (C-7), 155.9 (C-2), 159.6 (C-4′), 179.3 (C-4). ESIMS (+) *m/z* 419 $[M+H]^+$, 441 $[M+Na]^+$.

5.4.15. 3′-Aminomethyl-5-hydroxy-3,6,7,8,4′-pentamethoxy-flavone 20

A solution of compound **18** (21 mg, 0.05 mmol) in 10 mL MeOH was added with 15 equiv NH_4OAc (77 mg, 0.75 mmol) and $NaBH_3CN$ in excess, then stirred for 20 h at rt. Standard work-up of the reaction, then purification of the residue by tlc (silica gel, CH_2CH_2–MeOH 96.5–3.5) led to pure aminoflavone **20** (7 mg) in 34% yield. Compound **20** Amorphous; ^{1}H NMR ($CDCl_3$) δ ppm 3.86 (s, 3H, OMe-3), 3.90, 3.92 and 3.95 (3s, 9H, OMe-6, 8 and 4′), 3.94 (s, 2H, CH_2NH_2), 4.08 (s, 3H, OMe-7), 7.00 (d, J = 8.8 Hz, 1H, H-5′), 8.11 (dd, J = 8.8 and 2.3 Hz, 1H, H-6′), 8.15 (d, J = 2.3 Hz, 1H, H-2′), 12.38 (br s, 1H, 5-OH). ^{13}C NMR ($CDCl_3$) δ ppm 48.4 (CH_2NH_2), 55.6 (OMe-4′), 60.1 (OMe-3), 61.1, 61.7 and 62.0 (OMe-6, 7 and 8), 107.4 (C-10), 110.3 (C-5′), 122.7 (C-1′), 126.9 (C-3′), 129.3 (C-6′), 130.0 (C-2′), 132.8 (C-8), 136.1 (C-6),

138.2 (C-3), 144.9 (C-9), 149.1 (C-5), 152.8 (C-7), 156.1 (C-2), 159.9 (C-4′), 179.2 (C-4). HRESIMS (+) *m/z* $[M+Na]^+$ 440.1319 (calcd for $C_{21}H_{23}NO_8Na$, 440.1315).

5.4.16. 3′-Dimethylaminomethyl-5-hydroxy-3,6,7,8,4′-pentamethoxy-flavone 21

From **18** (21 mg), same work-up than for compound **20**, but with 6 equiv $(CH_3)_2NH$, HCl in place of NH_4OAc. Purification of the dried residue of the reaction by tlc (silica gel, CH_2CH_2–MeOH 94–6), led to **21** (5 mg) in 23% yield. Compound **21** Amorphous; ^1H NMR ($CDCl_3$) δ ppm 2.37 (s, 6H, $N(CH_3)_2$) 3.60 (s, 2H, C-3′-CH_2–), 3.87 (s, 3H, OMe-3), 3.94 (s, 3H, OMe-4′), 3.95 (s, 6H, OMe-6 and 8), 4.10 (s, 3H, OMe-7), 7.02 (d, *J* = 8.8 Hz, 1H, H-5′), 8.12 (dd, *J* = 8.8 and 2.3 Hz, 1H, H-6′), 8.13 (d, *J* = 2.3 Hz, 1H, H-2′), 12.42 (s, 1H, 5-OH). ^{13}C NMR ($CDCl_3$) δ ppm 45.2 ($N(CH_3)_2$), 55.5 (OMe-4′), 58.1 (C-3′–CH_2–), 60.0 (OMe-3), 61.2, 61.8 and 62.0 (OMe-6, 7 and 8), 107.7 (C-10), 110.9 (C-5′), 122.8 (C-1′), 126.7 (C-3′), 130.8 and 131.2 (C-2′ and 6′), 132.9 (C-8), 136.2 (C-6), 138.7 (C-3), 149.2 (C-5), 153.1 (C-7), 160.7 (C-4′); C-2, C-4 and C-9 not detected. ESIMS (+) *m/z* 446 $[M+H]^+$, 468 $[M+Na]^+$.

5.4.17. 3′-Cyano-5-hydroxy-3,6,7,8,4′-pentamethoxy-flavone 22

A solution of **18** (25 mg, 0.06 mmol) in anhydrous formic acid (5 mL) was added with 1.9 equiv NH_2OH, HCl (8 mg), then heated at reflux for 2 h. Standard work-up of the reaction gave a dried residue, which was purified by crystallization with MeOH, then flash chromatography (silica gel, CH_2CH_2–MeOH 99.5–0.5) to provide pure **22** (14 mg) in 56% yield. Compound **22** bright-yellow crystals: mp 148–149 °C; ^1H NMR ($CDCl_3$) δ ppm 3.91 (s, 3H, OMe-3), 3.94 (s, 6H, OMe-6 and 8), 4.03 (s, 3H, OMe-4′), 4.11 (s, 3H, OMe-7), 7.16 (d, *J* = 8.8 Hz, 1H, H-5′), 8.39 (dd, *J* = 8.8 and 2.3 Hz, 1H, H-6′), 8.40 (d, *J* = 2.3 Hz, 1H, H-2′), 12.17 (s, 1H, 5-OH). ^{13}C NMR ($CDCl_3$) δ ppm 56.5 (OMe-4′), 60.4 (OMe-3), 61.2, 61.8 and 62.2 (OMe-6, 7 and 8), 102.7 (C-3′), 107.5 (C-10), 111.7 (C-5′), 115.7 (CN), 123.6 (C-1′), 132.9 (C-8), 134.0 and 134.6 (C-2′ and 6′), 136.4 (C-6), 139.2 (C-3), 144.8 (C-9), 149.2 (C-5), 153.3 (C-7), 162.7 (C-4′), 179.2 (C-4); C-2 not detected. HRESIMS (+) *m/z* $[M+H]^+$ 414.1210 (calcd for $C_{21}H_{20}NO_8$, 414.1183), $[M+Na]^+$ 436.1032 (calcd for $C_{21}H_{19}NO_8Na$, 436.1003).

5.4.18. 4′-Benzyloxy-5-hydroxy-3,6,7,8-tetramethoxy-flavone (calycopterin 4′O-benzyl ether) 23

A solution of calycopterin **4** (0.3 g, 0.8 mmol) in DMF (10 mL) was added with $KHCO_3$ (0.1 g, 1 mmol) and benzyl bromide (0.19 mL, 1.6 mmol) and stirred for 1.5 h at 115 °C. New amounts of $KHCO_3$ (0.05 g, 0.5 mmol) and benzyl bromide (0.095 mL, 8 mmol) were added and the reaction was carried on for 1.5 h more. The reaction was diluted with CH_2Cl_2, filtered, and concentrated to dryness. The dried residue was taken up with CH_2Cl_2, and the organic phase submitted to the standard work-up. Crystallization of the residue with MeOH gave pure compound **23** (0.268 g, 72%). Compound **23** bright-yellow crystals: mp 117–118 °C; ^1H NMR ($CDCl_3$) δ ppm 3.89 (s, 3H, OMe-3), 3.98 (s, 6H, OMe-6 and 8), 4.11 (s, 3H, OMe-7), 5.17 (s, 2H, C_6H_5–CH_2) 7.11 (d, *J* = 8.7 Hz, 2H, H-3′ and 5′), 7.35–7.47 (m, 5H, C_6H_5–CH_2), 8.16 (d, *J* = 8.7 Hz, 2H, H-2′ and 6′), 12.40 (s, 1H, 5-OH).

5.4.19. 4′-Benzyloxy-3,6,7,8-tetramethoxy-5-trifluoromethanesulfonyloxy-flavone 24

A solution of **23** (0.26 g, 0.56 mmol) in dry THF (4 mL) was added at rt under N_2 with NaHMDS (2 M) in THF (0.4 mL, 0.8 mmol), and the mixture was stirred for 15 min. $PhN(Tf)_2$ (0.4 g, 1.12 mmol) was added and the reaction mixture stirred for 17 h more. New amounts of NaHMDS (0.2 mL, 0.4 mmol) and $PhN(Tf)_2$ (0.133 g, 0.37 mmol) were added and the reaction stirred for 5 h more. The reaction was diluted with H_2O, and thoroughly extracted with CH_2Cl_2. Standard work-up of the organic phase, then purification of the dried residue by flash chromatography (alumina, CH_2Cl_2–cyclohexane 1–1) gave pure triflate **24** which was crystallized with MeOH (0.249 g, 75%). Pale-yellow crystals: mp 120–121 °C.

5.4.20. 4′-Hydroxy-3,6,7,8-tetramethoxy-flavone (5-deoxy-calycopterin) 25

A solution of **24** (0.23 g, 0.38 mmol) was heated at reflux in MeOH (20 mL) till dissolution. Pd–C 10% (0.23 g) and $HCOONH_4$ (0.29 g, 4.5 mmol) were added, and the mixture heated at reflux for 1 h. The reaction was filtered, diluted with H_2O, brought to pH 6 with 2 N aqueous HCl, and extracted with CH_2Cl_2. Standard work-up of the organic phase gave a dried residue, which provided pure flavone **25** by crystallization in MeOH (0.11 g, 80%). Compound **25** pale-yellow crystals: mp 243–245 °C; ^1H NMR (DMSO-d_6) δ ppm 3.78 (s, 3H, OMe-3), 3.90 and 3.92 (2s, 6H, OMe-6 and 8), 4.00 (s, 3H, OMe-7), 6.97 (d, *J* = 8.7 Hz, 2H, H-3′ and 5′), 7.26 (s, 1H, H-5), 7.94 (d, *J* = 8.7 Hz, 2H, H-2′ and 6′). APCIMS (+) *m/z* 359 $[M+H]^+$.

5.4.21. 3′-Nitro-3,6,7,8,4′-pentamethoxy-flavone 27

A solution of **25** (0.093 g, 0.26 mmol) in trifluoroacetic acid (5 mL) at 0 °C was added with 11.19 N HNO_3 (1 equiv) then stirred for 0.5 h. The reaction mixture was taken up in iced water, then extracted with CH_2Cl_2. Standard work-up of the organic layer afforded a dried residue of crude crystallized nitroflavone **26** (0.094 g, 90%)., Without further purification, this residue was dissolved in DMF (5 mL), added twice with K_2CO_3 (0.035 g, 0.25 mmol) and iodomethane (0.1 mL, 1.6 mmol) at *t* = 0 and 2 h, and stirred for 6 h at rt. The mixture was diluted with H_2O, brought to pH 6 with 1 N aqueous HCl, and extracted with CH_2Cl_2. Standard work-up of the organic phase gave a dried residue, which led to pure compound **27** (0.075 g, 69% from **25**) by crystallization with MeOH and tlc (alumina, CH_2Cl_2). Compound **27** pale-yellow crystals: mp 188–189 °C; ^1H NMR ($CDCl_3$) δ ppm 3.96, 3.97, 4.05 and 4.08 (4s, 15H, OMe-3, 6,7,8 and 4′), 7.25 (d, *J* = 8.8 Hz, 1H, H-5′), 7.35 (s, 1H, H-5), 8.41 (dd, *J* = 8.8 and 2.3 Hz, 1H, H-6′), 8.71 (d, *J* = 2.3 Hz, 1H, H-2′). APCIMS (+) *m/z* 418 $[M+H]^+$.

5.4.22. 5,3′-Dinitro-3,6,7,8,4′-pentamethoxy-flavone 28

A solution of **27** (0.063 g, 0.15 mmol) in trifluoroacetic acid (5 mL) at 0 °C was added with 11.19 N HNO_3 (1.25 equiv) then stirred for 2 h. Same work-up as described for **27** led by crystallization with MeOH to dinitroflavone **28** (0.011 g) in 16% yield. Compound **28** pale-yellow crystals: mp 174–176 °C; ^1H NMR ($CDCl_3$) δ ppm 3.97, 3.99, 4.08 and 4.12 (4s, 15H, OMe-3, 6, 7, 8 and 4′), 7.25 (d, *J* = 8.8 Hz, 1H, H-5′), 8.40 (dd, *J* = 8.8 and 2.3 Hz, 1H, H-6′), 8.67 (d, *J* = 2.3 Hz, 1H, H-2′). APCIMS (+) *m/z* 463 $[M+H]^+$.

5.4.23. 5,3′-Diamino-3,6,7,8,4′-pentamethoxy-flavone 29

A solution of **28** (9.5 mg, 0.02 mmol) was heated at reflux in MeOH (5 mL) till dissolution. Pd–C 10% (10 mg) and $HCOONH_4$ (14 mg, 0.22 mmol) were added, and the mixture heated at reflux for 2 h. The reaction was filtered, diluted with H_2O, brought to pH 6 with 2 N aqueous HCl, and extracted with CH_2Cl_2. Standard work-up of the organic phase gave a dried residue, which was purified by tlc (silica gel, CH_2CH_2–MeOH 98–2) to give pure diaminoflavone **29** (7 mg, 85%). Compound **29** Amorphous; ^1H NMR ($CDCl_3$) δ ppm 3.82 (s, 3H, OMe-3), 3.84 (s, 3H, OMe-6), 3.91 (s, 3H, OMe-8), 3.93 (s, 3H, OMe-4′), 4.08 (s, 3H, OMe-7), 6.90 (d, *J* = 8.8 Hz, 1H, H-5′), 7.56 (d, *J* = 2.3 Hz, 1H, H-2′), 7.62 (dd, *J* = 8.8 and 2.3 Hz, 1H, H-6′). ^{13}C NMR ($CDCl_3$) δ ppm 55.7 (OMe-4′), 59.9 and 60.2 (OMe-3 and 6), 61.2 (OMe-7), 61.8 (OMe-8), 106.3 (C-10), 110.0 (C-5′), 114.4 (C-2′), 119.8 (C-6′), 123.6 (C-1′), 130.4 (C-8), 133.3 (C-6), 136.1 (C-3′), 139.2 (C-3), 149.1 (C-4′), 151.3

(C-7); C-2, C-4, C-5 and C-9 non detected. HRESIMS (+) *m/z* $[M+H]^+$ 403.1471 (calcd for $C_{20}H_{23}N_2O_7$, 403.1499).

5.4.24. 3′-Amino-3,6,7,8,4′-pentamethoxy-flavone 30

Hydrogenation of **27** (10 mg, 0.024 mmol) under same conditions of transfer hydrogenation as described from **29**, then purification of the dried residue by tlc (silica gel, CH_2CH_2–MeOH 97.5–2.5) led to pure crystallized aminoflavone **30** (8 mg, 86%). Compound **30** bright-yellow crystals: mp 193–195 °C; ^{1}H NMR ($CDCl_3$) δ ppm 3.87, 3.95, 3.97, 4.03 and 4.05 (5s, 15H, OMe-3, 6, 7, 8 and 4′), 6.92 (d, J = 8.8 Hz, 1H, H-5′), 7.40 (s, 1H, H-5), 7.56 (d, J = 2.3 Hz, 1H, H-2′), 7.62 (dd, J = 8.8 and 2.3 Hz, 1H, H-6′). ^{13}C NMR ($CDCl_3$) δ ppm 55.6 (OMe-4′), 59.9 (OMe-3), 56.3, 61.5 and 62.1 (OMe-6, 7, 8), 99.8 (C-5), 110.1 (C-5′), 114.4 (C-2′), 119.8 (C-6′), 120.1 (C-10), 123.8 (C-1′), 136.2 (C-3′), 140.5 (C-3), 142.0 (C-8), 144.8 (C-6), 147.0 (C-9), 149.3 (C-4′), 150.9 (C-7), 156.2 (C-2), 177.8 (C-4). HRESIMS (+) *m/z* $[M+H]^+$ 388.1416 (calcd for $C_{20}H_{22}NO_7$, 388.1396).

5.4.25. 3′-Amino-3,5,6,7,8,4′-hexamethoxy-flavone 31

Hydrogenation of **10** (10 mg, 0.022 mmol) under same conditions of transfer hydrogenation as described from **29**, then purification of the dried residue by tlc (silica gel, CH_2CH_2–MeOH 97.5–2.5) led to pure crystallized aminoflavone **31** (8 mg, 86%). Compound **5** light-yellow crystals: mp 160–162 °C; ^{1}H NMR ($CDCl_3$) δ ppm 3.86 (s, 3H, OMe-3), 3.94 (2s, 6H, OMe-6 and 4′), 3.97 (s, 3H, OMe-5), 3.99 (s, 3H, OMe-8), 4.09 (s, 3H, OMe-7), 6.91 (d, J = 8.8 Hz, 1H, H-5′), 7.56 (d, J = 2.3 Hz, 1H, H-2′), 7.62 (dd, J = 8.8 and 2.3 Hz, 1H, H-6′). ^{13}C NMR ($CDCl_3$) δ ppm 55.6 (OMe-4′), 59.8 (OMe-3), 61.3–61.8 (OMe-5, 6, 7, 8), 110.1 (C-5′), 114.3 (C-2′), 119.6 (C-6′), 123.1 (C-1′), 136.0 (C-3′), 137.6 (C-8), 140.5 (C-3), 143.4 (C-6), 147.9 (C-5), 149.3 (C-4′), 151.5 (C-7), 153.5 (C-2); C-4, C-9, C-10 not detected. APCIMS (+) *m/z* 418 $[M+H]^+$.

5.4.26. 3′-Amino-5,6-dihydroxy-3,7,8,4′-tetramethoxy-flavone 32 and 3′-amino-5,8-dihydroxy-3,6,7,4′-tetramethoxy-flavone 33

A solution of **9** (33 mg, 0.075 mmol) in trifluoroacetic acid (4 mL) at 0 °C was added with 11.19 N HNO_3 (1 equiv) then stirred for 0.5 h. The reaction mixture was taken up in iced water, then extracted with CH_2Cl_2. Standard work-up of the organic layer afforded a red dried residue, which was submitted to catalytic hydrogenolysis in DMF (4 mL) under 1 atm pressure hydrogen with 10% Pd–C (35 mg) at room temperature for 1.5 h. The catalyst was separated, and the filtrate concentrated to dryness (19 mg). The purification by tlc (silica gel, CH_2CH_2–MeOH 99–1) was very difficult owing to the very near R_f of two isomers, but it allowed isolation of pure **32** and **33** in very weak yields (6% for each isomer). Compound **32** Amorphous; ^{1}H NMR ($CDCl_3$) δ ppm 3.85 (s, 3H, OMe-3), 3.95 (s, 3H, OMe-4′), 3.96 (s, 3H, OMe-8), 4.13 (s, 3H, OMe-7), 6.91 (d, J = 8.8 Hz, 1H, H-5′), 7.55 (d, J = 2.3 Hz, 1H, H-2′), 7.63 (dd, J = 8.8 and 2.3 Hz, 1H, H-6′), 12.25 (s, 1 h, 5-OH). APCIMS (+) *m/z* 390 $[M+H]^+$. Compound **33** Amorphous; ^{1}H NMR ($CDCl_3$) δ ppm 3.84 (s, 3H, OMe-3), 3.94 (s, 3H, OMe-4′), 3.97 (s, 3H, OMe-6), 4.12 (s, 3H, OMe-7), 6.90 (d, J = 8.8 Hz, 1H, H-5′), 7.54 (d, J = 2.3 Hz, 1H, H-2′), 7.63 (dd, J = 8.8 and 2.3 Hz, 1H, H-6′), 12.18 (s, 1H, 5-OH). APCIMS (+) *m/z* 390 $[M+H]^+$.

5.4.27. 3′-Amino-5,7-dihydroxy-3,6,8,4′-tetramethoxy-flavone 34

A mixture of **5** (8 mg, 0.02 mmol) and 3.5 equiv LiCl (3 mg, 0.07 mmol) in DMF was stirred under N_2 at 180 °C for 17 h. The reaction was diluted with H_2O and extracted by *n*-butanol. Concentration to dryness of the organic phase and purification of the residue by tlc (silica gel, CH_2CH_2–MeOH 98–2) provided pure amorphous **34** in 26% yield. Amorphous; ^{1}H NMR ($CDCl_3$) δ ppm 3.85 (s, 3H, OMe-3), 3.95 (s, 3H, OMe-4′), 3.98 (s, 3H, OMe-6), 4.04 (s, 3H, OMe-8), 6.91 (d, J = 8.8 Hz, 1H, H-5′), 7.55 (d, J = 2.3 Hz, 1H, H-2′), 7.63 (dd, J = 8.8 and 2.3 Hz, 1H, H-6′), 12.65 (s, 1H, 5-OH). APCIMS (+) *m/z* 390 $[M+H]^+$.

5.5. Biological evaluation

5.5.1. Cell culture

Human cell lines were purchased from ATCC or ECACC or obtained from the NCI. The human cell lines KB, MiaPaca and HepG2 were cultured in D-MEM medium supplemented with 10% fetal calf serum, in the presence of penicillin, streptomycin and fungizone in 75 cm^2 flasks under 5% CO_2, whereas all other cell lines were cultured in complete RPMI medium.

5.5.2. Cell proliferation assay

Cells (600 cells/well) were plated in 96-well tissue culture microplates in 200 μL of medium and treated 24 h later with compounds dissolved in DMSO at concentrations that ranged 0.5 nM–10 μM with a Biomek 3000 automation workstation (Beckman-Coulter). Control cells received the same volume of DMSO (1% final volume). After 72 h exposure to the drug, MTS reagent (Promega) was added and incubated for 3 h at 37 °C. Experiments were performed in triplicate: the absorbance was monitored at 490 nm and results were expressed as the inhibition of cell proliferation calculated as the ratio $[(1 - (OD_{490}\ \text{treated}/OD_{490}\ \text{control})) \times 100]$. For IC_{50} determinations (50% inhibition of cell proliferation) experiments were performed in duplicate.

5.5.3. Activation of caspases 3/7

HL60 cells (20,000 cells/well) were plated in 96-well black tissue culture microplates and treated for 24 and 48 h with compounds dissolved in DMSO. Control cells received the vehicle only and positive control cells were treated with 1 μM doxorubicine. Cells were lysed with a buffer consisting in 25 mM HEPES, pH 7.5, 0.5 mM EDTA, 0.05% NP40, 0.001% SDS and 5 mM DTT containing 50 μM Ac-DEVD-AMC (Biomol). Fluorescence was monitored (λex = 360 nm, λ_{em} = 465 nm) over a 3 h period.

5.5.4. Inhibition of tubulin polymerization assay

Sheep brain microtubule proteins were purified by two cycles of assembly/disassembly at 37 °C/0 °C in MES buffer: 100 mM MES (2-[*N*-morpholino]-ethanesulfonic acid, pH 6.6), 1 mM EGTA (ethyleneglycol-bis[β-aminoethyl ether]-*N*,*N*,*N*′,*N*′-tetraacetic acid), 0.5 mM $MgCl_2$. All samples were dissolved in DMSO. The evaluated compound (1 μL) was added to microtubular solution (150 μL) that was incubated at 37 °C for 10 min and at 0 °C for 5 min. The tubulin polymerization rate was measured by turbidimetry at 350 nm according to Zavala and Guénard's protocol[26] using deoxypodophyllotoxin as reference compound. Compounds were tested at $\approx 2 \times 10^{-5}$ M, and results were given as the percentage of IPT or as IC_{50}, calculated for the most active compounds, and also expressed in relation to deoxypodophyllotoxin (DPPT) in terms of the $IC_{50}/IC_{50\ DPPT}$ ratio.

5.5.5. Measurement of annexin-V-PE/7-AAD staining

HL60 cells (5000 cells/well in 96-well microplates) were exposed for 24 and 48 h at 37 °C under 5% CO_2 with chemicals in 100 μl complete RPMI medium. Controls received the same volume of DMSO (1% final volume). 7-AAD (6.5 μl of 1 mg/ml ethanol solution) and human recombinant annexinV-PE (6.5 μl, Bender) were added to 1 ml binding buffer consisting in 30 mM Hepes buffer, pH7.4, 420 mM NaCl and 7.5 mM $CaCl_2$ immediately prior to addition to cells. After a 20 min incubation at room temperature in the dark, cells were analyzed by flow cytometry with a Guava EasyCyte plus cytometer (Millipore). Cells were classified according to their fluorescence and results expressed as the percentage of cells in each group, calculated on 5000 events.

5.5.6. Cell cycle analysis

KB cells (25,000 cells/well in 96-well microplates) were exposed for 24 and 48 h at 37 °C under 5%CO_2 to chemicals in 100 μl complete RPMI medium. Controls received the same volume of DMSO (1% final volume). Culture media were carefully collected and gently centrifuged to collect floating cells, adherent cells harvested after addition of trypsin, mixed with the pellet of floating cells, washed with PBS and fixed in ice-cold absolute ethanol. After 2 h at 4 °C, cells were spun down by centrifugation, washed with 2% FCS in PBS and stained with 50 μg/ml propidium iodide in hypotonic buffer in the presence of RNase A (50 μg/ml) for 30 min at room temperature shielding away from light, before be analyzed by flow cytometry with a Guava Easycyte cytometer (Millipore). Cell populations were quantified using Modfit LT (Verity Software House).

Acknowledgments

We thank J.-C. Jullian for NMR measurements, and Dr. F. Gueritte for a sample of **1**.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.11.035.

References and notes

1. Beutler, J. A.; Hamel, E.; Vlietinck, A. J.; Haemers, A.; Rajan, P.; Roitman, J. N.; Cardellina, J. H., II; Boyd, M. R. *J. Med. Chem.* **1998**, *41*, 2333.
2. Fang, N.; Leidig, M.; Mabry, T. J. *Phytochemistry* **1986**, *25*, 927.
3. Lichius, J. J.; Thoison, O.; Montagnac, A.; Païs, M.; Guéritte-Voegelein, F.; Sévenet, T. *J. Nat. Prod.* **1994**, *57*, 1012.
4. Shi, Q.; Li, L.; Chang, J.-J.; Autry, C.; Kozuka, M.; Konoshima, T.; Estes, J. R.; Lin, C. M.; Hamel, E.; McPhail, A. T.; McPhail, D. R.; Lee, K.-H. *J. Nat. Prod.* **1995**, *58*, 475.
5. Lewin, G.; Maciuk, A.; Thoret, S.; Aubert, G.; Dubois, J.; Cresteil, T. *J. Nat. Prod.* **2010**, *73*, 702.
6. Tron, G. C.; Pirali, T.; Sorba, G.; Pagliai, F.; Busacca, S.; Genazzani, A. A. *J. Med. Chem.* **2006**, *49*, 3033.
7. Karrer, W. *Helv. Chim. Acta* **1934**, *17*, 1560.
8. Ratnagiriswaran, A. N.; Sehra, K. R.; Venkataraman, K. *Biochem. J.* **1934**, *28*, 1964.
9. Sreekanth, P.; Narayana, K.; Shridhar, N. B.; Avinash, B. *J. Ethnopharmacol.* **2006**, *107*, 229.
10. Quintin, J.; Roullier, C.; Thoret, S.; Lewin, G. *Tetrahedron* **2006**, *62*, 4038.
11. Quintin, J.; Buisson, D.; Thoret, S.; Cresteil, T.; Lewin, G. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3502.
12. (a) Milligan, B. *J. Org. Chem.* **1983**, *48*, 1495; (b) Uemura, S.; Toshimitsu, A.; Okano, M. *J. Chem. Soc., Perkin Trans. 1* **1978**, 1076.
13. Sarin, P. S.; Seshadri, T. R. *Tetrahedron* **1960**, *8*, 64.
14. Gupta, R. S.; Seshadri, T. R.; Sood, G. R. *Phytochemistry* **1973**, *12*, 2539.
15. Ishiyama, T.; Murata, M.; Miyaura, N. *J. Org. Chem.* **1995**, *60*, 7508.
16. Goulette, G.; Gaux, B.; Le Henaff, P. *C.R. Acad. Sci., Ser. C* **1975**, *281*, 543.
17. Zembower, D. E.; Zhang, H. *J. Org. Chem.* **1998**, *63*, 9300.
18. Zheng, X.; Meng, W.-D.; Qing, F.-L. *Tetrahedron Lett.* **2004**, *45*, 8083.
19. Stavber, G.; Zupan, M.; Stavber, S. *Tetrahedron Lett.* **2007**, *48*, 2671.
20. (a) Akama, T.; Shida, Y.; Sugaya, T.; Ishida, H.; Gomi, K.; Kasai, M. *J. Med. Chem.* **1996**, *39*, 3461; (b) Akama, T.; Ishida, H.; Shida, Y.; Kimura, U.; Gomi, K.; Saito, H.; Fuse, E.; Kobayashi, S.; Yoda, N.; Kasai, M. *J. Med. Chem.* **1997**, *40*, 1894; (c) Akama, T.; Ishida, H.; Kimura, U.; Gomi, K.; Saito, H. *J. Med. Chem.* **1998**, *41*, 2056.
21. Lecoutey, C.; Fossey, C.; Demuynck, L.; Lefoulon, F.; Fabis, F.; Rault, S. *Tetrahedron* **2008**, *64*, 11243.
22. Li, S.; Pan, M.-H.; Lai, C.-S.; Lo, C.-Y.; Dushenkov, S.; Ho, C.-T. *Bioorg. Med. Chem.* **2007**, *15*, 3381.
23. Bernard, A. M.; Ghiani, M. R.; Piras, P. P.; Rivoldini, A. *Synthesis* **1989**, 287.
24. Lopez-Lazaro, M.; Galvez, M.; Martin-Cordero, C.; Ayuso, M. J. *Stud. Nat. Prod. Chem.* **2002**, *27*, 891.
25. Rodriguez, E.; Vander Velde, G.; Mabry, T. J.; Sankara Subramanian, S.; Nair, A. G. R. *Phytochemistry* **1972**, *11*, 2311.
26. Zavala, F.; Guénard, D.; Robin, J.-P.; Brown, E. *J. Med. Chem.* **1980**, *23*, 546.