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Influence of the skeleton on the cytotoxicity of flavonoids

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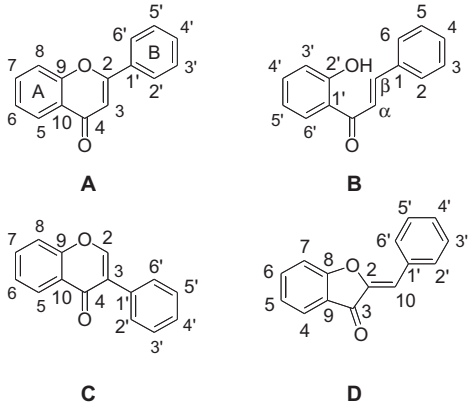
ABSTRACT

Analogs of 3′-amino-5-hydroxy-3,6,7,8,4′-pentamethoxy-flavone, a strongly cytotoxic and antimitotic semisynthetic flavone, were synthesized in the aurone, isoflavone and isoflavanone series. Comparison of the biological activity of these new compounds with the reference showed a potent cytotoxicity only in the flavone series. Influence of the hydroxy group (at C-5 in flavones, at C-4 in aurones) on the cytotoxicity, known to be favorable in flavones, was found to be detrimental in aurones. This observation was related to the hydrogen bonding formed with the carbonyl group, strong in the flavones, but of weak intensity in the aurones.

1. Introduction

Flavonoids, a widespread group, and isoflavonoids, a more limited one, are polyphenolic compounds with 15 carbons and two aromatic rings connected by a three carbon bridge. Their C_6–C_3–C_6 skeleton is derived from 1,3-diphenylpropane for flavonoids, and 1,2-diphenylpropane for isoflavonoids. These two groups include numerous members, plant secondary metabolites and synthetic analogs, which display significant cytotoxic properties.[1] Even if oxidation of the three carbons bridge to 1-propan- or propen-one units is not crucial for cytotoxicity, a carbonyl group is present in most of active compounds and seems important to increase the flavonoid activity.[1] For example, some flavones A,[2–4] chalcones B,[5–9] isoflavones C[10] (seldom), and aurones (D)[11,12] have been reported for their cytotoxicity. Structure–activity relationships within a structural type of flavonoids as well as between various structural types are known to be hard to lay down.[1] In previous studies on antiproliferative flavones, we showed that 5-hydroxy-6,7,8-trimethoxy groups on the A-ring and 3′-hydroxy-4′-methoxy or 3′-amino-4′-methoxy groups on the B-ring were substitution patterns the most favorable to the activity.[13–15]



These conclusions were in accordance with previous reports by the Sévenet[4] then Lee[3] groups of the strong cytotoxic and IPT (Inhibition of Polymerization of Tubulin) properties of 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*,[16] which remains the most antimitotic natural flavone isolated to date. So, taking as model the original synthetic flavone **2** prepared recently in the laboratory and almost as active as **1**,[13] we decided to evaluate cytotoxicity of chalcone **3**, isoflavone **4** and aurone **5**, three

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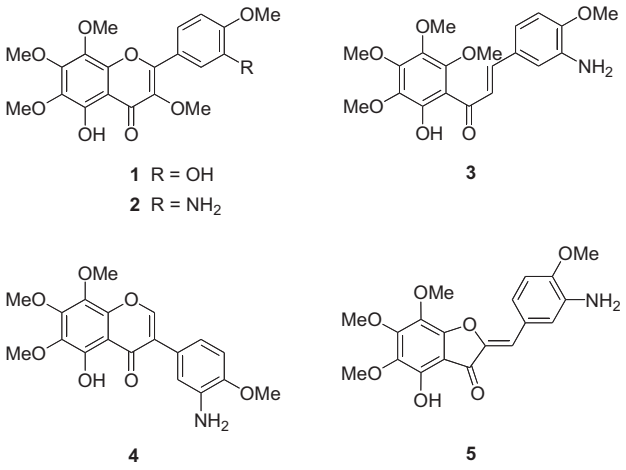
compounds with exactly the same substitution pattern on aromatic rings as **2**. Since chalcone **3** had been already obtained,[17] our study required synthesis of isoflavone **4** and aurone **5**.



1 R = OH
2 R = NH_2

3

4

5

2. Chemistry

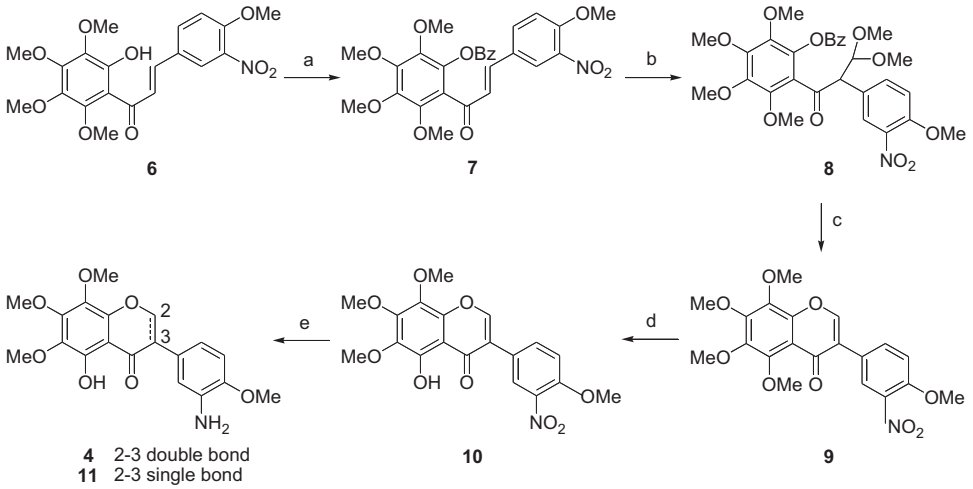
2.1. Synthesis of isoflavone 4

Access to **4** was achieved from the previously synthesized nitrochalcone **6**[17] by the following five-step process (Scheme 1): (a) benzoylation of **6** to chalcone **7**; (b) oxidative rearrangement of **7** to the dimethylacetal **8**; (c) cyclization of **8** to nitroisoflavone **9**; (d) selective demethylation of **9** at the 5-methoxy group to give **10**; (e) reduction of **10** to expected isoflavone **4** by hydrogenation of the nitro group (major), and to isoflavanone **11** by concomitant hydrogenation of the 2,3 double bond (minor). It is noteworthy that the oxidative rearrangement key step was carried out according to Kawamura et al.[18] with (diacetoxyiodo)benzene (DIB) less dangerous and providing a shorter time reaction than thallium(III) nitrate (TTN).

2.2. Synthesis of aurone 5 (Scheme 2)

Starting again from nitrochalcone **6**, which was submitted to oxidation by mercury(II) acetate in pyridine, led to the expected nitroaurone **12** (*Z* configuration thermodynamically more favorable) in excellent yield (90%).[19,20] The next step consisted of a regioselective demethylation of the C-4 methoxy group (biogenetically equivalent to C-5 in flavones or isoflavones): however, no reaction was observed with aluminium bromide or chloride, two reagents classically used for demethylation of the C-5 methoxy group of flavones or isoflavones.[21] Owing to this failure, nitroaurone **12** was reduced at the nitro group by tin(II)chloride, dihydrate in methanol to supply aminoaurone **13** as main compound (52%). A new attempt of regioselective demethylation at C-4 was then undertaken by heating **13** with lithium chloride in DMF, a system known to cleave alkylarylethers in *ortho* or *para* of electron-withdrawing substituents.[22] Two isomeric compounds were isolated (**14** and **5** in 57% and 9%, respectively), both resulting from loss of one methyl group located on the A-ring (a significant NOE correlation was still observed between OCH_3 signal at C-4′ and H-5′). In the ^{13}C NMR spectrum of the major compound **14**, comparison of the A-ring carbons signals with literature data concerning the four hydroxy-trimethoxy possible pattern substitutions in the flavone type was strongly in favor of a 6-hydroxy structure, indicating a *para* demethylation.[23] The expected 4-hydroxy structure **5** for the minor compound, corresponding to an *ortho* demethylation, will be proved correct afterwards, but it was here isolated in too small amounts for extensive structural analysis. We turned therefore to another approach to **5** by preparing an intermediate nitroaurone **17** bearing at C-4 a tosylate group, easily removable in alkaline medium.

The two first steps of this synthesis, which started again from nitrochalcone **6**, consisted of a tosylation-selective demethylation such as described by Horie et al. on acetophenones,[24] and led successively to **15** then to tosylchalcone **16**. Oxidative cyclization of **16** with mercury(II) acetate led to crystallized (*Z*)-nitroaurone **17** in 85% yield, while a careful examination of mother liquor allowed isolation of the very minor (*E*)-aurone **18** (2%). Comparison of **17** and **18** ^1H NMR spectra showed a deshielding of olefinic (0.12 ppm) and 2′,6′ (0.09 and 0.52 ppm) protons signals in the (*E*)-isomer **18**, as already mentioned in the literature.[25,26] Hydrolysis

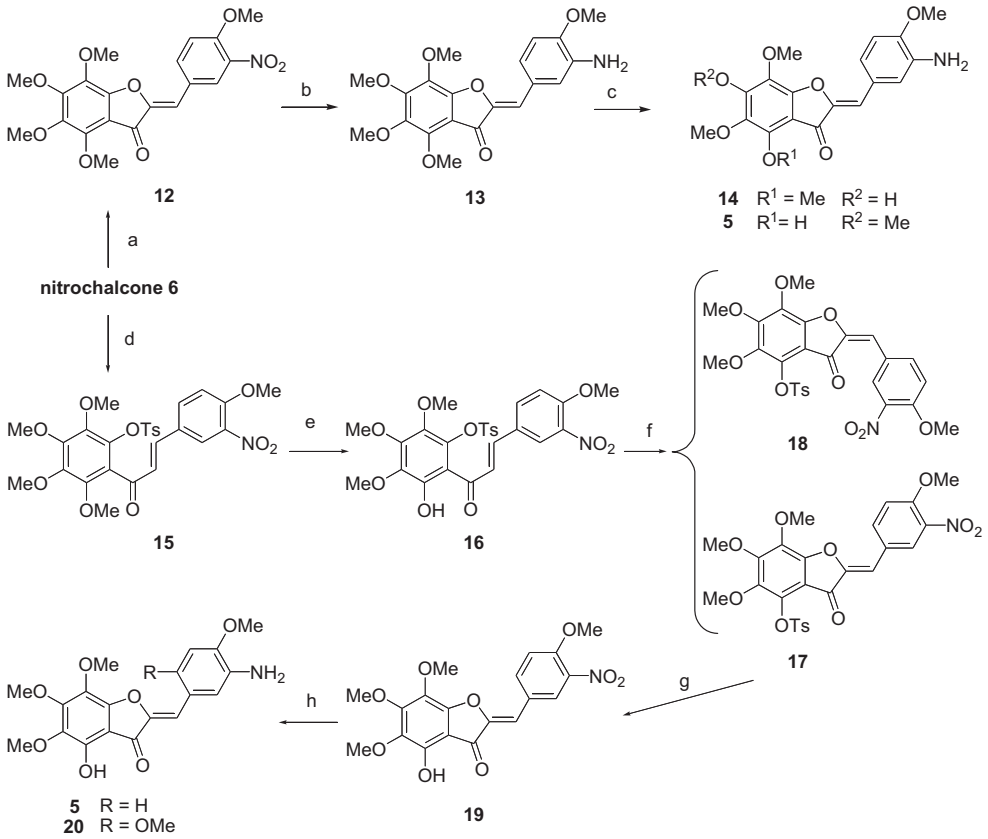


6 **7** **8**

9 **10**

4 2-3 double bond
11 2-3 single bond

Scheme 1. Reagents: (a) BzCl 1.5 equiv in pyridine, rt, 12 h, quantitative yield; (b) (diacetoxyiodo)benzene 1.5 equiv and PTSA 2 equiv in CH_2Cl_2–MeOH 1:3, rt, 60 h, quantitative yield; (c) MeOH–NaOH 1 N 1:1, 60 °C, 1 h; MeOH–HCl 1 N 6:1, reflux, 30 min, 70%; (d) $AlBr_3$ 3.1 equiv in acetonitrile, 0 °C, 30 min; addition HCl 1 N then 50 °C, 20 min, 49%; (e) H_2, Pd–C 10% in DMF, rt, 1.5 h, 35% **4** and 16% **11**.



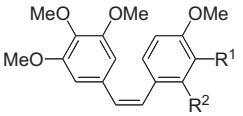
Scheme 2. Reagents: (a) $Hg(OAc)_2$ 1.9 equiv in pyridine, 60 °C, 2.5 h, 90%; (b) $SnCl_2$, 2 H_2O 5 equiv in MeOH, reflux, 8 h, 52%; (c) LiCl 3.3 equiv in DMF, 180 °C, 3 h, 57% **14** and 9% **5**; d) TsCl 1.5 equiv and K_2CO_3 10 equiv in acetone, reflux, 1.5 h, quantitative yield; (e) $AlBr_3$ 2 equiv in acetonitrile, 0 °C, 2 h; addition HCl 1 N then 0 °C, 20 min, 53%; (f) $Hg(OAc)_2$ 1.9 equiv in pyridine, 60 °C, 2.5 h, 85% **17** and 2% **18**; (g) K_2CO_3 12 equiv in MeOH, reflux, 1 h, quantitative yield; (h) $SnCl_2$, 2 H_2O 5 equiv in MeOH, reflux, 4 h, 41% **5** and 7% **20**.

of the tosylate group of **17** resulted in quantitative recovery of **19**, which was reduced by tin(II)chloride, dihydrate to expected aurone **5** (41%) and unexpected aurone **20** as minor compound (7%). Lack of a deshielded hydroxy-4 signal near 11–12 ppm in the ^1H NMR spectra of **19** and **5**, and the observation of a decreased R_f (TLC on silicagel) for aurone **5** versus the 4-methoxy analog **13** proved unambiguously the already reported weakness of the hydrogen bond between the 4-hydroxy group and the carbonyl in the aurone series.[27] That property which differentiates markedly 4-hydroxyaurones from 5-hydroxy-flavones, -flavanones, -isoflavones and 2′-hydroxychalcones (all displaying a typical strong hydrogen bond) accounts certainly for the above mentioned resistance of aurone **12** to demethylation reagents such as aluminium bromide or chloride. Structural analysis (MS, ^1H NMR) of the minor aurone **20** proved it to be the 6′-methoxy analog of **5**. In our opinion, this compound must result from a Bamberger rearrangement of the intermediate *N*-phenylhydroxylamine **21** (Scheme 3).

This rearrangement is known to provide generally *para*-aminophenols from *N*-phenylhydroxylamines with strong aqueous acid.[28] Under our conditions of reaction (weak acidity, methanol as solvent), rearrangement of **21** was only secondary versus expected reduction to amino group and led by O-protonation of hydroxylamine function, loss of water and nucleophilic attack by methanol at C-6′ to the *para*-methoxyaniline **20**.

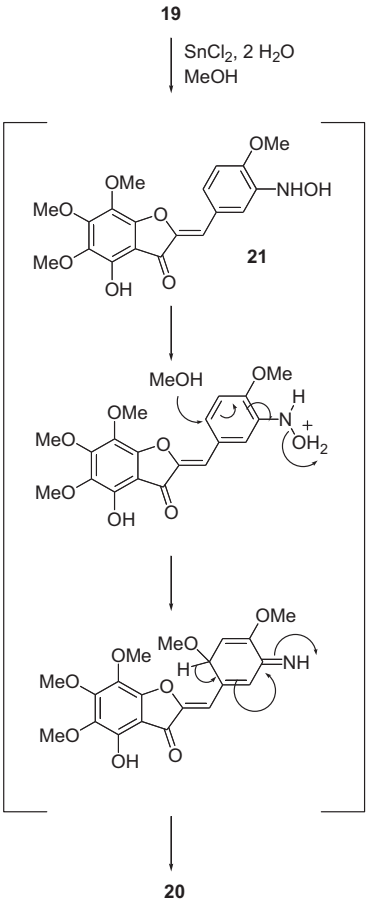
2.3. Synthesis of 22–24, three 4-unsubstituted analogs of aurone 5

In 2003, Lawrence et al. synthesized a series of aurones, including **22**, a strong enough cytotoxic compound with IPT property.[11] Aurone **22** displays the same substitution pattern as combretastatin A4 **25**, a powerful natural inhibitor of tubulin assembly now under clinical investigation in the cancer field as its phosphate prodrug. Two other combretastatins, deoxyaminocombretastatin **26** and combretastatin A1 (hydroxycombretastatin A4) **27**, possess the same activity profile and are also at present under clinical trials, as prodrugs (serine amide for **26**, diphosphate for **27**).[29] By analogy with the three different substitution patterns of **25–27**, we decided to prepare the Lawrence's aurone **22**, but also its original deoxyamino and hydroxy analogs **23** and **24** in order to include them in the biological evaluation.



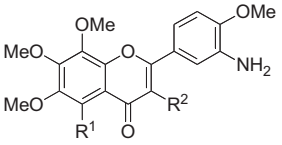
R^1 = OH R^2 = H **25**
R^1 = NH_2 R^2 = H **26**
R^1 = OH R^2 = OH **27**

According to scheme 4, these three aurones were synthesized from benzofuranone **28**,[11] which was alumina-mediated condensed with the adequately substituted benzaldehyde[30]: isovanillin for **22**, 4-methoxy-3-nitrobenzaldehyde for **23** and 2,3-bis-[(*tert*-butyldimethylsilyl)-oxy]-4-methoxybenzaldehyde[31] for **24**. Aurone **22** was then obtained in one step (31%), aurone **23** in two steps (36%) via nitroaurone **29**, subsequently reduced by



Scheme 3.

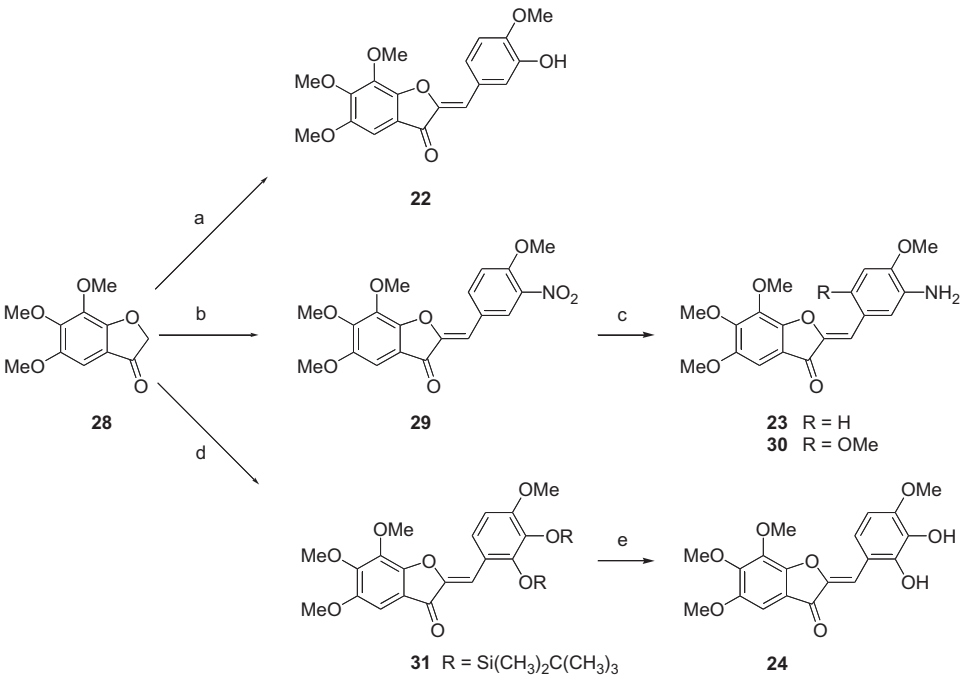
tin(II)chloride, dihydrate (the Bamberger rearrangement aurone **30** was also isolated in 7% yield), and aurone **24** in two steps (16%) after cleavage of the two silyl ether groups of the intermediate aurone **31**.



R^1 = OMe	R^2 = OMe	**32**
R^1 = H	R^2 = OMe	**33**
R^1 = OH	R^2 = H	**34**
R^1 = OMe	R^2 = H	**35**

3. Biology

In a first time, the antiproliferative effect of thirteen of the synthesized compounds in this study (ten aurones, two isoflavones, one isoflavanone), as well as six amino derivatives (flavones **2**, **32**–**35** and chalcone **3**) previously prepared in the laboratory,[13,15,17] was evaluated in a same set of experiments on KB human buccal carcinoma cells (Table 1). The comparison of aurone **5**, isoflavone **4**, isoflavanone **11**, flavones **2** and **34**, all bearing the same substitution pattern on aromatic rings, pointed out the two flavones **2** and **34** as the only strongly antiproliferative compounds. The enhanced response of **2** versus **34** confirmed the favorable effect of the 3-methoxy group on the activity.[1,2] Complete lack of activity of aurone **5** was unexpected and not in agreement with profile of **22**, previously described[11] and confirmed in our experiments as strongly cytotoxic. This discrepancy led us to explore the influence of the substituent group at C-5 or C-4 (in flavones



Scheme 4. Reagents: (a) isovanillin 1.1 equiv, basic alumina in CH_2Cl_2, rt, 48 h, 31%; (b) 4-methoxy-3-nitrobenzaldehyde 1.1 equiv, basic alumina in CH_2Cl_2, rt, 2 h, 81%; (c) $SnCl_2$, 2 H_2O 5 equiv in MeOH, reflux, 6 h, 45% **23** and 9% **30**; (d) 2,3-bis-[(*t*-butyldimethylsilyl)-oxy]-4-methoxybenzaldehyde 1.1 equiv, basic alumina in CH_2Cl_2, rt, 20 h, 23%; (e) TBAF 2.5 equiv in THF, rt, 15 min, 70%.

Table 1
Antiproliferative and IPT activities of synthesized compounds

Compd	Cytotoxicity on KB cells[a] IC_{50} (μM)	Cytotoxicity on HL60 cells[a] IC_{50} (μM)	IPT activity[b]
Aurones			
5	0		
13	0		
14	0		
19	0		
20	0		
22	100; IC_{50} = 0.11	IC_{50} = 0.12	21% inhibition
23	98; IC_{50} = 0.4	IC_{50} = 0.18	33% inhibition
24	nd	~100	8.6 μM (4)[c]
29	0		12% inhibition
30	0		12% inhibition
Isoflavones, *Isoflavanone*			
4	0		
10	18		
11	4		
Flavones			
2	99; IC_{50} = 0.025	IC_{50} = 0.055	9 μM (4)[c]
32	17		
33	84; IC_{50} = 0.078		
34	96; IC_{50} = 0.16		
35	6		
Chalcone			
3	11		

[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, and as IC_{50} (μM), calculated only for the most active compounds.
[b] Results are expressed as the percentage of IPT at ≈2 10^{-5} M, or as IC_{50} (μM).
[c] $IC_{50\ compound}/IC_{50\ deoxypodophyllotoxin}$.

or aurones numbering, respectively) on the cytotoxicity. Analysis of the biological response of 5 (or 4)-hydroxy, methoxy and unsubstituted derivatives in both structural types revealed a marked difference. In the flavone series, 5-hydroxylation is highly favorable (**2**, **34**) such as lack of substitution (**33** is only slightly less active than **2**), while 5-methoxylation (**32**, **35**) is highly detrimental to the activity, as previously reported.[13,32] In the aurone series, a positive response was still observed with 4-unsubstituted compounds (**23** and the reference **22**),[33] but 4-hydroxy and methoxy analogs **5** and **13** were both devoid of activity. We believe that the distinct behavior of the 5-hydroxyflavone **2** and the 4-hydroxyaurone **5** can be related to the nature of the intramolecular hydrogen bond of the *ortho*-hydroxy carbonyl group. In flavones, the strong intramolecular hydrogen bonding increases the lipophilic character, allowing a higher membrane permeability, which consequently accounts for the better activity of **2** compared to 5-unsubstituted analog **33**. On the contrary in the aurone series, the abovementioned weakness of this intramolecular hydrogen bonding makes **5** much polar than 4-unsubstituted analog **23**, which could explain (at least in part) loss of activity. So in our opinion, the well known positive influence on the cytotoxicity of the 5-hydroxy group in the flavone series[1,2] seems to be closely related to the strength of the intramolecular bond formed with the carbonyl group. Lastly about aurones, examination of 4-unsubstituted structures **22**, **23** and **30** indicates a slightly better cytotoxicity for 3′-hydroxy derivative **22** versus aminoaurone **23** (such as in the flavone series with **1** and **2**)[13] while an additional methoxy group at C-6′ removes the activity (**23** vs **30**). Evaluation of the cytotoxicity of 4-unsubstituted aurones **22**–**24** versus flavone **2** was also undertaken on HL60 human leukemia cells, which confirmed the better activity of flavone **2** over aurones **22**, **23**. Activity of **24** on this non adhesive cell line was measurable, and proved to be very weak (Table 1). The cytotoxic activity of flavone **2** was accompanied in HL60 by the externalization of phosphatidyl serine, an apoptosis landmark (Table 2) which was evident after a 24 h exposure and still increased at 48 h. Aurones **22–24** had only a modest effect upon apoptosis. Since flavone **2** is know to inhibit tubulin assembly,[13] we carried out, according to the Zavala and Guénard's method,[34] a comparative evaluation of the IPT effect of the five 4-unsubstituted aurones **22**–**24**, **29** and **30** versus **2**. As stated in Table 1, the good IPT property of flavone **2** was confirmed, and aurone **24** was slightly more active than **2** (a weak IPT effect was also observed with aminoaurone **23**). The effect of aurones **22**–**24** versus flavone **2** upon the cell cycle (on HL60 cell) was then evaluated (Table 3): only aurone **24** and flavone **2** (to a less extent) clearly showed to cause a blockade of cell cycle in phase G2/M after 24 h of treatment, in agreement with conclusions of IPT experiments, similar to the blockade elicited by DPPT. After 48 h cells entered the apoptotic Sub G1 phase, in contrast with aurones **22–23** which had no effect on the cell cycle and promote a modest apoptosis at 24 and 48 h. Similar data were collected on KB cells (data

Table 2
Early and late apoptosis in HL60 cells treated with **2**, **22**, **23** and **24**[a]

Compd	Viable [b]	% Cells	
		In early apoptosis [c]	In late apoptosis [d]
24 h			
Control	90.8	7.5	0.8
Doxorubicin (100 nM)	81.0	17.8	0.9
2 (100 nM)	74.2	22.8	2.1
22 (100 nM)	81.1	16.8	1.1
23 (100 nM)	85.4	12.1	1.5
24 (100 nM)	84.1	12.9	1.9
48 h			
Control	83.9	14.7	0.6
Doxorubicin (100 nM)	29.8	68.0	1.9
2 (100 nM)	56.3	41.0	1.9
22 (100 nM)	73.3	24.9	1.1
23 (100 nM)	81.7	16.4	1.1
24 (100 nM)	77.1	21.1	0.8

[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 positive.

Table 3
Cell cycle analysis of HL60 cells treated with **2**, **22**, **23** and **24**[a]

Compd	% Cells in			
	Sub G1	G0/G1	S	G2/M
24 h				
Control	2.3	50.6	22.3	24.8
DPPT[b] (20 nM)	7.7	25.2	9.5	57.6
2 (100 nM)	8.9	40.3	17.5	33.3
22 (100 nM)	14.4	40.3	20.6	24.7
23 (100 nM)	13.9	39.0	18.7	28.4
24 (100 nM)	14.0	9.8	23.7	52.5
48 h				
Control	4.0	51.2	20.9	23.9
DPPT (20 nM)	34.1	23.5	37.7	29.8
2 (100 nM)	19.1	33.1	26.5	21.3
22 (100 nM)	21.1	35.1	25.6	18.2
23 (100 nM)	14.0	42.6	22.5	20.8
24 (100 nM)	23.2	31.4	28.2	17.2

[a] Expressed as the percentage of HL60 cells in the various mitotic phases.
[b] DPPT: deoxypodophyllotoxin.

not shown). Concerning aurone **24**, a discrepancy between low cytotoxicity/apoptosis and IPT/cell cycle marked effects is to be noted. This observation that we cannot explain reminds us of combretastatin A1 **27** (same substitution pattern as **24**), which displays an as potent IPT effect as combretastatin A4 **25** in spite of a very low cytotoxicity on some cell lines (10^2–10^3 less active than **25**).

4. Conclusion

Unlike many structure–activity relationships (SARs) studies which are based on the variation of substituents of a same skeleton, our purpose consisted in a comparative evaluation of several chemical skeletons (aurone, isoflavone, isoflavanone, flavone, 2′-hydroxychalcone) having a same substitution pattern, known to be very favorable to cytotoxicity in the flavone series.[35] Comparison of the biological responses clearly demonstrated that this activity was no more present, or only at a small extent, in the other structural types. Important role of the intramolecular bonding related to the 5-hydroxy substituent of flavones was also pointed out. These conclusions were established for a cytotoxicity related to an IPT effect. Of course, they cannot be extended to any cytotoxic effect, since other mechanisms than IPT would lead certainly to other SARs.

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), and preparative tlc with 60 F 254 silica gel (5715 Merck).

5.2. Synthesis of isoflavone 4 from nitrochalcone 6

5.2.1. 1-(6-Benzoyloxy-2,3,4,5-tetramethoxyphenyl)-2-(4-methoxy-3-nitro)-3,3-dimethoxypropan-1-one 8

A solution of **6** (100 mg, 0.238 mmol) in pyridine (1 mL) at 0 °C was added with benzoyl chloride (54 mg, 0.38 mmol) then left at room temperature for 12 h. The reaction mixture was taken up in iced water, then extracted with Et_2O. Standard work-up of the organic layer afforded an amorphous residue of **7** (124 mg, quantitative yield), pure according to tlc (silica gel, CH_2CH_2–MeOH 99:1). A solution of **7** (78 mg, 0.15 mmol) in 12 mL of CH_2Cl_2–MeOH 1:3 was added with (diacetoxyiodo)benzene (75 mg, 0,23 mmol), then *p*-toluene sulfonic acid (57 mg, 0.3 mmol, in solution in 2 mL MeOH). The mixture was stirred for 60 h at rt, then taken up in iced water and extracted with CH_2Cl_2 to give a quantitative residue of pure **8** (87 mg). Amorphous; ^{1}H NMR ($CDCl_3$) δ ppm 3.11, 3.26 [2s, 6H, CH(OCH_3)$_2$], 3.66, 3.77, 3.89, 3.91 and 3.97 (5s, 15H, 5 aromatic methoxy groups), 4.64 and 4.88 [2d, J = 8.1 Hz, C*H*–C*H*(OCH_3)$_2$], 6.88 (d, J = 8.7 Hz, 1H, H-5′), 7.4–7.7 (m, 5H, benzoyl group), 7.84 (d, J = 2.3 Hz, 1H, H-2′), 8.08 (dd, J = 8.7 and 2.3 Hz, 1H, H-6′).

5.2.2. 3′-Nitro-5,6,7,8,4′-pentamethoxy-isoflavone 9

A solution of **8** (85 mg, 0.145 mmol) in MeOH–NaOH 1 N 1:1 (10 mL) was heated for 1 h at 60 °C under N_2, then brought to pH 1 with HCl 2 N and extracted with CH_2Cl_2. Standard work-up of the organic layer, then concentration to dryness afforded a residue which was heated at reflux for 0.5 h in MeOH–HCl 1 N 6:1 (7 mL). The reaction mixture was brought to pH 9, then thoroughly extracted with CH_2Cl_2. Standard work-up of the organic layer, then crystallization of the dried residue in MeOH led to pure nitroisoflavone **9** (43 mg, 70%). **9** Pale-yellow crystals: mp 146–148 °C; ^{1}H NMR ($CDCl_3$) δ ppm 3.90, 3.91, 3.93, 3.94 and 4.10 (5s, 15H, OMe-5, 6, 7, 8, 4′), 7.15 (d, J = 8.8 Hz, 1H, H-5′), 7.80 (dd, J = 8.8 and 2.3 Hz, 1H, H-6′), 7.97 (s, 1H, H-2), 8.02 (d, J = 2.3 Hz, 1H, H-2′). ESIMS (+) m/z 440 [M+Na]$^+$.

5.2.3. 5-Hydroxy-3′-nitro-6,7,8,4′-tetramethoxy-isoflavone 10

A stirred solution of **9** (40 mg, 0.096 mmol) in acetonitrile (3 mL) was cooled at 0 °C, then added with 0.5 M $AlBr_3$ in acetonitrile (0.6 mL, 0.3 mmol). After 30 min stirring, 0.35 mL HCl 1 N was added, then the resulting mixture was heated at 50 °C for 20 min. The reaction mixture taken up with water, adjusted to pH 6 with 0.1 N aqueous NaOH, and extracted with CH_2Cl_2. Standard work-up of the organic layer, purification of the dried residue by flash chromatography (silica gel, CH_2CH_2–MeOH 99.5:0.5), then crystallization with MeOH led to the pure 5-*O*-demethylated analog **10** (19 mg, 49%). Yellow crystals: mp: 151–153 °C (MeOH); ^{1}H NMR ($CDCl_3$) δ ppm 3.91, 3.92, 4.00, 4.13 (4s, 12H, OMe-6, 7, 8, 4′), 7.17 (d, J = 8.8 Hz, 1H, H-5′), 7.76 (dd, J = 8.8 and 2.3 Hz, 1H, H-6′), 8.04 (d, J = 2.3 Hz, 1H, H-2′ and s, 1H, H-2), 12.35 (s, 1H, OH). ESIMS (+) m/z 426 [M+Na]$^+$.

5.2.4. 3′-Amino-5-hydroxy-6,7,8,4′-tetramethoxy-isoflavone 4 and 3′-amino-5-hydroxy-6,7,8,4′-tetramethoxy-isoflavanone 11

A solution of **10** (17 mg, 0.042 mmol) in DMF (4 mL) was hydrogenated under 1 atm pressure hydrogen with 10% Pd–C (15 mg) at room temperature for 1.5 h. The catalyst was separated and the filtrate concentrated to a dried residue, which was purified by flash chromatography (silica gel, CH_2CH_2–MeOH 99.25–0.75) to provide 13 mg of a residue, homogeneous in tlc. As ^{1}H NMR spectrum of this product proved it to be a mixture of two compounds; their separation by tlc (polyamide, MeOH–H_2O 1:1) allowed isolation of expected **4** (5.5 mg) and **11** (2.5 mg). **4** (amorphous); ^{1}H NMR ($CDCl_3$) δ ppm 3.87, 3.91, 3.92, 4.10 (4s, 12H, OMe-6, 7, 8, 4′), 6.8–6.95 (m, 3H, H-2′, H-5′, H-6′), 7.96 (s, 1H, H-2), 12.65 (s, 1H, OH). ESIMS (+) m/z 374 [M+H]$^+$; HRESIMS (+) m/z [M+H]$^+$ 374.1219 (calcd for $C_{19}H_{20}NO_7$, 374.1234). **11** (amorphous); ^{1}H NMR ($CDCl_3$) δ ppm 3.84, 3.85, 3.86, 4.09 (4s, 12H, OMe-6, 7, 8, 4′), 4.09 (br s, 1H, H-3), 4.55–4.65 (m, 2H, 2H-2), 6.62 (s, 1H, H-2′), 6.65 and 6.75 (2d, J = 8.4 Hz, H-5′ and H-6′) 11.88 (s, 1H, OH). ESIMS (+) m/z 376 [M+H]$^+$.

5.3. Synthesis of aurone 5 from nitrochalcone 6

5.3.1. (*Z*)-3′-Nitro-4,5,6,7,4′-pentamethoxy-aurone 12

A solution of **6** (100 mg, 0.238 mmol) in pyridine (4 mL) was added with mercury(II)acetate (144 mg, 0.45 mmol), then stirred at 60 °C for 2.5 h. The reaction mixture was taken up in iced water, then extracted with CH_2Cl_2. Standard work-up of the organic layer, then crystallization in MeOH of the resulting dried residue afforded pure compound **12** (89 mg, 90%). Yellow crystals: mp: 183–185 °C (MeOH); ^{1}H NMR ($CDCl_3$) δ ppm 3.87, 4.05, 4.15 (×2), 4.19 (4s, 15H, OMe-4, 5, 6, 7, 4′), 6.73 (s, 1H, H-10), 7.17 (d, *J* = 8.8 Hz, 1H, H-5′), 7.93 (dd, *J* = 8.8 and 2.3 Hz, 1H, H-6′), 8.55 (d, *J* = 2.3 Hz, 1H, H-2′). ESIMS (+) *m/z* 456 $[M+K]^+$, 440 $[M+Na]^+$, 418 $[M+H]^+$.

5.3.2. (*Z*)-3′-Amino-4,5,6,7,4′-pentamethoxy-aurone 13

A mixture of **12** (72 mg, 0.17 mmol) in MeOH (30 mL) was heated at reflux till dissolving, then was added with tin(II)chloride, dihydrate (195 mg, 0.85 mmol). After 8 h stirring at the same temperature, the reaction mixture was taken up with a pH 4 buffer, then thoroughly extracted with CH_2Cl_2. Standard work-up of the organic layer, then crystallization in MeOH of the resulting dried residue provided pure compound **13** (35 mg, 52%). Yellow crystals: mp: 158–160 °C (MeOH); ^{1}H NMR ($CDCl_3$) δ ppm 3.85, 3.92, 4.09 (×2), 4.17 (4s, 15H, OMe-4, 5, 6, 7, 4′), 6.73 (s, 1H, H-10), 6.85 (d, *J* = 8.7 Hz, 1H, H-5′), 7.30 (dd, *J* = 8.7 and 2.3 Hz, 1H, H-6′), 7.34 (d, *J* = 2.3 Hz, 1H, H-2′). ESIMS (+) *m/z* 410 $[M+Na]^+$.

5.3.3. (*Z*)-3′-Amino-6-hydroxy-4,5,7,4′-tetramethoxy-aurone 14 and (*Z*)-3′-amino-4-hydroxy-5,6,7,4′-tetramethoxy-aurone 5

A solution of **13** (22 mg, 0.057 mmol) in DMF (3 mL) was added with lithium chloride (8 mg, 0.19 mmol), then stirred at 180 °C for 3 h under N_2. The reaction mixture was taken up in iced water, then extracted with CH_2Cl_2. Standard work-up of the organic layer gave a dried residue, which was purified by tlc (silica gel, cyclohexane–acetone 2:1) to provide a major compound **14** (12 mg, 57%) and a minor one **5** (2 mg, 9%). **14** Yellow crystals: mp: 185–187 °C (MeOH); ^{1}H NMR ($CDCl_3$) δ ppm 3.91 (s, 3H, OMe-4′), 3.93 (s, 3H, OMe-5), 4.14 (s, 3H, OMe-7), 4.18 (s, 3H, OMe-4), 6.71 (s, 1H, H-10), 6.85 (d, *J* = 8.2 Hz, 1H, H-5′), 7.28 (dd, *J* = 8.2 and 2.0 Hz, 1H, H-6′), 7.29 (d, *J* = 2.0 Hz, 1H, H-2′); ^{1}H NMR (DMSO-d_6) δ ppm 3.72 (s, 3H, OMe-5),3.84 (s, 3H, OMe-4′), 3.98 (s, 3H, OMe-7), 4.04 (s, 3H, OMe-4), 6.57 (s, 1H, H-10), 6.94 (d, *J* = 8.2 Hz, 1H, H-5′), 7.17 (dd, *J* = 8.2 and 2.0 Hz, 1H, H-6′), 7.20 (d, *J* = 2.0 Hz, 1H, H-2′). ^{13}C NMR ($CDCl_3$) δ ppm 55.6 (OMe-4′), 61.3 (OMe-7), 61.8 (OMe-5), 62.5 (OMe-4), 107.3 (C-9), 110.4 (C-5′), 112.6 (C-10), 116.9 (C-2′), 123.2 (C-6′), 125.4 (C-1′), 127.7 (C-7), 135.2 (C-5), 136.4 (C-3′), 146.0 (C-2), 146.5 (C-4), 148.9 (C-4′), 149.9 (C-8), 153.7 (C-6), 180.6 (C-3) ; ^{13}C NMR (DMSO-d_6) δ ppm 55.4 (OMe-4′), 61.0 (OMe-7), 61.1 (OMe-5), 61.9 (OMe-4), 105.6 (C-9), 110.7 (C-5′), 111.7 (C-10), 115.2 (C-2′), 121.1 (C-6′), 124.6 (C-1′), 128.4 (C-7), 136.4 (C-5), 138.0 (C-3′), 145.4 (C-2), 146.6 (C-4), 148.1 (C-4′), 152.5 and 153.8 (C-6 and C-8), 179.2 (C-3). ESIMS (-) *m/z* 372 $[M-H]^+$. **5** vide infra.

5.3.4. 3-Nitro-4,2′,3′,4′,5′-pentamethoxy-6′-tosyloxy-chalcone 15

A stirred solution of **6** (377 mg, 0.9 mmol) and tosyl chloride (256 mg, 1.35 mmol) in acetone (50 mL) was added with K_2CO_3 (1.24 g, 9 mmol), then heated at reflux for 1.5 h. The cooled reaction mixture was taken up in CH_2Cl_2, filtered and concentrated to a dried residue of pure tosylchalcone **15** (504 mg, quantitative yield). **15** Amorphous; ^{1}H NMR ($CDCl_3$) δ ppm 2.38 (s, 3H, CH_3 of tosyl group 3.77, 3.80, 3.94, 3.98 and 4.01 (5s, 15H, OMe-4, 2′, 3′, 4′, 5′), 6.70 (d, *J* = 16 Hz, 1H, Hα), 7.12 (d, *J* = 8.7 Hz, 1H, H-5′), 7.21 (d, *J* = 8.7 Hz, 2 H, tosyl protons), 7.24 (d, *J* = 16 Hz,1H, Hβ), 7.69 (dd, *J* = 8.7 and 2.3 Hz, 1H, H-6′), 7.75 (d, *J* = 8.7 Hz, 2 H, tosyl protons) 7.96 (d, *J* = 2.3 Hz, 1H, H-2′).

5.3.5. 2′-Hydroxy-3-nitro-4,3′,4′,5′-tetramethoxy-6′-tosyloxy-chalcone 16

A stirred solution of **15** (480 mg, 0.84 mmol) in acetonitrile (40 mL) was cooled at 0 °C, then added with 0.5 M $AlBr_3$ in acetonitrile (3.4 mL, 1.7 mmol). After 2 h reaction time, the mixture was taken up with iced water, adjusted to pH 1 with HCl 1 N, stirred at 0 °C for 20 min more, then thoroughly extracted with CH_2Cl_2. Standard work-up of the organic layer, then purification of the resulting dried residue by flash chromatography (silica gel, CH_2CH_2–MeOH 99.75:0.25), then crystallizaton with MeOH led to the pure 2′-*O*-demethylated tosylchalcone **16** (250 mg, 53%). Bright-yellow crystals: mp: 137–139 °C (MeOH); ^{1}H NMR ($CDCl_3$) δ ppm 2.36 (s, 3H, CH_3 of tosyl group), 3.71, 3.94, 4.04, 4.05 (4s, 12H, OMe-4, 3′, 4′, 5′), 7.14 (d, *J* = 8.8 Hz, 1H, H-5′), 7.17 (d, *J* = 8.7 Hz, 2 H, tosyl protons), 7.35 (d, *J* = 16 Hz, 1H, Hα), 7.56 (d, *J* = 16 Hz, 1H, Hβ), 7.70 (d, *J* = 8.7 Hz, 2 H, tosyl protons), 7.73 (dd, *J* = 8.8 and 2.3 Hz, 1H, H-6′), 7.99 (d, *J* = 2.3 Hz, 1H, H-2′), 12.33 (s, 1H, OH). ESIMS (+) *m/z* 582 $[M+Na]^+$

5.3.6. (*Z*)-3′-Nitro-5,6,7,4′-tetramethoxy-4-tosyloxy-aurone 17 and (*E*)-3′-nitro-5,6,7,4′-tetramethoxy-4-tosyloxy-aurone 18

From **16** (154 mg, 0.275 mmol), same work-up than for cyclization of **6** into **12** led to **17** (130 mg, 85%) by final crystallization of the dried residue with MeOH. Purification of the mother liquor by tlc (silica gel, CH_2CH_2–MeOH 99–1) provided the (*E*)-isomer **18** (3 mg, 2%). **17** Yellow crystals: mp: 169–170 °C (MeOH); ^{1}H NMR ($CDCl_3$) δ ppm 2.47 (s, 3H, CH_3 of tosyl group, 3.76, 4.03, 4.07, 4.20 (4s, 12H, OMe-5, 6, 7, 4′), 6.73 (s, 1H, H-10), 7.16 (d, *J* = 8.8 Hz, 1H, H-5′), 7.39 (d, *J* = 8.7 Hz, 2 H, tosyl protons), 7.86 (dd, *J* = 8.8 and 2.3 Hz, 1H, H-6′), 7.99 (d, *J* = 8.7 Hz, 2 H, tosyl protons), 8.55 (d, *J* = 2.3 Hz, 1H, H-2′). ESIMS (+) *m/z* 580 $[M+Na]^+$. **18** Amorphous; ^{1}H NMR ($CDCl_3$) δ ppm 2.47 (s, 3H, CH_3 of tosyl group), 3.76, 4.04, 4.07, 4.10 (4s, 12H, OMe-5, 6, 7, 4′), 6.85 (s, 1H, H-10), 7.10 (d, *J* = 8.8 Hz, 1H, H-5′), 7.39 (d, *J* = 8.7 Hz, 2 H, tosyl protons), 7.99 (d, *J* = 8.7 Hz, 2 H, tosyl protons), 8.38 (dd, *J* = 8.8 and 2.3 Hz, 1H, H-6′), 8.64 (d, *J* = 2.3 Hz, 1H, H-2′). ESIMS (+) *m/z* 580 $[M+Na]^+$.

5.3.7. (*Z*)-4-Hydroxy-3′-nitro-5,6,7,4′-tetramethoxy-aurone 19

A stirred solution of **17** (100 mg, 0.178 mmol) in MeOH (35 mL) was heated at reflux till dissolution, added with K_2CO_3 (300 mg), then left at reflux for 1 h more. The mixture was taken up with iced water, adjusted to pH 1 with HCl 1 N, then thoroughly extracted with CH_2Cl_2. Standard work-up of the organic layer afforded a dried quantitative residue of pure **19** (72 mg), which was crystallized with MeOH. **19** Bright-yellow crystals: mp: 202–204 °C (MeOH); ^{1}H NMR ($CDCl_3$) δ ppm 3.90 (s, 3H, OMe-5), 4.03 (s, 3H, OMe-4′), 4.10 (s, 3H, OMe-6), 4.11 (s, 3H, OMe-7), 6.73 (s, 1H, H-10), 7.17 (d, *J* = 8.8 Hz, 1H, H-5′), 7.33 (br s, 1H, OH), 7.93 (dd, *J* = 8.8 and 2.4 Hz, 1H, H-6′), 8.55 (d, *J* = 2.4 Hz, 1H, H-2′). ^{13}C NMR ($CDCl_3$) δ ppm 56.8 (OMe-4′), 61.5 (OMe-5), 61.5 and 61.7 (OMe-6 and OMe-7), 105.4 (C-9), 109.3 (C-10), 113.9 (C-5′), 124.9 (C-1′), 128.1 (C-2′), 131.2 (C-7), 136.1 (C-5), 136.8 (C-6′), 139.8 (C-3′), 144.6 (C-8), 147.2 (C-2), 151.6 (C-4), 153.7 (C-4′), 155.2 (C-6), 183.4 (C-3). ESIMS (+) *m/z* 426 $[M+Na]^+$.

5.3.8. (*Z*)-3′-Amino-4-hydroxy-5,6,7,4′-tetramethoxy-aurone 5 and (*Z*)-3′-amino-4-hydroxy-5,6,7,4′,6′-pentamethoxy-aurone 20

From **19** (34 mg, 0.084 mmol), same work-up than for reduction of **12** into **13** (4 h reaction time under N_2), then purification of the resulting dried residue by tlc (silica gel, CH_2CH_2–MeOH 99:1) led to **5** (13 mg, 41%) as major compound and **20** (2.5 mg, 7%) as minor

one. **5** Yellow ocher crystals: mp: 178–179 °C (MeOH); ^{1}H NMR ($CDCl_3$) δ ppm 3.89 (s, 3H, OMe-5), 3.91 (s, 3H, OMe-4′), 4.07 and 4.08 (2s, 6H, OMe-6 and OMe-7), 6.72 (s, 1H, H-10), 6.85 (d, J = 8.2 Hz, 1H, H-5′), 7.28 (dd, J = 8.2 and 2.0 Hz, 1H, H-6′), 7.30 (d, J = 2.0 Hz, 1H, H-2′). ^{13}C NMR ($CDCl_3$) δ ppm 55.6 (OMe-4′), 61.4, 61.4 and 61.7 (OMe-5, OMe-6, OMe-7), 106.0 (C-9), 110.4 (C-5′), 113.7 (C-10), 117.0 (C-2′), 123.7 (C-6′), 125.0 (C-1′), 131.2 (C-7), 135.7 (C-5), 136.5 (C-3′), 144.6 (C-8), 145.8 (C-2), 149.2 (C-4′), 151.6 (C-4), 154.8 (C-6), 184.0 (C-3). ESIMS (+) m/z 374 $[M+H]^+$; HRESIMS (+) m/z $[M+H]^+$ 374.1214 (calcd for $C_{19}H_{20}NO_7$, 374.1234) **20** Orange-colored crystals: mp: 214–218 °C (MeOH); ^{1}H NMR ($CDCl_3$) δ ppm 3.89, 3.90, 3.95, 4.07 (×2) (4s, 15H, OMe-5,6,7,3′,6′), 6.46 (s, 1H, H-10), 7.36 and 7.68 (2s, 2H, H-2′ and H-5′). ESIMS (+) m/z 404 $[M+H]^+$; HRESIMS (+) m/z $[M+H]^+$ 404.1321 (calcd for $C_{20}H_{22}NO_8$, 404.1339).

5.4. Synthesis of 4-unsubstituted aurones 22–24 from benzofuranone 28

5.4.1. (*Z*)-3′-Hydroxy-5,6,7,4′-tetramethoxy-aurone 22

A solution of benzofuranone **28** (45 mg, 0.2 mmol) and isovanillin (33 mg, 0.22 mmol) in CH_2Cl_2 (2 mL) was added with 1 g basic alumina (Brockmann activity I) and stirred for 48 h at rt. The reaction mixture was filtered and concentrated to dryness. Crystallization of the residue with MeOH provided pure aurone **22** (22 mg, 31%). **22** Golden yellow crystals: mp: 196–198 °C (MeOH) (lit 192–193 °C).[36] ^{1}H NMR ($CDCl_3$): identical to literature data.[36]

5.4.2. (*Z*)-3′-Nitro-5,6,7,4′-tetramethoxy-aurone 29

A solution of benzofuranone **28** (45 mg, 0.2 mmol) and 4-methoxy-3-nitrobenzaldehyde (41 mg, 0.22 mmol) in CH_2Cl_2 (2 mL) was added with 1 g basic alumina (Brockmann activity I) and stirred for 2 h at rt. The reaction mixture was filtered and concentrated to dryness. Crystallization of the residue with MeOH provided pure nitroaurone **29** (63 mg, 81%). **29** Golden yellow crystals: mp: 204–205 °C (MeOH); ^{1}H NMR ($CDCl_3$) δ ppm 3.88 (s, 3H, OMe-5), 4.02 (s, 3H, OMe-4′), 4.03 (s, 3H, OMe-6), 4.22 (s, 3H, OMe-7), 6.76 (s, 1H, H-10), 6.97 (s, 1H, H-4), 7.16 (d, J = 8.8 Hz, 1H, H-5′), 7.92 (dd, J = 8.8 and 2.1 Hz, 1H, H-6′), 8.57 (d, J = 2.1 Hz, 1H, H-2′). ^{13}C NMR ($CDCl_3$) δ ppm 56.4 (OMe-5), 56.7 (OMe-4′), 61.2 (OMe-7), 61.6 (OMe-6), 99.4 (C-4), 109.6 (C-10), 113.9 (C-5′), 116.2 (C-9), 125.0 (C-1′), 128.1 (C-2′), 136.8 (C-6′), 138.8 (C-7), 139.7 (C-3′), 147.2 (C-2), 149.3 (C-6), 150.7 (C-5), 153.6 (C-8), 153.7 (C-4′), 183.4 (C-3). ESIMS (+) m/z 388 $[M+H]^+$.

5.4.3. (*Z*)-3′-Amino-5,6,7,4′-tetramethoxy-aurone 23 and (*Z*)-3′-amino-5,6,7,4′,6′-pentamethoxy-aurone 30

From **29** (43 mg, 0.11 mmol), same work-up than for reduction of **12** into **13** (6 h reaction time under N_2), then purification of the resulting dried residue by tlc (silica gel, CH_2CH_2–MeOH 99:1) led to **23** (18 mg, 45%) as major compound and **30** (4 mg, 9%) as minor one. **23** Golden yellow crystals: mp: 169–171 °C (MeOH); ^{1}H NMR ($CDCl_3$) δ ppm 3.81, 3.84, 3.96 and 4.13 (4s, 12H, OMe-5, 6, 7, 4′), 6.72 (s, 1H, H-10), 6.78 (d, J = 8.7 Hz, 1H, H-5′), 6.92 (s, 1H, H-4), 7.23 (dd, J = 8.7 and 2.0 Hz, 1H, H-6′), 7.24 (d, J = 2.0 Hz, 1H, H-2′). ^{13}C NMR ($CDCl_3$) δ ppm 55.6 (OMe-4′), 56.4 (OMe-5), 61.2 (OMe-7), 61.6 (OMe-6), 99.3 (C-4), 110.4 (C-5′), 114.0 (C-10), 116.9 (C-9), 117.0 (C-2′), 123.6 (C-6′), 125.2 (C-1′), 136.5 (C-3′), 138.8 (C-7), 146.1 (C-2), 148.8 and 149.1 (C-6 and C-4′), 150.4 (C-5), 153.7 (C-8), 183.7 (C-3). ESIMS (+) m/z 358 $[M+H]^+$; HRESIMS (+) m/z $[M+H]^+$ 358. 1269 (calcd for $C_{19}H_{20}NO_6$, 358.1285). **30** Red orange-colored crystals: mp: 244–247 °C (MeOH); ^{1}H NMR ($CDCl_3$) δ ppm 3.88 (×2), 3.92, 4.02 and 4.19 (4s, 15H, OMe-5, 6, 7, 4′, 6′), 6.46 (s, 1H, H-10), 6.99 (s, 1H, H-4), 7.42 and 7.70 (2s, 2H, H-2′ and H-5′). ESIMS (+) m/z 388 $[M+H]^+$.

5.4.4. (*Z*)-2′,3′-Bis[(*t*-butyldimethylsilyl)-oxy]-5,6,7,4′-tetramethoxy-aurone 31

A solution of benzofuranone **28** (45 mg, 0.2 mmol) and 2,3-bis-[(*t*-butyldimethylsilyl)-oxy]-4-methoxybenzaldehyde (87 mg, 0.22 mmol) in CH_2Cl_2 (5 mL) was added with 1 g basic alumina (Brockmann activity I) and stirred for 20 h at rt. The reaction mixture was filtered and concentrated to dryness. Purification of the residue by flash chromatography (silica gel, CH_2CH_2–MeOH 99:1) provided pure aurone **31** (28 mg, 23%) and starting benzofuranone (22 mg). **31** Yellow crystals: mp: 179–181 °C (MeOH); ^{1}H NMR ($CDCl_3$) δ ppm 0.13 [s, 12H, 2Si–$(Me)_2$], 0.99 (s, 9H, *t*-butyl), 1.12 (s, 9H, *t*-butyl), 3.84, 3.87, 4.06, 4.13 (4s, 12H, OMe-5, 6, 7, 4′), 6.66 (d, J = 8.8 Hz, 1H, H-5′), 6.98 (s, 1H, H-4), 7.29 (s, 1H, H-10), 7.89 (d, J = 8.8 Hz, 1H, H-6′).

5.4.5. (*Z*)-2′,3′-Dihydroxy-5,6,7,4′-tetramethoxy-aurone 24

A stirred solution of aurone **31** (26 mg, 0.043 mmol) in THF (4 mL) was added with *n*-tetrabutyl ammonium fluoride 1 N in THF (0.11 mL, 0.11 mmol), then left under N_2 for 15 min. Standard work-up of the organic layer, then crystallization of the resulting dried residue with MeOH provided pure aurone **24** (11 mg, 70%). **24** Yellow ocher crystals: mp: 208–210 °C (MeOH); ^{1}H NMR ($CDCl_3$) δ ppm 3.83 (s, 3H, OMe-5), 3.85 (s, 3H, OMe-4′), 3.89 (s, 3H, OMe-6), 4.10 (s, 3H, OMe-7), 6.71 (d, J = 8.7 Hz, 1H, H-5′), 7.03 (s, 1H, H-4), 7.19 (s, 1H, H-10), 7.64 (d, J = 8.7 Hz, 1H, H-6′). ^{13}C NMR ($CDCl_3$) δ ppm 55.5 (OMe-4′), 56.0 (OMe-5), 60.9 (OMe-7), 61.2 (OMe-6), 99.2 (C-4), 103.8 (C-5′), 107.4 (C-10), 112.5 (C-1′), 132.1(C-2′), 133.2 (C-3′), 138.0 (C-7), 145.0 (C-2), 148.0 (C-6), 150.9 (C-4′), 150.2 (C-5), 152.5 (C-8), 181.9 (C-3), C-9 and C-6′ not detected. ESIMS (+) m/z 375 $[M+H]^+$; HRESIMS (+) m/z $[M+H]^+$ 375.1056 (calcd for $C_{19}H_{19}O_8$, 375.1074).

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 line KB was 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 to 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 (Celltiter 96AQeous One, 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 [$(1 - (OD_{490}$ treated/OD_{490} control)) × 100]. For IC_{50} determinations (50% inhibition of cell proliferation) experiments were performed in duplicate.

5.5.3. 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[ß-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[34] using deoxypodo-

phyllotoxin 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.

5.5.4. 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.

5.5.5. Cell cycle analysis

KB cells (25000cells/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).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.12.038.

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