Semisynthesis of Natural Flavones Inhibiting Tubulin Polymerization, from Hesperidin

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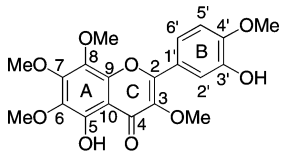
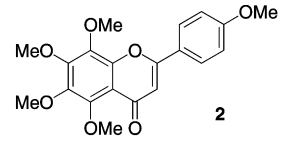
Semisynthesis of 5,3′-dihydroxy-3,6,7,8,4′-pentamethoxyflavone (**1**), a natural flavone that binds with high affinity to tubulin, was performed from hesperidin, the very abundant *Citrus* flavanone, by a five-step sequence. The last step of the synthesis also gave rise to 5,3′-dihydroxy-3,6,7,4′-tetramethoxyflavone (= casticin or vitexicarpin) (**10**), 5,3′-dihydroxy-3,7,8,4′-tetramethoxyflavone (= gossypetin 3,7,8,4′-tetramethyl ether) (**11**), and, unexpectedly, 5,7,3′-trihydroxy-3,6,8,4′-tetramethoxyflavone (**12**) and 5,3′-dihydroxy-8-dimethylamino-3,6,7,4′-tetramethoxyflavone (= 8-dimethylaminocasticin) (**13**). Cytotoxicity and antitubulin activity of these five flavones, as well as 5,3′-dihydroxy-3,7,4′-trimethoxyflavone (= ayanin) (**14**) and intermediate 6,8-dibromo-ayanin (**8**), were evaluated. Comparison of the responses confirmed and clarified the influence of the A-ring substitution pattern on the biological activity.

In 1998, Beutler et al. reported results of a comparative cytotoxicity screening and subsequent studies on tubulin binding carried out with a series of 79 natural and synthetic flavones.[1] Some structure−activity relationships for cytotoxicity and associated inhibitory effects on tubulin polymerization were drawn from these results. Maximum potencies for cytotoxicity and tubulin interaction were found only for 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 most potent compound in both tests was 5,3′-dihydroxy-3,6,7,8,4′-pentamethoxyflavone (**1**), a natural flavone first isolated by Mabry et al. in 1986 from *Gutierrezia microcephala.*[2] Its remarkable antimitotic and cytotoxic properties were reported in 1994 and in 1995 by the Sévenet[3] and Lee[4] groups, respectively. To the best of our knowledge, **1** remains the most antimitotic natural flavone isolated to date, thus appearing as a reference compound within this phytochemical class.



1

Our interest in antitumor natural compounds, especially flavonoids, led us to look for a ready access to **1**. Preparation of **1** could have been planned by total synthesis according to methods previously described,[5,6] but these multistep processes are excessively tedious, partly due to the tetramethoxylation pattern of ring A. Thus, we turned to semisynthetic methods starting from readily available natural sources. Tangeretin (**2**) appeared of interest, since its alkaline degradation is known to provide in a single step 2′-hydroxy-3′,4′,5′,6′-tetramethoxyacetophenone (**3**), a key intermediate compound toward the synthesis of **1**.[7] However, although **2** occurs in high concentration in the peel of various *Citrus* species such as sweet orange [*Citrus sinensis* (L.) Pers.] and mandarin (*Citrus reticulata* Blanco), commercial sources are scarce and the product is expensive. Thus we opted for hesperidin (**4**), the predominant flavonoid in sweet oranges, a very abundant and inexpensive starting material, which has the same B-ring substitution pattern as **1**.



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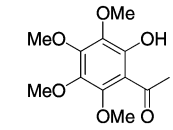

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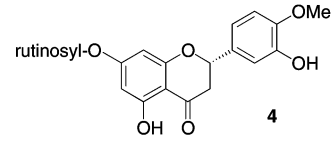

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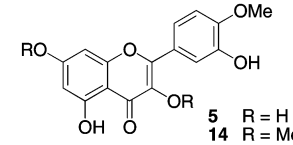

5 R = H
14 R = Me

Results and Discussion

Access to **1** from **4** was envisioned by the following five-step sequence: (a) oxidation to the 3-flavonol; (b) cleavage of the glycosidic bond; (c and d) selective methylation of phenol groups at C-3 and C-7, then 6,8-dibromination (or in reverse order); (e) nucleophilic aromatic substitution of bromide by methoxy groups (Scheme 1). The first step, using the Algar−Flynn−Oyamada (AFO) method[8] (oxidation with alkaline hydrogen peroxide of hesperidin chalcone, acyclic isomer of **4**), has been described by Pacheco et al.[9] and provided crude 7-*O*-rutinosyltamarixetin (**5**) (19%) by direct crystallization from the medium. This low yield, in accordance with previously reported results, can be explained mainly by the well-known competing oxidative cyclization of 6′-substituted chalcones (such as hesperidin chalcone) to aurone and coumaran-3-one skeletons.[10] The second step, hydrolysis of the 7-*O*-rutinosyl bond of **5**, was achieved under conditions (11 N HCl, 1.5 h at 55 °C) previously used in our laboratory with diosmin (the flavone analogue of hesperidin) derivatives,[11,12] providing tamarixetin (= 4′-*O*-methylquercetin) (**6**) in 73% yield. The methylation of only two (at C-3 and C-7) out of four OH groups was a crucial step since both free phenol groups at C-5 and C-3′ are known to be necessary for strong inhibition of tubulin polymerization.[1]

A recent study on selective methylations of quercetin by Rolando et al. gave the order of reactivity as 7 > 3 > 3′ > 5 for the phenol groups in tamarixetin (**6**).[13] We decided to confirm this theoretical order by a preliminary methylation study, carried out on a small scale (0.1 mM) with **6** and its 6,8-dibromo analogue (**7**). According to our previous studies[14,15] in the same field, attempts at bromination of **6** were undertaken with *N*-bromosuccinimide (2 equiv, rt) in a mixture of CH_2Cl_2−MeOH (2:1). Poor results, however, led to changing the solvent to trifluoroacetic acid, which gave 6,8-dibromotamarixetin (**7**) as the major product (72% yield from **6**).

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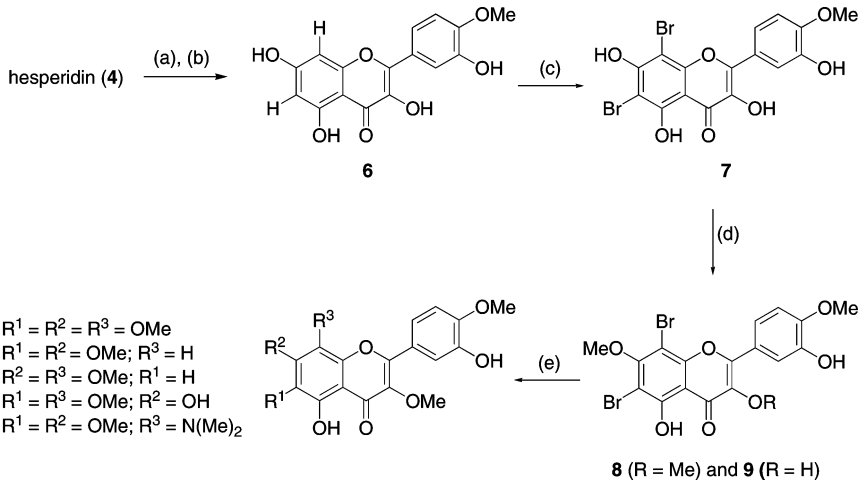
Scheme 1. Synthesis of **1** and Analogues from Hesperidin (**4**)[a]



[a] (a) NaOH 2 N, H_2O_2 30%, 0 °C, 48 h; AcOH to pH 6, then 0 °C, 24 h; sodium metabisulfite, reflux, 2 h; (b) HCl 11 N, 55 °C, 1.5 h, 14% (**6** from **4**); (c) TFA, NBS, rt, 4.5 h, 72% (**7**); (d) DMF, $KHCO_3$, MeI, rt, 2 h, then K_2CO_3, MeI, rt, 2.5 h, 39% (**8**), 6% (**9**); (e) DMF, MeOH, CuBr, MeONa, 130 °C, 22 h, 20% (**1**), 19% (**10**), 2% (**11**), 8% (**12**), 4% (**13**).

Brominations at both C-6 and C-8 were confirmed by ^{1}H NMR spectroscopy (loss of H-6 and H-8 signals, unchanged signals for the ABX spin system of the B ring). Methylations of tamarixetin (**6**) and its 6,8-dibromo derivative (**7**) in dimethylformamide with excess iodomethane and successive additions of $KHCO_3$ (2.2 equiv) and K_2CO_3 (1 equiv) gave best results in the case of **7**, which led to 6,8-dibromo-3,7-*O*-dimethyltamarixetin (= 6,8-dibromoayanin) (**8**) in 39% (the monomethylation product **9** was also isolated in 6% yield).

The expected reactivity of 7- and 3-hydroxy groups was proved by NMR experiments. Methylation of 7-OH was deduced from the observed HMBC correlation between signals of a methoxy group at δ 3.90 and C-7 at δ 159.0. Methylation of the 3-OH group was ascertained from comparison of the spectra with literature NMR data for 3,4′-dimethoxy-3′-hydroxyflavones[3,16,17] and 3′,4′-dimethoxy-3-hydroxyflavones.[18,19] Particularly significant in the ^{13}C NMR spectrum of **8** were the signals of C-2, C-2′, and 3-OMe at δ 156.2, 115.1, and 59.7, respectively. The remaining step was substitution of the bromines at C-6 and C-8 by methoxy groups, a reaction that has been described as a MeO^-/CuBr-promoted methanolysis in a mixture of MeOH−DMF.[20] In this paper, Bovicelli et al. reported excellent yields (89% and 80%) with two 6,8-dibromoflavones (1 h reaction times), but they stressed the versatility and the high dependence of the reaction on the experimental protocol, i.e., the quality of the CuBr catalyst and the composition of the solvent, which must contain a small percentage of water.[20,21] Unfortunately, in our hands, a first attempt with 6,8-dibromodiosmetin (used as a model) proved to be unsuccessful, since the dibromoflavone was fully recovered. Therefore, we decided to use the same reagents and solvent, but under harsher, more classical conditions such as those described by Guillaumet et al.[22] Methanolysis of 6,8-dibromoayanin (**8**) by heating it at 130 °C in DMF−MeOH with MeONa (10 equiv) and CuBr (0.026 equiv) for 22 h led to SNAr at both C-6 and C-8, giving the expected flavone (**1**) as the major compound (20%). Four other flavones, 5,3′-dihydroxy-3,6,7,4′-tetramethoxyflavone (= casticin or vitexicarpin) (**10**), 5,3′-dihydroxy-3,7,8,4′-tetramethoxyflavone (= gossypetin 3,7,8,4′-tetramethyl ether) (**11**), 5,7,3′-trihydroxy-3,6,8,4′-tetramethoxyflavone (**12**), and 5,3′-dihydroxy-8-dimethylamino-3,6,7,4′-tetramethoxyflavone (= 8-dimethylaminocasticin) (**13**), were also isolated in 19%, 2%, 8%, and 4% yields, respectively.

Formation of flavones **10** and **11** is consistent with the combination of a SNAr reaction at C-6 or C-8 and a reductive debromination on the other carbon. The side reaction, observed in this case mainly at C-8, is well known with aryl halogens *ortho* to a methoxy group.[23] The natural flavones **1**,[3,4] **10**,[16,24] and **11**[25−27] were identified unambiguously by comparison of their melting points and spectroscopic characteristics with literature values. Formation of the last two flavones, **12** and **13**, was rather unexpected. Compound **12** probably originates from **1** by nucleophilic attack at the C-7 methoxy group (nucleophilic cleavage of this methyl ether is facilitated by the *para* carbonyl function and the two adjacent methoxy groups), while **13** results from a nucleophilic substitution of the bromide at C-8 by dimethylamine. These two reactions can be explained by the reported property of DMF to decompose to dimethylamine by extended heating under some conditions.[28,29] The structure of **12**, a natural flavone, first isolated at the same time as **1**,[2] was established by mass, ^{1}H NMR, and ^{13}C NMR spectrometry and confirmed by comparison of these spectroscopic data with literature values.[30] Lastly, the presence of the dimethylamino group in **13** was deduced from the mass (pseudomolecular ion at $[M + H]^+$ 418) and ^{1}H NMR spectra (s, 6H at δ 2.96), and its position at C-8 was inferred from NOESY experiments (significant NOE correlations were observed between signals of the dimethylamino group and H-2′ and 6′ on one hand and signals of OH at C-5 and OCH_3 at C-6 on the other hand).

This chemical study led to the isolation of six flavones having in common OH groups at C-5 and C-3′ and OCH_3 groups at C-3 and C-4′, favorable structural requirements for cytotoxicity and inhibition of tubulin polymerization (ITP).[1,31] A seventh flavone with the same substituents, ayanin (**14**), was also prepared by hydrogenolysis of **8** (H_2, 10% Pd−C, rt, 48 h in DMF). As these seven flavones differ only in C-6, C-7, and C-8 substituents, we decided to measure their antiproliferative and ITP activities. We thought indeed that such a comparative study, in the same set of experiments, would improve knowledge about the influence of the A-ring substitution pattern on these biological properties. It is noteworthy that no comparison between activities of flavone **1** and casticin (**10**), a well-known cytotoxic and tubulin-binding agent,[24,32−35] had been reported until now. Furthermore, cytotoxicity and ITP have not been investigated for the two other naturally occurring flavones, gossypetin 3,7,8,4′-tetramethyl ether (**11**) and 5,7,3′-trihydroxy-3,6,8,4′-tetramethoxyflavone (**12**). The antiproliferative effect of flavones was assayed on KB human buccal carcinoma cells as well as the activation of caspase 3 with DEVD-AMC as substrate in HL60 human leukemia cells. Inhibition of tubulin polymerization was determined according to Zavala and Guenard's method.[36] Compounds were tested at 1 mg/mL ($\approx 2.5 \times 10^{-3}$ M),

Table 1. Antiproliferative, Proapoptotic, and Antitubulin Activities of Synthesized Flavones

compd	cytotoxicity on KB cells[a]	activation of apoptosis in HL60[b]	ITP activity[c]
	Natural Flavones		
1	IC_{50} = 8 nM	50 nM (×6.9)	1.3 μM (0.46)[d]
10	IC_{50} = 97 nM	500 nM (×5.3)	19 μM (6.8)[d]
11	9%	n.d.	0% inhibition
12	10%	n.d.	14 μM (5)[d]
14	3%	n.d.	0% inhibition
	Non-natural Flavones		
8	IC_{50} = 404 nM	500 nM (×3.2)	36% inhibition
13	40%	n.d.	0% inhibition

[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 three most active compounds. [b] Activation of caspases 3/7: optimal concentration of compound and fold-activation over control value after a 48 h exposure. [c] Results are expressed as the percentage of ITP at ~2.5 × 10^{-3} M or as IC_{50} (μM). [d] $IC_{50\ compound}/IC_{50\ DPPT}$. n.d.: not determined.

and results were given as the percentage of ITP 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. As depicted in Table 1, only flavone **1** and casticin (**10**) possess antiproliferative, proapoptotic, and ITP activities. The 10-fold higher activity of **1** versus **10** highlights the significant importance of an additional methoxy group at C-8 in **1**. Flavone **11**, an isomer of casticin, is devoid of both activities, like ayanin (**14**), previously reported[1] to be inactive. Comparison of isomeric flavones **10** (6-methoxylated) and **11** (8-methoxylated) corroborates the critical influence of the substitution pattern on the activity, while flavone **12**, the 7-*O*-demethyl analogue of **1**, displays a discrepancy between a very weak cytotoxicity and an ITP activity in the range of casticin. Lastly, responses of **8** and **13**, the two non-natural evaluated flavones, were rather unexpected: 8-dimethylaminocasticin (**13**), which differs from **1** and **10** only in the substituent at C-8, was weakly cytotoxic and completely inactive on ITP, whereas **8**, the 6,8-dibromo analogue of ayanin, showed significant antiproliferative and moderate ITP effects.

In conclusion, this study refines structure−activity relationships previously predicted.[1,31] Our biological results confirm that the most favorable A-ring substitution pattern for strong antiproliferative and ITP activities consists of an OH group at C-5 and OCH_3 groups at C-6, C-7, and C-8, as in **1**.

Experimental Section

General Experimental Procedures. Melting points were determined with a micro-Koffler apparatus and are uncorrected. NMR spectra, including NOESY and $^1H-^{13}C$ (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). Flash chromatography was performed with silica gel 60 (9385 Merck) and, for final purifications, with a Spot 1 flash chromatography integrated system and Reveleris silica 40 μm, 12 g cartridges. Preparative TLC was performed using 60 F 254 silica gel (5715 Merck). Hesperidin was purchased from Acros Organics.

Tamarixetin (6) from Hesperidin (4). The AFO oxidation of **4** was accomplished exactly under conditions described previously.[9] A solution of **4** (50 g, 82 mmol) in aqueous 2 N NaOH (1 L) was cooled at 0 °C, aqueous H_2O_2 30% (50 mL) was added, and the mixture was left for 24 h at 0 °C. To the mixture was added the same volume of aqueous H_2O_2 30% (50 mL), and the solution was left for 24 h more at 0 °C, adjusted to pH 6 with glacial acetic acid, then kept again for 24 h at 0 °C. Sodium metabisulfite (120 g) was then added, and the mixture was heated and stirred under reflux for 2 h. The abundant resulting yellow precipitate was recovered by filtration, washed with water, and dried with P_2O_5 under vacuum to provide crude 7-*O*-rutinosyltamarixetin (**5**) (9.6 g, 19%). Hydrolysis of crude **5** (9.2 g) was achieved by treatment with 11 N HCl (120 mL) at 55 °C for 1.5 h. The mixture was diluted with water (3 L), then extracted with EtOAc (3 × 500 mL). The organic phase was made clear by addition of MeOH (100 mL), dried with Na_2SO_4, filtered, and evaporated to dryness. The dried residue (5 g) was purified by flash chromatography (silica gel, CH_2CH_2−MeOH, 96:4) to provide pure tamarixetin (**6**) (3.37 g, 73%).

Tamarixetin (6): pale yellow crystals, mp 254−257 °C (lit.[13,37] 252−254 °C; 253−256 °C); ^1H NMR (DMSO-d_6) δ 3.82 (3H, s, OCH_3-4′), 6.18 (1H, d, J = 2 Hz, H-6), 6.42 (1H, d, J = 2 Hz, H-8), 7.04 (1H, d, J = 8.8 Hz, H-5′), 7.61 (2H, m, H-2′ and H-6′), 12.40 (1H, s, OH-5).

Bromination of Tamarixetin (6). NBS (3.47 g, 19.3 mmol) was added to a solution of **6** (3.05 g, 9.65 mmol) in TFA (100 mL), then stirred for 4.5 h at room temperature. The reaction mixture was diluted with iced water, and the resulting precipitate was filtered, washed with water, and dried with KOH pellets under vacuum overnight. The yellow crystallized residue (3.3 g, 72%) gave a homogeneous spot [TLC on silica gel (CH_2Cl_2−MeOH, 96:4)] and was identified as pure **7** according to NMR spectra.

6,8-Dibromotamarixetin (7): yellow crystals, mp 269−272 °C; ^1H NMR (DMSO-d_6) δ 3.87 (3H, s, OCH_3-4′), 7.14 (1H, d, J = 8.8 Hz, H-5′), 7.78 (1H, dd, J = 8.8 and 1.6 Hz, H-6′), 7.83 (1H, d, J = 1.6 Hz, H-2′), 9.39 and 9.90 (2H, 2s, OH-3 and OH-3′), 13.39 (1H, s, OH-5); ^{13}C NMR (DMSO-d_6) δ 55.2 (CH_3, OCH_3-4′), 87.4 (C, C-8), 93.1 (C, C-6), 103.8 (C, C-10), 111.4 (CH, C-5′), 114.3 (CH, C-2′), 119.5 (CH, C-6′), 122.7 (C, C-1′), 136.0 (C, C-3), 145.9 (C, C-3′), 146.8 (C, C-2), 149.3 (C, C-4′), 150.3 (C, C-9), 155.8 and 156.3 (2C, C-5 and C-7), 175.0 (C, C-4); ESIMS (−) m/z [M − H]$^-$ 471−473−475.

Methylation of 6,8-Dibromotamarixetin (7). To a solution of **7** (3.18 g, 6.71 mmol) in DMF (100 mL) at room temperature were added $KHCO_3$ (1.55 g, 15.5 mmol) and iodomethane (5 mL, 80 mmol), and the reaction mixture was stirred for 2 h at room temperature. K_2CO_3 (0.98 g, 7.1 mmol) and a new amount of iodomethane (5 mL) were added, and the medium was stirred for a further 2.5 h. The reaction mixture was filtered and evaporated to dryness. Crystallization of the dried residue from CH_2Cl_2−MeOH afforded 2.37 g of a mixture of two main compounds [TLC on silica gel (CH_2CH_2−MeOH, 99:1): two blue spots with $FeCl_3$]. Purification of this by flash chromatography (silica gel, CH_2CH_2−MeOH, 99:1), then crystallization from CH_2Cl_2−MeOH (**8**) or THF−EtOH (**9**), provided pure 6,8-dibromoayanin (**8**) (1.32 g, 39%) and **9** (0.205 g, 6%).

6,8-Dibromo-3,7-*O*-dimethyltamarixetin (6,8-dibromoayanin) (8): pale yellow crystals, mp 234−236 °C; ^1H NMR (DMSO-d_6) δ 3.83 (3H, s, OCH_3-3), 3.87 (3H, s, OCH_3-4′), 3.90 (3H, s, OCH_3-7), 7.14 (1H, d, J = 8.8 Hz, H-5′), 7.69 (1H, dd, J = 8.8 and 1.6 Hz, H-6′), 7.70 (1H, d, J = 1.6 Hz, H-2′), 9.53 (1H, s, OH-3′), 13.51 (1H, s, OH-5); ^{13}C NMR (DMSO-d_6) δ 55.7 (CH_3, OCH_3-4′), 59.7 (CH_3, OCH_3-3), 61.0 (CH_3, OCH_3-7), 94.4 (C, C-8), 99.6 (C, C-6), 108.7 (C, C-10), 112.1 (CH, C-5′), 115.1 (CH, C-2′), 120.7 (CH, C-6′), 121.7 (C, C-1′), 138.5 (C, C-3), 146.5 (C, C-3′), 150.9 (C, C-4′), 151.0 (C, C-9), 156.2 (C, C-2), 157.2 (C, C-5), 159.0 (C, C-7), 177.7 (C, C-4); ESIMS (−) m/z [M − H]$^-$ 499−501−503.

6,8-Dibromo-7-*O*-methyltamarixetin (9): yellow crystals, mp 257−260 °C; ^1H NMR (DMSO-d_6) δ 3.86 (3H, s, OCH_3-4′), 3.90 (3H, s, OCH_3-7), 7.13 (1H, d, J = 8.8 Hz, H-5′), 7.79 (1H, dd, J = 8.8 and 1.6 Hz, H-6′), 7.83 (1H, d, J = 1.6 Hz, H-2′); ^{13}C NMR (DMSO-d_6) δ 55.6 (CH_3, OCH_3-4′), 61.0 (CH_3, OCH_3-7), 94.8 (C, C-8), 98.6 (C, C-6), 107.4 (C, C-10), 111.8 (CH, C-5′), 114.8 (CH, C-2′), 120.1 (CH, C-6′), 122.9 (C, C-1′), 136.9 (C, C-3), 146.3 (C, C-3′), 148.0 (C, C-2), 149.9 and 150.4 (2C, C-4′ and C-9), 156.5 (C, C-5), 158.6 (C, C-7), 175.6 (C, C-4); ESIMS (−) m/z [M − H]$^-$ 485−487−489.

Methanolysis of 6,8-Dibromoayanin (8). To a solution of **8** (0.5 g, 1 mmol) in DMF (3.6 mL) in a test tube were added CuBr (0.038 g, 0.26 mmol), MeOH (1.7 mL), and 30% NaOMe in MeOH (1.9 mL, 10 mmol). The test tube was sealed, and the reaction mixture was stirred at 130 °C for 22 h. The cooled reaction mixture was taken up in water and extracted at pH 6 with CH_2Cl_2. Standard workup of the organic layer provided an amorphous residue (0.390 g). Purification of the residue by flash chromatography (silica gel, CH_2CH_2−MeOH, 99:1) provided a mixture of **1**, **10**, **11**, and **13** and pure compound **12** (0.03 g, 8%). A second flash chromatography (gradient elution from 100% cyclohexane to 50:50 cyclohexane−acetone in 30 min) led to isolation of pure **1** (0.081 g, 20%), **10** (0.07 g, 19%), and **13** (0.017 g, 4%), and

11 in a crude fraction. Separation of the latter by preparative TLC (silica gel, CH_2CH_2−MeOH, 99.5:0.5) gave pure **11** (0.007 g, 2%).

5,3′-Dihydroxy-3,6,7,8,4′-pentamethoxyflavone (1): yellow crystals, mp 169−171 °C (lit.[3] 170 °C); ^{1}H NMR ($CDCl_3$) δ 3.87 (3H, s, OCH_3), 3.96 (6H, s, 2 OCH_3), 3.99 (3H, s, OCH_3), 4.11 (3H, s, OCH_3), 5.73 (1H, s, OH-3′), 7.00 (1H, d, J = 8.8 Hz, H-5′), 7.78 (2H, m, H-2′ and H-6′), 12.38 (1H, s, OH-5); ESIMS (+) m/z $[M + Na]^+$ 427.

Casticin (10): pale yellow crystals, mp 187−189 °C (lit.[24] 188−190 °C); ^{1}H NMR ($CDCl_3$) δ 3.88 (3H, s, OCH_3), 3.93 (3H, s, OCH_3), 3.97 (3H, s, OCH_3), 4.00 (3H, s, OCH_3), 5.71 (1H, s, OH-3′), 6.51 (1H, s, H-8), 6.96 (1H, d, J = 8.8 Hz, H-5′), 7.68 (1H, d, J = 1.6 Hz, H-2′), 7.72 (1H, dd, J = 8.8 and 1.6 Hz, H-6′), 12.57 (1H, s, OH-5); ^{1}H NMR (DMSO-d_6) δ 3.75 (3H, s, OCH_3), 3.82 (3H, s, OCH_3), 3.89 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 6.89 (1H, s, H-8), 7.13 (1H, d, J = 8.8 Hz, H-5′), 7.61 (2H, m, H-2′ and H-6′), 9.45 (1H, br s, OH-3′), 12.60 (1H, br s, OH-5); ESIMS (+) m/z $[M + H]^+$ 375, $[M + Na]^+$ 397.

Gossypetin 3,7,8,4′-tetramethyl ether (11): yellow crystals, mp 185−187 °C (lit.[25,26] 184−185 °C; 185.5−186.5 °C); ^{1}H NMR (DMSO-d_6) δ 3.82 (3H, s, OCH_3), 3.83 (3H, s, OCH_3), 3.88 (3H, s, OCH_3), 3.92 (3H, s, OCH_3), 6.60 (1H, s, H-6), 7.16 (1H, d, J = 8.8 Hz, H-5′), 7.61 (2H, m, H-2′ and H-6′), 9.56 (1H, br s, OH-3′), 12.48 (1H, br s, OH-5); ESIMS (+) m/z $[M + Na]^+$ 397.

5,7,3′-Trihydroxy-3,6,8,4′-tetramethoxyflavone (12): yellow crystals, mp 184−186 °C (lit.[30] 182−184 °C); ^{1}H NMR (DMSO-d_6) δ 3.78 (3H, s, OCH_3-6), 3.80 (3H, s, OCH_3-3), 3.85 (3H, s, OCH_3-8), 3.87 (3H, s, OCH_3-4′), 7.13 (1H, d, J = 8.8 Hz, H-5′), 7.58 (2H, m, H-2′ and H-6′), 9.55 and 10.4 (2H, 2 br s, OH- 7 and 3′), 12.48 (1H, s, OH-5); ^{13}C NMR (DMSO-d_6) δ 55.6 (CH_3, OCH_3-4′), 59.7 and 61.0 (2 CH_3, OCH_3-3 and -7), 61.2 (CH_3, OCH_3-8), 103.4 (C, C-10), 112.0 (CH, C-5′), 114.8 (CH, C-2′), 120.2 (CH, C-6′), 122.4 (C, C-1′), 128.0 (C, C-8), 131.4 (C, C-6), 137.7 (C, C-3), 144.5 (C, C-9), 146.4 (C, C-3′), 147.9 (C, C-5), 150.3 (C, C-4′), 151.0 (C, C-7), 155.1 (C, C-2), 178.4 (C, C-4); ESIMS (+) m/z $[M + Na]^+$ 413.

5,3′-Dihydroxy-8-dimethylamino-3,6,7,4′-tetramethoxyflavone (13): yellow crystals, mp 160−162 °C; ^{1}H NMR ($CDCl_3$) δ 2.96 (6H, s, $N(CH_3)_2$), 3.87 (3H, s, OCH_3-3), 3.93 (3H, s, OCH_3-6), 3.99 (3H, s, OCH_3-4′), 4.12 (3H, s, OCH_3-7), 5.75 (1H, br s, OH-3′), 7.00 (1H, d, J = 8.8 Hz, H-5′), 7.77 (2H, m, H-2′ and H-6′), 12.64 (1H, s, OH-5); ^{13}C NMR ($CDCl_3$) main characteristic signals at δ 44.1 (2 CH_3, $N(CH_3)_2$), 56.0 (CH_3, OCH_3-4′), 60.1 (CH_3, OCH_3-3), 60.9 (CH_3, OCH_3-6), 61.7 (CH_3, OCH_3-7), 110.5 (CH, C-5′), 114.6 (CH, C-2′), 121.7 (CH, C-6′), 123.5 (C, C-1′), 124.9 (C, C-8), 136.4 (C, C-6), 138.6 (C, C-3), 145.6 (C, C-3′), 148.9 (C, C-4′), 157.0 (C, C-7); ESIMS (+) m/z $[M + H]^+$ 418.

Hydrogenolysis of 6,8-Dibromoayanin (8). A solution of **8** (20 mg, 0.04 mmol) in DMF (3 mL) was hydrogenated under 1 atm of hydrogen with 10% Pd−C (20 mg) at room temperature for 48 h. The catalyst was separated and the filtrate concentrated to dryness. Crystallization of the dried residue (MeOH) afforded pure ayanin (**14**) (0.010 g, 73%).

Ayanin (14): pale yellow crystals, mp 169−171 °C (lit.[38] 169 °C); ^{1}H NMR ($CDCl_3$) δ 3.88 (6H, 2s, OCH_3-3 and -4′), 3.99 (3H, s, OCH_3-7), 5.68 (1H, s, OH-3′), 6.35 (1H, d, J = 2 Hz, H-6), 6.46 (1H, d, J = 2 Hz, H-8), 6.96 (1H, d, J = 8.8 Hz, H-5′), 7.68−7.70 (2H, m, H-2′ and H-6′), 12.63 (1H, s, OH-5).

Cell Culture. The human cell lines KB (nasopharyngeal epidermoid carcinoma) and HL60 (promyelocytic leukemia) were purchased from ATCC. KB cells were cultured in D-MEM medium supplemented with 10% fetal calf serum, in the presence of penicillin, streptomycin, and fungizone in a 75 cm^2 flask under 5% CO_2, whereas HL60 cells were cultured in complete RPMI medium.

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 (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.

Activation of Caspase 3. 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 of 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 (λ_{ex} = 360 nm, λ_{em} = 465 nm) over a 3 h period.

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 (ethyleneglycolbis[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 a 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[36] using deoxypodophyllotoxin as reference compound.

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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*, 2333–2338.
(2) Fang, N.; Leidig, M.; Mabry, T. J. *Phytochemistry* **1986**, *25*, 927–934.
(3) Lichius, J. J.; Thoison, O.; Montagnac, A.; Païs, M.; Guéritte-Voegelein, F.; Sévenet, T. *J. Nat. Prod.* **1994**, *57*, 1012–1016.
(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*, 475–482.
(5) Horie, T.; Kawamura, Y.; Yamamoto, H.; Yamashita, K. *Chem. Pharm. Bull.* **1995**, *43*, 2054–2063.
(6) Fougerousse, A.; Gonzalez, E.; Brouillard, R. *J. Org. Chem.* **2000**, *65*, 583–586.
(7) Burnham, W. S.; Sidwell, R. W.; Tolman, R. L.; Stout, M. G. *J. Med. Chem.* **1972**, *15*, 1075–1076.
(8) Cummins, B.; Donnelly, D. M. X.; Eades, J. F.; Fletcher, H.; Cinneide, F. O'.; Philbin, E. M.; Swirski, J.; Wheeler, T. S.; Wilson, R. K. *Tetrahedron* **1963**, *19*, 499–512.
(9) (a) Pacheco, H.; Grouiller, A. *Bull. Soc. Chim. Fr.* **1966**, 3212–3217. (b) French Patent (Brevet Special de Médicament) 3.794 M, 1965.
(10) Adams, C. J.; Main, L. *Tetrahedron* **1991**, *47*, 4979–4990.
(11) Quintin, J.; Roullier, C.; Thoret, S.; Lewin, G. *Tetrahedron* **2006**, *62*, 4038–4051.
(12) Ferté, J.; Kühnel, J.-M.; Chapuis, G.; Rolland, Y.; Lewin, G.; Schwaller, M. *J. Med. Chem.* **1999**, *42*, 478–489.
(13) Bouktaib, M.; Lebrun, S.; Atmani, A.; Rolando, C. *Tetrahedron* **2002**, *58*, 10001–10009.
(14) Quintin, J.; Lewin, G. *Tetrahedron Lett.* **2005**, *46*, 4341–4343.
(15) Lewin, G.; Jullian, J.-C.; Rodrigo De Losada, J. *Tetrahedron Lett.* **2010**, *51*, 1145–1148.
(16) Barbera, O.; Marco, J. A.; Sanz, J. F.; Sanchez-Parareda, J. *Phytochemistry* **1986**, *25*, 2357–2360.
(17) Wang, Y.; Hamburger, M.; Gueho, J.; Hostettmann, K. *Phytochemistry* **1989**, *28*, 2323–2327.
(18) Dong, H.; Gou, Y.-L.; Cao, S.-G.; Chen, S.-X.; Sim, K.-Y.; Goh, S.-H.; Kini, R. M. *Phytochemistry* **1999**, *50*, 899–902.
(19) Xiong, Q.; Shi, D.; Mizuno, M. *Phytochemistry* **1995**, *39*, 723–725.
(20) Bovicelli, P.; D'Angelo, V.; Collalto, D.; Verzina, A.; D'Antona, N.; Lambusta, D. *J. Pharm. Pharmacol.* **2007**, *59*, 1697–1701.
(21) Bovicelli, P.; Antonioletti, R.; Barontini, M.; Borioni, G.; Bernini, R.; Mincione, E. *Tetrahedron Lett.* **2005**, *46*, 1255–1257.
(22) Besson, T.; Ruiz, N.; Coudert, G.; Guillaumet, G. *Tetrahedron* **1995**, *51*, 3197–3204.
(23) Lindley, J. *Tetrahedron* **1984**, *40*, 1433–1456.
(24) Diaz, F.; Chavez, D.; Lee, D.; Mi, Q.; Chai, H.-B.; Tan, G. T.; Kardono, L. B. S.; Riswan, S.; Fairchild, C. R.; Wild, R.; Farnsworth, N. R.; Cordell, G. A.; Pezzuto, J. M.; Kinghorn, A. D. *J. Nat. Prod* **2003**, *66*, 865–867, and references therein.
(25) Krishnamurti, M.; Seshadri, T. R.; Shankaran, P. R. *Indian J. Chem.* **1967**, *5*, 137–139.
(26) Farkas, L.; Nogradi, M.; Muszaki, E.; Szerves-Kem, T. *Magy. Kem. Foly.* **1967**, *73*, 472–474.
(27) Wollenweber, E.; Fischer, R.; Doerr, M.; Irvine, K.; Pereira, C.; Stevens, J. F. *Z. Naturforsch., C: Biosci.* **2008**, *63*, 731–739.
(28) Cho, Y. H.; Park, J. C. *Tetrahedron Lett.* **1997**, *38*, 8331–8334.
(29) Watanabe, T.; Tanaka, Y.; Sekiya, K.; Akita, Y.; Ohta, A. *Synthesis* **1980**, *3*, 9–41.

(30) Horie, T.; Kitou, T.; Kawamura, Y.; Yamashita, K. *Bull. Chem. Soc. Jpn.* **1996**, *69*, 1033–1041.
(31) Lopez-Lazaro, M.; Galvez, M.; Martin-Cordero, C.; Ayuso, M. J. *Stud. Nat. Prod. Chem.* **2002**, *27*, 891–932.
(32) Kobayakawa, J.; Sato-Nishimori, F.; Moriyasu, M.; Matsukawa, Y. *Cancer Lett.* **2004**, *208*, 59–64.
(33) Li, W.-X.; Cui, C.-B.; Cai, B.; Wang, H.-Y.; Yao, X.-S. *J. Asian Nat. Prod. Res.* **2005**, *7*, 615–626.
(34) Haidara, K.; Zamir, L.; Shi, Q.-W.; Batist, G. *Cancer Lett.* **2006**, *242*, 180–190.
(35) Shen, J.-K.; Du, H.-P.; Yang, M.; Wang, Y.-G.; Jin, J. *Ann. Hematol.* **2009**, *88*, 743–752.
(36) Zavala, F.; Guénard, D.; Robin, J.-P.; Brown, E. *J. Med. Chem.* **1980**, *23*, 546–549.
(37) Le Quesne, P. W.; Levery, S. B.; Menachery, M. D.; Brennau, T. F.; Raffauf, R. F. *J. Chem. Soc., Perkin Trans. 1* **1978**, 1572–1580.
(38) Jurd, L. *J. Org. Chem.* **1962**, *27*, 1294–1297.

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