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Synthesis and antifungal activities of natural and synthetic biflavonoids

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ABSTRACT

The synthesis of some natural and synthetic biflavonoids was performed in good overall yields starting from readily available materials via high yielding aldol and Ullmann condensations. Some of these compounds, especially bichalcones, display an interesting activity against fungi, higher than that of the corresponding monomers.

1. Introduction

The biflavonoids are dimers of C_4-carbonyl flavonoids (i.e., chalcones, flavanones, flavones, flavanols, flavonols, aurones, and isoflavones) which vary at the oxygenation pattern of their monomers, oxidation level of the C_3 moiety, and interflavonyl linkage.[1–4] The interflavonyl linkage may involve the A ring (at positions 5, 6, 7, or 8), the B ring (at positions 2′, 3′, 4′, 5′, or 6′) or the C ring (at positions 2 or 3), through C–C or C–O–C bonds. In this way, biflavonoids can be classified indicating the rings involved in the interflavonyl linkage (AA, BB, AB, CC, etc.).[5]

It has been reported that the class of biflavonoids represents a library of structurally diverse molecules, which remains to be fully exploited, since most of them have not yet been found in nature or else have not been synthesized or else its biological properties have not been described.[6]

Most natural biflavonoids contain an interflavonyl linkage between the B-ring of one and the A-ring of the other flavonoid moiety (AB type) or between two A rings (AA type) and are widely distributed in *Spermatophyta*. The first isolated biflavonoid was ginkgetin, **1**, by Furukawa in 1929 from *Ginkgo biloba* L. (a gymnosperm), (Fig. 1).[7–11] Also, cupressuflavone ([I-8, II-8]-biapigenin), **3**, and robustaflavone, ([I-6, II-3′]-biapigenin), **5**, were isolated from different species of *Gymnosperms.* In *Angiosperms*, the following biflavonoids were isolated from *Rhus succedanea* (Anacardiaceae) and *Garcinia multiflora* (Guttiferae): robustaflavone, amentoflavone ([I-8, II-3′]-biapigenin), **2** and agathisflavone ([I-6, II-8]-biapigenin), **4**. Biflavonoids of the BC type are found in *Angiosperms*. Biflavonoids with a 3,8″ interflavonyl linkage, **6**, are often found in different species of *Garcinia*. Ochnaflavone, **7**, and hinokiflavone, ([I-6-O-II-4′]-biapigenin), **8**, are examples of biflavonoids that contains C–O–C bonds.

The interflavonyl linkage between the two B-rings (BB type) is less common. In *Gymnosperms*, biflavonoids of this type are very rare. That is the case of 5′,5‴-bisdihydroquercetin, **9**, which has been found only in the Douglas-fir (*Pseudotsuga menziesii*; Pinaceae).[12] This type of biflavonoids can be found in mosses and ferns. For example, 3′,3‴-binaringenin, **10**, was isolated from *Homalothecium lutescens*,[13] *Selaginella chrysocaulos*,[14] some species of *Pilotrichella*[15,16] and *Thuidium kanedae*.[17]

Examples of biflavonoids of the 3,3″-CC type are chamaejasmine, **11**, and its derivatives.[18–20]

Biflavonoids display several biological activities, namely antifungal,[21–23] antiviral,[24–26] antibacterial,[27,28] antioxidant,[29,30] antitumor,[4,24,31–35] antiplasmodial,[36] antiallergenic, anti-inflammatory,[37,38] hepatoprotective,[39–41] vasodilating,[29,42,43] and hypotensive[26,43–46] activity, sometimes better than that of the corresponding monomers.[22,37]

There is a renewed interest in the biological activities of biflavonoids, since, as stated by Rahman et al. in a recent review,[6] 'the theoretical library of biflavanoids spans a wide range of configurational and conformational space suggesting that possibilities of interesting biological activity are strong, each of which is capable of multiple H-bonding and hydrophobic interactions'. In a recent paper, Kim et al. reported[37] that not only naturally-occurring biflavonoids but also synthetic biflavonoids show antibacterial, antifungal and antiviral activity and there have only been a few trials to synthesize a biflavonoid library. In that sense, Chen et al., prepared a C–C biflavonoid library and showed that the anti-inflammatory activity depends on the position of the C–C linkage.[47]

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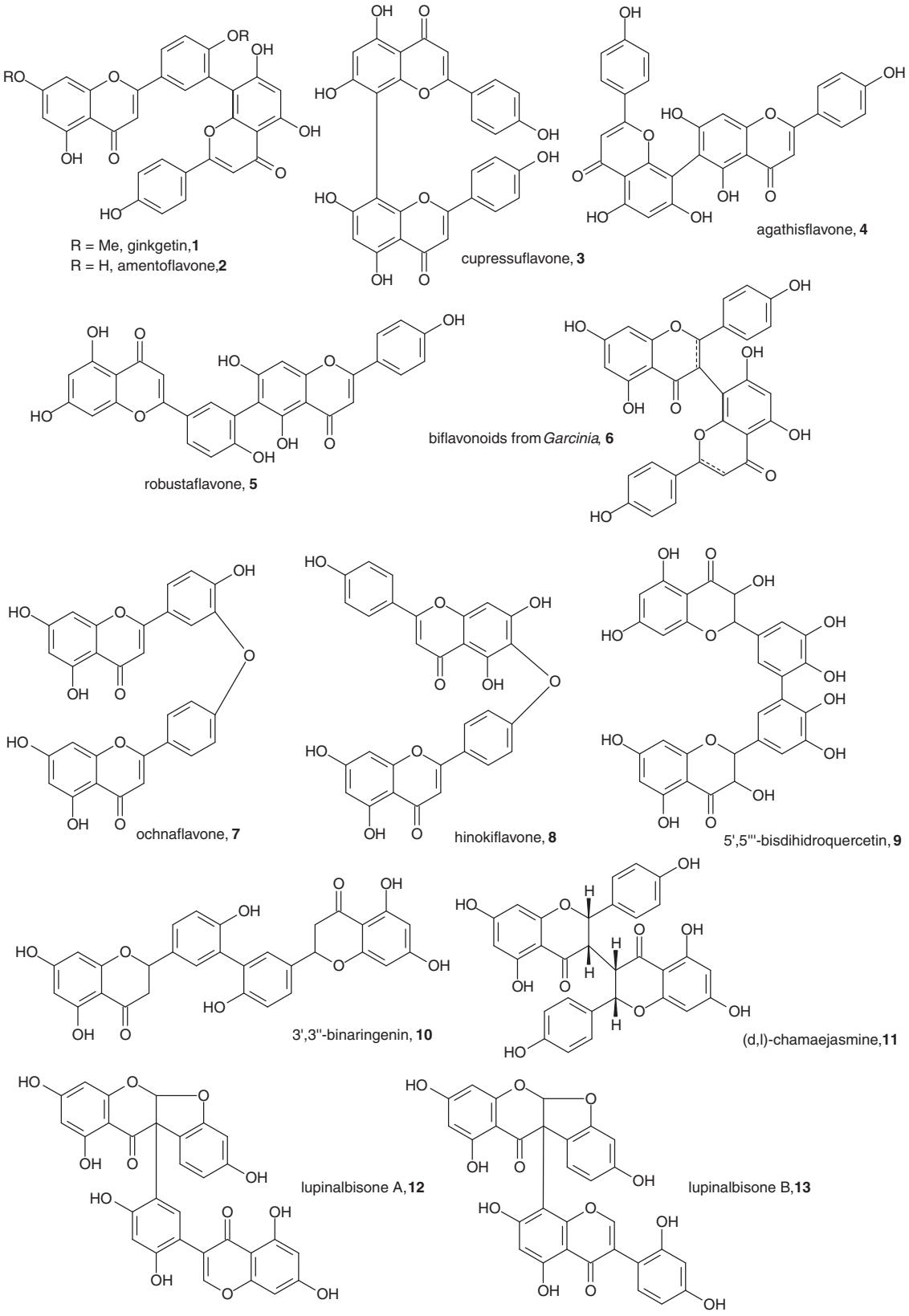


Figure 1. Some representative biflavonoids.

Also, it was reported that some synthetic biflavonoids have anti-inflammatory activity.[48,49]

Considering the antifungal properties, it was shown that amentoflavone,[21,50–54] isocryptomerin,[55] ginkgetin,[56] bilobetin,[56] and certain derivatives of chamaejasmine (neochamaejasmine B and chamaechromone)[23,57] have antifungal action.

In some cases the bioactivity of a biflavonoid is greater than that of the corresponding monomer.[22,37,58] It has been shown that

the dimerization of flavonoids can result in a remarkable increase of fungitoxicity. That is the case of lupinalbisones A, **12**, and B, **13** (two dimers of 2′-hydroxygenistein isolated from *Lupinus albus*),[22,59] Figure 1. These compounds are considered post-inhibitins[60] since are produced from less fungitoxic precursors in biologically and/or mechanically injured or stressed plants.

In the biflavone group, it was reported that some dimers of apigenin (6,6″-bigenkwanin; tetradimethoxybigenkwanin; amentoflavone and 7,7″-dimethoxyagathisflavone)[52] are inactive against *Aspergillus flavus* but inhibit the production of aflatoxins B_1 and B_2 by this fungus.[52] With respect to the inhibition of production of aflatoxins, the four compounds have similar activities, so the activities are more related to the presence of a dimer than to the particular location of the interflavonyl linkage.[61,62]

As part of an ongoing project on the synthesis and biological evaluation of phenolic compounds,[63–67] we became interested in the preparation of a biflavonoid library containing different interflavonyl linkages, different substitution patterns and different oxidation levels of the C_3 moiety, for the evaluation of its antifungal properties.

As a representative panel of fungi, we included *Candida albicans* (ATCC 90028), *Candida parapsilosis* (ATCC 22019), *Cryptococcus neoformans* (ATCC 14116), *Aspergillus niger* (ATCC 16404), *Aspergillus oryzae* (ATCC 10124), *Fusarium solani* (ATCC 36031), and *Rhizopus stolonifer* (+) (ATCC 6227b), according to the following reasons. The most common human fungal pathogens are species of the *Candida* and *Aspergillus* genera.[68,69] Mortality and morbility of infections caused by them have increased especially in immunocompromised and hospitalized patients. *C. neoformans* is considered as an opportunistic fungus; infections caused by this species are rare in those individuals with fully functioning immune system. *C. neoformans* causes primarily lung infections and also fungal meningitis, especially as a secondary infection in AIDS patients. *A. niger* can cause aspergillosis, which produces several pulmonary effects. *F. solani* is a plant pathogen and can cause several infections: superficial (queratitis, onicomycosis), localized (endoftalmitis, sinusitis) and disseminated. *R. stolonifer* (+), the bread mold fungus, is one of the most common fungi in the world. Many fruits and vegetables are susceptible to this pathogen, which causes postharvest diseases in them.

In a previous paper[66] we described the preparation of some natural and unnatural biflavonoids whose B rings are linked at position 3 (Fig. 2, structure **14**)

Herein we describe the preparation of some biflavonoids with other types of linkages: BB (2′,2‴-linkage), **15**, AA (6,6″-linkage), **16**, and CC (3,3″-linkage), **17**, using a reliable set of conditions from readily available reagents, and the antifungal activities of some of them.

2. Methods and results

2.1. Synthesis

2.1.1. Biflavonoids of the BB-type

For the synthesis of BB biflavonoids linked at 2′,2‴ (Fig. 2, general structure **15**) it was necessary to prepare the key intermediate 2,2′-diformylbiphenyl, **18**. The most direct route, consisting of the Ullmann condensation of 2-bromobenzaldehyde, **19**, formed a complex mixture from which only 21% of **18** was isolated. After extensive experimentation trying a number of conditions and reactants for the condensation,[70] the best procedure involved a clean condensation of the iodoester **21**, obtained from readily available 2-iodobenzoic acid, **20**, yielding a 73% of diester **22**, which was then reduced to the diol and oxidized to the desired dialdehyde **18** (Scheme 1). The overall yield of **18** from **20** was 50%.

With the dialdehyde **18** in hand, we prepared the flavanone **27** via aldol condensation with **24**, acidic cyclization and debenzylation (Scheme 2).

The unsubstituted 2′,2‴-biflavone, **33**, was obtained in 32% yield from **28** (Scheme 3). First, the iodoflavone **32** was obtained in three steps from **28** through esterification with **29**, Baker-Venkataraman rearrangement and acidic cyclization. Then, Ullmann condensation of 2-iodoflavone **32** formed **33**.

2.1.2. Biflavonoids of the AA-type

To obtain bichalcones linked in ring A, we envisioned a route involving the Ullmann condensation of A-ring iodinated 2′-hydroxychalcones, since it was reported that these compounds are easily obtained by direct iodination in positions 3′ and 5′, without formation of secondary products.[71] However, in our hands the treatment of 2′-hydroxy-4-methoxychalcone, **34**,[67] with the I_2–HIO_3 system formed different products by iodination of ring A in position 3′ or 5′ (*ortho* and *para* to free 2′-OH), of ring B in position 4 (*ortho* to OMe), addition of I_2 to the double bond and cyclization to the corresponding flavanone or flavone, depending on the conditions used (Scheme 4). Best conditions for the preparation of

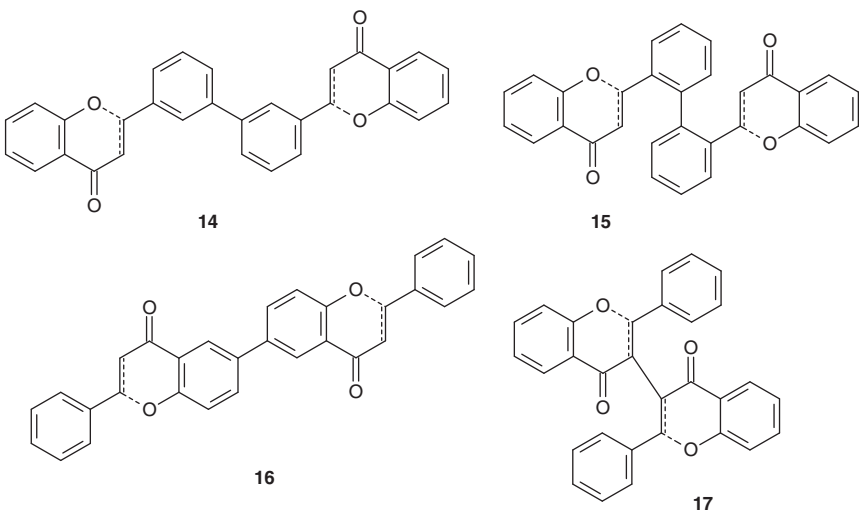
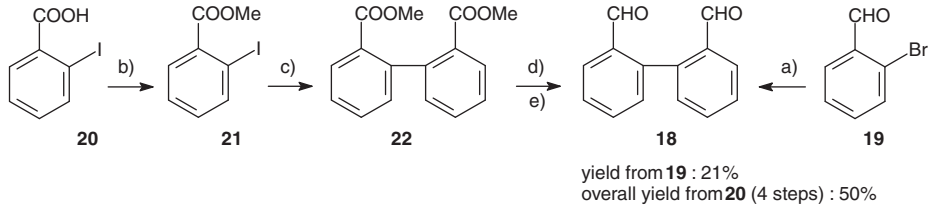
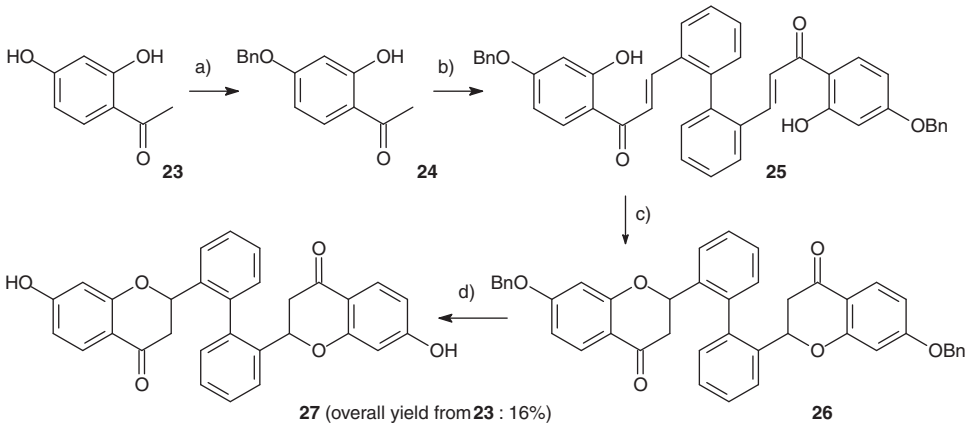


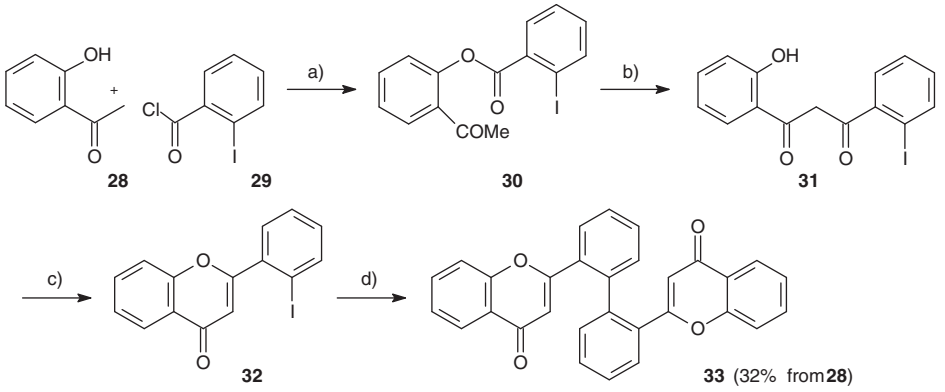
Figure 2. General structures of biflavonoids involved in this paper.



Scheme 1. Synthesis of dialdehyde **18**. Reagents and conditions: (a) Cu (activated), 200–220 °C, 30 min (21%); (b) MeOH–H_2SO_4, 95:5 v/v (86%); (c) Cu (activated), 200–220 °C, 30 min (73%); (d) $LiAlH_4$/THF, rt, 1 h (92%); (e) PDC/CH_2Cl_2, rt, 6 h (85%).



Scheme 2. Synthesis of biflavonoids of the BB (2′,2‴) type. Reagents and conditions: (a) BnCl (1.1 equiv)-K_2CO_3/DMF, 6 h, reflux, (93%); (b) **18**, NaH/THF, rt, 24 h (58%); (c) AcOH, reflux, 96 h (42%); (d) H_2–Pd/C, EtOAc, rt, 12 h (70%).



Scheme 3. Synthesis of 2′,2‴-biflavone, **33**. Reagents and conditions: (a) Py, 2 h, rt (87%); (b) KOH/Py, 1 h, 60 °C (72%); (c) AcOH/H_2SO_4, 95:5 v/v, reflux, 1 h (82%); (d) Cu (activated), 30 min, 200–220 °C (63%).

monoiodinated chalcone **35** involved the use of a 0.5:1:1 molar ratio of I_2/HIO_3: **34**, at 60 °C in aqueous acetic acid during 1 h, giving **35** in 45% isolated yield, together with 40% of **36** and trace amounts of products resulting from iodination of the double bond.[72]

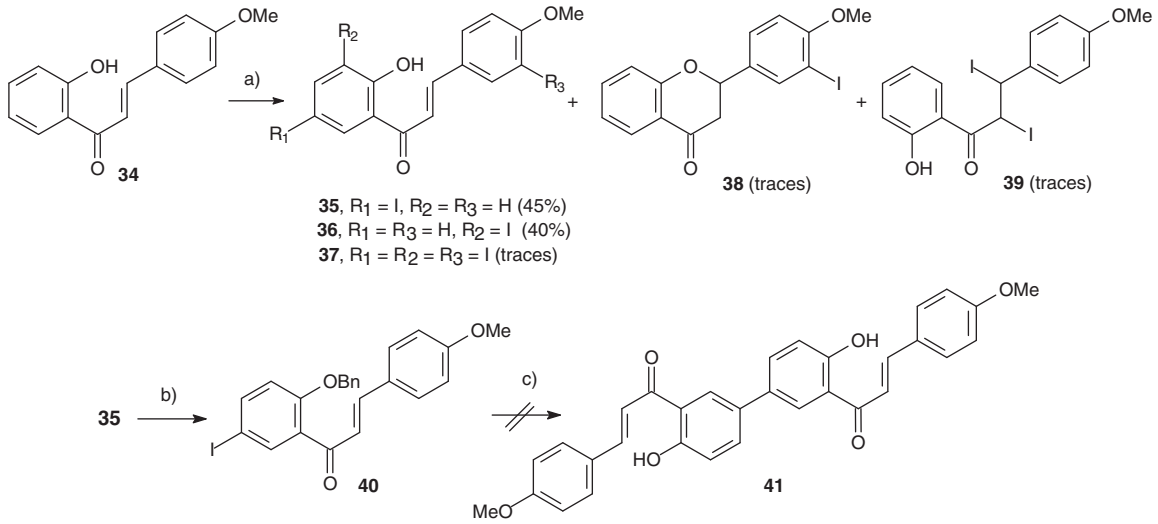
Treatment of chalcone **35** with benzyl chloride formed the corresponding protected chalcone **40**. Unfortunately, the Ullmann condensation of **40** in order to obtain **41**, was unsuccessful (Scheme 4).

In view of these results, we decided to try the synthesis of bichalcone **41** by first preparing the AA dimer **42**, followed by aldol condensation. This dimer was conveniently prepared in 42% overall yield from 4-aminophenol, **43** through a sequence involving diazotization and methylation to 4-iodoanisol, **44**, which gave 85% of the biphenyl **45** through Ullmann condensation, followed by formation of diacetate **46** and further Fries rearrangement to the desired dimer **42** (Scheme 5). It is worth mentioning that the more direct route involving the condensation of 5-iodo-2-benzyloxyacetophenone, **47**, gave only 5% of the desired dimer when submitted to the Ullmann condensation.

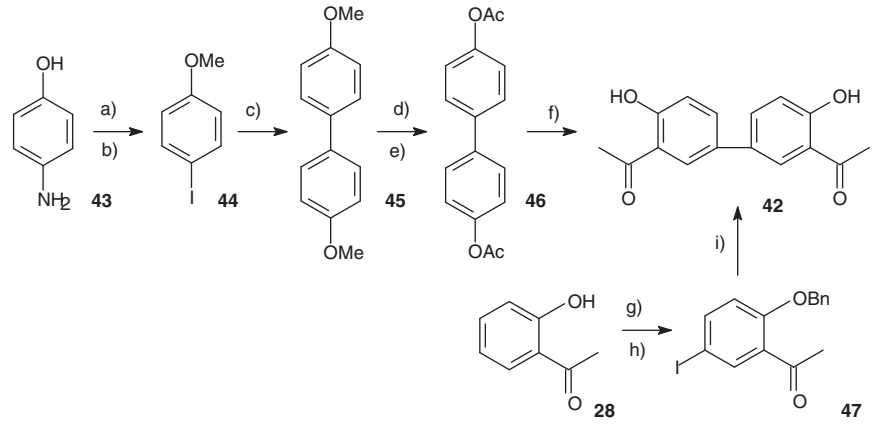
The aldol condensation of dimer **42** with *p*-anisaldehyde, **48**, yielded 2′,2‴-dihydroxy-4,4″-dimethoxy-5′,5‴-bichalcone, **41**. This compound was converted to the corresponding biflavone, **49** which was deprotected to give **50**. The overall yield of **50** from **42** was 55% (23% from **43**) (Scheme 6)

2.1.3. Biflavonoids of the CC-type

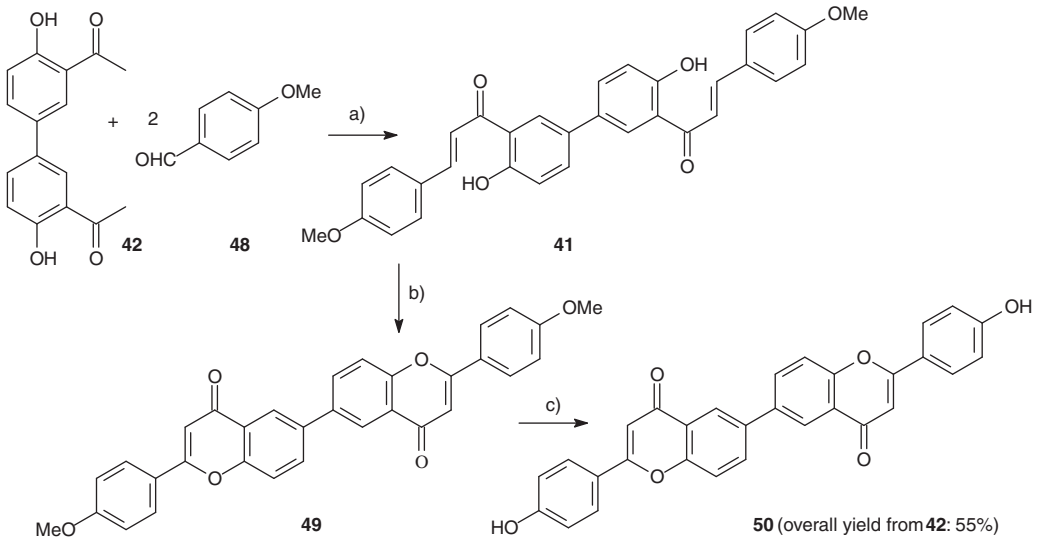
The synthesis of the 3,3″-biflavones **56**–**58** was performed as shown in Scheme 7. The treatment of chalcone **34** with the DMSO–I_2 system (using a 1:1 molar ratio of chalcone/I_2), formed the iodochalcone **53**, which was then submitted to an Ullmann



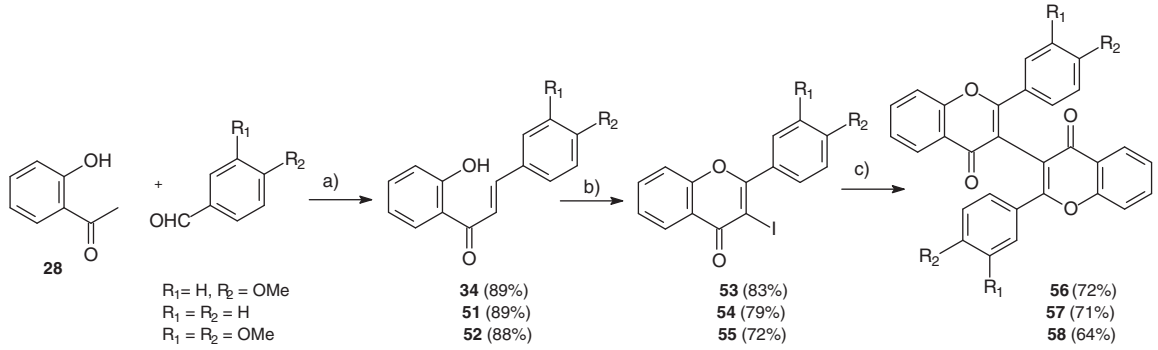
Scheme 4. Attempted preparation of bichalcone **41** via dimerization of chalcone **34**. Reagents and conditions: (a) I_2–HIO_3/AcOH–H_2O–EtOH (10:3:1 v/v), 50–60 °C, 1 h; (b) BnCl–K_2CO_3/DMF, reflux, 6 h (92%); (c) Cu, 200–220 °C, 2 h, no reaction.



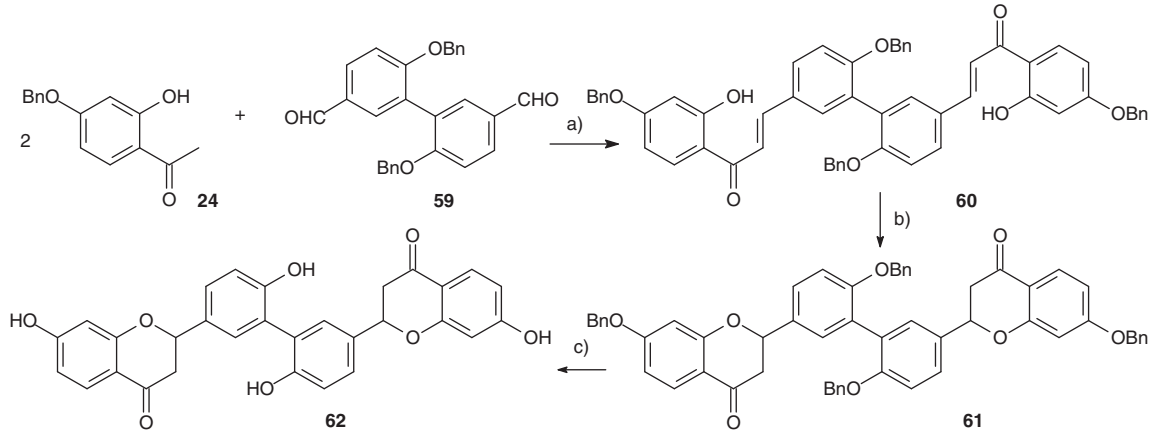
Scheme 5. Synthesis of dimer **42**. Reagents and conditions: (a_1) $NaNO_2$–HCl/H_2O, 0–5 °C, (a_2) KI/H_2O (79%); (b) Me_2SO_4–K_2CO_3/acetone, reflux, 24 h (98%); (c) Cu (activated), 200–220 °C, 2 h (85%); (d) BBr_3/CH_2Cl_2, −60 °C→TA, 6 h (90%); (e) Ac_2O–DMAP/Py, 60 °C, 3 h (97%); (f) $AlCl_3$/chlorobenzene, reflux 8 h (72%); (g) BnCl–K_2CO_3/DMF, reflux, 6 h (95%); (h) I_2–HIO_3/AcOH–H_2O–EtOH (10:3:1 v/v), reflux, 1 h (79%); (i) Cu, 200–220 °C, 2 h (5%).



Scheme 6. Synthesis of 4′,4‴-dihydroxy-5,5″-biflavone, **50**. Reagents and conditions: (a) NaH/THF, rt, 24 h (67%); (b) DMSO–I_2 (ratio chalcone/I_2 = 100:2), reflux, 1 h (89%); (c) BBr_3/CH_2Cl_2, −60 °C→rt, 7 days (92%).



Scheme 7. Synthesis of 3,3″-biflavones **56**–**58**. Reagents and conditions: (a) NaH/THF, rt, 16 h; (b) DMSO–I_2 (1 equiv), reflux, 12 h; (c) Cu (activated), 205–220 °C, 30 min.



Scheme 8. Synthesis of 5-deoxyanalog of 3′,3‴-binaringenin, **62**. Reagents and conditions: (a) NaH/THF, rt, 24 h, 72%; (b) AcOH, reflux, 96 h, 50%; (c) H_2–Pd/C 10%/EtOAc, rt, 6 h, 76%.

condensation to yield the biflavone **56**. In a similar way were obtained the biflavones **57** and **58** from chalcones **51** and **52**. The global yield of **56**–**58** from **28** was 40–53%.

Finally, using the methodology previously reported by us,[66] the 5-deoxyanalog of 3′,3‴-binaringenin was prepared (Scheme 8). To this end, protected resacetophenone, **24**, was condensed with dialdehyde **59** to form bichalcone **60**, which was then cyclized and debenzylated to biflavanone **62**. Dialdehyde **59** was prepared by us in a previous work,[66] by a four step synthesis from *p*-anisaldehyde (54%). Considering this fact, the global yield of **62** from readily available *p*-anisaldehyde was 15%.

2.2. Biological evaluation

In addition to compounds **25**–**27**, **33**, **49**, **50**, **57**, and **60**–**62** prepared in this work, the previously synthesized compounds **10** (binaringenin) and **63**–**69**,[66] were evaluated (Fig. 3). The figure also shows the corresponding monomers **70**–**86**, which are all known compounds and were prepared according to procedures previously reported by us.[65–67,73]

On the synthesized biflavonoids, as well as their monomers, a preliminary screening was carried out by a modified Agar Diffusion Bioautography method (Table 1).

None of the compounds were active against *A. oryzae* nor *F. solani* so these strains were not considered in further assays.

Then, the subset of active compounds was used in a quantitative screening by Microdilution to compare the antifungal activity of the biflavonoids to the corresponding monomers. Thus, the Minimum Inhibitory Concentration (MIC) of the compounds **25**, **33**, **50**, **57**, **62**, **63**, and **65**–**68** and their corresponding monomers were determined, as shown in Table 2. The MIC value of **60** against *C. neoformans* was not determined due to decomposition of the product during the assay.

3. Discussion

The antifungal activities (MIC values) are typical for a variety of flavonoids described in the literature.[74] Despite the reduced size of the set of compounds tested, some general trends seem to emerge from the inspection of Tables 1 and 2.

(a) Considering the microorganisms used:

The strains used showed differential susceptibility toward monomers and dimers, but no general trend (i.e., a higher susceptibility for either monomers o dimers) could be derived for any of the microorganism used. The most susceptible strain used was *A. niger*, and the majority of the biflavonoids that showed activity in the qualitative screening were active against this microorganism. Conversely, it is interesting to note that none of the compounds tested showed activity against *A. oryzae*.

Half of the biflavonoids whose MIC was determined (five out of ten) were active against only one of the five strain of the panel, and this strain was *A. niger* in four cases.

(b) Regarding the interflavonyl linkage:

Biflavonoids of the BB-type represented the biggest set of compounds assayed, comprising four compounds linked through the 2-position (compounds **25**–**27** and **33**) and eleven through the 3-position (compounds **10** and **60**–**69**). Two of the four representatives of the 2,2′-linkage and seven of the other group were active against

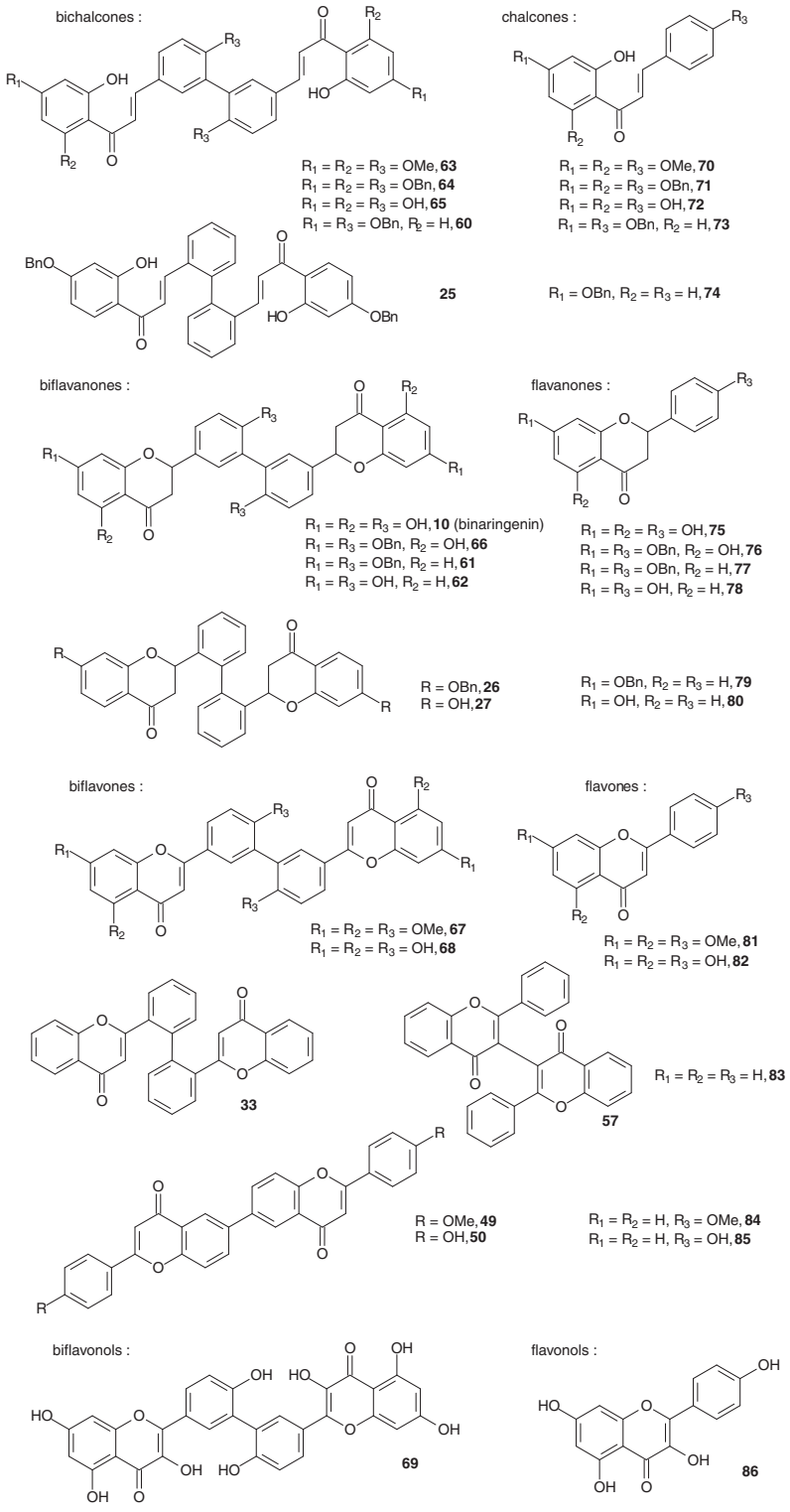


Figure 3. Biflavonoids tested for their antifungal activity and the corresponding monomers.

at least one microorganism of the panel, Table 1. The activity of the biflavonoids with the 2,2′-linkage was poor and lower than that of the corresponding monomers, Table 2. On the other hand, some members of the group linked through the 3-position showed an interesting activity, higher than that of the corresponding monomers (compounds **62**, **63**, **65**, and **67**), Table 2.

The only CC-type biflavonoid tested, **57**, was one of the two compounds showing activity against three strains in the qualitative screening, even though the MIC values were poor. The corresponding monomer displayed a better or similar activity, Table 2

Regarding the two AA-type biflavonoid tested, only one showed a slight activity against Cp, **50**, comparable to the corresponding monomer.

(c) Considering the C_3 moiety, five 2′,2‴-dihydroxybichalcones, six biflavanones, six biflavones, and one biflavonol were tested:

Table 1
Fungal activity of biflavonoids against seven isolates of pathogen fungi, determined by Agar Diffusion Bioautography method

Entry	Site of union	Ring A	Ring B	Compound	Ca	Cp	Cn	An	Ao	Fs	Rs
2′,2‴-Dihydroxybichalcones											
1	2,2″	4′-OBn	H	**25**	–	–	+	+	–	–	–
2	3,3″	4′-OBn	4-OBn	**60**	–	–	+	–	–	–	–
3	3,3″	4′,6′-DiOMe	4-OMe	**63**	–	–	–	+	–	–	–
4	3,3″	4′,6′-DiOBn	4-OBn	**64**	–	–	–	–	–	–	–
5	3,3″	4′,6′-DiOH	4-OH	**65**	+	–	+	–	–	–	+
Biflavanones											
6	3′,3‴	5,7-DiOH	4-OH	**10**	–	–	–	–	–	–	–
7	2′,2‴	7-OBn	H	**26**	–	–	–	–	–	–	–
8	2′,2‴	7-OH	H	**27**	–	–	–	–	–	–	–
9	3′,3‴	7-OBn	4-OBn	**61**	–	–	–	–	–	–	–
10	3′,3‴	7-OH	4-OH	**62**	–	–	–	+	–	–	–
11	3′,3‴	5-OH, 7-OBn	4-OBn	**66**	+	–	–	–	–	–	–
Biflavones											
12	2′,2‴	H	H	**33**	–	–	–	+	–	–	+
13	6,6″	H	4′-OMe	**49**	–	–	–	–	–	–	–
14	6,6″	H	4′-OH	**50**	–	+	–	–	–	–	–
15	3,3″	H	H	**57**	–	+	+	–	–	–	+
16	3′,3‴	5,7-DiOMe	4′-OMe	**67**	–	–	–	+	–	–	–
17	3′,3‴	5,7-DiOH	4′-OH	**68**	–	–	–	+	–	–	–
Biflavonols											
18	3′,3‴	5,7-DiOH	4-OH	**69**	–	–	–	–	–	–	–
Controls											
				Amphotericine B	+	+	+	+	+	+	+
				Acetone	–	–	–	–	–	–	–

Ca: *Candida albicans* (ATCC 90028), Cp: *Candida parapsilosis* (ATCC 22019), Cn: *Cryptococcus neoformans* (ATCC 14116), An: *Aspergillus niger* (ATCC 16404), Ao: *Aspergillus oryzae* (ATCC 10124), Fs: *Fusarium solani* (ATCC 36031), Rs: *Rhizopus stolonifer* (+) (ATCC 6227b).

Table 2
Minimum Inhibitory Concentration (MIC, μmol/mL) of selected biflavonoids and their corresponding monomers

Entry	Site of union	Ring A	Ring B	Compound	Ca	Cp	Cn	An	Rs
1	2,2″	4′-OBn	H	**25**			>0.8	>0.8	
				74			>0.8	0.1	
2	3,3″	4′,6′-DiOMe	4-OMe	**63**				0.013	
				70				>0.8	
3	3,3″	4′,6′-DiOH	4-OH	**65**	0.8		0.013		0.2
				72	>0.8		0.4		0.8
4	3′,3‴	7-OH	4-OH	**62**				0.2	
				78				>0.8	
5	3′,3‴	5-OH, 7-OBn	4-OBn	**66**	>0.8				
				76	>0.8				
6	2′,2‴	H	H	**33**				>0.8	0.8
				83				0.8	0.1
7	6,6″	H	4′-OH	**50**		0.4			
				85		0.2			
8	3,3″	H	H	**57**		0.4	>0.8		>0.8
				83		0.2	0.1		0.1
9	3′,3‴	5,7-DiOMe	4′-OMe	**67**				0.4	
				81				>0.8	
10	3′,3‴	5,7-DiOH	4′-OH	**68**				0.1	
				82				0.006	

Ca: *Candida albicans* (ATCC 90028), Cp: *Candida parapsilosis* (ATCC 22019), Cn: *Cryptococcus neoformans* (ATCC 14116), An: *Aspergillus niger* (ATCC 16404), Rs: *Rhizopus stolonifer* (+) (ATCC 6227b).

All assayed 2′,2‴-dihydroxybichalcones were active at least against one fungus, except for **64**, an hexabenzyloxy-substituted bichalcone, Table 1 This observation is consistent with the work of Zacchino and co-workers about the antifungal activity of different chalcones.[75] They found that the presence of the α,β-conjugated system and a high degree of coplanarity between the A- or B-rings and the C_3 moiety are required for the antifungal activity. The presence of electron donating groups in 4′ increases the planarity by favoring the electronic delocalization,[76] whereas substitution in 6′ diminishes the planarity by a steric effect.[77] Thus the presence of a bulky benzyloxy group in 6′ could be responsible for the loss of antifungal activity in **64**. In the same direction, the planarity could be related to the strength of the hydrogen bond of the 2′-OH proton, which is involved in intramolecular hydrogen bond to the carbonyl of the C_3 moiety. For this system, it is reported that the magnitude of the proton chemical shift is directly proportional to the strength of the hydrogen bond,[78,79] so a comparison of the 2′-OH shifts in different 2′-hydroxychalcones could give an indication of the planarity of the system through the strength of the hydrogen bond. In our case (using $CDCl_3$ as solvent) we observed the 2′-OH protons as sharp singlets for compounds **63** and **65**, at δ >14 ppm, whereas the corresponding proton in **64** appears as a broad singlet at δ <14 ppm, indicating a smaller degree of coplanarity, resulting in the loss of antifungal activity. In addi-

tion to weaken the hydrogen bond, the existence of other effects on the antifungal activity caused by the presence of six benzyloxy groups can not be ruled out.

All biflavanones were inactive, except for **66** and **62** which were active against only one microorganism, Table 1. The poor antifungal activity of these compounds can be explained by the absence of a double bond in the C_3 moiety. This observation is consistent with the fact that compounds obtained by conjugate addition of diethyl malonate or malononitrile to a chalcone were inactive.[77] On the other hand, it was reported a slight activity of 7-hydroxyflavanone, **80**, against different fungi.[80] However biflavanone **27**, dimer of **80**, displayed no activity when submitted to the panel, Table 1.

All assayed biflavones were active at least against one fungus, except for **49**, Table 1.

Biflavonol **69** resulted inactive against the whole panel. This observation is consistent with the fact that monomeric flavonols such as 3-hydroxyflavone and quercetin are inactive or slightly active against different fungi.[81]

Considering the MIC values, Table 2, bichalcones (entries 1–3) gave the smallest values of MIC and were generally more active than their corresponding monomers (compounds **63**, **65**, exception: **25**). On the other hand, biflavones (entries 6–10) show an opposite trend, being the monomers generally more active than the dimers (compounds **33**, **50**, **57**, **68**, exception: **67**). For the biflavanones, **66** and **62** (entries 4 and 5), no trend could be derived.

(d) The most active biflavonoids tested were two bichalcones of the BB-type, **63** and **65**, showing different activity profiles: whereas **65** was active against three microorganisms of the panel (*C. albicans*, *C. neoformans*, and *R. stolonifer* (+)), **63** was active only against *A. niger*. Both dihydroxybichalcones were more active than the corresponding monomers and displayed the same substitution pattern (4,4′,6′), but the substituents are OH for **65** and MeO for **63**.

4. Conclusions

We have prepared a small library of natural and unnatural biflavonoids in good yields using a reliable set of conditions, based on aldol and Ullmann condensations of suitable precursors. Both reactions were optimized according to the type of desired biflavanoid. In particular, the order of events is dependent on the structure of the target compound (AA-, BB-, or CC-type), that is, the Ullmann condensation can be performed either on the whole monomer or an early intermediate previous to the aldol reaction.

The results of the antifungal activity on 18 biflavonoids (and their monomers) indicate that various compounds such as bichalcones **63** and **65** display interesting in vitro activity against some human pathogenic fungi. The pronounced differential activity found between monomers and dimers in the more active bichalcones merits further studies, which are being conducted in our laboratory.

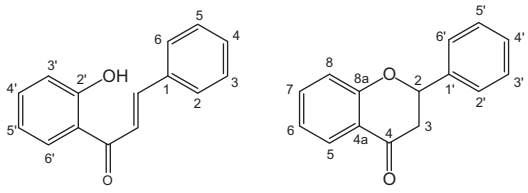
5. Experimental

5.1. Chemistry

All reagents were purchased from commercial sources and used without purification, unless otherwise indicated. All solvents were dried and distilled prior to use. THF was dried by refluxing with benzophenone over sodium wire until a blue color persisted, then distilled and collected. CH_2Cl_2 was dried by refluxing over P_2O_5 for 3 h, then distilled and collected. All reactions were monitored by TLC. TLC was carried out on Alugram® Sil G/UV_{254} on polyester plates using different solvent systems. Column chromatography (CC) was carried out on silica gel (Merck, 60–230 mesh) using hexanes as initial eluent followed by different eluent mixtures (CH_2Cl_2/hexanes; ethyl acetate/hexanes or ethyl acetate/CH_2Cl_2/hexanes).

^1H and ^{13}C NMR spectra were recorded at 30 °C on a Bruker DPX-400 spectrometer (at 400 and 100 MHz, respectively). ^1H NMR were recorded using TMS as the internal reference. ^{13}C NMR were recorded using the residual solvent as the internal reference (the central peak of the $CDCl_3$ triplet was assigned to 77.00 ppm). Assignments of H and C were done on the basis of 2D NMR (^1H–^1H COSY, ^1H,^{13}C HMQC and ^1H,^{13}C HMBC) experiments. Mass spectra were recorded on a Shimadzu GC–MS QP 1100 EX spectrometer at 70 eV. HRMS were recorded on an AutoSpecQ spectrometer at 70 eV (EI, positive mode). Melting points were determined using a Gallenkamp capillary melting point apparatus and are uncorrected. Elemental analyses were obtained from vacuum-dried samples (over phosphorus pentoxide at 3–4 mmHg, 24 h at room temperature) and performed on a Fisons EA1108 CHNS–O analyzer.

Assignments of H and C are given according to the following numbering scheme (in flavanones, C=O means C_4):



5.1.1. Synthesis of chalcones and 2′-hydroxychalcones. General procedure 1 (adapted from a procedure reported by Stout)[82]

This procedure is suitable to compounds that not contain free phenolic groups or when the phenolic groups are protected as methyl or benzylethers.

To a solution of the corresponding 2′-hydroxyacetophenone (10 mmol) in dry THF (25 mL), NaH (25 mmol, 1 g of 60% dispersion in mineral oil) was added in portions, under a nitrogen atmosphere and with vigorous stirring. When the evolution of H_2 ceased, a solution of the corresponding benzaldehyde (10 mmol) in dry THF (25 mL) was added dropwise over 15 min and the reaction mixture was stirred at room temperature for 16 h, except otherwise stated. After this, the mixture was poured cautiously over ice-water (100 mL) to destroy the excess of NaH and stirred until the evolution of H_2 subsided. The mixture was acidified with 25% HCl and extracted with ethyl acetate (3×50 mL). The organic layer was washed with water (3×50 mL), brine (50 mL), and dried over anhydrous $MgSO_4$. The solution was concentrated under vacuum at 40 °C, until it reached 1/3 of the original volume. After cooling at 0 °C for 12 h the crystalline product was separated by filtration. The 2′-hydroxychalcones obtained in this way are pure enough for most purposes. Analytical samples were obtained after purification by column chromatography.

Using this procedure, good yields of chalcones were obtained. An additional amount of product can be obtained from the mother liquors. To this end, the solvent was removed under vacuum and the compound was purified by column chromatography.

5.1.2. Synthesis of polyhydroxychalcones. General procedure 2 (adapted from a procedure reported by Sogawa)[83]

This procedure is suitable to compounds with phenolic groups protected as tetrahydropyranylethers.

5.1.2.1. Synthesis of tetrahydropyranylethers. To a stirred suspension of the corresponding phenol (0.1 mol) in dry CH_2Cl_2 (100 mL), dry *p*-TsOH (0.85 g, 0.005 mol) and dry pyridine (recently distilled from KOH) (0.40 mL, 0.39 g, 0.005 mol) were added under a nitrogen atmosphere. After this, 3,4-dihydro-α-pyran (DHP, 10.5 g, 0.125 mol, 11.4 mL per OH group) was added dropwise. The reaction mixture was stirred at room temperature for 30 min, and after this time, a clear solution was obtained. The solvent was removed under vacuum and the residue was dissolved in EtOAc (500 mL). The organic layer was washed with water (3 × 100 mL), brine (100 mL), separated and dried over anhydrous $MgSO_4$. The solvent was removed under vacuum and the compound was purified by column chromatography.\

5.1.2.2. Synthesis of 2′-hydroxy-tetrahydropyranyloxychalcones. To a solution of the corresponding 2′-hydroxyacetophenone (10 mmol) and benzaldehyde (10 mmol) in absolute MeOH (25 mL), barium hydroxide octahydrate (3.2 g, 0.01 mol) was added under a nitrogen atmosphere and with vigorous stirring. The reaction mixture was stirred at 60 °C for 12 h. After this, the mixture was neutralized with 10% HCl and extracted with ethyl acetate (3 × 50 mL). The organic layer was washed with water (3 × 50 mL), brine (50 mL), and dried over anhydrous $MgSO_4$. The solvent was removed under vacuum and the compound was purified by column chromatography.

5.1.2.3. Deprotection of 2′-hydroxy-tetrahydropyranyloxychalcones. To a solution of the corresponding 2′-hydroxy-tetrahydropyranyloxychalcone (1 mmol) in absolute MeOH (20 mL), *p*-toluenesulfonic acid (10 mg, 0.058 mmol) was added. The reaction mixture was stirred for 2 h at room temperature and then the solvent was removed under vacuum. After this, water (50 mL) was added and the mixture was extracted with EtOAc (3 × 50 mL). The organic layer was washed with water (3 × 50 mL), brine (50 mL), and dried over anhydrous $MgSO_4$. The solvent was removed under vacuum and the compound was purified by column chromatography.

5.1.3. Synthesis of flavanones using AcOH as solvent. General procedure 3

A solution of the corresponding 2′-hydroxychalcone (1 mmol) in glacial AcOH (15 mL) was refluxed for 72 h under nitrogen atmosphere. Then, the solution was poured in 100 mL of cold water and extracted with ethyl acetate (3 × 50 mL). The organic layer was washed with water (3 × 50 mL), brine (50 mL), separated and dried over anhydrous $MgSO_4$. Cyclohexane (10 mL) was added and the solvent was removed under vacuum. The compound was purified by column chromatography.

5.1.4. Synthesis of flavanones by microwave heating. General procedure 4[65]

This procedure is suitable for compounds that not contain benzyloxy groups.

To a solution of desired chalcone (0.1 mmol) in dry CH_2Cl_2 (5 mL), TFA (0.2 mL, 0.307 g, 2.69 mmol) and 1 g of silica gel (1 g) were added. The mixture was evaporated under vacuum and the resulting powder was poured into a loosely stoppered test tube. The tube was placed vertically in the center of the microwave oven besides a vial with ice (20 g), and irradiated for three successive periods of 3 min with cooling intervals of 5 min. After irradiation was finished, the solid mixture was extracted with EtOAc (3 × 15 mL) and filtered. The organic layer was washed with water, brine, separated and dried over anhydrous $MgSO_4$. The mixture was purified by column chromatography.

5.1.5. Synthesis of flavones. General procedure 5 (adapted from a procedure reported by Song et al.)[84]

To a solution of the corresponding 2′-hydroxychalcone (1 mmol) in DMSO (10 mL), iodine (2.5 mg, 0.01 mmol) was added. The reaction mixture is placed in a pre-heated sand bath (190 °C) and refluxed for 1 h. After this time, water (50 mL) was added and the mixture was extracted with EtOAc (3 × 50 mL). The organic layer was washed with a 10% solution of $NaHSO_3$ (50 mL), water (3 × 50 mL), brine (50 mL), and dried over anhydrous $MgSO_4$. The solvent was removed under vacuum and the compound was purified by column chromatography.

5.1.6. Synthesis of flavonols. General procedure 6

To a solution of the corresponding flavone (1 mmol) in MeOH (15 mL), a solution of KOH (170 mg, 3 mmol) in MeOH (15 mL) was added with stirring at room temperature. After this, iodobenzene diacetate (IBD) (350 mg, 1.1 mmol) was added in four portions. The reaction mixture was stirred at room temperature for 72 h. After this, water (50 mL) was added and the mixture was extracted with EtOAc (3 × 50 mL). The organic layer was washed with water (3 × 50 mL), brine (50 mL), and dried over anhydrous $MgSO_4$. The solvent was removed under vacuum and the compound was purified by column chromatography.

5.1.7. Deprotection of methoxy groups in methylphenylethers. General procedure 7

The corresponding methoxyflavone or methoxyflavonol (1 mmol) was dissolved in dry CH_2Cl_2 (15 mL) under a nitrogen atmosphere. The solution was cooled to −60 °C and BBr_3 (3 mmol per OMe group; 3 mL of a 1 M solution in CH_2Cl_2) was added dropwise from an equalizer. The reaction mixture was stirred at room temperature for the indicated time. After this, the mixture was poured cautiously over ice-water (50 mL) and extracted with EtOAc (3 × 50 mL). The organic layer was washed with water (3 × 50 mL), brine (50 mL), and dried over anhydrous $MgSO_4$. The solvent was removed under vacuum and the compound was purified by column chromatography.

5.1.8. Hydrogenation of benzyloxychalcones and benzyloxyflavanones. General procedure 8

To a solution of the corresponding benzyloxychalcone or benzyloxyflavanone (0.5 mmol) in ethyl acetate (50 mL), 50 mg of 10% Pd–C was added and the mixture was stirred at room temperature under a H_2 atmosphere. After 1 h, the mixture was filtered and washed with ethyl acetate (3 × 50 mL). The solvent was removed under vacuum. The compound was purified by column chromatography.

5.1.9. Synthesis of alkylethers. General procedure 9

To a solution of the corresponding phenol (0.01 mol) in anhyd DMF (15 mL), the suitable alkyl halide (0.011 mol per OH group) and K_2CO_3 (0.011 mol per OH group) were added. The reaction mixture is vigorously stirred and refluxed for 6 h under a nitrogen atmosphere. After this, ice-water (100 mL) was added and the mixture was stirred until all carbonate was dissolved. Concd HCl was added until pH 1 and stirred until the evolution of CO_2 subsided. The mixture was extracted with EtOAc (3 × 100 mL). The organic layer was washed with water (3 × 100 mL), brine (100 mL), and dried over anhydrous $MgSO_4$. The solvent was removed under vacuum and the compound was purified by column chromatography.

5.1.10. Synthesis of aryl iodides. General procedure 10

To a solution of the corresponding arene (0.1 mol) in a mixture of AcOH (250 mL), H_2O (75 mL) y EtOH (25 mL), I_2 (0.06 mol), and HIO_3 (0.1 mol) were added. The reaction mixture was refluxed for 3 h. After this, the mixture was poured cautiously over ice-water

(500 mL) and the solid was separated by means of a Buchner funnel. The solid is dissolved in EtOAc (150 mL) and a saturated solution of Na_2SO_3 (50 mL) was added. The organic layer was separated and washed with water (3 × 50 mL), brine (50 mL), and dried over anhydrous $MgSO_4$. The solvent was removed under vacuum and the compound was purified by column chromatography.

5.1.11. Synthesis of biaryls by Ullmann reaction. General procedure 11

To a solution of the corresponding aryl iodide (0.01 mol) in EtOAc (50 mL), activated Cu[85] (2.5 g, 0.04 mol) was added and the solvent was removed under vacuum.† A reflux condenser was connected and the mixture was heated for 30 min at 205–220 °C, under N_2 atm, with stirring. The mixture was allowed to cool under N_2 atm. EtOAc (100 mL) was added and the mixture was vigorously stirred at rt under N_2 atm until a fine suspension was obtained. The suspension was filtered through a sintered glass pad and washed with EtOAc (250 mL, in portions) until absence of product (checked by TLC in the mother liquors). The solvent was removed under vacuum and the compound was purified by column chromatography.

5.1.12. Synthesis of flavones by the Baker–Venkataraman method. General procedure 12

5.1.12.1. Synthesis of esters of 2′-hydroxyacetophenones. A solution of the corresponding 2′-hydroxyacetophenone (10 mmol) and acid chloride (10 mmol) in dry pyridine (15 mL), was stirred at rt for 2 h. After this time, the mixture was neutralized with 10% HCl and extracted with EtOAc (3 × 50 mL). The organic layer was washed with water (3 × 50 mL), brine (50 mL), and dried over anhydrous $MgSO_4$. The solvent was removed under vacuum and the compound was purified by column chromatography.

5.1.12.2. Synthesis of 1,3-diaryl-1,3-diketones by Baker-Venkataraman rearrangement. To a solution of the corresponding ester of 2′-hydroxyacetophenone (1 mmol) in dry pyridine (15 mL), KOH (850 mg, 15 mmol) was added. The reaction mixture was heated at 60 °C for 30 min.

After this time, the mixture was neutralized with 10% HCl and extracted with ethyl acetate (3 × 50 mL). The organic layer was washed with water (3 × 50 mL), brine (50 mL), and dried over anhydrous $MgSO_4$. The solvent was removed under vacuum and the compound was purified by column chromatography.

5.1.12.3. Acid cyclization of 1,3-diaryl-1,3-diketones. A solution of the corresponding 1,3-diaryl-1,3-diketone (1 mmol) in AcOH–H_2SO_4 (10 mL; 95:5 v/v), was refluxed for 1 h. After this time, water (100 mL) was added the mixture was extracted with ethyl acetate (3 × 50 mL). The organic layer was washed with water (3 × 50 mL), brine (50 mL), and dried over anhydrous $MgSO_4$. The solvent was removed under vacuum and cyclohexane (10 mL) was added. The solvent was removed under vacuum and the compound was purified by column chromatography.

5.1.13. Acetylation of phenols. General procedure 13

To a solution of the corresponding phenol (1 mmol) in dry pyridine (5 mL) Ac_2O (freshly distilled, 130 mg, 1.25 mmol per OH group) and *N*,*N*-dimethylaminopyridine (DMAP, 10 mg), were added. The mixture was heated at 60 °C for 3 h with stirring. After this time, H_2O (50 mL) was added and the mixture was stirred for 15 min to destroy the excess of Ac_2O. The mixture was extracted with EtOAc (3 × 30 mL). The organic layer was washed with water (3 × 30 mL), brine (30 mL), and dried over anhydrous $MgSO_4$. The solvent was removed under vacuum and the compound was purified by column chromatography.

5.1.14. 2′,2‴-Dihydroxy-4′,4‴-dibenzyloxy-2,2″-bichalcone, 25

Prepared according general method 1, from **24** (484 mg, 2 mmol) and **18** (210 mg, 1 mmol), stirred at rt for 24 h. Purified by CC (hexanes→EtOAc/CH_2Cl_2/hexanes 1:5:5). Yield: 382 mg (58%).

Yellow solid, mp: 129.0–130.5 °C.

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 13.28 (1H, s, 2′-OH), 8.21 (1H, d, $J_{\beta\alpha}$ = 15.4, H_β), 7.81 (1H, d, $J_{6'5'}$ = 8.0, $H_{6'}$), 7.72 (1H, dd, J_{65} = 7.8, J_{64} = 1.4, H_6), 7.64 (1H, dd, J_{34} = 8.0, J_{35} = 1.0, H_3), 7.49 (1H, d, $J_{\alpha\beta}$ = 15.4, H_α), 7.44–7.32 (6 H, m, phenyl + H_5), 7.25 (1H, ddd, J_{43} = 8.0, J_{45} = 7.3, J_{46} = 1.6, H_4), 6.57–6.54 (2H, m, $H_{3'}$ + $H_{5'}$), 5.11 (2H, s, CH_2).

^{13}C NMR ($CDCl_3$, 100 MHz): δ = 191.5 (C=O), 166.7 ($C_{4'}$), 165.5 ($C_{2'}$), 150.0 (C_2), 142.7 (C_1), 135.8 (C_β), 135.0 ($C_{1phenyl}$), 133.6 (C_4), 131.4 (C_6), 128.7 ($C_{6'}$), 128.3, 128.0, 127.7, 127.5, 126.0 (C_3 + C_5 + $(C_2$–$C_6)_{phenyl}$), 123.3 (C_α), 114.2 ($C_{1'}$), 108.3 ($C_{5'}$), 102.2 ($C_{3'}$), 70.3 (CH_2).

MS (EI, 70 eV): *m*/*z* (%) = 658 (26.2, M^+), 568 (15.4, M^+–$C_6H_5CH_2^+$), 478 (2.4, M^+–$2C_6H_5CH_2^+$), 91 (100.0, $C_6H_5CH_2^+$).

Anal. Calcd for $C_{44}H_{34}O_6$: C, 80.23; H, 5.20. Found: C, 80.41; H, 5.11.

5.1.15. 7,7″-Dibenzyloxy-2′,2‴-diflavanone, 26

Prepared according to general method 3, from **25** (329 mg, 0.5 mmol) and refluxing for 96 h. Purified by CC (hexanes→EtOAc/CH_2Cl_2/hexanes 1:5:5). Yield: 138 mg (42%).

White solid, mp: 118–120 °C.

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.85 (1H, d, J_{56} = 8.7, H_5), 7.79–7.60 (2H, m, $H_{3'}$ + $H_{6'}$), 7.45–7.31 (6 H, m, phenyl + $H_{5'}$), 7.28 (1H, ddd, $J_{4'5'}$ = 7.2, $J_{4'3'}$ = 7.9, $J_{4'6'}$ = 1.6, $H_{4'}$), 6.60 (1H, dd, J_{65} = 8.7, J_{68} = 2.3, H_6), 6.45 (1H, d, J_{86} = 2.3, H_8), 5.44 (1H, dd, $J_{2,3ax}$ = 13.2, $J_{2,3eq}$ = 2.9 H_2), 5.13 (2H, s, CH_2), 3.04 (1H, dd, $J_{3ax,3eq}$ = 16.8, $J_{3ax,2}$ = 13.2, H_{3ax}), 2.83 (1H, dd, $J_{3eq,3ax}$ = 16.8, $J_{3eq,2}$ = 2.9 H_{3eq}).

^{13}C NMR ($CDCl_3$, 100 MHz): δ = 190.2 (C=O), 164.6 (C_7), 163.9 (C_{8a}), 148.2 ($C_{2'}$), 140.3 ($C_{1'}$), 136.1 ($C_{1phenyl}$), 131.0 ($C_{3'}$), 129.3, 128.6, 128.3, 128.1, 128.0, 127.5, 127.1, ($C_{4'}$ + $C_{5'}$ + $C_{6'}$ + C_5 + $((C_2$–$C_6)_{phenyl}$), 116.2 (C_{4a}), 107.4 (C_6), 100.3 (C_8), 78.3 (C_2), 70.0 (CH_2), 44.1 (C_3).

MS (EI, 70 eV): *m*/*z* (%) = 658 (21.2, M^+), 568 (10.1, M^+–$C_6H_5CH_2^+$), 91 (100.0, $C_6H_5CH_2^+$).

Anal. Calcd for $C_{44}H_{34}O_6$: C, 80.23; H, 5.20 Found: C, 80.05; H, 5.35.

5.1.16. 7,7″-Dihydroxy-2′,2‴-diflavanone, 27

Prepared according to general method 8, from **26** (132 mg, 0.2 mmol) with 12 h of stirring at rt Purified by CC (hexanes→EtOAc/hexanes 1:1). Yield: 67 mg (70%).

White solid, mp: 135–136.5 °C.

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.83 (1H, d, J_{56} = 8.7, H_5), 7.50–7.30 (4H, m, $H_{3'}$–$H_{6'}$), 6.55 (1H, dd, J_{65} = 8.7, J_{68} = 2.3, H_6), 6.40 (1H, d, J_{86} = 2.3, H_8), 5.43 (1H, dd, $J_{2,3ax}$ = 13.2 $J_{2,3eq}$ = 3.0, H_2), 3.03 (1H, dd, $J_{3ax,3eq}$ = 16.8, $J_{3ax,2}$ = 13.2, H_{3ax}), 2.83 (1H, dd, $J_{3eq,3ax}$ = 16.8, $J_{3eq,2}$ = 3.0, H_{3eq}).

^{13}C NMR ($CDCl_3$, 100 MHz): δ = 190.3 (C=O), 166.7 (C_7), 162.9 (C_{8a}), 148.5 ($C_{2'}$), 142.5 ($C_{1'}$), 131.4 ($C_{3'}$), 128.2, 128.0, 127.6, 127.2 ($C_{4'}$, $C_{5'}$, $C_{6'}$, C_5), 117.1 (C_{4a}), 107.4 (C_6), 101.0 (C_8), 77.9 (C_2), 44.3 (C_3).

MS (EI, 70 eV): *m*/*z* (%) = 659 (25.7, M^++1), 658 (100.0, M^+), 657 (58.4, M^+−1), 137 (33.2), 136 (10.0), 121 (10.1), 129 (63.8).

Anal. Calcd for $C_{30}H_{22}O_6$: C, 75.30; H, 4.63. Found: C, 75.58; H, 4.38.

† This step is performed to obtain an intimate mixture of the reactants and is advantageous over the use of a mortar.

5.1.17. Iodination of 2′-hydroxy-4-methoxychalcone

According to general method 10, **34** (254 mg, 1 mmol) was treated with iodine (254 mg, 1 mmol) and HIO_3 (90 mg, 0.5 mmol) and heated at 50–60 °C for 1 h. The mixture was separated by CC (hexanes→EtOAc/hexanes 2:8). The following products were obtained: **35** (45%), **36** (40%), **37** (1%), **38** (2%), and **39** (1%).

5.1.18. 2′-Hydroxy-5′-iodo-4-methoxychalcone, 35

Yellow solid, mp: 178–180 °C (lit.: 175 °C).[86]

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 12.88 (1H, s, 2′-OH), 8.16 (1H, d, $J_{6'5'}$ = 2.1, $H_{6'}$), 7.92 (1H, d, $J_{\beta\alpha}$ = 15.4, H_β), 7.71 (1H, dd, $J_{4'3'}$ = 8.8, $J_{4'6'}$ = 2.1, $H_{4'}$), 7.67–7.62 (2H, m, H_2 + H_6), 7.42 (1H, d, $J_{\alpha\beta}$ = 15.3, H_α), 7.01–6.94 (2H, m, H_3 + H_5), 6.81 (1H, d, $J_{3'4'}$ = 8.8, $H_{3'}$), 3.88 (3H, s, OMe).

^{13}C NMR ($CDCl_3$, 100 MHz): δ = 192.5 (C=O), 163.1 ($C_{2'}$), 162.4 (C_4), 146.4 ($C_{4'}$), 144.3 (C_β), 137.8 ($C_{6'}$), 130.8 (C_2 + C_6), 127.1 (C_1), 122.3 ($C_{1'}$), 121.0 (C_α), 116.9 ($C_{3'}$), 114.6 (C_3 + C_5), 79.6 ($C_{5'}$), 55.5 (OMe).

MS (EI, 70 eV): *m*/*z* (%) = 381 (10.2, M^++1), 380 (100.0, M^+), 254 (M^+−I, 23.2), 127 (9.4, I).

Anal. Calcd for $C_{16}H_{14}IO_3$: C, 50.55; H, 3.45. Found: C, 50.72; H, 3.28.

5.1.19. 2′-Hydroxy-3′-iodo-4-methoxychalcone, 36

Yellow solid, mp: 153–154 °C (lit.: 152.0–152.5 °C).[87]

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 13.92 (1H, s, 2′-OH), 7.98–7.92 (3H, m, H_β + $H_{6'}$ + $H_{4'}$), 7.66–7.58 (2H, m, H_2 + H_6), 7.52 (1H, d, $J_{\alpha\beta}$ = 15.4, H_α), 7.00–6.93 (2H, m, H_3 + H_5), 6.74 (1H, t, $J_{5'4'}$ = $J_{5'6'}$ = 7.8, $H_{5'}$), 3.88 (3H, s, OMe).

^{13}C NMR ($CDCl_3$, 100 MHz): δ = 191.4 (C=O), 160.0 (C_4 + $C_{2'}$), 146.4 ($C_{4'}$), 145.4 (C_β), 130.7 ($C_{6'}$), 129.7 (C_2 + C_6), 120.4 (C_1 + $C_{5'}$), 127.0 (C_α), 116.8 ($C_{1'}$), 114.6 (C_3 + C_5), 86.9 ($C_{3'}$), 55.5 (OMe).

MS (EI, 70 eV): *m*/*z* (%) = 381 (14.2, M^++1), 380 (100.0, M^+), 254 (M^+−I, 17.9), 127 (6.7, I).

5.1.20. 2′-Hydroxy-3,3′,5′-triiodo-4-methoxychalcone, 37

Yellow solid, mp: 97.5–100 °C.

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 13.79 (1H, s, 2′-OH), 8.23 (1H, d, $J_{4'6'}$ = 2.0, $H_{4'}$), 8.16–8.14 (2H, m, H_2 + $H_{6'}$), 7.86 (1H, d, $J_{\beta\alpha}$ = 15.3, H_β), 7.62 (1H, dd, J_{65} = 8.5, J_{62} = 2.1, H_6), 7.39 (1H, d, $J_{\alpha\beta}$ = 15.3, H_α), 6.87 (1H, d, J_{56} = 8.5, H_5), 3.96 (3H, s, OMe).

^{13}C NMR ($CDCl_3$, 100 MHz): δ = 191.8 (C=O), 162.0 ($C_{2'}$), 160.7 (C_4), 152.4 ($C_{4'}$), 145.7 (C_β), 139.5 (C_2), 138.0 ($C_{6'}$), 131.5 ($C_{6'}$), 128.9 (C_1), 121.7 ($C_{1'}$), 117.2 (C_α), 110.9 (C_5), 88.0 ($C_{3'}$ o $C_{5'}$), 86.9 (C_3), 80.2 ($C_{3'}$ o $C_{5'}$), 56.6 (OMe).

MS (EI, 70 eV): *m*/*z* (%) = 633 (13.2, M^++1), 632 (100.0, M^+), 254 (M^+−3I, 16.2), 127 (7.4, I).

Anal. Calcd for $C_{16}H_{11}I_3O_3$: C, 30.41; H, 1.75. Found: C, 30.50; H, 2.01.

5.1.21. 3-Iodo-4-methoxyflavanone, 38

Yellowish solid, mp: 162–163 °C (lit.: 160 °C).[88]

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.93 (1H, dd, J_{56} = 7.6, J_{57} = 1.6, H_5), 7.92 (1H, d, $J_{2'6'}$ = 2.2, $H_{2'}$), 7.51 (1H, ddd, J_{78} = 8.3, J_{76} = 7.2, J_{75} = 1.7, H_7), 7.41 (1H, dd, $J_{6'5'}$ = 8.5, $J_{6'2'}$ = 2.1, $H_{6'}$), 7.09–7.01 (2H, m, H_6 + H_8), 6.86 (1H, d, $J_{5'6'}$ = 8.4, $H_{5'}$), 5.39 (1H, dd, $J_{2ax,3ax}$ = 13.3, $J_{2ax,3eq}$ = 2.8, H_2), 3.91 (3H, s, OMe), 3.06 (1H, dd, $J_{3ax,3eq}$ = 16.8, $J_{3ax,2ax}$ = 13.3, H_{3ax}), 2.86 (1H, dd, $J_{3eq,3ax}$ = 16.8, $J_{3eq,2ax}$ = 2.9, H_{3eq}).

^{13}C NMR ($CDCl_3$, 100 MHz): δ = 191.7 (C=O), 161.4 ($C_{4'}$), 158.5 (C_{8a}), 139.5 ($C_{2'}$), 137.6 (C_7), 132.9 ($C_{1'}$), 127.6 ($C_{6'}$), 127.1 (C_5), 121.7 (C_{4a}), 120.9 (C_6), 118.1 (C_8), 110.8 (C_5), 86.3 ($C_{3'}$), 78.4 (C_2), 56.5 (OMe), 44.5 (C_3).

MS (EI, 70 eV): *m*/*z* (%) = 381 (11.6, M^++1), 380 (100.0, M^+), 254 (M^+−I, 13.2), 127 (3.7, I).

Anal. Calcd for $C_{16}H_{13}IO_3$: C, 50.55; H, 3.45. Found: C, 50.72; H, 3.68.

5.1.22. 2′-Hydroxy-α,β-diiodo-4-methoxy-dihydrochalcone, 39

Yellowish solid, mp: 95–97 °C.

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 11.86 (1H, s, 2′-OH), 7.76 (1H, dd, $J_{6'5'}$ = 8.1, $J_{6'4'}$ = 1.1, $H_{6'}$), 7.50 (1H, ddd, $J_{4'3'} \approx J_{4'5'}$ = 7.8, $J_{4'6'}$ = 1.3, $H_{4'}$), 7.42–7.38 (2H, m, H_2 + H_6), 7.01 (1H, d, $J_{3'4'}$ = 8.3, $H_{3'}$), 6.95–6.88 (3H, m, H_3 + H_5 + $H_{5'}$), 5.53 (1H, d, $J_{\alpha\beta}$ = 8.5, H_α), 5.34 (1H, d, $J_{\beta\alpha}$ = 8.5, H_β), 3.82 (3H, s, OMe).

^{13}C NMR ($CDCl_3$, 100 MHz): δ = 204.3 (C=O), 158.2 ($C_{2'}$), 157.0 (C_4), 132.8 ($C_{6'}$), 131.3 ($C_{4'}$), 130.4 (C_1), 122.1 (C_2 + C_6), 119.4 ($C_{5'}$), 116.6 ($C_{1'}$), 115.4 ($C_{3'}$), 111.2 (C_3 + C_5), 56.0 (OMe), 36.2 (C_α), 31.4 (C_β).

MS (EI, 70 eV): *m*/*z* (%) = 509 (5.4, M^++1), 508 (33.6, M^+), 507 (14.8, M^+−1), 127 (8.8, I), 121 (100.0, $C_6H_4(OH)CO^+$).

Anal. Calcd for $C_{16}H_{14}I_2O_3$: C, 37.82; H, 2.78. Found: C, 37.60; H, 2.66.

5.1.23. 2′,2‴-Dihydroxy-4,4″-dimethoxy-5′,5‴-dichalcone, 41

Prepared according to general method 1, from **42** (135 mg, 0.5 mmol) and **48** (136 mg, 1 mmol), with stirring at rt for 24 h. Yield: 169 mg (67%). An analytical sample was obtained by purification by CC (hexanes→EtOAc/CH_2Cl_2/hexanes 1:5:5).

Yellow solid, mp: 131–132.5 °C.

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 12.85 (1H, s, 2′-OH), 8.25 (1H, d, $J_{6'4'}$ = 2.2, $H_{6'}$), 7.80 (1H, dd, $J_{4'3'}$ = 8.7, $J_{4'6'}$ = 2.2, $H_{4'}$), 7.74 (1H, d, $J_{\beta\alpha}$ = 15.5, H_β), 7.68–7.59 (2H, m, H_2 + H_6), 7.52 (1H, d, $J_{\alpha\beta}$ = 15.5, H_α), 7.13 (1H, d, $J_{3'4'}$ = 8.7, $H_{3'}$), 6.94–6.81 (2H, m, H_3 + H_5), 3.89 (s, 3H, OMe).

^{13}C NMR ($CDCl_3$, 100 MHz): δ = 193.5 (C=O), 161.9, 161.8 ($C_{2'}$, C_4), 145.8, 145.5, 145.2 (C_β, $C_{4'}$, $C_{5'}$), 131.0 ($C_{6'}$), 130.9 (C_2 + C_6), 127.1 (C_1), 119.1 ($C_{3'}$), 119.3 ($C_{1'}$), 117.1 (C_α), 114.8 (C_3 + C_5), 55.9 (OMe).

MS (EI, 70 eV): *m*/*z* (%) = 507 (15.0, M^++1), 506 (83.1, M^+), 134 (100.0), 161 (13.2), 147 (17.8), 119 (10.1), 121 (52.3).

Anal. Calcd for $C_{32}H_{26}O_6$: C, 75.88; H, 5.17. Found: C, 75.59; H, 5.31.

5.1.24. 4′,4‴-Dimethoxy-6,6″-diflavone, 49

Prepared according to general method 5, from **41** (152 mg, 0.3 mmol). Purified by CC (hexanes→EtOAc/hexanes 2:8). Yield: 134 mg (89%).

Yellowish solid, mp: 310 °C (dec) (lit.: 315–320 °C).[87,89]

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.35 (1H, d, J_{57} = 2.1, H_5), 7.92–7.86 (2H, m, $H_{2'}$ + $H_{6'}$), 7.75 (1H, dd, J_{78} = 8.7, J_{75} = 2.1, H_7), 7.28 (1H, d, J_{87} = 8.6, H_8), 7.08–7.01 (2H, m, $H_{3'}$ + $H_{5'}$), 6.71 (1H, s, H_3), 3.88 (3H, s, OMe).

^{13}C NMR ($CDCl_3$, 100 MHz): δ = 178.2 (C=O), 162.7 ($C_{4'}$), 162.0 (C_2), 159.4 (C_{8a}), 149.0 (C_6), 145.2 (C_7), 128.3 ($C_{2'}$ + $C_{6'}$), 125.3 (C_5), 124.5 ($C_{1'}$), 123.5 (C_{4a}), 117.0 (C_8), 114.0 ($C_{3'}$ + $C_{5'}$), 106.8 (C_3), 55.9 (OMe).

MS (EI, 70 eV): *m*/*z* (%) = 503 (13.8, M^++1), 502 (100.0, M^+), 501 (34.5, M^+−1), 251 (5.6, M^+/2).

Anal. Calcd for $C_{32}H_{22}O_6$: C, 76.48; H, 4.41. Found: C, 76.25; H, 4.69.

5.1.25. 4′,4‴-Dihydroxy-6,6″-diflavone, 50

Prepared according to general method 7, from **49** (50 mg, 0.1 mmol), with stirring at rt for 7 days. Purified by CC (hexanes→EtOAc/hexanes 1:1). Yield: 43 mg (92%).

Whitish solid, mp: 265–267 °C.

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 9.88 (1H, s, OH), 8.34 (1H, d, J_{57} = 2.2, H_5), 7.90–7.85 (2H, m, $H_{2'}$ + $H_{6'}$), 7.76 (1H, dd, J_{78} = 8.6, J_{75} = 2.2, H_7), 7.27 (1H, d, J_{87} = 8.6, H_8), 6.98–7.03 (2H, m, $H_{3'}$ + $H_{5'}$), 6.70 (1H, s, H_3).

^{13}C NMR ($CDCl_3$, 100 MHz): δ = 177.9 (C=O), 162.5, 162.1 ($C_{4'}$, C_2), 160.7 (C_{8a}), 149.0 (C_6), 144.9 (C_7), 127.8 ($C_{2'}$ + $C_{6'}$), 126.1 (C_5), 125.0 ($C_{1'}$), 121.5 (C_{4a}), 117.5 (C_8), 113.1 ($C_{3'}$ + $C_{5'}$), 106.2 (C_3).

MS (EI, 70 eV): m/z (%) = 476 (11.4, M^++2), 475 (23.0, M^++1), 474 (100.1, M^+), 474 (50.3, M^+-1), 120 (17.4).

Anal. Calcd for $C_{30}H_{18}O_6$: C, 75.94; H, 3.82. Found: C, 76.15; H, 3.49.

5.1.26. 3′,3‴,4′,4‴-Tetramethoxy-3,3″-biflavone, 58

Prepared according to general method 11, from **55** (41 mg, 0.1 mmol). Purified by CC (hexanes→EtOAc/hexanes 2:8). Yield: 18 mg (64%)

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.30 (1H, dd, J_{56} = 8.0, J_{57} = 1.5, H_5), 7.70 (1H, ddd, J_{78} = 8.6, J_{76} = 7.1, J_{75} = 1.5, H_7), 7.63–7.58 (4H, m, H_6 + H_8 + $H_{2'}$ + $H_{6'}$), 7.02 (1H, d, $J_{5'6'}$ = 8.2, $H_{5'}$), 3.97 (3H, s, OMe), 3.95 (3H, s, OMe).

^{13}C NMR ($CDCl_3$, 100 MHz): 175.8 (C=O), 173.6 (C_2), 155.4 (C_{8a}), 150.3 ($C_{3'}$), 146.4 ($C_{4'}$), 134.1 (C_7), 126.7, 126.4, 125.0, 123.8, 123.0 (C_6, $C_{1'}$, C_5, C_{4a}, C_3), 119.4, 119.2 ($C_{6'}$, C_8), 110.7, 109.2 ($C_{2'}$, $C_{5'}$), 56.9 (OMe), 56.7 (OMe).

MS (EI, 70 eV): m/z (%) = 564 (3.8, M^++2), 563 (27.2, M^++1), 562 (100.0, M^+), 561 (57.4, M^+-1), 544 (11.4, M^+-H_2O), 281 (10.0, M^+/2), 121 (47.2, $^{1,3}A^+$), 92 (17.4, $C_6H_5O^+$).

Anal. Calcd for $C_{34}H_{26}O_8$: C, 72.59; H, 4.66. Found: C, 72.25; H, 4.92.

5.1.27. 2′,2‴-Dihydroxy-4,4′,4″,4‴-tetrabenzyloxy-3,3″-dichalcone, 60

Prepared according to general method 1, from **24** (484 mg, 2 mmol) and **59** (422 mg, 1 mmol), with stirring at rt for 24 h. Yield: 626 mg (72%). A sample for elemental analysis was purified by CC (hexanes→EtOAc/CH_2Cl_2/hexanes 1:5:5).

Yellow solid, mp: 143–144.5 °C.

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 13.45 (1H, s, 2′-OH), 7.78 (1H, d, $J_{6'5'}$ = 8.0, $H_{6'}$), 7.77 (1H, d, $J_{\beta\alpha}$ = 15.5, H_β), 7.48–7.29 (11 H, m, phenyl × 2 + H_α)[‡], 7.22 (1H, d, J_{26} = 1.8, H_2), 7.19 (1H, dd, J_{65} = 8.3, J_{62} = 1.7, H_6), 6.95 (1H, d, J_{56} = 8.3, H_5), 6.57–6.55 (2H, m, $H_{3'}$ + $H_{5'}$), 5.21 (2H, s, CH_2), 5.11 (2H, s, CH_2).

^{13}C NMR ($CDCl_3$, 100 MHz): δ = 191.8 (C=O), 166.6, 165.2 ($C_{2'}$ $C_{4'}$), 161.0 (C_4), 151.6 (C_1), 144.3 (C_β), 137.0, 136.7, 135.9, 131.1, 128.7, 128.6, 128.3, 128.0, 127.5, 127.4, 127.2 ($C_{6'}$ + C_2 + C_3 + C_5 + $(C_1-C_6)_{phenyl}$), 123.6 (C_6), 118.4 (C_α), 114.4 ($C_{1'}$), 108.1, 102.1, ($C_{3'}$, $C_{5'}$), 71.7 (CH_2), 71.0 (CH_2).

MS (EI, 70 eV): m/z (%) = 690 (15.2, M^+-2Bn), 600 (8.7, M^+-3Bn), 510 (30.1, M^+-4Bn), 91 (100.0, Bn^+).

Anal. Calcd for $C_{58}H_{46}O_8$: C, 79.98; H, 5.32. Found: C, 80.09; H, 5.12.

5.1.28. 7,7″,4′,4‴-Tetrabenzyloxy-3′,3‴-biflavanone, 61

Prepared according to general method 3, from **60** (435 mg, 0.5 mmol), refluxing for 96 h. Purified by CC (hexanes→EtOAc/CH_2Cl_2/hexanes 1:5:5). Yield: 217 mg (50%).

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.86 (1H, d, J_{56} = 8.8, H_5), 7.48–7.24 (10 H, m, phenyl × 2), 7.07 (1H, m, $H_{5'}$), 6.96 (2H, m, $H_{2'}$ + $H_{6'}$), 6.68 (1H, dd, J_{65} = 8.8, J_{68} = 2.3, H_6), 6.53 (1H, d, J_{86} = 2.3, H_8), 5.33 (1H, dd, $J_{2,3ax}$ = 13.1, $J_{2,3eq}$ = 2.8, H_2), 5.17 (2H, s, CH_2), 5.08 (2H, s, CH_2), 2.96 (1H, dd, $J_{3ax,3eq}$ = 16.8, $J_{3ax,2}$ = 13.1, H_{3ax}), 2.74 (1H, dd, $J_{3eq,3ax}$ = 16.9, $J_{3eq,2}$ = 2.9, H_{3eq}).

^{13}C NMR ($CDCl_3$, 100 MHz): δ = 190.6 (C=O), 165.2 (C_{8a}), 163.4 (C_7), 149.5 ($C_{4'}$), 137.1, 137.0, 135.9, (($C_{1phenyl}$) × 2 + $C_{i'}$), 131.9 ($C_{3'}$), 128.8, 128.7, 128.5, 128.3, 128.0, 127.9, 127.8, 127.5, 127.4 ($C_{2'}$ + $C_{6'}$ + C_5 + $(C_{2-6})_{phenyl}$ × 2), 115.0 (C_{4a}), 113.5 (C_5), 110.8 (C_6), 101.9 (C_8), 79.8 (C_2), 71.5 (CH_2), 71.3 (CH_2), 44.1 (C_3).

MS (EI, 70 eV): m/z (%) = 690 (13.0, M^+-2Bn), 600 (7.8, M^+-3Bn), 510 (25.3, M^+-4Bn), 91 (100.0, Bn^+).

Anal. Calcd for $C_{58}H_{46}O_8$: C, 79.98; H, 5.32. Found: C, 79.65; H, 5.21.

5.1.29. 7,7″,4′,4‴-Tetrahydroxy-3′,3‴-biflavanone, 62

Prepared according to general method 8, from **61** (87 mg, 0.1 mmol), with stirring at rt for 6 h. Purified by CC (hexanes→EtOAc). Yield: 39 mg (76%).

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.85 (1H, d, J_{56} = 8.8, H_5), 7.03 (1H, m, $H_{5'}$), 6.95 (2H, m, $H_{2'}$ + $H_{6'}$), 6.64 (1H, dd, J_{65} = 8.7, J_{68} = 2.3, H_6), 6.51 (1H, d, J_{86} = 2.3, H_8), 5.32 (1H, dd, $J_{2,3ax}$ = 13.0, $J_{2,3eq}$ = 2.8, H_2), 2.96 (1H, dd, $J_{3ax,3eq}$ = 16.8, $J_{3ax,2}$ = 13.0, H_{3ax}), 2.75 (1H, dd, $J_{3eq,3ax}$ = 16.8, $J_{3eq,2}$ = 2.9, H_{3eq}).

^{13}C NMR (acetone-d_6, 100 MHz): δ = 190.4 (C=O), 161.7, 161.3 (C_{8a}, C_7), 155.6 ($C_{4'}$), 135.0 ($C_{i'}$), 131.1 ($C_{2'}$), 128.7 (C_5), 124.8 ($C_{3'}$ + $C_{6'}$), 118.0 ($C_{5'}$), 116.0 (C_{4a}), 113.9 (C_6), 99.1 (C_8), 76.6 (C_2), 44.7 (C_3).

MS (EI, 70 eV): m/z (%) = 511 (17.4, M^++1), 510 (100.0, M^+), 509 (56.3, M^+-1), 492 (47.9, M^+-18), 255.8 (7.1, M^+/2).

Anal. Calcd for $C_{30}H_{22}O_8$: C, 70.58; H, 4.34. Found: C, 70.25; H, 4.23

5.2. Biology

For the antifungal evaluation fresh cultures of *C. albicans* (Ca) ATCC 90028, *C. parapsilosis* (Cp) ATCC 22019, *C. neoformans* (Cn) ATCC 14116; *A. niger* (An) ATCC 16404, *R. stolonifer* (Rs) ATCC 6227b were used. For each extract, the MIC value was determined by using broth microdilution techniques according to the guidelines of the NCCLS for yeasts (M27-A2) and for filamentous fungi (M 38 A).[90,91] MIC values were determined in Sabouraud dextrose broth (SDB) (Sigma, St. Louis, MO, USA) buffered to pH 7.0 with MOPS. Microtiter trays were incubated at 28–30 °C for all strains in a moist, dark chamber. MICs were visually recorded at 48 h for yeasts, 72 h for *C. neoformans*.

For the assay, 40 μL of a stock solution of each extract in DMSO (50 mg/mL) was diluted with 960 μL of SDB giving a solution of 2 mg/mL. Two hundred microliters of this new solution was poured into the first well and then, 100 μL were transferred to the next well containing 100 μL of SDB. The same procedure was performed for all wells of the same file obtaining twofold dilutions of the extract. A volume of 100 μL of inoculum suspension was added to each well (with the exception of the sterility control where sterile water was added to the well instead) giving extract's concentrations from 1000 to 0.98 μg/mL and a final DMSO concentration ⩽1%. Amphotericin B was used as positive control for yeasts and *Aspergillus* spp. End-points were defined as the lowest concentration of drug resulting in total inhibition (MIC) of visual growth compared to the growth in the control wells containing no antifungal. Tests were carried out by duplicate.

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Supplementary data

Supplementary data (experimental procedures and spectral data for known compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.04.010.

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