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Total synthesis, antiprotozoal and cytotoxicity activities of rhuschalcone VI and analogs

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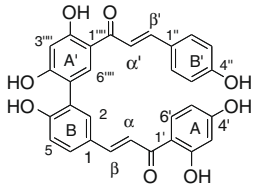
ABSTRACT

The total synthesis of a potent antiplasmodial natural bichalcone, rhuschalcone VI, is described starting from simple and available resorcinol and 4-hydroxybenzaldehyde. Key steps include the solvent-free Aldol syntheses of chalcones, and the successful application of the Suzuki–Miyaura coupling reaction in the synthesis of bichalcones. The present work constitutes a general method for the rapid syntheses of a number of bichalcones related to rhuschalcone VI. Some of the bichalcones showed moderate antiprotozoal activities against *Bodo caudatus*, a preliminary screening system for antitrypanosomal activities, most of them with little or no cytotoxicity.

1. Introduction

Rhus pyroides Burch. (Anacardiaceae) is a shrub to a medium-sized tree widely distributed in the eastern part of Botswana and South Africa, and is used against epilepsy in traditional medicine.[1,2] *R. pyroides* is an exceptionally rich source of bioactive bichalcones with novel structural characteristics.[1] These bichalcones exhibit potent antiplasmodial (unpublished results) and moderate antiproliferative properties against various strains and cancer cell lines in standard assays.[1] The Rhus bichalcones are basically of two structural types: the bi-aryl ether type and the bi-aryl type; the latter having been reported from *Rhus* only. These compounds possess unique molecular structures and hence pose as worthwhile targets for a detailed assessment of their biological properties. However, their extreme scarcity renders any rational effort to evaluate their anti-malarial activities difficult if one relies on extraction from the producing *Rhus* species only. In an attempt to circumvent this challenge, we have developed an efficient total synthesis of rhuschalcone VI (**1**) using palladium-catalyzed Suzuki–Miyaura cross-coupling reaction. Moreover, from purely chemical perspective, this endeavor provided an excellent opportunity to achieve the first use of Suzuki–Miyaura reaction in the synthesis of bichalcones.



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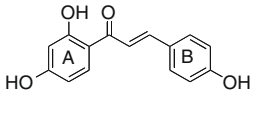

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Rhuschalcone VI (**1**) is a naturally occurring bichalcone composed of two molecules of isoliquiritigenin (**2**) joined via a bi-aryl linkage between the C-5′ of the A-ring of one and the C-3 of the B-ring of a second molecule of isoliquiritigenin. The natural material was reported for the first time by Mdee et al.[1] from the root bark of *R. pyroides* and was shown to have a strong antiplasmodial activity and a moderate antiproliferative activity against two colorectal cancer cells.[1] However, the quantities obtained from natural sources were limited. As far as we are aware, rhuschalcone VI (**1**) is the first and unique example of a natural dimer in which two chalcones are linked by a C–C bond; and no group has reported the total synthesis of C–C AB bichalcones. It is remarkable that whereas (a) the total synthesis of the bi-aryl ether-type bichalcones (rhuschalcones and verbenachalcone) have been reported,[1,3,4] by employing a novel application of the Ullmann synthesis and through anodic oxidation, respectively, and (b) a

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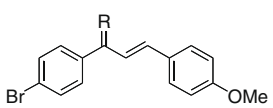
number of flavonoids have been employed in Suzuki reactions,[5] the use of chalcones and the synthesis of any bi-aryl type rhus bichalcones has not yet been reported. In order to provide ready access to sufficient quantities of material for more complete biological studies, as well as a general route for the preparation of rhuschalcone VI and its structural analogs, a total synthesis of rhuschalcone VI is described herein. Most of the compounds prepared in the course of this work were evaluated for their antiprotozoal activities using a free-living, non-pathogenic protozoa *Bodo caudatus* (Bodonidae) as a model. *B. caudatus* as well as the pathogenic human parasites *Trypanosoma cruzi* and *Trypanosoma brucei* (Trypanosomatidae) fall under the same order of Kinetoplastida.[6]

2. Results and discussion

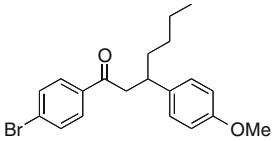
Among the various methodologies available to accomplish C–C bond formation, we chose the Suzuki–Miyaura reaction, one of the most popular and powerful methods for the coupling of aryl–aryl moieties. This methodology has gained prominence[7,8] and found many applications[7,9,10] both in research laboratories and for large-scale industrial processes due to its compatibility with a variety of functional groups, the stability and the commercial availability of a wide-range of organoboron starting materials, and the ease of working up the reaction mixtures.[7,11] The retrosynthetic analysis of rhuschalcone VI (**1**) is shown in Scheme 1. Because phenols are very sensitive to autoxidation even under slightly basic conditions[12] we decided to protect the phenolic hydroxyl groups as their methyl ethers. This would lead to a permethylated bichalcone as the penultimate target (not shown in Scheme 1), which would require complete demethylation to give bichaclone **1**. We anticipated that incomplete demethylation during the last step of the synthesis may result in analogs of the targeted bichalcone (**1**), which may provide a diversity of the natural product for biological evaluation.

The syntheses of bromochalcones **5** and **6** are shown in Scheme 2. Thus, protection of the hydroxyl groups of resorcinol (**3**) as methyl ethers and Friedel–Crafts acylation afforded compound **3a**, which was subsequently brominated with molecular bromine to give **3b** in 99% yield. The condensation of equimolar quantities of **3a** and **4b** in the presence of NaOH, in methanol according to a reported procedure,[13] furnished **6** in 75% yield. Substantial improvement in yields (90–97%) were obtained by running the aldol condensation (to synthesize **5**, **6** as well as many other chalcones described later) under solvent free conditions, confirming reports by others that this methodology is superior to the synthesis in solution, faster, cleaner and eco-friendly.[14,15] Thus, solvent-free aldol condensation, by grinding the mixture of equimolar quantities of **3b** with anisaldehyde (**4a**) and NaOH, in a porcelain mortar, gave bromochalcone **5** in 90% yield. Also, 3-bromoanisaldehyde (**4b**) was subjected to solvent-free aldol condensation with **3a** to give bromochalcone **6** in 97% yield (Scheme 2).

With bromochalcones **5** and **6** at hand the preparation of the key intermediates (i.e., chalconylboronate esters, e.g., **7**) for the coupling reactions, was to be undertaken. The synthesis of boronate ester **7** was envisaged via bromine-lithium exchange with *n*-BuLi, followed by a reaction with 2-isopropoxy-4,4,5,5-tetramethyl-1,3,2-dioxaborolane in THF. The feasibility of this approach was tested by reacting bromochalcone **8** (itself prepared by the aldol condensation of *p*-bromoacetophenone and anisaldehyde) with *n*-BuLi and the dioxoborolane mentioned above. However, the expected boronate ester was not obtained; instead the 1,2- and 1,4-addition products (**9a** and **9b**) of *n*-BuLi to the α,β-unsaturated carbonyl group of the chalcone were isolated.

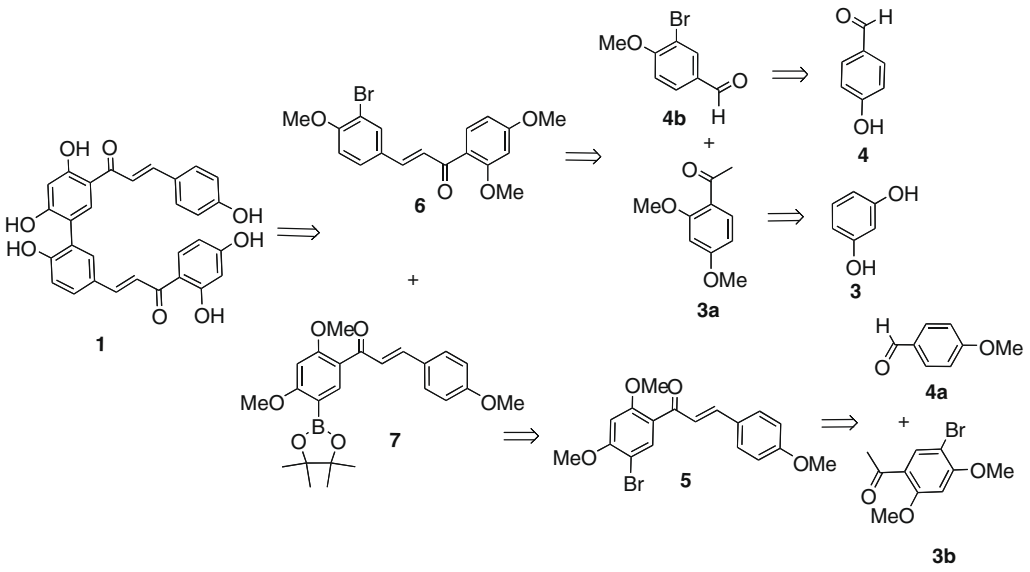


8 R = O
9b R = OH, *n*-Bu

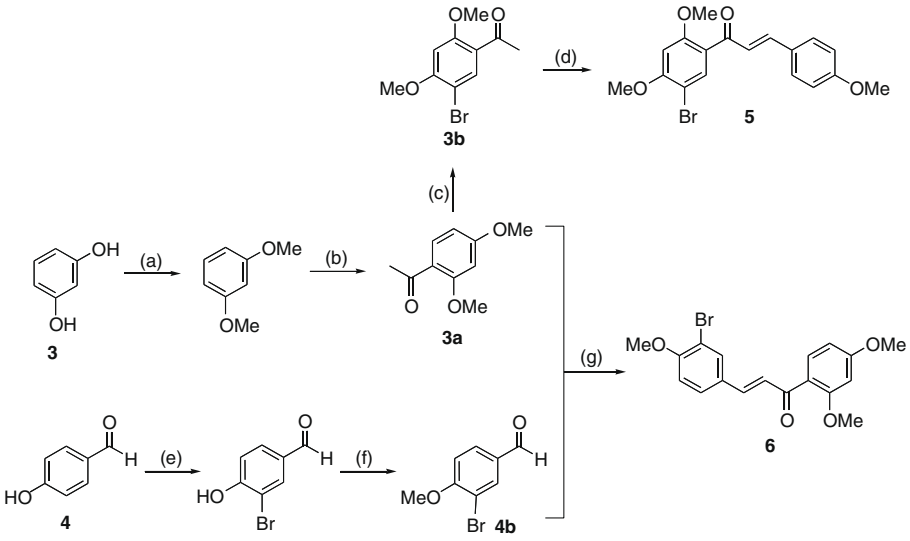


9a

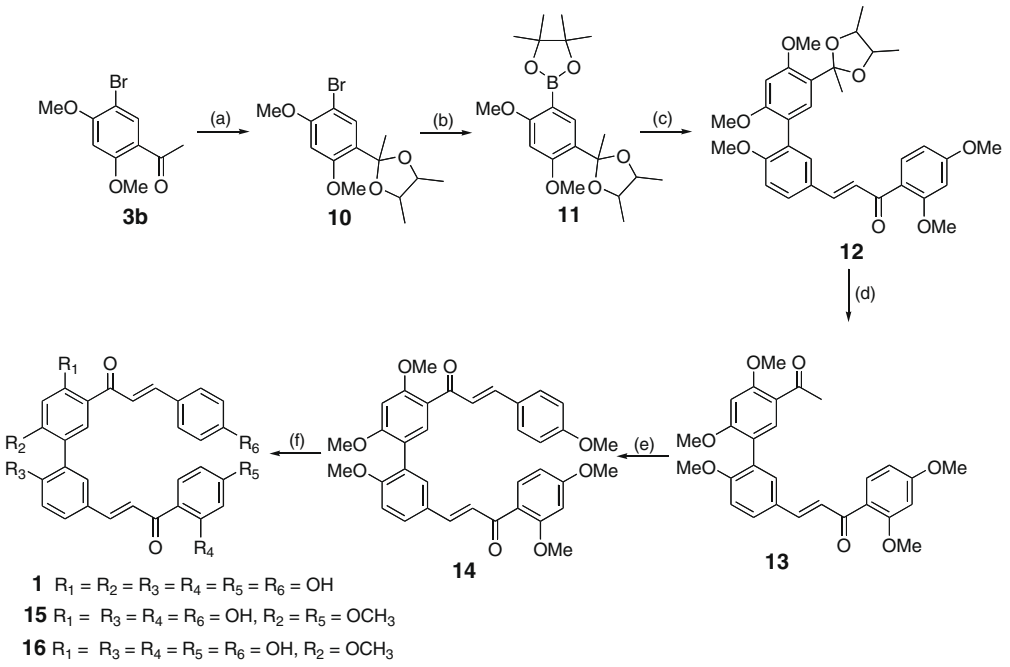
Attempts to protect the carbonyl groups of the chalcones, using ethylene glycol, 1,2-propanediol, 1,3-propanediol or 2,3-butanediol and *p*-TsOH (or H_2NSO_3H) in benzene (or toluene) were not successful. Failure to convert the bromochalcones to the corresponding boronate esters forced us to look for an alternative strategy. At this point it was decided to form the boronate ester at an early stage of the ketal of bromoacetophenone **10** to give **11** and then perform the Suzuki–Miyaura coupling with bromochalcone **6.** This would then allow the synthesis of various bichalcones by aldol condensation of the acetophenone derivative **13** with variously substituted benzaldehydes. This approach was found successful and the details are presented in Scheme 3. The synthesis com-



Scheme 1. Retrosynthetic analysis of rhuschalcone VI (**1**).



Scheme 2. Syntheses of bromochalcones **5** and **6**. Reagents, conditions and (yields): (a) K_2CO_3, Me_2SO_4, acetone, reflux, 4 h (94%); (b), CH_3COCl, $AlCl_3$, dichloromethane, reflux, 30 min (60%); (c) Br_2, AcOH, −20 °C, 43 min (99%); (d) **4a**, NaOH, 23 min (90%); (e) Br_2, methanol, −5 °C, 5 min (49%); (f) K_2CO_3, Me_2SO_4, acetone, reflux, 2.5 h (69%); (g) NaOH, 10 min (97%).



Scheme 3. Synthesis of rhuschalcone VI (**1**) and bichalcones **15** and **16**. Reagents, conditions and (yields): (a) 2,3-butanediol, H_2NSO_3H, toluene, reflux, 20 h (96%); (b) *n*-BuLi, 2-isopropoxy-4,4,5,5-tetramethyl-1,3,2-dioxaborolane, THF, −78 °C (92%); (c) **6**, $Pd(PPh_3)_4$, toluene, reflux, 7 h (73%); (d) I_2, acetone, reflux, 10 min (77%); (e) **3a**, NaOH, rt, 8 min (98%); (f) BBr_3, CH_2Cl_2, reflux, 3 h (52%).

menced with a smooth transformation of acetophenone **3b** to ketal **10** using 2,3-butanediol/H_2NSO_3H in toluene.[16] Metal-halogen exchange of **10** with *n*-BuLi followed by addition of 2-isopropoxy-4,4,5,5-tetramethyl-1,3,2-dioxaborolane gave boronate ester **11** in 92% yield. Compound **11** was successfully coupled with bromochalcone **6** under Suzuki–Miyaura conditions using tetrakis(triphenylphosphine)Pd(0) as catalyst to give ketal **12** in 73% yield. This was in turn deprotected by refluxing with 10 mol % of molecular iodine in acetone[17] to give methyl ketone **13** in 77% yield. Solvent-free aldol condensation of compound **13** with 6.3 equiv of **4a** in presence of NaOH gave the hexamethoxy-bichalcone **14** in 98% yield. Finally, bichalcone **14** was demethylated using BBr_3 in refluxing dichloromethane to give Rhuschalcone VI (**1**) in 52% yield together with partially demethylated rhuschalcone VI derivatives **15** (18%) and **16** (6%).

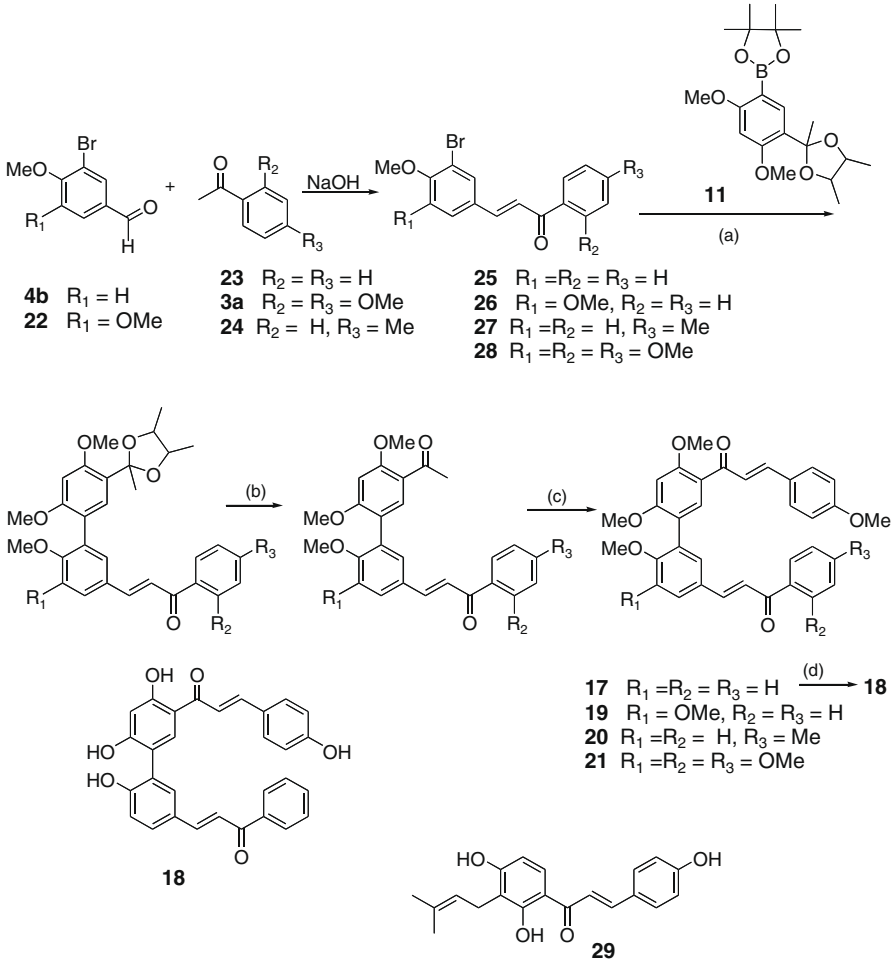
Since a previous report on rhuschalcone VI (**1**) has shown that it possesses interesting biological activities, unnatural rhuschalcone VI analogs may show similar and/or other pharmacological activities, which would warrant additional investigation. Within this context, and in order to demonstrate the applicability of our strategy, we undertook and successfully completed the preparation of a few such rhuschalcone VI analogues that would be of value in SAR studies. These are 4,4″,2‴,4‴-tetramethoxy-3,5‴-bichalcone (**17**), 4,4″,2‴,4‴-tetrahydroxy-3,5‴-bichalcone (**18**), 4,5,4″,2‴,4‴-penta-

methoxy-3,5‴-bichalcone (**19**), 4,4″,2‴,4‴-tetramethoxy-4′-methyl-3,5‴-bichalcone (**20**), and 4,5,2′,4′,4″,2‴,4‴-heptamethoxy-3,5‴-bichalcone (**21**). Scheme 4 outlines the syntheses of bichalcones **17**–**21**. Thus, bromochalcones **25**–**28** were synthesized by solvent-free aldol condensation reactions between the appropriate aldehydes and acetophenone derivatives as shown in Scheme 4. Coupling of these bromochalcones with **11** followed by deprotection of the resulting chalconylketals, and finally aldol condensation of the acetophenone derivatives with anisaldehyde yielded compounds 17–21. Removal of protecting groups in **17** gave **18** in 22% yield (Scheme 4).

The structure of the synthetic rhuschalcone VI was determined mainly from ^{1}H and ^{13}C NMR spectral data that are similar to those reported in the literature for the natural product. To further confirm the structure of the synthetic **1** an authentic natural product was sought to make direct comparison possible. Such a sample was not available and hence the natural compound was isolated from the root barks of *R. pyroides* following the published procedure.[18] The ^{1}H and ^{13}C NMR spectroscopic data generated for the natural product and the synthetic material were in complete agreement (Table 1). Complete spectroscopic data (1D and 2D-NMR data, HRMS) were generated for the rhuschalcone VI analogs **14, 17, 19, 20** and **21**. ^{1}H and ^{13}C NMR data assignments were achieved by analysis of HMBC and HMQC correlations as well as by comparison of NMR data to that of rhuschalcone VI and the synthetic intermediates. The permethylated compound **14** showed signals for six methoxyl groups as expected. The partial demethylation products, **15** and **16**, showed a 6H (2 × OMe) singlet at δ 3.89 for **15** and a methoxy singlet (3H) at δ 3.89. The location of the two methoxy signals in the bichalcone skeleton of **15** and one OMe group in **16** were deduced from HMBC analyses of the corresponding spectra. For compound **15** HMBC correlations were observed between the methoxy signal and C-4‴ at δ 164.0, which in turn showed correlation to H-3‴ at δ 6.63.

3. Antiprotozoal activity and cytotoxicity

The antiprotozoal activities of compounds generated in this study, **1, 14**–**21** were evaluated using the free living protozoa *B. caudatus*. This organism is a non-pathogenic member of the family Bodonidae, which is related to the genera *Leishmania* and *Trypanosoma* (Trypanosomatidae) by belonging to the same order of Kinetoplastida.[16] *Leishmania* and *Trypanosoma* include the pathogenic human parasites such as *T. cruzi* and *T. brucei*.[6] *B. caudatus* therefore can be considered as a preliminary screening system for antitrypanosomal activity. Compound **29** a prenylated chalcone (isobavachalcone) isolated from *Dorstenia kameruniana*[19] which has shown activity on other organisms[20] was included in this study for comparison purposes. As shown in Figure 1, compounds **1**, **16**, **29** and **18** induced the largest reduction in viability of protozoa with an inhibition of 75–83% compared to controls after 24 h of exposure to compounds at a concentration of 0.6 mg/mL. Inhibition was higher after exposure of *B. caudatus* to compounds after 24 h (Fig. 1 B) compared to a 4-h incubation with respective compounds (Fig. 1, A). This most likely reflects the time period needed for uptake and metabolism of compounds by the organism.



Scheme 4. Synthesis of bichalcones **17**–**21**. Reagents and conditions: (a) $Pd(PPh_3)_4$, toluene, reflux; (b) I_2, acetone, reflux; (c) anisaldehyde, NaOH, rt; (d) BBr_3, CH_2Cl_2, reflux.

Table 1
^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) data for compounds **1**, **14** and **17–21**

Position	**1** (acetone-d_6)			**14** ($CDCl_3$)		**17** ($CDCl_3$)		**19** ($CDCl_3$)		**20** ($CDCl_3$)		**21** ($CDCl_3$)	
	δ_H	δ_C		δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
		Synthetic	Natural										
1	–	125.8	126.4	–	127.8	–	127.3	–	126.3	–	127.5	–	130.9
2	7.89(2.1)	132.7	132.8	7.53(1.6)	131.6	7.63(m)	131.3[a]	6.84	113.8	7.62(m)	131.2	7.15	124.2
3	–	127.8	128.1	–	127.1	–	127.4	–	133.4	–	130.4	–	132.0
4	–	160.8	161.1	–	158.9	–	159.4	–	151.5	–	159.3	–	149.1
5	6.96(8.4)	117.8	117.3	6.99(8.6)	111.1	7.01(9.1)	111.1	–	148.5	7.01(9.1)	111.1	–	152.7
6	7.71(8.4, 2.1)	129.5	129.8	7.58(8.5)	129.8	7.63(m)	130.5[a]	7.33	108.2	7.62(m)	131.2	7.16	111.4
1′	–	113.7	113.6	–	122.6	–	138.5	–	138.5	–	135.9	–	122.4
2′	–	166.7[a]	166.7[a]	–	160.1	8.05(7.3)	128.5	7.96(7.4)	128.4	7.96(8.0)	128.6	–	160.2
3′	6.37(2.3)	102.9	102.9	6.50(1.5)	98.7	7.51(m)	128.6	7.48(7.7)	128.5	7.31(8.0)	129.3	6.51(1.9)	98.7
4′	–	164.8[a]	164.8[a]	–	163.9	7.59(8.5)	132.6	7.55(7.3)	132.5	–	143.4	–	164.0
5′	6.45(8.9, 2.4)	107.8	107.9	6.57(8.6, 1.9)	105.0	7.51(m)	128.6	7.48(7.7)	128.5	7.31(8.0)	129.3	6.58(8.6, 2.0)	105.1
6′	8.18(8.9)	132.4	132.5	7.74(8.7)	132.6	8.05(7.3)	128.5	7.96(7.4)	128.4	7.96(8.0)	128.6	7.73(8.6)	132.7
1″	–	126.9	126.7	–	128.2	–	128.2	–	128.1	–	128.2	–	128.2
2″, 6″	7.74(8.6)	130.8	131.0	7.58(8.5)	130.0	7.59(8.5)	130.0	7.58(8.6)	130.1	7.59(8.7)	130.0	7.58(8.8)	130.0
3″, 5″	6.89(8.6)	115.8	115.9	6.94(8.6)	114.3	6.95(8.6)	114.3	6.92(8.6)	114.3	6.95(8.6)	114.3	6.93(8.7)	114.3
4″	–	160.0	160.1	–	161.2	–	161.3	–	161.3	–	161.3	–	161.3
1‴	–	112.6	113.3	–	121.6	–	121.6	–	121.9	–	121.6	–	121.5
2‴	–	166.7[b]	166.7[b]	–	160.2[a]	–	160.2[b]	–	160.3[a]	–	160.2[a]	–	160.2
3‴	6.39	104.2	103.7	6.60	95.3	6.61	95.3	6.59	95.4	6.61	95.3	6.60	95.2
4‴	–	166.4[b]	166.2[b]	–	161.2[a]	–	161.3[b]	–	160.8[a]	–	161.3[a]	–	161.0
5‴	–	120.5	119.2	–	119.7	–	119.7	–	121.3	–	119.7	–	119.8
6‴	8.20	133.4	133.7	7.73	134.1	7.76	134.1	7.66	134.2	7.75	134.1	7.72	133.7
C=O	–	192.0	192.0	–	191.0	–	190.6	–	190.8	–	190.1	–	190.9
α	7.84(15.2)	116.6	117.1	7.38(15.7)	125.2	7.47(15.6)	119.9	7.33(15.6)	120.3	7.47(15.5)	119.9	7.39(15.7)	126.5
β	7.93(15.2)	145.1	144.7	7.68(15.4)	142.4	7.85(15.7)	144.8	7.62(15.7)	144.5	7.83(15.5)	144.3	7.63(15.7)	142.3
C=O′	–	191.3	191.9	–	190.4	–	190.4	–	190.5	–	190.4	–	190.3
α′	7.92(15.4)	118.1	117.8	7.46(15.7)	125.0	7.48(15.8)	125.0	7.45(15.9)	124.9	7.48(15.7)	125.0	7.45(15.7)	124.9
β′	7.84(15.2)	143.4	144.1	7.71(15.5)	142.1	7.72(15.7)	142.1	7.71(15.7)	142.4	7.72(15.7)	142.1	7.70(15.8)	142.2
OMe-4				3.83	55.9	3.85	55.9	3.94	56.0	3.84	55.9	3.71	60.9
OMe-5				–	–	–		4.03	56.0	–	–	3.96	55.9
OMe-2′	13.79[a]			4.03	56.1	–		–	–	–	–	3.90	55.8
OMe-4′				3.88	55.5[b]	–		–	–	–	–	4.03	56.1
OMe-4″				3.89	55.8[b]	3.87[a]	55.4[c,d]	3.86	55.4	3.90[a,b]	55.9[a,b]	3.87	55.4
OMe-2‴	13.73[a]			3.87	55.4	4.03[a]	56.1[c]	4.03[a]	56.0[b]	4.03[a]	56.1[a]	3.89[a]	55.6[a]
OMe-4‴				3.90	55.8[b]	3.90[a]	56.0[d]	3.84[a]	55.9[b]	3.87[b]	55.4[b]	3.90[a]	55.8[a]
CH3-4′										2.45	21.6		

Values with the same letter superscripts in the same column are interchangeable.
Figures in parentheses are *J* values in hertz.

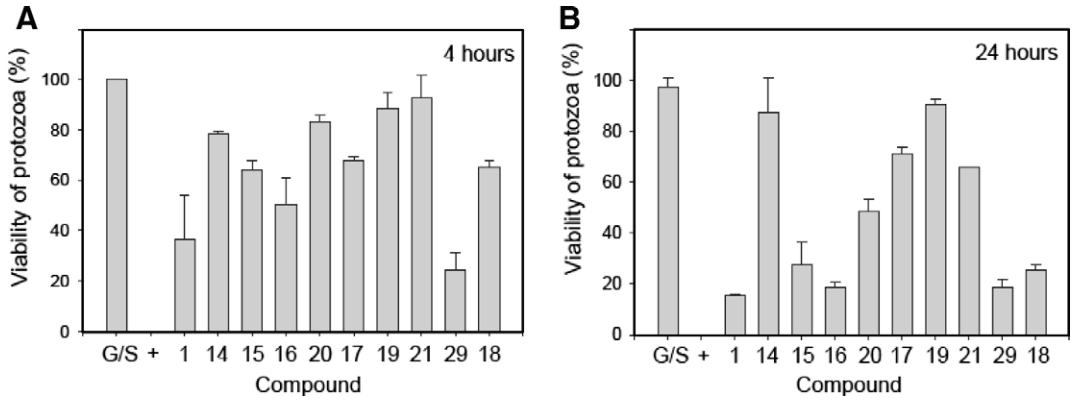


Figure 1. Growth inhibition of *Bodo caudatus*. Protozoa were exposed for 4 h (A) and 24 h (B) to compounds **1**, **14–21** and **29** at a concn of 0.6 mg/mL. G/S represents growth control of protozoa in solvent (end concn 8% DMSO). (+) is positive control with $CuSO_4$ in the same concn as compounds. Note 100% inhibition of protozoa growth with $CuSO_4$, while compounds **1**, **16**, **18**, **29** and **18** showed growth inhibition in a time-dependent manner of 75–83%. Assays were performed in triplicates, bars represent mean ± SD.

However, it must be noted that none of the compounds were as active as $CuSO_4$, which served as positive control killing 100% of the protozoa at the same concentration. LC_{50} concentrations were determined for the four most active compounds. As is evident from Figure 2, the most active compound (**29**) displayed LC_{50} concentration of 4.36 μg/mL, which was comparable to LC_{50} concentration of 2.30 μg/mL for $CuSO_4$ (data not shown). The other compounds killed protozoa with lesser efficacy at LC_{50} concentrations of 20.59 μg/mL (**18**), 53.32 μg/mL (**1**) and 118.20 μg/mL (**16**), respectively. Compounds **1** and **29** induced significant cell death of BHK cells (Fig. 3) after exposure for 48 h at a concentration of 100 μg/mL, which corresponded to a CC_{50} value of 97.59 μg/mL and

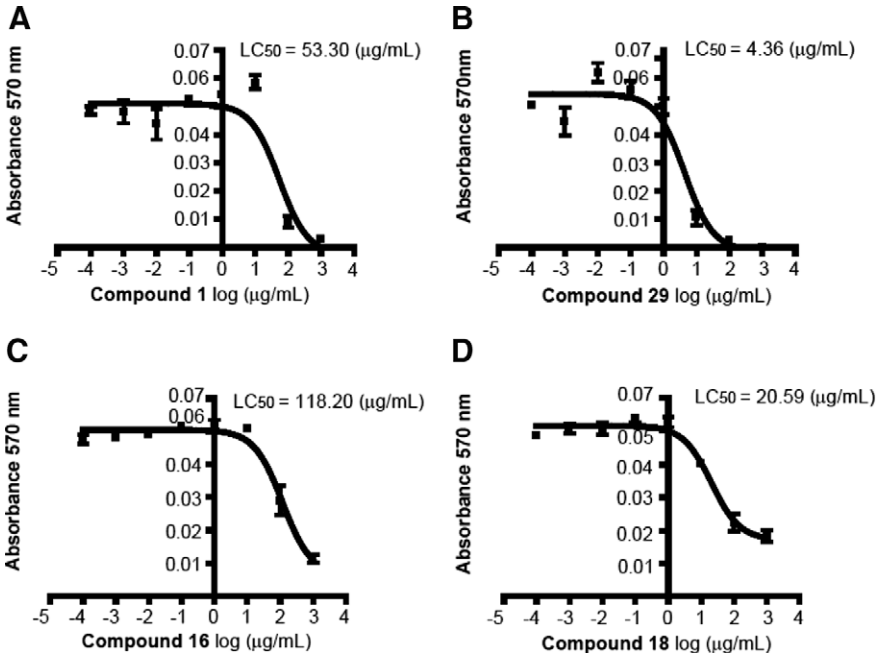


Figure 2. Antiprotozoal activity of compounds **1**, **29**, **16** and **18**. The lethal concn of compounds for 50% of *Bodo caudatus* cells (LC_{50}) was determined by the MTT-viability assay. Compound **29** was the most active with a LC_{50} concentration of 4.36 (μg/mL) (B), followed by compound **18** (LC_{50} = 20.59 μg/mL, D), compound **1** (LC_{50} = 53.30 μg/mL, A) and compound **16** (LC_{50} = 118.20 μg/mL, C), respectively. Data are presented as the mean ± SD of triplicate determinations.

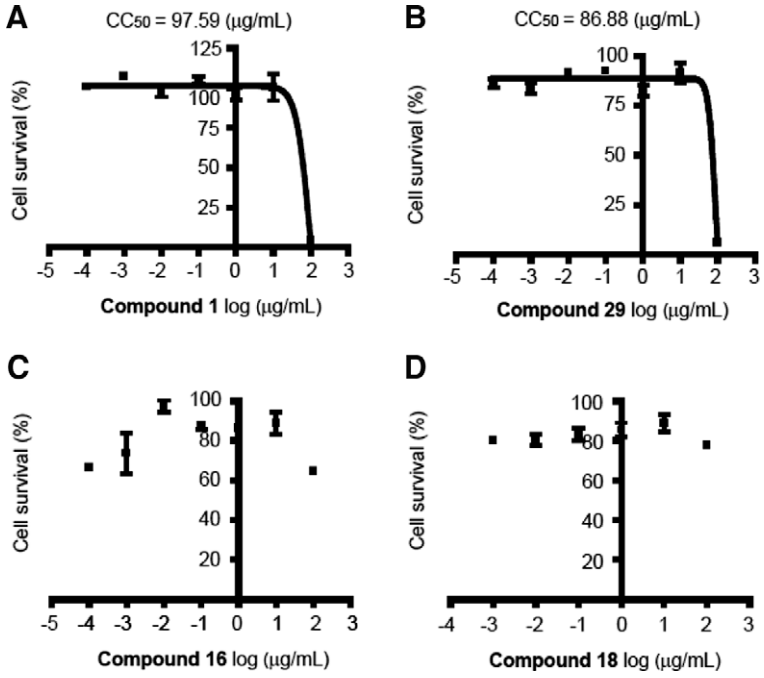


Figure 3. Cytotoxicity of compounds with antiprotozoal activity. (A–D) BHK cells in exponential growth phase were exposed to compounds **1**, **29**, **16** and **18** in a concn range of 0.0001–100 μg/mL for 48 h. Cell survival was determined in comparison to untreated cells by the MTT assay. Note that compounds **1** and **29** induced significant cell death at a concn of 100 μg/mL corresponding to a CC_{50} value of 97.59 μg/mL and 86.88 μg/mL, respectively (A, B), while cell viability was maintained after exposure to compounds **16** and **18** at the same concn (C, D). Data are presented as means ± SD from triplicate determinations.

86.88 μg/mL, respectively. The CC_{50} concentration of compound **29** is approximately 20 times higher than its antiprotozoal concentration (LC_{50} = 4.36 μg/mL), while antiprotozoal activity of compound **1** is in the same range as its cytotoxic concentration. Compounds **16** and **18** induced a small decrease in cell viability compared to non-treated cells, but did not exhibit significant cytotoxicity up to a concentration of 100 μg/mL.

4. Conclusions

The total synthesis of rhuschalcone VI (**1**) has been achieved in 12 steps starting from resorcinol and 4-hydroxybenzaldehyde. This synthesis constitutes the first application of the Suzuki–Miyaura reaction in the synthesis of C–C AB linked bichalcones. Convergence of the strategy enables the syntheses of a wide-range of

bichalcones bearing a C–C linkage by simple structural modification of the starting acetophenone and benzaldehyde derivatives. The first total syntheses of eight (**14**–**21**) rhuschalcone VI-type bichalcones were achieved, indicating that the general methodology developed here is of practical use in the syntheses of more congeners carrying the same carbon-framework.

5. Experimental

5.1. General

Commercially available reagents and solvents were used without further purification other than those detailed below. THF was distilled from sodium–benzophenone under nitrogen immediately before use. Analytical thin layer chromatography was carried out using aluminum or glass-backed plates coated with Merck Kieselgel 60 GF_{254}. Developed plates were visualised under ultra-violet light (254 nm) and/or sprayed with vanillin-sulfuric acid. Column chromatography was conducted on columns of different sizes using Silica Gel 60, particle size 0.040–0.063 mm (Merck). Fully characterized compounds were chromatographically homogeneous. Melting points were determined using a Büchi mp B545 apparatus and are uncorrected. The ultraviolet and visible (UV–vis) spectra were measured on a Shimadzu UV-2101PC UV–VIS scanning spectrometer. Infrared (IR) spectra were measured on Perkin Elmer System 2000 FT-IR spectrometer as KBr pellets. NMR spectra were recorded on Bruker Avance 300, 400 or 600 MHz spectrometers. Low-resolution mass spectra were obtained on Finnigan MAT LCQ^{DECA} instrument and high-resolution mass spectra were obtained on GCT Premier Instrument.

Dimethoxybenzene was prepared by methylation[21] of resorcinol. 2,4-Dimethoxyacetophenone (**3a**) was prepared by Friedel–Crafts acetylation of dimethoxybenzene using standard procedures.[22] 5-Bromo-2,4-dimethoxyacetophenone (**3b**) and 5′-Bromo-2′,4′,4-trimethoxychalcone (**5**) were prepared as reported previously.[18] 3-Bromo-4-methoxybenzaldehyde (**4b**) was prepared by methylation of 3-bromo-4-hydroxybenzaldehyde, which was itself prepared by bromination[23] of 4-hydorxybenzaldehyde.

5.2. 3-Bromo-2′,4′,4-trimethoxychalcone (6)

A mixture of **4b** (3.00 g, 13.95 mmol, 1 equiv), **3a** (2.51 g, 13.94 mmol, 1 equiv), and NaOH (6 pellets) was ground in a porcelain mortar at room temperature. After 10 min, the mixture turned to a yellow solid which was treated with water (60 mL) and filtered to give bromochalcone **6** (5.12 g, 97%) as a yellow solid. Mp 152.1–153.3 °C); IR (KBr) ν_{max} 2943, 1649, 1593, 1508, 1460, 1423, 1389, 1323, 1271, 1215, 1166, 1020, 978, 816, 650 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 3.90 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 3.96 (3H, s, OCH_3), 6.52 (1H, d, J = 2.1 Hz, H-3′), 6.59 (1H, dd, J = 2.1, 8.4 Hz, H-5′), 6.93 (1H, d, J = 8.4 Hz, H-5), 7.41 (1H, d, J = 15.8 Hz, H_α), 7.52 (1H, dd, J = 1.8, 8.4 Hz, H-6), 7.60 (1H, d, J = 15.8 Hz, H_β), 7.77 (1H, d, J = 8.4 Hz, H-6′), 7.84 (1H, d, J = 1.8 Hz, H2).

5.3. 2-(5-Bromo-2,4-dimethoxyphenyl)-2,4,5-trimethyl-1,3-dioxolane (10)

In a round-bottomed flask equipped with a Dean-Stark trap and a reflux condenser, compound **3b** (13.87 g, 53.55 mmol, 1 equiv) was treated with 2,3-butanediol (6.26 g, 69.55 mmol, 1.3 equiv) and H_2NSO_3H (0.52 g, 5.36 mmol, 10 mol % relative to the diol) in refluxing toluene (50 mL) until the reaction was complete (TLC monitoring, 20 h). The mixture was filtered and the residue was washed with diethyl ether (30 mL). After evaporation of the solvent, the resulting solid was dried overnight in a vacuum oven (55 °C) to give ketal **10** as pink solid (17.02 g, 96%). Mp 73.7–76.4 °C; ^1H NMR (400 MHz, $CDCl_3$): δ 1.23 (3H, d, J = 6 Hz), 1.30 (3H, d, J = 6 Hz), 1.73 (3H, s), 3.52 (1H, m), 3.75 (1H, m), 3.89 (3H, s), 3.90 (3H, s), 6.51 (1H, s), 7.12 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ 16.47, 16.79, 26.78, 56.24, 56.37, 78.20, 78.90, 97.79, 101.14, 106.72, 126.17, 130.71, 156.19, 157.43. HRMS (TOF EI^+) m/z: M^+ calcd for $C_{14}H_{19}BrO_4$ 330.0467; found 330.0465.

5.4. 2-(2,4-Dimethoxy-5-(2,4,5-trimethyl-1,3-dioxolan-2-yl)phenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (11)

To a cooled (−78 °C) solution of ketal 10 (4.00 g, 12.08 mmol, 1 equiv) in THF (10 mL) under nitrogen atmosphere was added 1.6 M *n*-BuLi (8.31 mL, 0.85 g, 13.29 mmol, 1.1 equiv) and the mixture was stirred for about 5 min. 2-Isopropoxy-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (4.94 g, 26.58 mmol, 2.2 equiv) was added quickly and the mixture was left to warm to room temperature overnight. The resulting yellowish solution was poured onto water (50 mL) and extracted with diethyl ether (5 × 20 mL). The combined extract was dried (Na_2SO_4) and the solvent was removed and the resulting solid was dried overnight in a vacuum oven (55 °C) to afford boronate ester **11** (4.21 g, 92%) as a whitish solid. Mp 134.9–137.6 °C; ^1H NMR (400 MHz, $CDCl_3$): δ 1.24 (3H, d, J = 6 Hz), 1.31 (3H, d, J = 6 Hz), 1.34 (12H, m), 1.75 (3H, s), 3.53 (1H, m), 3.79 (1H, m), 3.88 (3H, s), 3.91 (3H, s), 6.44 (1H, s), 7.90 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ 16.63, 16.80, 24.74, 24.81, 2 × 24.92, 26.85, 55.68, 56.14, 77.95, 78.86, 2 × 83.08, 95.74, 107.46, 123.91, 135.22, 160.85, 165.91. HRMS (TOF EI^+) m/z: M^+ calcd for $C_{20}H_{31}BO_6$ 378.2214; found 378.2235.

5.5. Tetrakis(triphenylphosphine)Pd(0)

Tetrakis(triphenylphosphine)palladium(0) was prepared following a modified literature[24] procedure. Thus, triphenylphosphine (7.40 g, 28.24 mmol, 5 equiv) was added to a solution of palladium chloride (1.00 g, 5.64 mmol, 1 equiv) in DMSO (70 mL) under nitrogen atmosphere and the resulting mixture was refluxed in an oil bath until complete solubilization (140 °C, 2.4 h). Then the oil bath was removed and the solution was rapidly stirred for 5 min after which hydrazine hydrate (1.2 mL) was slowly added over 1 min during which a vigorous reaction took place with evolution of gas. The resulting dark solution was then cooled with a water bath for about 5 min and left to crystallize at rt, filtered under nitrogen atmosphere on a sintered-glass funnel and was washed successively with absolute ethanol (2 × 15 mL) and diethyl ether (2 × 15 mL), then dried to give tetrakis(triphenylphosphine)Pd(0) as a yellow crystalline solid (6.39 g, 98%).

5.6. (*E*)-1-(2,4-Dimethoxyphenyl)-3-(2′,4′,6-trimethoxy-5′-(2,4,5-trimethyl-1,3-dioxolan-2-yl)biphenyl-3-yl)prop-2-en-1-one (12)

A mixture of **6** (1.50 g, 3.98 mmol, 1 equiv), **11** (1.50 g, 3.98 mmol, 1 equiv), and $Pd(PPh_3)_4$ (5 mol %, relative to **6**) in toluene (15 mL) was refluxed under nitrogen atmosphere for 10 min. A 20% aqueous solution of tetraethylammonium hydroxide (12.25 mL, 2.45 g, 16.71 mmol, 4.2 equiv) was added and the resulting mixture was refluxed following the progress of reaction by TLC. After 5 h, the mixture was cooled to room temperature and water (10 mL) was added and the mixture extracted with diethyl ether (4 × 20 mL) and dried (Na_2SO_4). The solvent was removed and the residue was chromatographed over silica gel (petroleum ether/ethyl acetate 2:3–1:1). The resulting solid was dried overnight in a vacuum oven to afford ketal **12** (1.59 g, 73%). ^1H NMR (400 MHz, $CDCl_3$): δ 1.25 (3H, d, J = 6 Hz), 1.32 (d, J = 6 Hz), 1.83 (3H, s), 3.60 (1H, m), 3.78 (1H, m), 3.81 (3H, s), 3.82 (3H, s), 3.85 (3H, s), 3.88 (3H, s), 3.96 (3H, s), 6.50 (1H, d, J =

2.0 Hz), 6.56 (1H, dd, J = 2.0, 8.4 Hz), 6.62 (1H, s), 6.99 (1H, d, J = 8.8 Hz), 7.41 (1H, d, J = 15.6 Hz), 7.52 (1H, s), 7.54 (1H, d, J = 2.0 Hz), 7.58 (1H, dd, J = 2.0, 8.4 Hz), 7.71 (1H, d, J = 15.6 Hz), 7.74 (1H, d, J = 8.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 16.54, 16.83, 27.00, 55.52, 55.77, 55.77, 55.87, 56.01, 78.15, 78.84, 97.67, 98.63, 105.10, 107.37, 111.20, 117.77, 122.53, 124.04, 124.99, 127.69, 128.13, 128.81, 129.11, 132.08, 132.65, 142. 51, 157.54, 157.69, 159.01, 160.22, 163.93, 190.84.

5.7. (*E*)-3-(5′-Acetyl-2′,4′,6-trimethoxybiphenyl-3-yl)-1-(2,4-dimethoxyphenyl)prop-2-en-1-one (13)

Ketal **12** (3.73 g, 6.80 mmol) was treated with molecular iodine (0.150 g, 10 mol %) in refluxing acetone (30 mL) for 10 min.[17] After cooling to room temperature, acetone was distilled off and the residue suspended in CH_2Cl_2 (30 mL) and washed successively with 5% aq $Na_2S_2O_3$ (20 mL), water (2 × 20 mL) and brine (20 mL). The resulting solution was dried ($MgSO_4$), filtered and the solvent evaporated in vacuo to give chalconylacetophenone **13** (2.51 g, 77%) as a dark solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.63 (3H, s), 3.82 (3H, s), 3.87 (3H, s), 3.88 (3H, s), 3.89 (3H, s), 4.01 (3H, s), 6.50 (1H, d, J = 2.4 Hz), 6.56 (1H, s), 6.57 (1H, dd, J = 2.4, 8.8 Hz), 6.98 (1H, d, J = 8.8 Hz), 7.37 (1H, d, J = 15.6 Hz), 7.49 (1H, d, J = 2.0 Hz), 7.58 (1H, dd, J = 2.0, 8.4 Hz), 7.67 (1H, d, J = 15.6 Hz), 7.73 (1H, d, J = 8.4 Hz, H), 7.82 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ 31.99, 55.55, 55.67, 55.81, 55.81, 55.90, 94.82, 98.66, 104.99, 111.02, 119.57, 120.11, 122.54, 125.14, 127.02, 127.81, 129.75, 131.52, 132.63, 133.93, 142.33, 158.90, 160.19, 160.99, 161.81, 163.85, 190.94, 197.55. HRMS (TOF EI$^+$) m/z: M$^+$ calcd for $C_{28}H_{28}O_7$ 476.1835; found 476.1870.

5.8. (*E*)-1-(2,4-Dimethoxyphenyl)-3-(2′,4′,6-trimethoxy-5′-((*E*)-3-(4-methoxyphenyl)acryloyl)biphenyl-3-yl)prop-2-en-1-one (14)

A mixture of chalconylacetophenone **13** (0.12 g, 0.25 mmol, 1 equiv), anisaldehyde (0.21 g, 1.58 mmol, 6.3 equiv), and NaOH (1 pellet) was ground in a porcelain mortar at room temperature. After 8 min, a yellowish solid was formed which was treated with water (100 mL) and filtered to give **14** (0.146 g, 98%) as a dark-yellow solid. Mp 184.9–187.1 °C; IR (KBr) ν_{max} 2916, 1649, 1597, 1502, 1458, 1423, 1254, 1202, 1159, 1024, 986, 823 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1. HRMS (TOF EI$^+$) m/z: M$^+$ calcd for $C_{36}H_{34}O_8$ 594.2254; found 594.2256.

5.9. Rhuschalcone VI (1)

To a solution of **14** (0.050 g, 0.08 mmol, 1 equiv) in CH_2Cl_2 (5 mL), 1 M BBr_3 in CH_2Cl_2 (1.5 mL, 0.380 g, 1.52 mmol, 6 equiv) was added. The resulting mixture turned pink and was heated under reflux, for 3 h. After cooling to room temperature, and the mixture was quenched by slow addition of methanol, evaporated in vacuo and the resulting orange solid was dissolved in KOH (2.5% in water). The resulting yellowish solution was acidified to pH 6 by drop wise addition of 3 M HCl and a precipitate formed. This was filtered, dried, and purified by preparative TLC (0.25 mm silica gel plates) using chloroform/methanol (46:04) as eluent to afford rhuschalcone VI **1** (22 mg, 52%, R_f = 0.20), **15** (8 mg, 18%, R_f = 0.27), and **16** (3 mg, 6%, R_f = 0.35). Rhuschalcone VI (**1**): yellow solid; Mp 192–198 °C; IR (KBr) ν_{max} 3429, 2924, 1626, 1555, 1502, 1362, 1219, 1171 cm^{-1}; UV (MeOH) λ_{max} (log ε) 370 (4.54), 275 (4.25) nm, (MeOH + NaOMe) λ_{max} (log ε) 414 (4.56), 305 (4.32) nm, (MeOH + NaOAc) λ_{max} (log ε) 378 (4.68), 304 (4.52) nm, (MeOH + $AlCl_3$) λ_{max} (log ε) 374 (4.76), 236 (4.50) nm, (MeOH + $AlCl_3$ + HCl) λ_{max} (log ε) 375 (4.72) nm. ^{1}H and ^{13}C NMR see Table 1.

5.10. 4,2′,4″,2‴-Tetrahydroxy-4′,4‴-dimethoxy-3,5‴-bichalcone (15)

Yellow solid; IR (KBr) ν_{max} 3423, 2926, 1628, 1555, 1502, 1450, 1367, 1276, 1209, 1155, 1130 cm^{-1}; UV (MeOH) λ_{max} (log ε) 375 (4.38), 262 (4.03) nm, (MeOH + NaOMe) λ_{max} (log ε) 433 (4.44), 279 (3.99) nm, (MeOH + NaOAc) λ_{max} (log ε) 374 (4.36), 306 (4.02) nm, (MeOH + $AlCl_3$) λ_{max} (log ε) 373 (4.36), 288 (3.95) nm, (MeOH + $AlCl_3$ + HCl) λ_{max} (log ε) 377 (4.32) nm; ^{1}H NMR (acetone-d_6, 300 MHz) δ 13.89 (1H, s), 13.73 (1H, s), 8.20 (1H, d, J = 9 Hz, H-6′), 8.15 (1H, s; H-6‴), 7.91–7.89 (4H, overlapping multiplet; H-α, H-α′, H-β, H-β′), 7.78–7.74 (4H, overlapping multiplet; H-2, H-6, H-2″, H-6″), 7.05 (1H, d, J = 9 Hz, H-5), 6.90 (2H, d, J = 9 Hz, H-3″, H-5″), 6.63 (1H, s, H-3‴), 6.52–6.47 (2H, overlapping multiplet, H-5′, H-3′), 3.89 (6H, s, –OMe-4′, –OMe-4‴).

5.11. 4,2′,4′,4″,2‴-Pentahydroxy-4‴-methoxy-3,5‴-bichalcone (16)

Yellow solid; IR (KBr) ν_{max} 3424, 2924, 1631, 1555, 1502, 1366, 1275, 1209, 1157 cm^{-1}; UV (MeOH) λ_{max} (log ε) 374 (4.70), 241 (4.45) nm, (MeOH + NaOMe) λ_{max} (log ε) 433 (4.78), 293 (4.26) nm, (MeOH + NaOAc) λ_{max} (log ε) 379 (4.70), 377 (4.65) nm, (MeOH + $AlCl_3$) λ_{max} (log ε) 375 (4.68), 273 (4.19) nm, (MeOH + $AlCl_3$ + HCl) λ_{max} (log ε) 377 (4.65) nm; ^{1}H NMR (acetone-d_6, 300 MHz) δ 13.88 (1H, s), 13.66 (1H, s), 8.15–8.13 (2H, overlapping multiplet; H-6‴, H-6′), 7.91–7.86 (4H, overlapping multiplet; H-α, H-α′, H-β, H-β′), 7.76–7.74 (4H, overlapping multiplet; H-2, H-6, H-2″, H-6″), 7.05 (1H, d, J = 6 Hz, H-5), 6.90 (2H, d, J = 9 Hz, H-3″, H-5″), 6.63 (1H, s, H-3‴), 6.45 (1H, dd, J = 3 Hz, 9 Hz, H-5′), 6.38 (1H, dd, J = 3 Hz, 9 Hz, H-3′), 3.89 (3H, s, –OMe-4‴).

5.12. Synthesis of rhuschalcone VI (1) analogs

5.12.1. General procedure

A solution of boronate ester **11**, the appropriate chalcone (**25–28**), and $Pd(PPh_3)_4$ (5 mol %, relative to **11**) in toluene (20 mL) was refluxed under nitrogen atmosphere for 10 min. A 20% aqueous solution of tetraethylammonium hydroxide (4.2 equiv) was added and the resulting mixture refluxed following the progress of reaction by TLC (6.5 h for **17** and **19;** 5 and 7 h for **20** and **21**, respectively), the mixture was cooled to room temperature and water (10 mL) was added and the mixture extracted with diethyl ether (4 × 20 mL) and dried ($MgSO_4$). The solvent was removed and the crude product passed through a column of silica gel (hexane/ethyl acetate 3.5:1.5), and the purified solid product was dried overnight in a vacuum oven to afford coupled intermediates **17a**, **19a**, **20a** and **21a** in 86%, 80%, 95% and 79%, respectively. One equivalent of each of these intermediates (1.32 g of **17a**; 1.20 g of **19a**; 0.37 g of **20a** and 1.12 g of **21a**) was refluxed with I_2 (10 mol %) in acetone (20 mL) for 12 min (5 min for **20a**). After evaporation of solvent, the resulting residue was dissolved in dichloromethane (40 mL) and washed successively with 5% aq $Na_2S_2O_3$ (3 × 20 mL), water (2 × 20 mL), brine (30 mL) and dried over anhydrous $MgSO_4$. After evaporation of solvent, the crude product was purified over silica gel column chromatography (petroleum ether/ethyl acetate 3.5:1.5) to afford the deketalized ketones **17b** (900 mg, 80%), **19b** (588 mg, 57%), **20b** (118 mg, 37%) and **21b** (302 mg, 79%). One equivalent of each of **17b** (100 mg, 1 equiv), **19b** (50 mg, 1 equiv), **20b** (110 mg, 1 equiv) and **21b** (50 mg, 1 equiv) were mixed with anisaldehyde (5, 6, 1, 8 equiv, respectively), and NaOH (1 pellet). The mixtures were ground in a porcelain mortar at room temperature. After 8, 6, 13, 10 min, a yellowish solid was formed, treated with water and filtered. The resulting solid was purified using prep TLC (petroleum ether/ethyl acetate 4:2).

5.12.2. 4,4″,2‴,4‴-Tetramethoxy-3,5‴-bichalcone (17)

The crude **17** obtained following the general procedure above was purified using prep TLC (petroleum ether/ethyl acetate 4:2) to afford compound **17** (104 mg, 81%): yellow solid; Mp 155.8–157.1 °C; IR (KBr) ν_{max} 2934, 1655, 1593, 1499, 1280, 1252, 1205, 1144, 1020, 822 cm^{-1}; UV ($CHCl_3$) λ_{max} (log ε) 342 (4.40) nm; ^1H and ^{13}C NMR see Table 1. ; HRMS (TOF EI$^+$) *m/z*: M^+ calcd for $C_{34}H_{30}O_6$ 534.2043; found 534.2065.

5.12.3. 4,4″,2‴,4‴-Tetrahydroxy-3,5‴-bichalcone (18)

Yellow solid; IR (KBr) ν_{max} 3429, 2924, 1631, 1555, 1502, 1450, 1371, 1280, 1209, 1163, 1030, 825 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 13.71 (1H, s), 8.19–8.14 (3H, overlapping multiplet; H-3′, H-5′, H-6‴), 7.89–7.80 (5H, overlapping multiplet; H-β , H-β′, H-2, H-2″, H-6″), 7.75–7.70 (3H, overlapping multiplet; H-2′, H-4′, H-6′), 7.55–7.51 (2H, overlapping multiplet, H-α, H-α′), 7.00 (1H, d, *J* = 9 Hz, H-5), 6.94 (3H, overlapping multiplet, H-6, H-3″, H-5″).

5.12.4. 4,5,4″,2‴,4‴-Pentamethoxy-3,5‴-bichalcone (19)

The crude **19** obtained following the general procedure above was purified using prep TLC (petroleum ether/ethyl acetate 6.3:3.7) to afford compound **19** (49.8 mg, 79%): yellow solid; Mp 76.5–78.3 °C; IR (KBr) ν_{max} 2934, 1653, 1597, 1502, 1454, 1258, 1202, 1163, 1016, 827 cm^{-1}; ^1H- and ^{13}C NMR see Table 1. HRMS (TOF EI$^+$) *m/z*: M^+ calcd for $C_{35}H_{32}O_7$ 564.2148; found 564.2170.

5.12.5. 4,4″,2‴,4‴-Tetramethoxy-4′-methyl-3,5‴-bichalcone (20)

The crude **20** obtained following the general procedure above was purified using prep TLC (petroleum ether/ethyl acetate 5.3:4.5) to afford compound **20** (25 mg, 18%): yellow solid; Mp 112.4–114.1 °C; IR (KBr) ν_{max} 2934, 1653, 1597, 1454, 1253, 1202, 1024, 814 cm^{-1}; ^1H and ^{13}C NMR See Table 1. HRMS (TOF EI$^+$) *m/z*: M^+ calcd for $C_{35}H_{32}O_6$ 548.2199; found 548.2213.

5.12.6. 4,5,2′,4′,4″,2‴,4‴-Heptamethoxy-3,5‴-bichalcone (21)

The crude **21** obtained following the general procedure above was purified using prep TLC (petroleum ether/ethyl acetate 3:2) to afford compound **21** (34 mg, 54%): yellow solid; Mp 113.1–114.9 °C; IR (KBr) ν_{max} 2924, 1649, 1597, 1460, 1414, 1261, 1205, 1167, 1115, 1020, 825 cm^{-1}; ^1H and ^{13}C NMR δ see Table 1.

5.13. Isolation of natural of rhuschalcone VI (1) from *R. pyroides*

The air-dried and powdered *R. pyroides* (root bark, 1.6 kg) was extracted exhaustively by cold percolation with a mixture of methylene chloride–methanol (1:1) followed by 100% methanol. The combined extracts (167.02 g), obtained by evaporation under reduced pressure was suspended in a mixture of water–methanol (1:1; 800 mL) and extracted with methylene chloride (4 × 300 mL) to give, after concentration in vacuo, 16.32 g of a methylene chloride soluble extract. This extract was adsorbed on 25 g of silica gel and chromatographed through a column packed with 200 g silica gel in chloroform. The column was eluted using the following solvent systems, and 19 fractions of ca. 500 mL each were collected: (a) neat chloroform: fractions 1–3, (b) chloroform–methanol (96:04): fractions 4–11, (c) chloroform–methanol (93:07): fractions 12–14, (d) chloroform–methanol (90:10): fractions 15–17, and (e) chloroform–methanol (96:04): fractions 18–19. TLC monitoring using synthetic rhuschalcone VI (**1**) as reference showed that fractions 7 (0.375 g), and 8–11 (1.045 g) contained the sought after substance (rhuschalcone VI (**1**)), which were independently subjected to chromatographic separations on Sephadex LH-20 followed by preparative TLC (chloroform–methanol 46:04) to give **1** (24 mg and 79 mg, respectively).

5.14. Protozoa and mammalian cells

B. caudatus protozoa cultures were a generous gift of the Global Institute for Bioexploration (Gibex, Rutgers University, New Brunswick, NJ, USA). Protozoa were maintained at 30 °C in cereal grass medium (Wards, Rochester, NY, USA) diluted 1:1 with sterile water, supplemented with a liquid *E. coli* culture (strain DH5α was donated by G. Joseph, Gibex, Rutgers University, New Brunswick, NJ, USA) grown in LB medium (Sigma) and subcultured every 7–12 days. Baby hamster kidney (BHK) cells were kindly donated by Botswana Vaccine Institute. BHK cells were grown in DMEM/F10 1:1 (Sigma) supplemented with 10% fetal calf serum (Highveld Biological, South Africa) and antibiotics penicillin and streptomycin (Sigma) in a 37 °C/5% CO_2 incubator.

5.15. *B. caudatus* viability assay

The growth inhibition assay was performed as described by Joseph[25] with some modifications. 100 μL of a fresh subculture containing 10,000 protozoa were transferred into wells of a flat-bottomed 96-well plate and 10 μL of a mixture of 5000 units/mL penicillin, streptomycin and 100 μg/mL kanamycin were added. *B. caudatus* were then exposed to different compounds for 4 h or 24 h at a final concentration of 0.6 mg/mL. $CuSO_4$ served as positive control at the same concentration. Finally, 5 μL of a 5 mg/mL stock solution of 3-(4,6-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added. After 2 h incubation, supernatant was carefully removed from each well and 100 μL DMSO was added to dissolve MTT crystals. Absorbance at 570 nm indicative of viability of protozoa was measured with a multiwell-plate reader (Tecan Sunrise, Waesi Pharmaceuticals, Gaborone, Botswana). Viability assays were performed in triplicates.

5.16. Determination of LC_{50} concentrations and cytotoxicity

Lethal concentration of compounds for 50% of *B. caudatus* (LC_{50}) and general cytotoxicity of compounds (CC_{50}) were determined by the MTT viability assay as described by Mosmann[26] with some modifications. Briefly, BHK cells or *B. caudatus* protozoa were adjusted to 1×10^5/mL in their respective growth media. 100 μL of BHK cell suspension was transferred to wells of a 96-well plate and left to adhere overnight. 100 μL of *B. caudatus* cells were used directly after adding 20 μL of a mixture of 5000 units/mL penicillin, streptomycin and 100 μg/mL kanamycin into wells. Cells or protozoa were exposed for 48 h to serial dilutions of compounds with the respective growth media to an end concentration ranging from 0.0001 to 1000 μg/mL in a 37 °C/5% CO_2 incubator. 10 μL of a MTT (Sigma) stock solution of 5 mg/mL was added to each well and the plate was incubated for further 4 h. Supernatant was removed carefully and MTT crystals were dissolved by adding 100 μL DMSO. Absorbance was measured at 570 nm using a microplate reader (Tecan Sunrise, Waesi Pharmaceuticals, Gaborone, Botswana). Assays were performed in triplicates and best-fit curves were generated using GraphPad Prism 4.0 software (GraphPad Software, La Jolla, CA, USA).

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