

Biflavanones, Chalconoids, and Flavonoid Analogues from the Stem Bark of *Ochna holstii*

Thobias M. Kalenga, Monica M. Ndoile, Yoseph Atilaw, Pieter J. Gilissen, Joan J. E. Munissi, Anastasia Rudenko, Catarina Bourgard, Per Sunnerhagen, Stephen S. Nyandoro,* and Mate Erdelyi*

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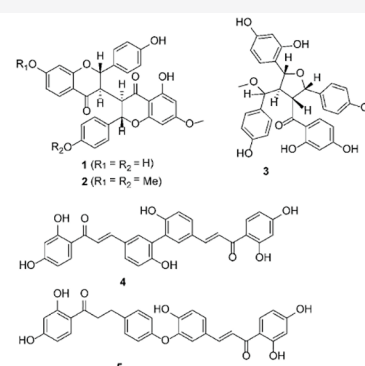
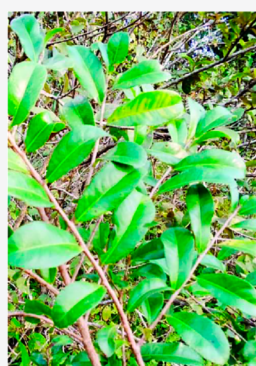
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ABSTRACT: Two new biflavanones (**1** and **2**), three new bichalconoids (**3–5**), and 11 known flavonoid analogues (**6–16**) were isolated from the stem bark extract ($\text{CH}_3\text{OH}-\text{CH}_2\text{Cl}_2$, 7:3, v/v) of *Ochna holstii*. The structures of the isolated metabolites were elucidated by NMR spectroscopic and mass spectrometric analyses. The crude extract and the isolated metabolites were evaluated for antibacterial activity against *Bacillus subtilis* (Gram-positive) and *Escherichia coli* (Gram-negative) as well as for cytotoxicity against the MCF-7 human breast cancer cell line. The crude extract and holstinone A (**1**) exhibited moderate antibacterial activity against *B. subtilis* with MIC values of 9.1 $\mu\text{g}/\text{mL}$ and 14 μM , respectively. The crude extract and lophirone F (**14**) showed cytotoxicity against MCF-7 with EC_{50} values of 11 $\mu\text{g}/\text{mL}$ and 24 μM , respectively. The other isolated metabolites showed no significant antibacterial activities ($\text{MIC} > 250 \mu\text{M}$) and cytotoxicities ($\text{EC}_{50} \geq 350 \mu\text{M}$).



The genus *Ochna* (Ochnaceae) comprises about 86 species of evergreen trees or shrubs distributed in Madagascar and in tropical Africa, Asia, and the America.¹ Members of this genus are used in traditional medicine to treat malaria, diarrhea, and hemorrhoids, as well as microbial and helminthic ailments.^{1,2} Many species from the genus *Ochna* are known to metabolize biflavanones, chalcones, and related compounds^{3,4} which are reported to exhibit antimalarial and antibacterial activity and cytotoxicity against cancer cells.^{3–8} In our endeavor to search for bioactive natural products from Tanzanian medicinal plants, we investigated extracts of *O. holstii*. It is a shrub or a medium-sized tree up to 15 m high occurring in Southern Asia, Republic of South Africa, Ethiopia, Zimbabwe, Kenya, and Tanzania.^{9–11} The plant is locally known as “mkumbi” in Tanzania. There is no ethnomedical information reported for this plant species, apart from its use to relinquish evil spirits from children. The crude extract of *O. holstii* has been reported to exhibit anti-HIV and contraceptive activities.^{12,13} Previous phytochemical investigations of *O. holstii* revealed the presence of dimeric and monomeric flavonoids.¹⁴ Herein, we evaluated the crude extract of the stem bark of *O. holstii* and its isolated constituents (**1–16**) for antibacterial activity against *Bacillus subtilis* and *Escherichia coli* and for cytotoxicity against the MCF-7 human breast cancer cell line.

RESULTS AND DISCUSSION

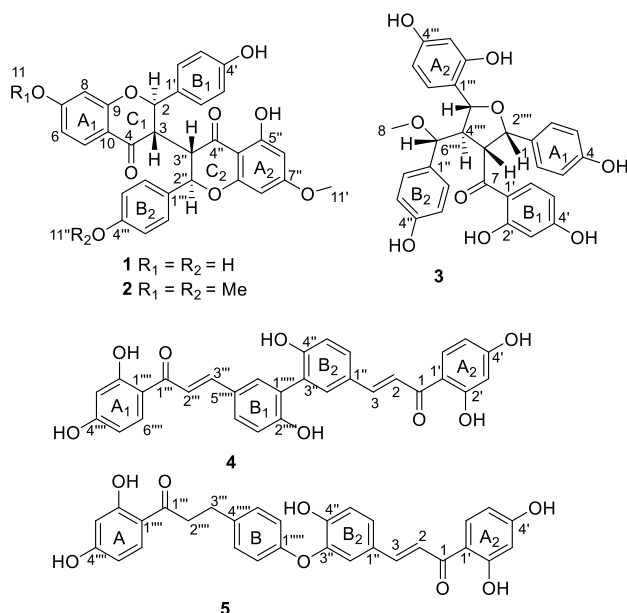
Systematic purification of the $\text{CH}_3\text{OH}-\text{CH}_2\text{Cl}_2$ (7:3, v/v) extract of the stem bark of *O. holstii* using silica gel 60 (230–400 mesh) column chromatography, followed by gel filtration on a Sephadex LH-20 column and preparative reverse-phase HPLC, yielded five new metabolites (**1–5**) and 11 known compounds (**6–16**) (Figures S1–S84, Supporting Information). The structures of the isolated compounds were elucidated by 1D and 2D NMR spectroscopic and mass spectrometric analyses. The known compounds were identified as ouratein D (**6**),¹⁵ isochamaejasmin A (**7**),^{16,17} 7',7"-di-O-methylisochamaejasmin (**8**),¹⁸ campylospermone A (**9**),¹⁹ liquiritigeninyl-(1-3,II-3)-naringenin (**10**),⁸ isoliquiritigenin (**11**),²⁰ terminalionone (**12**),²¹ flavumchalcone (**13**),²² lophirone F (**14**),²³ 2,3-dihydrocalodenin B (**15**),³ and calodenin B (**16**),^{24,25} by comparison of their spectroscopic data with reported data.

Compound **1** was isolated as a pale white solid and was assigned the molecular formula $\text{C}_{31}\text{H}_{24}\text{O}_9$ based on HRESIMS ($[\text{M} + \text{H}]^+$ m/z 541.1487, calcd 541.1499, Figure S8,

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Supporting Information) and NMR data (Table 1, Figures S1–S7, Supporting Information). It showed a specific rotation $[\alpha]_D^{24}$ of -115 (c 0.013, CH_3OH). The IR spectrum revealed the presence of hydroxy (3396 cm^{-1}) and aromatic $C=C$ (1604 , 1516 , and 1453 cm^{-1}) groups. UV absorption maxima

at 262 and 330 nm indicated the presence of a flavanone-type structure.^{15,26}

The 1H NMR spectrum (Table 1, Figure S1, Supporting Information) of **1** suggested that the compound has four distinct aromatic systems. Ring A_1 contained an ABX spin system resonating at δ_H 7.50 (H-5), 6.54 (H-6), and 6.45 (H-8). The substitution pattern of ring A_2 was deduced from the coupling constant ($^4J_{6'',8''} = 2.4\text{ Hz}$) of the protons at δ_H 6.12 (H-6'') and 6.16 (H-8''). Furthermore, compound **1** contained two protons of a *p*-disubstituted aromatic system resonating at δ_H 7.28 (H-2'/6') and 6.76 (H-3'/5') for ring B_1 and at δ_H 7.25 (H-2'''/6''') and 6.74 (H-3'''/5''') for ring B_2 . In addition, the 1H NMR spectrum displayed methoxy group protons at δ_H 3.77 (H₃-11), two oxymethine protons at δ_H 5.62 (H-2) and 5.58 (H-2''), and two methine protons at δ_H 4.50 (H-3) and 4.46 (H-3''). The 2,3-relative configuration of compound **1** was determined via the 1H NMR coupling constants and splitting patterns. The splitting patterns of the resonances assigned to H-2 (d, $J = 10.2\text{ Hz}$), H-2'' (d, $J = 10.0\text{ Hz}$), H-3 (dd, $J = 10.2$, 7.8 Hz) and H-3'' (dd, $J = 10.0$, 7.8 Hz) suggested rings C_1 (H-2/H-3) and C_2 (H-2''/H-3'') to have *trans*-relative configurations. This conclusion is corroborated by the observed $^3J_{2,3}$ being larger than that (2.3 Hz) reported by Rocha et al. for the 2,3-*cis*-configured analogues, but similar to the 9.2 and 9.9 Hz reported for the *trans* relative configured analogues.¹⁵ Protons H-3 and H-3'' showed $^3J_{3,3''} = 7.8\text{ Hz}$, which is comparable to the $^3J_{3,3''} = 8.5\text{ Hz}$ previously reported

Table 1. NMR Spectroscopic Data (500 MHz) for Holstiinone A (**1**) and Holstiinone B (**2**)

position	holstiinone A (methanol- d_4)			holstiinone B ($CDCl_3$)		
	δ_C , type	δ_H (J in Hz)	HMBC, H $\rightarrow$ C	δ_C , type	δ_H (J in Hz)	HMBC, H $\rightarrow$ C
2	86.7, CH	5.62, d (10.2)	C-2'/6', C-3'', C-4, C-9	85.3, CH	5.66, d (10.1)	C-3, C-3', C-2''/6'', 4, 4'
3	60.4, CH	4.50, dd (10.2, 7.8)	C-2', C-4, C-4', C-10	59.8, CH	4.49, dd (10.1, 8.2)	C-3'', C-1'', C-2, C-4, C-4''
4	196.0, C			194.2, C		
5	131.9, CH	7.50, d (8.2)	C-4, C-7, C-9	131.1, CH	7.66, d (8.8)	C-4, C-7, C-9
6	112.8, CH	6.54, dd (8.2, 2.4)	C-7, C-8, C-10	111.0, CH	6.62, dd (8.8, 2.4)	C-7, C-8, C-10
7	165.3, C			164.9, C		
8	107.9, CH	6.45, d (2.4)	C-7, C-9, C-10, C-6	105.1, CH	6.55, d (2.4)	C-6, C-7, C-10
9	166.5, C			165.7, C		
10	122.1, C			121.9, C		
1'	132.1, C			132.0, C		
2'/6'	129.3, CH	7.25, AA'	C-2'', C-3'/5', C-4'	128.2, CH	7.34, AA'	C-2, C-3'/5', C-4'
3'/5'	116.3, CH	6.76, XX'	C-2'/6', C-1', C-4'	114.1, CH	6.85, XX'	C-2'/6', C-4', C-1'
4'	158.7, C			159.7, C		
2''	86.4, CH	5.58, d (10.0)	C-2'''/6''', C-3'', C-4'', C-9''	85.2, CH	5.74, d (10.1)	C-2'''/6''', C-9'', C-4'', C-3''
3''	60.2, CH	4.46, dd (10.0, 7.8)	C-2, C-2'', C-4, C-4'', C-1'''	59.5, CH	4.45, dd (10.1, 8.2)	C-2, C-3, C-4, C-4'', C-1'''
4''	200.5, C			198.6, C		
5''	165.3, C			164.6, C		
6''	97.7, CH	6.12, d (2.4)	C-7'', C-8'', C-10''	96.9, CH	6.14, d (2.4)	C-5'', C-10'', C-8'', C-7''
7''	167.9, C			166.4, C		
8''	99.9, CH	6.16, d (2.4)	C-6'', C-7'', C-10''	99.1, CH	6.11, d (2.4)	C-10'', C-6'', C-7''
9''	166.9, C			165.1, C		
10''	109.7, C			108.8, C		
1'''	131.8, C			132.5, C		
2'''/6'''	129.5, CH	7.28, AA'	C-2, 3'/5', C-4'	128.7, CH	7.36, AA'	C-2'', C-4''', C-3'''/5'''
3'''/5'''	116.2, CH	6.74, XX'	C-2'''/6''', C-4''', C-1'''	115.5, CH	7.79, XX'	C-2'''/6''', C-1''', C-4'''
4'''	158.7, C			155.7, C		
OCH ₃ -11	56.2, CH	3.77, s	C-7''	55.8, CH ₃	3.76, s	C-7
OCH ₃ -11''				55.4, CH ₃	3.79, s	C-7''
OCH ₃ -7'''				55.8, CH ₃	3.78, s	C-4'''
OH-5'					11.9, s	C-5', C-6', C-10'

for the *gauche* conformation, but dissimilar to the $^3J_{3,3'} = 12.0$ Hz of the *anti*-oriented protons of structurally closely related compounds.⁶ Based on this observation, **1** is proposed to possess *syn* H-3/H-3'' geometry. The HMBC cross-peaks (Table 1, Figure S5, Supporting Information) of H-11 (δ_{H} 3.77), H-6'' (δ_{H} 6.12), and H-8'' (δ_{H} 6.16) to C-7'' (δ_{C} 167.9) corroborated the location of the methoxy group at C-7'' of ring A₂. In addition, the HMBC cross-peaks of proton resonances at δ_{H} 4.50 (H-3) to C-2'' (δ_{C} 86.4), C-4 (δ_{C} 196.0), C-4'' (δ_{C} 200.5), and C-10 (δ_{C} 122.1) and those at δ_{H} 5.62 (H-2) to C-3'' (δ_{C} 60.2), C-4 (δ_{C} 196.0), C-9 (δ_{C} 166.4), and C-2'/6'' (δ_{C} 129.5) established the C-3–C-3'' linkage of the two flavanone units. Similar C-3–C-3'' linkages were previously reported from other plant species including campylospermone B from *Campylospermum mannii*,¹⁹ ouratein D from *Ouratea spectabilis*,¹⁵ 4,4,7-tri-*O*-methylisocampylospermone A from *Ochna serrulata*,²⁷ chamaejasmin from *Ormocarpum kirkii*,⁸ and chamaejasmin D from *Stellera chamaejasme*.²⁸ The NMR spectroscopic data for compound **1** closely resembled those of campylospermone B¹⁹ and liquiritigeninyl-(1-3,II-3)-naringenin,⁸ but had a methoxy group resonating at δ_{H} 3.77 (H-11) and attached to C-7'' (δ_{C} 167.9), indicated by the HMBC cross-peak of H-11 (δ_{H} 3.77) with C-7'' (δ_{C} 167.9). The ECD spectrum of **1** (Figure 1) showed a weak positive Cotton effect

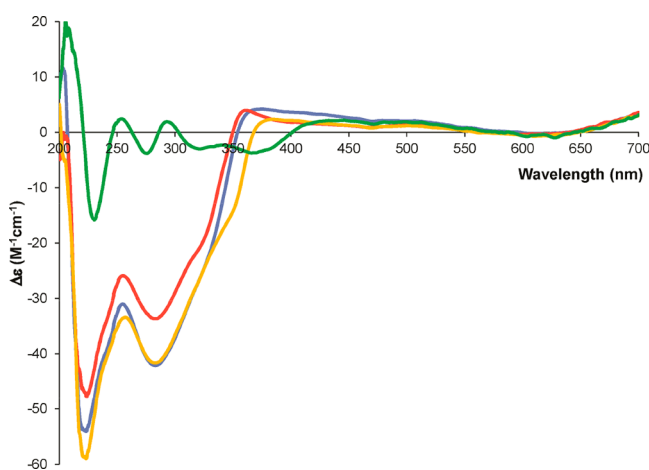


Figure 1. ECD spectra of holstiinone A (**1**, blue), holstiinone B (**2**, red), holstiichalcone I (**3**, yellow), and holstiichalcone II (**4**, green) in MeOH.

for the $\pi \rightarrow \pi^*$ transition at ca. 360 nm, a negative Cotton effect for the $n \rightarrow \pi^*$ transition at ca. 300 nm, and a negative Cotton effect at ca. 210 nm for the ^1La electronic transition, which indicated a (2*R*) absolute configuration for each flavanone moiety.¹⁵ The absolute configuration at C-3 was determined based on the *trans* relative configurations of the neighboring H-2 and H-3 protons. Based on the above spectroscopic data, the new compound, holstiinone A (**1**), was characterized as (2*R*,2''*R*,3*S*,3''*S*)-5'',7-dihydroxy-2,2''-bis-(4',4'''-hydroxyphenyl)-7''-methoxy-[3,3''-bichromane]-4,4''-dione.

Compound **2** was isolated as a white solid. The molecular formula $\text{C}_{33}\text{H}_{28}\text{O}_9$ was assigned based on HRESIMS ($[\text{M} + \text{H}]^+$ m/z 569.1741, calcd 569.1812, Figure S16, Supporting Information) and NMR data (Table 1, Figures S9–S15, Supporting Information). It showed a specific rotation $[\alpha]_{\text{D}}^{24}$ of -92 (c 0.013, CH_3OH). IR spectroscopy indicated the presence of hydroxy (3320 cm^{-1}), conjugated carbonyl (1698

cm^{-1}), and aromatic $\text{C}=\text{C}$ (1598 and 1515 cm^{-1}) groups. The UV spectrum showed absorption maxima at 258 and 340 nm, conforming the presence of a flavanone-type structure.^{26,29}

The NMR spectroscopic data of **2** (Table 1, Figures S9–S15, Supporting Information) are closely related to those of compound **1**. Compound **2** contained two additional methoxy groups with protons resonating at δ_{H} 3.76 (δ_{C} 55.8) and 3.78 (δ_{C} 55.8) and attached to C-7 (δ_{C} 165.1) and C-4'' (δ_{C} 155.7), respectively. For compound **2**, the attachments of the three methoxy groups at rings A₁, A₂, and B₂ were established based on the HMBC cross-peaks (Figure S13, Supporting Information) of H₃-11 (δ_{H} 3.76) and H-5 (δ_{H} 7.66) to C-7 (δ_{C} 164.9); of H-11' (δ_{H} 3.79), H-6'' (δ_{H} 6.14), and H-8'' (δ_{H} 6.11) to C-7'' (δ_{C} 166.4); and of H-11'' (δ_{H} 3.78) and H-2'''/6'' (δ_{H} 7.36) to C-4'' (δ_{C} 155.7). The 2,3-relative configuration of structure **2** was determined based on the ^1H NMR coupling constants and splitting patterns. The resonances assigned to H-2 and H-2'' were doublets with a coupling constant of 10.2 Hz each, indicating 2,3- and 2'',3''-*trans* relative configurations. The ECD spectrum of **2** (Figure 1) closely resembled that of **1** and was thus compatible with a (2*R*,2''*R*,3*S*,3''*S*) absolute configuration. Based on the above spectroscopic evidence, the new compound holstiinone B (**2**) was characterized as (2*R*,2''*R*,3*S*,3''*S*)-5''-hydroxy-2-(4'-hydroxyphenyl)-7,7''-dimethoxy-2''-(4'''-methoxyphenyl)-[3,3''-bichromane]-4,4''-dione. It is a methoxy derivative of **1**, which is in agreement with the enzymatic *O*-methylation, typical of flavonoids, by *O*-methyltransferases.³⁰

Compound **3** was obtained as a pale yellow amorphous solid and was assigned the molecular formula $\text{C}_{31}\text{H}_{28}\text{O}_9$ based on HRESIMS ($[\text{M} + \text{Na}]^+$ m/z 567.1634, calcd 567.1631 (Figure S24, Supporting Information) and NMR data (Table 2, Figures S17–S23, Supporting Information). It showed a specific rotation $[\alpha]_{\text{D}}^{24}$ of -176.9 (c 0.23, CH_3OH). IR absorptions at 3305, 1700, and 1598 cm^{-1} indicated the presence of hydroxy, conjugated carbonyl, and aromatic moieties, respectively. The UV spectrum showed absorption maxima at 340 and 264 nm, corresponding to aromatic moieties of a flavanone. The ^1H NMR spectrum (Table 2; Figure S17, Supporting Information) showed 10 distinct aromatic proton signals. COSY and TOCSY correlations (Figures S19 and S23, Supporting Information) corroborated the presence of two *p*-disubstituted aromatic rings (A₁ and B₂), two ABX aromatic ring systems (A₂ and B₁), and one tetrahydrofuran ring system, reminiscent of flavumchalcone,²² the only difference being the presence of a signal corresponding to a methoxy group, the protons of which resonate at δ_{H} 3.28. The attachment of the methoxy group to C-6''' (δ_{C} 83.6) was established based on the HMBC (Table 2, Figure S21, Supporting Information) cross-peaks of H₃-8 (δ_{H} 3.28) to C-6''' (δ_{C} 83.6). Further important HMBC cross-peaks that confirmed the molecular framework included those supporting the assignment of the tetrahydrofuran ring, namely, H-3''' (δ_{H} 4.69) to C-6''' (δ_{C} 83.6), C-1' (δ_{C} 113.6), and C-5''' (δ_{C} 81.3), H-2''/6'' (δ_{H} 6.94) to C-6''' (δ_{C} 83.6), H-2''' (δ_{H} 3.22) to C-4''' (δ_{C} 58.5) and C-7 (δ_{C} 204.5), and H-4''' (δ_{H} 3.22) to C-1'' (δ_{C} 131.9), C-1''' (δ_{C} 117.5), and C-7 (δ_{C} 204.5). The placement of protons in ring B₁ and A₁ was supported by the HMBC cross-peak of H-6' (δ_{H} 7.54) to C-7 (δ_{C} 204.5) and H-2/6 (δ_{H} 7.07) to C-2''' (δ_{C} 84.4), respectively. In addition, the protons of rings A₂ and B₂ were assigned based on the HMBC cross-peak of H-6''' (δ_{H} 7.02) to C-5''' (δ_{C} 81.3) and that of H-2''/6'' (δ_{H}

Table 2. NMR Spectroscopic Data (500 MHz, Methanol- d_4) for Holstiichalcone I (3)

position	δ_C , type	δ_H (J in Hz)	HMBC, H $\rightarrow$ C
1	129.8, C		
2/6	129.2, CH	7.07, AA'	C-2'', C-4, C-1
3/5	115.4, CH	6.54, XX'	C-1, C-4, C-2''
4	157.7, C		
1'	113.6, C		
2'	170.1, C		
3'	103.7, CH	5.90, d (2.1)	C-5', C-1', C-4'
4'	166.6, C		
5'	110.4, CH	6.19, dd (8.8, 2.1)	C-1', C-3', C-4'
6'	134.3, CH	7.54, d (9.0)	C-2', C-4', C-7
1''	131.9, C		
2''/6''	128.9, CH	6.94, AA'	C-4'', C-6'', C-1''
3''/5''	116.0, CH	6.51, XX'	C-1'', C-4'', C-5''
4''	157.9, C		
1'''	117.5, C		
2'''	157.6, C		
3'''	103.9, CH	6.31, d (2.1)	C-5''', C-4''', C-2'''
4'''	159.4, C		
5'''	107.8, CH	6.26, dd (8.2, 2.1)	C-1''', C-3''', C-4'''
6'''	131.4, CH	7.02, d (8.2)	C-2''', C-4''', C-5'''
2'''	84.4, CH	5.27, d (8.2)	C-2, C-3'', C-6, C-7
3'''	52.9, CH	4.69, dd (8.2, 5.5)	C-2''', C-4''', C-1', C-7
4'''	58.5, CH	3.22, ddd (9.2, 5.5, 5.4)	C-3''', C-1'', C-5''', C-6''', C-7
5'''	81.3, CH	5.12, d (9.2)	C-4''', C-6''', C-2'''
6'''	83.6, CH	4.27, d (5.4)	C-2'', C-3''', C-4''', C-5''', C-6'', C-8
7	204.5, C		
8	57.0, CH	3.28, s	C-6'''

6.94) to C-6''' (δ_C 57.0), respectively (Table 2, Figure S21, Supporting Information).

The protons belonging to the spin system of the tetrahydrofuran ring were assigned based on the COSY and TOCSY correlations (Figure S19 and S22, Supporting Information) of H-2''' (δ_H 5.27) to H-3''' (δ_H 4.69), H-3''' (δ_H 4.69) to H-4''' (δ_H 3.22), and H-4''' (δ_H 3.22) to H-5''' (δ_H 5.12) and H-6''' (δ_H 4.27). The relative configurations at the carbon atoms of the tetrahydrofuran ring were assigned based on the NOESY correlations (Figure 2) of H-2''' (δ_H

5.27) and H-5''' (δ_H 5.12), H-2''' (δ_H 5.27) and H-3''' (δ_H 4.69), H-5''' (δ_H 5.12) and H-6''' (δ_H 4.27), and H-4''' (δ_H 3.22) with H-6''' (δ_H 4.27). Based on the above spectroscopic data, the new compound, holstiichalcone I (3), was characterized as (2'',4''-dihydroxyphenyl)-[(2''S*,3''R*,4''S*,5''R*)-5''-(2',4'-dihydroxyphenyl)-2''-(4-hydroxyphenyl)-4''-{(4'-hydroxyphenyl)(methoxy)methyl}tetrahydrofuran-3'''-yl]methanone. Despite repeated attempts, no crystals suitable for X-ray crystallography to determine the absolute configuration of 3 were obtained. The ECD spectrum is shown in Figure 1. Similar structures have previously been reported from various *Ochna*,^{27,31,32} *Campylospermum*,^{22,33} and *Lophira*^{34,35} species, however without their absolute configuration having been determined.

Compound 4 was isolated as a yellow solid and was assigned the molecular formula $C_{30}H_{22}O_8$ based on HRESIMS ($[M + H]^+$ m/z 511.1478, calcd 511.1393, Figure S32, Supporting Information) and NMR data (Table 3, Figures S25–S31, Supporting Information). It showed a specific rotation $[\alpha]_D^{24}$ of -21 (c 0.013, CH_3OH). IR vibrations at 3382 and 1604 cm^{-1} suggested the presence of hydroxy and $C=C$ groups, respectively. The UV spectrum displayed absorption bands centered at 368, 340, and 284 nm that were in agreement with a chalcone skeleton.⁷ The ^{13}C NMR spectrum (Table 3, Figure S26, Supporting Information) showed only 15 instead of 30 resonances, revealing compound 4 to be a symmetric dimeric molecule. The 1H NMR data (Table 3, Figures S25 and S27, Supporting Information) indicated the presence of two distinct ABX spin systems at δ_H 7.99 (H-6'/6'''), 6.42 (H-5'/5'''), and 6.30 (H-3'/3''') and at 7.70 (H-4'/4'''), 7.68 (H-6''/6'''), and 7.01 (H-3''/3'''). In addition, *trans* olefinic protons resonating at δ_H 7.88 (H-3/3'') and 7.67 (H-2/2'') suggested the presence of a chalcone moiety. Furthermore, the ^{13}C NMR spectrum (Table 3, Figure S26, Supporting Information) consisted of signals due to a carbonyl function at δ_C 193.6 (C-1/1''), three oxygenated aromatic carbons at δ_C 167.5 (C-2'/2'''), 166.4 (C-4'/4'''), and 158.9 (C-2''/2'''), and 11 other sp^2 -hybridized carbons between 145.6 and 103.8 ppm. The locations of the *trans* olefinic systems and the ABX spin systems were corroborated by HMBC cross-peaks (Table 3, Figure S29, Supporting Information) of H-6'/6'''' (δ_H 7.99) and H-3/3''' (δ_H 7.88) to C-1/1'' (δ_C 193.6) and of H-2/2'' (δ_H 7.67) and H-3'/3''' (7.01) to C-1'/1''' (δ_C 128.2). In

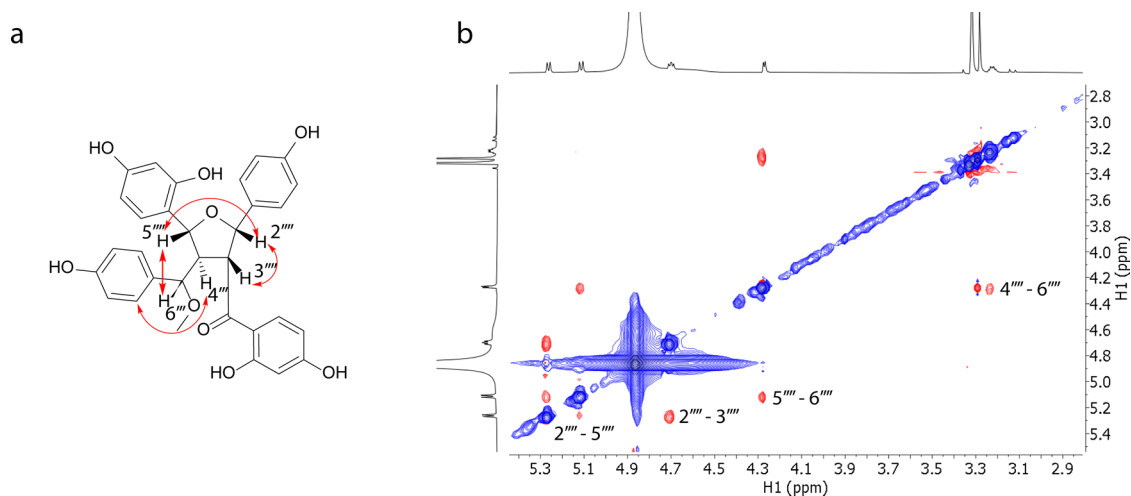
**Figure 2.** Key NOE correlations allowing the determination of the relative configuration of the tetrahydrofuran ring of holstiichalcone I (3).

Table 3. NMR Spectroscopic Data (500 MHz, Methanol-*d*₄) for Holstiichalcone II (4)

position	δ_C , type	δ_H (J in Hz)	HMBC, H $\rightarrow$ C
1	193.6, C		
2	118.7, CH	7.67, m	C-1, C-5'', C-1'
3	145.6, CH	7.88, d (15.3)	C-1, C-4'', C-6''
1'	114.7, C		
2'	167.5, C		
3'	103.8, CH	6.30, d (2.3)	C-1', C-5'
4'	166.4, C		
5'	109.2, CH	6.42, d (2.4)	C-3', C-1'
6'	133.5, CH	7.99, d (8.8)	C-1, C-2', C-4'
1''	128.2, C		
2''	158.9, C		
3''	117.6, C	7.01, d (8.8)	C-1'', C5''
4''	130.8, CH	7.70, m	C-3, C-2'', C-6''
5''	127.5, C		
6''	133.8, CH	7.68, m	C-2'', C-4''
1'''	193.6, C		
2'''	118.7, CH	7.67, m	C-1''', C-4''', C-5'''
3'''	145.6, CH	6.42, d (15.3)	C-1''', C-4''', C-6'''
1'''	114.7, C		
2'''	167.5, C		
3'''	103.8, CH	6.30, d (2.3)	C-1''', C-5'''
4'''	166.4, C		
5'''	109.2, CH	6.42, d (2.4)	C-3''', C-1'''
6'''	133.5, CH		C-1''', C-2''', C-4'''
1''''	128.2, C		
2''''	158.9, C		
3''''	117.6, C	7.01, d (8.8)	
4''''	130.8, CH	7.70, m	C-3''', C-2''', C-6'''
5''''	127.5, C		
6''''	133.8, CH	7.68, m	C-2''', C-4'''

addition, the HMBC cross-peaks of H-6'/6''' (δ_H 7.99) to C-1/1''' (δ_C 193.6), C-4'/4''' (δ_C 166.4), and C-2'/2''' (δ_C 167.5) and of H-3/3''' (δ_H 7.88) to C-1/1''' (δ_C 193.6) and C-4'/4''' (δ_C 130.8) (Table 3, Figure S29, Supporting Information) confirmed the connection of the ABX rings A₁/A₂ and *trans* olefinic units to carbonyl groups. Furthermore, the common HMBC cross-peak of H-3''/3''' (δ_H 7.01) and H-2/2''' (δ_H 7.67) to C-5''/5''' (δ_C 127.5) (Table 3, Figure S29, Supporting Information) and H-3/3''' (δ_H 7.01) to C-1''/1''' (δ_C 128.2) supported the assignment of rings B₁/B₂. Compound 4 exhibited optical activity because of its axis of chirality (atropisomerism), supporting its dimeric nature, which resembles previously reported dimeric chalconoids.^{7,36–39} The ECD spectrum (Figure 1) shows positive Cotton effects at 204, 253, 293, and 436 nm and negative Cotton effects at 230, 274, 320, and 373 nm. As there are no published ECD spectra available for bichalcones with known absolute configuration, the ECD of the related biflavones may provide a handle on the structure determination of 4. Hence the spectrum of 4 shows resemblance to the ECD spectrum of (–)-(S_a)-agatisflavone,³⁶ whereas little to none to that of (–)-(R_a)-4',4'',7,7''-tetra-O-methylcupressuflavone.⁴⁰ Its negative specific rotation ($[\alpha]^{24}_D$ of –21) and negative Cotton effects at 373 and 320 nm therefore suggest that 4 most likely has an (S_a)-bichalcone unit and thus a *P*-configuration.^{41,42} Based on the above spectroscopic features, the new compound holstiichalcone II (4) was characterized as (–)-(S_a)-(2*E*,2''*E*)-3,3''-(2'',2'''-dihydroxy-[1,1'''-biphenyl]-3,3'''-diyl)bis[1-

(2',4'-dihydroxyphenyl)prop-2-en-1-one]. Similar compounds have been previously reported from *Rhus pyroides*.⁷

Compound 5 was isolated as a yellow solid and was assigned the molecular formula C₃₀H₂₄O₈ based on HRESIMS ([M + H]⁺ *m/z* 513.1575, calcd 513.1549, Figure S40, Supporting Information) and NMR data (Table 4, Figures S33–S39,

Table 4. NMR Spectroscopic Data (500 MHz, Methanol-*d*₄) for Holstiichalcone III (5)

position	δ_C , type	δ_H (J in Hz)	HMBC, H $\rightarrow$ C
1	193.3, C		
2	119.4, CH	7.57, d (15.4)	C-2', C-2 C-6', C-1
3	144.9, CH	7.72, d (15.4)	C-3, C-1', C-1, C-4''
1'	114.6, C		
2'	166.6, C		
3'	103.8, CH	6.27, AA'	C-1', C-5'
4'	167.5, C		
5'	109.4, C	6.38, dd (8.8, 2.4)	
6'	133.4, CH	7.91, XX'	C-1, C-2', C-4'
1''	128.7, C		
2''	122.1, CH	7.31, d (2.4)	C-6'', C-4''
3''	145.7, C		
4''	153.3, C		
5''	118.5, CH	7.00 d (8.8)	C-1'', C-3''
6''	127.4, CH	7.46, dd (8.8, 2.4)	C-2'', C-4''
1'''	205.2, C		
2'''	40.6, CH ₂	3.24, t (7.6)	C-1''', C-4''', C-3''', C-1'''
3'''	31.0, CH ₂	3.00, t (7.6)	C-3'''/5''', C-2''', C-1'''
1''''	114.0, C		
2''''	166.4		
3''''	103.7, CH	6.24, d (2.4)	C-1''', C-5'''
4''''	166.8, C		
5''''	109.3, CH	6.26, dd (8.8, 2.4)	C-3''', C-1'''
6''''	133, CH	7.73, dd (8.8)	C-2''', C-2''', C-1'''
1'''''	157.6, C		
2'''''/6'''''	118.3, CH	6.89, d (8.6)	C-3'''''/5'''''', C-1'''''', C-4'''''
3'''''/5'''''	130.7, CH	7.23, d (8.6)	C-2'''''/6'''''', C-1'''''', C-3'''
4'''''	136.9, C		

Supporting Information). IR absorptions at 3326 and 1709 cm^{–1} indicated the presence of hydroxy and conjugated carbonyl moieties, respectively. IR absorptions at 1631 and 1506 cm^{–1} are typical of C=C stretches. The UV spectrum with absorption maxima centered at $\lambda_{\max}$ 264, 320, and 346 nm indicated the presence of a chalconoid skeleton.^{29,43} The ¹H NMR spectrum (Table 4, Figure S33, Supporting Information) displayed signals of a *p*-disubstituted aromatic ring resonating at δ_H 7.23 (H-3''''/5''''') and 6.89 (H-2''''/6'''''), three sets of ABX spin systems at 7.73 (H-6'''), 6.24 (H-3'''), and 6.26 (H-5''') for ring A, at δ_H 7.31 (H-2''), 7.00 (H-5''), and 7.46 (H-6'') for ring B₂, and at 7.91 (H-6'), 6.38 (H-5'), and 6.27 (H-3') for ring A₂. The ¹H NMR spectrum consisted also of signals corresponding to *trans*-olefinic protons at δ_H 7.72 (H-3) and 7.57 (H-2) and ethylene protons at δ_H 3.24 (H-2''') and 3.00 (H-3'''), which are common features of chalcone and dihydrochalcone moieties, respectively. The ¹³C NMR spectrum (Table 4, Figure S34, Supporting Information) of 5 showed a total of 30 carbons comprising two carbonyl carbons at δ_C 193.3 (C-1) and 205.3 (C-1'''), six oxygenated aromatic carbons (167.6–145.7 ppm), 20 sp²-hybridized carbons (144.9–103.7 ppm), and two methylene carbons at δ_C 40.6 (C-2'') and 31.0 (C-3'''). Analysis of the HMBC

(Figure S37, Supporting Information) cross-peaks of H-2''' (δ_{H} 3.24), H-3''' (δ_{H} 3.00), and H-6''' (δ_{H} 7.73) to C-1'' (δ_{C} 205.2) and that of H-2''' (δ_{H} 3.24), H-3''' (δ_{H} 3.00), H-2'''/6''' (δ_{H} 6.89), and H-3'''/5''' (δ_{H} 7.23) to C-1''' (δ_{C} 157.6) allowed the assignment of the dihydrochalcone moiety possessing rings A and B. In addition, the HMBC cross-peaks of H-3 (δ_{H} 7.72), H-2 (δ_{H} 7.57), and H-6' (δ_{H} 7.91) to C-1 (δ_{C} 193.3) and that of H-5'' (δ_{H} 7.00) to C-1'' (δ_{C} 128.7) and C-3'' (δ_{C} 145.7) and H-6'' (δ_{H} 7.46) to C-3 (δ_{C} 144.9) allowed the assignment of the chalcone moiety possessing ring A₂ and ring B₂. The NOESY correlation of H-2'' (δ_{H} 7.31) to H-2'''/6''' (δ_{H} 6.89) corroborated the connectivity of the chalcone and dihydrochalcone moieties. Based on the above spectroscopic analyses, the new compound holstiichalcone III (5) was characterized as (E)-1-(2',4'-dihydroxyphenyl)-3-(3''-[4'''-{1'''-(2'''',4'''',dihydroxyphenyl)-5'''',oxopropyl}phenoxy]-4''-hydroxyphenyl)prop-2'-en-1-one. Similar compounds constituting chalcone and dihydrochalcone moieties have been reported from *Rhus pyroides*,⁷ *Luxemburgia octandra*,⁴⁴ and *Schinopsis brasiliensis*.⁴⁵

In addition to the five new compounds, 11 known flavonoid analogues, 6–16 (Figures S41–S84, Supporting Information), were isolated from the stem bark of *O. holstii*. Previously related secondary metabolites have also been reported from the Thymelaeaceae and Anacardaceae families.^{16,17,28,46–48,7,36,44,45,49} Such interfamily phytochemical similarities are indicative of phylogenetic relationships and imply that the plant families possess similar enzymatic systems.

The crude extract of the stem bark of *O. holstii* and its constituents were evaluated for antibacterial activity against *B. subtilis* (Gram-positive) and *E. coli* (Gram-negative), as well as for cytotoxicity against the MCF-7 human breast cancer cell line (Figures S85–S88, Table 5, Supporting Information). The crude extract exhibited significant activity against *B. subtilis* with an MIC value of 9.1 $\mu\text{g/mL}$. Holstiinone A (1) showed moderate antibacterial activity (MIC = 14.0 μM), whereas the other tested compounds showed no significant activity (MIC > 250 μM) against *B. subtilis*. Furthermore, none of the investigated compounds showed substantial antibacterial activity against *E. coli*. Antibacterial activity dose–response curves for compounds 1, 5, 6, 8, 9, and 14 against Gram-positive *B. subtilis* and that of compound 14 against Gram-negative *E. coli* are summarized in Figure S87 (Supporting Information). The compounds were also tested for cytotoxicity against the MCF-7 human breast cancer cell lines. Most of these did not show substantial cytotoxicity ($\text{EC}_{50} \geq 350 \mu\text{M}$), while the crude extract and lophirone F (14) exhibited moderate cytotoxicity with EC_{50} values of 11 $\mu\text{g/mL}$ and 24 μM , respectively (Figure S88, Supporting Information). Previous pharmacological studies have indicated that biflavonones and chalconoids possess antibacterial,¹⁸ antimalarial,^{6,50} anti-inflammatory,¹⁵ cytotoxic,⁴⁷ anti-HIV,⁵¹ and antifungal²⁸ activities, indicating their broad spectrum of biomedical potential, warranting further exploration of the genus *Ochna* and the Ochnaceae family in general for drug discovery. Antibacterial activity of compounds 1, 5, 6, 8, 9, and 14 against Gram-positive *B. subtilis* and 14 against Gram-negative *E. coli*, showing the EC_{50} , EC_{90} , and MIC values (in μM), is summarized in Table S5 (Supporting Information).

In conclusion, five new natural products (1–5) were isolated from the stem bark extract of *O. holstii* along with 11 known compounds. The biflavonones (1, 2, 6–10) and bichalconoids (3, 4, 5, 15, 16) isolated from this species are in line with the

richness of the Ochnaceae family in this type of compounds. The compounds also highlight the chemotaxonomic relationship of the Ochnaceae family with the Anacardaceae and Thymelaeaceae families. Holstiinone A (1) and lophirone F (14) exhibited moderate antibacterial activity against *B. subtilis* and showed weak cytotoxicity against MCF-7, respectively.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were determined using a 341 LC OROT polarimeter (589 nm, 24.0 °C). ECD experiments were performed on a Jasco model J-810, Rev.1.00, spectropolarimeter. UV spectra were obtained using CH₃OH as the solvent using a Shimadzu UV-1650PC UV/vis spectrophotometer. Infrared (IR) spectra were recorded on a PerkinElmer Spectrum FT-IR spectrometer using liquid samples. NMR spectra were acquired on either a Bruker Avance NEO 500 MHz (TXO cryogenic probe) or a 600 MHz (TCI cryogenic probe) spectrometer and were processed using the MestreNova (v14.0.0) software. Chemical shifts were referenced to the carbon and residual proton signals of the deuterated solvents (CD₃CN δ_{H} 1.94 and δ_{C} 118.26, methanol-*d*₄ δ_{H} 3.30 and δ_{C} 49.00, CDCl₃ δ_{H} 7.26 and δ_{C} 77.16) as internal standard. Assignments were based on ¹H, ¹³C, COSY, HSQC, HMBC, TOCSY, and NOESY NMR spectra. LC-ESIMS spectra were acquired on a Micromass GC-TOF micromass spectrometer (Micromass, Wythenshawe, Waters, Inc. UK), recorded on a direct inlet, with 70 eV ionization voltage. Elution was performed with a gradient mobile phase (H₂O–CH₃CN, 95:05–5:95, v/v) with a run time of 4–10 min. HRESIMS spectra were obtained with a Q-TOF-LC/MS spectrometer using a 2.1 × 30 mm 1.7 μm RPC₁₈ and H₂O–CH₃CN gradient (5:95–95:5 in 0.2% formic acid, v/v) at Sternhagen Analys Lab AB, Gothenburg, Sweden. Thin layer chromatography (TLC) was performed on silica gel 60 F₂₅₄ (Merck, Darmstadt, Germany) using precoated aluminum plates to monitor isolation processes. Various appropriate solvent systems were used to develop TLC plates, which were visualized under UV light (254 and 366 nm) and spraying with an anisaldehyde reagent (prepared by mixing 3.5 mL of 4-anisaldehyde with 2.5 mL of concentrated H₂SO₄, 4 mL of glacial HOAc, and 90 mL of CH₃OH) followed by heating (80–100 °C). Column chromatography (CC) was run on silica gel 60 (230–400 mesh). Gel filtration was performed on Sephadex LH-20 (GE Healthcare). Preparative reversed-phase HPLC was performed on an Interchim Ultra Performance Flash Purification (PF-430) system using Interchim v 5.1d.02 software and an RP-C₈ Kromasil column (250 mm × 25 mm, 5 μm).

Plant Material. The stem bark of *Ochna holstii* was collected in April 2017 from the Mkwagulo and Mtakayo clans' sacred forest graveyards (6°24'51.918" S; 38°40'19.308" E, altitude 78.80 m) in Fukayosi Village, Bagamoyo District, Pwani Region in Tanzania. The plant was identified and authenticated by F. M. Mbago, a senior taxonomist of the herbarium at the Botany Department of the University of Dar es Salaam, where a voucher specimen (FMM. 3809) was deposited.

Extraction and Isolation. The air-dried and ground stem bark (1.7 kg) of *O. holstii* was extracted with CH₃OH–CH₂Cl₂ (7:3, v/v) for 72 h at room temperature by percolation (3 × 3 L) to yield a dark brown crude extract (86 g) after evaporation in a rotary evaporator at 40 °C. A portion of the crude extract (63 g) was subjected to column chromatography over silica gel 60 (400 g), and gravity elution was performed with a mixture of isohexane containing increasing amounts of EtOAc (0–100%), followed by 5% CH₃OH in EtOAc. A total of 180 fractions were collected and combined to 20 fractions, F1–F20, on the basis of TLC analyses. F1 was subjected to a Sephadex LH-20 column (CH₃OH–CH₂Cl₂, 8:2) to afford white solids holstiinone B (2, 8 mg) and ouratein D¹⁵ (6, 6 mg). F2 was subjected to silica gel column chromatography (30% EtOAc in isohexane) followed by purification on Sephadex LH-20 (CH₃OH–CH₂Cl₂, 8:2) to give a pale yellow solid, holstiinone A (1, 6 mg). F3 and F4 were further combined and purified on Sephadex LH-20 (CH₃OH–CH₂Cl₂, 1:1) to afford isochamaejasmin A^{16,17} (7, 6 mg). Purification of F5 on

Sephadex LH-20 (CH₃OH) gave a yellow solid, calodenin B^{24,25} (16, 50 mg). F6 and F7 were further combined and purified by a Sephadex LH-20 column (CH₃OH–CH₂Cl₂, 1:1) followed by preparative HPLC (CH₃OH–MeCN, 85:15, for 40 min, with a flow rate of 4 mL/min) to afford holstiichalcone I (3, 12 mg) and flavumchalcone (13, 4 mg).²² F8 was purified with a Sephadex LH-20 column (CH₃OH) to afford a yellow solid, holstiichalcone II (4, 7 mg). F9 was subjected to silica gel column chromatography (EtOAc–CH₂Cl₂, 1:1) followed by repeated purification on Sephadex LH-20 (CH₃OH–CH₂Cl₂, 8:2) to give a yellow solid, holstiichalcone III (5, 5 mg). F17 and F18 were combined and purified on a Sephadex LH-20 column (CH₃OH–CH₂Cl₂, 1:1) followed by silica gel column chromatography (EtOAc–CH₂Cl₂, 1:1) to afford a yellow solid, lophirone F²³ (14, 40 mg), and dihydrocalodenin B³ (15, 14 mg). Purification of F10 on a Sephadex LH-20 column (CH₃OH) gave a white solid, campylospermonone A¹⁹ (9, 15 mg). Purification of F11 by preparative TLC (EtOAc–CH₂Cl₂, 30:70) afforded a white solid, 7,7'-di-O-methylchamaejasmin¹⁸ (8, 4 mg). F12 and F15 were combined and purified on a Sephadex LH-20 column (CH₃OH–CH₂Cl₂, 1:1) followed by further purification by preparative HPLC (H₂O–CH₃CN, 85:15, for 60 min at a flow rate of 4 mL/min) to give liquiritigeninyl-(1-3,II-3)-naringenin⁸ (10, 4 mg). F16 was purified by preparative TLC (CH₃OH–CH₂Cl₂, 5:95) to give a white solid, isoliquitigenin²⁰ (11, 4 mg), and a yellow solid, terminalionone²¹ (12, 6 mg).

Holstiinone A (1): pale white, amorphous solid; $[\alpha]_D^{24}$ –115 (c 0.013, CH₃OH); UV (CH₃OH) $\lambda_{\max}$ 330 and 262 nm; ECD (c 0.05, CH₃OH) $\lambda_{\max}$ ($\Delta\epsilon$) 360 (6.0), 300 (–45.0), 210 (–50.0); IR $\nu_{\max}$ 3396, 1604, 1516, 1453, cm^{–1}; ¹H and ¹³C NMR see Table 1; HRESIMS m/z 541.1487 [M + H]⁺ (calcd for C₃₁H₂₅O₉, 541.1499).

Holstiinone B (2): white solid; $[\alpha]_D^{24}$ –92 (c 0.013, CH₃OH); UV (CH₃OH) $\lambda_{\max}$ 340 and 258 nm; ECD (c 0.05, CH₃OH) $\lambda_{\max}$ ($\Delta\epsilon$) 360 (6.0), 300 (–45.0), 210 (–50.0); IR $\nu_{\max}$ 3320, 1698, 1598, 11515, cm^{–1}; ¹H and ¹³C NMR data see Table 1; HRESIMS m/z 569.1741 [M + H]⁺ (calcd for C₃₃H₂₉O₉, 569.1812).

Holstiichalcone I (3): yellow, amorphous solid; $[\alpha]_D^{24}$ –176.9 (c 0.23 CH₃OH); UV CH₃OH $\lambda_{\max}$ 340 and 264 nm; ECD (c 0.013, CH₃OH) $\lambda_{\max}$ ($\Delta\epsilon$) 224 (–47), 284 (–33), 361 (+4) nm; IR $\nu_{\max}$ 3305, 1700, 1598, 1513, cm^{–1}; ¹H and ¹³C NMR see Table 2; HRESIMS m/z 567.1634 [M + Na]⁺ (calcd for C₃₁H₂₈O₉, 567.1631).

Holstiichalcone II (4): yellow solid; $[\alpha]_D^{24}$ –21 (c 0.013, CH₃OH); UV CH₃OH $\lambda_{\max}$ 368, 340, and 284 nm; ECD (c 0.05, CH₃OH) $\lambda_{\max}$ ($\Delta\epsilon$) 436 (–7), 373 (–6), 320 (–6.0), 293 (4.0), 274 (–4), 253 (5.0), 230 (–18), 204 (20); IR $\nu_{\max}$ 3382, 1604, 1554, 1515, 1444, 1363 cm^{–1}; ¹H and ¹³C NMR see Table 3; HRESIMS m/z 511.1478 [M + H]⁺ (calcd for C₃₀H₂₃O₈, 511.1393).

Holstiichalcone III (5): yellow solid; UV CH₃OH $\lambda_{\max}$ 336, 320, and 264 nm; IR $\nu_{\max}$ 3326, 1709, 1506, 1515 cm^{–1}; ¹H and ¹³C NMR data see Table 4; HRESIMS m/z 513.1575 [M + H]⁺ (calcd for C₃₀H₂₅O₈, 513.1549).

Antibacterial Assays. The antibacterial activity of the crude extract and the isolated compounds was evaluated against two bacterial strains, *B. subtilis* (Gram-positive) and *E. coli* (Gram-negative). Each test sample was first dissolved at 10 mg/mL in 100% DMSO and stored at –20 °C. The bacterial strains were cultured by standard procedures as previously reported.⁵² Briefly, a culture of bacterial cells was grown to optical density (OD) 600_{nm} = 0.5, then diluted (10X) with prewarmed medium. The substances to be tested were added to culture medium for a final concentration of 30 µg/mL, each at 100 µL in a 96-well microtiter plate, then incubated for 24 h at 37 °C without agitation. The cell viability was measured by a resazurin-based assay as previously described.⁵³ Subsequently, 12 µL of AlamarBlue solution was added to each well and incubated at 37 °C continuously for 1 h. The fluorescence was measured using a POLARstar Omega microplate reader from BMG Labtech with the excitation filter set to 544 nm and emission filter to 590 nm. Ampicillin, a standard antibiotic, was used as a positive control, while DMSO, the solvent used to dissolve the test substances, was used as a negative control. In order to prevent fluorescence bleed-through from the resorufin dye, the assays performed in 384-well plates were set up

with an empty well in-between. Certain substances were checked for possible fluorescent or fluorescence-quenching properties by adding the substance after the culture was incubated with the resazurin dye only. The results were then compared to the fluorescence values obtained initially without the addition of the compound/extract. All experiments were performed in triplicate. The test compound/extracts that caused a reduction of fluorescence by at least 50% relative to the solvent control in any of the species were followed up by additional tests to accurately determine antibacterial activity.

Cytotoxicity Assay. MCF-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and kept in exponential growth as described previously.⁵⁴ Briefly, cells were seeded in 96-well plates at a density of 5 × 10³ per well allowing continued exponential growth overnight.⁵⁵ Stock solutions of the test sample dissolved in DMSO were added for a final concentration of 0.3% v/v of the solvent in the culture medium. Cell viability was assayed using PrestoBlue cell viability reagent (ThermoFisher) according to the manufacturer's instructions following 24 h of incubation in the presence of the compounds/extracts. A POLARstar Omega plate reader (BMG Lab Tech) was used to measure resorufin fluorescence at 544 nm excitation/590 nm emission. The cells treated only with DMSO were used as a negative control.⁵⁵ Three independent replicate experiments were used to calculate the EC₅₀ value for each compound/extract using 2-fold serial dilution.

■ ASSOCIATED CONTENT

Supporting Information

The original NMR spectra for all compounds, along with the corresponding NMReDATA⁵⁶ for the new compounds 1–5, are available free of charge on Zenodo as DOI: 10.5281/zenodo.4030263. The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jnatprod.0c01017>.

NMR and MS data for the isolated compounds (1–16) (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Mate Erdelyi – Department of Chemistry – BMC, Uppsala University, SE-751 23 Uppsala, Sweden; orcid.org/0000-0003-0359-5970; Phone: +46-72-9999166; Email: mate.erdelyi@kemi.uu.se

Stephen S. Nyandoro – Chemistry Department, College of Natural and Applied Sciences, University of Dar es Salaam, Dar es Salaam, Tanzania; Phone: +255-754-206560; Email: nyandoro@udsm.ac.tz

Authors

Thobias M. Kalenga – Chemistry Department, College of Natural and Applied Sciences, University of Dar es Salaam, Dar es Salaam, Tanzania

Monica M. Ndoile – Chemistry Department, College of Natural and Applied Sciences, University of Dar es Salaam, Dar es Salaam, Tanzania

Yoseph Atilaw – Department of Chemistry – BMC, Uppsala University, SE-751 23 Uppsala, Sweden

Pieter J. Gilissen – Institute for Molecules and Materials, Radboud University, 6525 AJ Nijmegen, The Netherlands

Joan J. E. Munissi – Chemistry Department, College of Natural and Applied Sciences, University of Dar es Salaam, Dar es Salaam, Tanzania

Anastasia Rudenko – Department of Chemistry and Molecular Biology, University of Gothenburg, SE-405 30 Gothenburg, Sweden; Centre for Antibiotic Resistance

Research (CARE) at the University of Gothenburg, 405 30 Gothenburg, Sweden

Catarina Bourgard – Department of Chemistry and Molecular Biology, University of Gothenburg, SE-405 30 Gothenburg, Sweden; Centre for Antibiotic Resistance Research (CARE) at the University of Gothenburg, 405 30 Gothenburg, Sweden

Per Sunnerhagen – Department of Chemistry and Molecular Biology, University of Gothenburg, SE-405 30 Gothenburg, Sweden; Centre for Antibiotic Resistance Research (CARE) at the University of Gothenburg, 405 30 Gothenburg, Sweden;

orcid.org/0000-0002-0967-8729

Complete contact information is available at:

<https://pubs.acs.org/10.1021/acs.jnatprod.0c01017>

Notes

The authors declare no competing financial interest.

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