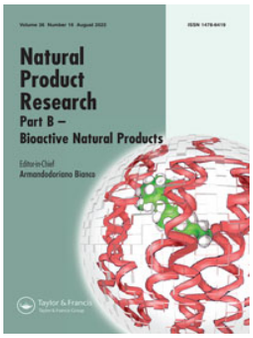

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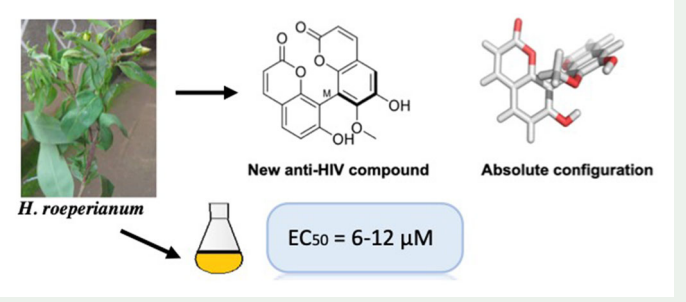
Bichromonol, a dimeric coumarin with anti-HIV activity from the stem bark of *Hypericum roeperianum*

Serge A. T. Fobofou[a,b], Katrin Franke[a], Wolfgang Brandt[a], Aldo Manzin[c], Silvia Madeddu[c], Gabriele Serreli[c], Giuseppina Sanna[c#] and Ludger A. Wessjohann[a#]

[a]Department of Bioorganic Chemistry, Leibniz Institute of Plant Biochemistry, Halle (Saale), Germany; [b]Institute of Pharmaceutical Biology, Technische Universität Braunschweig, Braunschweig, Germany; [c]Department of Biomedical Sciences, University of Cagliari, Monserrato (CA), Italy

ABSTRACT

Infectious diseases caused by viruses like HIV and SARS-COV-2 (COVID-19) pose serious public health threats. In search for new antiviral small molecules from chemically underexplored *Hypericum* species, a previously undescribed atropisomeric C8-C8′ linked dimeric coumarin named bichromonol (**1**) was isolated from the stem bark of *Hypericum roeperianum*. The structure was elucidated by MS data and NMR spectroscopy. The absolute configuration at the biaryl axis was determined by comparing the experimental ECD spectrum with those calculated for the respective atropisomers. Bichromonol was tested in cell-based assays for cytotoxicity against MT-4 ($CC_{50} = 54\,\mu M$) cells and anti-HIV activity in infected MT-4 cells. It exhibits significant activity at $EC_{50} = 6.6–12.0\,\mu M$ against HIV-1 wild type and its clinically relevant mutant strains. Especially, against the resistant variants A17 and EFV^R, bichromonol is more effective than the commercial drug nevirapine and might thus have potential to serve as a new anti-HIV lead.



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CONTACT Serge A. T. Fobofou s.fobofou@tu-bs.de; Giuseppina Sanna g.sanna@unica.it
#These authors share senior authorship.

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1. Introduction

Viral infections as those caused by the human immunodeficiency (HIV) or the new severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) are among the major diseases enumerated by the World Health Organization (WHO) on its recent list of ten global health threats. There is a need for new antiviral drugs (UNAIDS Report 2020; Adamson et al. 2021).

Members of the *Hypericum* genus are well known for their biological activities against a range of diseases including depression, viral and microbial infections, skin wounds, gastrointestinal disorders, and intestinal worms (Toniolo et al. 2014; Mandrone et al. 2015; Caprioli et al. 2016; Fobofou et al. 2016; Venditti and Bianco 2018). During our collection of Cameroonian *Hypericum* species for bioactive compound discovery, we heard from the local population that the plants are traditionally used against mental disorders and by AIDS patients. This raised our curiosity, and we decided to evaluate their anti-HIV properties to determine whether *Hypericum* species can really help HIV-infected people and ultimately might hint toward a new anti-HIV lead compound from a medicinal plant (Fobofou et al. 2015). *Hypericum roeperianum* Schimp. ex A. Rich is one of the plant species that have recently been the focus of several investigations (Fobofou et al. 2014; 2015; Guefack et al. 2020; Damen et al. 2021; Demgne et al. 2021; Damen et al. 2022). Herein, the isolation, characterization, and anti-HIV activity of bichromonol (**1**) from the stem bark of *H. roeperianum* is reported. Previous studies on this plant (including our own investigations) reported the isolation of xanthones, polyketides, coumarins, and acylphloroglucinol derivatives as well as their cytotoxic, antibacterial, and anthelmintic activities (Fobofou et al. 2014; 2015; Guefack et al. 2020; Damen et al. 2021; Demgne et al. 2021; Damen et al. 2022). However, none of these compounds were found or described to exhibit anti-HIV activity. In our studies on the leaves of *H. roeperianum*, we could not find anti-HIV activity for the leaves extract and isolated constituents (prenylated acylphloroglucinol derivatives) at concentrations below cytotoxicity for exponentially growing MT-4 cells (Fobofou et al. 2015).

2. Results and discussions

The $CHCl_3$ extract obtained from the stem bark of *H. roeperianum* exhibits significant anti-HIV-1_{IIIB} activity (EC_{50}= 0.4 μg/ml) associated with moderate cytotoxicity (CC_{50}= 6 μg/ml) against MT-4 cells. MT-4 cells represent a HTLV-1-transformed human T-cell line sensitive to HIV infection. A portion of the $CHCl_3$ extract (2.7 g) was subjected to repeated column chromatography to afford bichromonol (**1**) (1.4 mg, Rf = 0.34 in CH_2Cl_2-MeOH (90:10)).

Bichromonol (**1**) was isolated as an optically active, yellow, and amorphous substance, $[\alpha]^{25}_D$ −81.7 (*c* 6.0 mM, MeOH). The HR-ESI-FTMS indicates a deprotonated molecule at *m/z* 351.0525 ($[M\text{-}H]^-$) consistent with the molecular formula $C_{19}H_{11}O_7^-$. The MS^2 spectrum of the $[M\text{-}H]^-$ ion shows a base peak ion resulting from the loss of MeOH. Further fragmentations are mainly characterized by successive losses of CO_2 and CO as previously described for MS/MS data of coumarin derivatives (Figure S4) (Heinke et al. 2012; Fobofou et al. 2014; Sun et al. 2020). The IR spectrum exhibits

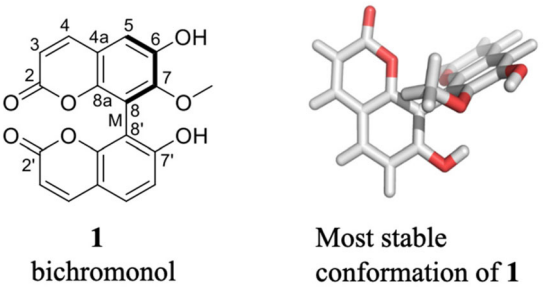

Figure 1. Structure of bichromonol (**1**) from *Hypericum roeperianum* and its most stable conformation with the S_a (or M) atropisomer configuration (dihedral angles 8a-8-8′-8á = −67.9°, 8-7-O-C = −71.4°) constructed using MOE 2013.0801 (see Supplemental Information).

absorption bands of hydroxy (3353 cm^{-1}) and carbonyl (1698 cm^{-1}) groups. The ^1H NMR spectrum (Table S1) shows the presence of eight proton signals including one methoxyl group (δ_H 3.69, 7-OMe). The observation of four distinct doublets at δ_H 6.31 (1H, *d*, *J* = 9.7 Hz, H-3), δ_H 7.90 (1H, *d*, *J* = 9.7 Hz, H-4), δ_H 6.17 (1H, *d*, *J* = 9.7 Hz, H-3′), δ_H 7.93 (1H, *d*, *J* = 9.7 Hz, H-4′) corresponding to two coumarin units (Liu et al. 2007), suggests that **1** is an unsymmetrical coumarin dimer. The ^1H NMR spectrum (Table S1) also shows two signals at δ_H 6.95 (1H, *d*, *J* = 8.3 Hz, H-6′) and δ_H 7.57 (1H, *d*, *J* = 8.3 Hz, H-5′), characteristic of aromatic protons of a 1,2,3,4-tetrasubstituted benzene ring as well as one signal at δ_H 7.14 (1H, *s*, H-5) attributable to the proton of a 1,2,3,4,5-pentasubstituted benzene nucleus. The ^1H and ^{13}C NMR (Table S1) signals as well as the connectivity between the two coumarin units through a C-8/C-8′ biaryl axis were assigned unambiguously using DEPT, ROESY, COSY, HSQC, and HMBC spectroscopy. The ^{13}C NMR spectrum reveals nineteen carbon signals including two carbonyl groups (δ_C 163.8, C-2; δ_C 163.7, C-2′), one methoxy group (δ_C 61.1, 7-OMe) as well as five oxygenated aromatic carbon signals (δ_C 148.8, C-6; δ_C 151.9, C-7; δ_C 147.6, C-8a; δ_C 161.6, C-7′; δ_C 155.0; C-8á). The COSY spectrum reveals correlations between H-3 and H-4, H-3′ and H-4′ as well as between H-5′ and H-6′. Key interactions are observed in the ROESY spectrum (Figure S15) between H-4 and H-3/H-5, H-4′ and H-3′/H-5′ as well as between H-5′ and H-6′. In addition, no ROESY correlation of the OMe group could be detected. These findings indicate the attachment of the methoxyl group to C-7 and the connection of the two coumarin units via C-8 and C-8′. This was further supported by the HMBC spectrum which reveals correlations from 7-OMe to C-7, from H-5 to C-4, C-8a, C-7, and C-6, from H-5′ to C-4′, C-8á, and C-7′ as well as from H-6′ to C-4á and C-8′ (Figure S15). Based on the above spectroscopic data, the structure of compound **1** (Figure 1) was characterized as 6,7′-dihydroxy-7-methoxy-8,8′-biscoumarin and trivially named bichromonol (derived from bis-2*H*-chromen-2-one alcohol, **1**).

Only a few simple coumarin derivatives were previously reported from the genus *Hypericum* (Ang'edu et al. 1999; Nedialkov et al. 2007). However, *H. roeperianum* is the sole producer of dimeric coumarins so far described from this genus (Fobofou et al. 2014). Furthermore, bichromonol (**1**) belongs to C8-C8′ linked dimeric coumarins and is reported herein as a new compound for the first time. The absolute configuration of bichromonol (**1**, Figure 1) was determined by comparing its experimental ECD spectrum with those calculated by quantum mechanical methods (see details in supplemental

Table 1. Cytotoxicity and antiviral activity of bichromonol (1) against HIV-1 and its NNRTI-(N119, A17, EFV^R) and NRTI-(AZT^R, MDR) resistant variants.

Bioassay (μM)		Bichromonol (1)	Nevirapine[c]	Azidothymidine[c]	Efavirenz[c]
Cytotoxicity (CC_{50})[a]	MT-4	54.0	>100	>100	40
Anti-HIV activity against HIV-1_{IIIB} and its resistant variants (EC_{50})[b]	HIV-1_{IIIB}	**8.7**	0.08	0.02	0.002
	N119 (Y1811C)	**6.7**	6.3	0.02	0.03
	A17 (K103N, Y181C)	**8.3**	80	0.01	0.08
	EFV^R (K103R, V179D, P225H)	**6.6**	100	0.02	12.0
	AZT^R (67N, 70R, 215F, 219Q)	**6.6**	0.07	0.3	0.003
	MDR (74V, 41L, 106A, 215Y)	**12.0**	5.0	0.08	0.01

[a]Cytotoxicity concentration (CC): Compound concentration (μM) required to reduce the viability of mock-infected MT-4 cells by 50%, as determined by the MTT method.
[b]Effective concentration (EC): Compound concentration (μM) required to achieve 50% protection of MT-4 cells from HIV-1 induced cytopathogenicity, as determined by the MTT method.
[c]Efavirenz, azidothymidine, and nevirapine are the reference anti-HIV-1 drugs. Data represent mean values ± SD for three independent determinations. Variation among replicate samples was less than 15%.

information) for both configurations S_a (or M) and R_a (or P) for 30 singlet and triplet states. The comparison of the calculated with the experimental ECD spectrum shows that the compound is preferentially populated in the S_a-configuration (Figure S3). In general, dimeric coumarins were reported as racemic mixtures or optically pure compounds with either M or P absolute configuration (Baba et al. 1990; Hussain et al. 2012).

The $CHCl_3$ extract and bichromonol (**1**) from the stem bark of *H. roeperianum* were tested in a cell-based assay against the human immunodeficiency virus type-1 (HIV-1), using efavirenz as reference inhibitor. The cytotoxicity against the uninfected MT-4 cells was evaluated in parallel with the antiviral activity. The $CHCl_3$ extract displays substantial anti-HIV-1_{IIIB} (EC_{50} = 0.4 μg/ml) activity associated with a moderate cytotoxicity (CC_{50} = 6 μg/ml) against the uninfected MT-4 cells. As reported in Table 1, bichromonol (**1**) shows a relevant activity (EC_{50} = 8.7 μM) associated with a lower cytotoxicity (CC_{50} = 54 μM). In contrast, prenylated acylphloroglucinol derivatives obtained from the same species were not active against HIV-1 (Fobofou et al. 2015). Since a critical issue in the long-term clinical management of HIV diseases is the development of drug resistance, bichromonol (**1**) was evaluated against a panel of viruses possessing mutations that confer selective resistance either to nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) or non-nucleoside reverse transcriptase inhibitors (NNRTIs) that often appear during highly active antiretroviral therapy (HAART), reducing its effectiveness. Bichromonol (**1**) shows significant activity with EC_{50} values ranging from 6.6 to 12.0 μM on all the clinically relevant HIV-1 mutant strains tested, as depicted in Table 1. It is noteworthy to mention that its activity on some of the resistant strains is higher than that of the reference anti-HIV drug nevirapine (Table 1). The activity exhibited by the new compound (**1**) demonstrates its potential as anti-HIV drug and the role of natural products to provide unique chemical entities in drug discovery programs. Recently, also ethanolic extracts of *Hypericum hircinium* and its components betulinic acid and 5,7,3′,5′-tetrahydroxyflavanone 7-*O*-glucoside were reported as active against HIV (Esposito et al. 2013; Ornano et al. 2018).

The safety profile of Bichromonol was analyzed through transepithelial electrical resistance (TEER) assay (Figure S18). It was observed that the tested concentration (30 μM) did not affect the TEER over the time of the experiment (144 h), keeping values like those of

the untreated cells. Furthermore, the mode of action of **1** was preliminarily assessed in an enzymatic assay aimed at evaluating its capability to inhibit HIV-1 reverse transcriptase (RT) activity in vitro. Interestingly, unlike Efavirenz, a known RT inhibitor (NNRTI), the compound was inactive against RT (Figure S19), suggesting a different mode of action.

Studies based on various coumarins from plant sources and their synthetic analogs indicate that some of them behave like potent non-nucleoside RT-inhibitors and others as inhibitors of HIV-integrase or HIV-protease (Kostova 2006). However, the major approved anti-HIV drugs fall into five categories: the nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), the non-nucleoside reverse transcriptase inhibitors (NNRTIs), the protease inhibitors (PIs), entry inhibitors, and integrase inhibitors (Zhuang et al. 2020). Studies to identify the mode of action for dimeric coumarins as new anti-HIV-leads are ongoing. In addition, bichromonol (**1**) was evaluated for antibacterial activity against representative human pathogenic Gram negative (*Escherichia coli*), and Gram positive (*Staphylococcus aureus,* and *Enterococcus faecalis*) bacteria. Ciprofloxacin was used as reference compound. The tested compound does not show significant inhibitory activity (MIC $>$ 1 mg/L). The lack of antibacterial activities against a panel of microorganisms might support the specific inhibitory effect of bichromonol as antiviral agent.

3. Experimental (see supporting information)

Supporting Information. Plant material, extraction and isolation, bioassay and ECD calculation methods, 1 D and 2 D NMR spectra, MS/MS fragmentation behavior for compound **1**. This material is available free of charge via the Internet.

Bichromonol (1), 6,7′-dihydroxy-7-methoxy-8,8′-biscoumarin: Yellow amorphous powder; $[\alpha]^{25}{}_{D}$ −81.7 (*c* 6 mM, MeOH); UV (MeOH), λ_{max} (log ε): 287 (1.49), 321 (1.51) nm; IR (ATR) υ_{max} (cm^{-1}): 3353, 2940, 2937, 1698, 1599, 1503, 1452, 1426, 1398, 1316, 1240, 1144, 1121, 1018, 834, 759; ^{1}H NMR (600 MHz, CD_3OD) ppm: 7.93 (1H, *d* *J* = 9.65 Hz, H-4′), 7.90 (1H, *d* *J* = 9.65 Hz, H-4), 7.57 (1H, *d* *J* = 8.33 Hz, H-5′), 7.14 (1H, *s*, H-5), 6.95 (1H, *d* *J* = 8.33 Hz, H-6′), 6.31 (1H, *d* *J* = 9.65 Hz, H-3), 6.17 (1H, *d* *J* = 9.65 Hz, H-3′), 3.69 (3H, s, 7-OMe), see Table S1; ^{13}C NMR (150 MHz, CD_3OD) ppm: 163.7 (C-2′), 163.3 (C-2), 161.6 (C-7′), 155.0 (C-8a′), 151.9 (C-7), 148.8 (C-6), 147.6. (C-8a), 146.5 (C-4′), 145.9 (C-4), 130.5 (C-5′), 116.6 (C-4a), 116.2 (C-8), 115.1 (C-3), 114.6 (C-5), 114.4 (C-6′), 113.1 (C-4a′), 112.0 (C-3′), 108.9 (C-8′), 61.1 (7-OMe), see Table S1; negative ion ESI-FTMS [*m/z* (rel. int., %)]: [M-H]$^-$ at *m/z* 351.0525 (calcd. for $C_{19}H_{11}O_7^-$, 351.0510). MS^2 [*m/z* 351 (48)]: *m/z* 319.0253 [M-MeOH]$^-$ (100, calcd for $C_{18}H_7O_6^-$, 319.0248). MS^3 [*m/z* 319 (100)]: *m/z* 291.0305 [M-MeOH-CO]$^-$ (100, calcd for $C_{17}H_7O_5^-$, 291.0299), 275.0357 [M-MeOH-CO_2]$^-$ (16, calcd for $C_{17}H_7O_4^-$, 275.0305). MS^4 [*m/z* 291 (100)]: *m/z*263.0357 [M-MeOH-CO-CO]$^-$ (100, calcd for $C_{16}H_7O_4^-$, 263.0305), 247.0400 [M-MeOH-CO-CO_2]$^-$ (14, calcd for $C_{16}H_7O_3^-$, 247.0401).MS^5 [*m/z* 263 (100)]: *m/z* 235.0409 [M-MeOH-CO-CO-CO]$^-$ (100, calcd for $C_{15}H_7O_3^-$, 235.0401).

4. Conclusion

Finally, the significant anti-HIV-1 activity of *H. roeperianum* stem bark extract supports its use in Cameroonian traditional medicine by AIDS patients. The compound

bichromonol (**1**) was isolated as active principle and might provide a new anti-HIV lead. However, further studies are still required to produce derivatives with improved therapeutic index, to evaluate its toxicity, pharmacodynamics, andelucidate its mode of action.

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Disclosure statement

The authors declare that they have no conflict of interest.

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ORCID

Wolfgang Brandt http://orcid.org/0000-0002-0825-1491

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