Structure-Activity Relationship Study of Biflavonoids on the Dengue Virus Polymerase DENV-NS5 RdRp

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Key words
- biflavonoids
- *Dacrydium araucarioides*
- *Dacrydium balansae*
- Podocarpaceae
- antiviral
- Dengue virus
- NS5 RdRp
- SAR study

Abstract

Dengue virus is the world's most prevalent human pathogenic arbovirus. There is currently no treatment or vaccine, and solutions are urgently needed. We previously demonstrated that biflavonoids from *Dacrydium balansae*, an endemic gymnosperm from New Caledonia, are potent inhibitors of the Dengue virus NS5 RNA-dependent RNA polymerase. Herein we describe the structure-activity relationship study of 23 compounds: biflavonoids from *D. balansae* (**1**–**4**) and from *D. araucarioides* (**5**–**10**), hexamethyl-amentoflavone (**11**), cupressuflavone (**12**), and apigenin derivatives (**13**–**23**). We conclude that 1) over the four different biflavonoid skeletons tested, amentoflavone (**1**) and robustaflavone (**5**) are the most promising ones for antidengue drug development, 2) the number and position of methyl groups on the biflavonoid moiety modulate their inhibition of Dengue virus NS5 RNA-dependent RNA polymerase, and 3) the degree of oxygenation of flavonoid monomers influences their antidengue potential. Sotetsuflavone (**8**), with an $IC_{50} = 0.16\,\mu M$, is the most active compound of this series and is the strongest inhibitor of the Dengue virus NS5 RNA-dependent RNA polymerase described in the literature.

Abbreviations

NC:	New Caledonia
SAR:	structure-activity relationship
DENV:	Dengue virus
BVDV:	bovine viral diarrhea virus
RdRp:	RNA-dependent RNA polymerase
DMF:	dimethylformamide
MeI:	methyl iodide

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

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Bibliography
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Introduction

Since a few decades DENV has become the most widespread and prevalent human pathogenic arbovirus, and no treatment or vaccine is available yet [1]. For these reasons, the research for a vaccine or chemotherapy is currently a priority for public health. The non structural protein NS5, which is essential for virus replication [2] and highly conserved between the four virus serotypes of the virus [3], is an appropriate target for the research of non cytotoxic antidengue compounds [4], particularly because there is no enzyme with RNA-dependent RNA polymerase activity in humans [5]. In a previous study, the bioguided fractionation of the leaf extract obtained from *Dacrydium balansae* (Podocarpaceae) led to the identification of three biflavonoids derived from amentoflavone (**1**–**3**) with high antidengue potential [6,7]. Herein we present an extended study of the DENV-NS5 RdRp inhibitory activity of biflavonoids. Among the tested biflavonoids, six were isolated from *D. araucarioides* (Podocarpaceae), another endemic gymnosperm from New Caledonia (**5**–**10**), one was obtained by hemisynthesis (**11**), and one was commercially purchased (**12**). Apigenin (**13**) and ten related flavonoid monomers (**14**–**23**) were also tested on the DENV-NS5 RdRp.

Results and Discussion

Four different biflavonoid skeletons (**1, 4, 5**, and **12**), characterized by the bond linking the two apigenin units, were tested. All biflavonoids iso-

lated from *Dacrydium* spp. (**1, 4**, and **5**) showed strong inhibition of the DENV-NS5 RdRp (IC_{50} comprised between 0.26 and 1.3 µM). Hinokiflavone (**4**), with an IC_{50} = 0.26 µM, is the most potent DENV polymerase inhibitor, but it was cytotoxic on COS and BHK cells [6]. Robustaflavone (**5**) also strongly inhibits the DENV-NS5 RdRp (CI_{50} = 0.33 µM) and is not cytotoxic according to results exposed in the literature [12]. This compound was also described as a potent HBV replication inhibitor [13]. Amentoflavone (**1**) was previously shown to be a strong and specific noncytotoxic inhibitor of the DENV-NS5 RdRp [6]. Cupressuflavone (**12**) was significantly less active (CI_{50} > 5 µM).

Comparison of the DENV-NS5 RdRp inhibitory activities of amentoflavone (**1**) and its methylated derivatives **2, 3**, and **6**–**11** (see ◉ **Table 1**) shows that the position and number of methoxy groups modulate the biological activity of the compounds. Thus, all monomethylated compounds (**2, 6, 7**, and **8**) displayed a lower IC_{50} on the DENV polymerase (respectively, 0.75, 0.46, 0.64, and 0.16 µM) than amentoflavone (**1**) (IC_{50} = 1.3 µM). The presence of two or three methoxy groups at C-7 and/or C-4 positions of both apigenin units (**3, 9**, and **10**) appears to slightly decrease the activity (IC_{50} comprised between 1.0 to 3.1 µM). Methylations in positions 7 and 7″ on the amentoflavone skeleton seem to be the most favorable to inhibit the DENV-NS5 activity, since compounds **6** and **8** were more active than **2** and **7**. These slight modulations of the activity by the number and position of methoxy groups could be attributed to the modification of the polarity and of the capacity to establish hydrogen bonds with DENV polymerase as suggested in other studies [14]. On the contrary, methylation of both 5-OH groups clearly decreases the inhibitory activity on the DENV-RdRp, since compound **11** displayed an IC_{50} equal to 50 µM probably due to steric and electronic modifications of **11** vs. other amentoflavone derivatives, related to the lack of the well-known hydrogen bond between the 5-OH group and carbonyl function in **11**.

In order to extend our comprehension of the SAR of flavonoids on the DENV-NS5 RdRp, apigenin (**13**) and 10 related flavonoids (**14**–**23**) were tested on the DENV polymerase. These compounds only weakly inhibited DENV-NS5, justifying that their inhibitory activities are, for most of them, only described at the single concentration of 50 µM. Again, the number and position of methylations on the genin influenced the ability of flavonoids to inhibit the DENV-NS5 RdRp. However, dimethoxylation was here the most favorable since the 7,4′-dimethylapigenin (**16**) was the strongest inhibitor (92% inhibition at 50 µM) among the tested flavonoids monomers. The results of polymerase inhibition for other related flavonoid monomers suggest that the degree and the position of oxygenation also influence their inhibitory activity. Thus, quercetin (**21**), rhamnetin (**22**), and kaempferol (**23**), which inhibit more than 90% of the enzyme activity at 50 µM, were significantly more active than apigenin (**13**), pinocembrin (**18**), naringenin (**19**), and galangin (**20**). Compounds **21, 22**, and **23** belong to the 3-flavonol subtype, such as galangin (**20**). They are unsubstituted on the lateral ring and present a weaker activity than **20**. These results suggest that the simultaneous presence of oxygenated substituents on the three rings of the flavone increases the inhibitory activity on the DENV-NS5 RdRp.

The SAR study of biflavonoids and related flavonoid monomers on the DENV-NS5 RdRp confirms that the biflavonoid skeleton is a promising template for the design of an antidengue compound. Their antidengue potential is modulated by the biflavonoid skeleton and by the number and position of methylation groups. We also correlated the modulation of antiviral potential with the oxygenated substitution patterns. Robustaflavone (**5**), characterized by a C3′-C6″ linkage, is newly described as a strong inhibitor of the DENV-NS5 RdRp (IC_{50} = 0.33 µM). Sotetsuflavone (IC_{50}= 0.16 µM), the 7″-O-methylamentoflavone (**8**), is the strongest inhibitor of the DENV-NS5 RdRp among the tested compounds. It is also one of the strongest nonnucleotide inhibitors of the DENV-NS5 RdRp described in the literature so far [7, 15, 16]. Inhibition assays of robustaflavone and amentoflavone derivatives on infected cells should be performed to complete the evaluation of the antidengue potential of these compounds.

Materials and Methods

Plant material and crude extracts

Leaves of *Dacrydium araucarioides* were harvested in Prony in April 2009 (22°16′02″S; 166°50′07″E), in the South province of New Caledonia (Province Sud authorization N°10919–2009). A voucher specimen (reference: Cou 11), identified by Dr. Jérome Munzinger (IRD), was deposited at the herbarium of the IRD center of Nouméa.

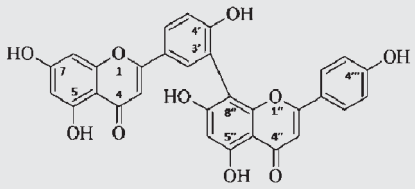
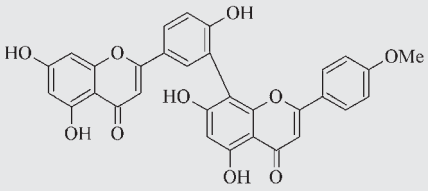
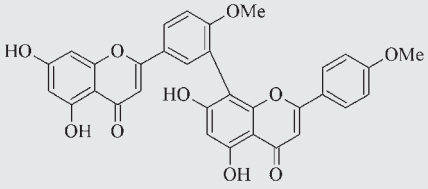
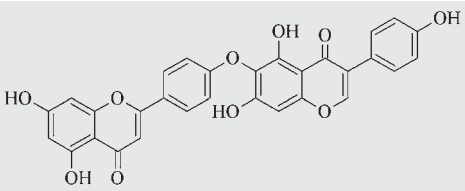
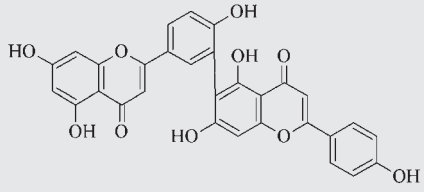
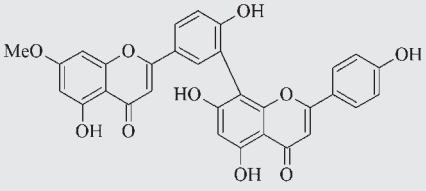
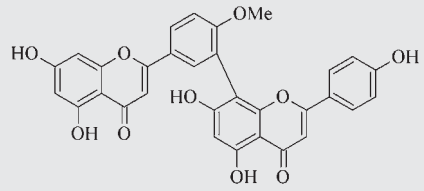
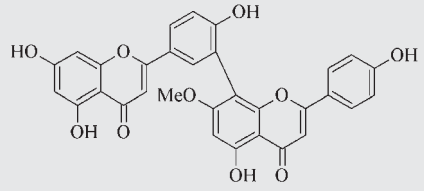
General experimental procedures

Cupressuflavone (**12**), apigenin (**13**), pinocembrin (**18**), naringenin (**19**), galangin (**20**), quercetin (**21**), rhamnetin (**22**), and kaempferol (**23**) were purchased from Extrasynthèse. The structure of all isolated compounds from *D. araucarioides* was determined by NMR and HRMS data analysis and by comparison with data from the literature [8–10]. Spectral data are available in the Supporting Information. 1D and 2D NMR spectra were recorded on a Bruker AM-300 and Bruker AM-400 instruments, at room temperature. APCI and ESIMS spectra were recorded on an Agilent MSD G1946D spectrometer and HRESIMS spectra on a Bruker MicroTOF Q-II spectrometer. Copies of the original spectra are available from the corresponding author. Analytical HPLC-UV were performed on an Agilent HP1100 instrument equipped with a Waters Sunfire C18 column (4.6 × 150 mm, 5 µm), at a flow rate of 1 mL/min. Preparative HPLC was performed on a Waters Deltaprep equipped with a sunfire RP-18 column (19 × 250 mm, 10 µm; Waters) with a flow rate of 17 mL/min. The purity of all tested compounds (> 95%) was verified by HPLC-UV/MS. All chemical structures are given in ◉ **Table 1**.

Compound isolation

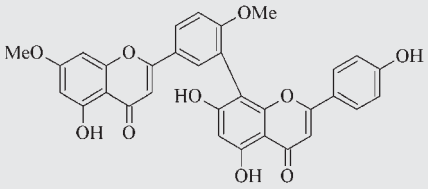
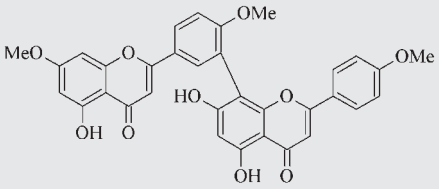
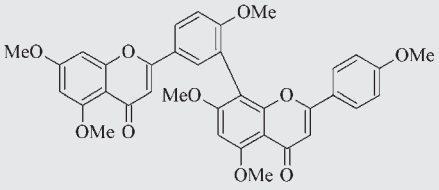
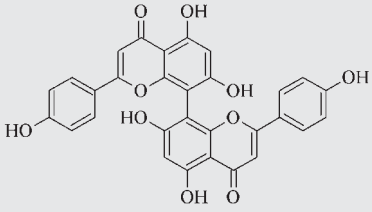
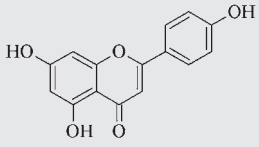
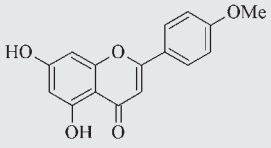
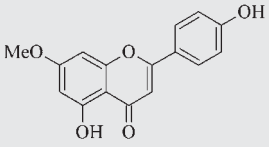
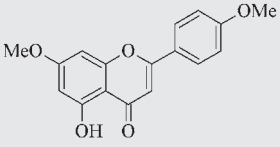
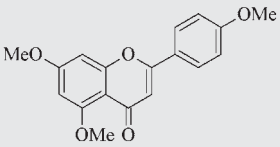
A crude extract was obtained from maceration of the 1200 g dried leaf powder from *D. araucarioides* in methanol under agitation and sonication at room temperature (3 × 4 L, 6 h each). The methanolic extract was dried and dissolved in 1 L of MeOH 80% to be successively extracted by C_6H_{12} (3 × 1 L) and CH_2Cl_2 (3 × 1 L). The methylene chloride fraction was reduced to 1 L and washed with water (2 × 1 L) to obtain the total biflavonoid extract (20 g). This extract was then subjected to silica gel chromatography (63–200 µm; 5 × 60 cm; Merck) eluted with a step gradient of CH_2Cl_2/MeOH (90 : 10; 75 : 25 and 0 : 100) to obtain 10 fractions (F1–F10). F3 (300 mg) was then fractionated by Sephadex LH20 chromatography (2.5 × 40 cm; Sigma-Aldrich) with CH_2Cl_2/MeOH 3 : 1 to obtain 5 fractions (F3.1–F3.5). F3.3 (40 mg) was finally separated by preparative HPLC on an RP-18 column eluted with an isocratic mix of TFA(0.5%)/MeOH 22 : 78 to obtain **9** (14.9 mg) and **10** (3.7 mg). F5 (1.8 g) was fractionated by silica gel chromatography (63–200 µm; 2.5 × 30 cm; Merck) with a step gradient of CH_2Cl_2/MeOH (92 : 8; 75 : 25 and 0 : 100) to obtain 8

Table 1 Inhibitory activity of biflavonoids and flavonoid monomers against the DENV-NS5 RdRp. Percentage inhibition of Dengue 2 NS5 RNA-dependent RNA polymerase (RdRp) by isolated compounds from *D. balansae* at 50 µg/mL and IC_{50} of DENV-NS5 RdRp (in µM). Positive control was measured with the 3′dGTP.

Compounds	Structure	Inhibitory potency on the DENV-NS5 RdRp	
		at 50 µM (%)	IC_{50} (µM)
1		100	1.3 ± 0.1
2		100	0.75 ± 0.03
3		100	3.12 ± 0.09
4		100	0.26 ± 0.01
5		100	0.33 ± 0.01
6		100	0.46 ± 0.01
7		100	0.64 ± 0.05
8		100	0.16 ± 0.02

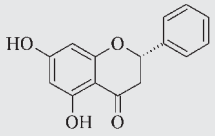
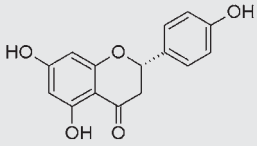
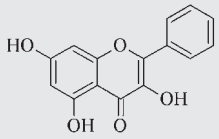
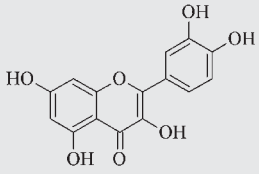
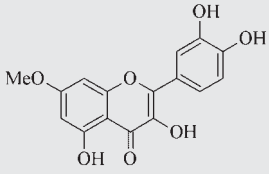
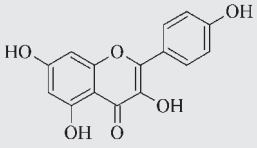
continued

Table 1 *Continued*

Compounds	Structure	Inhibitory potency on the DENV-NS5 RdRp	
		at 50 µM (%)	IC_{50} (µM)
9		98	1.6 ± 0.1
10		100	1.0 ± 0.1
11		52 ± 4	–
12		80 ± 4	> 5
13		> 50	–
14		31 ± 5	–
15		48 ± 6	–
16		92 ± 2	13.8 ± 1.9
17		21 ± 12	–

continued

Table 1 *Continued*

Compounds	Structure	Inhibitory potency on the DENV-NS5 RdRp	
		at 50 µM (%)	IC_{50} (µM)
18		35 ± 9	–
19		33 ± 4	–
20		67 ± 3	–
21		100 ± 0	3.1 ± 0.4
22		91 ± 8	2.2 ± 0.2
23		99 ± 0	5.5 ± 1.9
3′dGTP	–	100	0.02

fractions (F5.1–F5.8). F5.2 (45 mg) was then separated by preparative HPLC with an isocratic elution mix of TFA(0.5%)/MeOH 30 : 70 to obtain **4** (22 mg) and **6** (3.8 mg). F5.6 (28 mg) was also separated by preparative HPLC with an isocratic elution mix of TFA(0.5%)/MeOH 30 : 70 to obtain **7** (3.1 mg) and **8** (0.7 mg). 500 mg of F7 (2.0 g) were separated on a Sephadex LH20 column eluted with CH_2Cl_2/MeOH 3 : 1 to obtain 5 fractions (F7.1-F7.5). F7.3 (70 mg) was then fractionated by preparative HPLC using an isocratic mix of TFA(0.5%)/MeOH 30 : 70 to obtain **1** (36 mg). Compounds **2** and **3** were previously isolated from *D. balansae* [6] and were not detected in *D. araucarioides*.

Preparation of O-methyl flavonoids

O-methylated flavonoids were prepared following a protocol adapted from the literature [11]. Amentoflavone (**1**) was placed in a solution with DMF, a large excess of MeI and K_2CO_3 for three days under agitation, to obtain hexametylamentoflavone (**11**). Acacetin (**14**), genkwanin (**15**), 7,4′-dimethylapigenin (**16**), and trimethylapigenin (**17**) were prepared with the same procedure using apigenin as a precursor, and the time of the reaction ranged from 2 hours for monomethylated compounds to 3 days for permethylation. Apigenin derivatives were then separated on a silica gel column using an isocratic mix of $CHCl_3$/EtOAc/CH_3COOH 60 : 35 : 5.

Dengue NS5 polymerase assay

DENV-NS5 RdRp activity was assayed by monitoring the incorporation of radiolabeled guanosine (Amersham-Bioscience) into a homopolymeric cytosine RNA template obtained from Amersham-Pharmacia as previously described [6]. Positive and negative controls consisted of a reaction mix supplemented by 10 µM [^{3}H]-GTP (> 98%, 5.1 Ci/mmol, from Amersham-Bioscience) and 5% DMSO for the positive control, and 20 mM EDTA or 10 µM 3′ dGTP (> 90%; Trilink Biotech, N-3002–5) for the negative controls.

Supporting information

All spectroscopic data of biflavonoids isolated from *Dacrydium spp.* are available as Supporting Information.

Acknowledgments

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Conflict of Interest

The authors state that there is no conflict of interests.

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