EurJOC European Journal of Organic Chemistry

FULL PAPER

DOI: 10.1002/ejoc.201201421

Dilobenol A–G, Diprenylated Dihydroflavonols from the Leaves of *Dilobeia thouarsii*

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Keywords: Natural products / Medicinal chemistry / Antimicrobials / Cytotoxicity / Structure elucidation

The study of the EtOAc extract of the leaves of *Dilobeia thouarsii* led to the isolation and identification of seven new diprenylated dihydroxyflavonols named dilobenol A–G (**1–7**). Their structures were elucidated by spectroscopic analysis including UV, IR, 1D and 2D NMR and MS as well as by chemical hydrolysis. The isolated compounds were assessed for their antibacterial, antiplasmodial and cytotoxic activities. They exhibited moderate growth inhibitory activities against *Staphylococcus aureus, Vibrio* spp., *Bacillus* spp, and *Plasmodium falciparum* without significant toxicity against mammalian cell line L-6.

Introduction

Dilobeia thouarsii Roem & Schult (Proteaceae) syn *D. madagascariensis* Chancerel or *D. boiviniana* Baill. is a tree endemic to Madagascar growing in forest and is characterized by male and female dioecious individuals bearing slightly different leaves.[1] Its wood is resistant to pathogens such as fungi or insects and is used in house construction and carpentry. The leaves are used in traditional medicine for the treatment of infected wounds and as an anthelmintic, diuretic and tonic. It is also used to prevent the risks of abortion.[2] A literature survey revealed that no phytochemical or pharmacological studies have been reported from the genus *Dilobeia* that is made up of two species endemic to Madagascar: *D. thouarsii* and *D. tenuinervis*. The family Proteaceae with 75 genera is represented by 1500 species and found in the southern hemisphere, particularly in Australia, New Caledonia, Madagascar and South Africa. These species are less common in South America.[3]

Early phytochemical investigations of other Proteaceae showed them to be rich in the biosynthesis of tropane alkaloids,[4] naphtoquinones,[5] phenols and macrocyclic phenols,[6–8] aryl and cyanogenic glycosides.[9–12] These compounds displayed a wide spectrum of biological activities, for example, antimicrobial,[13–14] antioxidant,[15] cytotoxic,[8] anti-HIV,[16] anti-inflammatory[17] and antiplasmodial.[18]

In our search for bioactive metabolites from plant extracts, the species *Dilobeia thouarsii* was selected for screening to find new and active constituents and to explore further their antibacterial properties towards a wide range of Gram-positive and Gram-negative bacteria.

The current study reports the isolation and structural elucidation of seven new flavonol derivatives **1**–**7** from the crude extract to their in vitro antimicrobial activity against seven strains of Gram-negative bacteria, four Gram-positive ones and *Plasmodium falciparum*. Their cytotoxic activity was also evaluated.

Results and Discussion

Air-dried and ground leaves of male and female individuals of *D. thouarsii* were extracted separately and successively with cyclohexane, EtOAc and MeOH. The two resulting EtOAc extracts displayed antimicrobial activity and had comparable profiles by TLC. A phytochemical investigation was performed with the EtOAc extract from female individuals. Fractionation was performed with silica gel followed by Sephadex LH-20 to yield seven new dihydroflavonol (or flavanonol) derivatives **1**–**7** (Figure 1).

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/ejoc.201201421.

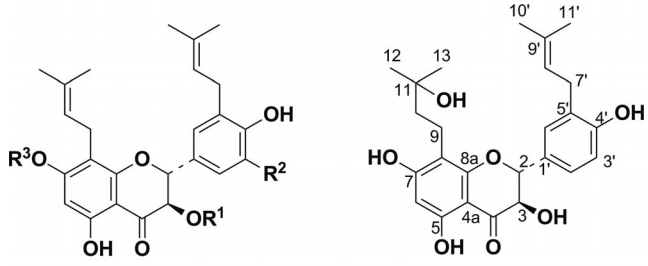

Figure 1. Structures of compounds **1**–**7** isolated from the leaves of *Dilobeia thouarsii*.

Dilobenol A (**1**) was isolated as a yellow amorphous powder. Its HRMS spectrum showed a deprotonated molecular ion at *m*/*z* 439.1740 $[M - H]^-$ corresponding to a molecular formula of $C_{25}H_{28}O_7$ that implied twelve degrees of unsaturation. The UV spectrum showed absorption maxima at 219 and 296 nm indicating a dihydroflavonol derivative.[19–20] The IR spectrum exhibited bands at 3394, 1635 and 1292 cm^{-1} characteristic of hydroxy, saturated carbonyl and olefinic groups.

The ^{13}C NMR spectrum showed the presence of 23 carbons suggesting, from the molecular formula, there are some overlapping signals. They were assigned to a carbonyl carbon, eleven quaternary carbons (including five oxygenated), seven methine carbons (of which two are oxymetines), two methylene carbons and two methyl groups.

The ^1H NMR spectrum recorded in [D_6]DMSO indicated the presence of an intense deshielded broad singlet at δ = 11.84 ppm, three aromatic protons as singlets at δ = 6.74, 6.64 and 5.97 ppm, two olefinic protons at δ = 5.23 (t, J = 7.2 Hz) and 5.10 (t, J = 7.0 Hz) ppm and two oxymethine protons at δ = 4.88 (d, J = 10.9 Hz) and 4.37 (m) ppm. In addition, two methylenes were observed at δ = 3.01 and 3.19 ppm and the four signals between δ = 1.49 and 1.66 ppm are characteristic of methyl groups. Three phenolic hydroxy protons were observed at δ = 10.78, 9.28 and 8.27 ppm as broad singlets together with a hydroxy group at δ = 5.69 (d, J = 5.9 Hz) ppm as suggested by an absence of correlation on the heteronuclear single quantum coherence (HSQC) spectrum. This deshielded proton at δ = 11.84 ppm arises from an intramolecular hydrogen bond between a hydroxy and a ketone group, as determined from HMBC connectivity studies with a carbon at δ = 197.8 ppm. Two spin systems corresponding to isoprenyl groups (9-H_2, 10-H, 12-CH_3) and (7′-H_2, 8′-H, 10′-CH_3) were identified from the ^1H–^1H COSY spectrum. The first system was supported by long-range connectivities of methyl protons at δ = 1.57 ppm (12-H_3) with carbon atoms at δ = 25.2 (12-CH_3), 122.5 (C-10) and 130.2 ppm (C-11). The second spin system (2-H/3-H/3-OH) was revealed by the coupling between oxymethine protons 2-H and 3-H, in which 3-H is coupled with a hydroxy group at δ = 5.69 ppm. These data suggest that compound **1** is a prenylated dihydroflavonol derivative.

Because the aromatic protons resonated as singlets, the connectivity of 2′-H (δ = 6.74 ppm) with carbon atoms at 127.5 (C-1′), 119.7 (C-6′), 143.1 (C-4′), 144.4 (C-3′) and 83.1 (C-2) ppm established that the ring B was oxygenated at C-4′ and C-3′ and corresponded to a 1,3,4,5-tetrasubstituted benzene. Further correlations between 7′-H and C-4′ and C-6′ demonstrated a link to the isoprenyl group at C-5′.

The remaining proton at δ = 5.97 (6-H) ppm correlated with the aromatic carbon at δ = 95.5 ppm on the HSQC spectrum. The upfield shift of this carbon suggested the proximity of two oxygenated aromatic carbons and was further confirmed by its long-range correlations with signals at 160.9 (C-5) and 164.6 (C-7) ppm. The proton also showed connectivity to two other quaternary carbons at δ = 100.4 (C-4a) and 106.9 (C-8) ppm. Correlation of the hydroxy proton at δ = 11.84 ppm with C-6, C-5 and C-4a proved the presence of a penta-substituted benzene ring. Proton 9-H correlated with C-7 and the downfield aromatic carbon at δ = 159.2 ppm (C-8a) on the HMBC spectrum that indicated the attachment of the prenyl moiety at C-8 of ring A. Vicinal coupling between 2-H and 3-H along with their connectivity with a ketone at δ = 197.8 (C-4) ppm defined the ring C. The junction of ring C to B was determined by the interaction of 2′-H and 6′-H to C-2 and by those of 2-H and 3-H to C-1′. A β-axial position was attributed to 2-H with a *trans*-diaxial relationship to 3-H as suggested by the value of their coupling constant (J = 10.9 Hz). The NOE interaction between 2-H and 3-OH confirmed their orientation on the same side of the molecule. NOE correlations between 3-H, 2′-H and 6′-H indicated the proximity of these protons.

This compound is a prenylated derivative at position 8 and 5′ of dihydroquercetin or taxifolin. From the above evidence, the structure of **1** was elucidated as 3,5,7,3′,4′-pentahydroxy-8,5′-bis(3-methylbut-2-enyl)flavanone and was named dilobenol A.

Dilobenol B (**2**) was obtained as a yellow amorphous powder, optically active $[\alpha]_D$ = –5.3 (c = 0.33, MeOH). A molecular formula was deduced as $C_{25}H_{30}O_7$ from the HRMS spectrum with a molecular ion at 441.1904 $[M - H]^-$ compatible with eleven degrees of unsaturation, and 2 atomic mass units (amu) higher than compound **1**. The IR absorption bands at 3416, 1715 and 1684 cm^{-1} revealed the presence of hydroxy and carbonyl functionalities, including a α,β-unsaturated ketone.

The ^1H and ^{13}C NMR spectroscopic data showed a pattern corresponding to a prenylated flavanonol for compound **2** (see Tables 1 and 2).

Relative to compound **1**, differences were noticed for the aromatic protons as well as the disappearance of an olefinic proton suggesting the modification of one of the prenyl moieties. The ^1H NMR spectrum showed signals for three protons characteristic of a trisubstituted aromatic ring at δ = 7.23 (d, J = 2.2 Hz, 6′-H), 7.18 (dd, J = 2.2, 8.2 Hz, 2′-

Table 1. ^{13}C NMR spectroscopic data for compounds **1**–**7**.

Pos.	**1**[a]	**2**[b]	**3**[b]	**4**[b]	**5**[b]	**6**[b]	**7**[b]
2	83.1	84.9	84.0	83.9	85.4	84.2	84.0
3	71.7	73.8	78.5	78.7	73.9	78.7	78.8
4	197.8	198.7	196.2	196.2	197.5	197.2	197.2
4a	100.4	102.2	102.6	102.5	103.2	103.9	104.0
5	160.9	162.9	163.2	163.2	163.1	163.1	163.2
6	95.5	96.8	96.7	96.8	96.7	96.7	96.7
7	164.6	166.5	166.3	166.4	164.9	164.8	164.8
8	106.9	110.0	109.2	109.2	110.0	111.3	111.3
8a	159.2	161.2	160.8	160.8	160.6	160.2	160.2
9	21.0	18.6	22.3	22.4	22.6	22.6	22.6
10	122.5	43.8	123.6	123.7	123.7	123.6	123.6
11	130.2	71.7	131.8	131.7	131.2	132.0	132.0
12	25.2	28.9	25.9	25.9	25.9	25.9	25.9
13	17.6	28.7	17.9	17.9	17.9	17.9	18.1
1′	127.5	129.4	128.5	128.6	129.0	128.3	128.4
2′	112.5	127.4	112.7	127.1	113.3	112.8	127.2
3′	144.4	115.6	143.8	115.8	144.0	146.0	115.8
4′	143.1	156.0	146.0	156.9	145.9	145.0	157.0
5′	133.0	129.4	129.8	129.6	129.5	129.8	129.7
6′	119.7	130.0	120.8	129.7	121.3	120.9	129.8
7′	28.2	29.4	29.1	29.1	29.3	29.1	29.2
8′	122.9	123.9	123.7	123.6	123.9	123.7	123.7
9′	130.9	133.1	133.8	133.4	132.3	133.3	133.4
10′	25.4	17.8	25.9	25.9	25.9	25.9	25.9
11′	17.7	17.7	17.9	17.9	17.9	17.9	18.1
1′′			101.9	101.9		102.0	102.0
2′′			71.8	71.9		71.8	71.8
3′′			72.2	72.2		72.2	72.2
4′′			73.9	73.9		73.8	73.8
5′′			70.4	70.5		70.5	70.5
6′′			17.8	17.8		17.8	17.9
1′′′					101.5	101.4	101.5
2′′′					78.3	78.2	78.3
3′′′					74.9	74.8	74.9
4′′′					78.3	78.2	78.3
5′′′					71.2	71.1	71.2
6′′′					62.4	62.4	62.4

[a] Spectra were recorded in [D_6]DMSO. [b] Spectra were recorded in CD_3OD.

H) and 6.78 (d, J = 8.2 Hz, 3′-H) ppm. The singlet observed at δ = 5.97 (6-H) ppm belongs to a penta-substituted aromatic ring. The proton of a prenyl moiety was observed at δ = 5.31 (t, J = 8.6 Hz) ppm coupled to a multiplet at δ = 3.31 and overlapped singlets at δ = 1.72 ppm. Two doublets were present at δ = 4.45 and 4.93 (J = 11.2 Hz) ppm corresponding to 3-H and 2-H, two methyl groups at δ = 1.13 and 1.12, a multiplet at δ = 3.31 and a triplet at δ = 2.51 ppm.

Proton 2′-H is shifted downfield and its connectivity with the carbon at δ = 156.0 ppm as well as its coupling with a proton at δ = 6.78 ppm carried by a carbon at δ = 115.6 (C-3′) ppm were indicative of a single oxygenated quaternary carbon for ring B instead of the two present in compound **1**.

In addition, from analysis of the HSQC spectrum, the carbon at δ = 71.7 ppm is an oxygenated quaternary carbon. The proton at δ = 2.51 (9-H_2) coupled with those at δ = 1.52 (10-H_2) ppm correlated with the carbon at δ = 71.7 ppm according to the HMBC spectrum.

The connectivity of two methyl protons with the same carbon suggested that hydroxylation of the prenyl moiety occurred at C-11. This 3-hydroxy-3-methylbutyl substituent was linked to aromatic ring A at position 8 as supported by long-range interaction of protons 10-H_2 with C-8.

The HMBC correlations of 6′-H with C-2, C-2′, C-4′ and C-7′ substantiated that the prenyl group is located at C-5′. The coupling constant of 11.2 Hz established a *trans*-diaxial orientation of protons 2-H and 3-H. Thus, compound **2** is a prenylated derivative at position 8 and 5′ of dihydrokaempferol. Indeed, dilobenol B (**2**) was identified as 3,5,7,11,4′-pentahydroxy-8-(3-hydroxy-3-methylbutyl)-5′-(3-methylbut-2-enyl)flavanone.

Dilobenol C (**3**) was isolated as an amorphous powder. The HRMS data for **3** gave the molecular formula $C_{31}H_{38}O_{11}$ based on a pseudomolecular $[M - H]^-$ ion peak at m/z 585.2317, indicating thirteen degrees of unsaturation. The ^{13}C NMR spectrum was similar to that for compound **1** and showed five additional oxymethine carbons between δ = 70.4 and 101.9 ppm. Its ^1H NMR spectrum also exhibited signals for five additional oxymethine protons at δ = 3.29 and 4.22 ppm together with a doublet at δ = 1.20 ppm that account for three protons. These NMR spectroscopic data suggest the presence of a sugar moiety as confirmed by the correlation of an anomeric proton at δ = 4.08 ppm with a carbon signal at δ = 101.9 ppm on the HSQC spectrum. Furthermore, the coupling of the methyl protons at δ = 1.20 (d, J = 6.2 Hz) ppm with a carbon at δ = 17.8 ppm indicates the presence of a 6-deoxy sugar unit.

The ^1H–^1H COSY spectrum established the spin system 1′′-H/2′′-H/3′′-H/4′′-H/5′′-H/6′′-H_3. Proton 5′′-H correlated with carbons C-1′′, C-3′′, C-4′′ and C-6′′ whereas 1′′-H is connected to C-3, C-2′′ and C-5′′. This result suggested an ether linkage from the sugar with the flavanonol through C-3 and was supported by the downfield chemical shift of this oxymethine carbon (+7.0 ppm) relative to compound **1**.

The relative configuration for the sugar moiety was elucidated by NOESY experiment along with analysis of the vicinal ^1H–^1H coupling constants as shown in Figure 2.

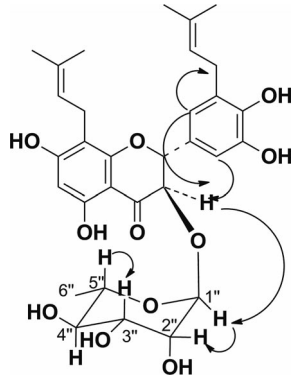

Figure 2. Selected NOE correlations of dilobenol C (**3**).

A β-axial position was attributed to 3′′-H owing to the large coupling constant (J = 9.5 Hz) with 4′′-H whereas it showed a gauche coupling (J = 3.3 Hz) with equatorial 2′′-H. Protons 4′′-H and 5′′-H adopted a *trans*-diaxial orientation in agreement with the correlation of 3′′-H with 5′′-H

Table 2. ^{1}H NMR spectroscopic data for compounds **1**–**7**.

Pos.	**1**[a] δH (*J* in Hz)	**2**[b] δH (*J* in Hz)	**3**[b] δH (*J* in Hz)	**4**[b] δH (*J* in Hz)	**5**[b] δH (*J* in Hz)	**6**[b] δH (*J* in Hz)	**7**[b] δH (*J* in Hz)
2	4.88, d (10.9)	4.93, d (11.2)	4.99, d (10.4)	5.09, d (10.4)	4.86, d (10.5)	5.01, d (10.7)	5.12, d (10.7)
3	4.37, m	4.45, d (11.2)	4.49, d (10.4)	4.53, d (10.4)	4.49, d (10.5)	4.56, d (10.7)	4.60, d (10.7)
6	5.97, s	5.97, s	5.96, s	5.96, s	6.33, s	6.33, s	6.32, s
9a	3.01, d (7.0)	2.51, t (9.7)	3.14, m	3.14, d (7.4)	3.31, m	3.31, m	3.31, m
9b					3.13, m	3.15, dd (7.2, 13.9)	3.15, dd (7.3, 13.4)
10	5.1, t (7.0)	1.52, m	5.12, t (7.3)	5.14, t (7.4)	5.15, t (7.4)	5.13, t (7.2)	5.16, t (7.3)
12	1.57, s	1.13, s	1.61, s	1.61, s	1.60, s	1.61, s	1.61, s
13	1.49, s	1.12, s	1.54, s	1.53, s	1.53, s	1.57, s	1.55, s
2′	6.74, s	7.18, dd (2.2, 8.2)	6.82, d (2.0)	7.15, dd (2.1, 8.2)	6.84, d (2.0)	6.83, d (1.9)	7.16, dd (1.8, 8.2)
3′	–	6.78, d (8.2)	–	6.79, d (8.2)	–	–	6.79, d (8.2)
6′	6.64, s	7.23, d (2.2)	6.71, d (2.0)	7.19, d (2.1)	6.76, d (2.0)	6.73, d (1.9)	7.21, d (1.8)
7′	3.19, d (7.2)	3.31, m	3.32, m	3.27, m	3.31, m	3.31, m	3.31, m
8′	5.23, t (7.2)	5.31, t (8.6)	5.30, t (8.7)	5.31, t (7.3)	5.32, t (7.2)	5.31, t (7.4)	5.32, t (7.3)
10′	1.66, s	1.72, s	1.73, s	1.75, s	1.73, s	1.74, s	1.75, s
11′	1.64, s	1.72, s	1.71, s	1.71, s	1.73, s	1.72, s	1.72, s
1′′			4.08, d (1.7)	4.07, d (1.7)		4.05, d (1.2)	4.04, s
2′′			3.56, dd (1.7, 3.3)	3.53, dd (1.7, 3.3)		3.51, dd (1.2, 3.2)	3.50, m
3′′			3.65, dd (3.3, 9.5)	3.64, dd (3.3, 9.5)		3.65, dd (3.2, 9.6)	3.64, dd (3.6, 9.6)
4′′			3.29, m	3.30, m		3.31, m	3.31, m
5′′			4.22, m	4.19, dd (6.2, 9.5)		4.21 dd (6.2, 9.6)	4.19, m
6′′			1.2, d (6.2)	1.17, d (6.2)		1.18, d (6.2)	1.17, d (6.2)
1′′′					4.99, d (7.2)	5.00, d (7.0)	4.99, d (7.2)
2′′′					3.47, m	3.46, m	3.47, m
3′′′					3.48, m	3.37, m	3.47, m
4′′′					3.48, m	3.46, m	3.47, m
5′′′					3.40, m	3.37, m	3.40, m
6′′′a					3.70, dd (5.2, 12.2)	3.70, dd (5.2, 12.2)	3.70, dd (5.1, 12.1)
6′′′b					3.88, dd (2.0,12.2)	3.89, dd (1.7, 12.2)	3.88, d (1.5, 12.1)
3-OH	5.69, d (5.9)						
5-OH	11.84, br. s						
OH	10.78, br. s						
OH	9.28, br. s						
OH	8.27, br. s						

[a] Spectra were recorded in DMSO d_6. [b] Spectra were recorded in CD_3OD.

indicating that they are both on the same side of the molecule. Proton 1′′-H adopted an α-equatorial position and a coupling constant (J = 1.7 Hz) with 2′′-H. The α-glycoside linkage was supported by the NOE correlation between 1′′-H and 3-H. The sugar moiety was identified as α-rhamnose. The presence of a L-rhamnopyranosyl unit was confirmed by acidic hydrolysis of **3** with 2 N TFA followed by TLC analysis of the components after their separation from the mixture by extraction with CH_2Cl_2.[21] The derivative that co-eluted with compound **1** was found in the CH_2Cl_2 layer and the sugar present in the aqueous fraction showed physical data (TLC analysis, MS and $[\alpha]_D^{20}$) identical to those of monosaccharide L-rhamnose. Accordingly, the structure of compound **3** was determined as 5,7,3′,4′-tetrahydroxy-8,5′-bis(3-methylbut-2-enyl)flavanone-3-*O*-α-L-rhamnopyranoside.

Dilobenol D (**4**) was isolated as an amorphous powder. Its molecular formula $C_{31}H_{38}O_{10}$ was deduced from its HRMS deprotonated molecular ion at *m*/z 569.2364 [M – H]$^-$. The ^{1}H and ^{13}C NMR spectroscopic data (see Tables 1 and 2) of **4** were closely related to those of **3** and the difference of 16 amu suggested that it is deoxygenated. The substitution pattern of the aromatic benzene ring B of compound **4** seemed different and allowed the two compounds to be distinguished. The signals of the aromatic protons are typical to a trisubstituted ring B with three protons at δ = 7.19 (d, J = 2.1 Hz, 6′-H), 7.15 (dd, J = 2.1, 8.2 Hz, 2′-H) and 6.79 (d, J = 8.2 Hz, 3′-H) ppm suggesting a dihydrokaempferol derivative.

Careful analysis of the 2D NMR experiments (^{1}H–^{1}H COSY, HSQC and HMBC) established that the genuine sample of **4**, corresponding to 3,5,7,4′-tetrahydroxy-8,5′-bis(3-methylbut-2-enyl)flavanone, is identical to lespedezaflavanone C isolated from *Lespedeza davidii*.[22] The coupling constant of 1.7 Hz between 1′′-H and 2′′-H was in good accordance with its linkage in the α position with a 6-deoxy sugar unit corresponding to the rhamnopyranoside. From the NOESY spectrum, it was observed that compound **4** shares the same relative configuration as **3**. Therefore, dilobenol D (**4**) was identified as 5,7,4′-trihydroxy-8,5′-bis(3-methylbut-2-enyl) flavanone-3-*O*-α-L-rhamnopyranoside.

The HRMS spectrum of dilobenol E (**5**) shows a deprotonated molecular ion at *m*/*z* 601.2259 [M – H]$^-$, which is

consistent with the molecular formula $C_{31}H_{38}O_{12}$ with 13 degrees of unsaturation. The ^1H and ^{13}C NMR spectroscopic data were similar to those of **3**. The disappearance of the methyl doublet at δ = 1.2 ppm, characteristic of rhamnose, as well as the difference of molecular weight suggested a modification of the sugar moiety. Furthermore, the upfield chemical shift of the oxymethine carbon C-3 (–4.6 ppm) relative to **3** indicated the presence of a free hydroxy group at this position, whereas 6-H was shifted downfield (+0.37 ppm).

Analysis of ^1H–^1H COSY spectrum established the spin system 1‴-H/2‴-H/3‴-H/4‴-H/5‴-H/6‴-H_2. The occurrence of two non-equivalent protons at δ = 3.70 and 3.88 ppm connected with a carbon at δ = 62.4 ppm combined with that of a slightly downfield anomeric proton at δ = 4.99 (d, J = 7.2 Hz) ppm carried by a carbon at δ = 101.5 ppm were indicative of a pyranose moiety with β-configuration.

The linkage of the diprenylated dihydroflavonol with the glucosyl unit was evidenced by the long-range connectivity deduced from the HMBC data. The anomeric proton 1‴-H correlated with C-2‴ and C-7, whereas 4‴-H showed cross-peaks with C-3‴ and C-5‴. Further support was obtained from the NOESY experiment that revealed the interaction of 6-H and 1″-H. The coupling constant of 7.2 Hz at δ = 4.99 ppm is in agreement with a *trans*-diaxial between protons 1″-H and 2″-H in a β attached D-glucopyranose. Although there was insufficient quantity of the compound to allow for acidic hydrolysis, dilobenol E (**5**) was characterized as 3,5,3′,4′-tetrahydroxy-8,5′-bis(3-methylbut-2-enyl)flavanone-7-*O*-β-D-glucopyranoside.

Dilobenol F (**6**) was isolated as an amorphous powder that was optically active $[\alpha]_D$ = –35 (c = 0.1, MeOH). Its HRMS spectrum exhibited a deprotonated molecular ion at m/z 747.2836 $[M - H]^-$ leading to a molecular formula of $C_{37}H_{48}O_{16}$. The ^1H and ^{13}C NMR data of **6** displayed similarities to those of **3** (see Tables 1 and 2). Five additional oxymethine carbons and one oxymethylene carbon were noted on the ^{13}C spectrum relative to **3**. Furthermore, a difference of 162 amu suggested that compound **6** is constituted of disaccharide moieties. Analysis of their ^1H NMR spectra showed additional protons between δ = 3.15 and 5.01 ppm and 6-H was shifted downfield (+ 0.37 ppm) for **6**.

Analysis of HMBC and NOESY spectra established that the two glycosyl moieties were linked with the diprenylated dihydroflavonol. Correlation of the proton at δ = 4.05 (1″-H, d, J = 1.2 Hz) ppm with C-3 together with NOE interactions of 1″-H, 2′-H and 3′-H with 3-H revealed a α linkage of rhamnose at C-3. On the other hand, connectivity of 1‴-H with C-7 along with the cross-peak of 6-H and 1‴-H observed on the NOESY spectrum confirmed the β linkage of glucose at C-6. Acid hydrolysis was performed on compound **6**. It afforded the diprenylated dihydroflavanone corresponding to compound **1** and the monosaccharide components were identified as D-glucose and L-rhamnose. Thus, the structure of dilobenol F was established as 5,3′,4′-trihydroxy-8,5′-bis(3-methylbut-2-enyl)flavanone-3-*O*-α-L-rhamnopyranoside-7-*O*-β-D-glucopyranoside.

Dilobenol G (**7**), an amorphous powder, had a molecular formula of $C_{37}H_{48}O_{15}$ that was deduced from the deprotonated HRMS ion peak at m/z 731.2888 $[M - H]^-$. Its ^1H NMR spectrum was almost identical to that of dilobenol F (**6**) except the presence of a signal relating to three aromatic protons instead of two, indicating the presence of a trisubstituted aromatic ring B, as for compound **4**. The full assignment of all proton and carbon signals of **7** was done by 2D NMR experiments. The signal for a β-glucoside (J = 7.2 Hz) moiety was observed in the ^1H and ^{13}C NMR spectra (see Tables 1 and 2). As a consequence of a small coupling between 1″-H and 2″-H ($J \approx 0$ Hz), 1″-H, which is in an equatorial position, appears as a singlet suggesting a α-rhamnoside. Thus, compound **7** was identified as 5,4′-dihydroxy-8,5′-bis(3-methylbut-2-enyl)flavanone-3-*O*-α-L-rhamnopyranoside-7-*O*-β-D-glucopyranoside.

Compounds **1**–**7** were evaluated for their antibacterial activities against a range of seven strains of Gram-negative bacteria including *Pseudomonas aeruginosa*, *Vibrio harveyi* and *V. fischeri*, *Salmonella antarctica* and *Salmonella enterica* Typhimurium, *Escherichia coli*, and *Klebsiella pneumoniae* because the crude EtOAc extract was active against these strains.

Activity was also evaluated against *Bacillus cereus*, *B. megaterium*, *Enterococcus faecalis* and *Staphylococcus aureus*, which are Gram-positive bacteria. The results are summarized in Table 3. The cyclohexane extract, which was not active against any of the strains tested, is not shown. The disc diffusion assay showed that the EtOAc extract was more effective than the MeOH extract for the majority of strains.[13–14] They both displayed similar effect towards *V. fischeri* and *Bacillus* spp. The EtOAc extract exhibited similar activity towards *Vibrio harveyi* as standard antibiotics gentamicin and tetracycline, which were used as controls. Moreover, all isolated compounds were inactive against the majority of strains (Table 3).

All compounds, except **3** and **5**, displayed moderate activity against *Vibrio harveyi*. Compounds **1**, **3**, **4**, **6** and **7** were also active against *V. fischeri*. Compounds **1**, **2** and **4** were active against *Bacillus cereus* and *Staphylococcus aureus*, whereas **1** displayed some activity on *B. megaterium*. Relative to the controls, these compounds exhibited moderate antibacterial activity at the tested concentration (30 μg/disc). Perry and Brennan have shown that the inhibition diameter on solid cultures is dose dependent.[14] The active phenolic glycoside ester isolated from *Toronia toru* (Proteaceae) inhibited the growth of *B. subtilis*, *E. coli* and *P. aeruginosa* at 120 μg/disc (inhibition diameters of 5, 4 and 3 mm, respectively) but not at 30 μg/disc.

This species of Proteaceae contains other antibacterial compounds such as hydroquinone and tulipalin A that showed larger inhibition zones towards *B. subtilis* (9 and 10 mm, respectively).

Overall the crude extract showed larger growth inhibition than pure compounds **1**–**7** for all strains suggesting synergy between compounds. It has been reported in the literature that the inhibitory activity of a crude plant extract results from a complex interaction between its different constitu-

Table 3. Antibacterial activity of extracts and compounds **1**–**7**.[a]

Microorganism	Gram negative bacteria							Gram positive bacteria			
Samples[a]	Pa	Vh	Vf	ST	San	Ec	Kp	Bc	Bm	Ef	Sau
EtOAc ext.	7 ± 0.0	19 ± 0.0	11 ± 0.0	8 ± 0.1	7 ± 0.1	7 ± 0.4	7 ± 0.6	11 ± 0.0	11 ± 0.1	9 ± 0.6	10 ± 0.0
MeOH ext.	–	9 ± 0.1	10 ± 0.2	–	–	–	–	10 ± 0.0	10 ± 0.1	–	–
1	–	7 ± 2.2	7 ± 0.0	–	–	–	–	7 ± 2.0	8 ± 0.1	–	7 ± 1.0
2	–	7 ± 1.3	–	–	–	–	–	8 ± 0.0	–	–	8 ± 0.3
3	–	–	8 ± 0.0	–	–	–	–	–	–	–	–
4	–	7 ± 0.0	9 ± 1.5	–	–	–	–	7 ± 1.0	–	–	7 ± 0.0
5	–	–	–	–	–	–	–	–	–	–	–
6	–	9 ± 0.0	9 ± 1.6	–	–	–	–	–	–	–	–
7	–	8 ± 0.0	8 ± 0.0	–	–	–	–	–	–	–	–
Gentamycin	19.0 ± 0.0	13.0 ± 0.0	19.5 ± 0.0	19.0 ± 0.0	26.0 ± 0.0	30.5 ± 0.5	19.5 ± 0.5	25.5 ± 0.5	22.5 ± 0.2	20.0 ± 0.0	20.0 ± 0.5
Tetracycline	n.d.	19.5 ± 0.0	–	14.0 ± 0.0	22.0 ± 0.0	11 ± 0.2	13.0 ± 0.5	20.5 ± 0.5	18.0 ± 0.5	8 ± 0.5	22.0 ± 0.5

[a] Samples: crude extract was tested at 1 mg/disc, compounds **1**–**7** at 30 μg/disc, gentamycin at 10 μg/disc, tetracycline at 30 μg/disc. Results are expressed as zones of growth inhibition (mm). (–): no zone of inhibition. n.d.: not determined. [b] Microorganisms: Pa: *Pseudomonas aeruginosa*; Vh: *Vibrio harveyi*; Vf: *Vibrio fischeri*; ST: *Salmonella enterica* Typhimurium; San: *Salmonella antarctica*; Ec: *Escherichia coli*; Kp: *Klebsiella pneumoniae*; Bc: *Bacillus cereus*; Bm: *Bacillus megaterium*; Ef: *Enterococcus faecalis*; Sau: *Staphylococcus aureus.*

ents, which may produce, additive, synergistic or antagonistic effects, even for compounds present at low concentrations.[23,24]

Because it was reported in the literature that some plants of the Proteaceae family exhibited significant antiplasmodial and cytotoxicity activities,[8,16] all compounds were tested for their inhibitory capacity against the in vitro development of the chloroquine-resistant strain FcB1 of *Plasmodium falciparum* (Table 4).

Table 4. In vitro antiplasmodial and cytotoxic activities of compounds **1**–**7**.

	IC_{50} ± SD (μM) FcB1	IC_{50} ± SD (μM) L-6
1	24.8 ± 0.5	64.3 ± 1.0
2	34.3 ± 0.6	62.9 ± 9.0
3	16.0 ± 2.6	88.6 ± 6.1
4	15.8 ± 1.4	58.8 ± 0.4
5	25.3 ± 1.0	91.0 ± 5.6
6	30.8 ± 2.9	> 134
7	20.8 ± 1.8	> 137
CQ	0.103 ± 0.015	nd

[a] Results are expressed as IC_{50} values (μM) ± standard deviations. All experiments were performed in triplicate. Chloroquine (CQ) was used as a positive control for antiplasmodial activity. nd: not determined.

Compounds **3** and **4** were the most active with similar IC_{50} values (ca. 16 μM). All other compounds had IC_{50} values ranging 20–34 μM. They all displayed moderate antiplasmodial activity relative to the control, chloroquine. The evaluation of their cytotoxicity against the rat cell line L-6 showed that they were slightly cytotoxic with IC_{50} values up to 58.8 μM.

Conclusions

Seven new diprenylated flavanonols (dilobenol A–G) including four dihydroquercetin and three dihydrokaempferol derivatives were isolated from the leaves of female individuals of *Dilobeia thouarsii*. This is the first report of constituents from the genus *Dilobeia*.

Experimental Section

General Experimental Procedures: Optical rotations were measured with a Perkin–Elmer model 341 polarimeter at 20 °C. IR spectra were recorded with a Shimadzu FTIR-8400S spectrophotometer. Mass spectra data were recorded with an electrospray time-of-flight mass spectrometer operating in the negative mode (QSTAR Pulsar I of Applied Biosystems). ^{13}C NMR spectra were recorded with an AC 300 BRUKER spectrometer operating at 75.47 MHz (for ^{13}C). ^{1}H and 2D-NMR spectra were recorded with an Avance-400 Bruker spectrometer operating at 400.13 MHz, equipped with ^{1}H-broad-band reverse-gradient probe head. The temperature was controlled with a Bruker BCU-05 refrigeration unit and a BVT 3000 control unit. The ^{1}H and ^{13}C NMR chemical shifts are given relative to TMS. For the HMBC experiments, the delay (1/2 *J*) was 70 ms and for the NOESY experiments the mixing time was 150 ms. Analytical and preparative TLC were carried out on precoated Si gel 60 F_{254} plates (Merck). Compounds were detected under UV (254 and 366 nm) before spraying with either a vanillin/sulfuric acid solution in EtOH followed by heating the plate at 110 °C or with 2% ethanolic ferric chloride reagent. Column chromatography was performed on 200–400 mesh silica gel 60 (Merck) and on Sephadex LH-20 (25–100 μm; Pharmacia Biotech Ltd). Preparative medium-pressure liquid chromatography (MPLC) was performed with a pump K-120 (Knauer) and Flash-smart cartridges (Si and C-18 gels 20–40 μm, AIT, France).

Plant Material: The leaves of *Dilobea thouarsii*, Proteaceae were collected in the Mandraka area at 70 km off Antananarivo in April and November 2008. This species was identified by Dr Rabarison Harison, Department of Biology and Plant Ecology, University of Antananarivo. A voucher specimen was deposited in the Herbarium of the University under number HERB/DBEV/4708.

Extraction and Isolation: Air-dried powdered leaves of *Dilobeia thouarsii* (275 g) were extracted successively with cyclohexane, EtOAc and MeOH to afford, after evaporation of solvent, 1.78 g, 9.75 g and 32 g of extracts, respectively. A portion (6 g) of the EtOAc extract was purified by chromatography on a silica gel col-

umn with a mixture of cyclohexane/EtOAc/MeOH, increasing in polarity, as eluant to give 12 fractions. Fraction F4 (102 mg) was purified by silica gel eluted with CH_2Cl_2/MeOH (gradient 98:2 to 90:10) to yield 14 sub-fractions. Sub-fractions F4–5 (21 mg) and F4–8 (8 mg) were subjected to Sephadex® LH-20 eluted with MeOH to furnish compounds **1** (2 mg) and **2** (4 mg). Fraction F6 (239 mg) was submitted successively to MPLC eluted with CH_2Cl_2/MeOH (gradient 95:5 to 90:10) and to silica gel eluted with CH_2Cl_2/MeOH (95:5) to afford dilobenol D (**4**, 4 mg). Fraction F8 (73 mg) was purified by chromatography on silica gel eluted with CH_2Cl_2/MeOH (first 90:10, then 80:20) to give **3** (10 mg). Fraction F9 (1156 mg) was subjected to silica gel chromatography eluted with CH_2Cl_2/MeOH, increasing in polarity, to provide 18 fractions. Compounds **7** (7 mg) and **5** (2 mg) were obtained from F9–16 (245 mg) through Sephadex® LH-20 eluted with MeOH/H_2O (90:10) followed by MPLC on R**P-1**8 silica gel eluted with MeOH/H_2O (gradient 30:70 to 80:20). Purification by chromatography of sub-fraction F9–17 (200 mg) by repeated Sephadex® LH-20 eluted with MeOH/H_2O (90:10) furnished **6** (40 mg).

Dilobenol A (1): Yellow amorphous powder. $[\alpha]_D^{20}$ = –23.3 (*c* = 0.12, MeOH). UV/Vis (MeOH): λ (ε, L mol^{-1} cm^{-1}) = 219 (4.04), 296 (3.79), 345 (sh) nm. IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3394, 2924, 1635, 1446, 1292, 1080, 999 cm^{-1}. ^{1}H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2. HRMS (ESI): calcd. for $C_{25}H_{27}O_7$ [M – H]$^-$ 439.1742; found 439.1740.

Dilobenol B (2): Yellow amorphous powder. $[\alpha]_D^{20}$ = –5.3 (*c* = 0.325, MeOH). UV/Vis (MeOH): λ (ε, L mol^{-1} cm^{-1}) = 220 (3.75), 294 (3.52), 347 (sh) nm. IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3360, 2924, 1635, 1508, 1439, 1261, 1134, 1076 cm^{-1}. ^{1}H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2. HRMS (ESI): calcd. for $C_{25}H_{29}O_7$ [M – H]$^-$ 441.1898; found 441.1904.

Dilobenol C (3): White amorphous powder. $[\alpha]_D^{20}$ = –25.5 (*c* = 0.26, MeOH). UV/Vis (MeOH): λ (ε, L mol^{-1} cm^{-1}) = 219 (4.03), 294 (3.77), 347 (sh) nm. IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3410, 2924, 1640, 1446, 1284, 1080 cm^{-1}. ^{1}H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2. HRMS (ESI): calcd. for $C_{31}H_{37}O_{11}$ [M – H]$^-$ 585.2314; found 585.2317.

Acid Hydrolysis of 3: Compound **3** (6 mg) was heated to reflux in 2 N aqueous CF_3COOH (2 mL) for 3 h. After cooling, the reaction mixture was diluted with H_2O (2 mL) and extracted with CH_2Cl_2. The organic layer was washed with a saturated solution of $NaHCO_3$, dried with Na_2SO_4, filtered, and concentrated under reduce pressure. The acidic aqueous layer was co-evaporated twice with MeOH/H_2O (1:1) until neutral to afforded L-rhamnose (2 mg). The compounds obtained were analyzed by TLC with CH_2Cl_2/MeOH (90–10) and MS and compared to authentic samples. L-Rhamnose $[\alpha]_D^{20}$ = +15 (*c* = 0.10, MeOH).

Dilobenol D (4): White amorphous powder. $[\alpha]_D^{20}$ = –12.7 (*c* = 0.23, MeOH). UV/Vis (MeOH): λ (ε, L mol^{-1} cm^{-1}) = 220 (3.82), 296 (3.57), 347 (sh) nm. IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3371, 2920, 1635, 1438, 1261, 1080 cm^{-1}. ^{1}H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2. HRMS (ESI): calcd. for $C_{31}H_{37}O_{10}$ [M – H]$^-$ 569.2366; found 569.2364.

Dilobenol E (5): Colorless amorphous powder. $[\alpha]_D^{20}$ = –24 (*c* = 0.06, MeOH). UV/Vis (MeOH): λ (ε, L mol^{-1} cm^{-1}) = 218 (3.84), 289 (3.53), 347 (sh) nm. IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3379, 2924, 1635, 1446, 1076 cm^{-1}. ^{1}H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2. HRMS (ESI): calcd. for $C_{31}H_{37}O_{12}$ [M – H]$^-$ 601.2262; found 601.2259.

Dilobenol F (6): Colorless amorphous powder. $[\alpha]_D^{20}$ = –35 (*c* = 0.1, MeOH) UV/Vis (MeOH): λ (ε, L mol^{-1} cm^{-1}) = 220 (4.19), 290 (3.95), 344 (3.27) nm. IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3371, 2920, 1635, 1585, 1438, 1072 cm^{-1}. ^{1}H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2. HRMS (ESI): calcd. for $C_{37}H_{47}O_{16}$ [M – H]$^-$ 747.2834; found 747.2836.

Acid Hydrolysis of 6: This reaction was performed as described for compound **3** with compound **6** (10 mg). After neutralization and concentration of the acidic aqueous layer, the resulting soluble fraction (4 mg) was purified on Sephadex® LH-20 eluted with MeOH to afford D-glucose (1.9 mg) and L-rhamnose (1.4 mg). The optical rotations taken were similar with those of authentic samples. D-glucose: $[\alpha]_D^{20}$ = +120 (*c* = 0.19, MeOH).

Dilobenol G (7): White amorphous powder. $[\alpha]_D^{20}$ = –27.9 (*c* = 0.19, MeOH). UV/Vis (MeOH): λ (ε, L mol^{-1} cm^{-1}) = 219 (3.93), 290 (3.66), 340 (3.03) nm. IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3379, 2924, 1635, 1585, 1438, 1373, 1072 cm^{-1}. ^{1}H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2. HRMS (ESI): calcd. for $C_{37}H_{47}O_{15}$, [M – H]$^-$ 731.2886; found 731.2888.

Biological Activities

Antimicrobial Assays: Four Gram-positive (*Bacillus cereus* LMG 6910, *Bacillus megaterium* LMG 7127, *Staphylococcus aureus* ATTC 25920, *Enterococcus faecalis* ATTC 29212) and seven Gram-negative bacteria (*Vibrio harveyi* ATCC 14126, *Vibrio fischeri* ATCC 49387, *Salmonella enterica* Typhimurium ATCC 14028, *Salmonella antarctica* LMG 3264, *Escherichia coli* CCM 451, *Klebsiella pneumoniae* ATTC 13883 and *Pseudomonas aeruginosa* LMG 1242) were used to study the antibacterial activity. The bacteria were obtained from the collections of both the University of La Réunion (LCSNSA: Laboratoire de Chimie des Substances Naturelles et des Sciences des Aliments, Saint Pierre) and Cirad (Montpellier, France). Susceptibility-screening tests were performed by using the disc diffusion method.[25] Each microorganism was suspended in brain-heart infusion broth (Difco, Detroit, MI) and diluted with peptone water to provide initial cell counts of the inoculum of about 10^6 CFU/mL. Bacterial strains were inoculated on duplicate plates of marine agar for vibrios and Mueller–Hinton agar for the other strains. Sterilized filter paper discs of 6 mm (Biomérieux, Marcy l'Etoile, France) were saturated with 10 µL of the ethyl acetate/methanol extract and pure compounds **1**–**7** (in distillated water). The soaked discs were then placed on the plates and incubated for 24 h, after which the diameter of the inhibitory zone was measured (mm). Negative controls were prepared by using the same solvents employed to dissolve the plant-extract samples. Each assay was repeated three times. The results were expressed as the mean value (mm ± SD). The reference antibiotics tetracycline and gentamycin (30 and 10 µg, respectively; Bio-Rad, Marnes-la-Coquette, France) were used as positive controls.

In Vitro Antiplasmodial Assay: The in vitro antiplasmodial tests, based on the inhibition of [^{3}H]-hypoxanthine uptake by *P. falciparum* cultured on human red blood cells were performed as described previously.[26] The concentration causing 50% growth inhibition (IC_{50}) was obtained from the drug concentration-response curve, and the results were expressed as the mean from three independent experiments. Chloroquine diphosphate (Sigma Aldrich Chimie SARL, St Quentin Fallavier, France) was used as positive control for antiplasmodial activity.

In Vitro Cytotoxicity Assay on Mammalian Cells: The cytotoxicity was evaluated by using a rat myoblast-derived cell line (L-6) as described previously.[26] Cells were obtained from ATCC (Rockville, Maryland, USA). They were maintained 5 days in culture in the presence of the tested compounds and the cytotoxicity was determined by using the colorimetric MTT assay according to the

manufacturer's recommendations (cell proliferation kit I, Roche Applied Science, France). The IC_{50} was obtained from the drug concentration-response curve, and the results were expressed as the mean from three independent experiments.

Supporting Information (see footnote on the first page of this article): ^1H NMR and HMBC spectra of compounds **1**–**7**.

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Received: October 24, 2012
Published Online: February 13, 2013

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Highlights

South African Journal of Botany xxx (2013) xxx–xxx

Antimicrobial activities of *Dilobeia thouarsii* Roemer and Schulte, a traditional medicinal plant from Madagascar

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- Leaf extraction (solvent extraction of varying polarity) of *Dilobeia thouarsii*
- Purified compounds (**1**, **2**) from leaf ethyl acetate extracts were more active.
- Compound **1** is a 4-aminophenol, compound **2** is 4-hydroxybenzaldehyde.
- Good antimicrobial activity against *Staphylococcus aureus* (MIC < 0.1 mg/ml)

0254-6299/$ – see front matter © 2013 Published by Elsevier B.V. on behalf of SAAB.
http://dx.doi.org/10.1016/j.sajb.2013.02.171