DNA Topoisomerase I Inhibitors: Cytotoxic Flavones from *Lethedon tannaensis*

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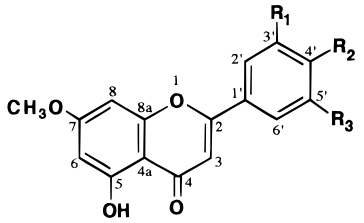
From ethyl acetate and methanolic extracts of *Lethedon tannaensis* leaves, which were cytotoxic against murine leukemia (P-388) and human nasopharynx carcinoma (KB) cells, one new and six known 5-hydroxy-7-methoxyflavones variously substituted on the B ring were isolated and their structures determined by spectral analysis. Compounds active against KB cells were velutin (**4**) (IC_{50} 4.8 μM), 7,3′,5′-tri-*O*-methyltricetin (**2**) (IC_{50} 22.2 μM), genkwanin (**6**) (IC_{50} 30.6 μM), and the novel compound, 7,3′,4′-tri-*O*-methyltricetin, named lethedocin (**1**) (IC_{50} 47.6 μM). These flavones required the presence of hydroxyl groups at C-5 and C-4′ and methoxyl groups at C-7 and C-3′ for inhibition of calf thymus DNA topoisomerase I activity.

Lethedon tannaensis Forst. (Thymelaeaceae) (= *Microsemma salicifolia*) is a tree from New Caledonia,[1] known as "wao" in folk medicine, and used as an antibacterial (Cosson, J. P. Unpublished observations). In our search for naturally occurring antitumor agents from plants, a methanolic extract of the leaves of *L. tannaensis* was found to display cytotoxic activity against murine leukemia (P-388) cells and inhibited DNA topoisomerase I activity.

DNA topoisomerases regularize the topological states of DNA during cellular events such as replication, transcription, and recombination, by transient cleavage of single or double DNA strands, by swiveling, and by religation of initial bonds. A substance that can stabilize DNA topoisomerase cleavable complex to stop the progression of DNA processes may be useful in cancer chemotherapy. Main anticancer drugs targeting topoisomerase I are camptothecin and topotecan, and those acting on topoisomerase II are doxorubicin, etoposide, and teniposide.[2,3]

Previous phytochemical studies on plants of the Thymelaeaceae have described the isolation of flavonoids[4] and biflavonoids such as chamaejasmins and chamaejasmenin from *Stellera chamaejasme*,[5] genkwanols from *Daphne genkwa*,[6] and daphnodorins from *Daphne odora*.[7] Here we report on the isolation of a new flavone, 7,3′,4′-tri-*O*-methyltricetin (**1**), named lethedocin, from *L. tannaensis*, together with six 5-hydroxy-7-methoxyflavones (**2**−**7**) variously substituted in the B ring. Some of these flavones exhibited significant cytotoxic activity against tumor cells.

The EtOAc and MeOH extracts of the leaves of *L. tannaensis* were significantly cytotoxic for P-388 murine leukemia cells, showing 45% and 65% inhibition at 100 μg/mL, respectively. TLC of the extracts indicated several Dragendorff reagent-positive spots, suggesting the presence of alkaloids, but it was later shown that such coloration was due to flavonoids. Bioassay-directed fractionation by reversed-phase chromatography of the MeOH extract yielded two groups of active fractions. The less polar active fractions and the EtOAc extract, purified by Si gel column chromatography, followed by preparative TLC, yielded seven flavones (**1**−**7**).



	R_1	R_2	R_3
1	OCH_3	OCH_3	OH
2	OCH_3	OH	OCH_3
3	OCH_3	OCH_3	OCH_3
4	OCH_3	OH	H
5	OCH_3	OCH_3	H
6	H	OH	H
7	H	OCH_3	H

Lethedocin (**1**) crystallized as colorless needles from MeOH, mp 194−195 °C. The HREIMS showed the molecular ion $[M]^+$ at *m/z* 344.0891, corresponding to a molecular formula of $C_{18}H_{16}O_7$. UV, IR, and NMR data suggested a fully aromatic flavone ring system. The ^{1}H-NMR spectrum displayed signals for three methoxyl groups between δ 3.8 and 4.0, five aromatic protons constituted by one singlet at δ 6.56, four doublets characteristic of two independent *meta*-protons on tetrasubstituted benzene rings, and two hydroxyl groups at δ 5.85 and 12.70, with the latter strongly chelated (HO-5) by a vicinal carbonyl group. Assignment of the 18 carbon signals of the ^{13}C-NMR spectrum was enabled from an analysis of the ^{1}H−^{13}C COSY and HMBC data. Long-range correlations were observed between the carbon atom at δ 165.6 (C-7) and both the methoxyl at δ 3.87 and the protons at δ 6.36 (H-6) and 6.47 (H-8), between the carbon at δ 138.9 (C-4′) and the methoxyl

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Table 1. ^{1}H-NMR Data for Compounds **1** and **2** and ^{13}C-NMR Data for Compounds **1**−**7** ($CDCl_3$)

	1		**2**		**1**	**2**	**3**	**4**	**5**	**6**[a]	**7**
position	δ_H	*J* (Hz)	δ_H	*J* (Hz)	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C
2					163.8	164.1	163.7	164.5	163.8	166.0	163.9
3	6.56 s		6.55 s		105.6	104.8	105.5	103.8	104.5	104.2	104.2
4					182.4	182.2	182.2	182.4	182.3	181.4	182.3
4a					105.6	105.5	105.5	105.2	105.4	106.5	105.5
5					162.1	162.2	162.1	161.5	162.0	159.0	162.1
6	6.36 d	2.2	6.35 d	2.2	98.2	98.1	98.1	98.0	98.0	99.2	98.0
7					165.6	165.5	165.6	165.4	165.4	167.1	165.4
8	6.47 d	2.2	6.47 d	2.2	92.7	92.7	92.6	92.9	92.6	93.7	92.5
8a					157.7	157.7	157.6	157.6	157.6	157.4	157.6
1′					126.9	122.4	126.4	122.5	123.6	122.9	123.4
2′	6.94 d	2.0	7.08 s		102.4	103.5	103.8	108.9	108.6	129.6	127.9
3′					152.5	147.4	153.5	147.5	149.2	117.2	114.4
4′					138.9	138.5	141.6	150.0	152.2	162.8	162.5
5′					149.6	147.4	153.5	115.3	111.0	117.2	114.4
6′	7.16 d	2.0	7.08 s		106.7	103.5	103.8	120.6	120.0	129.6	127.9
OH-5	12.70 s		12.75 s								
OMe-7	3.87 s		3.87 s		55.8	55.8	55.8	55.6	55.7	56.7	55.7
OMe-3′	3.94 s		3.97 s		56.1	56.5	56.3	55.9	56.0		
OMe-4′	3.97 s				61.2	OH	61.0	OH	56.0	OH	55.4
OMe-5′			3.97 s		OH	56.5	56.3				
OH-4′			5.90 s								
OH-5′	5.85 s										

[a] Spectrum run in CD_3OD.

Table 2. Cytotoxic Activity, *in Vitro*, of Compounds **1**−**7** for KB Cells and Inhibition of Calf Thymus DNA Topoisomerase I (Topo I) Activity

compd	P-388 IC_{50}, μg/mL	KB IC_{50}, μg/mL (μM)	Topo I MED,[a] μg/mL
1	*b*	16.4 (47.6)	*c*
2	*b*	7.7 (22.2)	1.0
3	*b*	19.4 (54.2)	*c*
4	10	1.5 (4.8)	0.1
5	*b*	19.4 (59.1)	*c*
6	10	8.7 (30.6)	>10.0
7	*b*	16.4 (55.0)	*c*
doxorubicin	0.05	0.005 (0.009)	
camptothecin	*c*	0.01 (0.03)	0.03

[a] MED, minimum efficient dose. [b] No inhibition at 100 μg/mL. [c] Not determined.

at δ 3.97 as well as the two aromatic protons at δ 6.94 (H-2′) and 7.16 (H-6′), and finally between the carbon atom at δ 152.5 (C-3′) and the methoxyl at δ 3.94 and the proton at δ 6.94 (H-2′). The carbon atom at C-5′ (δ 149.6) was only correlated with H-6′ (δ 7.16). The C-5-hydroxyl proton also showed cross-peaks with C-5, C-4a, and C-6. These results allowed the assignment of the three methoxyl groups on a basic tricetin flavone skeleton. Lethedocin (**1**) was thus established as 7,3′,4′-tri-*O*-methyltricetin.

The six other flavones were identified as 7,3′,5′-tri-*O*-methyltricetin (**2**),[8] 7,3′,4′,5′-tetra-*O*-methyltricetin (corymbosin) (**3**),[9] 7,3′-di-*O*-methylluteolin (velutin) (**4**),[10] 7,3′,4′-tri-*O*-methylluteolin (**5**),[11] 7-*O*-methylapigenin (**6**),[12] and 7,4′-di-*O*-methylapigenin (genkwanin) (**7**)[13] from analysis of their mass spectra and from 2D-NMR experiments. Full ^{13}C-NMR unambiguous assignments for these flavones are summarized in Table 1, together with the ^{1}H NMR data of compound **2**, previously isolated from *Betonica officinalis*.[8]

Velutin (**4**) exhibited demonstrable *in vitro* cytotoxicity against human nasopharynx carcinoma (KB) cells (IC_{50} 4.8 μM), while flavones **2**, **6**, and **1** were less active (IC_{50} values of 22.2, 30.6, and 47.6 μM, respectively) (Table 2). The other flavones were only slightly active with IC_{50} values of 54.2 μM for **3** and 55.0 μM for **7**. Compounds **4** and **6** were cytotoxic against P-388 (IC_{50} values of 10 μg/mL). Investigation of the possible molecular targets revealed that flavones **2** and **4** inhibit the calf thymus DNA topoisomerase I activity, but not tubulin assembly activity. Several natural cytotoxic flavonoids were recently shown to inhibit protein kinase C,[14] reverse transcriptase,[15] topoisomerase I,[16] topoisomerase II,[17] and tubulin polymerization into microtubules.[18,19] Myricetin, quercetin, and fisetin were described as potent inhibitors of topoisomerase I and II enzymes. The activities of these flavones are related to the presence of a ketone at C-4 and hydroxyl groups at C-3, C-7, C-3′, and C-4′.[17]

Flavones **4** and **2** efficiently inhibited calf thymus DNA topoisomerase I activity at concentrations of 0.1 and 1.0 μg/mL, respectively. Flavone-stabilized DNA topoisomerase I cleavable complexes are similar to those formed with camptothecin on agarose electrophoretic gels.[20] The structural requirements for topoisomerase I inhibition by the flavones of *L. tannaensis* are the presence of 5-hydroxy and 7-methoxy groups in the A ring and 4′-hydroxyl and 3′-methoxyl groups in the B ring. Flavones possessing only a 4′-hydroxyl or a 4′-methoxyl substituent were much less active (Table 2). Flavones **2**, **4**, and **6** displayed a significant correlation between *in vitro* antitumor activity on KB cell lines and DNA topoisomerase I inhibition. Although these flavones are weaker tumor cell-growth inhibitors than tubulin assembly inhibitors such as centaureidin,[18] and related polyhydroxyl and methoxyl flavonols[19] (CI_{50} 0.04−6.0 μg/mL), *L. tannaensis* flavones are of interest for their topoisomerase I inhibition and may have activities against slow-growing human tumor cells.[3]

Experimental Section

General Experimental Procedures. NMR spectra were recorded at 300 MHz (^{1}H) and 75 MHz (^{13}C) on a Bruker AC 300 spectrometer. CIMS and EIMS were obtained from a Nermag R 10-10 mass spectrometer. Column chromatography was carried out on Si gel (Merck, 0.2−0.63 mm) or reversed-phase Si gel (Merck, Lichroprep RP-2) and TLC on Si gel 60 F_{254} Merck.

Plant Material. The leaves of *L. tannaensis* were

collected near Goro, Noumea (New Caledonia), in May 1992. A voucher specimen (COPI 842) has been deposited at the Herbarium of the Forest Montravel Park, Noumea, New Caledonia.

Extraction and Isolation. The air-dried and pulverized leaves of *L. tannaensis* (460 g) were extracted at room temperature with EtOAc and MeOH and the extracts concentrated to dryness under reduced pressure, yielding residues of 56 and 45 g, respectively. Extensive Si gel column chromatography of the EtOAc extract furnished dotriacontanol (35 mg), 1-undecaprenol (130 mg), **7** (200 mg), **2**, (10 mg), and **3** (35 mg).

The MeOH extract was fractionated by reversed-phase chromatography (RP-2) eluted with a H_2O/MeOH gradient starting with H_2O and afforded 29 fractions. Bioactive fractions 17−23 and 24−29, cytotoxic against P-388 and KB cells, and significantly inhibiting topoisomerase I activity, were combined, and fractions 24−29 were chromatographed on a Si gel column eluted with a CH_2Cl_2/MeOH gradient, starting with CH_2Cl_2, and yielded eight fractions. Fraction 1 afforded **7** (99 mg), and fraction 8 gave **6** (90 mg). Fractions 2−7 were further purified by preparative TLC (CH_2Cl_2−MeOH, 95:5) to furnish the following pure flavones: **1** (13 mg), **2** (7 mg), **3** (35 mg), **4** (45 mg), and **5** (6 mg).

7,3′,4′-Tri-*O*-methyltricetin (**1**): $C_{18}H_{16}O_7$; yellow needles, mp 194−195 °C (MeOH); UV (EtOH) λ max (log ϵ) 334 (4.2), 270 (4.1), 240 (4.2), 210 (4.6) nm; IR (KBr) ν max 3398, 1654, 1639, 1389, 1164, 1114 cm^{-1}; ^1H and ^{13}C-NMR see Table 1; EIMS *m*/z 344 (M^+, 100), 329 (11), 315 (6), 301 (17), 283 (3), 273 (11), 258 (5), 227 (11), 167 (13), 158 (9), 135 (5), 113 (3), 95 (6); HREIMS *m*/*z* 344.0891 $[M]^+$ (calcd for $C_{18}H_{16}O_7$, 344.0896).

7,3′,5′-Tri-*O*-methyltricetin (**2**): $C_{18}H_{16}O_7$; not crystallized (lit.[8] mp 218−220 °C); IR (KBr) ν max 3443, 2935, 2855, 1658, 1620, 1505, 1466, 1383, 1345, 1255, 1200, 1165, 1121, 781, 743, 660 cm^{-1}; ^1H- and ^{13}C-NMR see Table 1; EIMS *m*/*z* 344 (M^+, 100), 328 (8), 315 (49), 301 (21), 295 (11), 285 (9), 273 (16), 255 (37), 236 (15), 227 (26), 167 (18).

1-Undecaprenol: $C_{55}H_{90}O$; ^1H NMR ($CDCl_3$) δ 5.43 (1 H, td, 7.2, 1.3, =C*H*CH_2OH), 5.11 (10 H, s br, =CH−), 4.07 (2 H, d, J = 7.2 Hz, −C*H*$_2$OH), 2.03 (40 H, m, −$[CH_2]_{20}$−), 1.73 (3H, d, 1.0, −CH_3), 1.67, (24 H, s br, 8 × −CH_3), 1.58, (9H, s br, 3 × −CH_3); CIMS (NH_3) *m*/*z* 784 $[MNH_4]^+$, 767 $[MH]^+$.

Bioassays. Bioassays were carried out at the Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France, and the Research Center of Rhône-Poulenc Rorer, Vitry-sur-Seine, France. The cytotoxic potential of the flavones was measured on *in vitro* cultured P-388 and KB cells.[21] Inhibition of calf thymus DNA topoisomerase I activity was performed with circular pBR322 DNA as substrate.[20]

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