Pharmaceutical Institute, University of Bonn, Germany

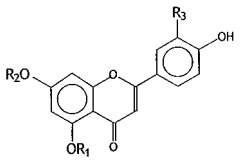
Isolation and structure elucidation of ligustroflavone, a new apigenin triglycoside from the leaves of *Ligustrum vulgare* L.

A. Pieroni and P. Pachaly

A new flavone, apigenin-7-O-β-(2″,6″-di-α-rhamnopyranosyl)-glucopyranoside, named ligustroflavone, was isolated from the leaves of common privet (*Ligustrum vulgare* L., Oleaceae), whose popular use was well known in the Mediterranean historical medicine and ethnomedicine as anti-inflammatory. The structures of other five apigenin and luteolin derivates, isolated from the polar fractions of the methanolic leaf extracts, were elucidated.

1. Introduction

Common privet (*Ligustrum vulgare* L., Oleaceae) represents a quite common shrub in Southern, Western and Middle Europe; its medicinal attitudes are only known in the Mediterranean regions. Already Disoscorides (1st century a. C.) described the use of the leaves of this plant, which were chewed against smooth inflammations, while a decoction of the aerial parts had to be active against burns and headache. Notes from the 16th century written by the Tuscanian physician Mattioli [1] confirmed such a utilisation and Cazin [2] in the last century quoted its water decoctions, sweetened by honey, in gurgles as remedy against inflammation. In ethnobotanical researches of the last decades only very few reports about the use of privet leaves can be found: in a very closed area in Southern Italy decoctions should be still used as smooth anti-inflammatory [3], while in Cyprus the plant is considered anti-rheumatic [4] and in Azerbaijan anti-hypertensive [5]. Phytochemical studies on aerial parts of *Ligustrum* ssp. were carried out on Asiatic species and they mainly studied the occurrence or iridoids [6–15] while for the European common privet only the fruits were investigated recently [16–19]. Nevertheless, common privet extracts showed strong anticomplementary activity in preliminary studies [20].



Compounds	R_1	R_2	R_3
Compound 1 (Apigenin)	H	H	H
Compound 2 (Luteolin)	H	H	OH
Compound 3 (Apigenin-7-O-β-glucoside)	H	glc	H
Compound 4 (Apigenin-5-O-β-glucoside)	glc	H	H
Compound 5 (Luteolin-7-O-β-glucoside)	H	glc	OH
Compound 6 (Apigenin-7-O-β-rutinoside)	H	glc(6→1) rha	H
Compound 7 (Apigenin-7-O-β-(2″,6″-α-rhamnosyl)-glucoside)	H	glc(2→1) rha, (6→1) rha	H

Fig. 1: Isolated flavons

2. Investigations, results and discussion

Seven compounds were isolated by CC from a methanolic extract of privet leaves.

NMR spectra (^{1}H NMR and APT-NMR data) of compounds **1**–**6** were compared with literature data [21–24] and identified as apigenin, luteolin, apigenin-7-O-β-glucoside, apigenin-5-O-β-glucoside, luteolin-7-O-β-glucoside and apigenin-7-O-β-rutinoside (Fig. 1).

Compound **7** showed a brown fluorescence under UV light at 366 nm and after spraying with NP reagent a

Table: ^{13}C NMR data of ligustroflavone (compound 7) and reference data of apigenin-7-O-β-rutinoside and apigenin-7-O-β-neohesperidoside

	Compound 7, ligustroflavone	Apigenin-7-O-β-rutinoside	Apigenin-7-O-β-neohesperidoside
^{13}C NMR	δ (ppm)	δ (ppm)	δ (ppm)
C-2	164.2	164.1	164.4
C-3	102.9	102.8	103.3
C-4	181.6	181.8	181.8
C-5	161.0	161.5[a]	161.2
C-6	99.2	99.6[aa]	99.9[b]
C-7	162.2	163.7	162.7
C-8	94.2	94.8	94.8
C-9	156.7	157.3	157.0
C-10	105.3	105.4	105.6
C-1′	120.5	121.3	121.2
C-2′	128.3	128.4	128.4
C-3′	116.0	116.0	116.1
C-4′	161.5	161.1[a]	161.2
C-5′	116.0	116.0	116.1
C-6′	128.3	128.4	128.4
C-1″ (glc)	99.7	100.7[aa]	100.6[b]
C-2″ (glc)	76.9	73.2	77.0[bb]
C-3″ (glc)	75.4	76.6[aaa]	77.0[bb]
C-4″ (glc)	69.7	70.0[aaaa]	70.2[bbb]
C-5″ (glc)	76.1	77.2[aaa]	77.3[bb]
C-6″ (glc)	66.5	66.7	61.0
C-1‴ (rha1)	100.3	100.3[a]	100.6[b]
C-2‴ (rha1)	70.2*	70.4[aaaa]	70.6[bbb]
C-3‴ (rha1)	70.4*	70.8[aaaa]	70.9[bbb]
C-4‴ (rha1)	71.8**	72.1	72.3
C-5‴ (rha1)	68.2	68.1	68.4
C-6‴ (rha1)	17.7***	17.5	17.9
C-1⁗ (rha2)	100.4	–	–
C-2⁗ (rha2)	70.2*	–	–
C-3⁗ (rha2)	70.7*	–	–
C-4⁗ (rha2)	72.0**	–	–
C-5⁗ (rha2)	68.2	–	–
C-6⁗ (rha2)	18.0***	–	–

Assignments with the same superscripts may be interchanged

green colour. Its ^{1}H NMR spectrum presented in the region of the aromatic protons the typical signals of an apigenin derivate: proton signals of H-6 and H-8 were recognisable at 6.38 and 6.70 ppm respectively, with a *meta* coupling constant of 2.1 Hz; signals of the protons H-2′, H-3′, H-5′ and H-6′ were located at 6.96 and 7.90 ppm respectively, with a coupling constant of 8.7 Hz, what spoke for a 1,4-disubstituted B ring. The single signal at 6.81 ppm, was to assign to the H-3 proton. The couplings between H-6 and H-8 and H-2′/H-6′ and H-3′/H-5′ were also confirmed by H,H-COSY spectrum.

In the field of the sugar protons signals a big band was recovered between 3 and 4 ppm. Anomeric proton signals were found at 5.21 (coupling constant: 7.2 Hz, what indicated a β-glycosidic linkage), at 5.13 ppm (coupling constant: 1.2 Hz, which is typical for α-glycosidic linkage) and at 4.55 ppm (coupling constant: 1.2 Hz, speaking for an other α-glycosidic linkage). The occurrence of two possible molecules of rhamnose were confirmed by two sugar methyl signals at 1.08 ppm and 1.20 ppm. Such observations pointed out that compound **7** is an apigenin triglycoside.

In the Table, ^{13}C NMR data are reported. In the field between 65 and 80 ppm it is possible to recognise twelve CH group signals and one CH_2 group signal; together with three anomeric signals at 99.8, 100.3 and 100.4 ppm and with the CH_3 signals at 17.7 and 18.0 ppm there are sexteen sugar signals. The elucidation of the sugar component could be carried out the comparison with literature data of apigenin-7-O-β-rutinoside (apigenin-7-O-β-(1→6)glucopyranoside) and apigenin-7-O-β-neohespeidoside (apigenin-7-O-β-(1→2)glucopyranoside) [21–24]. In compound **7** both glucose signals of C-2″ and C-6″ (at 76.9 and 66.5 ppm respectively) are shifted about 5 ppm down-fields towards free glucose data. This demonstrates the occurrence of an O-interglycosidic linkage in both positions.

The glucose moiety is linked in 6 and in 2 α-glycosidic with a rhamnose and represents a tryglicosidic derivate of rutinose (or neohesperidose). Moreover, the typical shifts of the signals of C-6, C-8 and C-10 (if compared with data of a free apigenin) showed that the triglycoside is linked to the apigenin skeleton in C-7.

Coupling of protons and carbon signals, especially for the anomeric ones, was confirmed by HETCOR experiments (Fig. 2).

(−)-FAB-MS data of compound **7** showed a molecule signal at m/z 723 $[M-H]^-$ and an important fragment at m/z 577 $[M-H\text{-rhamnose}]^-$. Such evidence agreed with the molecular formula $C_{33}O_{18}H_{40}$, which correspond to the

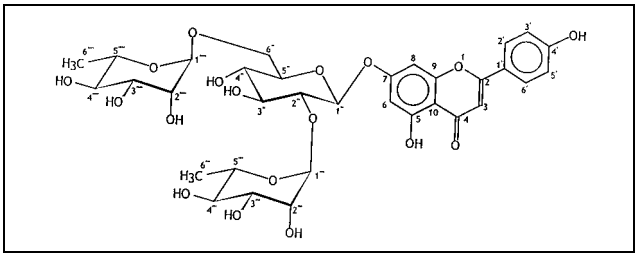


Fig. 3: Ligustroflavone

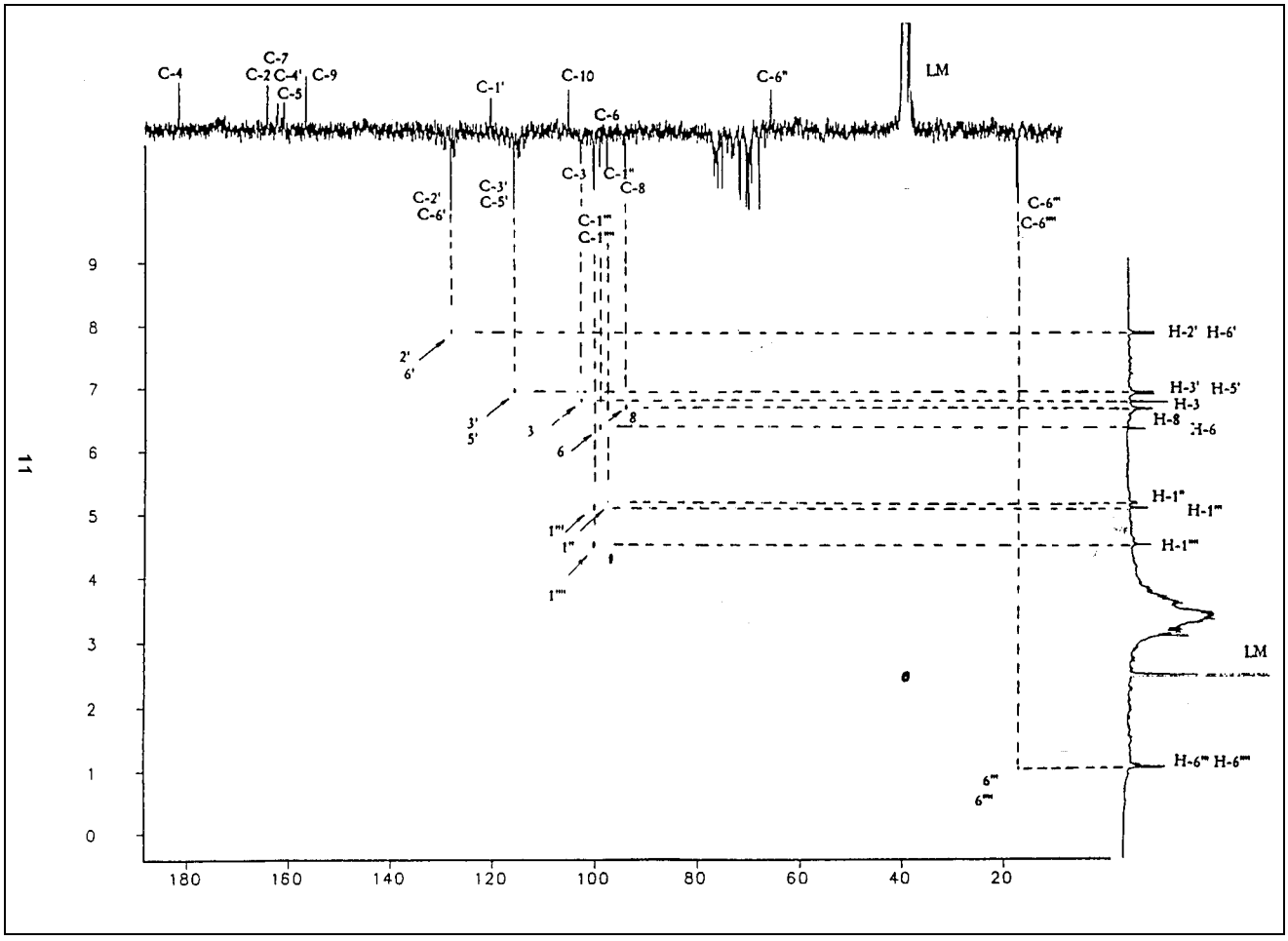


Fig. 2: HETCOR spectrum of ligustroflavone

structure of a new flavone triglycoside: apigenin-7-O-β-(2″,6″-di-α-rhamnopyranosyl)-glucopyranoside. It represents a new natural product, for which the name ligustroflavone was adopted (Fig. 3).
Flavonoids with triglycoside components are quite rare in nature and only in recent years other similar structures with a flavonol skeleton were isolated and elucidated [25–27].

3. Experimental

3.1. Instruments

CC was carried out on silica gel (63–100 μm, Merck) and on Sephadex LH-20® (Pharmacia) columns. TLC plates used were silica gel 60 F_{254} layers (Merck). ^{1}H NMR and ^{13}C NMR spectra were recorded in DMSO-d_6 (300 and 75 MHz respectively) with a Varian XL 300 instrument.
UV spectrometer was a Hewlett Packard 8452A Dioden-Array, while FAB-MS spectra were recorded in positive and negative ion mode (matrix: glycerol, NBA) by a FAB-MS Kratos instrument.

3.2. Plant material

Leaves of *Ligustrum vulgare* L. were collected in Garfagnana Valley, Lucca, Tuscany, central Italy, at the end of September, 1994. A voucher speciment is deposited at the author address.
The leaves were dried at room temperature for three weeks.

3.3. Extraction and isolation procedures

Dried and powdered leaves (1950 g) were macerated repeatedly with MeOH (23 l). The dried residue (354 g) was suspended in water and extracted successively in petroleum ether (8 l), $CHCl_3$ (7 l), EtOAc (8 l) and n-BuOH (4 l).
The EtOAc fraction (9.1 g) was subjected to CC on silica gel (63–100 μm) and eluted with $CHCl_3$/MeOH 4 : 1 ≫ 1 : 1 (1670 ml), giving subfractions **I**–**IV**. These subfractions were chromatographed on Sephadex LH-20® columns, using MeOH or MeOH/H_2O 4 : 1, 3 : 1 and 2 : 1 as eluents.
From the subfraction **I** constituents **1** (7.0 mg) and **2** (9.2 mg), from subfraction **II**, constituents **3** (11.5 mg) and **4** (6.0 mg), from **III** compund **5** (10.3 mg) and from **IV** substance **6** (9.6 mg) were isolated.
The n-BuOH fraction (7.1 g) was eluted directly by CC on Sephadex LH-20® with MeOH/H_2O 3 : 1 ≫ 1 : 1 at first (520 ml) and than with MeOH/H_2O 1 : 1 (510 ml), giving compound **7** (7.8 mg).
Isolated compounds were identified as flavonoidic structures, detecting them on silica gel layers and using EtOAc/H_2O/HCOOH/CH_3COOH (100 : 27 : 11 : 11) as eluent and natural product reagent, NP (5% methanolic solution of diphenylboric acid aminoethyl ester) as spray reagent. Green and yellow coloured spots were obtained.

3.4. Ligustroflavone

Apigenin-7-O-β-(2″,6″-di-α-rhamnopyranosyl)-glucopyranoside (**7**). Brown amorphous powder, 7.8 mg (representing 6.8 mg/kg dried material), soluble in MeOH/H_2O 4 : 1, less soluble in MeOH; R_f value on silica gel 60 F_{254} layers, eluent: EtOAc/H_2O/HCOOH/CH_3COOH (100 : 27 : 11 : 11): 0.40; brown fluorescence under UV light at 366 nm and green fluorescence after spraying with NP reagent. UV (1.0 mg/10 ml. MeOH, λ_{max}, nm): 268, 336; (−)-FAB-MS (NBA, % relative abundance): m/z 723 $[M-H]^-$ (1.7), m/z 577 $[M-H\text{-rhamnose}]^-$ (15.1); ^{1}H NMR (DMSO-d_6, 300 MHz, δ, ppm): 7.90 (d, J = 8.7 Hz, H-2′, H-6′), 6.96 (d, J = 8.7 Hz, H-3′, H-5′), 6.81 (s, H-3), 6.70 (d, J = 2.1 Hz, H-8), 6.38 (d, J = 2.1 Hz, H-6), 5.21 (d, J = 7.2 Hz, H-1″), 5.13 (d, J = 1.2 Hz, H-1‴), 4.55 (d, J = 1.2 Hz, H-1⁗), 3.95–3.10 (m, H-2″-H-6″, H-2‴-H-5‴, H-2⁗-H-5⁗), 1.20 (d, J = 6.3 Hz, H-6‴), 1.20 (d, J = 6.3 Hz, H-6⁗); ^{13}C NMR (DMSO-d_6, 75 MHz): see Table.

Acknowledgements: Special thanks are due to the European Commission in Brussels, for the 24 months Marie Curie Fellowship assigned to Dr. Andrea Pieroni (CT-94-8047) inside the Programm FAIR; to Ms. Ilona Knoblauch, Pharmaceutical Institute, Bonn University for the technical assistance in NMR spectroscopy; to Dr. Hans-Martin Schiebel, Institut for Organic Chemistry, Braunschweig University, Germany, for recording MS spectroscopic data.

References

1 Mattioli, P.: I Discorsi di M. Pietro Andrea Matthioli, p. 187, Appresso Vincenzo Valgrisi, Venice, 1578 (Reprint 1968)
2 Cazin, F.-J.: Traité pratique et raisonné des plantes médicinales indigènes, p. 1074, P. Asselin, Paris, 1868 (Reprint: éditions de l'envol, Mane, France, 1997)
3 Antonone, R.; De Simone, F.; Morrica, P.; Ramundo, E.: J. Ethnopharmacol. **22**, 295 (1988)
4 Arnold, N.; Arnold, H.-J.; Gehu, J.-M.; Gehu-Franck, J.; in: Ethnopharmacologie: sources, méthodes, objectifs; Actes du 1er Colloque Européen d'Ethnopharmacologie, Metz, 22.-25. März 1990, pp. 179–181, ORSTOM, Paris, 1993
5 Hammermann, A. F.; Damirov, J. A.; Sokolw, W. S.: Planta Med. **20**, 347 (1971)
6 Kikuchi, M.; Yamauchi, Y.: Yakugaku Zasshi **105**, 142 (1985)
7 Inoue, K.; Nishioka, T.; Tanahashi, T.; Inouye H.: Phytochemistry **21**, 2305 (1982)
8 Kikuchi, M.; Yamauchi, Y.: Nippon Nogei Kagaku Kaishi **56**, 939 (1988)
9 Fukuyama, Y.; Koshino, K.; Hasegawa, T.; Yamada, T.; Nakagawa, K.: Planta Med. **53**, 427 (1987)
10 Kikuchi, M.; Yamauchi, Y.; Takahashi, Y.: Yakugaku Zasshi **109**, 460 (1989)
11 Kuwajima, H.; Matsuuchi, K.; Takishi, K.; Inoue, K.; Fujita, T.; Inouye, H.: Phytochemistry **28**, 1409 (1989)
12 Inouye, H.; Nishioka, T.: Tetrahedron **28**, 4231 (1972)
13 Kikuchi, M.; Yamauchi, Y.; Nagaoka, I.; Sugiyama, M.; Takahashi, Y.: Yakugaku Zasshi **108**, 647 (1988)
14 He, Z. D.; Ueda, S.; Akaji, M.; Fujita, T.; Inoue, K.; Yang, C. R.: Phytochemistry **36**, 709 (1994)
15 Fukuda, T.; Kitada, Y.; Chen, X.-M.; Yang, L.; Miyase, T.: Chem. Pharm. Bull. **44**, 2173 (1996)
16 Willems, M.: Arch. Pharm. **320**, 1245 (1987)
17 Willems, M.: Arch. Pharm. **321**, I, 229 (1988)
18 Willems, M.: Arch. Pharm. **321**, II, 357 (1988)
19 Willems, M.: Planta Med. **54**, 66 (1988)
20 Pieroni, A.; Van Poel, B.; Vlietinck, A.J.; Heimler, D.; in: Healing yesterday and today. Tomorrow? Proceedings of the 3rd European Ethnopharmacological Colloquium, Genoa, Italy, 29. 05.–02. 06. 1996, CD-ROM, Edizioni Erga, Genoa, Italy, 1997
21 Harborne, J. B.: The Flavonoids: Advances in research since 1990, Chapman & Hall, London, 1993
22 Harborne, J. B., The Flavonoids: Advances in research since 1982, Chapman & Hall, London, 1986
23 Mabry, T. J.; Markham, K. R.; Thomas, M. B.: The systematic Identification of Flavonoids, Springer Verlag, Berlin, 1970
24 Agrawal, P. K.: Carbon-13 NMR of Flavonoids, Elsevier, Amsterdam, 1989
25 Kijima, H.; Ide, T.; Otsuka, H.: J. Nat. Prod. **58**, 1753 (1995)
26 Kamusiime, H.; Pedersen, A. T.; Andresen, Ø. M.; Kiremire, B.: Int. J. Pharmacognosy **34**, 370 (1996)
27 Cimanga, K.; de Bruyne, T.; Van Poel, B.; Ma, Y.; Claeys, M.; Pieters, L.; Kambu, K.; Tona, L.; Bakana, P.; Vanden Berghe, D.; Vlietinck, A. J.: Planta Med. **63**, 220 (1997)

Received April 23, 1999
Accepted June 29, 1999

Dr. Andrea Pieroni
Dipartimento di Nutrizione della Pianta
Università degli Studi di Firenze
Venloer Str. 233a
D-50823 Köln
experiences@netcologne.de