Statin-like Principles of Bergamot Fruit (*Citrus bergamia*): Isolation of 3-Hydroxymethylglutaryl Flavonoid Glycosides

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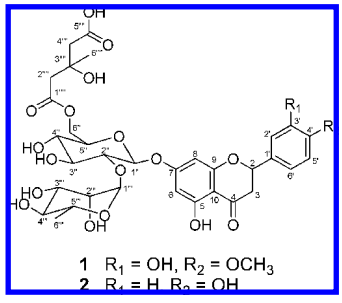
The 3-hydroxy-3-methylglutaryl neohesperidosides of hesperetin (brutieridin, **1**) and naringenin (melitidin, **2**) were isolated and detected from the fruits of bergamot (*Citrus bergamia*). The structures of these compounds were determined by spectroscopic and chemical methods.

Bergamot is the common name of the fruit *Citrus bergamia* Risso, which belongs to the family Rutaceae, subfamily Esperidea. The uniqueness of bergamot trees is represented by a habitat that is virtually restricted to the coastal region of the Ionian Sea in the southern Calabrian region of Italy.[1] Hence, the production of bergamot has been a flagship product of Calabrian agriculture for many years, and its volatile fraction is still used in the cosmetic and perfumery industries,[2−6] despite the presence on the market of synthetic surrogates. Another peculiarity of the fruit is the considerable abundance and variety of nutraceuticals, such as naringin, neoeriocitrin, and neohesperidin, which are present in the juice on the order of hundreds of ppm. Other flavonoids, such as rhoifolin, neodiosmin, and some chryosoeriol derivatives, are present in smaller amounts.[7−9] Bergamot juice has not reached the popularity of other citrus juices in the daily diet for its organoleptic properties, but it is used to fortify fruit juice in place of synthetic additives. Different tissues of the fruit also produce compounds such as diosmin and poncirin.[10] Rare higher molecular weight flavonoids are present in bergamot fruit juice[9] and may play a role in the anticholesterolemic activity known in the local folk medicine.[11]

Apart from the well-known components of the fruit of *C. bergamia* thoroughly investigated in the last three or four decades, a detailed profiling of bergamot extract by tandem mass spectrometry (MS/MS) has led to the discovery of some flavonoid diglycosides carrying the 3-hydroxy-3-methylglutaric acid (HMG) moiety. In the present study, two new molecules have been isolated and identified as HMG conjugates of neohesperidin and naringin, namely, brutieridin (**1**, hesperetin 7-(2″-α-rhamnosyl-6″-(3‴′-hydroxy-3‴′-methylglutaryl)-β-glucoside)) and melitidin (**2**, naringenin 7-(2″-α-rhamnosyl-6″-(3‴′-hydroxy-3‴′-methylglutaryl)-β-glucoside)).



The HPLC-UV and (−) ESIMS chromatograms of the bergamot extract showed compound **1**, eluting at 10.05 min; its structure determination was performed on a pure sample, isolated by semipreparative HPLC MS separation of the whole extract of the peeled fruit.

Several analytical experiments were performed to assess the structure of **1**. The positive HRESIMS provided the $[M + H]^+$ of 755.2387, corresponding to the elemental composition $C_{34}H_{43}O_{19}$ with −0.80 ppm accuracy. The ESIMS/MS obtained from both protonated and deprotonated molecular ions showed highly diagnostic fragment ions. In particular, a structural feature of the positive-ion MS/MS suggested that a HMG moiety may be selectively located on the glucosyl moiety since the peaks at *m/z* 453.1610 (B_2^+)[12] and *m/z* 609.1836 are produced by loss of the aglycon and of the rhamnose units, respectively. The gas-phase chemistry of the deprotonated molecular $[M - H]^-$ ions showed an ion at *m/z* 489.1357, which is a typical fragment of flavonoid diglycosides[13] originating from the breakage of the $O-C_1$ and C_2-C_3 bonds of the hexose directly attached to the aglycon. The formation of this ion suggested that (i) the rhamnose is connected at the C-2 position of the glucose and (ii) the HMG moiety might be linked at either C-3, C-4, or C-6 of the glucose unit.

The IR spectrum of **1** revealed typical absorption bands for hydroxy, carboxyl, and carbonyl ketone groups. The UV spectrum showed two maxima at 285 and 324 nm. The ^{1}H NMR data (summarized in Table 1) clearly showed the structure of hesperetin as the aglycon. The presence of a singlet (δ_H 3.85, 3H, s) could be assigned unambiguously to the methoxy protons of the flavonol moiety of compound **1**. The aromatic protons of the B ring appeared as multiplets (δ_H 6.96, 1H, m, H-2′; δ_H 6.90, 1H, m, H-5′; and δ_H 6.92, 1H, m, H-6′), while the other multiplets present (δ_H 6.17, 1H, m, H-6 and δ_H 6.14, 1H, m, H-8) were assigned to the aglycon protons at positions 6 and 8, respectively (A ring). The HSQC spectrum correlated the aromatic protons described above to signals at δ_C 114.6 (d, C-2′), 112.6 (d, C-5′), 119.1 (d, C-6′), 98.1 (d, C-6), and 99.3 (d, C-8).

The protons at positions 2 and 3 of the aglycon provided resonances at δ_H 5.38 (1H, dd, J = 2.9, 12.7 Hz, H-2) and δ_H 2.77−3.09 (1H, dd, J = 2.9, 17.2 Hz, H-3b)−(1H, dd, J = 12.7, 17.2 Hz, H-3a), which were found to be correlated in the COSY experiment. The hesperidose protons were recognized in the spectrum range δ_H 5.1−3.3. The typical signal of the acetal moiety (doublet) of the glucose unit was identified at δ_H 5.07 (1H, d, J = 7.6 Hz, H-1″) correlated to C-1″ at δ_C 96.8 (d, C-1″) (Table 1). The other protons and carbons of the glucose ring showed the expected correlations in the 2D NMR experiments performed. The shift to a lower field of the methylene proton resonance at δ 4.19 with respect to that of the equivalent protons of the neohesperidin standard found in the range 3.3−3.9 ppm suggested that the primary alcoholic function of the glucose unit of **1** is esterified with the HMG moiety.

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Table 1. ^{1}H NMR and ^{13}C NMR Spectroscopic Data for Compounds **1** and **2**

	brutieridin (**1**)		melitidin (**2**)	
position	δ_H (J in Hz)	δ_C mult.	δ_H (J in Hz)	δ_C mult.
OCH_3		56.5, CH_3		
2	5.38 dd (2.9, 12.7)	80.5, CH	5.40 dd (2.7, 13.0)	80.7, CH
3a	3.09 dd (2.9, 17.2)	44.3, CH_2	3.14 dd (2.7, 17.2)	44.3, CH_2
3b	2.77 dd (12.7, 17.2)			
	2.73 dd (13.0, 17.2)			
4		198.3, qC		198.5, qC
5		164.5, qC		164.6, qC
6	6.17 m, ar	98.1, CH	6.17 m, ar	98.0, CH
7		164.9, qC		164.9, qC
8	6.14 m, ar	99.3, CH	6.14 m, ar	99.3, CH
9		166.4, qC		166.4, qC
10		105.0. qC		105.0, qC
1′		132.9, qC		130.9, qC
2′	6.96 m, ar	114.6, CH	6.96 m, ar	129.1, CH
OCH_3-3′	3.85 s	147.8, qC		116.4, CH
H-3′	6.70 m, ar			
4′		149.4, qC		159.1, qC
5′	6.90 m, ar	112.6, CH	6.70 m, ar	116.4, CH
6′	6.92 m, ar	119.1, CH	6.97 m, ar	129.3, CH
1″	5.07 d (7.6)	96.8, CH	5.08 d (7.6)	96.8, CH
2″	3.66 dd (7.7, 9.0)	78.9, CH	3.66 dd (7.6, 9.1)	78.9, CH
3″	3.60 dd (9.0, 9.1)		3.39 dd (9.0, 9.1)	79.0, CH
4″	3.35 dd (9.1, 9.8)	70.0, CH	3.37 dd (9.0, 9.6)	70.0, CH
5″	3.69 ddd (2.2, 7.1, 9.8)	75.4, CH	3.67 ddd (1.8, 7.2, 9.6)	75.4, CH
6″a	4.44 dd (2.0, 11.9)	64.6, CH_2	4.43 dd (1.8, 11.2)	64.6, CH_2
6″b	4.19 dd (7.1, 11.9), 4.20 dd (7.1, 11.2)			
1‴	5.25 d (1.6)	102.5, CH	5.25 d (1.3)	102.5, CH
2‴	3.93 dd (1.6, 3.4)	73.9, CH	3.93 dd (1.6, 3,2)	73.9, CH
3‴	3.59 dd (3.4, 9.4)	72.1, CH	3.59 dd (3,2, 9.5)	72.2, CH
4‴	3.39 dd (9.4, 9.5)	78.8, CH	3.66 dd (9.3, 9.5)	78.8, CH
5‴	3.90 dq (6.2, 9.5)	71.7, CH	3.90 m	71.7, CH
6‴	1.30 d (6.2)	18.3, CH_3	1.29 d (6.1)	18.2, CH_3
1⁗		172.5, qC		172.5, qC
2⁗	2.65−2.52 m	46.0, CH_2	2.65−2.52 m	45.9, CH_2
3⁗		70.8, qC		70.8, qC
4⁗	2.65−2.52 m	46.3, CH_2	2.65−2.52 m	46.3, CH_2
5⁗		175.7, qC		175.2, qC
6⁗	1.26 s	27.7, CH_3	1.26 s	27.8, CH_3

COSY, HSQC, and TOCSY experiments were used to corroborate the assignments made for the glucose protons of **1**. Similarly, the structure of the rhamnose moiety was supported by COSY data: in particular, the acetal proton provided a signal at δ_H 5.25 (1H, d, J = 1.6 Hz, H-1‴), and the 6‴ methyl protons gave a doublet at δ_H 1.30 (3H, d, J = 6.2 Hz, H-6‴). The latter were correlated by COSY to the 5‴ proton at δ_H 3.90 (1H, dq, H-5‴) (Table 1).

The presence of the hesperidin core was confirmed by two independent hydrolysis experiments. First, a conventional basic hydrolysis in saturated carbonate solution, monitored by HPLC-UV-MS (see Supporting Information), showed after 20 h the complete disappearance of **1** and the formation of a product whose deprotonated molecular ion appeared at m/z 609, whereas its retention time coincides with that of the hesperidin standard. A second reaction product, characterized by the m/z value of 161, appeared in the chromatographic run with t_R 0.5 min. This species corresponded to the HMG acid released by basic hydrolysis. The molecule was collected by semipreparative HPLC and confirmed by NMR analysis (see Experimental Section).

Enzymatic hydrolysis was carried out on **1** in the presence of hesperidinase and monitored by HPLC-MS/UV (Supporting Information) in the conditions previously reported. After 4 h a chromatographic peak was observed, corresponding to a compound with a deprotonated molecular ion of 607 amu. A high-resolution MS experiment gave an elemental composition that corresponded to that of the starting molecule (**1**) less a rhamnose moiety. After 300 h of reaction, the predominant product was hesperetin.

Compound **2** was characterized by the same procedure as described for **1**. The chromatogram of the fruit extract injected into the HPLC showed a peak at a retention time of 9.85 min. A yellowish powder was obtained after semipreparative HPLC purification. The HRESIMS showed a $[M + H]^+$ ion at m/z 725.2290, which corresponded to the elemental composition $C_{33}H_{41}O_{18}$ (0.36 ppm error); the UV and IR spectra were similar to those of compound **1**. All the chemicophysical parameters (Table 1 and Supporting Information) strongly suggest the proposed structure. The main difference in the ^{1}H NMR spectrum is the lack of the methyl signal at δ_H 3.85 and the different shift of the C-3′ aromatic carbon atom in the ^{13}C NMR spectrum (Table 1).

The hydrolysis experiments, described above, were performed also on compound **2**. The enzymatic treatment showed after 4 h an intermediate product, which, when submitted to HRMS experiments, confirmed the loss of the rhamnose moiety. The final product of the enzymatic reaction was the naringin aglycon, as confirmed by the mass spectrum and by matching the retention time of the standard naringenin. Basic hydrolysis was also performed and gave naringin as a final product, confirming the ester-like nature of the side chain (Supporting Information).

Brutieridin (**1**) and melitidin (**2**) are present in bergamot fruit in concentrations ranges of approximately 300−500 and 150−300 ppm, respectively, as a function of the ripening stage; these compounds may be found either in the juice or in the albedo and flavedo of bergamot. To the best of our knowledge, the identification of similar HMG conjugates in plants has been described only for Roman chamomile (*Chamaemelum nobile*).[14]

Experimental Section

General Experimental Procedures. UV spectra were recorded in MeOH solution on a Varian Cary 50 spectrophotometer. IR spectra

were acquired on a PerkinElmer Spectrum One FT-IR instrument. LC-MS/UV chromatograms were obtained using a Fractionlynx system from Waters (Milford, MA) working in analytical mode and equipped with a ZMD mass spectrometer and a 486 UV detector. A monolithic column ONYX-C_{18} (100 × 3 mm) from Phenomenex (Torrance, CA) was used for the run. Purification of compounds **1** and **2** from Castagnaro bergamot samples was performed with the same system, working in the semipreparative mode (21 mL/min) and using an AXIA synergy fusion column (21.2 × 100 mm) from Phenomenex. The UV detector was set at 280 nm. ^{1}H NMR spectra were recorded at 25 °C on a Bruker Avance 500 MHz (^{1}H: 500.13 MHz, ^{13}C: 125.77 MHz) instrument (Rheinstetten, Germany), dissolving purified samples in CD_3OD, except where otherwise stated. HRMS were acquired on a Q-star pulsar-*i* (MDS Sciex Applied Biosystems, Toronto, Canada), equipped with an ion-spray source on samples collected from the analytical chromatographic runs. The MS/MS data were obtained at 15 and −40 eV collision energy voltages in the positive and negative mode, respectively, using N_2 as collision gas.

Basic hydrolyses were performed by dissolving 1 mg of purified samples in 1 mL of saturated Na_2CO_3 water solution. The reaction lasted 20 h, and the formation of products was followed by HPLC UV/MS until the disappearance of the reactant. NMR data (DMSO-d_6) of the 3-hydroxymethylglutaric acid obtained after hydrolysis: CH_3-3, δ_H 1.22 (s), δ_C 28.0 (q), CH_2-2,4, δ_H 2.46 (m), δ_C 46.5 (q), 1,5-COOH, δ_H 8.21 (s), δ_C 173.0 (s), OH-3, δ_H not detected, δ_C 69.0 (s).

Enzymatic hydrolyses were performed as follows: 1 mg of purified product and 1 mg of hesperidinase were dissolved in 1 mL of McIlvaine's buffer at pH 3.5. The reaction temperature was maintained at 37 °C. The reaction lasted until the appearance of the aglycon moiety as the major product. The product formation was followed by HPLC UV/MS until the aglycon was evident.

Plant Material. Bergamot fruits (*Citrus bergamia*) of three different cultivars (Castagnaro, Fantastico, and Femminello) were harvested in different periods (October, November, and December 2007) by Unionberg Association, located at Condofuri Marina (RC, Italy). Fruit samples were classified by Furfari nursery as number 02004450801/814/06/ZP.

Chemicals. HPLC grade solvents were purchased from Carlo Erba (Milan, Italy). Standard flavonoids were purchased from Extrasynthese (Genay, France). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Extraction and Isolation. The flavonoid extract of bergamot was obtained by chopping the fruit in a ternary mixture of methanol, ethanol, and chloroform, 65:30:5 (v/v/v); subsequently, it was filtered on a Buchner funnel, concentrated, and passed through a C_{18} cartridge (Supelclean LC-18, 60 mL, 10 g; Supelco, St. Louis, MO), previously activated with MeOH and washed with water. The loaded material was washed with 15 mL of water and then eluted with 2 × 15 mL of MeOH. The eluate was evaporated under vacuum to dryness. For analytical chromatography a solution of 1000 ppm was injected into the HPLC. The run time was 30 min, the flow rate was 1.5 mL/min, and the gradient was built using 0.1% HCOOH in H_2O (solvent A) and CH_3CN (solvent B) as eluting phase. The solvent run was composed by the following steps: linear gradient from 95% A to 5% A in 20 min; linear gradient from 5% A to 95% A in 5 min; equilibration of the column for 5 min. For semipreparative purposes, 500 mg of the dried residue were dissolved in 1 mL of H_2O-CH_3OH (1:1) and submitted to HPLC purification. The semipreparative run was performed at a 21 mL/min flow rate, using 0.1% HCOOH in H_2O (solvent A) and CH_3CN (solvent B) in two steps. The first step was performed to partially purify each compound; it consisted in an isocratic run of 12 min (55% A, 45% B). The collected fractions of interest were mixed, lyophilized, and dissolved again (100 mg in 1 mL of H_2O-CH_3OH, 1:1) for further purification. The second step, carried out to obtain the pure compounds, consisted in an isocratic run of 15 min (77% A, 23% B). In the two steps of the semipreparative purification, fractions were collected every 18 s. After the second step, the fractions corresponding to the UV and mass signal of each product were mixed and the solvent was evaporated under vacuum. Finally, the residual water was lyophilized to yield the powdered compounds. The semipreparative steps were repeated until an appropriate amount was recovered for each experiment.

Hesperetin 7-[2″-α-rhamnosyl-6″-(3‴″-hydroxy-3‴-methylglutaryl)-β-glucoside] (brutieridin) (1): pale yellow powder; UV (MeOH) λ_{max} (log ϵ) nm 285 (3.80), 324 (3.12); IR (KBr) ν_{max} 3304, 1720, 1641, 1574, 1515, 1440, 1295, 1274, 1202, 1129, 1085 cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz) see Table 1; ^{13}C NMR (CD_3OD, 125 MHz) see Table 1; (+) HRESIMS *m/z* 755.2387 $[M + H]^+$, calcd for $C_{34}H_{43}O_{19}$, 755.2404; (−) HRESIMS/MS *m/z* 691.2277 $[(M - CO_2 - H_2O) - H]^-$, calcd for $C_{33}H_{39}O_{16}$, 691.2243; *m/z* 651.1890 $[(M - C_4H_6O_3) - H]^-$, calcd for $C_{30}H_{35}O_{16}$, 651.1930; *m/z* 609.1836 $[(M - C_6H_8O_4) - H]^-$, calcd for $C_{28}H_{33}O_{15}$, 609.1824; *m/z* 489.1357 $[(M - C_{10}H_{16}O_8) - H]^-$ or $[^{0,2}X_0]^-$, calcd for $C_{24}H_{25}O_{11}$, 489.1397; *m/z* 301.0715 $[(M - Rha - Glc - HMG) - H]^-$, calcd for $C_{16}H_{13}O_6$, 301.0717; HPLC (−) ESIMS t_R 10.05 min, *m/z* 753 $[M - H]^-$.

Naringenin 7-[2″-α-rhamnosyl-6″-(3‴″-hydroxy-3‴-methylglutaryl)-β-glucoside] (melitidin) (2): pale yellow powder; UV (MeOH) λ_{max} (log ϵ) 283 (3.80), 327 (3.23); IR (KBr) ν_{max} 3348, 1717, 1642, 1520, 1177, 1087, 834 cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz) see Table 1; ^{13}C NMR (CD_3OD, 125 MHz) see Table 1; (+) HRESIMS *m/z* 725.2290 $[M + H]^+$, calcd for $C_{33}H_{41}O_{18}$, 725.2287; (−) HRESIMS/MS *m/z* 661.2114 $[(M - CO_2 - H_2O) - H]^-$, calcd for $C_{32}H_{37}O_{15}$, 661.2137; *m/z* 621.1864 $[(M - C_4H_6O_3) - H]^-$, calcd for $C_{29}H_{33}O_{15}$, 621.1824; *m/z* 579.1764 $[(M - C_6H_8O_4) - H]^-$, calcd for $C_{27}H_{31}O_{14}$, 579.1719; *m/z* 459.1311 $[(M - C_{10}H_{16}O_8) - H]^-$ or $[^{0,2}X_0]^-$, calcd for $C_{23}H_{23}O_{10}$, 459.1291; *m/z* 271.0601 $[(M - Rha - Glc - HMG) - H]^-$, calcd for $C_{15}H_{11}O_5$, 271.0611; HPLC (−) ESIMS t_R 9.85 min, *m/z* 723 $[M - H]^-$.

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Supporting Information Available: ESIMS/MS of compounds **1** and **2**; ^{1}H and ^{13}C 1D and 2D NMR spectra of compounds **1** and **2**; LC MS chromatograms of basic and enzymatic hydrolysis of compounds **1** and **2**; ESIMS/MS of product from enzymatic reaction on compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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