

New Phenylethanoid Glycosides from *Cistanche phelypaea* and Their Activity as Inhibitors of Monoacylglycerol Lipase (MAGL)

Authors

Khadidja Aya Beladjila¹, Djemaa Berrehal¹, Nunziatina De Tommasi², Carlotta Granchi³, Giulia Bononi³, Alessandra Braca^{3,4}, Marinella De Leo^{3,4}

Affiliations

- 1 Laboratoire d'Obtention des Substances Thérapeutiques (LOST), Département de Chimie, Université des Frères Mentouri-Constantine, Constantine, Algeria
- 2 Dipartimento di Farmacia, Università di Salerno, Fisciano, Italy
- 3 Dipartimento di Farmacia, Università di Pisa, Italy
- 4 Centro Interdipartimentale di Ricerca "Nutraceutica e Alimentazione per la Salute", Università di Pisa, Italy

Key words

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Bibliography

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Correspondence

Prof. Alessandra Braca

Dipartimento di Farmacia, Università di Pisa

Via Bonanno 33, 56126 Pisa, Italy

Phone: + 39 05 02 21 96 88, Fax: + 39 05 02 22 06 80

alessandra.braca@unipi.it



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ABSTRACT

Four new phenylethanoid glycosides (1–4), 1- β -*p*-hydroxyphenyl-ethyl-2-*O*-acetyl-3,6-di- α -L-rhamnopyranosyl- β -D-glucopyranoside (1), 1- β -*p*-hydroxyphenyl-ethyl-3,6-di- α -L-rhamnopyranosyl- β -D-glucopyranoside (2), 1- β -*p*-hydroxyphenyl-ethyl-2-*O*-acetyl-3,6-di- α -L-rhamnopyranosyl-4-*p*-coumaroyl- β -D-glucopyranoside (3), and 1- β -*p*-hydroxyphenyl-ethyl-3,6-di- α -L-rhamnopyranosyl-4-*p*-coumaroyl- β -D-glucopyranoside (4), together with three known compounds, were isolated from the *n*-butanol extract of *Cistanche phelypaea* aerial parts. The structural characterization of all compounds was performed by spectroscopic analyses, including 1D and 2D NMR, and HRESIMS experiments. The isolated compounds were assayed for their inhibitory activity on two enzymes involved in the peculiar glycolytic or lipidic metabolism of cancer cells, human lactate dehydrogenase (LDH), and monoacylglycerol lipase (MAGL), respectively. All the compounds showed negligible activity on LDH, whereas some of them displayed a certain inhibition activity on MAGL. In particular, compound 1 was the most active on MAGL, showing an IC₅₀ value of 88.0 μ M, and modeling studies rationalized the supposed binding mode of 1 in the MAGL active site.

Introduction

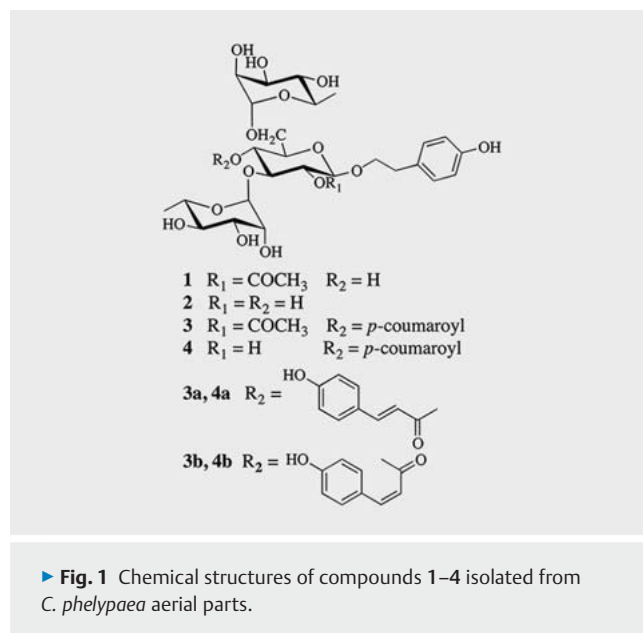
Cistanche is a genus of Orobanchaceae family including about 20 species, usually holoparasitic desert plants, that lack chlorophyll and obtain their nutrients and water from the roots of host plants that they parasitize. This genus is distributed to certain arid and semiarid regions of Africa, Asia, and the Mediterranean area, including parts of southern Europe [1]. Some *Cistanche* species are used for food application and in the traditional Chinese medicine as tonic for the treatment of kidney deficiency, impotence, infertility, profuse metrorrhagia, and chronic constipation [2]. The secondary metabolites produced by the representatives of the *Cistanche* genus include phenylethanoid glycosides, lignans, iridoids,

and oligosaccharides [3–5] some of which have been shown to possess interesting biological activity such as anti-inflammatory, neuroprotective, immunomodulatory, antioxidative, antibacterial, and antiviral properties [2]. *Cistanche phelypaea* (L.) Coutinho syn. *Cistanche tinctoria* (Desf.) Beck is an erect perennial parasite plant, with numerous yellow flowers and absent roots: the plant attaches itself to its host (*Tamarix gallica* L. [Tamaricaceae], *Calligonum comosum* L'Hér [Polygonaceae], and *Pulicaria* sp. [Asteraceae]) through small tubers. In North Africa traditional medicine, it is used for diarrhea, diabetes, intestinal troubles, infection, and as a diuretic [6]. Previous chemical studies revealed the presence of iridoids and phenylethanoids as main constituents [7, 8].

As a part of our research program for characterization of the biological activity of different classes of natural products [9, 10], herein we report the isolation and the structure characterization of four new phenylethanoid glycosides (1–4) (► Fig. 1), along with three known compounds from *C. phelypaea* aerial parts. The isolates were assayed for their lactate dehydrogenase (LDH) and monoacylglycerol lipase (MAGL) inhibitory activity. In fact, tumor cells exhibit progressive metabolic changes that are correlated with malignancy. In particular, glycolytic and lipidic pathways are reprogrammed in order to support the rapid growth of proliferating cells [11]. These dysregulated metabolic pathways are typical features of cancer, and at the same time, they may offer therapeutic windows for anticancer agents that target them. In this context, LDH and MAGL are two enzymes overexpressed in tumor tissues, which play key roles in the typical glycolytic and lipidic cancer metabolism, respectively [12, 13]. In particular, the human isoform 5 of the enzyme LDH (*hLDH5*), made of four A subunits, catalyzes the final step of glycolysis, which consists in the reduction of pyruvate to lactate, thus allowing the adenosine triphosphate production in cancer cells that rely on glycolysis for their survival. Glycolytic cancer cells display high glucose uptake and elevated lactate production, and the overexpression of specific enzymes such as LDH is necessary to assure a sufficient energy production. Therefore, the inhibition of this crucial enzyme may be considered a promising strategy to counteract cancer invasiveness, without affecting normal cells, which instead use oxidative phosphorylation for most of their purposes. On the other hand, MAGL is a serine hydrolase that cleaves monoacylglycerols into fatty acids and glycerol. In particular, MAGL is the lipolytic enzyme that is mainly responsible for the degradation of the endocannabinoid 2-arachidonoylglycerol, which is a neurotransmitter and an important intermediate in lipid metabolism involved in many physiological processes. Moreover, the intensified production of fatty acids in cancer cells, generated by MAGL activity, increases the formation of protumorigenic signaling molecules [14]. Therefore, MAGL represents a potential target to treat diverse pathological conditions, including cancer. In the last decades, a great effort has been directed toward the identification of small molecules that are able to inhibit LDH [15, 16] or MAGL [17, 18], and many compounds displaying efficient inhibitory activity on these targets were either discovered from natural sources or obtained by chemical synthesis. Therefore, the search for LDH or MAGL inhibitors represents an attractive goal to find new effective anticancer drugs interfering with the peculiar cancer metabolism and, therefore, has prompted us to carry out the investigation herein reported.

Results and Discussion

The phytochemical study of *C. phelypaea* *n*-butanol extract, after submission to flash column chromatography followed by RP-HPLC, afforded four new phenylethanoid glycosides (1–4) (► Fig. 1) and three known compounds, named brandioside [19], pinoresinol 4-*O*- β -D-glucopyranoside [20], and apigenin 7-*O*- β -glucuronopyranoside [21] by comparison of NMR and MS data with those reported in the literature.



Compound 1 was obtained as a brownish amorphous solid with a molecular formula $C_{28}H_{42}O_{16}$, as determined by ^{13}C NMR and HRESIMS analyses (m/z 657.2353 $[M + Na]^+$). The positive ESIMS spectrum showed a sodiated molecular ion peak at m/z 657 $[M + Na]^+$, while its fragmentation pattern was characterized by peaks at m/z 597 $[M + Na - 60]^+$, 511 $[M + Na - 146]^+$, and 365 $[M + Na - 146 - 146]^+$, due to the subsequent loss of an acetyl group and two deoxyhexose units, respectively. The 1H NMR spectra of 1 (► Table 1) exhibited characteristic signals of *ortho*-coupled A_2B_2 type aromatic protons at δ 6.71 (2H, d, $J = 8.0$ Hz) and 7.04 (2H, d, $J = 8.0$ Hz), three anomeric protons at δ 4.44 (1H, d, $J = 8.0$ Hz), 4.76 (1H, d, $J = 1.3$ Hz), and 4.80 (1H, d, $J = 1.5$ Hz), an acetyl group at δ 2.00 (3H, s), two methyl groups at δ 1.26 (3H, d, $J = 6.3$ Hz) and 1.27 (3H, d, $J = 6.5$ Hz), and two methylenes at δ 4.04 (1H, m), 3.62 (1H, m), and 2.80 (2H, m). This evidence suggested the presence of a phenylethanol residue and three sugar moieties [5]. The observation of ^{13}C NMR spectrum (► Table 1) confirmed these findings. The structures of the sugar units were assigned by 1D TOCSY and 2D NMR experiments, indicating the presence of one β -glucopyranoside and two α -rhamnopyranosyl residues. The complete assignments of all protons and carbons were based by the analysis of HSQC, COSY, and HMBC data, indicating that the two rhamnopyranosyl units were terminal units. The relative configuration of the anomeric carbon of the glucose residue was deduced to be β from the coupling constant ($J = 8.0$ Hz) of the anomeric proton. In the case of the rhamnose residue, the α anomeric configuration was derived by comparison of the pertinent ^{13}C NMR data with those reported in literature [9]. The configuration of the sugar units was assigned after hydrolysis of 1 with 1 N HCl. The hydrolyzate was trimethylsilylated, and GC retention times of each sugar were compared with those of authentic samples prepared in the same manner. The HMBC spectrum of 1 displayed the correlations between the proton signal at δ_H 4.44 (H-1_{glc}) and carbon resonance at δ_C 72.5 (C-8), confirming that the phenylethanol moiety was linked to C-1_{glc}. Simi-

► **Table 1** ^1H and ^{13}C -NMR data of compounds 1–4 (CD_3OD , 600 MHz, J in Hz).

Position	1		2		3a/3b		4a/4b	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}
1		131.0		131.0		131.7		130.7
2/6	7.04 d (8.0)	131.2	7.07 d (8.0)	131.5	7.05 d (8.0)	131.3	7.10 d (8.0)	130.9
3/5	6.71 d (8.0)	116.3	6.72 d (8.0)	116.4	6.73 d (8.0)	116.2	6.73 d (8.0)	116.2
4		157.0		157.1		157.0		157.1
7	2.80 m	36.4	2.85 m	37.6	2.78 m	36.0	2.86 m	36.7
8a	4.04 m	72.5	3.99 m	72.0	4.06 m	72.3	4.02 m	72.7
8b	3.62 m		3.74 m		3.65 m		3.75 m	
glc-1	4.44 d (8.0)	103.2	4.34 d (7.8)	104.6	4.55 d (7.8)	102.1	4.40 d (8.0)	104.6
2	4.78 dd (9.0, 8.0)	75.0	3.29 dd (9.5, 7.8)	75.0	4.88 dd (9.5, 7.8)	74.3	3.40 dd (9.5, 8.0)	76.4
3	3.65 t (9.0)	83.1	3.50 t (9.5)	83.8	4.03 t (9.5)	80.8	3.83 t (9.5)	81.9
4	3.44 t (9.0)	70.1	3.35 ^a	70.0	5.12 t (9.5)	69.3	5.00 t (9.5)	70.2
5	3.46 m	77.1	3.43 m	76.3	3.77 m	75.2	3.70 m	74.9
6a	4.00 dd (12.0, 3.0)	68.0	3.99 dd (12.0, 3.0)	68.0	3.79 dd (12.0, 3.0)	67.5	3.77 dd (12.0, 3.5)	67.8
6b	3.68 dd (12.0, 4.5)		3.65 dd (12.0, 5.0)		3.50 dd (12.0, 5.0)		3.49 dd (12.0, 4.5)	
COCH ₃		171.3				171.6		
COCH ₃	2.00 s	21.2			1.98 s	21.4		
rha _I - 1	4.76 d (1.3)	102.1	5.21 d (2.0)	100.8	4.77 d (1.8)	103.3	5.21 d (2.0)	102.7
2	3.83 dd (3.0, 1.8)	72.8	3.96 dd (3.0, 2.0)	71.6	3.74 dd (3.0, 1.8)	71.7	3.94 dd (3.0, 2.0)	72.4
3	3.96 dd (9.0, 3.0)	72.0	3.70 dd (9.0, 3.0)	70.7	3.54 dd (9.0, 3.0)	72.0	3.60 dd (9.0, 3.0)	72.7
4	3.39 t (9.0)	74.3	3.39 t (9.0)	73.4	3.29 t (9.0)	73.0	3.31 t (9.0)	74.2
5	3.68 m	70.7	3.69 m	71.2	3.54 m	70.2	3.58 m	70.6
6	1.26 d (6.3)	18.1	1.26 d (6.5)	18.3	1.08 d (6.5)	18.7	1.09 d (6.0)	18.3
rha _{II} - 1	4.80 d (1.5)	102.5	4.76 d (1.8)	101.6	4.63 d (2.0)	102.5	4.65 d (2.0)	101.1
2	3.83 dd (3.0, 1.8)	72.6	3.85 dd (3.5, 1.8)	71.5	3.89 dd (3.5, 2.0)	71.4	3.86 dd (3.5, 2.0)	72.3
3	3.96 dd (9.0, 3.0)	72.4	3.70 dd (9.0, 3.5)	72.3	3.70 dd (9.0, 3.5)	72.0	3.75 dd (9.0, 3.5)	72.6
4	3.39 t (9.0)	74.0	3.40 t (9.0)	74.0	3.37 t (9.0)	73.2	3.34 ^a	74.0
5	3.68 m	70.6	3.99 m	70.0	3.64 m	69.3	3.66 m	70.7
6	1.27 d (6.5)	18.3	1.27 d (6.5)	18.1	1.22 d (6.0)	18.3	1.21 d (6.0)	18.5
<i>p</i> -coum-1						127.3		127.4
2/6 <i>trans</i>					7.44 d (8.0)	131.3	7.50 d (7.8)	130.9
2/6 <i>cis</i>					7.75 d (8.0)	134.6	7.72 d (7.8)	133.4
3/5 <i>trans</i>					6.84 d (8.0)	116.2	6.83 d (7.8)	116.2
3/5 <i>cis</i>					6.80 d (8.0)	115.3	6.80 d (7.8)	115.2
4						162.0		161.7
α - <i>trans</i>					6.37 d (16.0)	114.9	6.38 d (16.0)	114.0
α - <i>cis</i>					5.83 d (12.0)	115.9	5.83 d (12.0)	115.2
β - <i>trans</i>					7.70 d (16.0)	148.1	7.71 d (16.0)	147.3
β - <i>cis</i>					6.97 d (12.0)	147.7	6.99 d (12.0)	146.4
COO						168.0		168.0

J values are in parentheses and reported in Hz. Chemical shifts are given in ppm. Assignments were confirmed by COSY, 1D-TOCSY, HSQC, and HMBC experiments. Glc: β -D-glucopyranoside; rha: α -L-rhamnopyranosyl; *p*-coum: *p*-coumaroyl; ^a overlapped signal.

larly, the correlation between signals at δ_{H} 4.78 (H-2_{glc}) and δ_{C} 171.3 suggested that the acetyl group was linked to C-2_{glc}. Likewise, the long-range correlations between δ_{H} 4.76 (H-1_{rhai}) and

carbon resonance δ_{C} 83.1 (C-3_{glc}), δ_{H} 4.80 (H-1_{rhai}), and δ_{C} 68.0 (C-6_{glc}) allowed to establish that the two α -L-rhamnopyranosyl moieties were linked at C-3_{glc} and C-6_{glc}, respectively. Thus, the

structure of **1** was established as 1- β -*p*-hydroxyphenyl-ethyl-2-*O*-acetyl-3,6-di- α -L-rhamnopyranosyl- β -D-glucopyranoside.

The molecular formula of compound **2**, isolated as a brownish amorphous solid, was assigned C₂₆H₄₀O₁₅, as deduced by HRESIMS analyses (m/z 615.2232 [M + Na]⁺), as well as from ¹³C NMR data. The ESIMS/MS spectrum displayed two fragments at m/z 469 [M + Na - 146]⁺ and 323 [M + Na - 146 - 146]⁺ attributable to the subsequent loss of two deoxyhexose units, respectively. Comparison of ¹H and ¹³C NMR spectra of **2** (► **Table 1**) with those of **1** revealed **2** to differ from **1** only for the absence of the acetyl group at C-2 of glucose unit. Thus, the structure of **2** was determined as 1- β -*p*-hydroxyphenyl-ethyl-3,6-*O*-di- α -L-rhamnopyranosyl- β -D-glucopyranoside.

Compound **3** displayed a molecular formula C₃₇H₄₈O₁₈ by HRESIMS (m/z 803.2715 [M + Na]⁺). The ESIMS of **3** showed a sodiated molecular ion peak at m/z 803 [M + Na]⁺ and a fragmentation pattern with peaks at m/z 743 [M + Na - 60]⁺, 657 [M + Na - 146]⁺, and 511 [M + Na - 146 - 146]⁺, corresponding to the subsequent loss of an acetyl moiety and two deoxyhexose units. Analysis of NMR data of **3** (► **Table 1**), besides chemical shifts similar to those of **1**, displayed the presence of two couples of signals attributable to *trans*-olefinic protons at δ 6.37 (1H, d, J = 16.0 Hz, H- α_{trans}) and 7.70 (1H, d, J = 16.0 Hz, H- β_{trans}), *cis*-olefinic protons at δ 5.83 (1H, d, J = 12.0 Hz, H- α_{cis}), and 6.97 (1H, d, J = 12.0 Hz, H- β_{cis}), and two couples of *ortho*-coupled A₂B₂ aromatic protons at δ 6.84 (2H, d, J = 8.0 Hz, H-3/H-5_{*trans*}) and 7.44 (2H, d, J = 8.0 Hz, H-2/H-6_{*trans*}), and 6.80 (2H, d, J = 8.0 Hz, H-3/H-5_{*cis*}) and 7.75 (2H, d, J = 8.0 Hz, H-2/H-6_{*cis*}). Also in this case the assignments of all proton and carbon signals were deduced from a combined analysis of 1D and 2D NMR experiments. Moreover, key correlation peaks in the HMBC spectrum of **3** between the proton signal at δ_H 5.12 (H-4_{glc}) and carbon resonance at δ_C 168.0 (COO), δ_H 4.88 (H-2_{glc}), and δ_C 171.6 (COCH₃) were present, confirming that the *p*-coumaroyl group was linked to C-4_{glc} while the acetyl group at C-2_{glc}. Thus, compound **3** was identified as an inseparable mixture 7:3 of 1- β -*p*-hydroxyphenyl-ethyl-2-*O*-acetyl-3,6-di- α -L-rhamnopyranosyl-4-*trans*-*p*-coumaroyl- β -D-glucopyranoside (**3a**) and 1- β -*p*-hydroxyphenyl-ethyl-2-*O*-acetyl-3,6-di- α -L-rhamnopyranosyl-4-*cis*-*p*-coumaroyl- β -D-glucopyranoside (**3b**).

The HRESIMS of compound **4**, a brownish amorphous solid, showed a sodiated molecular ion peak at m/z 761.2604 [M + Na]⁺, corresponding to the molecular formula C₃₅H₄₆O₁₇. In the ESIMS/MS fragmentation pattern of m/z 761 [M + Na]⁺, peaks at m/z 615 [M + Na - 146]⁺ and 469 [M + Na - 146 - 146]⁺, due to the subsequent loss of two deoxyhexose units, respectively, were evident. Comparison of NMR data of **4** (► **Table 1**) with those of **3** showed in **4** the absence of the acetyl group at C-2_{glc}. In the light of these data, compound **4** was established to be an inseparable mixture 7:3 of 1- β -*p*-hydroxyphenyl-ethyl-3,6-di- α -L-rhamnopyranosyl-4-*trans*-*p*-coumaroyl- β -D-glucopyranoside (**4a**) and 1- β -*p*-hydroxyphenyl-ethyl-3,6-di- α -L-rhamnopyranosyl-4-*cis*-*p*-coumaroyl- β -D-glucopyranoside (**4b**).

Phenylethanoids **1–4**, pinosresinol 4-*O*- β -D-glucopyranoside, brandioside, and apigenin 7-*O*- β -glucuronopyranoside, were assayed on human LDH5 and MAGL purified isoforms to determine their inhibition potencies (► **Table 2**). All the compounds were inactive on *h*LDH5, showing IC₅₀ values greater than 200 μ M, thus

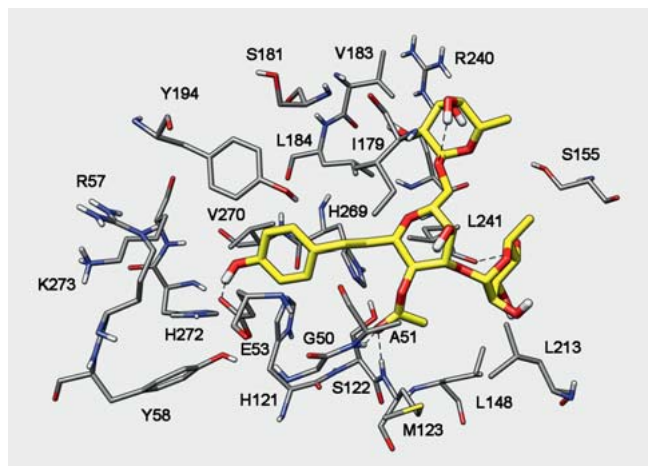
► **Table 2** *h*LDH5 and *h*MAGL inhibition potencies.

Compound	<i>h</i> LDH5	IC ₅₀ (μ M)		
		CI (95%, n = 3)	<i>h</i> MAGL	CI (95%, n = 3)
1	> 200	–	88.0	[60.7, 127.6]
2	> 200	–	117.4	[95.0, 145.1]
3a/3b	> 200	–	113.9	[93.8, 138.4]
4a/4b	> 200	–	> 200	–
pinosresinol 4- <i>O</i> - β -D-glucopyranoside	> 200	–	130.2	[110.2, 153.8]
brandioside	> 200	–	156.1	[151.1, 164.7]
apigenin 7- <i>O</i> - β -glucuronopyranoside	> 200	–	> 200	–
galloflavin	110.1	[82.9, 146.2]	– ^a	–
CL6a	– ^a	–	12.1	[8.9, 16.3]

CI: confidence interval; ^a not tested.

being less potent than reference compound galloflavin [22]. In *h*MAGL enzymatic assays, only two derivatives (compound **4** and apigenin 7-*O*- β -glucuronopyranoside) proved to be inactive, whereas pinosresinol 4-*O*- β -D-glucopyranoside and brandioside exhibited IC₅₀ values of 130.2 and 156.1 μ M, respectively. Compounds **2** and **3** showed a better inhibition activity, with similar IC₅₀ values of 117.4 and 113.9 μ M, respectively. The best inhibition potency on *h*MAGL was demonstrated by compound **1**, with an IC₅₀ value in the low micromolar range (88.0 μ M), and it proved to be selective for *h*MAGL over *h*LDH. Although its inhibition activity was lower than reference MAGL synthetic inhibitor (4-[4-chlorobenzoyl]piperidin-1-yl)(4-methoxyphenyl)-methanone (compound “CL6a” [17]), this natural compound is the first phenylethanoid, bearing β -D-glucopyranosyl and α -L-rhamnopyranosyl sugar units, displaying a promising activity on *h*MAGL. Presently, only few compounds derived from natural sources have shown an inhibitory activity on this enzyme [23]; however, they belong to different chemical classes.

In order to better characterize the binding mode of compound **1** into MAGL, its complex with the target protein was subjected to docking calculations. In particular, the putative binding pose emerging from these modeling studies showed that the sugar moiety lies in the wide lipophilic cavity of the protein forming lipophilic interactions with L148, L213, L241, and V183, whereas the *p*-hydroxyphenyl-ethyl ring lies into the small pocket of the binding site and forms lipophilic interactions with residues Y194 and V270 (► **Fig. 2**). A high number of H-bonds stabilizes the binding disposition of compound **1**: the carbonyl group of the *O*-acetyl group linked to the glucopyranoside forms two H-bonds with the backbone nitrogen of A51 and M123 and the 4-OH of



► **Fig. 2** View of the most relevant ligand-receptor interactions of the 1-*h*MAGL complex. Hydrogen bonds are represented as black dashed lines.

the 3- α -L-rhamnopyranoside forms an H-bond with the oxygen backbone of L241. Compound **1** is also characterized by an intramolecular H-bond between the ethereal oxygen of the 6- α -L-rhamnopyranoside and the 4-OH of the same portion. Furthermore, the OH group of the *p*-hydroxyphenyl-ethyl portion forms an H-bond with residue E53, in which it behaves as a H-bond donor.

In summary, seven compounds, including four new structures (1–4), were isolated and characterized from the aerial parts of *C. phelypaea*. The isolation and structural characterization of the new phenylethanoids 1–4 and the known compound pinoresinol 4-*O*- β -D-glucopyranoside are completely in agreement with the available information on the chemical types of secondary metabolites typical of *Cistanche* species [24, 25], while brandioside and apigenin 7-*O*- β -glucuronopyranoside are reported from this genus for the first time here. Moreover, to our knowledge this is the first report of naturally occurring phenyletanoid as a MAGL inhibitor. This study will open the way for future investigation on plant glycosylated small molecules as potential MAGL inhibitors. HRESIMS and NMR spectra of compounds 1–4 and data for the IC₅₀ determination of compound **1** in MAGL enzymatic assays are available as Supporting Information.

Materials and Methods

General experimental procedures

Optical rotations were measured on an Atago AP-300 digital polarimeter equipped with a sodium lamp (589 nm) and a 1-dm microcell. NMR experiments were recorded at 300 K in CD₃OD on a Bruker DRX-600 spectrometer (Bruker BioSpin GmbH) equipped with a Bruker 5 mm TCI CryoProbe as reported previously [26]. HRESIMS were acquired in the positive ion mode on a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific). ESIMS were obtained from an LCQ Advantage ThermoFinnigan spectrometer (ThermoFinnigan), equipped with a Xcalibur software. Column

chromatography was performed over Sephadex LH-20 (40–70 μ m, Pharmacia) at a flow rate 0.8 mL/min. HPLC separations were conducted on a Shimadzu LC-8A series pumping system equipped with a Shimadzu RID-10A refractive index detector and Shimadzu injector on a C₁₈ μ -Bondapak column (30 cm \times 7.8 mm, 10 μ m Waters, flow rate 2.0 mL/min). TLC analyses were carried out using glass-coated silica gel 60 F₂₅₄ (0.20 mm thickness) plates (Merck) with CHCl₃-MeOH-H₂O (70:30:3) as eluent and cerium sulphate as spray reagent. GC analysis was performed using a Dani GC 1000 instrument on a L-CP-Chirasil-Val column (0.32 mm \times 25 m) working with the following temperature program: 100 °C for 1 min, ramp of 5 °C/min up to 180 °C; injector and detector temperature 200 °C; carrier gas N₂ (2.0 mL/min); detector dual FID; split ratio 1:30; injection 5 μ L.

Plant material

C. phelypaea aerial parts were collected in March 2012 in the southwest of Algeria. The plant was identified by Prof. Gérard De Bélair, Faculty of Sciences, University of Annaba, Algeria. A voucher specimen (number Ct.03.12) has been deposited in the Herbarium of the Department of Chemistry, Université des Frères Mentouri-Constantine, Constantine, Algeria.

Extraction and isolation

The dried and powdered aerial parts (4 kg) of *C. phelypaea* were extracted with MeOH-H₂O (4:1) at room temperature for 48 h. The extraction was repeated three times by changing the solvent. The obtained extract was filtered, concentrated, and successively extracted with petroleum ether, chloroform, ethyl acetate and *n*-butanol, to give 3.27, 4.87, 10.96, and 36.86 g of the respective residues. The *n*-butanol extract (10.59 g) was subjected to column chromatography over Sephadex LH-20 in MeOH. Fractions of 12 mL were collected, analyzed by TLC, and grouped to give 11 major fractions (A–K). Fraction K contained pure apigenin 7-*O*- β -D-glucuronopyranoside (28 mg). Fraction B (245.1 mg) was purified by RP-HPLC with MeOH-H₂O (3:7) as eluent to obtain pure compound **1** (7.9 mg, *t*_R 21 min). Fraction C (896.8 mg) was purified by RP-HPLC with MeOH-H₂O (25:75) as eluent to afford pure compound **2** (6.3 mg, *t*_R 15 min). Fraction E (638.9 mg) was purified by RP-HPLC with MeOH-H₂O (45:55) as eluent to give brandioside (7 mg, *t*_R 15 min) and compounds **4** (14.9 mg, *t*_R 20 min) and **3** (11.1 mg, *t*_R 34 min). Fraction G (172.6 mg) was purified by RP-HPLC with MeOH-H₂O (35:65) as eluent to obtain pinoresinol 4-*O*- β -D-glucopyranoside (3.6 mg, *t*_R 18 min).

Compound 1: brownish amorphous solid; [α]_D²⁵ – 58.5 (c 0.1, MeOH); UV (MeOH) λ_{\max} (log ϵ): 223 (4.20), 280 (3.50), 321 (3.06); ¹H and ¹³C NMR data, see ► **Table 1**; ESIMS *m/z* 657 [M + Na]⁺, 597 [M + Na – 60]⁺, 511 [M + Na – 146]⁺, 451 [M + Na – 60 – 146]⁺, 365 [M + Na – 146 – 146]⁺; HRESIMS: *m/z* 657.2353 [M + Na]⁺ (calcd. for C₂₈H₄₂NaO₁₆, *m/z* 657.2371).

Compound 2: brownish amorphous solid; [α]_D²⁵ – 7.0 (c 0.1, MeOH); UV (MeOH) λ_{\max} (log ϵ): 222 (4.09), 280 (3.55), 315 (3.10); ¹H and ¹³C NMR data, see ► **Table 1**; ESIMS *m/z* 615 [M + Na]⁺, 469 [M + Na – 146]⁺, 323 [M + Na – 146 – 146]⁺; HRESIMS: *m/z* 615.2232 [M + Na]⁺ (calcd. for C₂₆H₄₀NaO₁₅, *m/z* 615.2265).

Compound 3: brownish amorphous solid; [α]_D²⁵ – 96 (c 0.1, MeOH); UV (MeOH) λ_{\max} (log ϵ): 225 (4.26), 316 (3.87); ¹H and

¹³C NMR data, see ► **Table 1**; ESIMS *m/z* 803 [M + Na]⁺, 743 [M + Na - 60]⁺, 657 [M + Na - 146]⁺, 511 [M + Na - 146 - 146]⁺; HRESIMS: *m/z* 803.2715 [M + Na]⁺ (calcd. for C₃₇H₄₈NaO₁₈, *m/z* 803.2738).

Compound 4: brownish amorphous solid; [α]_D²⁵ - 85.5 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 224 (4.30), 315 (3.85); ¹H and ¹³C NMR data, see ► **Table 1**; ESIMS *m/z* 761 [M + Na]⁺, 615 [M + Na - 146]⁺, 469 [M + Na - 146 - 146]⁺; HRESIMS: *m/z* 761.2604 [M + Na]⁺ (calcd. for C₃₅H₄₆NaO₁₇, *m/z* 761.2633).

Acid hydrolysis of compounds 1–4

A solution of each compound (2.0 mg) in 1 N HCl (1 mL) was stirred at 80 °C in a stoppered reaction vial for 4 h. After cooling, the solution was evaporated under a stream of N₂. The residue was dissolved in 1-(trimethylsilyl)imidazole and pyridine (0.2 mL, 1 : 1), and the solution was stirred at 60 °C for 5 min. After drying the solution, the residue was partitioned between H₂O and CHCl₃. The CHCl₃ layer was analyzed by GC using a L-CP-Chirasil-Val column (0.32 mm × 25 m). Temperatures of both the injector and detector was 200 °C. A temperature gradient system was used for the oven, starting at 100 °C for 1 min and increasing up to 180 °C at a rate of 5 °C/min. Peaks of the hydrolysate were detected by comparison with retention times of authentic samples of D-glucose and L-rhamnose (Sigma-Aldrich) after treatment with 1-(trimethylsilyl)imidazole in pyridine.

Enzymatic assays

All the compounds were assayed against purified hLDH5 (Lee Biosolution Inc.) and hMAGL (Cayman Chemical), as previously reported [15, 17]. The reference MAGL synthetic inhibitor (4-[4-chlorobenzoyl]piperidin-1-yl)(4-methoxyphenyl)-methanone (compound “CL6a”) was synthesized in our laboratory (purity percentage 99% by HPLC analysis), as previously reported [17]. Both assays were performed in 96-well plates at room temperature at a final volume of 200 μL. Compounds were dissolved in DMSO stock solutions at the maximum concentration of 625 μM (the concentration of DMSO did not exceed 4% during the measurements), and this solution was diluted to seven different concentrations (from 625 to 0.86 μM, in duplicate for each concentration) that were used to generate the concentration-response curve. All the IC₅₀ values are the mean of three independent experiments. Maximum and minimum controls were also included in each plate. The enzymatic reaction for LDH assays was performed in the “forward” direction (pyruvate to lactate), and the amount of consumed NADH was monitored (340 nm). Assays were performed in 100 mM phosphate buffer (pH = 7.4) in the presence of 200 μM pyruvate and 40 μM NADH. After 15 min of incubation, the final measurements were carried out by using a Victor X3 microplate reader (PerkinElmer). IC₅₀ values were derived from experimental data using the sigmoidal dose-response fitting of GraphPad Prism software. The enzymatic reaction for MAGL assays was based on the conversion of the substrate 4-nitrophenylacetate (4-NPA) to 4-nitrophenol and the amount of produced 4-nitrophenol was monitored (405 nm), as previously reported [27]. Assays were performed in 10 mM Tris buffer (pH = 7.2), containing 1 mM EDTA and 0.1 mg/mL BSA, in the presence of 100 μM 4-NPA. After the reaction had proceeded for 30 min, absorbance

values were then measured by using a Victor X3 microplate reader (PerkinElmer). IC₅₀ values were derived from experimental data using the sigmoidal dose-response fitting of GraphPad Prism software.

Docking studies

The crystal structure of hMAGL (pdb code 3PE6 [28]) was sourced from the Protein Data Bank [29]. First we added hydrogen atoms. Then the protein complexed with its reference inhibitor was minimized using Amber14 software [30] and ff14SB force field at 300 K. The complex was placed in a rectangular parallelepiped water box, an explicit solvent model for water (TIP3P) was used, and the complex was solvated with a 10 Å water cap. To neutralize the system, sodium ions as counterions were added. Then two steps of minimization were carried out. First, the protein was kept fixed with a position restraint of 500 kcal/mol-Å² and only the positions of the water molecules were minimized. Second, the entire system through 5000 steps of steepest descent followed by a CG was minimized until a convergence of 0.05 kcal/Å-mol was reached. The ligands were built using Maestro [31] and minimized by means of MacroModel [32] in a water environment, following the CG method to obtain a convergence value of 0.05 kcal/Å-mol, using the MMFF's force field and a distance-dependent dielectric constant of 1.0. Automated docking was carried out by means of the AutoDock 4.0 program [33]; AutoDock Tools [34] was used to identify the torsion angles in the ligands, add the solvent model, and assign the Kollman atomic charges to the protein. The ligand charge was calculated using the Gasteiger method. By considering the ZYH [(2-cyclohexylbenzo[d]oxazol-6-yl)(3-(4-(pyrimidin-2-yl)piperazin-1-yl)azetid-1-yl)methanone] reference inhibitor as the central group, the regions of interest used were defined by AutoDock; in particular a grid of 82, 40, and 30 points in the x, y, and z directions, centered on the core of the mass of this compound, was constructed. For the energetic map calculations, a grid spacing of 0.375 Å and a distance-dependent function of the dielectric constant were used. With the aid of the Lamarckian Genetic Algorithm, the docked compounds were subjected to 100 runs of the AutoDock search, using 500 000 steps of energy evaluation and the default values of the other parameters. The cluster analysis on the results was performed using an RMS tolerance of 2.0 Å.

Supporting Information

HRESIMS and NMR spectra of compounds 1–4 and data for the IC₅₀ determination of compound 1 in MAGL enzymatic assays are available as Supporting Information.

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Conflict of Interest

The authors declare no conflicts of interest.

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