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Structure and absolute configuration of new acidic metabolites from *Stachys ehrenbergii*

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ABSTRACT

Two novel metabolites have been isolated from the aerial parts of *Stachys ehrenberiigii*. Their structures and stereochemistry were elucidated using a combination of ^{13}C and ^{1}H homo and heteronuclear 2D NMR experiments and mass analysis. The development of an enantioselective synthesis of 3-(2′-acetoxy-4-phenylbut-3′-enoylamino)propionic acid allowed to confirm the structure and assign the (*R*) absolute configuration at C-2′ of the natural product.

The genus *Stachys* is one of the largest of the Lamiaceae (Labiatae) family and includes about 200–300 species, in the subtropical and tropical regions of both hemispheres.[1,2] The investigated species contain many kinds of secondary metabolites, mainly flavonoids, iridoids, and terpenoids from the aerial parts and the roots.[3]

As a part of a program aimed to identify new acidic metabolites from plants of the genus *Stachys*, we investigated the aerial parts of *Stachys ehrenbergii* Boiss, a species native of Lebanon.

Extensive chromatographic separation of the acetone extracts led to the isolation of two new metabolites (**1**, **10**) soluble in aqueous basic solution, thus containing acidic functional groups.

Structure assignments of the isolated compounds were based on the interpretation of spectroscopic data from MS and NMR analysis. Compound **1** was isolated as a white solid, mp 84 °C; $[\alpha]_D^{25}$ −43.2 (*c* 0.2, $CHCl_3$); ^{1}H and ^{13}C NMR spectra disclosed signals and correlations for 15 C and 17 H atoms (Table 1). In the ^{13}C NMR spectrum, three signals at 168.6, 169.6, 175.6 ppm suggested the presence of three carbonyl groups. The presence of a monosubstituted aromatic ring was clearly inferred from a set of signals at 7.30–7.42 ppm in the ^{1}H NMR spectrum, integrating for five hydrogens, and from a set of six carbons in the region between 127.1 and 135.9 ppm in the ^{13}C NMR spectrum.

The ^{1}H NMR spectrum revealed two signals at 6.77 ppm (d) and 6.28 ppm (dd) with a coupling constant of 16.0 Hz, providing evidence of a *trans* double bond. Information from ^{13}C NMR spectrum in conjunction with DEPT showed only one methine group at 74.8 ppm. The hydrogen of this group appeared as a double doublet in the ^{1}H spectrum at 5.75 ppm. A vicinal coupling (J = 6.9 Hz) with the olefinic proton at 6.28 ppm and an allylic coupling (J = 1.4 Hz) with the olefinic proton at 6.77 ppm allowed us to connect this moiety to the double bond. Moreover, the shift of this methine suggested that it was oxygenated.

Table 1
^{1}H NMR and ^{13}C NMR Spectroscopic Data (600 MHz, $CDCl_3$) for compound **1**

Position	δ_H ppm (J in Hz)	δ_C ppm	HMBC[a]
1		175.6, C	
2	2.66, t (5.9)	33.5, CH_2	1, 3
3	3.60, m	34.9, CH_2	1, 2, 1′
NH	6.77, br s		
1′		168.6, C	
2′	5.75, dd (6.9, 1.4)	74.8, CH	4′,3′, 1′
3′	6.28, dd (16.0, 6.9)	122.4, CH	2′, 1′,1″,
4′	6.77, dd (16.0, 1.4)	135.3, CH	2′, 2″, 6″
1″		135.9, C	
2″	7.42, m	127.1, CH	4′, 6″, 4″
3″	7.35, m	128.9, CH	4″,1″
4″	7.30, m	128.8, CH	2″, 6″
5″	7.35, m	128.9, CH	4″, 1″
6″	7.42, m	127.1, CH	4′, 6″, 4″
$COCH_3$		169.6, C	
$COCH_3$	2.22, s	21.3, CH_3	$COCH_3$

[a] HMBC correlations, optimized for 6 Hz, are from proton(s) stated to the indicated carbon.

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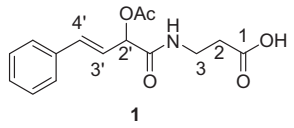

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Figure 1. Structure of compound **1**.

A broad signal that overlapped the olefinic signal at 6.77 ppm, integrating for one proton, was present in the ^{1}H NMR spectrum. The absence of any carbon corresponding to this hydrogen in the HMBC experiment, along with the disappearance of this signal in the ^{1}H spectrum after exchange with D_2O and trifluoroacetic acid, led us to hypothesize the presence of an amidic bond. In addition, the analysis of the COSY spectrum showed that the NH group was coupled with a methylene resonating at 3.60 ppm. (δ C 34.9), on its turn coupled with a second methylene group resonating as a triplet (J = 5.9 Hz) at 2.66 ppm. The chemical shift and the multiplicity of this signal suggested that it was linked to a carbonyl group.

The most upfield signal in the ^{1}H spectrum was a singlet at 2.22 ppm, corresponding to a methyl group. Examination of HMBC and HSQC spectra showed that this methyl resonated at 21.3 ppm and was close to the carbonyl at 169.6 ppm, thus corroborating the presence of an acetyl group.

Upon rationalizing the above data we established that the three carbonyl groups at 168.6, 169.6, and 175.6 ppm corresponded to an amide, to an acetyl group, and to a carboxylic acid, respectively. The above information allowed the structure assignment as depicted in Figure 1. The structure was supported by the ESI mass spectra. Indeed, the ESI positive mass spectrum showed peaks at 314.0997 $[M+Na]^+$, 627.1959 $[2M+1]^+$, and 649.1779 $[2M+3Na-2]^+$ m/z, while the ESI negative spectrum showed a peak at 290.1024 $[M-1]^-$ m/z.

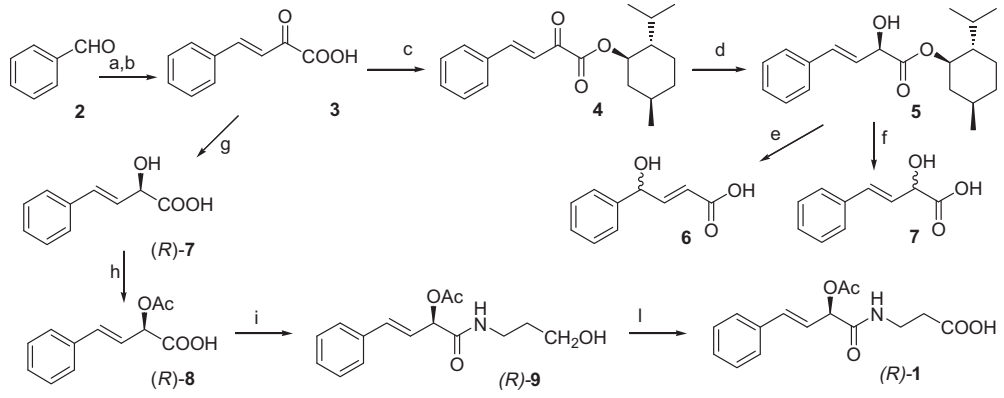
Compound **1** is the amide of a substituted glycolic acid with β-alanine. This appears to be a remarkable occurrence, as, to the best of our knowledge, the only other example of this kind of product in plants is the well-known pantothenic acid (vitamin B5),[4a] the essential precursor to CoA.[4b] As several analogues of this metabolite have been demonstrated to exert an antimicrobial effect against a range of microorganisms,[4c] evaluation of biological activity of compound **1** is in progress.

To unambiguously confirm the proposed structure, a total enantioselective synthesis of **1** was carried out (Scheme 1). For the synthesis of enantiomerically pure 2-hydroxy-4-phenylbut-3-enoic acid or close derivatives, numerous strategies have been devised, including enzymatic resolution of esters[5] and diastereoselective reduction of α-oxo esters[6] or amides[7] using chiral auxiliaries. A first attempt to prepare *E*-2-oxo-4-phenylbut-3-enoic acid following a reported enzymatic resolution[5] failed, giving only the recovery of starting material. Thus, we planned to use (−)-menthol as a chiral auxiliary for the asymmetric reduction of α-oxo ester. Compound **3** was obtained by reacting benzaldehyde with pyruvic acid enolate in methanol, followed by acidification with HCl.[8] The treatment with α,α-dichloromethyl methyl ether, TEA and (−)-menthol afforded compound **4**, that was reduced to α-hydroxyester **5** (Scheme 1). Very high diastereoselectivity was achieved with L-selectride[9] in diethylether at −78 °C (% de: 97%, determined by 300 MHz ^{1}H NMR). However, hydrolysis of the ester to remove the chiral auxiliary under basic conditions (LiOH, THF) gave partial racemization (determined by comparison of optical rotation with that reported in the literature[5]) whereas under acidic conditions, (HCl 37% in dioxane, reflux) the isomerized product **6**[10] was formed. Thus, we turned back to compound **3**. An enantioselective reduction of this ketoacid was performed using a chiral complex $NaBH_4$-L-proline, prepared as described by Schmutzler.[11] The absolute configuration (*R*) and the enantiomeric excess (92%) of the α-hydroxy acid **7** were determined by comparison with the optical rotation of (*R*)-2-hydroxy-4-phenylbut-3-enoic acid obtained by enzymatic resolution.[5] Acetylation of (*R*)-**7** performed either with Ac_2O/Py at 0 °C or with AcCl in THF gave partial racemization, confirming the sensitivity of this α-hydroxy acid to acidic and basic conditions. Finally, it was found that treatment with AcCl as a solvent at room temperature[12] gave the desired acetylated compound without the loss of optical purity. This was condensed with 3-amino-1-propanol at room temperature[13] to give compound **9** in good yield. Oxidation of **9** with Jones reagent[14] at 0–5 °C afforded acid **1** in 56% yield and 75% ee. The spectroscopic properties and the sign of optical rotation matched with those found for the natural compound. As the absolute configuration at C-2 in **7** is (*R*) and during the following steps no inversion of configuration occurs, we may safely deduce that the absolute configuration of the natural compound must be (*R*). Chiral HPLC analysis[15] showed two peaks at t_R = 34.0 min and at t_R = 49.2 min. HPLC analysis of the natural sample using the same conditions showed a single peak at t_R = 34.7 min.

Compound **10** was isolated as a yellow powder, mp 173 °C; $[\alpha]_D^{25}$ −16.9 (*c* 0.1, ethanol); ^{1}H and ^{13}C NMR spectra disclosed signals and correlations for 43 C and 36 H atoms, ten of which were exchanged with D_2O (Table 2).

The ^{13}C NMR spectrum revealed two signals at 182.0 and 182.06 ppm, suggesting the presence of two α,β-unsaturated carbonyl groups, whereas the presence of a signal at 170.3 ppm, led to hypothesize the presence of a carboxylic acid, confirmed by the solubility in aqueous sodium bicarbonate.

The presence of aromatic rings was clearly deduced from a set of signals in the region between 7.95 and 6.46 ppm in the ^{1}H



Scheme 1. Synthesis of compound **1**. Reagents and conditions: (a) Pyruvic acid, KOH, MeOH, 30 °C, 1 h, 70%; (b) HCl 1.6 M, 60%; (c) α,α-dichloromethyl methyl ether, 50 °C, 1 h, then (−)-menthol, TEA, CH_2Cl_2, rt, overnight, 61% (d) L-selectride, Et_2O, −78 °C, 1 h, 76%; (e) HCl 32%, dioxane, reflux, 6 h, 29%; (f) LiOH, THF, 60 °C, 4 h, 60%; (g) $NaBH_4$-L-proline, dry THF, rt, 24 h, 84%; (h) AcCl, rt, 2 h, 82%; (i) 3-amino-1-propanol, WSC, HOBt, dry THF, rt, overnight, 74%; (j) Jones reagent, acetone, 0 °C, 1 h, 56%.

Table 2
^{1}H NMR and ^{13}C NMR Spectroscopic Data (600 MHz, DMSO-d_6) for compound **10**

Position	δ_H ppm (J in Hz)	δ_C ppm	HMBC[a]
2		164.2, C	
2″		164.3, C	
3	6.99, s	103.5, CH	4, 2, 1′
3″	6.87, s	103.1, CH	4″, 2″, 1‴
4		182.06, C	
4″		182.0, C	
4a, 4″a		105.5, C	
5, 5″		161.2, C	
6	6.46, d (2.3)	99.19, CH	8, 4, 7
6″	6.47, d (2.3)	99.25, CH	8″,4″,7″
7, 7″		162.5, C	
8	6.85, d (2.3)	94.6, CH	6, 7
8″	6.88, d (2.3)	94.8, CH	6″, 7″
8a, 8″a		156.9, C	
1′		121.3, C	
2′	7.58, m	110.3, CH	6′, 3′, 4′, 2
3′		148.0, C	
4′		151.0, C	
5′	6.95, m	115.8, CH	4′, 3′, 1′
6′	7.59, m	120.5, CH	2′, 4′, 2
1‴		121.0, C	
2‴,6‴	7.95, m	128.6, CH	4‴, 2″
3‴, 5‴	6.94, m	116.0, CH	1‴
4‴		161.4, C	
5′′′′, 5′′′′′	4.02, d (9.6)	75.3, CH	COOH, 4′′′′, 4′′′′′, 3′′′′, 3′′′′′
1′′′′, 1′′′′′	5.26, d (7.4)	99.37, CH	7, 7″
2′′′′, 2′′′′′	3.30, dd (8.7, 7.4)	72.8, CH	1′′′′, 1′′′′′, 3′′′′, 3′′′′′
3′′′′, 3′′′′′	3.33, dd (8.7, 8.8)	75.7, CH	4′′′′, 4′′′′′, 2′′′′, 2′′′′′
4′′′′, 4′′′′′	3.39, dd (8.8, 9.6)	71.3, CH	COOH, 3′′′′, 3′′′′′, 5′′′′, 5′′′′′
OMe	3.89, s	56.0, CH_3	3′, 2′
COOH		170.3, C	
OH	12.99, s		4, 5, 4a, 6
OH	12.98, s		4″, 5″, 4″a, 6″

[a] HMBC correlations, optimized for 6 Hz, are from proton(s) stated to the indicated carbon.

NMR spectrum. First, a *para*-substituted aromatic ring, with two sets of chemically equivalent protons shifting at 7.95 and 6.94 ppm, was detected. From the HSQC spectrum it was possible to identify the chemical shift of the corresponding carbons, at 128.6 and 116.0 ppm, respectively.

A further 1,3,4 trisubstituted aromatic ring was identified from the analysis of a double doublet at 7.59 ppm (δ C 120.5) coupled with one hydrogen at 6.95 ppm whose carbon resonated at 115.8 ppm, and another hydrogen at 7.58 ppm whose carbon appeared at 110.3 ppm.

Two singlets, each integrating for one hydrogen, were identified at 6.87 ppm (^{13}C δ 103.1 ppm) and 6.99 ppm (δ C 103.85). Moreover, it was possible to detect two doublets at 6.88 ppm (^{13}C δ 94.8 ppm) and 6.85 ppm (δ C 94.6), each integrating for one hydrogen and coupled, respectively, with a doublet at 6.47 ppm (δ C 99.25) and a doublet at 6.46 ppm (δ C 99.19) with a *meta* coupling constant (J = 2.3 Hz). These data, together with the presence of two carbonyl groups in the ^{13}C NMR spectrum as reported above, allowed to identify **10** as a biflavone derivative.

The identification in the ^{1}H spectrum of two strongly deshielded singlets at 12.98 and 12.99 ppm indicated the presence of two chelated hydroxy groups. Moreover, the identification in the ^{1}H spectrum of a singlet at 3.89 ppm (δ C 56.0) allowed the placement of a methoxy group on the trisubstituted aromatic ring. HMBC analysis allowed to conclude that the compound was a biflavone (Fig. 2) with either 4′–4‴ or 3′–4‴ interflavonoid ether linkage.

Okigawa[16] and Gunasekar[17] have reported the characterization of biflavonoids from *Ochna squarrosa* and *Ochna obtusata* with a 3′–4‴ ether linkage, whereas Kumar,[18] Shen[19] and Bahia[20] identified biflavonoids with a 4′–4‴ ether linkage. Comparison of ^{13}C NMR spectroscopic data of compound **10** with those reported by these Authors, showed that C-4′ should be involved in the interflavonoid ether linkage with C-4‴. This was further confirmed by analyzing NOESY spectroscopic data. The position of the methoxy group on C-3′ was established by the positive nOe effect between H-2′ and 3′-OCH_3. Further, in the HMBC spectrum, the correlations among C-3′ and 3′-OCH_3, C-4′ and H-5′ and H-6′; C-4‴ and H-2‴ and H-6‴; C-2 and H-6′ and H-2′, confirmed the 4′–4‴ ether linkage. Examination of the remaining signals in NMR spectra led to identify two sugar moieties: in fact, in ^{13}C NMR, a signal at 99.37 ppm whose hydrogen resonated as a doublet at 5.26 ppm (2H, J = 7.4 Hz), was clearly attributable to two overlapped anomeric carbons. The remaining oxygenated carbons showed signals in the range 71.3–75.7 ppm (^{1}H signals in the range 3.39–4.02 ppm).

The absence of methylenic groups, deduced from the DEPT spectrum, led to hypothesize that the molecule contained two uronic acid portions. This hypothesis was confirmed by HMBC analysis, showing a clear correlation between the signal of the hydrogen at 4.02 ppm and the carboxylic group at 170.3 ppm. The position of the sugar linkage was confirmed by the correlation between C-7, C-7″ and H-1′′′′, H-1′′′′′. Finally, the configurations of anomeric protons were both deduced to be β on the basis of coupling constants of H-1′′′′ and H-1′′′′′ with their adjacent protons (J = 7.4 Hz). The large values of all the remaining H, H coupling constants in the sugar moieties (J in the range 8.7–9.6 Hz) indicative of axial–axial couplings, confirmed the presence of two glucuronic acid portions. After acid hydrolysis of **10**, the aqueous layer was separated to give only D-glucuronic acid, as determined by optical rotation measurement.

Based on the above data, the structure of compound **10** was determined as a Chrysoeriol-7-*O*-β-D-glucuronopyranoside-(4′-*O*-4‴)-chrysin-7″-*O*-β-D-glucuronopyranoside. In accordance, positive ESI-MS spectrum of compound **10** showed a peak at 491.05541 m/z [B−H+2Na]$^+$, 521.06603 [A−H+2Na]$^+$, whereas negative ESI-MS spectrum showed peaks at 445.07660 m/z [B−H]$^-$ and 475.08679 [A−H]$^-$.

While the biflavonoid 5,5″,7,7″-tetrahydroxy-3′-methoxy-4′,4‴-biflavonylether (3′-*O*-methyl loniflavone) has previously been

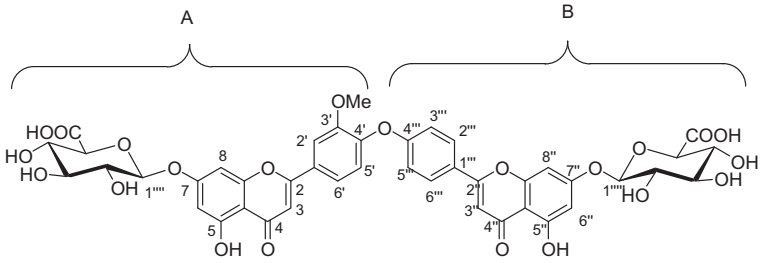


Figure 2. Structure of compound **10**.

isolated from *Lonicera japonica* (Caprifoliacee)[18] this is, to our knowledge, the first occurrence of the corresponding 7,7″-diglucuronopyranoside.

Acknowledgments

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Supplementary data

Supplementary data (experimental procedures for the synthesis of compound **1**, ^{1}H, ^{13}C, HSQC, HMBC, COSY NMR spectra of natural compounds **1** and **10**) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2011.08.143.

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