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Unprecedented Ultra-High-Resolution Hydroxy Group ^{1}H NMR Spectroscopic Analysis of Plant Extracts

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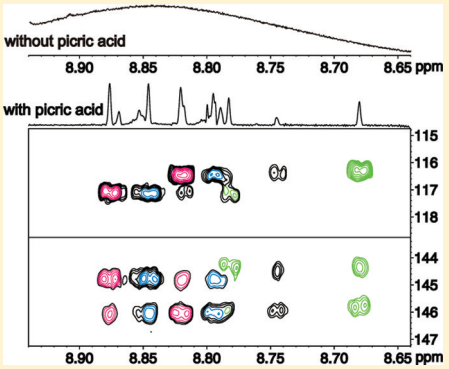
Supporting Information

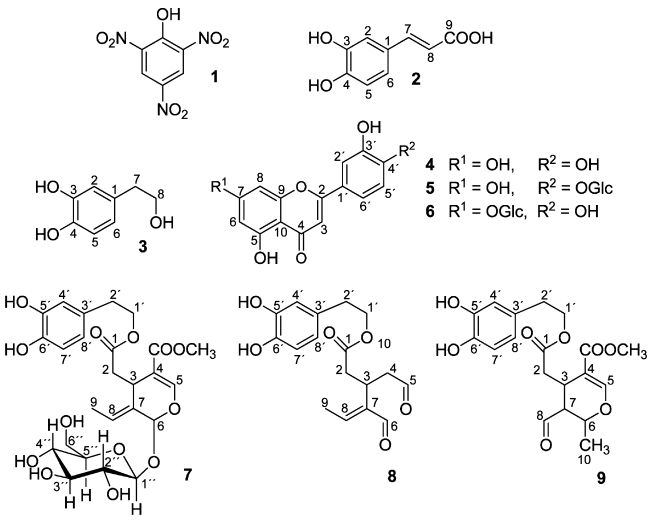
ABSTRACT: A general method is demonstrated for obtaining ultra-high resolution in the phenolic hydroxy group ^{1}H NMR spectroscopic region, in DMSO-d_6 solution, with the addition of picric acid. Line-width reduction by a factor of over 100 was observed, which resulted in line-widths ranging from 1.6 to 0.6 Hz. This unprecedented resolution, in combination with the shielding sensitivity of the hydroxy group absorptions to substituent effects at least up to 11 bonds distant and the application of 2D ^{1}H−^{13}C HMBC techniques, allows the unequivocal structure analysis of natural products with phenolic hydroxy groups in complex plant extracts.



Analysis of plant extracts is an active research area that is based on quite time-consuming chromatographic techniques and/or the use of specialized hyphenated NMR and MS techniques.[1,2] In the past few years, however, significant effort has been given to the development of new NMR methodologies that can be applied successfully to the analysis of various constituents of complex plant extracts without any previous separation or isolation of the individual components.[3−7]

Among the most important and frequently encountered functionalities conferring bioactivity in natural products are phenolic hydroxy groups.[8,9] The ^{1}H NMR resonances of these hydroxy groups appear at room temperature as broad signals, usually due to intermolecular proton exchange.[10] This exchange rate depends upon the concentration of the solute molecule and of the residual H_2O and is acid- or base-catalyzed.[11] Recently, it has been demonstrated that the use of DMSO-d_6 solution having the pH value adjusted with HCl for a minimum hydroxy group proton exchange results in significantly enhanced resolution in model compounds or their artificial mixtures.[12]

Herein, we describe a general procedure for the observation of ultra-high-resolution resonance absorptions ($\Delta\nu_{1/2} \leq 1.6$ Hz) of the hydroxy group NMR region in DMSO-d_6 solution with the addition of picric acid (**1**) to eliminate fast intermolecular proton exchange. The sensitivity of the −OH shieldings is demonstrated in terms of substitution at least up to 11 bonds distant, and the straightforward application is shown of the ^{1}H−^{13}C HMBC experiment for the unequivocal assignment of naturally occurring antioxidants in plant extracts, without the need for the isolation of the individual components. Furthermore, this method is shown to be more effective for the analysis of complex plant extracts when compared to the use of HCl[12] or other acids.



The 1D ^{1}H NMR spectrum of caffeic acid (**2**, $c = 5.7 \times 10^{-3}$ M) in DMSO-d_6 exhibits extremely broad resonances of the C-4 and C-3 OH groups, with line-widths of 203 and 164 Hz,

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respectively (Figure 1A). The addition of a progressively increased amount from a stock solution of 2.1×10^{-3} M picric

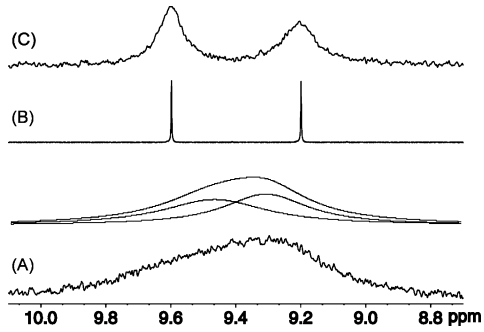

Figure 1. 500 MHz 1D ^{1}H NMR spectra of the hydroxy group region of caffeic acid (**2**) ($c = 5.7 \times 10^{-3}$ M, $T = 292$ K, number of scans = 32), in (A) DMSO-d_6; upper trace: simulated Lorentzian line-shapes using a peak-fitting routine; (B and C) with a molar ratio [**1**]/[**2**] of 12×10^{-3} and 219×10^{-3}, respectively.

acid (**1**) in DMSO-d_6 demonstrated a very significant reduction in these line-widths (Figure 1B). Picric acid is a strong organic acid (pK_a 0.42),[13] which interacts with numerous aromatic systems, adopting a variety of stacking modes,[14] hydrogen-bonding interactions,[15,16] and charge-transfer complexes.[17] For a molar ratio [**1**]/[**2**] $\approx 12 \times 10^{-3}$, a minimum in the line-widths of the C-4 ($\Delta\nu_{1/2} \approx 1.2$ Hz) and C-3 ($\Delta\nu_{1/2} \approx 1.1$ Hz) hydroxy group resonance absorptions was obtained, with a reduction in the line-widths by a factor of more than 100. The minimum line-width achieved for the COOH group was ~20 Hz, while that of H_2O was approximately the same as that of the C-4 and C-3 OH resonance absorptions. Further increases of the amount of **1** resulted in an increase in the line-widths of the absorptions (Figure 1C). It has been demonstrated that the chemical exchange of −OH and −NH protons proceeds by way of distinct chemical reactions that may involve, in the role of catalysts, H_3O^+ and OH^-, and general acids and bases including H_2O.[11,18,19] A U-shaped curve is predicted with a minimum in aqueous solutions at pH 4.5.[18,19] A similar minimum in the exchange rate has been observed for a mixture of H_2O and organic solvents.[18] It is evident therefore that at pH values below this minimum the exchange rate is acid-catalyzed, while above this it is OH^--catalyzed.

The 1D ^{1}H NMR spectrum in DMSO-d_6 of hydroxytyrosol (**3**) (Figure 2A), which is one of the major constituents of the olive [*Olea europea* L. (Oleaceae)] leaf methanol extract, demonstrated a composite broad signal ($\Delta\nu_{1/2} \approx 47.6$ Hz) of the C-3 and C-4 OH groups. Addition of 6 μL of 2.1×10^{-3} M **1** resulted in a very significant reduction in the line-widths of the two phenolic −OH, the −CH_2OH, and the H-8 resonance absorptions (Figure 2B). The sharp −OH resonances ($\Delta\nu_{1/2} \approx$ 1.0 Hz) allowed the application of the 2D ^{1}H−^{13}C HMBC method, which resulted in a significant number of $^nJ(^1H,^{13}C)$ cross-peaks of the C-3, C-4, and C-8 OH groups (Figure 3). For example, the common $^{2,3}J(^1H,^{13}C)$ cross-peaks of the C-4 and C-3 hydroxy protons to carbons C-4 and C-3 indicated that these two OH groups are in an *ortho* position with respect to each other (Figure 3A). The $^3J(^1H,^{13}C)$ cross-peak of the C-3 OH group to C-2 is of particular interest, since it allows further connectivity to the H-7 proton and, thus, through $^2J(^1H,^{13}C)$ to the C-8 carbon and, then, to the C-8 OH group (Figure 3B).

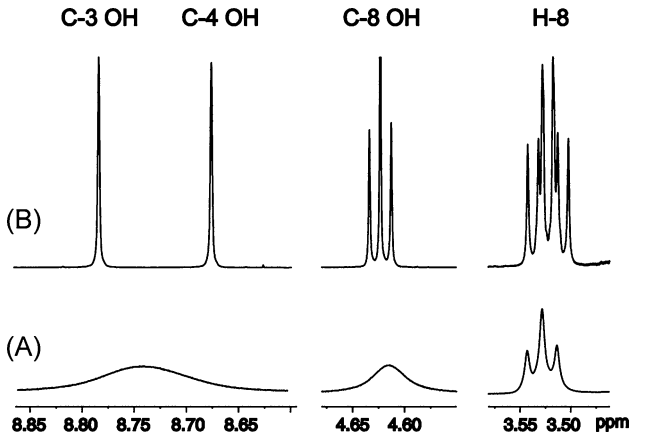

Figure 2. 500 MHz 1D ^{1}H NMR spectra of hydroxytyrosol (**3**) ($c = 19.6 \times 10^{-3}$ M, $T = 288$ K, number of scans = 16), in (A) DMSO-d_6; (B) with a molar ratio of [**1**]/[**3**] of 1.3×10^{-3}.

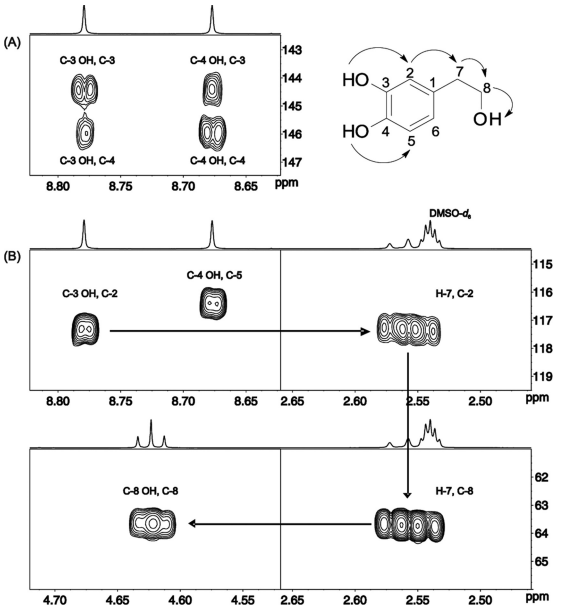

Figure 3. Selected regions of the 500 MHz 2D ^{1}H−^{13}C HMBC NMR spectrum of the solution of Figure 2B ($T = 288$ K, number of scans = 8, experimental time = 1 h).

Figure 4A illustrates selected regions of the ^{1}H NMR spectrum of the olive leaf methanol extract in DMSO-d_6. Several extremely deshielded resonances were observed in the region above 12.5 ppm with line-widths ranging from 10.0 to 5.0 Hz. These resonances could be attributed to the C-5 OH protons of flavonoids that participate in a strong intramolecular hydrogen bond with the oxygen atoms of the C-4 carbonyl group,[20] thus resulting in relatively sharp resonances. No resonance absorptions were observed in the region δ_H 10.5−11.5 ppm, where the C-7 OH groups of flavonoids are expected to give rise to ^{1}H NMR signals. In the region δ_H 8.0−9.0 ppm (Figure 4A), a strong and broad resonance ($\Delta\nu_{1/2} \approx 250$ Hz) was observed, which was attributed to oleuropein 6-*O*-β-D-glucopyranoside (**7**) and its derivatives, as the main constituents of the extract.[21] Addition of **1** (Figure 4B) in combination with dilution (Figure 4C) resulted in excellent resolution of all the hydroxy group resonances. More specifically, in the region above 11.5 ppm, three major peaks at δ_H 13.03 ($\Delta\nu_{1/2} \approx 0.7$ Hz), 13.02 ($\Delta\nu_{1/2} \approx 0.7$ Hz), and

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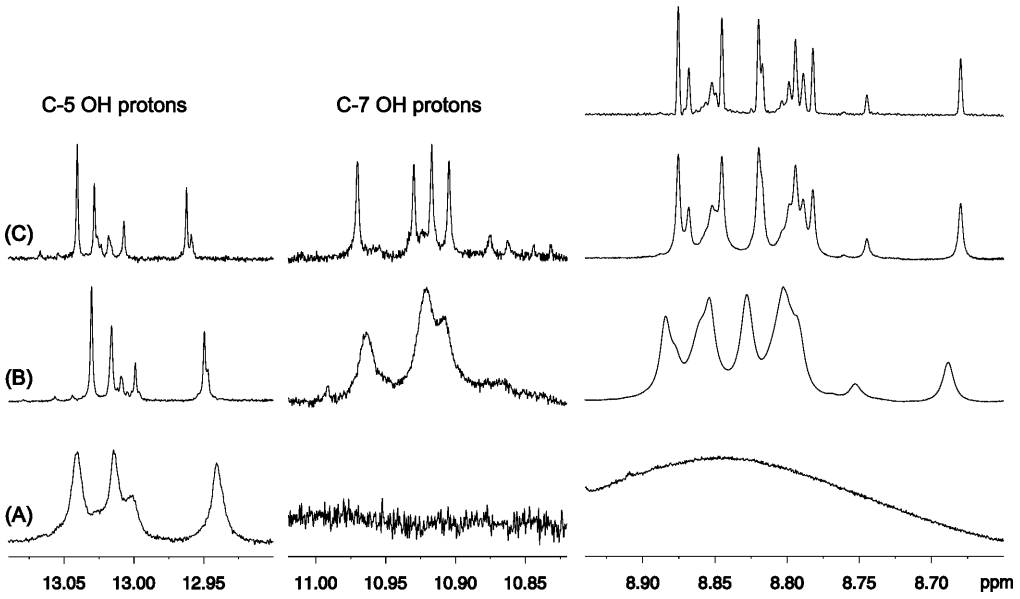

Figure 4. Selected regions of the 500 MHz 1D ^{1}H NMR spectra of 20 mg of an olive leaf methanol extract in 0.6 mL of DMSO-d_6 (T = 288 K): (A) without the addition of **1**; (B) with a mass ratio of [**1**]/[extract] of 49.3 × 10^{-3}; (C) the same solution as in (A) with a dilution factor of 8 and mass ratio of [**1**]/[extract] of 49.4 × 10^{-3}; upper trace, the same spectrum as in (C) with the application of Lorentz-to-Gauss transformation.

12.95 ($\Delta\nu_{1/2} \approx 0.6$ Hz) and several minor peaks were observed clearly (Figure 4C). In the shielding region of the C-7 hydroxy groups, four major peaks at δ_H 10.97 ($\Delta\nu_{1/2} \approx 1.1$ Hz), 10.93 ($\Delta\nu_{1/2} \approx 1.1$ Hz), 10.92 ($\Delta\nu_{1/2} \approx 1.1$ Hz), and 10.91 ($\Delta\nu_{1/2} \approx 1.1$ Hz) were seen clearly (Figure 4C), which were previously undetected (Figure 4A). It should be emphasized that the addition of **1** has a minor effect on ^{1}H NMR chemical shifts, which was found to be below 0.03 ppm (Figures 2 and 4). This demonstrates that the ^{1}H shieldings of the phenolic −OH groups can be of high diagnostic value for identifying individual components in complex plant extracts.

Application of the 2D ^{1}H−^{13}C HMBC method (Figure 5A) resulted in a significant number of $^{n}J(^{1}H,^{13}C)$ cross-peaks of the C-5 and C-7 hydroxy groups, which allowed the assignments of compounds **4**−**6**. For example, the common cross-peaks of the C-5 and C-7 OH groups to C-6 (99.92 ppm), C-5 (162.55 ppm), and C-7 (165.05 ppm) of luteolin-4′-*O*-*β*-D-glucopyranoside (**5**) and the common cross-peaks of the C-5 and C-7 OH groups to C-6 (99.42 ppm) and C-7 (165.01 ppm) of luteolin (**4**) are of particular diagnostic value (Figure 5A).

Addition of **1** in combination with dilution (Figure 4C) resulted in excellent resolution of the hydroxy group region of oleuropein 6-*O*-*β*-D-glucopyranoside (**7**) and its derivatives in the region δ_H 8.6−8.9 ppm.[22] Application of the 2D ^{1}H−^{13}C HSQC and HMBC pulse sequences (Figure 5B) showed a significant number of cross-peaks of the OH groups that allowed the complete assignment of the carbon skeletal of compounds **3**, **7**, and **9**. The resonance absorptions at δ_H 8.66 and 8.77 were attributed to the C-4 and C-3 hydroxy protons, respectively, of hydroxytyrosol (**3**), since the cross-peaks to carbons resonating at δ_C 116.28, 144.37, and 145.83 and 117.22, 144.37, and 145.83, respectively, corresponded to those of the model compound [within ±0.01 ppm (^{1}H) and ±0.2 ppm (^{13}C)]. In addition, the C-4 and C-3 hydroxy protons exhibited common cross-peaks to the C-4 and C-3 signals at δ_C 144.37 and 145.83, respectively, which are of particular diagnostic value (Figure 5B). Similarly, the resonance

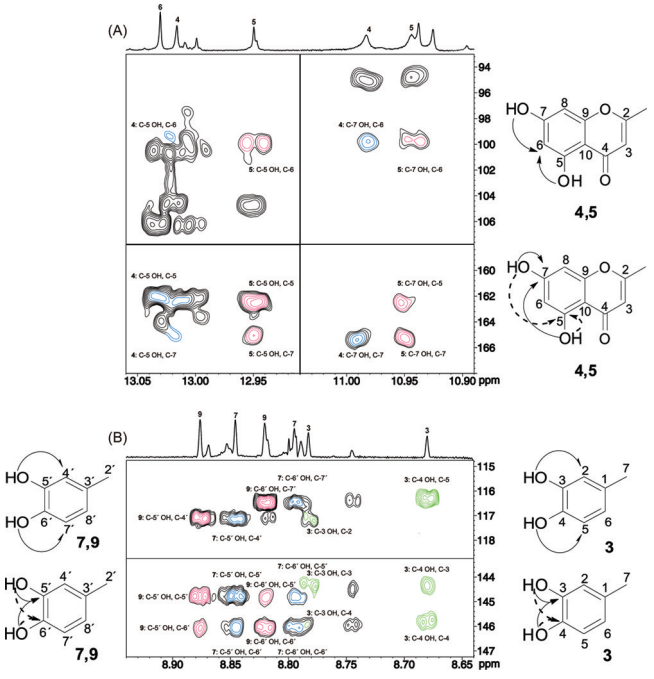

Figure 5. 500 MHz 2D ^{1}H−^{13}C HMBC NMR spectrum of 10 mg of an olive leaf methanol extract in 0.6 mL of DMSO-d_6 with a mass ratio of [**1**]/[extract] of 49.3 × 10^{-3} (T = 288 K, number of scans = 88, experimental time = 11 h and 34 min). (A) The common cross-peaks of the C-5 and C-7 hydroxy protons to carbons C-6, C-5, and C-7 of luteolin-4′-*O*-*β*-D-glucopyranoside (**5**) and the common cross-peaks of the C-5 and C-7 hydroxy protons to carbons C-6 and C-7 of luteolin (**4**) are illustrated in red and blue, respectively. (B) The common cross-peaks of the C-5′ and C-6′ hydroxy protons to carbons C-4′ and C-7′, respectively (upper trace), and the common cross-peaks to carbons C-5′ and C-6′ (lower trace) are illustrated in blue for oleuropein 6-*O*-*β*-D-glucopyranoside (**7**), green for hydroxytyrosol (**3**), and red for oleuropein (aldehyde form) (**9**).

absorptions at δ_H 8.80 and 8.85 and those at δ_H 8.82 and 8.88 were assigned to the C-6′ and C-5′ hydroxy protons of

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Table 1. Concentration Levels[a,b] of Compounds 3−9 in Olive Leaf Extracts

extract	3	4	5	6	7	8	9
methanol	7.0 ± 0.4	1.6 ± 0.1	2.9 ± 0.3	3.4 ± 0.2	36.9 ± 3.0	ND[c]	32.9 ± 2.0
aqueous	1.3 ± 0.1	ND[c]	0.2 ± 0.05	0.4 ± 0.05	9.2 ± 1.1	7.2 ± 0.2	ND[c]

[a]mg g^{-1} extract. [b]Mean value of three measurements on three different extracts of the same sample ± SD (standard deviation). [c]ND = not detected.

oleuropein 6-*O*-*β*-D-glucopyranoside (**7**) and oleuropein (aldehyde form) (**9**), respectively. Similar methodology was used for the identification of the compounds **3** and **5**−**8** of an olive leaf aqueous extract in DMSO-d_6. In Table 1, quantitative results for compounds **3**−**9** are provided for these two extracts.

The experimental protocol for sequential −OH and carbon skeletal resonance assignment is exemplified in the case of compounds **3**, **5**, **7**, and **9** for the olive leaf methanol extract and **8** for the aqueous extract and is illustrated in the Supporting Information. It is of interest that the ^{13}C shieldings of **7**, **8**, and **9** are nearly identical, while those of the C-5′ and C-6′ hydroxy protons are considerably different and distinct. The same phenomenon could also be observed for the case of flavonoids **4**−**6** (luteolin, luteolin-4′-*O*-*β*-D-glucopyranoside, and luteolin-7-*O*-*β*-D-glucopyranoside, respectively), where the C-5 OH shielding is very sensitive to substitution at position C-4′. This demonstrates the shielding sensitivity of the −OH absorptions to the substituent effect for at least up to 11 bonds distant and, thus, the excellent diagnostic value of the −OH shielding range. Identifying individual components in complex mixtures of structurally related compounds could be of importance, since they might possess significantly different biological activities.[2,9,21]

Several comparative experiments were performed with the pH value adjusted for a minimum in the −OH proton exchange rate with HCl,[12] CCl_3COOH (pK_a 0.66), CF_3COOH (pK_a 0.52), and **1**. Picric acid (**1**) and CF_3COOH were found to be more effective when compared to the use of HCl[12] or CCl_3COOH (see Supporting Information). CF_3COOH was shown to be less effective when compared to **1** in achieving line-widths in the range 0.6 to 1.5 Hz. This result could be attributed to stacking modes of interaction of **1** with aromatic systems.[14]

In this study, a general method has been described for obtaining ultra-high-resolution −OH ^1H NMR resonances in DMSO-d_6 with the addition of picric acid (**1**), which results in a minimum −OH proton exchange rate. This method in combination with the 2D $^1H-^{13}C$ HMBC technique allows the rapid, systematic, and complete assignments of phenol-type compounds in complex plant extracts and may open a new avenue in structural analysis without the need for laborious isolation of the individual components. Since the −OH ^1H NMR spectroscopic region is much simpler than the overcrowded aromatic region, and −OH shieldings are sensitive to substituent effects at least up to 11 bonds distant, the presently described methodology could easily be implemented in combination with principal component analysis in metabolomics.[23]

EXPERIMENTAL SECTION

General Experimental Procedures. NMR experiments were performed on a Bruker AV-500 spectrometer equipped with a TXI cryoprobe (Bruker BioSpin, Rheinstetten, Germany). Samples were dissolved in 0.6 mL of DMSO-d_6 and transferred to 5 mm NMR tubes. All chemical shifts were measured with reference to the internal standard, $Me_3SiCD_2CD_2COONa$, TSP-d_4 (δ_H = 0.000 ppm), of a given concentration (0.02 mM). The amount of added picric acid (**1**) (as an agent capable of considerably lowering the −OH proton exchange rate and, thus, sharpening the ^1H NMR signals of the hydroxy groups) was 5 to 15 μL from a stock solution of 2.1×10^{-3} M **1** in DMDO-d_6 for model compounds and 40 to 60 μL from a stock solution of 0.1 M **1** in DMDO-d_6 for plant extracts. The NMR system was controlled by the software TopSpin 2.1. All spectra were acquired with an acquisition time of 2.499 s, 64K data points, and 90° pulse length. To ensure adequate relaxation of the −OH protons used for quantification, their T_1 was measured by the null point method. T_1 was found to be 2 to 3 s, and thus the acquisition time plus the relaxation delay was set at 4 times longer. Quantification was achieved using TSP-d_4. The $^1H-^{13}C$ HSQC and HMBC experiments were carried out using standard Bruker software, and parameters were optimized for 145.0 and 8.0 to 2.5 Hz, respectively.

Plant Material and Extraction Procedures. An olive tree (*O. europaea*) was located in northwest Greece, and the leaves were collected in November 2005. The leaves were washed, dried in the open air, and stored in a freezer. Reference specimens are retained in the herbarium of the University of Ioannina with voucher accession number UOI051108. Fresh whole leaves (50 g) were boiled with 250 mL of distilled water for 1 h and filtered. The water was totally removed with a freeze-dryer (CHRIST Alpha 1-2, Osterode, Germany) to obtain the aqueous olive leaf dried extract. Approximately 4.2 g of purified material was obtained. In order to prepare the olive leaf methanol extract, 50 g of the leaves was macerated in 250 mL of methanol for 7 days in the dark at room temperature. The extract was separated by filtration, and the solvent was evaporated under a vacuum. Approximately 6.7 g of purified material was obtained.

Chemicals. DMSO-d_6 (NMR quality) was purchased from Deutero (Kastellaun, Germany). Oleuropein 6-*O*-*β*-D-glucopyranoside, hydroxytyrosol, luteolin-7-*O*-*β*-D-glucopyranoside, luteolin-4′-*O*-*β*-D-glucopyranoside, and luteolin were obtained from Extrasynthese (Genay, France). Picric acid was obtained from Ferak (Berlin, Germany), CF_3COOH from Fluka Chemie (Buchs, Switzerland), and CCl_3COOH from Merck (Darmstadt, Germany).

ASSOCIATED CONTENT

Supporting Information

Experimental protocol for the assignment of the HMBC spectra for compounds **3**, **5**, and **7**−**9**. This material is available free of charge via the Internet at http://pubs.acs.org.

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