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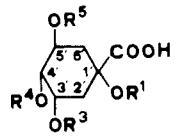
Food-Related Applications of One- and Two-Dimensional High-Resolution Proton Nuclear Magnetic Resonance: Structure and Conformation of Cynarin

Ian Horman,* Raphael Badoud, and Willi Ammann

Cynarin, a depside isolated from artichokes, is shown to be 1′,3′-di-*O*-caffeoyl-D-(−)-quinic acid. This confirms one of the two structures suggested in earlier literature. The two caffeoyl residues are axially disposed on the cyclohexane ring of quinic acid. These results were obtained from 1 mg of cynarin in acetone solution and from only 90 μg in D_2O solution, showing the capabilities of modern NMR techniques in providing extensive structural information on the small quantities of food constituents typically isolated by current high-performance separation techniques.

If the volatiles of foods have received much attention during the past decade, the same cannot be said for food solids. For these, a major barrier has been the availability of methods capable of providing comprehensive structural and conformational information on the small quantities of substances typically isolated. This problem has been accentuated by the development of separation techniques like high-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC) that permit isolation of constituents in a highly pure state but often in submilligram amounts. Recent advances in one-dimensional (1-D) and two-dimensional (2-D) NMR methods have opened up new fields of exploitation of this technique, and we report here a study on cynarin using a 2-D homonuclear correlated (COSY) proton experiment along with the conventional 1-D spectrum, both recorded at 300 MHz. Cynarin provides a good example because it is of limited solubility, giving saturated solution at 20 °C in acetone-d_6 of 6.8 millimolar, and in D_2O of 0.58 millimolar. The maximum quantities of sample analysed thus correspond to 1 mg in acetone-d_6 and 90 μg in D_2O.

Cynarin is a depside diester isolated from artichokes. It belongs to the chlorogenic acid family, which are shikimic acid metabolites in plants (Bu'Lock, 1965), and was first reported by Panizzi and Scarpati (1954) to 1′,4′-di-caffeoylquinic acid (I). Panizzi and Scarpati (1965) subsequently published a revised structure, namely, 1′,3′-dicaffeoylquinic acid (II), based on oxidative cleavage, derivatization, and specific synthesis. Our results have confirmed structure II and have demonstrated the conformation of the quinic acid ring, which places the two bulky caffeoyl groups in axial positions.



	I	II
R^1	CAFFEOYL	CAFFEOYL
R^3	H	CAFFEOYL
R^4	CAFFEOYL	H
R^5	H	H

It should be noted that throughout this article, numbering of the quinic acid ring follows IUPAC nomenclature recommendations and goes around the ring in the opposite direction to the original literature nomenclature: II was originally labeled 1,5-dicaffeoylquinic acid.

EXPERIMENTAL SECTION

Cynarin, isolated from artichokes, was purchased from Roth Products (Basel, CH) and had mp 229 °C [cf. lit. mp 227–228 °C with decomposition, (Panizzi and Scarpati, 1954)]. It was used without further purification. Acetone-d_6 and D_2O from Stohler Isotopes (Innerberg, CH) were at least 99.85% pure. Cynarin was dissolved to give a saturated solution at 20 °C either in acetone-d_6 (3.5 mg·mL^{-1}) or in D_2O (300 μg·mL^{-1}), and a 0.3-mL solution in a 5 mm diameter sample tube was used for the NMR

Nestlé Research Laboratories, 1814 La Tour de Peilz, Switzerland (I.H. and R.B.), and Varian NMR Applications Laboratories, 6500 Zug, Switzerland (W.A.).

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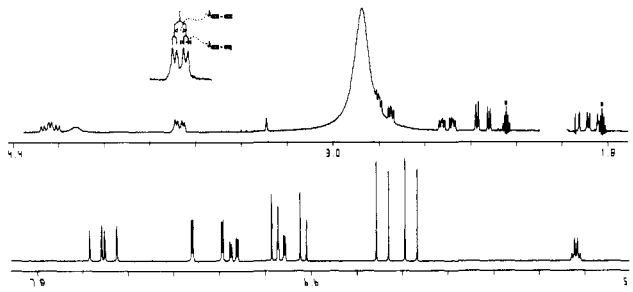


Figure 1. ^1H NMR spectrum (300 MHz) of cynarin in acetone-d_6 (solvent signal at 2.04 ppm): 2400-Hz spectral width; 4.0-s acquisition time; zero-filled to 32K data points; 0.147-Hz digital resolution; 90° observe pulse angle; 240 transients accumulated in 16 min. Exponential resolution enhancement and Gaussian apodization were applied. The two quintets at 1.83 and 2.25 ppm, separated by 126 Hz, represent the ^{13}C satellite signals of the solvent peak.

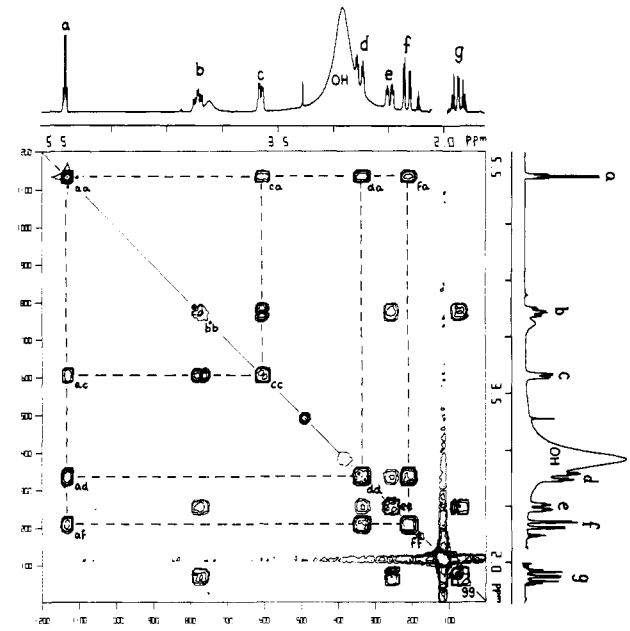


Figure 2. Homonuclear correlated ^1H NMR spectrum (300 MHz) of cynarin in acetone-d_6: 2200 × 2200 Hz spectral widths; 512 × 512 data points; 0.12-s acquisition time; 3.0-s equilibrium delay between acquisitions; 4 transients completed for each of the 256 traces; total measuring time 55 min. The absolute-value intensity contour plot of the 1.5–6.0-ppm region is shown. The strong signal at 2.04 ppm arises from residual acetone-d_5.

experiments. Spectra were obtained on a Varian XL-300 NMR spectrometer using standard software. The homonuclear COSY experiment employed the pulse sequence

equilibration–90° $(+X)$–T_1–90° (ϕ)–acquisition (T_2)

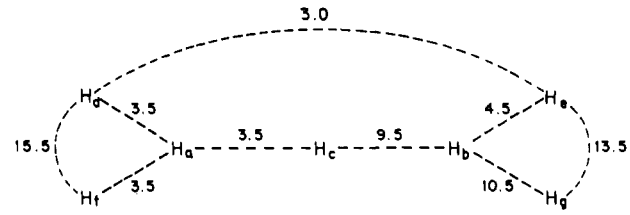
where $\phi = +X, +Y, -X, -Y$.

The phase cycling in the experiment was based on that given by Bax et al. (1981a), permitting quadrature detection in both frequency dimensions. Pseudoecho data processing (Bax et al., 1981b) of the 2-D data was used to improve peak definition and triangular folding for improved sensitivity and spectral quality.

RESULTS AND DISCUSSION

The 300-MHz proton spectrum of cynarin in acetone-d_6 is shown in Figure 1. Signals for the protons in the caffeoyl moities (excluding phenolic protons) appear between δ 6.0 and 8.0, and seven multiplets for the protons in the quinic acid cycle (excluding hydroxyl protons) are observed between δ 1.5 and 5.7. For this latter group of signals, a 2-D COSY proton spectrum is presented in Figure 2 in the

Scheme I. Coupling Network of Quinic Acid Ring Protons with Coupling Constants (J_{HH}) in Hertz

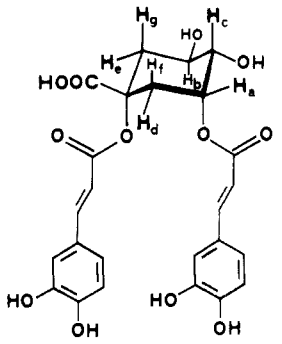


form of a contour plot. In such a 2-D spectrum, the base line of the conventional 1-D spectrum as in Figure 1 is replaced by a base plane, and areas of signal density are shown as spots on the contour plot, in a manner analogous to that in which a cartographer represents mountains. Both the horizontal and vertical axes of Figure 2 are composed of the segment of Figure 1 corresponding to the seven quinic acid protons, and the seven corresponding multiplets are lettered a–g: spots with coordinates (a,a), (b,b), ..., (g,g) in the contour plot lie on the diagonal and represent correlation peaks for a–g that relate their positions on the horizontal and vertical axes. The off-diagonal signals in Figure 2 establish correlations between protons that couple: thus all two-bond and longer range couplings can be observed. Coupling relationships are revealed as squares symmetrically disposed about the diagonal, and three such squares from proton a are shown in Figure 2, establishing its coupling to protons c, d, and f. In the same manner, all other coupling relationships can be established unambiguously to give the coupling network shown in Scheme I. Here, it is important to note that the sequence of hydrogen atoms, which corresponds to the sequence of CH_2 and CH groups in quinic acid, its unequivocally established by the correlation peaks in the 2-D spectrum, requiring no previous knowledge of the quinic acid structure.

The absolute values of the coupling constants, as indicated, were classically measured from the 1-D spectrum as shown in insert in Figure 1. The largest couplings in Scheme I, $J_{df} \sim 15.5$ Hz and $J_{eg} \sim 13.5$ Hz, must be attributed to geminal couplings in the methylene groups. Because there is only one coupling, $J_{ed} \sim 3.0$ Hz, between these methylene groups, they cannot be directly bound and must be separated by a nonprotonated nucleus, in this case C-1′ of the quinic acid cycle.

The deshielded position of the signal for proton H_a (δ 5.43) must result from the presence of an *O*-caffeoyl residue attached to the same carbon. The more shielded positions of signals for protons H_b (δ 4.23) and H_c (δ 3.67) suggest that the hydroxyl groups attached to the corresponding carbons are free. This excludes I as being the structure of cynarin but does not as yet prove structure II, because the carbon bearing H_a and hence the caffeoyl group could be either C-3′ or C-5′.

The total structure is established by determining the conformation of the cyclohexane ring of quinic acid through analysis of the vicinal ($^3J_{H-H}$) and longer range ($^4J_{H-H}$) coupling constants. Their relation to molecular conformation is described in the literature in several texts [e.g., Jackman and Sternhell (1969)]. A complete analysis for the seven-proton network of quinic acid is presented in Table I. Proton H_c, which must be attached to C-4′, is seen to have an axial–axial coupling, $J_{bc} \sim 9.5$ Hz, with proton H_b. H_c also gives a coupling constant of ~3 Hz, typical of an axial–equatorial interaction, and indicating that its second neighboring proton H_a is equatorial. This immediately establishes the conformation of the quinic acid ring as shown in structure III and demonstrates the



III

position of the *O*-caffeoyl residue at C-3′. H_a shows three vicinal couplings with its neighbors, all of about 3.5 Hz and corresponding to axial–equatorial and equatorial–equatorial interactions. Because all three ^3J values are about the same, multiplet a appears as a quartet in the 1-D spectrum. In structure III, the quinic acid cycle is turned through 180° relative to structures I and II. This is done to represent the naturally occurring D-(−) enantiomer of quinic acid found in cynarin and not the L-(+) enantiomer as would be implied by I and II.

The signals of the methylene protons H_e and H_g at C-6′ are readily assigned: H_g is the axial proton, having a large trans diaxial vicinal coupling, J_{bg} ~10.5 Hz, with H_b. H_e is then equatorial as confirmed by the vicinal coupling J_{be} ~4.5 Hz. Observation of a relatively large longer range coupling between H_e and H_d ($^4J_{de}$ ~3.0 Hz) can only be explained by a W-type four-bond coupling between two equatorial protons (Barfield and Chakrabarti, 1969), and this establishes the assignments of the methylene protons at C-2′: H_d equatorial; H_f axial.

Thus, we establish III as the conformational structure of cynarin, showing that the two *O*-caffeoyl groups occupy axial positions on the quinic acid ring. Intuitively, in view of the bulk of these two substituents, we might have expected them to be equatorially disposed around the quinic acid cycle. It may be argued that the conformation could have been determined from the coupling constants of Table I alone, as Corse et al. (1966) have done for other chlorogenic acid isomers. However, the 2-D NMR spectrum of Figure 2 facilitated the creation of Table I by providing a totally unambiguous picture of the coupling network of Scheme I from which it was a trivial matter to rationalize the coupling constants. Further, running proton spectra at 300 MHz disperses the signals and facilitates spectral assignments. For example, the four methylene protons of cynarin that give well-separated signals in 300-MHz spectrum of Figure 1 give unresolved signals that are overlapped by the solvent peak (acetone) when observed at 80 MHz.

Spectral sensitivity is also important. The results described here were obtained from only 1 mg of cynarin in 3 h, of which about half the time was spent in recording and plotting data and the other half in interpretation. More striking, the same results can be obtained on only 90 μg (175 nmol) of cynarin, which represents its solubility limit in 0.3 mL of D_2O, in about 18 h of which 16 h are spent recording data, and the conformation of cynarin is seen to be the same in D_2O solution as in acetone-d_6.

These experiments serve to demonstrate the power of modern 2-D NMR techniques and high-resolution 1-D NMR measurements at high field strength in characterizing small amounts of individual food components. In addition to the COSY experiment described, other forms of 2-D experiments exist. Ammann et al. (1982) have described the application of several 2-D techniques in the structural investigation of the natural product lupane, and in a treatise dealing with the instrumental analysis of foods, Horman (1984) has described food applications of NMR, including possible areas where 2-D NMR at high field will be important in the future. An immediate application will be to confirm structures of known compounds like cynarin where there is doubt and to confirm and complete spectral assignments that have often been tentatively based on the incomplete information available from earlier techniques.

Table I. Compilation of ^{1}H NMR Data for the Quinic Acid Protons of Cynarin in Acetone-d_6 Solution

signal	assignment	shift,[a] ppm	multiplicity[b]	J_{H-H},[c] Hz	
a	3′, equatorial	5.43	ddd	$^3J_{H_a-H_c}$	~ 3.5
				$^3J_{H_a-H_d}$	~ 3.5
				$^3J_{H_a-H_f}$	~ 3.5
b	5′, axial	4.23	ddd	$^3J_{H_b-H_c}$	~ 9.5
				$^3J_{H_b-H_e}$	~ 4.5
				$^3J_{H_b-H_g}$	~ 10.5
c	4′, axial	3.67	dd	$^3J_{H_c-H_a}$	~ 3.5
				$^3J_{H_c-H_b}$	~ 9.5
d	2′, equatorial	2.77	ddd	$^3J_{H_d-H_a}$	~ 3.5
				$^2J_{H_d-H_f}$	~ 15.5
				$^4J_{H_d-H_e}$	~ 3.0
e	6′, equatorial	2.50	ddd	$^3J_{H_e-H_b}$	~ 4.5
				$^2J_{H_e-H_g}$	~ 13.5
				$^4J_{H_e-H_d}$	~ 3.0
f	2′, axial	2.34	dd	$^3J_{H_f-H_a}$	~ 3.5
				$^2J_{H_f-H_d}$	~ 15.5
g	6′, axial	1.88	dd	$^3J_{H_g-H_b}$	~ 10.5
				$^2J_{H_g-H_e}$	~ 13.5

[a] In ppm, relative to residual acetone-d_5 at 2.04 ppm. [b] dd = doublet of doublet; ddd = doublet of doublets of doublet. [c] Absolute value of coupling constants measured to the nearest 0.5 Hz.

Registry No. Cynarin, 1182-34-9.

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Received for review July 20, 1983. Accepted December 29, 1983.