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Characterization of Two Unknown Compounds in Methanol Extracts of Rosemary Oil

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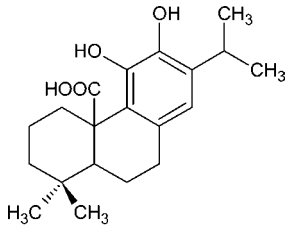
In this study, two unknown compounds in rosemary oil, containing 3% carnosic acid and 0.3% carnosol, were identified and characterized. After methanol extraction, purification, and analysis by reversed-phase HPLC and LC-MS, a recovery of 92% (±8%) of carnosic acid was obtained, but no carnosol was found. However, two unknown compounds with a molecular weight of 330.2 and 302.2 were consistently detected. From additional LC-MS-MS, ^{1}H NMR, and elemental analyses, it became clear that the first compound (M_w = 330.2) could not be carnosol. It was hypothesized that it originated from the breakdown of the intramolecular bond of carnosol, followed by the addition of a water molecule. Possibly, an unsaturated double bond was formed after dehydration. Assuming that this compound was an intermediate in the conversion to rosmanol, the second unknown compound (M_w = 302.2) may have resulted from the breakdown of the intramolecular bond of rosmanol. Similarly, an unsaturated double bond may have been formed. After splitting off carbon oxide, a detectable molecule with a molecular weight of 302.2 was observed.

KEYWORDS: Rosemary; carnosic acid; LC-MS-MS; ^{1}H NMR

INTRODUCTION

Rosemary is a well-known spice often used in cooking for its specific flavor. However, because of the presence of phenolic diterpenes with a high antioxidant activity, interest has grown in using rosemary as a natural antioxidant in food (*1*). The compound with the strongest antioxidant activity is carnosic acid, which was reported to have an even higher antioxidant activity than the most commonly used synthetic antioxidants BHT and BHA (*2*, *3*). **Figure 1** shows the chemical structure of carnosic acid (*4*).



Carnosic acid

Figure 1. Chemical structure of carnosic acid.

Research has demonstrated the instability of this phenolic diterpene in the presence of oxygen by identifying compounds originating from the breakdown of carnosic acid (*5*−*8*). Some of these breakdown compounds have been identified (e.g., carnosol, rosmanol, epi-rosmanol, epi-isorosmanol, rosmadial, and methyl carnosate). The type and number of breakdown compounds as well as their amount depend on the source, the extraction method, and the analytical techniques used for identification (*4*, *9*−*11*). However, extraction conditions such as solvent, temperature, and type of glassware also influence the stability of carnosic acid and the breakdown compounds (*12*). Quantification of the breakdown compounds of carnosic acid is further hampered by the lack of available standards and by insufficient knowledge about the molecular absorption coefficient of these compounds (*11*).

This study is part of a larger investigation aimed at screening different plant extracts for their antioxidant activity. Furthermore, these extracts were investigated for their effect on the oxidative

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stability of feed for farm animals and animal tissues. Within this framework, rosemary oil was investigated in more detail. In particular, we have focused on two unknown compounds, present in methanol extracts of rosemary oil, which were identified and characterized using different analytical techniques.

MATERIALS AND METHODS

Samples. Carnosic acid (Sigma Aldrich, Steinheim, Germany) was used for the characterization. Rosemary oil (Nutri-Ad, Dendermonde, Belgium) was investigated, and according to the information from the supplier, this rosemary oil was suspended in a vegetable oil and contained 3% carnosic acid, 0.3% carnosol, and 0.03% methylcarnosate.

Solvents. Methanol (Lab-Scan, Stillorgan, Ireland) was used as the extraction solvent. During the purification step of the samples, HPLC grade methanol, *n*-hexane, and ethyl acetate (Biosolve, Valkenswaard, The Netherlands) were used. LC-MS grade solvents (Biosolve, Valkenswaard, The Netherlands) were used for the LC-MS analyses. $CDCl_3$ and tetramethylsilane (TMS), both purchased from Aldrich (St. Louis, MO), were chosen as solvent and internal standard, respectively, for measuring ^{1}H NMR spectra.

Extraction Procedure. The extraction procedure was fully described by Smet et al. (*13*). Briefly, 0.1−1.0 mg of carnosic acid or 1.6−16.0 mg of rosemary oil were mixed with 30 mL of methanol. After shaking for 3 h at 200 rpm on a Heidolph Unimax apparatus (Model 2010, Schwabach, Germany), the mixture was filtered using a Rotilabo 130s, size 150 mm Roth filter. The filter was further washed with 30 mL of methanol. The total filtrate was then evaporated to dryness by a rotary evaporator at 30 °C. The residue was dissolved in 10 mL of methanol and stored overnight at 4 °C.

Purification. The day after extraction, the sample was purified using solid-phase extraction (SPE), as described by Mateos et al. (*14*). Briefly, under a light vacuum, the Discovery DSC-diol 3 mL tube, 500 mg packing cartridges (Supelco, Bellefonte, PA) were conditioned with 6 mL of methanol followed by 6 mL of *n*-hexane. The evaporated sample was dissolved in 6 mL of *n*-hexane and applied on the SPE column. The column was subsequently washed twice with 3 mL of *n*-hexane and once with 4 mL of *n*-hexane/ethyl acetate (90:10, v/v). Afterward, the polar fraction was eluted with 10 mL of methanol. The solvent was evaporated under reduced pressure until the sample was completely dry. The residue was dissolved in 0.5 mL of methanol, filtered over an Acrodisc CR 13 mm, 0.45 μm pore size, PTFE-membrane syringe filter, injected in a clear glass vial protected from the light with aluminum foil, and finally analyzed by LC-MS. On the basis of the stability studies of carnosic acid, described by Thorsen and Hildebrandt, the use of amber glassware was avoided to preserve the maximum amount of carnosic acid (*12*). An aliquot of 20 μL was injected into the LC-MS system.

LC-MS Analysis. Reversed-phase HPLC was performed on an Agilent 1100 LC-MSD system (Agilent Technologies, Waldbronn, Germany), which was equipped with an autosampler, a quaternary pump, a vacuum degasser, and an Agilent 1100 injection valve (20 μL loop). The system was controlled by Agilent software v. A.09.03. A C_{18} ODS precolumn was used followed by a 250 mm × 4.6 mm i.d. Luna C_{18} column (Phenomenex, Amstelveen, The Netherlands). The column was maintained at 35 °C. The mobile phase was a mixture of 0.2% acetic acid in water (solvent A) with a pH of 3.1, acetonitrile (solvent B), and methanol (solvent C). During the first 5 min, the solvent gradient was changed linearly from 95% A, 2.5% B, and 2.5% C to 50% A, 25% B, and 25% C. Between 5 and 20 min, the gradient was changed to 30% A, 35% B, and 35% C. During the next 10 min, the gradient was changed to 0% A, 50% B, and 50% C. This gradient lasted for 12 min, and during the last 3 min of the sequence, the starting conditions were linearly re-established. The elution was performed at a flow rate of 1.0 mL/min.

Detection was accomplished by a DAD detector at wavelengths of 230 and 280 nm because carnosic acid and its breakdown compounds show absorption maxima at those wavelengths (*3*, *10*, *15*).

Electrospray ionization (ESI) mass spectrometry was used because of its soft ionization, which allows for the production of ions, even of labile compounds, such as carnosic acid (*16*). For this ESI method, an Agilent G1946D (SL) mass spectrometry system was used. The eluted compounds were mixed with nitrogen in the heated nebulizer interface. Adequate calibration of ESI parameters was required to optimize the response and to obtain a high sensitivity of the molecular ion. The ESI parameters were as follows: needle potential 4100 V, gas temperature 350 °C, drying gas 13 mL/min, and nebulizer pressure 50 psi. The full scan mass spectra of the phenolic compounds were measured from *m*/*z* 100−1000. The data were acquired in the negative ionization mode to increase the sensitivity for acidic compounds (e.g., carnosic acid (*11*)).

Structural Identification Using Preparative HPLC, ^{1}H NMR, and Elemental Analysis. For the preparative HPLC, the same method was used as for LC-MS with the difference being that fractions were collected each minute, for several cycles. These fractions were evaporated and dissolved in $CDCl_3$ for ^{1}H NMR analysis. High-resolution, one-dimensional, ^{1}H NMR spectra were obtained by a JEOL Eclipse FT nuclear magnetic resonance spectrometer (300 Hz), using 16 scans per spectra, a relaxation delay of 1 s, and a sequence duration of 3.6 s. Afterward, the spectra were used for structural identification of two unknown breakdown compounds, present in the extracts of both rosemary oil and carnosic acid. Combustion analyses for elemental composition were performed for both compounds, using a CHNS/O analyzer 2400 series II (PerkinElmer, Waltham, MA).

Identification of Daughter Fragments Using LC-MS-MS. An Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters, Milford, MA), equipped with an autosampler, a vacuum degasser, and an injection valve (full loop) was controlled by the Masslynx 4.0 sp4.525.532 software. For the separation of the different compounds the column, the mobile phase, solvent gradient, and flow rate were the same as described previously for LC-MS analysis. For the identification, a Waters Quattro Micro was used, equipped with an electrospray ionizer used in the negative ionization mode.

First, a full scan mass spectrum of carnosic acid (0.50 mg/mL) was measured from *m*/*z* 200−400 for the complete run. During the analysis, the cone voltage was 25 V. Afterward, the daughter scans were taken to obtain a fragmentation pattern of carnosic acid and its metabolites. Therefore, masses from *m*/*z* 50−400 were measured in the specific time intervals for carnosic acid and its metabolites. The collision energy to obtain the daughter fragments was set to 20 eV.

RESULTS

On the basis of the information of the supplier of the rosemary oil, it was suggested that after extraction, purification, and analysis of an amount of this specific oil, carnosic acid, carnosol, and methyl carnosate would have been found. However, except for carnosic acid, which is the compound with the highest antioxidant activity and present in the highest amount (*2*), carnosol and methyl carnosate were not found with our procedure. A calibration curve of carnosic acid (0.1−1.0 mg/mL) was made using the available standard, and an R^2 value of 0.995 was acquired. The recovery of carnosic acid from the rosemary oil after extraction and purification was found to be 92% (±8%). However, after the extraction and purification of an amount of rosemary oil, different analyses on LC-MS of those extracts showed two unknown peaks (**Figure 2**), which consistently appeared. Information about the composition of the rosemary oil did not explain the presence of those compounds. However, the same was observed with the standard carnosic acid, preserved in methanol for several weeks at −21 °C. This indicates the low stability of carnosic acid. Those two compounds, one with a retention time of 26.632 min (**1**) and the other with a retention time of 29.880 min (**2**), had a similar UV spectrum to carnosic acid (**Figure 3**) and an UV absorption maximum at 280 nm. This indicates that the molecular structure of those two unknown compounds was similar to carnosic acid. The molecular weights of those two compounds were 330.2 and 302.2, respectively. They were isolated from the oil extract using preparative HPLC, further evaporated, and then dissolved in

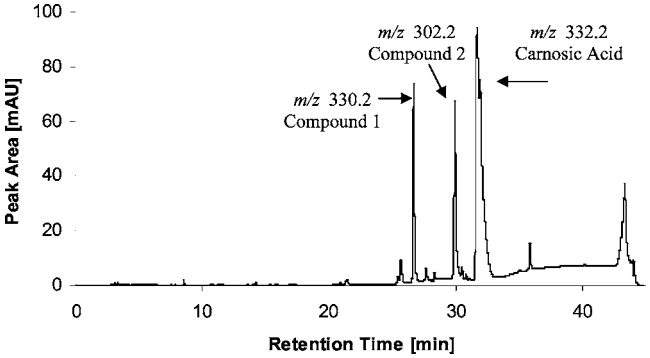

Figure 2. Chromatogram from extracted carnosic acid (1.00 mg/mL), LC-DAD at 280 nm, **1** (T_r 26.632 min; *m*/*z* 330.2), **2** (T_r 29.880 min; *m*/*z* 302.2), and carnosic acid (T_r 31.669 min; *m*/*z* 332.2). Other peaks are unidentified.

$CDCl_3$ to obtain a ^{1}H NMR spectrum. For **1**, the ^{1}H NMR (300 MHz, $CDCl_3$, ppm) was δ 0.92 (s, 3, C−C$\underline{H}_3$), 1.03 (s, 3, C−C$\underline{H}_3$), 1.20 (d, 3, J = 7 Hz, CH−C$\underline{H}_3$), 1.22 (d, 3, J = 7 Hz, CH−C$\underline{H}_3$), 1.60−2.30 (m, 7, (C$\underline{H}_2$)$_3$−C−C$\underline{H}$), 3.15 (septet, 1, C$\underline{H}$−(CH$_3$)$_2$), 5.37 (m, 1, =C$\underline{H}$), 5.68 (m, 1, =C$\underline{H}$), 5.65 (s, 1, Ar−$\underline{H}$), and 7.3 ($CDCl_3$). For **2**, the ^{1}H NMR (300 MHz, $CDCl_3$, ppm) was described as δ 0.91 (s, 3, C−C$\underline{H}_3$), 1.01 (s, 3, C−C$\underline{H}_3$), 1.19 (d, 3, CH−C$\underline{H}_3$), 1.23 (d, 3, CH−C$\underline{H}_3$), 1.30−1.90 (m, 7, (C$\underline{H}_2$)$_3$−C$\underline{H}$), 2.55 (m, 1, C$\underline{H}$−C=O), 2.85 (m, 2, C$\underline{H}_2$−C=O), 3.18 (septet, 1, C$\underline{H}$−(CH$_3$)$_2$), 6.56 (s, 1, Ar−$\underline{H}$), and 7.3 ($CDCl_3$). The elemental composition calculated for **1** ($C_{20}H_{26}O_4$): C, 72.69; H, 7.95. Found: C, 72.86; H, 7.84. Calculated for **2** ($C_{19}H_{26}O_3$): C, 75.45; H, 8.68. Found: C, 75.67; H, 8.58. Additional analyses were performed by LC-MS-MS, aiming at fragmentation spectrum of these two unknown compounds. Unfortunately, only one main daughter fragment was found for **1**, namely, a structure with *m*/*z* 287.2. No results were obtained for **2**, probably because the collision energy of 20 eV was too low to fragment this compound. Finally, the interpretation of the ^{1}H NMR spectra combined with the outputs from LC-MS and LC-MS-MS led to the structural formula of those two unknown compounds.

DISCUSSION

The composition of the analyzed rosemary oil, according to the supplier, was the following: 3% carnosic acid, 0.3% carnosol, and 0.03% methyl carnosate. However, after extraction, purification, and analysis, only carnosic acid was found in the methanol solution. This can be explained by the fact that carnosol is less stable in methanol, the extraction solvent, than carnosic acid, as reported by Thorsen and Hildebrandt (*12*). These authors found that carnosol showed a 40% reduction of its peak area after 3 days of preservation at room temperature in methanol as compared to carnosol just after manufacture (*12*). In contrast to this, carnosic acid, preserved under the same conditions, showed a reduction of only 10%. Bicchi et al. (*10*) and Troncoso et al. (*15*) reported that the main breakdown product of carnosic acid is carnosol, which can be further reformed into other phenolic diterpenes (e.g., rosmanol, epi-rosmanol, and epi-isorosmanol). In spite of the breakdown of carnosol and carnosic acid in methanol, it is still preferred as the extraction solvent because of its good extraction efficiency (*17*−*19*). This information and the data and spectra obtained were used to identify the two unknown compounds present in the extracts.

Compound 1. On the basis of the ^{1}H NMR spectrum of the compound eluted at 26.632 min, combined with its mass spectrum obtained by analysis on LC-MS-MS, it was concluded that the compound with a molecular weight of 330.2 was not carnosol. First, there appeared to be three peaks in the ^{1}H NMR spectrum of this compound between 5 and 6 ppm, which means that more than one proton was present that was bound to an unsaturated carbon atom. Therefore, besides the aromatic proton, other unsaturated carbon bonds with their protons should also occur; this was not the case for carnosol. Another reason as to why this unknown compound was not carnosol was deduced from its MS spectrum. The fragmentation of this compound showed a daughter ion with *m*/*z* 286.7. This fragment could have been formed after the cleavage of a carbon dioxide ion from the structure. The molecular structure of carnosol with its intramolecular bond does not allow this kind of cleavage. However, the unknown compound contained an isopropyl group, represented in the ^{1}H NMR spectrum by two doublets on 1.20 and 1.22 ppm, respectively, and a septet at 3.15 ppm. There was also one aromatic proton at 5.65 ppm, seven protons between 1.60 and 2.30 ppm, as well as two methyl groups at 0.92 and 1.03 ppm, respectively. These peaks correspond to the ^{1}H NMR spectrum of carnosic acid. There were also two unknown multiplets present, namely, at 5.37 and 5.68 ppm, which means that two unsaturated carbon atoms were present, each bound to one proton.

Therefore, it was hypothesized that during the methanol extraction and due to the acid mixture of the LC-MS, the lactone bond of carnosol could have been broken, as is shown in **Figure 4**. This also could have allowed the addition of a water molecule. Possibly, after dehydration, an unsaturated double bond was formed, having one proton on each carbon atom. These protons explain the two unidentified peaks in the ^{1}H NMR spectrum between 5 and 6 ppm. Because the carbonyl group is free in this structure, it also explains the daughter fragment with a

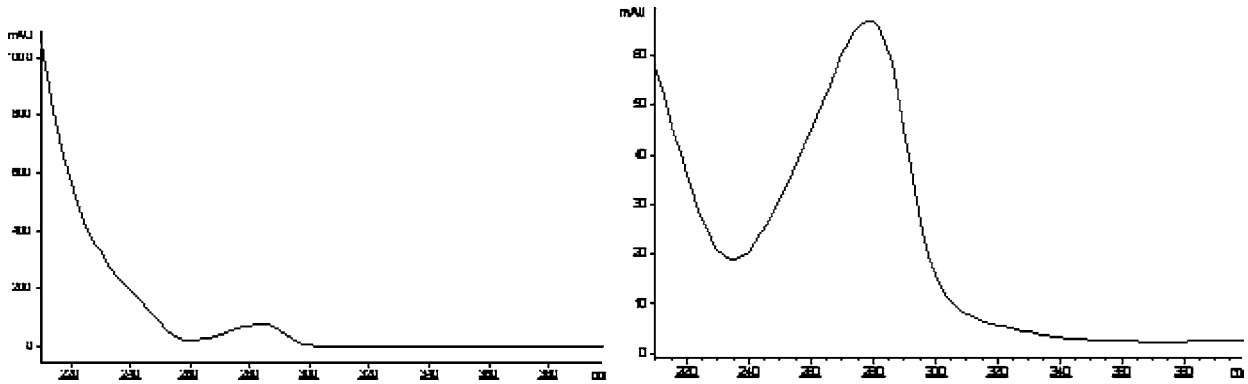

Figure 3. UV spectra from **1** (left) and **2** (right).

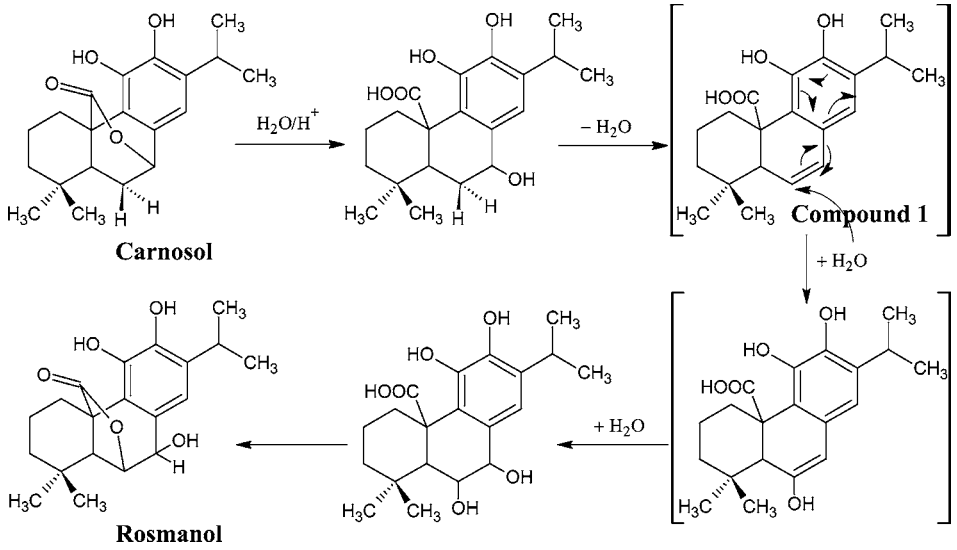

Figure 4. Hypothesis for formation of **1**, a possible intermediate in the conversion of carnosol to rosmanol.

molecular weight of 286.7 that developed after splitting off carbon dioxide. Further, this compound also could be an intermediate in the conversion reaction from carnosol to rosmanol (**Figure 4)**. After hydration, another intramolecular bond could be formed, to give rise to rosmanol. The ^{1}H NMR spectrum of **1** corresponds to the ^{1}H NMR spectrum of the compound, obtained after preparative HPLC, eluting at 26.632 min.

Compound 2. As mentioned previously, Bicchi et al. (*10*) described rearrangements of carnosic acid into carnosol. Carnosol, may be converted into other phenolic diterpenes (e.g., rosmanol, epi-rosmanol, and epi-isorosmanol). In the ^{1}H NMR spectrum, an isopropyl group was present, represented by two methyl groups at 1.19 and 1.23 ppm, respectively, and a tertiary carbon at 3.18 ppm. Also, one aromatic proton at 6.56 ppm and two methyl groups at 0.91 and 1.01 ppm, respectively, were identified. Furthermore, the UV spectrum had an absorbance pattern comparable to carnosic acid and **1** (**Figure 3**).

Assuming that rosmanol is present in the sample due to the oxidation of carnosol, it was hypothesized that the intramolecular bond of rosmanol and its isomers could have been broken in the acidified environment of the extraction solvent and the solvent mixture of LC-MS (**Figure 5**). This could again have been the start of the addition of a water molecule that after dehydration formed an unsaturated double bound. As an intermediate, an intramolecular bond could have been formed because an intramolecular reaction with a phenolic OH is much faster than the reaction with methanol. Decarboxylation of this tertiary carboxylic enol could readily occur through neighborhood participation of the phenolic OH function. After the cleavage of the carbon oxide ion, a fragment ion of *m*/*z* 302.2 and two additional peaks in the ^{1}H NMR spectrum at 2.55 and 2.85 ppm from the carbon bonds next to the carboxyl group may have been formed.

In conclusion, the analytical procedure including extraction, purification, LC-MS-MS, and ^{1}H NMR to isolate and measure the antioxidative diterpenes in a rosemary extract revealed some breakdown compounds of carnosic acid. Two of these compounds were identified (i.e., eluting at 26.632 and 29.880 min). These compounds have molecular weights of 330.2 and 302.2, respectively.

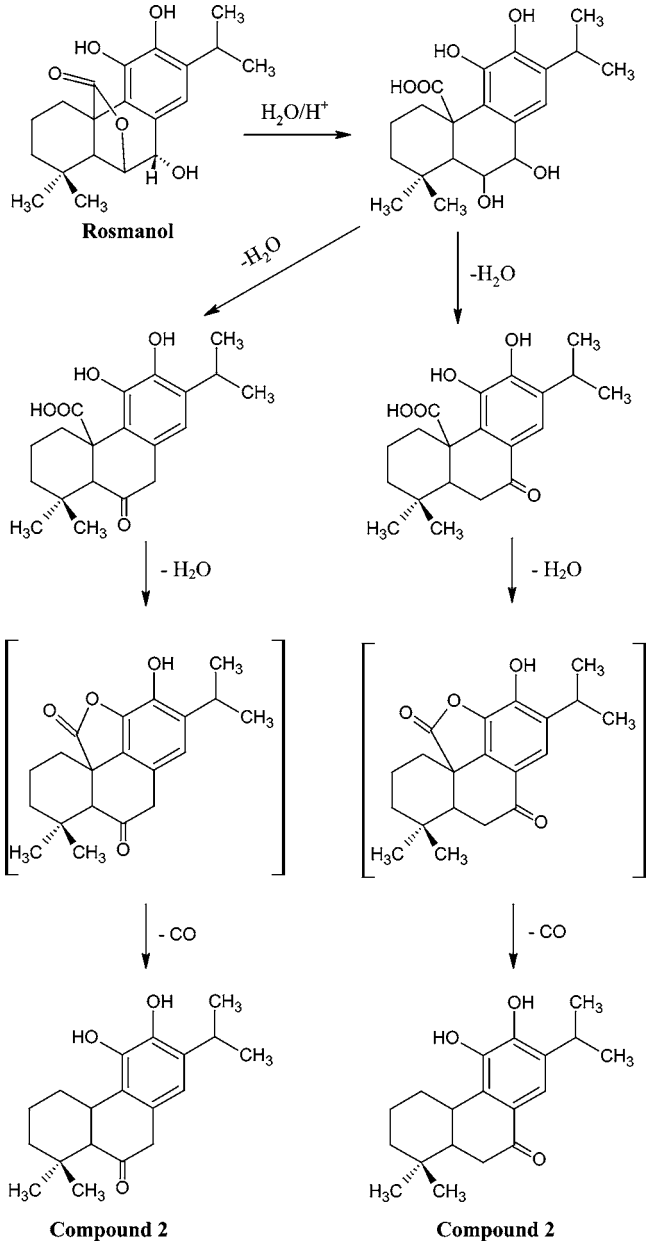

Figure 5. Hypothesis for formation of **2**.

ACKNOWLEDGMENT

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