JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Biological Activity of Acetylated Phenolic Compounds

ELIZABETH FRAGOPOULOU,† TZORTZIS NOMIKOS,† HARALABOS C. KARANTONIS,† CONSTANTINOS APOSTOLAKIS,† EMMANUEL PLIAKIS,† MARTINA SAMIOTAKI,§ GEORGE PANAYOTOU,§ AND SMARAGDI ANTONOPOULOU*,†

Department of Science of Nutrition-Dietetics, Harokopio University of Athens, 17671 Athens, Greece, and Protein Chemistry Laboratory B.S.R.C. "Alexander Fleming", Athens, Greece

In recent years an effort has been made to isolate and identify biologically active compounds that are included in the Mediterranean diet. The existence of naturally occurring acetylated phenolics, as well as studies with synthetic ones, provide evidence that acetyl groups could be correlated with their biological activity. Platelet activating factor (PAF) is implicated in atherosclerosis, whereas its inhibitors seem to play a protective role against cardiovascular disease. The aim of this study was to examine the biological activity of resveratrol and tyrosol and their acetylated derivatives as inhibitors of PAF-induced washed rabbit platelet aggregation. Acetylation of resveratrol and tyrosol was performed, and separation was achieved by HPLC. Acetylated derivatives were identified by negative mass spectrometry. The data showed that tyrosol and its monoacetylated derivatives act as PAF inhibitors, whereas diacetylated derivatives induce platelet aggregation. Resveratrol and its mono- and triacetylated derivatives exert similar inhibitory activity, whereas the diacetylated ones are more potent inhibitors. In conclusion, acetylated phenolics exert the same or even higher antithrombotic activity compared to the biological activity of the initial one.

KEYWORDS: HPLC separation; acetylation; platelet activating factor; electrospray mass spectrometry; resveratrol; tyrosol

INTRODUCTION

Diet is the cornerstone of cardiovascular disease prevention. Although Mediterranean diets are associated with a low incidence of atherosclerotic disease, data about the specific dietary constituents involved and the mechanisms concerning cardioprotection are still sparse (*1*). Nutritional constituents that typically occur in small quantities in foods may be responsible for this effect (*2*). In recent years an effort has been made to isolate and identify a variety of biologically active compounds from foods included in the Mediterranean diet. Phenolic compounds present in all plants are considered to be biologically active constituents and have been studied extensively.

Platelet activating factor (1-*O*-alkyl-2-acetyl-sn-glycero-3-phosphocholine, PAF) (*3*) is a potent inflammatory phospholipid mediator that is implicated in the initiation of the inflammation as well as in the process of atherogenesis (*4*). PAF and PAF-like lipids are degraded in plasma by the lipoprotein-bound enzyme, PAF acetyl hydrolase, leading to completely inactive compounds.

In our previous studies we have isolated and characterized bioactive lipids from vegetable oils, olive oil, red/white wine, red/white must, fish, milk, and yogurt (*5*−*10*). These compounds had antithrombotic and anti-inflammatory actions because they inhibited or antagonized PAF (*4*). Even though these molecules have different origins, the biological activity has always been detected in the polar lipid fraction. Specifically, these molecules belong to phospholipids, glycolipids, phenolics, and phenolic glycosides. It should be noted that despite the different skeletons of these molecules, the majority of them had acetyl groups, which seems to be essential for their biological activity. Moreover, our data showed that the hydrolysis as well as the addition of acetyl groups in their structures led to differentiation of their biological action.

The existence of acetylated phenolic compounds in natural sources is also established from other research groups. Acetylated glycosides of flavonoids (quercetin, kaempferol, apigenin, luteolin) (*11*−*14*) and resveratrol (*15*, *16*) have been found in plants, triacetylated resveratrol has been found in marine invertebrates (*17*), and acetylated tyrosol has been found in black olives (*18*) and olive oils (*19*). These data provide evidence about the existence of acetyl groups in naturally occurring compounds.

Recently, attempts were made to correlate biological activity with the molecular structure. Structural modification of natural phenolics is expected to produce analogues that may be useful tools to study the structure−activity relationships. The synthesis of acetylated coumarins (*20*), acetylated *O*-glycosides (*21*), acetylated resveratrol (*22*) and its oligomers (*23*, *24*), and

* Address correspondence to this author at 70 El. Venizelou St., Athens 17671, Greece (telephone 0030-2109549230; fax 0030-2109577050; e-mail antonop@hua.gr).
† Harokopio University of Athens.
§ Protein Chemistry Laboratory B.S.R.C. "Alexander Fleming".

10.1021/jf0627221 CCC: $37.00
Published on Web 01/03/2007

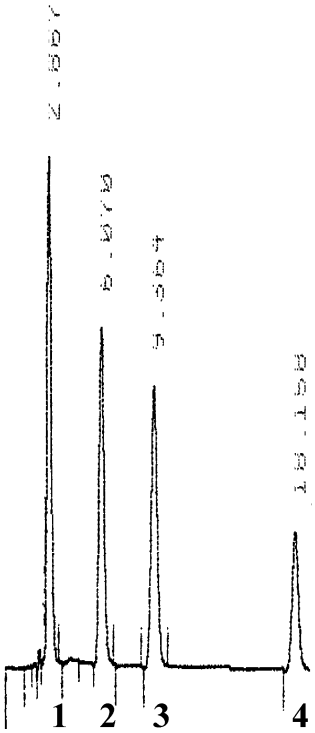


Figure 1. Typical HPLC separation of tyrosol acetylated derivatives. The separation was made on C8 reverse phase HPLC using an isocratic elution system consisting of 40% aqueous methanol. Flow = 1 min/mL, and UV detection was at 256 nm.

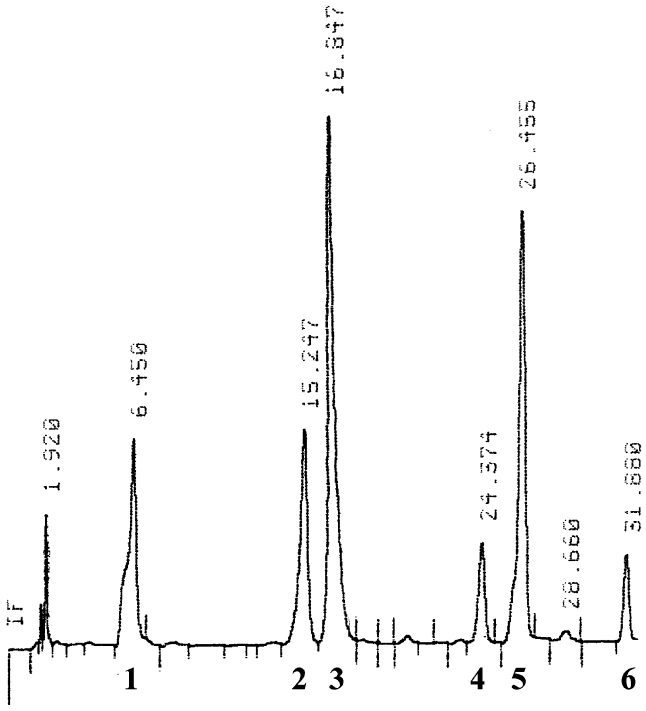


Figure 2. Typical HPLC separation of resverartol acetylated derivatives. The separation was made on C8 reverse phase HPLC using a gradient elution system from 40% aqueous methanol to 60% aqueous methanol in 30 min. Flow = 1 min/mL, and UV detection was at 256 nm.

acetylated quercetin (*25*) has already been reported. In all studies the acetylated phenolics revealed the same or higher biological activity compared with the one of the initial phenolic compound, concerning the inhibition of lipid peroxidation (*21*), the inhibition of cancer cells growth (*22*), the cytotoxicity against human tumor cell lines (*23*), and the down-regulation of NO synthase (iNOS) in leukemia cells (*24*) and macrophages (*25*).

Table 1. Phenolic Determination of Tyrosol Acetylated Derivatives before and after Mild Alkaline Hydrolysis

HPLC fraction	tyrosol (μmol) before hydrolysis	tyrosol (μmol) after hydrolysis	% recovery
1	2.95	3.22	22.2
2	ND[a]	3.08	21.6
3	2.95	2.75	19.0
4	ND	1.60	11.0
total		10.6	73.5

[a] Not detected.

On the other hand, although the use of phenolic compounds in pharmaceuticals as well as in food preparations is very promising, limitations occur with regard to their weak solubility and stability in a lipophilic environment. The existence of lipophilic derivatives of flavonoids via esterification of the hydroxyl functions with aliphatic molecules can be used as a tool to increase their lipophilicity and therefore improve their intestinal absorption and cell permeability of the compounds (*26*).

We have previously demonstrated that acetylation of standard sphingophospholipids as well as phosphoglycolipids resulted in compounds with significant biological activity that also exist in nature (*27*−*29*).

On the other hand, acetylation occurs as a modification of proteins (histones and transcription factors) inside the cell. Even though the acetyl-CoA is usually the acetyl donor for acetylation reactions, it has also been reported that nonenzymatic transfer of the acetyl group from aspirin to cyclooxygenase exists, resulting in inhibition of prostaglandin synthesis (*30*). Recently, evidence for the existence of an enzyme that transfers acetyl groups from polyphenolic acetates to proteins has beenfound, and this mechanism appears to be analogous to phosphorylation/dephosphorylation regulation of enzyme activity (*31*). It seems that the acetyl group, despite improving the cell permeability of compounds, may also serve as a messenger group.

Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene) is a common phytoalexin found in a few edible materials, such as grape skins, peanuts, and red wine, and seems to protect against oxidation, inflammation, platelet aggregation (*8*), and thrombus formation (*32*, review). Tyrosol is also a phenolic compound found in olive oil and wine (especially white wine) and seems to have antioxidant properties and anti-inflammatory actions (*33*, *34*).

The aforementioned data support the prospect that the acetyl group is a structural characteristic that provides significant biological activity. The majority of these studies investigate the activity of acetylated phenolics in cancer cells. The aim of this study is to examine the antithrombotic activity of resveratrol and tyrosol as well as their acetylated derivatives with respect to platelet aggregation and more specifically their ability to act as PAF antagonists or inhibitors.

MATERIALS AND METHODS

Materials. All reagents and chemicals were of analytical grade supplied by Merck (Darmstadt, Germany). Resveratrol and tyrosol were purchased from Sigma (St. Louis, MO). High-performance liquid chromatography (HPLC) solvents were from Merck (Walkerburn, Peebleshire, U.K.). Semisynthetic PAF (80% C-16PAF and 20% C-18PAF) was synthesized in our laboratory as previously described (*4*). Bovine serum albumin (BSA) and BN 52021 were obtained from Sigma.

Table 2. Phenolic Determination of Resveratrol Acetylated Derivatives before and after Mild Alkaline Hydrolysis

HPLC fraction	resveratrol (μmol) before hydrolysis	resveratrol (μmol) after hydrolysis	% recovery
1	1.92	1.88	15.9
2	0.83	1.05	8.9
3	2.09	2.85	24.0
4	0.17	0.50	4.2
5	0.61	1.49	12.6
6	ND[a]	0.82	6.9
total		8.59	72.5

[a] Not detected.

Instrumentation. HPLC was performed on a Hewlett-Packard (Avondale, PA) series 1100, supplied with a 100 μL loop Rheodyne (i 7725) injector. An 1100 HP UV spectrometer was used as detector. The spectrometer was connected to a Hewlett-Packard model HP-3396A integrator−plotter.

Separation of lipids was carried out on a 15 cm × 4.6 mm i.d. reverse phase Zobax Eclipse XDB-C8 column (Agilent Technologies) at room temperature.

The PAF-induced aggregation was measured in a Crono-Log (Havertown, PA) aggregometer coupled to a Crono-Log recorder.

The electrospray ionization (ESI) mass spectrometry experiments were performed on an Electronspray MS-LCQ-Deca (Thermo-Finnigan), low-flow mass spectrometer. Samples were dissolved in a small volume of HPLC grade methanol/water (70:30, v/v). Electrospray samples were typically introduced into the mass analyzer at a rate of 3 μL/min. Nitrogen of purity 99.99% was used as nebulizing gas and as bath gas.

The spectrum analysis was contacted in the ion space of *m*/*z* 50−1500, and the conditions for the highest intensity of the summits with the lowest rupture were as follows: spray voltage, 5 kV; capillary voltage, 7 V; capillary temperature, 275 °C; lens-entrance voltage, −54 V; lens voltage, −22 V.

Methods. *Acetylation Reaction.* The acetylated phenolics were prepared under standard conditions using acetic anhydride. Specifically, a mixture of tyrosol or resveratrol (2−3 mg) and acetic acid anhydride (3 mL) was warmed at 60 °C for 45 min. The reaction mixture was evaporated to dryness under N_2 and was dissolved in ethanol. A part of it was kept for biological assay, and the rest was subjected to HPLC separation.

HPLC Separation. The reaction mixture was separated on a reverse phase C8 HPLC column. An elution system consisting of 40% aqueous methanol was used to separate the tyrosol reaction mixture. The resveratrol reaction mixture separation was achieved by an elution system consisting of a gradient from 40% aqueous methanol to 60% aqueous methanol in 30 min and finally a hold for 5 min. The flow rate was 1 mL/min, and the detection was at 256 nm.

Biological Assay. PAF and the examined samples were dissolved in 2.5 mg of BSA/mL of saline (solution of 0.9% w/v of NaCl in water). Various concentrations of the examined sample were added into the aggregometer cuvette, and the aggregation induced by the sample was studied in a Crono-Log aggregometer (*3*). Experiments with PAF specific inhibitor BN 52021 0.1 mM (0.3% aqueous DMSO) were also performed. The inhibitor was added to washed rabbit platelets 1 min prior to the addition of the examined sample into the aggregometer cuvette. In cross-desensitization experiments platelets were desensitized by the addition of the examined lipid to the platelet suspension at a concentration that caused reversible aggregation. A second stimulation with PAF inducing the same height of aggregation as the tested compound was performed immediately after complete disaggregation. The platelet aggregation induced by PAF (2.5 × 10−11 M, final concentration) was measured as PAF-induced aggregation in washed rabbit platelets before (considered as 0% inhibition) and after the addition of various concentrations of the examined sample. Consequently, the plot of percent inhibition (ranging from 0 to 100%) versus different concentrations of the sample is linear. From this curve, the concentration of the sample that inhibited 50% PAF-induced aggregation is calculated. This value is defined as IC_{50}, namely, inhibitory concentration 50.

Mild Alkaline Hydrolysis. This procedure was carried out according to the method of Demopoulos et al. (*3*). Briefly, the sample was dissolved in 1 mL of chloroform/methanol (1:4 v/v), and then 0.1 mL of 1.2 N NaOH 1 N 50% methanol was added and kept for 20 min at 60 °C. The mixture was neutralized with 0.15 mL of 1 N acetic acid, evaporated to dryness, and dissolved in ethanol.

Phenolic Determination. The phenol content was determined using a modified method of Singleton and Rossi (*35*). Samples were dried under a stream of nitrogen and dissolved in 3.5 mL of water. An amount of 0.1 mL of Folin−Ciocalteu reagent was added, and after 3 min, 0.4 mL of 35% aqueous Na_2CO_3 was added. The reaction mixture was rested for 1 h, and the intensity of the blue color was measured at 725 nm. Standards of tyrosol or resveratrol were prepared similarly.

Statistical Analysis. Data were expressed as the mean value ± standard deviation (SD), and *t* test analysis was used for the comparison.

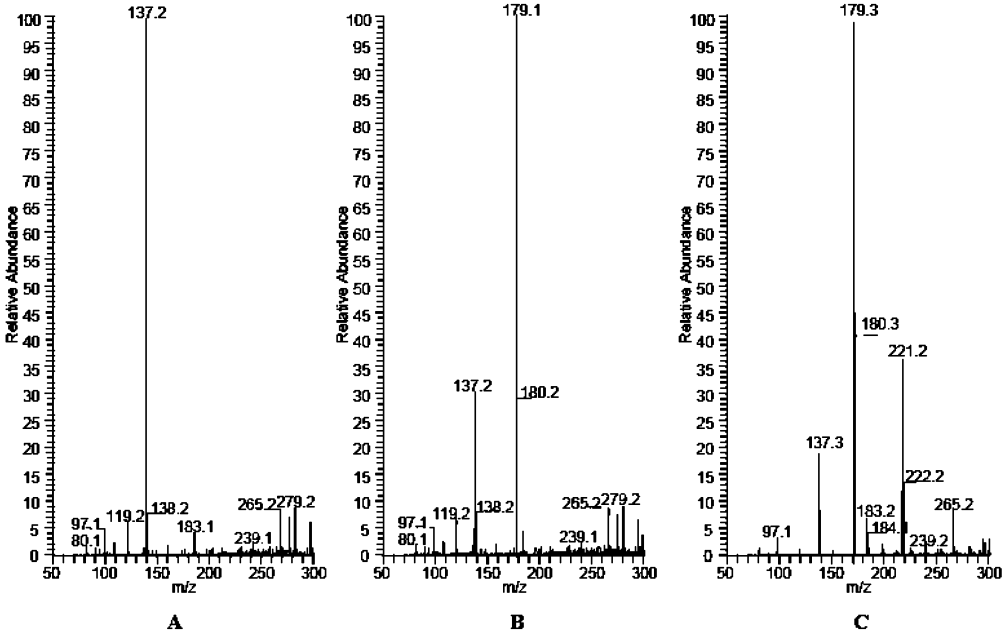


Figure 3. Negative ion mass spectra of tyrosol acetylated derivatives: fraction 1 (**A**); fraction 2 (**B**); fraction 4 (**C**).

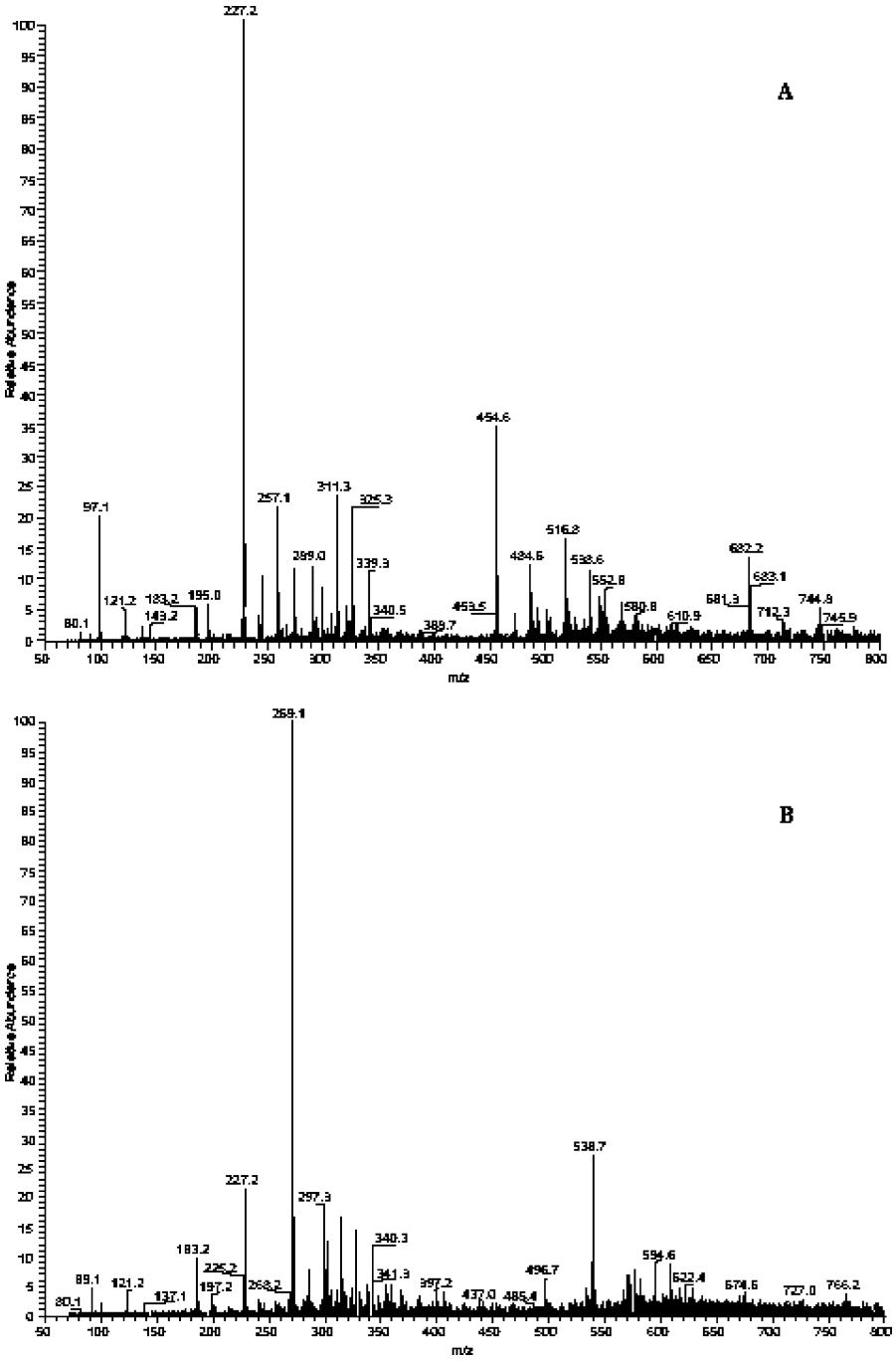


Figure 4. Negative ion mass spectra of resveratrol acetylated derivatives: fraction 1 (**A**); fraction 2 (**B**).

All analyses were done with the Statistical Package for Social Sciences (SPSS, version 10.0, SPSS Inc., Chicago, IL). Differences were considered to be statistically significant at the 5% level.

RESULTS

Acetylation and HPLC Separation of Phenolic Compounds. To prepare the acetylated derivatives of tyrosol and resveratrol, acetic acid anhydride was used. Several conditions were tested including different quantities of acetic anhydride, temperatures, and incubation times. Finally, 1 mL of acetic acid anhydride was used per milligram of phenolic compound, and the reaction was performed at 60 °C for 45 min. Initial amounts of 2 and 2.7 mg were used for the acetylation of tyrosol and resveratrol, respectively. After the reaction, the mixture was evaporated to air N_2 and dissolved in ethanol.

Two hydroxyl groups exist in the tyrosol structure, and therefore three acetylated derivatives were expected, specifically, two isomers of a monoacetylated derivative and one diacetylated derivative. To separate them, an isocratic elution system was developed consisting of 40% aqueous methanol. A typical profile of HPLC separation of acetylated tyrosol derivatives is shown in **Figure 1**. Four fractions were selected, evaporated to air N_2, and dissolved in ethanol. Standard tyrosol on this system is eluted at 2.8 min, corresponding to fraction 1. Under the above conditions of acetylation, an amount of 69.8 ± 1.2% of tyrosol is acetylated on the basis of phenolic determination, performed on each HPLC fraction separately.

Resveratrol shares three hydroxyl groups and therefore five acetylated derivatives were expected, specificcally, two isomers of monoacetylated derivatives, two isomers of diacetylated derivatives, and one triacetylated derivative. Their separation was finally achieved by a gradient elution system from 40% aqueous methanol to 60% aqueous methanol in 30 min. A

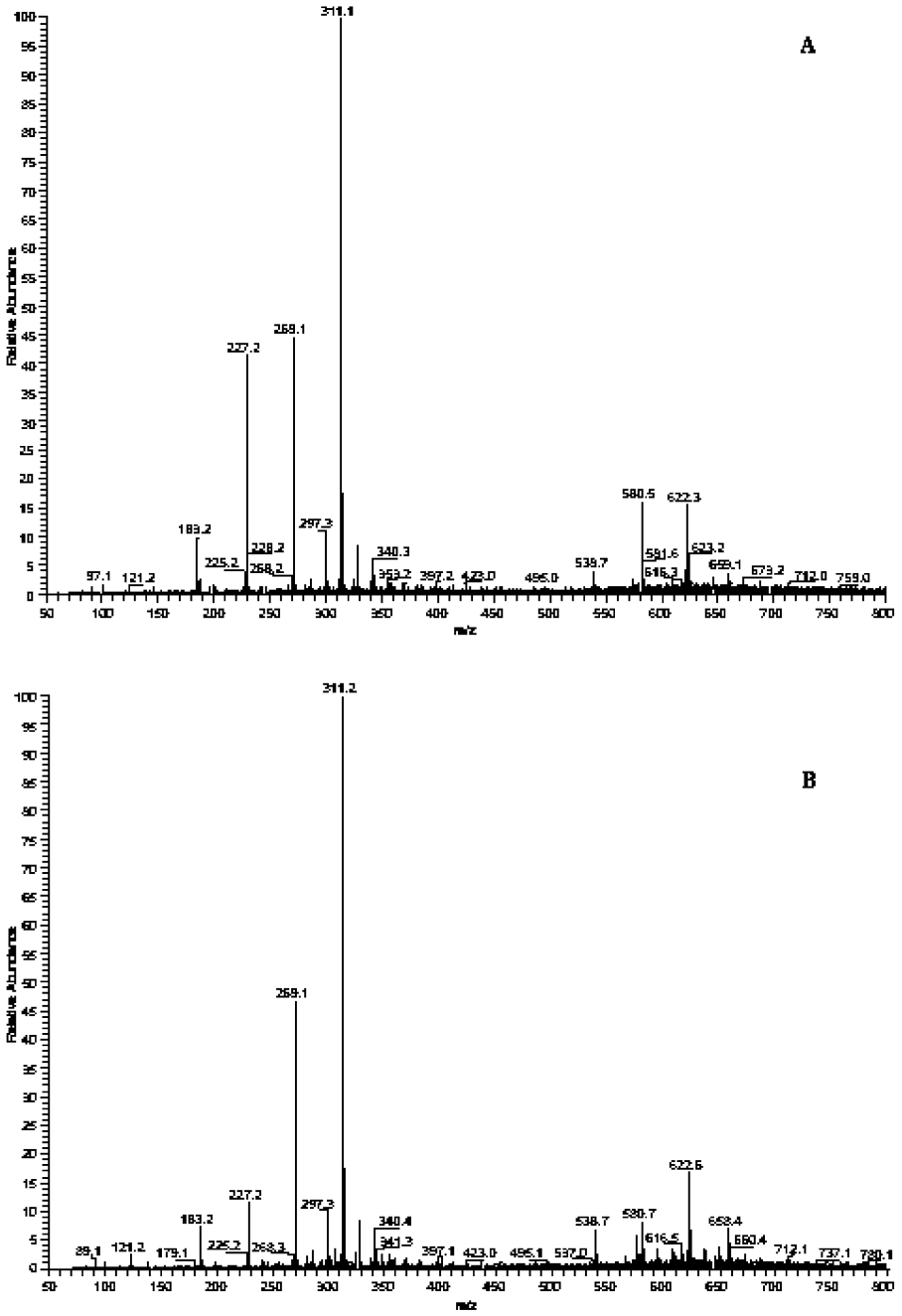


Figure 5. Negative ion mass spectra of resveratrol acetylated derivatives: fraction 4 (**A**); fraction 5 (**B**).

typical profile of HPLC separation of acetylated resveratrol derivatives is shown in **Figure 2**. Six fractions were selected, evaporated to air N_2, and dissolved in ethanol. The elution time for standard resveratrol on this system is 6.5 min, corresponding to fraction 1. Under the above conditions of acetylation, an amount of 78.1 ± 1.6% of resveratrol is acetylated on the basis of phenolic determination, performed on each HPLC fraction.

Phenolic Determination. The phenolic determination requires at least one free hydroxyl group on the phenolic ring that is oxidized to give a quinoid structure. The substitution of this type of hydroxyl group by an acetyl group results in partial or complete inhibition of phenolic determination. For this reason, phenolic determinations were performed in equal amounts of all HPLC fractions including phenolic standards before and after mild alkaline hydrolysis. Moreover, these data provide evidence about the chemical structure of tyrosol and resveratrol acetylated derivatives. The results of phenolic determination before and after the mild alkaline hydrolysis of acetylated tyrosol derivatives are summarized in **Table 1**. The data show that the phenolic determination of fractions 2 and 4 was blocked before the mild alkaline hydrolysis, indicating that the acetylation took place at the phenolic hydroxyl group. Their chromatographic behavior indicates that fraction 2 corresponds to monoacetylated derivative and fraction 4 should be the diacetylated one.

All of the hydroxyl groups of resveratrol are on the phenolic ring and participate in the phenolic determination. The results of the phenolic determination of resveratrol derivatives before and after mild alkaline hydrolysis are shown in **Table 2**. Only fraction 8 gave a negative phenolic determination before mild alkaline hydrolysis, indicating that this is the triacetate derivative.

ESI-MS. To establish the structure of phenolic derivatives, ESI-MS of all fractions was performed. Standard tyrosol gave only an $[M - 1]^-$ fragment at *m*/*z* 137 (data not shown). The

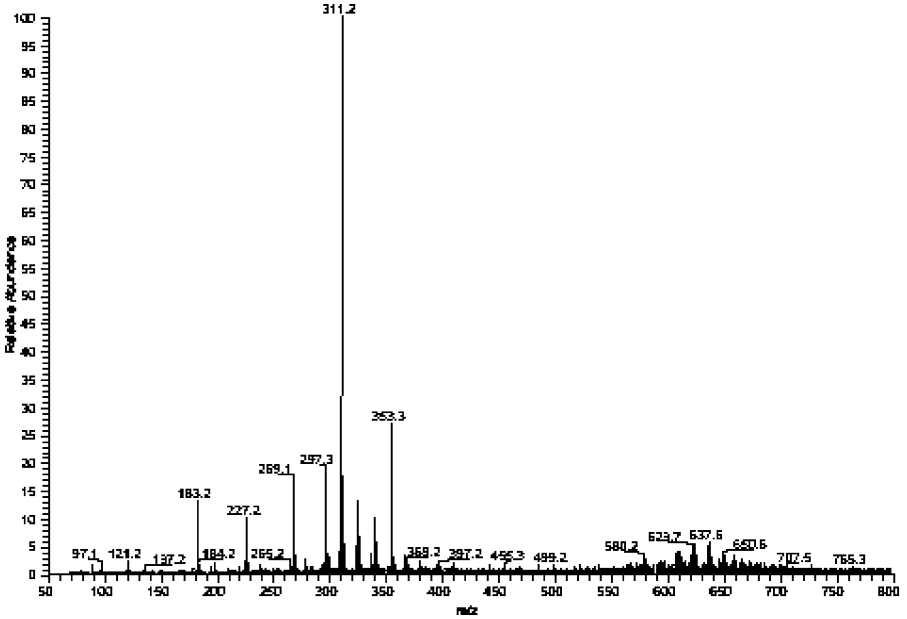


Figure 6. Negative ion mass spectrum of resveratrol acetylated derivatives: fraction 6.

negative ion mass spectrum of fraction 1 gave a peak at *m*/*z* 137 (**Figure 3A**), indicating that this is the nonacetylated tyrosol. Fractions 2 and 3 showed almost identical negative mass spectra (**Figure 3B**). The predominant peak at *m*/*z* 179 $[M - 1]^-$ corresponds to the molecular weight of a tyrosol molecule plus an acetyl group, whereas the removal of the acetyl group gave rise to the peak at *m*/*z* 137. These data are in accordance with the results of the phenolic determination and lead to the conclusion that fractions 2 and 3 are the monoacetylated derivatives (**Figure 3B**). The negative ion ESI-MS of fraction 4 gave two peaks at *m*/*z* 137 and 179 as well as at *m*/*z* 221, corresponding to the molecular weight of tyrosol plus two acetyl groups. These data in combination with phenolic determination results lead to the conclusion that fraction 4 is the diacetyl derivative of tyrosol (**Figure 3C**).

Fraction 1 of resveratrol derivatives gave a negative ion mass spectrum (**Figure 4A**) identical with the one of the standard resveratrol. Specifically, the predominant peak at *m*/*z* 227 corresponds to fragment $[M - 1]^-$. Moreover, peaks at *m*/*z* 455 and 683 correspond to fragments $[2M - 1]^-$ and $[3M - 1]^-$, respectively. Negative ESI-MS of fraction 2 showed an intense peak at *m*/*z* 269 $[M - 1]^-$ corresponding to the molecular weight of the resveratrol molecule plus an acetyl group, the peak at *m*/*z* 227 resulting from the cleavage of the acetyl group $[M - 1 - COCH_3 + H]-$ (**Figure 4B**). Moreover, the peaks at *m*/*z* 539 and 497 represent the fragments $[2M - 1]^-$ and $[2M - 1 - COCH_3 + H]^-$, respectively. The above data confirm that fraction 2 is a monoacetylated derivative of resveratrol. Negative ESI-MS of fraction 3 gave fragments identical with the ones of fraction 2, showing that it is also a monoacetylated derivative (data not shown). Fractions 4 and 5 gave a predominant peak at *m*/*z* 311 corresponding to the fragment $[M - 1]^-$ that is the molecular weight of resveratrol plus two acetyl groups, indicating that they are the diacetylated derivatives of resveratrol (**Figure 5**). Cleavage of the first and second acetyl groups gave rise to fragments at *m*/*z* 269 $[M - 1 - COCH_3 + H]^-$ and 227 $[M - 1 - 2(COCH_3 + H)]^-$. The mass spectrum of fraction 6 showed a peak at *m*/*z* 353 corresponding to fragment $[M - 1]^-$ that is the molecular weight of resveratrol plus three acetyl groups (**Figure 6**). Peaks at *m*/*z* 311, 269, and 227 arise after the cleavage of the first $[M - 1 - COCH_3 + H]$, the second $[M - 1 - 2(COCH_3 + H)]^-$, and the third $[M - 1 - 3(COCH_3 + H)]^-$ acetyl group, respectively. These data in combination with the negative phenolic determination led to the conclusion that fraction 8 is the triacetate derivative of resveratrol.

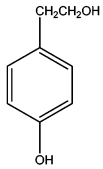
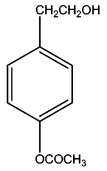
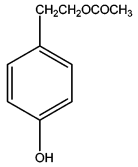
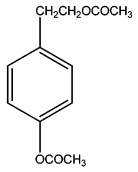
Biological Activity. The acetylated derivatives of tyrosol and resveratrol as well as standards of tyrosol and resveratrol were tested for their ability to induce washed rabbit platelet aggregation and/or to inhibit PAF-induced washed rabbit platelet aggregation. The results are summarized in **Tables 3** and **4**, respectively.

Data showed that tyrosol inhibits PAF-induced rabbit platelet aggregation with an IC_{50} value of $(21.6 \pm 3.98) \times 10^{-4}$ M. The monoacetylated derivatives (fractions 2 and 3) were more potent inhibitors of PAF-induced rabbit platelet aggregation because IC_{50} values were $(0.40 \pm 0.08) \times 10^{-4}$ and $(0.27 \pm 0.05) \times 10^{-4}$ M, respectively, that is, 2 orders of magnitude more potent ($p < 0.05$). The diacetylated derivative (fraction 4) induced aggregation of washed rabbit platelet with an EC_{50} value of $(0.14 \pm 0.02) \times 10^{-4}$ M. The addition of the second acetyl group inverts the biological activity from inhibition to aggregation. Cross-desensitization experiments and experiments with specific PAF inhibitor were performed on this case. Fraction 4 seems to act through the PAF pathway because it desensitized platelets against PAF and its aggregation was fully inhibited by the specific inhibitor of PAF, namely, BN 52021.

Resveratrol also inhibited PAF-induced rabbit platelet aggregation as we have previously shown (*8*) with an IC_{50} value $(0.52 \pm 0.12) \times 10^{-4}$ M. The monoacetylated derivatives of resveratrol (fractions 2 and 3) had a similar inhibitory activity as resveratrol. More specifically, as shown in **Table 4** the IC_{50} values of these fractions have the same order of magnitude as that of resveratrol.

Diacetylated derivatives of resveratrol (fractions 4 and 5) also showed inhibitory activity. The IC_{50} values of these fractions of $(0.08 \pm 0.02) \times 10^{-4}$ and $(0.24 \pm 0.05) \times 10^{-4}$ M, respectively, were significantly different from that of resveratrol ($p < 0.05$). Even more, the IC_{50} value of fraction 4 is different by 1 order of magnitude, indicating that the entrance of the second acetyl group is capable of improving the inhibitory activity of resveratrol.

Table 3. Biological Activity of Tyrosol and Its Acetylated Derivatives[a]

	Number of acetyl-group	Structure	Biological activity	IC_{50} PAF (10^{-4} M)	EC_{50} (10^{-4} M)
Tyrosol	-		Inhibition	21.6 ± 3.98	-
Fraction 2	1		Inhibition	0.40 ± 0.08	-
Fraction 3	1		Inhibition	0.27 ± 0.05	-
Fraction 4	2		Aggregation	-	0.14 ± 0.02

[a] Data are expressed as mean values ± SD derived from three experiments performed on different platelet preparations.

The triacetylated derivative of resveratrol, fraction 6, gave inhibitory activity of the same order of magnitude as resveratrol.

DISCUSSION

It is now common knowledge that the consumption of a Mediterranean diet is protective against cardiovascular diseases (*1*). Responsible for this protection are thought to be biological active compounds that typically occur in small quantities in foods (*2*). Among them, phenolic compounds are thought to be important constituents and have been found to exert many biological activities.

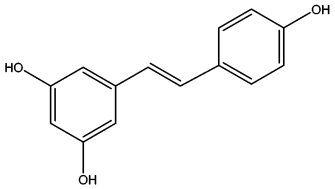
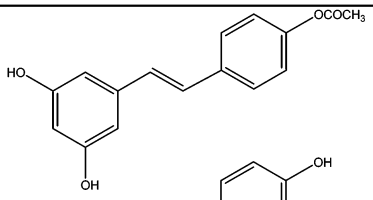
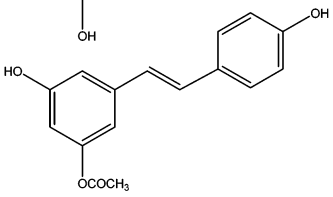
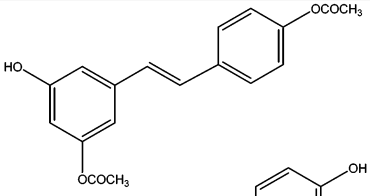
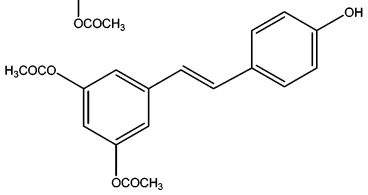
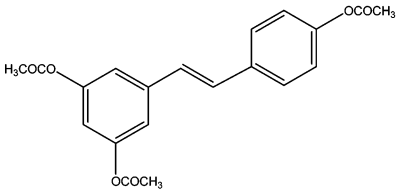
In addition, data indicate the existence of acetyl groups in naturally occurring compounds. Acetyl groups seem to be a structural characteristic that provides biological activity, and therefore recently an attempt was made to synthesize acetylated derivatives (*20*−*25*).

In this study the semisynthesis of acetylated resveratrol and tyrosol derivatives was performed using acetic anhydride. Acetylation reaction products were separated on HPLC. The identification of the acetylated derivatives has been made according to their chromatographic behavior and the phenolic determination before and after mild alkaline hydrolysis and mainly from negative ESI-MS of each fraction. The combination of these data leads to the conclusion that HPLC fractions 2 and 3 are the monoacetylated derivatives and fraction 4 is the diacetylated derivative of tyrosol. ESI-MS analysis of tyrosol as well as of its monoacetylated derivatives gave predominant peaks corresponding to their $[M - H]^-$. The mass spectrum of the diacetylated derivatives showed an $[M - H]^-$ fragment, but the predominant peak was the one produced after the loss of one acetyl group. To our knowledge, no report of the mass spectrometry analysis of tyrosol exists, with the exception of one publication in which no clearly distinctive molecular ions were observed for tyrosol (*36*). Fractions 2 and 3 derived from HPLC are the monoacetylated derivatives, fractions 4 and 5 are the diacetylated derivatives, and fraction 6 is the triacetylated derivative of resveratrol. Resveratrol as well as the mono- and diacetylated derivatives gave predominant ions corresponding to $[M - H]^-$. Our results are in accordance with a previous finding of negative ion mass spectrometry of resveratrol showing an ion at *m*/*z* 227 (*37*). In addition, under our ESI-MS conditions fragments corresponding to oligomerics of resveratrol were also observed. Even though the ESI-MS analysis of the triacetylated showed the $[M - H]^-$ fragment, the predominant ion was the one produced after the loss of one acetyl group.

Previous studies reported that acetylated phenolics exert the same or higher biological activity compared with the initial phenolic compound (*21*−*25*). The majority of these studies investigated the activity of acetylated phenolics in cancer cells.

In this study the antithrombotic activity of resveratrol and tyrosol as well as their acetylated derivatives with respect to platelet aggregation and more specifically their ability to act as

Table 4. Biological Activity of Resveratrol and Its Acetylated Derivatives[a]

	Number of acetyl-group	Structure	Biological activity	IC_{50} PAF (10^{-4} M)
Resveratrol	-		Inhibition	0.52 ± 0.12
Fraction 2	1		Inhibition	0.41 ± 0.08
Fraction 3	1		Inhibition	0.28 ± 0.09
Fraction 4	2		Inhibition	0.08 ± 0.02
Fraction 5	2		Inhibition	024 ± 0.05
Fraction 6	3		Inhibition	0.35 ± 0.09

[a] Data are expressed as mean values ± SD derived from three experiments performed on different platelet preparations.

PAF antagonists or inhibitors was investigated. Resveratrol is a stilbene that has been exhaustively studied, and it is known to have many biological cardioprotective actions (*34*, review). Tyrosol is a simple phenolic compound that is reported to have antioxidant activity (*33*, *34*).

Our results showed that tyrosol inhibited PAF-induced washed rabbit platelet aggregation with an IC_{50} value of 21.6×10^{-4} M. As far as we know, there are no other data concerning tyrosol-induced inhibition of platelet aggregation. The monoacetylated derivatives were more potent inhibitors of PAF-induced rabbit platelet aggregation, indicating that the addition of an acetyl group on the tyrosol structure results in phenolic compounds that are 2 orders of magnitude more potent. The exact position of the acetyl group on the structure of tyrosol does not seem to be crucial. The diacetylated derivative induced washed rabbit platelet aggregation. According to these data, the addition of two acetyl groups on the structure of tyrosol is capable of inverting the biological activity from inhibition to aggregation.

Resveratrol inhibited PAF-induced washed rabbit platelet aggregation as we have previously reported (*8*). The monoacetylated derivatives of resveratrol had inhibitory activity similar to that of resveratrol. Diacetylated derivatives of resveratrol also exerted more potent inhibitory activity, indicating that the entrance of the second acetyl group is capable of improving the inhibitory activity of resveratrol. The triacetylated derivative of resveratrol gave an inhibitory activity of the same order of magnitude as resveratrol.

In conclusion, acetylation of phenolics leads to compounds with more potent antithrombotic activity. Further investigation is needed to elucidate the exact role of acetylated phenolics in cardiovascular disease.

LITERATURE CITED

(1) Keys, A. Coronary heart disease in seven countries. 1970. *Nutrition* **1997**, *13*, 250−252.

(2) Kris-Etherton, P. M.; Hecker, K. D.; Bonanome, A.; Coval, S. M.; Binkoski, A. E.; Hilpert, K. F.; Griel, A. E.; Etherton, T. D. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. *Am. J. Med.* **2002**, *113* (Suppl. 9B), 71S−88S.

(3) Demopoulos, C. A.; Pinckard, R. N.; Hanahan, D. J. Platelet-activating factor. Evidence for 1-*O*-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine as the active component (a new class of lipid chemical mediators). *J. Biol. Chem.* **1979**, *254*, 9355−9358.

(4) Demopoulos, C. A.; Karantonis, H. C.; Antonopoulou, S. Platelet activating factor—a molecular link between atherosclerosis theories. *Eur. J. Lipid Sci. Technol.* **2003**, *105*, 705−716.

(5) Karantonis, H. C.; Antonopoulou, S.; Demopoulos, C. A. Antithrombotic lipid minor constituents from vegetable oils. Comparison between olive oils and others. *J. Agric. Food Chem.* **2002**, *50*, 1150−1160.

(6) Karantonis, H. C.; Antonopoulou, S.; Perrea, D. N.; Sokolis, D. P.; Theocharis, S. E.; Kavantzas, N.; Iliopoulos, D. G.; Demopoulos, C. A. In vivo antiatherogenic properties of olive oil and its constituent lipid classes in hyperlipidemic rabbits. *Nutr. Metab. Cardiovasc. Dis.* **2006**, *16*, 174−185.

(7) Fragopoulou, E.; Antonopoulou, S.; Nomikos, T.; Demopoulos, C. A. Structure elucidation of phenolic compounds from red/white wine with antiatherogenic properties. *Biochim. Biophys. Acta* **2003**, *1632*, 90−99.

(8) Fragopoulou, E.; Nomikos, T.; Antonopoulou, S.; Mitsopoulou, C. A.; Demopoulos, C. A. Separation of biologically active lipids from red wine. *J. Agric. Food Chem.* **2000**, *48*, 1234−1238.

(9) Rementzis, J.; Antonopoulou, S.; Argyropoulos, D.; Demopoulos, C. A. Biologically active lipids from *S. scombrus*. *Adv. Exp. Med. Biol.* **1996**, *416*, 65−72.

(10) Antonopoulou, S.; Semidalas, C. E.; Koussissis, S. G.; Demopoulos, C. A. A. Platelet-activating factor (PAF) antagonists in foods. A study of lipids, with PAF or anti-PAF like-activity, in cow's milk and yoghurt. *J. Agric. Food Chem.* **1996**, *44*, 3047−3051.

(11) Shang, X. Y.; Wang, Y. H.; Li, C.; Zhang, C. Z.; Yang, Y. C.; Shi, J. G. Acetylated flavonol diglucosides from *Meconopsis quintuplinervia*. *Phytochemistry* **2006**, *67*, 511−515.

(12) Li, Y.; Chen, X.; Satake, M.; Oshima, Y.; Ohizumi, Y. Acetylated flavonoid glycosides potentiating NGF action from *Scoparia dulcis*. *J. Nat. Prod.* **2004**, *67*, 725−727.

(13) Gabrieli, C.; Kokkalou, E. A new acetylated glucoside of luteolin and two flavone glucosides from *Lavandula stoechas* ssp. *stoechas*. *Pharmazie* **2003**, *58*, 426−427.

(14) Fico, G.; Braca, A.; Bilia, A. R.; Tome, F.; Morelli, I. Flavonol glycosides from the flowers of *Aconitum paniculatum*. *J. Nat. Prod.* **2000**, *63*, 1563−1565.

(15) Okasaka, M.; Takaishi, Y.; Kogure, K.; Fukuzawa, K.; Shibata, H.; Higuti, T.; Honda, G.; Ito, M.; Kodzhimatov, O. K.; Ashurmetov, O. New stilbene derivatives from *Calligonum leucocladum*. *J. Nat. Prod.* **2004**, *67*, 1044−1046.

(16) Munkombwe, N. M. Acetylated phenolic glycosides from *Harpagophytum procumbens*. *Phytochemistry* **2003**, *62*, 1231−1234.

(17) Jayatilake, G. S.; Baker, B. J. Isolation and identification of a stilbene derivative from the antarctic sponge *Kirkpatrickia variolosa*. *J. Nat. Prod.* **2006**, *58*, 1958−1960.

(18) Romero, C.; Brenes, M.; Garcia, P.; Garcia, A.; Garrido, A. Polyphenol changes during fermentation of naturally black olives. *J. Agric. Food Chem.* **2004**, *52*, 1973−1979.

(19) Christophoridou, S.; Dais, P.; Tseng, L. H.; Spraul, M. Separation and identification of phenolic compounds in olive oil by coupling high-performance liquid chromatography with postcolumn solid-phase extraction to nuclear magnetic resonance spectroscopy (LC-SPE-NMR). *J. Agric. Food Chem.* **2005**, *53*, 4667−4679.

(20) Tyagi, Y. K.; Kumar, A.; Raj, H. G.; Vohra, P.; Gupta, G.; Kumari, R.; Kumar, P.; Gupta, R. K. Synthesis of novel amino and acetyl amino-4-methylcoumarins and evaluation of their antioxidant activity. *Eur. J. Med. Chem.* **2005**, *40*, 413−420.

(21) Ponticelli, F.; Trendafilova, A.; Valoti, M.; Saponara, S.; Sgaragli, G. Synthesis and antiperoxidant activity of new phenolic *O*-glycosides. *Carbohydr. Res.* **2001**, *330*, 459−468.

(22) Cardile, V.; Lombardo, L.; Spatafora, C.; Tringali, C. Chemoenzymatic synthesis and cell-growth inhibition activity of resveratrol analogues. *Bioorg. Chem.* **2005**, *33*, 22−33.

(23) Ohyama, M.; Tanaka, T.; Ito, T.; Iinuma, M.; Bastow, K. F.; Lee, K. H. Antitumor agents 200. Cytotoxicity of naturally occurring resveratrol oligomers and their acetate derivatives. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 3057−3060.

(24) Quiney, C.; Dauzonne, D.; Kern, C.; Fourneron, J. D.; Izard, J. C.; Mohammad, R. M.; Kolb, J. P.; Billard, C. Flavones and polyphenols inhibit the NO pathway during apoptosis of leukemia B-cells. *Leuk. Res.* **2004**, *28*, 851−861.

(25) Chen, Y. C.; Shen, S. C.; Lee, W. R.; Hou, W. C.; Yang, L. L.; Lee, T. J. Inhibition of nitric oxide synthase inhibitors and lipopolysaccharide induced inducible NOS and cyclooxygenase-2 gene expressions by rutin, quercetin, and quercetin pentaacetate in RAW 264.7 macrophages. *J. Cell Biochem.* **2001**, *82*, 537−548.

(26) Riva, S.; Monti, D.; Luisetti, M.; Danieli, B. Enzymatic modification of natural compounds with pharmacological properties. *Ann. N. Y. Acad. Sci.* **1998**, *864*, 70−80.

(27) Zanglis, A.; Lianos, E. A.; Demopoulos, C. A. The biological activity of acetylated sphingosylphosphorylcholine derivatives. *Int. J. Biochem. Cell Biol.* **1996**, *28*, 63−74.

(28) Avramopoulou, V.; Antonopoulou, S.; Argyropoulos, D.; Froussios, C.; Demopoulos, C. A. Synthesis of a new phosphoglycolipid with biological activity towards platelets. *Int. J. Biochem. Cell Biol.* **1997**, *29*, 767−774.

(29) Antonopoulou, S.; Oikonomou, A.; Karantonis, H. C.; Fragopoulou, E.; Pantazidou, A. Isolation and structural elucidation of biologically active phospholipids from *Scytonema julianum* (cyanobacteria). *Biochem. J.* **2002**, *367*, 287−293.

(30) Vane, J. R. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat. New Biol.* **1971**, *231*, 232−235.

(31) Khurana, P.; Kumari, R.; Vohra, P.; Kumar, A.; Seema; Gupta, G.; Raj, H. G.; Dwarakanath, B. S.; Parmar, V. S.; Saluja, D.; Bose, M.; Vij, A.; Chaudhary, N. K.; Adhikari, J. S.; Tyagi, Y. K.; Kohli, E. Acetoxy drug: protein transacetylase catalyzed activation of human platelet nitric oxide synthase by polyphenolic peracetates. *Bioorg. Med. Chem.* **2006**, *14*, 575−583.

(32) Delmas, D.; Jannin, B.; Latruffe, N. Resveratrol: preventing properties against vascular alterations and ageing. *Mol. Nutr. Food Res.* **2005**, *49*, 377−395.

(33) Giovannini, C.; Straface, E.; Modesti, D.; Coni, E.; Cantafora, A.; De Vincenzi, M.; Malorni, W.; Masella, R. Tyrosol, the major olive oil biophenol, protects against oxidized-LDL-induced injury in Caco-2 cells. *J. Nutr.* **1999**, *129*, 1269−1277.

(34) Giovannini, L.; Migliori, M.; Filippi, C.; Origlia, N.; Panichi, V.; Falchi, M.; Bertelli, A. A.; Bertelli, A. Inhibitory activity of the white wine compounds, tyrosol and caffeic acid, on lipopolysaccharide-induced tumor necrosis factor-α release in human peripheral blood mononuclear cells. *Int. J. Tissue React.* **2002**, *24*, 53−56.

(35) Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic−phosphotungstic acid reagents. *Am. J. Enol. Vitic* **1965**, 144−158.

(36) La Torre, G. L.; Saitta, M.; Vilasi, F.; Pellicano, T.; Dugo, G. Direct determination of phenolic compounds in Sicilian wines by liquid chromatography with PDA and MS detection. *Food Chem.* **2006**, *94*, 640−650.

(37) Wang, D.; Hanga, T.; Wu, C.; Liu, W. Identification of the major metabolites of resveratrol in rat urine by HPLC-MS/MS. *J. Chromatogr. B* **2005**, *829*, 97−106.

Received for review September 22, 2006. Revised manuscript received November 6, 2006. Accepted November 8, 2006.

JF0627221