Natural Polyphenols as Direct Trapping Agents of Lipid Peroxidation-Derived Acrolein and 4-Hydroxy-*trans*-2-nonenal

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Acrolein (ACR) and 4-hydroxy-*trans*-2-nonenal (HNE) are two cytotoxic lipid-derived α,β-unsaturated aldehydes which have been implicated as causative agents in the development of carbonyl stress-associated pathologies. In this study, 21 natural polyphenols were screened to identify effective scavenging agents of ACR and/or HNE in simulated physiological conditions. It was found that flavan-3-ols, theaflavins, cyanomaclurin, and dihydrochalcones effectively trapped ACR and HNE by working as sacrificial nucleophiles. The most effective one was phloretin, which quenched up to 99.6% ACR in 90 min and 90.1% HNE in 24 h. Subsequent LC-MS/MS analysis showed that these effective polyphenols formed adducts with ACR and HNE. A major adduct formed from phloretin and ACR was purified, and its structure was characterized by LC-MS and NMR spectroscopy as diACR-conjugated phloretin. The chemical nature of interactions between ACR and polyphenols was proposed as the Michael addition reaction of phloretin to the C═C double bond of ACR, followed by the formation of hemiacetal between the hydroxyl group in the A ring of phloretin and the C═O carbonyl group in ACR, thus yielding more stable products. Findings of the present study highlighted certain classes of polyphenols as promising sequestering agents of α,β-unsaturated aldehydes to inhibit or restrain carbonyl stress-associated diseases.

Introduction

Exposure of lipid molecules to various free radicals and oxidants could cause lipid peroxidation and give rise to a wide range of reactive carbonyl species (RCS[1], mainly unsaturated aldehydes) through a series of cascade oxidation and cleavage reactions (*1, 2*). In physiological systems, reactive carbonyls might arise as a consequence of oxidative stress, and emerging evidence suggests that it is the presence of reactive carbonyls rather than the initial oxidative injury that possesses high potential to cause cellular damage (*3*). In this regard, α,β-unsaturated aldehydes have attracted the most attention because of the simultaneous occurrence of two reactive sites (a C═C and a C═O group), which render these aldehydes capable of forming cyclic adducts or cross-links with nucleophilic cellular components (*4*). Acrolein (ACR) and 4-hydroxy-*trans*-2-nonenal (HNE) are two of the most important α,β-unsaturated aldehydes. ACR is considered as the strongest electrophilic α,β-unsaturated aldehyde and therefore exhibits the highest reactivity toward nucleophiles such as thiol or amino compounds (*5*). However, HNE is the most abundant α,β-unsaturated aldehyde formed by β-cleavage of omega-6 polyunsaturated fatty acids and is also one of the best recognized and most extensively studied cytotoxic products of lipid peroxidation (*6, 7*).

Covalent modifications of biomolecules such as proteins, DNA, and phospholipids by the reactive carbonyls at the thiol or amino group(s) have been implicated in various pathophysiological conditions (*3, 8, 9*). For example, HNE- and ACR-bound proteins were suggested to be potential markers of oxidative stress states and long-term damage to proteins in some of the brain or liver diseases, and increased levels of HNE and ACR were observed in patients with Alzheimer's disease (*10*−*12*). In addition, high reactivity of ACR and HNE toward thiols means that they might easily form conjugates with glutathione (GSH), an antioxidant that plays an important role in protecting cells from toxicants such as free radicals *in vivo*. As a consequence, cellular GSH might be depleted and redox status perturbed (*13*−*15*). HNE-GSH has been reported to be a major stable hepatic metabolite of HNE and is considered a marker of oxidative stress in the rat liver and hepatocytes (*16*). The use of chemical agents to quench RCS has been one of the most widely recognized approaches to attenuate RCS-associated deleterious effects. Most of these chemical agents act as sacrificial nucleophiles by adding to RCS such as ACR and HNE, thus preventing the addition of the RCS to biomolecules. Effective scavengers identified so far are mainly amino compounds, including aminoguanidine, carnosine, pyridoxamine, and hydralazine (*17*).

Traditionally, the application of polyphenols in the context of prevention of lipid peroxidation typically aimed to harness their antioxidant activity to counteract the action of pro-oxidation factors, which may initiate or accelerate lipid peroxidation (*18*). However, this approach to arrest lipid peroxidation did not take into consideration the probable harmful effects of secondary products, especially RCS generated from oxidative stress (*19*). The recent discovery of EGCG (epigallocatechin-3-gallate) as a novel trapping agent of ACR has revealed the probable

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[1] Abbreviations: ACR, acrolein; CM, cyanomaclurin; DNPH, 2,4-dinitrophenylhydrazine; EC, epicatechin; ECG, epicatechin-3-gallate; EGCG, epigallocatechin-3-gallate; HNE, 4-hydroxynonenal; PBS, phosphate buffered saline; PE, phloretin; PZ, phloridzin; RCS, reactive carbonyl species; TF, theaflavin; TFG, theaflavin-3,3′-digallate.

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existence of a large class of promising RCS scavengers, natural polyphenols (*20, 21*). Nevertheless, the mechanism of action remains to be clearly defined. It is possible that certain polyphenols could directly interact with toxic RCS and thus spare biomolecules the attack by the RCS. On the basis of the above considerations, the discovery of special polyphenols that possess both antioxidant/free radical-scavenging and RCS-trapping activities is of tremendous significance, and this represents a new therapeutic approach that may contribute to the amelioration of health disorders associated with lipid peroxidation and/or RCS (*22*). In addition, natural polyphenols occur widely in plants (fruits, vegetables, spices, etc.), and many have thus become an integral part of the human diet (*23*). With the discovery of their dual antioxidant and RCS-scavenging activity, polyphenols will be of practical use for the prevention of age-related diseases.

In this study, 21 natural polyphenols with diverse structural characteristics were compared for their ACR-/HNE-trapping capacities under simulated physiological conditions. The test compounds included members of different categories of phenolic compounds, such as flavones, flavonols, flavanones, flavanonols, flavan-3-ols, theaflavins, cyanomaclurins, dihydrochalcones, simple phenolic acids, and others. Apart from the analysis of the structure−activity relationship of these polyphenols in the trapping of ACR/HNE, this study also aimed to examine the mechanism of action by means of which particular polyphenols scavenge ACR/HNE. The latter purpose was largely facilitated with the application of LC-MS/MS in the structural identification of compounds postulated to arise from the interaction between the added polyphenols and ACR/HNE in the reaction systems. Eventually, isolation and purification of a key reaction product and the subsequent structural elucidation by NMR spectroscopy have provided more solid evidence to support the proposed mechanism of action in the trapping of the α,β-unsaturated RCS.

Experimental Procedures

Solvents and Reagents. 4-Hydroxy-*trans*-2-nonenal was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Acrolein, acrolein-2,4-dinitrophenylhydrazon, 2,4-dinitrophenylhydrazine (DNPH), phosphate buffered saline (PBS, pH 7.4), dimethyl sulfoxide, naringenin, taxifolin, phloretin (PE), phloridzin (PZ), quercetin, epicatechin (EC), rosmarinic acid, thymol, 2-hydroxycinnamic acid, and oleuropein were purchased from Sigma-Aldrich (St. Louis, MO, USA). Apigenin, norartocarpetin, steppogenin, dihydromorin, kaempferol, epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG), theaflavin (TF), theaflavin-3,3′-digallate (TFG), and cyanomaclurin(CM) were isolated with high purity (>95% by LC-DAD) from natural sources in our lab. All solvents used were of analytical grade and were obtained from BDH Laboratory Supplies (Poole, UK).

Quantification of Residual ACR and HNE in Model Reactions by HPLC. The test polyphenols were predissolved in DMSO. The ACR-reaction system was adopted from our previous study (*20*). The derivatization agent, DNPH solution, was freshly prepared weekly by dissolving 62.5 mg of recrystallized DNPH and 3 mL of HCl (1.0 M) in 50 mL of acetonitrile. Briefly, reaction mixtures, each containing ACR (0.5 mM) with or without a polyphenol (1 mM) in PBS (0.01M, pH 7.4), were incubated in a water bath at 37 °C with constant shaking (120 rpm) for 90 min. The concentration of apigenin and norartocarpetin was reduced to 0.2 mM and that of ACR to 0.1 mM because of the low solubility of these two polyphenols in PBS. After 90 min, 0.5 mL of each of the above reaction mixtures was allowed to react with 0.3 mL of acidified DNPH solution in darkness for 2 h. This derivatization reaction led to the formation of ACR−DNPH complexes. HPLC-DAD was applied to identify and quantify ACR−DNPH complexes formed in the reaction mixtures. This was performed on a Shimadzu HPLC system with a separation module (LC-20AT), an autosampler (SIL-20A), a degasser (DGU-20A3), and a photodiode array detector (SPD-M20A). Separation of the reaction mixtures was carried out with a Phenomenex Luna C_{18} (*2*) column (250 × 4.6 mm, 5 μm). The mobile phase was composed of 0.1% formic acid in Milli-Q water (solvent A) and acetonitrile (solvent B). Isocratic elution (1 mL/min) was used with 40% A and 60% B, and detection wavelength was set at 372 nm.

For HNE, the reaction system was adopted from that described in Beretta et al.'s study (*21*). Reaction mixtures containing HNE (0.2 mM) and a phenolic compound (2 mM) were incubated in a water bath (37 °C) with constant shaking (120 rpm) for 24 h. For the same reason as that stated above, the concentration of apigenin and norartocarpetin was reduced to 0.2 mM and that of HNE to 0.02 mM. The residual HNE was analyzed by HPLC to compare HNE-trapping capacities of the polyphenols. The mobile phase was composed of 0.1% formic acid in Milli-Q water (solvent A) and acetonitrile (solvent B) with an isocratic flow (1 mL/min) of 50% A and 50% B. Detection wavelength was set at 221 nm. All analyses were performed in triplicate.

LC-MS Analysis of Reaction Products of Polyphenols with ACR/HNE. LC-MS/MS analysis was carried out on an instrument equipped with an electrospray ionization source interfaced to an API 2000 QTRAP mass spectrometer (Applied Biosystems). Liquid chromatography was performed on an Agilent HPLC system with a degasser (G1379A), a quaternary pump (G1311A), a thermostatted autosampler (G1329A), and a diode array detector (G1315B). Separation of reaction products was carried out on a Waters RP-C_{18} column (150 × 4.6 mm, 3.5 μm). The mobile phase was composed of 0.1% formic acid water (solvent A) and acetonitrile (solvent B) of the following gradients: 0 min, 80% A/20% B; 30 min, 40% A/60% B; 35 min, 20% A/80% B; 40 min, 80% A/20% B. Effluent from the LC system was split 4:1 with one part (200 μL/min) directed to the MS for spectrometric analysis and the remaining to waste. MS operational parameters were as follows: negative ion mode; spray voltage, 4 kV; scan range, 200−1000 Da; ion source temperature, 300 °C. MS/MS was conducted with the precursor ion set according to the predicted molecular weight of adducts formed between the polyphenols and ACR/HNE and with collision energy at 30 eV.

Purification of di-ACR Conjugated Phloretin As a Major Adduct from the Reaction between Phloretin and ACR. Phloretin was dissolved in DMSO and diluted in 200 mL of phosphate buffer (0.01 M, pH 7.4). ACR was added so that the final concentration of phloretin and ACR was 2 mM and 4 mM, respectively. The reaction mixture was incubated in a water bath (37 °C) with constant shaking (120 rpm) for 2 h. The reaction mixture was extracted with ethyl acetate. The ethyl-acetate extract was evaporated to a minimal volume on a rotary evaporator under vacuum. The residue was dissolved in methanol and loaded onto a Sephadex LH-20 column (40 cm × 4 cm). Elution was performed with 70% methanol and the eluate was collected using an automatic fraction collector. Profiles of the fractions were checked by HPLC, and similar fractions were combined. This chromatographic process led to the isolation of compound **1** (light yellow powders) with high purity (>95% by LC-DAD).

NMR Analysis and Spectral Data of Compound 1. The purified adduct was dissolved in DMSO. ^{1}H (600 MHz), ^{13}C (150 MHz), and 2D NMR spectra (DEPT, ^{1}H−^{1}H COSY, HSQC, and HMBC) were acquired on a Bruker, AVANCE 600 instrument.

Compound **1**: ^{1}H NMR (600 MHz, DMSO): δ = 14.00 (s, 1H, OH-6), 9.11 (s, 1H, OH-4′), 7.25 (m, 2H, OH-12 and OH-15), 7.05 (d, J = 8.1 Hz, 2H, H-2′ and H-6′), 6.67 (d, J = 8.1 Hz, 2H, H-3′ and H-5′), 5.53 (m, 2H, H-12 and H-15), 3.31 (m, 2H, H-8), 2.80 (m, 2H, H-9), 2.50 (m, 4H, H-10 and H-13), 1.82 ppm (m, 4H, H-11 and H-14). ^{13}C NMR (150 MHz, DMSO): δ = 204.8 (C-7), 160.9 (C-6), 156.7, 153.3 (C-2 and C-4), 155.3 (C-4′), 131.5 (C-1′), 129.2 (C-2′ and C-6′), 115.0 (C-3′ and C-5′), 104.4 (C-1), 101.5

(C-5), 101.1 (C-3), 92.8, 92.2 (C-12 and C-15), 45.6 (C-8), 29.5 (C-9), 26.7, 26.3 (C-11 and C-14), 15.1, 14.3 ppm (C-10 and C-13).

Results

Activities of Polyphenols in the Trapping of ACR and HNE. Twenty-one different polyphenols, including members of flavones, flavonols, flavanones, flavanonols, flavan-3-ols, theaflavins, cyanomaclurin, dihydrochalcones, simple phenolic acids, and others (structures are shown in Figure 1) were compared for their relative ACR/HNE-trapping capacities. Quantitative HPLC-DAD analysis showed that nine of them effectively scavenged ACR (27.4−99.6%, Figure 2), and eight of them, effectively scavenged HNE (13.4−90.1%, Figure 3). The effective scavengers of ACR, epicatechin, epigallocatechin, epicatechin-3-gallate, epigallocatechin-3-gallate, theaflavin, theaflavin-3,3′-digallate, cyanomaclurin, phloretin from the groups of flavan-3-ols, theaflavins, cyanomaclurin, and dihydrochalcones, were also effective HNE scavengers. Interestingly, phloridzin effectively scavenged ACR but not HNE. Nevertheless, phloridzin only demonstrated about a quarter of phloretin's ACR-scavenging capacity and thus pointed to an important role of a free hydroxyl group on C-6 of the dihydrochalcone in ACR-trapping. Notablely, the aglycone of phloridzin, phloretin, demonstrated the highest activity in trapping both ACR in 90 min and HNE in 24 h with over 90% of the RCS scavenged. To better understand the reaction kinetics, a time-course study was carried out to monitor the rate of ACR (Figure 4) and HNE (Figure 5) scavenging by dihydrochalcones. Although in the phloretin−HNE reaction system, the molar ratio of phenol to HNE was much higher than that in the phloretin−ACR system, the former showed a much lower rate of RCS scavenging than the latter. In particular, phloretin scavenged more than 50% of the ACR added in 10 min, but it took more than 10 h to scavenge this amount of HNE, suggesting that different reactive carbonyl species have different reactivity with polyphenols.

LC-MS Analysis of Polyphenol-ACR/HNE Adducts. To gain insight into the mechanism of action by means of which polyphenols scavenge α,β-unsaturated aldehydes, LC-MS analysis was performed to identify reaction products that may arise from reactions between ACR/HNE and polyphenols. The total ion chromatograms (TIC) of the reaction mixtures that contain one of the effective scavengers mentioned above plus ACR/HNE showed some distinct peaks, which were absent from TIC of the control samples. The predicted molecular weights of these peaks matched well with molecular compositions that correspond to adducts formed between the added polyphenols and ACR/HNE. Consequently, key adducts formed from some of the effective ACR/HNE scavengers (phloretin, epicatechin-3-gallate, and cyanomaclurin) were further examined by tandem MS. Results showed that phloretin reacted with ACR, giving rise to two major adducts of $[M - H]^-$ *m/z* 329.3 and *m/z* 385.1, assignable to monoACR-conjugated phloretin and diACR-conjugated phloretin, respectively (Figure 6). Collision-induced dissociation (CID) of the *m/z* 385.1 ion gave a major product ion at *m/z* 279.2 $[M - 106 - H]^-$, which is an ion produced by a typical loss of the B ring unit of phloretin, and thus precluded the B ring as the active trapping site for ACR. As to the reaction of ACR and epicatechin-3-gallate, there was a set of peaks in TIC with the same molecular weight which might represent isomeric products, assignable to monoACR-conjugated epicatechin-3-gallate ($[M - H]^-$ at *m/z* 497.1) (Supporting Information, Figure 1). On the basis of the MS/MS analysis, the product ion of *m/z* 345.0 generated from the precursor ion of *m/z* 497.1 suggested the typical loss of a galloyl moiety which excluded this galloyl moiety as the trapping site for ACR.

Major adducts arising from the reactions between HNE and phloretin appeared at *m/z* 429.1 and 411.1, which correspond to a molecular composition of phloretin + HNE and phloretin + HNE-H_2O, respectively. As shown in Supporting Information, Figure 2, CID of the *m/z* 411.1 ions led to *m/z* 305.1 fragment ions, which is assignable to the ion produced by a typical loss of the B ring unit of phloretin, suggesting that the A ring of phloretin is the trapping site for HNE. For the cyanomaclurin−HNE reaction system, adducts with a predicted molecular weight of 426.1 ($[M - H]^-$ at *m/z* 425.1) predominated, and these could be formed by direct combination of cyanomaclurin and HNE, followed by the elimination of a water molecule. MS/MS of the *m/z* 425.1 ions showed fragmentation behavior (Supporting Information, Figure 3) similar to that of the phloretin−HNE product ions.

Isolation and Structural Elucidation of diACR-Conjugated Phloretin. Isolation and characterization of major adduct(s) formed from the above proposed trapping reactions would provide stronger evidence about the mechanism of action involved. Although both monoACR- and diACR-substituted phloretin were identified from reaction systems comprising ACR and phloretin, when the molar proportion of ACR was increased and the reaction time prolonged, the latter (compound **1**) was found to be the dominant adduct formed. It was purified by chromatographic methods and then subjected to spectral analysis for its detailed structure. On the basis of the information obtained from NMR data, its molecular formula might be $C_{21}H_{22}O_7$, which was also in agreement with the molecular ion peak $[M - H]^-$ at *m/z* 385.1. The ^1H and ^{13}C NMR spectra suggested the presence of a dihydrochalcone skeleton similar to phloretin. The proton signals at δ 7.05 (d, $J = 8.1$ Hz, 2H) and 6.67 (d, $J = 8.1$ Hz, 2H) were indicative of an aromatic A_2B_2 system and are typical of a 4-substituted B ring, similar to that of phloretin. A singlet at δ 14.00 (1H, OH) indicated that there is hydroxyl coupling with a carbonyl group. No other aromatic proton signals were observed in the ^1H NMR spectrum, suggesting a fully substituted A ring moiety for compound **1**. From the $^1H-^1H$ COSY spectrum, two additional spin systems comprising H-10 or H-13 [δ 2.50 (m, 4H)], H-11 or H-14 [δ 1.82 (m, 4H)], and H-12 or H-15 [δ 5.53 (m, 2H)] were identified, suggesting thaht compound **1** comprises two moieties of $-CHCH_2CH_2-$. In the HMBC (Figure 7) spectrum, correlations between H-11 and C-3, H-10 and C-3, H-13 and C-5, and H-14 and C-5 confirmed that the two side chains were linked at C-3 and C-5, respectively. In addition, the correlations between H-12 and C-2, and H-15 and C-4 suggested that the two side chains were linked to the aromatic ring and formed two cyclic hemiacetal partial structures. Thus, compound **1** was identified as the diACR-conjugated phloretin hemiacetal structure, as shown in Figure 8.

Discussion

In recent years, emerging evidence suggests that polyphenols could exhibit multiple mechanisms of action. Of particular interest and to a certain extent, complementary to the traditional view of these phytochemicals predominantly as antioxidants, is the identification of certain polyphenols as effective scavenging agents of RCS, including those derived from lipid peroxidation. Nonetheless, these previous studies either lack comprehensive structure−activity relationship analysis or provide only limited information regarding the chemistry of the reaction(s) proposed (*20, 21*). In the present study, a large number of natural polyphenols with widely diverse structural features were screened to identify effective scavengers of α,β-unsaturated

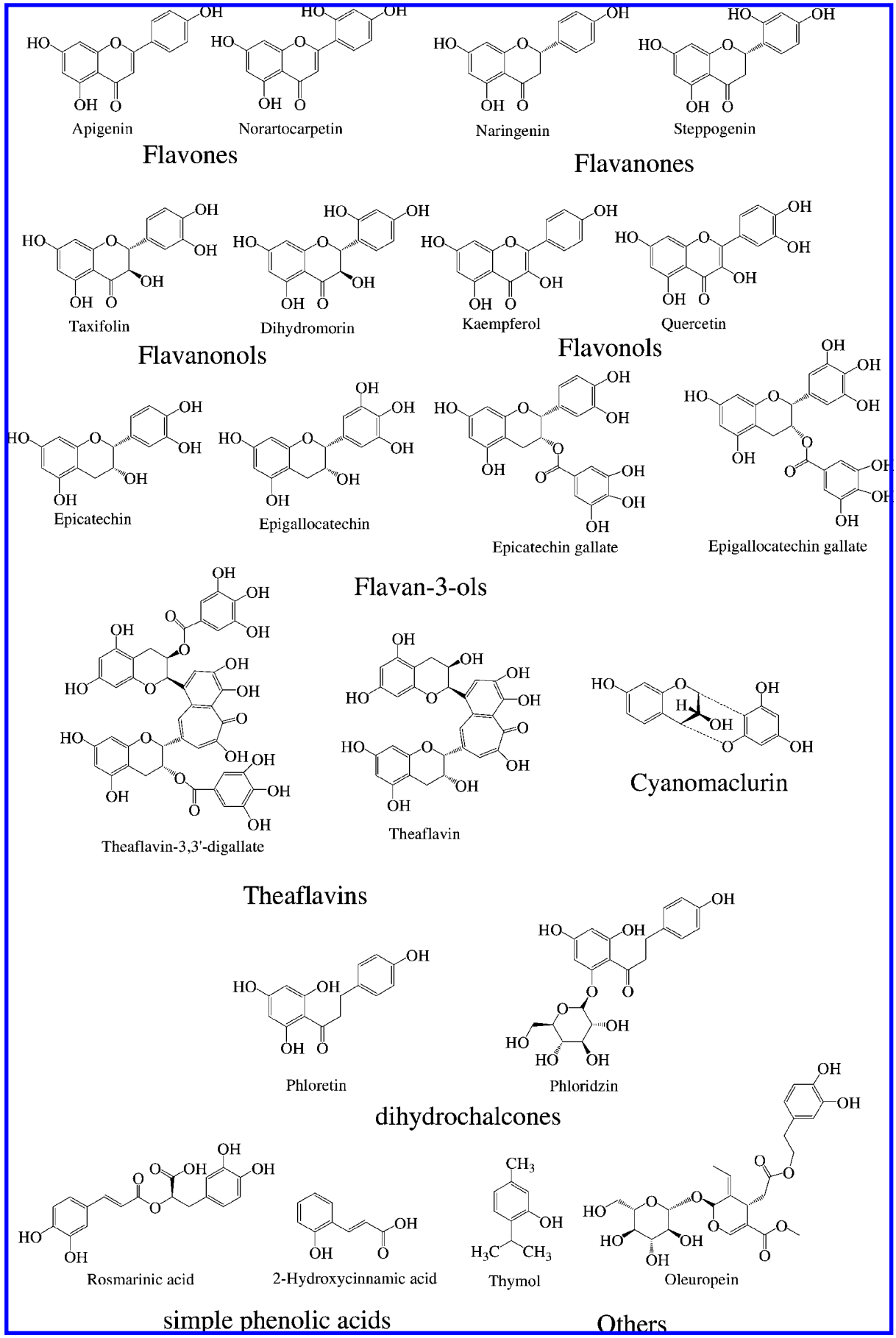

Figure 1. Chemical structures of 21 kinds of polyphenols represent the groups of flavones, flavonols, flavanones, flavanonols, flavan-3-ols, theaflavins, cyanomaclurin, dihydrochalcones, simple phenolic acids, and others.

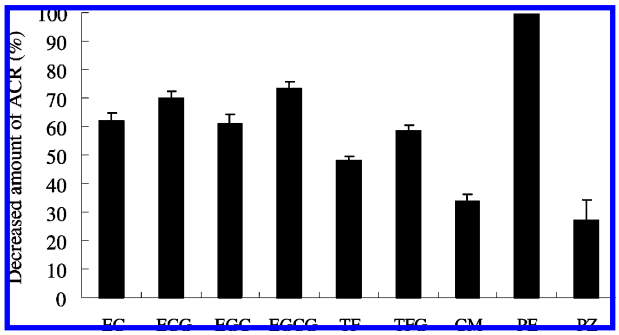

Figure 2. Percentage of decreased ACR compared with that of the control when incubated with effective ACR-trapping polyphenols in pH 7.4 PBS for 1.5 h at 37 °C. Each value is expressed as the mean ± standard error of 3 replications.

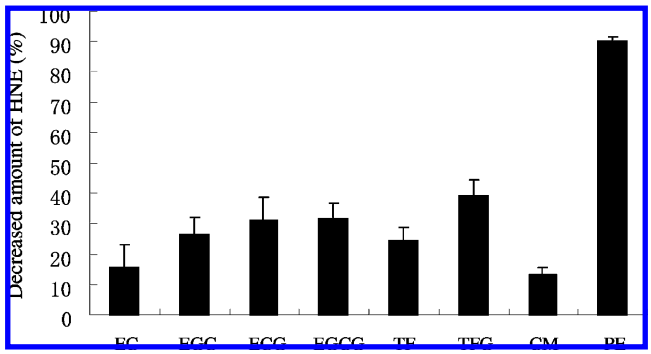

Figure 3. Percentage of decreased HNE compared with that of the control when incubated with effective HNE-trapping polyphenols in pH 7.4 PBS for 24 h at 37 °C. Each value is expressed as the mean ± standard error of 3 replications.

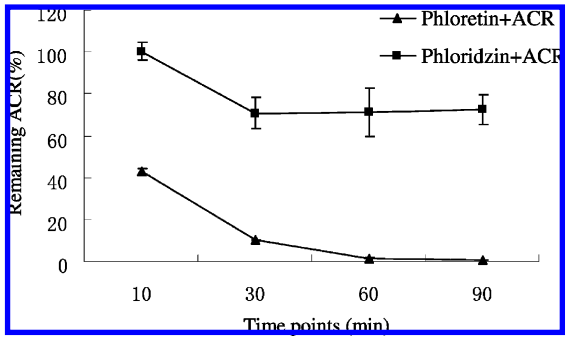

Figure 4. Trapping of ACR by phloretin and phloridzin in phosphate buffer (pH 7.4, 37 °C) at different time points. Each value is expressed as the mean ± standard error of 3 replications.

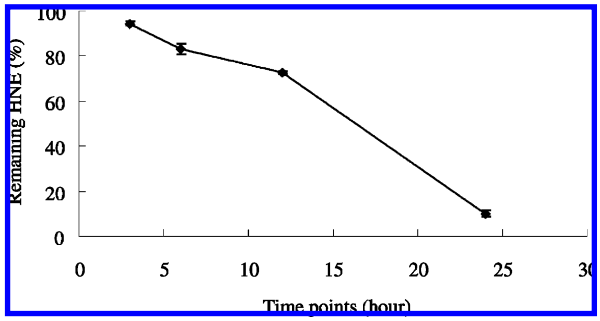

Figure 5. Trapping of HNE by phloretin in phosphate buffer (pH 7.4, 37 °C) at different time points. Each value is expressed as the mean ± standard error of 3 replications.

RCS. Only polyphenols from the categories of flavan-3-ols, theaflavins, cyanomaclurins, and dihydrochalcones were found to effectively trap ACR and/or HNE, while flavones, flavanones, flavanonols, flavonols, and other phenolics were ineffective,

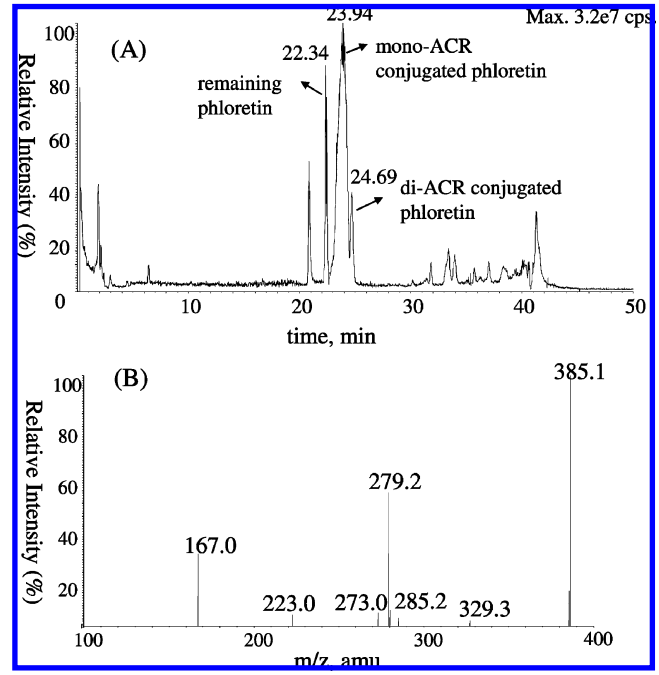

Figure 6. Total ion chromatogram of the reaction products in the incubation of phloretin and ACR together (A). MS/MS spectrum of the precursor ion of *m*/*z* $[M - H]^-$ 385.1 as diACR-conjugated phloretin (B).

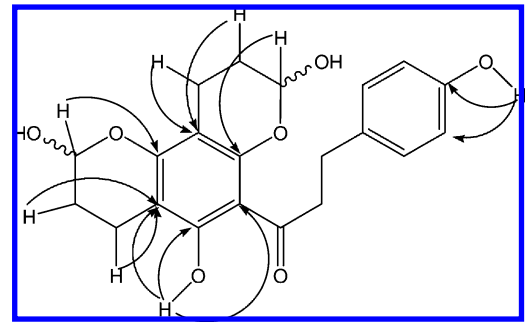

Figure 7. Key HMBC correlations of compound **1**.

suggesting that special structural moieties are needed for phenolics to show ACR and/or HNE trapping capacity. Careful examination of the structures of the active ones revealed that one structural characteristic that is shared among the effective scavengers is a phloroglucinol moiety (usually called an A ring in these structures). This is in agreement with our previous study, in which we found that when ACR was incubated individually with the analogues of the A (phloroglucinol), B (1,2,3-trihydroxybenzene), and C (methyl gallate) rings of EGCG, only phloroglucinol effectively scavenged ACR (*20*). The three electron-donating hydroxyl groups in the meta configuration on phloroglucinol might generate electron-rich centers at the unsubstituted carbon sites, thus facilitating electrophilic substitution reactions with electrophiles such as RCS (*24*). In addition, further comparison of flavan-3-ols, theaflavins, and cyanomaclurins with the ineffective scavengers such as flavones, flavanones, flavanonols, and flavonols suggested that the absence of an electron-withdrawing carbonyl group on the C ring of these structures was probably an additional requirement for significant ACR/HNE-trapping activity to effect. Another influencing factor was the substitution situation of the proton on the hydroxyl group. As elucidated by phloretin, the existence of three free hydroxyl groups in the A ring contributed to phloretin's highest activities among all in trapping of ACR/HNE, while substitution of the proton in any of the hydroxyl

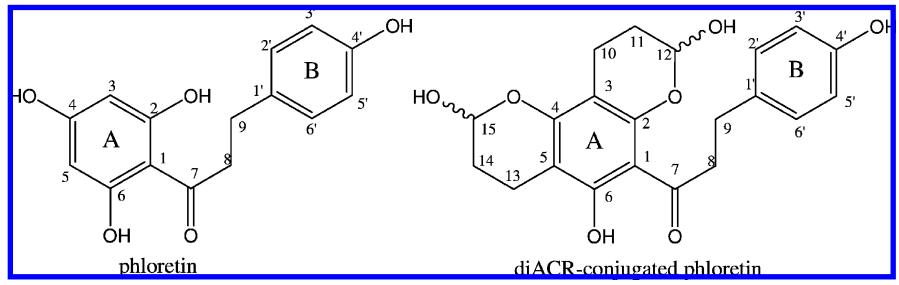

Figure 8. Structures of phloretin and diACR-conjugated phloretin (compound **1**).

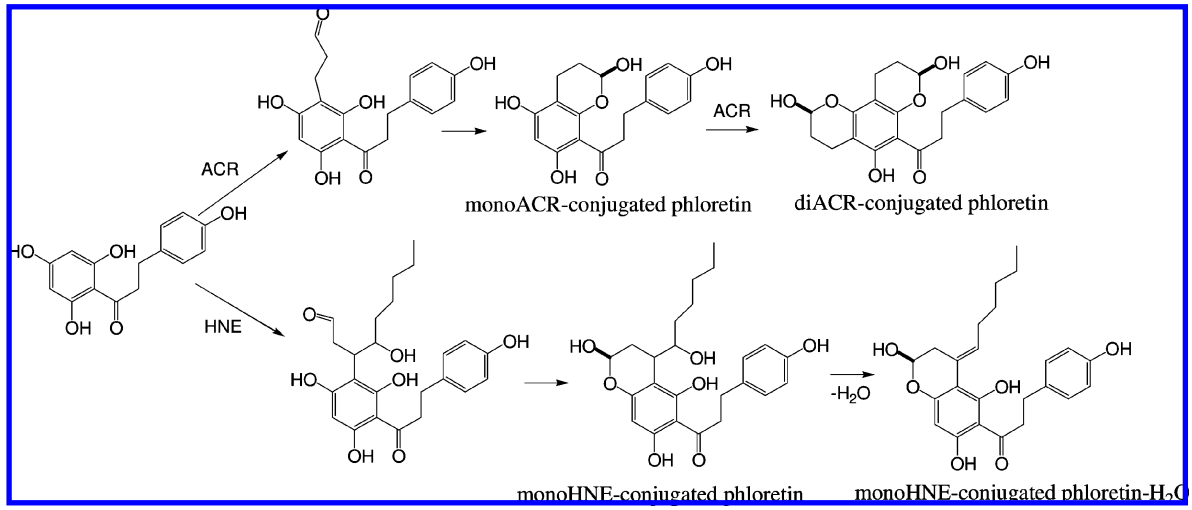

Figure 9. Proposed mechanism of the reaction between phloretin and ACR/HNE.

groups such as phloridzin and compounds in the groups of flavan-3-ols, theaflavins, and cyanomaclurin weakened the activities for electrophilic aromatic substitution. Whether this was a consequence of unfavorable factors such as steric hindrance or perturbation of electron delocalization over the RCS-trapping moiety remains to be clarified. In conclusion, three factors at least might contribute to RCS-trapping activities which included the number and pattern of hydroxyl substituents on the A ring of these phenolics, presence of a carbonyl group in conjugation with the A ring, and integrity of the A-ring hydroxyl groups.

After identifying some promising scavengers of α,β-unsaturated aldehydes (i.e., ACR and HNE), LC-DAD and LC-MS analyses were carried out to investigate the mechanism(s) of action involved. Comparison of chromatograms obtained from different chemical model reactions revealed the generation of new derivatives in reaction systems intervened with certain polyphenols. Consequently, tandem MS was executed to acquire more structural information on the derivatives. MS/MS spectra of the certain derivatives formed in the following reaction systems, phloretin and ACR (Figure 6), epicatechin-3-gallate and ACR (Supporting Information, Figure 1), phloretin and HNE (Supporting Information, Figure 2), and cyanomaclurin and HNE (Supporting Information, Figure 3) suggested that ACR/HNE likely undergo electrophilic substitution on the A ring of these polyphenols. These data were in agreement with a previous study which identified the A ring of EGCG as the active moiety in the trapping of ACR and HNE (*20, 21*). Besides α,β-unsaturated aldehydes, the A ring of certain flavonoids including green tea catechins could undergo nucleophilic substitution of acetaldehyde and glyoxylic acid at C-6 or C-8 of the A ring (*25, 26*). Tea catechins, together with phloridzin and phloretin, were also identified to be involved in the scavenging of RCS such as methylglyoxal (MGO) and glyoxal (GO) at the same position of the A ring (*27*−*29*). A recent study also demonstrated that naringenin, a citrus flavanone, was capable of scavenging phenylacetaldehyde via electrophilic substitution reactions at C-6 or C-8 of the A ring (*30*) under heating conditions. All of this literature and our data suggest that the A ring of these phenolics is the trapping site for reactive carbonyls, including ACR and HNE.

It was proposed that the high reactivity of ACR and HNE toward thiol and amino groups of biomolecules accounted for most of their potential health hazard. There are basically two active sites which contribute to ACR's high reactivity: a C═C double bond and a C═O carbonyl group, whereas in HNE, an additional hydroxyl group at C-4 might lead to polarization of the C═C bond that might facilitate internal cyclization reactions (*10, 31*). The C═C double bond in an α,β-unsaturated carbonyl compound can undergo Michael addition. Several studies have reported that Michael addition was responsible for ACR/HNE's modification of thiol and amino groups in proteins and DNA (*32*−*36*). Taking the literature data and the structure of the adduct purified in this study together, we proposed a chemical rationale for the probable nature of interactions between ACR and phloretin. Phloretin likely underwent Michael addition to the C═C bond of ACR, which mainly involves C-3 and/or C-5 of the A ring. Subsequently, nucleophilic attack at the terminal aldehyde carbon by a nearby hydroxyl group at C-2 and/or C-4 leads to the formation of cyclic hemiacetal(s) as more stable final products. Similar reactions could proceed between certain classes of polyphenols and HNE with an additional step of dehydration which occurred at the side chain of the HNE moiety. (A trapping mechanism of phenolics on ACR and HNE is proposed in Figure 9. In order to simplify the complicated situations in a reaction which might include the formation of isomeric monoACR- or monoHNE-conjugated phloretin, only one possible pathway was listed for ACR and HNE for

reference). Hence, certain polyphenols can be anticipated to act as sacrificial nucleophiles and consequently render the active sites of α,β-unsaturated RCS unavailable to covalently modify critical biomolecules. Other than the involvement of the C−N bond between amino groups and ACR and/or HNE, the adducts that formed with a highly stable C−C bond by natural polyphenols via the same mechanism of Michael addition (more than 99% of the protein modification occurred through Michael addition) made them strong competitors with biomolecules (*21, 37*).

Supporting Information Available: Figures showing total ion chromatogram of the reaction products in the incubation of ACR with epicatechin-3-gallate, HNE with phloretin, and HNE with cyanomaclurin as well as the MS/MS spectrum of a certain adduct in each reaction. This material is available free of charge via the Internet at http://pubs.acs.org.

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