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Angiotensin-Converting Enzyme Inhibitory Effects by Plant Phenolic Compounds: A Study of Structure Activity Relationships

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Supporting Information

ABSTRACT: In this study, 22 phenolic compounds were investigated to inhibit the angiotensin-converting enzyme (ACE). Tannic acid showed the highest activity (IC_{50} = 230 μM). The IC_{50} values obtained for phenolic acids and flavonoids ranged between 0.41 and 9.3 mM. QSAR analysis confirmed that the numbers of hydroxyl groups on the benzene ring play an important role for activity of phenolic compounds and that substitution of hydroxyl groups by methoxy groups decreased activity. Docking studies indicated that phenolic acids and flavonoids inhibit ACE via interaction with the zinc ion and this interaction is stabilized by other interactions with amino acids in the active site. Other compounds, such as resveratrol and pyrogallol, may inhibit ACE via interactions with amino acids at the active site, thereby blocking the catalytic activity of ACE. These structure−function relationships are useful for designing new ACE inhibitors and potential blood-pressure-lowering compounds based on phenolic compounds.

KEYWORDS: *hypertension, angiotensin-converting enzyme, phenolic compounds, molecular docking, structure−activity relationship*

INTRODUCTION

Hypertension or high blood pressure is a rising health concern since it is a major risk factor for cardiovascular disease and related complications, such as kidney damage and heart attack.[1] Globally, about one-quarter of the adult population suffers from hypertension. This percentage is likely to increase unless significant improvements are made in the prevention and treatment of hypertension.[2] Hypertension medication includes the use of drugs of which inhibitors of the angiotensin-converting enzyme (ACE) are considered as one of the most important classes.[3] ACE catalyzes the conversion of the precursor angiotensin I into angiotensin II, a peptide responsible for triggering vasoconstrictive effects,[4] and it degrades bradykinin, which is a potent vasodilator.[5] Therefore, inhibition of ACE has become a promising way for regulation and treatment of high blood pressure. Although several synthetic ACE inhibitors, such as lisinopril, captopril, and enalapril, are widely used for successful treatment of hypertension, the chronic use of these synthetic inhibitors may be associated with many undesirable side effects, such as persistent cough, postural hypotension, renal failure, and angioedema.[6,7] Extensive research has been carried out to look for ACE inhibitors from natural products as the latter might have better drug profiles and fewer side effects. Natural substances targeting ACE inhibition, such as peptides[8,9] and triterpenes,[10] have been described in the literature. Recent studies also demonstrated phenolic compounds isolated from different plants as effective ACE inhibitors in vitro.[11−13] Although these compounds have a poor solubility and subsequent restriction in bioavailability, different experiments with spontaneous hypertensive rats confirmed that phenolic compounds, such as quercetin,[14] ferulic acid,[15] and tannic acid,[16] gave a blood-pressure-lowering effect in vivo. These confirming data form a basis for our study with phenolic compounds and also underline the interest to investigate the structural differences in terms of ACE inhibition and thereby blood-pressure-reducing potency, especially because only a few studies have been done to address the relationship between the activity of phenolic compounds and their structures. Recently, Guerrero et al.[17] reported on the key structural elements of flavonoids that contribute to their ACE inhibitory activity.

The objectives of this study were first to evaluate the ACE inhibitory activity of a wide range of 22 phenolic compounds belonging to different classes and subclasses (Figure 1). Second, results were used to identify possible mechanisms of action based on structure−activity relationships and molecular docking. We believe that these new insights about the structure−function relationships may be useful for designing new ACE inhibitors based on phenolic compounds.

MATERIALS AND METHODS

Products. ACE from rabbit lung, hippuryl-histidyl-leucine (HHL), *o*-phthaldialdehyde (OPA), HCl, BSA, tannic acid, gallic acid, benzoic acid, *p*-hydroxybenzoic acid, syringic acid, vanillic acid, ellagic acid, protocatechuic acid, catechol, pyrogallol, caffeic acid, ferulic acid, *p*-coumaric acid, *trans*-cinnamic acid, quercetin, rutin, kaempferol, resveratrol, *trans*-stilbene,

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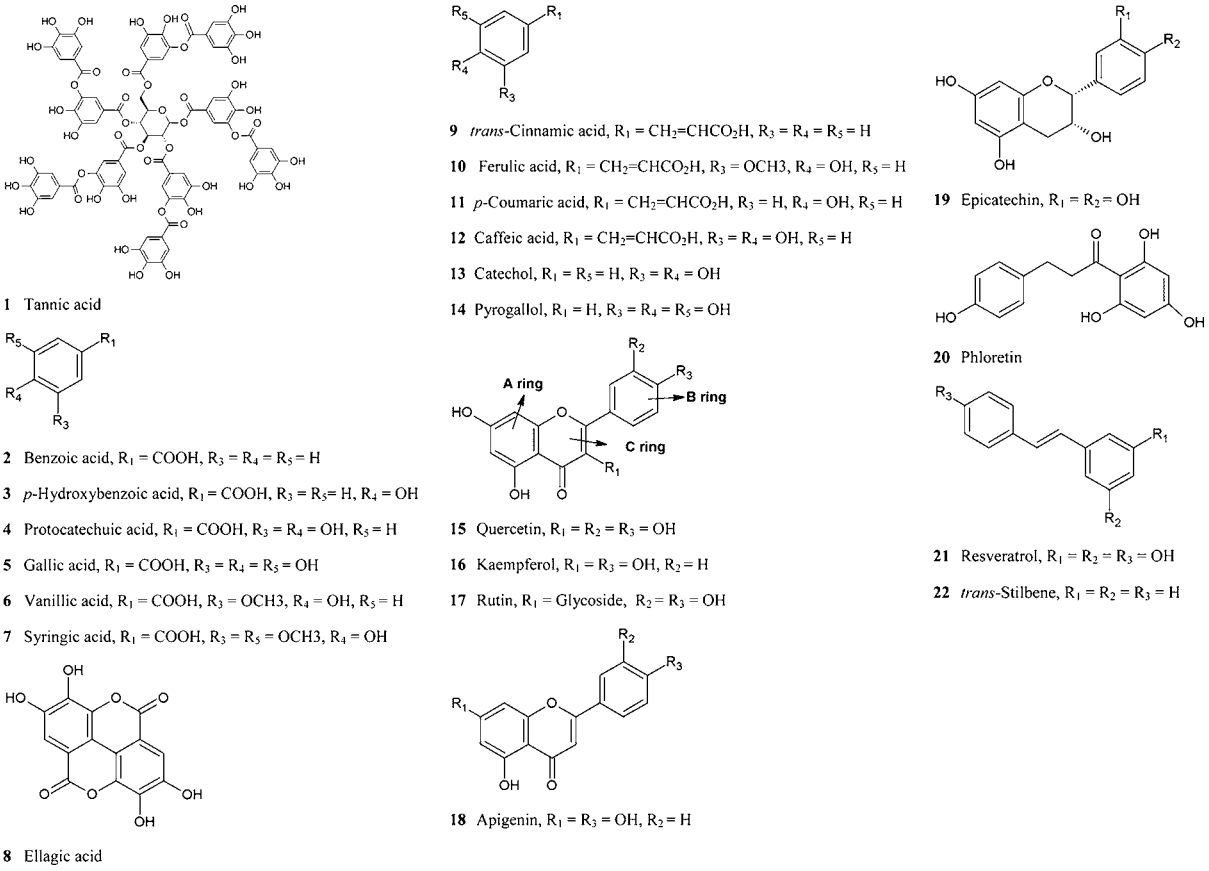

Figure 1. Structure of the 22 phenolic compounds tested in this study.

apigenin, epicatechin, phloretin, and ninhydrin were purchased from Sigma-Aldrich (Bornem, Belgium; St. Louis, MO). Lisinopril was provided by Merck & Co. (Rahway, NJ, USA). Sodium tetraborate ($Na_2B_4O_7{\cdot}10H_2O$) was purchased from Acros Organics (Geel, Belgium), while NaOH and NaCl were from Chem Lab (Lichtervelde, Belgium).

In Vitro ACE Inhibitory Activity Assay. For the selection of 22 phenolic compounds, the ACE inhibitory activity was measured using the colorimetric method previously described[18] with slight modifications. In brief, the ACE-catalyzed reaction was performed in cuvettes containing 100 μL of sample solution, 100 μL of ACE solution, and 100 μL of HHL solution. The phenolic compounds were dissolved in 100% ethanol, and different concentrations were tested with a maximum of 10% ethanol in the final reaction volume. Tannic acid was dissolved in water. Appropriate control and blank reactions were performed concurrently. The reaction mixtures were incubated for 2 h at 37 °C, and the reaction was stopped by adding 2 mL of *o*-phthaldialdehyde reagent. After incubation for 20 min at 25 °C, the absorbance was measured at 390 nm.

The concentrations of the phenolic compounds (mM) were plotted versus the corresponding ACE inhibitory activity values (%), and the dose−response curves were obtained by the nonlinear sigmoid regression with Prism v4 (GraphPad Prism, La Jolla, CA). The IC_{50} value, expressing the concentration of the phenolic compound inhibiting 50% of ACE activity, together with the corresponding 95% confidence interval and the R^2 of the curve fitting were determined as previously described.[19] However, 50% inhibition was not always reached due to a lower solubility of some phenolic compounds at high concentrations. In that case, IC_{50} values were extrapolated from the linear regression plot of the percentage of ACE inhibition versus the concentrations used.[20]

Molecular Docking and Quantitative Structure−Activity Relationship Analysis. Molecular docking of the phenolic compounds **1**−**7**, **9**−**15**, **17**, **19**, **21**, and **22** into testicular ACE (tACE) was performed with Discovery Studio (DS) 2.5 (Accelrys, San Diego, CA) using a CHARMm-based protocol, CDOCKER. The crystal structure of tACE (1UZF) was obtained from the Protein Databank, and the ligands (phenolic compounds) were sourced from PubChem. The receptor protein and the ligands were subjected to protein and ligand preparations to simulate the conditions used in the in vitro analysis (pH 8.3) using the Prepare Protein and Prepare Ligand protocols. The best conformation based on docking score and literature reports were used in subsequent analyses. In an attempt to establish a linear relationship between the IC_{50} values and docking scores, the generated docking poses were ranked by each of the four individual scores (shape, hydrogen bound, protein desolvation, and ligand desolvation). The sum of the normalized scoring functions generated the FRED Chemgauss4 score, using the OEDocking suite for OSX (ver. 3.0.1) (OpenEye, Santa Fe, NM).

Multiple linear regression (MLR) analysis of the tested phenolic compounds was used to determine the molecular properties influencing the ACE inhibitory activity of the test phenolic compounds. However, only the structure−activity relationship of phenolic acids was analyzed due to the limited number of flavonoids tested in this study because of their low solubility. Molecular descriptors consisting of 2D molecular properties and functional group counts were obtained from DS and E-Dragon 1.1.[21] MLR was performed using SPSS 20 (IBM, Armonk, NY).

RESULTS

Selection of Solvent to Dissolve Phenolic Compounds. Since many phenolic compounds are poorly soluble in water, a suitable organic solvent to dissolve the phenolic compounds was needed. With the use of 10% methanol and 10% DMSO in the enzyme solution, there was an inhibition of the ACE enzyme activity by 25% and 16%, respectively. In contrast, no loss of ACE enzyme activity was recorded with 10% ethanol in the reaction mixture. Therefore, the phenolic compounds were dissolved in 100% ethanol and their effect on ACE inhibition was tested with an

Table 1. Percent Inhibition of ACE Activity at Different Concentrations Together with Calculated IC_{50} Values Obtained for Tannic Acid, Phenolic Acids, Catechol, and Pyrogallol[a]

class	subclass	compound	% inhibition (conc., mM))	% inhibition (conc., mM)	IC_{50} value (mM)	95% CI (mM)	R^2
tannins	tannins	tannic acid	88 ± 2 (0.90)	97 ± 1 (1.9)	0.23	0.08−0.69	0.79
phenolic acids	hydroxybenzoic acids	benzoic acid	1 ± 4 (0.85)	90 ± 4 (13.6)	6.20	5.78−6.66	0.99
		p-hydroxybenzoic acid	3 ± 9 (0.75)	94 ± 2 (12.0)	5.95	5.65−6.26	0.99
		protocatechuic acid	9 ± 1 (0.80)	91 ± 2 (10.8)	5.07	2.70−9.24	0.97
		gallic acid	26 ± 2 (0.80)	84 ± 1 (10.0)	3.70	1.86−7.29	0.93
		vanillic acid	9 ± 7 (1.50)	85 ± 3 (11.8)	8.00	7.60−9.23	0.98
		syringic acid	4 ± 3 (1.50)	54 ± 4 (10.0)	9.30	8.35−10.42	0.98
		ellagic acid	32 ± 1 (1.00)	ND	2.00*		0.78
	hydroxycinnamic acids	*trans*-cinnamic acid	10 ± 4 (1.50)	ND	8.50*		0.54
		ferulic acid	18 ± 2 (0.85)	ND	4.40*		0.82
		p-coumaric acid	19 ± 1 (1.00)	ND	2.80*		0.76
		caffeic acid	34 ± 2 (0.93)	ND	2.10*		0.56
other polyphenols	other polyphenols	catechol	18 ± 1 (1.50)	64 ± 3 (12.0)	7.70	5.96−9.90	0.98
		pyrogallol	47 ± 5 (1.20)	90 ± 4 (10.0)	1.12	0.76−1.64	0.98

[a]Data are expressed as means ± SD based on three biological replicates. "*" indicates that IC_{50} values were extrapolated from the linear regression plot of the percentage of ACE inhibition versus the concentrations used. ND: not determined due to poor solubility of the sample at a concentration in the range of 10−14 mM.

Table 2. Effect of Different Flavonoids and Stilbenes on ACE Activity

class	subclass	phenolic compounds	% of ACE inhibition at 0.75 mM[a]	IC_{50} value (mM)[b]	95% CI (mM)	R^2
flavonoids	flavonols	quercetin	37 a ± 2.1	0.415	0.18−0.97	0.96
		kaempferol	29 b ± 4.4	0.512	0.19−1.40	0.94
	flavanones	rutin	21 c ± 3.7	0.472	0.14−1.55	0.85
	flavones	apigenin	13 d ± 3.3	0.667*		0.88
	flavanols	epicatechin	19 c ± 2.8	1.381*		0.82
	dihydrochalcones	phloretin	8 d ± 3.4	1.110	0.71−1.70	0.98
stilbenes	stilbenes	resveratrol	15 ± 1.3	0.970	0.70−1.33	0.99
		trans-stilbene	no activity	no activity		

[a]The values are expressed as means ± SD based on three biological replicates. Means with different superscripts are significantly different at P values < 0.05 using one-way ANOVA. [b]Activity to inhibit ACE is expressed as the concentration of phenolic compound (mM) to inhibit 50% of ACE enzyme activity; the IC_{50} value is calculated from the sigmoid curve in Prism and is given together with the 95% confidence interval. The goodness of curve fitting is given with the R^2 value. "*" indicates that the IC_{50} values were extrapolated.

amount of 10% ethanol present in the final reaction mixtures. Tannic acid, having a higher water solubility, was dissolved in water.

ACE Inhibitory Activity by Tannic Acid, Different Phenolic Acids, Pyrogallol, and Catechol. Before testing the phenolic compounds, we evaluated the ACE inhibitory activity of different concentrations of the synthetic ACE inhibitor lisinopril that were prepared in water. After sigmoid curve fitting, the IC_{50} of lisinopril was 1.00 nM (95% confidence interval = 0.85−1.26 nM; R^2 = 0.99), confirming the reliability of the ACE inhibitory bioassay.[19]

Table 1 demonstrates the ACE inhibitory effects by tannic acid, phenolic acids, pyrogallol, and catechol. It was clear that, among the 22 different phenolic compounds tested, tannic acid, **1**, was the most active with the lowest IC_{50} value (230 μM). Both subgroups of phenolic acids, i.e., hydroxybenzoic acids (compounds **2**−**8**) and hydroxycinnamic acid (compounds **9**−**12**), showed ACE inhibition in a range from 2.1 to 9.3 mM. Among all hydroxybenzoic acids, only ellagic and gallic acid showed some ACE inhibition at a concentration between 0.75 and 1.5 mM, whereas the other hydroxybenzoic acids at this concentration range did not affect the ACE activity. However, at a concentration in the range of 10−14 mM, all hydroxybenzoic acids, except syringic acid, resulted in ≥84% ACE inhibition. Among the hydroxycinnamic acids, caffeic acid caused the highest ACE inhibition at a concentration in the range of 0.85−1.5 mM. As the hydroxycinnamic acids have a poor solubility at high concentrations, even in a 10% ethanol solution, a concentration in the range of 10−14 mM could not be tested. Furthermore, catechol and pyrogallol, **13** and **14**, were also tested. While these compounds both showed a concentration-dependent ACE inhibition, the IC_{50} value obtained for pyrogallol was 7-fold lower than that of catechol.

ACE Inhibitory Activity of Flavonoids and Stilbenes. Six flavonoids representing four subclasses (flavonol, flavanol, flavones, and dihydrochalcone) (compounds **15**−**20**) and two stilbenes (compounds **21** and **22**), were tested. As presented in Table 2, all tested flavonoids showed ACE inhibitory activity with IC_{50} values ranging between 0.415 and 1.381 mM. At a concentration of 0.075 mM, quercetin and kaempferol showed the highest activity. Rutin and epicatechin appeared to have an intermediate ACE inhibitory activity, whereas the lowest ACE inhibitory activity was observed with apigenin and phloretin.

Regarding stilbenes, resveratrol showed a concentration-dependent ACE inhibition, giving an IC_{50} value of 0.970 mM (Table 2). *trans*-Stilbene did not show any activity at the concentrations tested (0.05−0.1 mM).

Quantitative Structure Activity Relationship for Phenolic Acids. The best multiple linear regression (MLR) analysis equation obtained for the log IC_{50} values of the phenolic acids (Compounds **2**−**7** and **9**−**12**) is as follows

$$\log IC_{50} = 4.618(\pm 0.54) - 4.042(\pm 0.54)\ A\log P + 0.07(\pm 0.01)\text{vol} - 1.63(\pm 0.21)\text{HBA}$$

$$\text{with } n = 10,\ F = 18.83,\ p = 0.002,\ R^2 = 0.904$$

where *A*log *P* is a measure of hydrophobicity based on the octanol–water partition coefficient, vol is the calculated 3D volume of each molecule, and HBA is the number of hydrogen-bond acceptors. A high correlation ($R^2 = 0.950$) between observed and predicted IC_{50} values (from MLR analysis equation) of the phenolic acids was obtained. It should be noticed that tannic acid and ellagic acid were not included in the analysis since they have a different and more polymerized structure compared to the other phenolic acids.

Molecular Docking Experiments. Here, tACE was used since it is the only available structure and it is highly identical to the C terminal half of the somatic ACE that has been shown to be sufficient for blood pressure regulation.[22]

In addition, the ligand–protein interaction was evaluated on the basis of the Chemgauss4 score. As shown in Figure 2, a

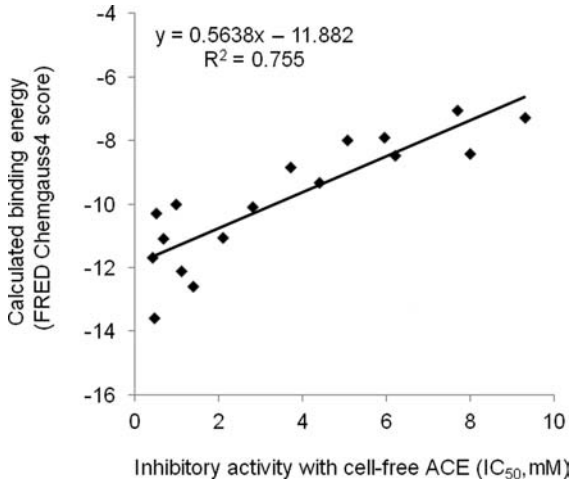

Figure 2. Linear correlation between docking scores and IC_{50} values, with $R^2 = 0.755$, for 17 phenolic compounds (compounds **2**–**7**, **10**–**13**, and **15**–**21**). The ligand–protein interaction was evaluated based on the calculated binding energy as the FRED Chemgauss4 score, and the calculated IC_{50} values represent the concentration of phenolic compound inhibiting 50% of free ACE enzyme activity as calculated in this study (from Tables 1 and 2).

significant linear correlation exists between this docking score and the IC_{50} values with $R^2 = 0.755$. Typically, the three most active compounds in the ACE cell-free assay, namely, quercetin, rutin, and kaempferol, were also ranked in the top based on the Chemgauss4 score.

Phenolic Acids, Catechol, and Pyrogallol. Table 3 represents the intermolecular interactions between ACE binding site residues and predicted poses for most of the tested phenolic compounds (compounds **2**–**7**, **9**–**15**, **17**, **19** and **21**–**22**). It can be seen that all phenolic acids formed charge–charge interactions with the zinc ion in the active site of ACE. Additionally, different types of molecular interactions with the amino acids in the active site were found for all phenolic compounds tested. Figure 3A–D shows the docking results from the predicted poses for gallic, protochatechuic, caffeic, and syringic acids at the ACE binding site. From these results, it could be clearly observed that gallic, protochatechuic, and caffeic acids are able to make interaction with the zinc ion in the active site via their carboxylate group. On the other hand, syringic acid interacts with the zinc ion via its hydroxyl group. Caffeic acid interacts with its acrylic acid group with amino acid residues at both the S2′ and S1 binding sites, whereas, for protocathechuic acid, the carboxylate group only interacts with the S2′ binding site. It should also be noticed that tannic acid could not fit into the active site of ACE.

Concerning pyrogallol and catechol, neither of these compounds interacted with the zinc in the active site. However, these compounds formed interactions with amino acids in the active site of ACE (Table 3).

Flavonoids and Stilbenes. As shown in Table 3, quercetin, rutin, and epicatechin formed molecular interactions with the ACE binding site residues. The quercetin molecule docks into ACE, interacting with the zinc via the 3-hydroxyl group in the C-ring (Figure 3E). Epicatechin is able to form an interaction with the zinc ion via the A-ring hydroxyl on position 7 (Figure 3F). On the other hand, rutin did not show any interaction with the zinc in the active center of ACE.

With regard to stilbenes, resveratrol did not show interaction with the zinc metal, but it establishes some interactions via its hydroxyl groups with amino acids in the active site of ACE (Figure 3G). In contrast, with the exception of π-stacking, no interactions were observed between *trans*-stilbene and both zinc and amino acids in the active site of ACE (Figure 3H).

DISCUSSION

The importance of phenolic compounds and their extracts on ACE inhibition has gained more and more interest during the last 10 years. Several studies demonstrated that phenolic compounds isolated from different plants and some pure flavonoids can inhibit ACE activity and reduce blood pressure. Indeed, blood-pressure-lowering effects in vivo with spontaneous hypertensive rats have been confirmed for quercetin,[14] ferulic acid,[15] and tannic acid.[16] These data in vivo form a strong basis for our study with phenolic compounds and also underline the interest to investigate the structural differences in terms of ACE inhibition and thereby blood pressure-reducing potency, especially because only a few studies have been done so far. We, therefore, gave attention in this paper to pure phenolic compounds with regards to their potency to inhibit ACE activity. The ACE inhibitory properties of 22 pure phenolic compounds representing different classes and subclasses were evaluated. Together with the obtained results, structure–activity relationship analysis and molecular docking were used for a better understanding of how these phenolic compounds interact with the ACE enzyme. To discuss and analyze the results obtained, the phenolic compounds as included in this study were categorized into three groups.

The first group includes tannic acid that was proposed to inhibit ACE. Considering all the results reported in this study, tannic acid exhibited the highest ACE inhibitory activity. Tannic acid is available in high quantities and is well-known to bind and/or precipitate proteins.[23−27] Indeed, some studies showed that the high ACE inhibitory activity found for gallotannin[28] and procyanidin hexamer [29] was reduced by 86% and 65% after addition of BSA and albumin, respectively. To a similar extent, our earlier unpublished data confirmed that tannic acid can cause 88 ± 2% precipitation of BSA protein at a concentration of 3.5 mM, which is >10 times higher than the calculated IC_{50} value (0.23 mM) for inhibition of ACE. Also, it should be mentioned that the precipitation of BSA by tannic acid was only seen at very high concentrations and that precipitation with the ACE protein has never been confirmed in our own experiments testing ACE

Table 3. Intermolecular Interactions between ACE Inhibitors and the ACE Binding Site[a]

	the active site												
		S1			S2′						other		
ligands	zinc	Tyr523	Ala354	Glu384	His353	Lys511	Gln281	His513	Asp453	Tyr520	Glu411	Asp415	Lys454
2	C	H			Pi, C								
3	C				Pi, C	C	H						
4	C				Pi, C	C	H			H			
5	C	H			C	C					C		
6	C	H			H, C	C	H			H	C		
7	C				Pi, C						C		
9	C				Pi						C		
10	C				Pi		H				C		
11	C				Pi		H				C		
12	C		H		Pi						C		
13		H		H	H			H					
14		H		H	Pi			H					
15	C				Pi, C, H	C	H				C		
17		H	H		Pi, H			H					
19	C				C, Pi						C	H, H	H
21					H	H	H					H	H
22					Pi								
lisinopril	C		H		H, C	H, C	H				C	C	

[a]C, charge−charge interactions; H, hydrogen bonding; Pi, π (stacking) interaction.

inhibition. Additionally, our docking experiments demonstrated that tannic acid did not fit into the active site of ACE due to its too large polymer size. However, it has been reported for phlorotannin, belonging together with tannic acid to the group of tannins, that the hexameric phenol, dieckol, also inhibited the activity of ACE and this by binding on the outside of the ACE molecule. [30] As such, although we cannot explain the exact mechanism(s) of ACE inhibition by tannic acid, this compound showed a high activity, which is in line with the blood-pressure-lowering activity found for tannic acid in spontaneously hypertensive rats.[16]

The second group includes phenolic acids and flavonoids, such as quercetin, that are expected to inhibit ACE activity via interaction with the zinc ion in the active site. All the tested phenolic acids exhibited ACE inhibition with IC_{50} values ranging between 2 and 9.3 mM. Our structure−activity relationship analysis helped us to understand the key structural elements influencing ACE inhibitory activity of the phenolic acids. It was observed that the hydroxybenzoic acids, differing only in the numbers of hydroxyl groups on the benzene ring, showed a significant increase in ACE inhibitory activity with increasing numbers of hydroxyl groups. A similar observation was seen with the hydroxycinnamic acids. Indeed, previous studies reported the significance of hydroxyl groups on phenolic compounds for zinc metalloproteinase inhibition.[31,32] In addition, the 2-fold lower IC_{50} value of caffeic acid compared to that of protocatechuic acid and the similar observation seen when comparing ferulic acid with vanillic acid confirm the importance of the acrylic group for ACE inhibition.[33] Another functional group that seems to influence the ACE inhibitory activity of phenolic acids is a methoxy group. Methylation of 3- and 5-hydroxyl groups of gallic acid to generate syringic acid caused a 3-fold decrease in ACE inhibitory activity. A similar reduction in ACE inhibitory activity was observed when comparing caffeic acid and ferulic acid. The reduced ACE inhibitory activity seen in the presence of a methoxy group might be explained by the steric hindrance caused by this structure, which might hamper the binding to the active site of ACE.

To determine the molecular properties that influence the ACE inhibitory activity of the phenolic acids under consideration, MLR analysis was done using molecular descriptors. It was clear that the presence of certain functional groups, such as hydroxyl, carboxyl, and acrylic acid groups, which can act as hydrogen-bond acceptors or donors, seems to increase the potency to inhibit ACE. On the other hand, the presence of methoxy groups negatively influences its ACE inhibitory activity. For instance, syringic acid, which is the least active phenolic acid, contains two methoxy groups, which makes the number of hydrogen-bond acceptors low. In contrast, gallic acid, possessing five hydrogen-bond acceptor groups, showed a higher activity. This suggests that the overall contribution, rather than the presence or absence of certain functional groups (hydroxyl, carboxyl, etc), influences the potential of phenolic acids to inhibit ACE activity. Furthermore, our docking studies revealed that most of the phenolic acids included in this study formed charge−charge interactions with the zinc ion in the active site of ACE via the oxygen atom in the carboxylate moiety. Additionally, these compounds made different molecular interactions with the amino acids at the active site of ACE, which gives rise to a stable complex between the phenolic acid molecule and ACE. This interaction with the zinc ion, stabilized by other interactions with amino acids in the active site, may have caused the ACE inhibitory activity seen by phenolic acids. It is well-known that the synthetic inhibitor lisinopril complexes the zinc via its carboxylic moiety and, subsequently, different interactions with key amino acids in the active center are established to stabilize this complex.[22] Therefore, it can be suggested that the phenolic acids inhibit ACE activity in a manner similar to that of lisinopril.

With regard to the flavonoids included in this study, these compounds all inhibited ACE, but with different potencies. It is known that flavonoids have a wide range of structures differing in substitution patterns to the A-, B-, and C-ring[34] which might influence their bioactivity. However, it was not possible to test a

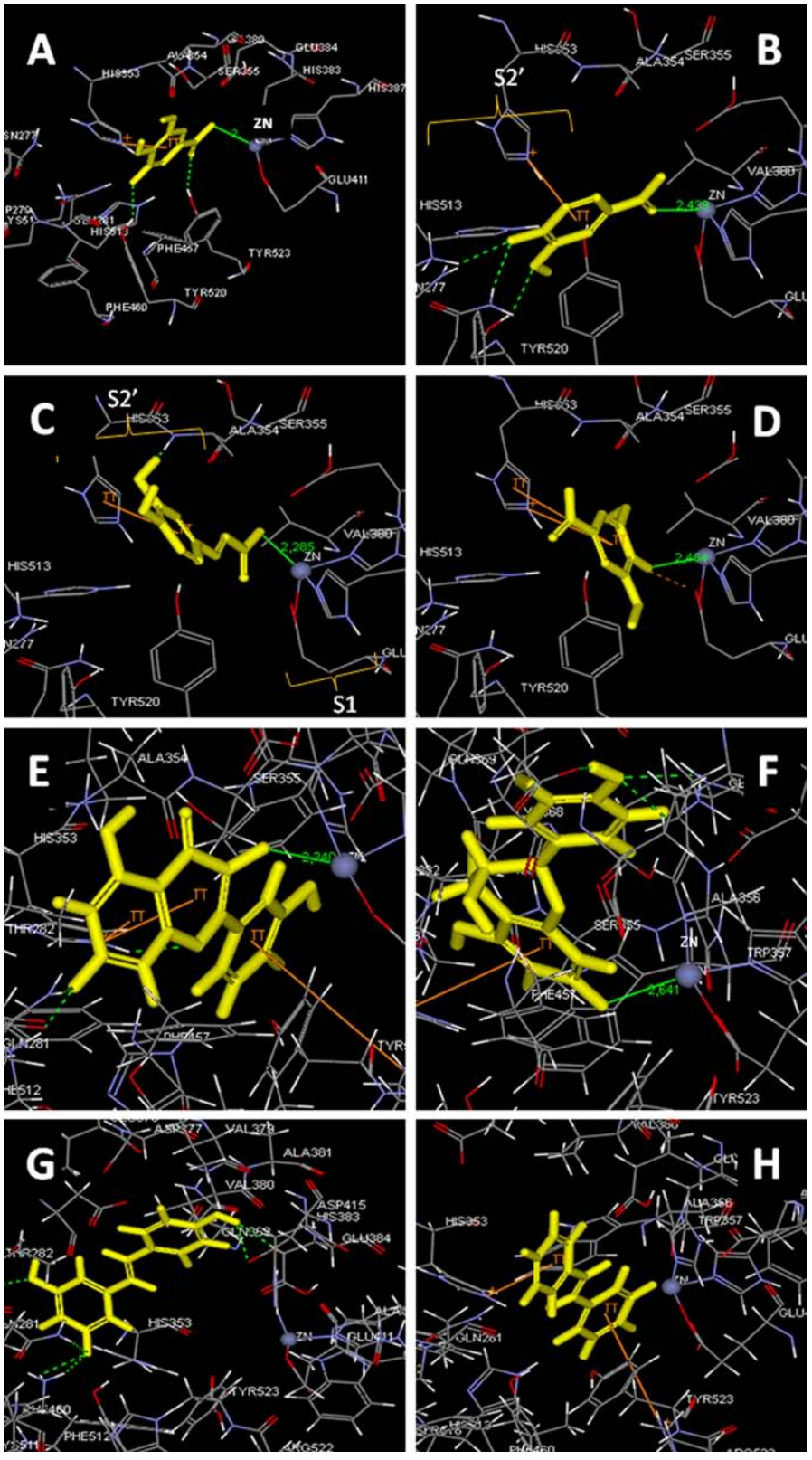

Figure 3. Docking of (A) gallic acid, (B) protocatechuic acid, (C) caffeic acid, (D) syringic acid, (E) quercetin, (F) epicatechin, (G) resveratrol, and (H) *trans*-stilbene in the binding cavity of ACE, showing interactions with zinc and amino acids in the active site. The ligands in the cavity are shown in yellow and the zinc ion in blue. Green dashed lines are used to show hydrogen bonds, while the full green ones represent charge−charge interaction. Orange stacks were used to show the π-stacking. Chemical structures were drawn not to scale.

wider range of flavonoids for ACE inhibitory activity due to the poor water solubility of flavonoids. As a consequence, a larger structure−activity relationship analysis could not be established in this project. Recently, a study reported on the importance of the catechol group on the B-ring in flavonoids for ACE inhibition.[17] In agreement, we believe that this might explain the higher

activity seen with quercetin compared to kaempferol and apigenin in which the catechol group is lacking. With respect to the flavonol quercetin, the flavanol epicatechin exerted a significantly lower activity at 0.075 mM and a 3-fold increase in IC_{50} value. Epicatechin lacks the keto group in C4 and the C2−C3 double bond, causing the flavonoid skeleton to lose its planar structure and, subsequently, changing the molecular electronic distribution. Previous studies reported that the planar structure of flavonoids[35] and cyanidins[13] is an important factor for ACE inhibition. Docking results of both quercetin and epicatechin showed that the absence of the keto group and the C2−C3 double bond shifts the zinc binding site to the 7-OH moiety. Tsutsumi et al.[36] reported on the importance of the hydroxyl group on position 7 in the structure of flavonoids for inhibiting ACE enzyme activity. However, according to studies pertaining to metal chelation of phenolic compounds in solution, zinc and other metal ions have a stronger binding affinity to the 3-hydroxy-keto group, which is absent in epicatechin.[37,38] This may justify the higher activity seen for quercetin than for epicatechin.

The third group includes other flavonoids (rutin), stilbenes (resveratrol), and the other phenolic compounds (catechol and pyrogallol) that may inhibit ACE activity via only interactions with amino acids in the active site. ACE is a zinc metallopeptidase, in which the zinc ion at the active site is essential for ACE catalysis. Thus, the interaction with zinc in the active site of ACE is considered an important mechanism in ACE inhibition. However, on the basis of a quantum mechanical and molecular mechanical study, the interaction with the zinc ion does not seem to be the only mechanism, since it has been shown that ACE may cleave a substrate without interaction between the substrate and zinc.[39] The third group of our phenolic compounds did inhibit ACE activity but did not show any interaction with the zinc in the active site. Therefore, we believe that the ACE inhibitory activity seen by this group may be due to the interactions established via hydrogen bonds between the hydroxyl groups and amino acids in the active site that block the catalytic activity of ACE enzyme.

Finally, the results in this study need to be placed in a larger perspective. To exert an ACE inhibitory effect in vivo, phenolic compounds have to be transported through the intestinal wall to reach the bloodstream. The IC_{50} values for phenolic compounds in this study are higher than the concentrations reported in blood plasma.[40] Nevertheless, in previous studies, several phenolic compounds were shown to have antihypertensive effects in vivo.[14−16] To explain these ACE inhibitory activities in vivo, we agree with the statement of Actis-Goretta et al.[11] that a local enrichment of phenolic compounds near the membrane surface in the vascular endothelial cells is possible since ACE is a membrane-bound enzyme. As such, repeated exposure to phenolic compounds coming from plant-based diets[41,42] might result in an accumulation near the endothelial lining with concentrations higher than those circulating in the blood, hereby enhancing the interaction with ACE. Additionally, the existence of ACE activity in the intestinal mucosa was demonstrated in some studies.[43−45] This might suggest that, in the case of high concentrations of phenolic compounds present in the gastrointestinal tract, enzyme inhibition at the intestinal level may occur.

In conclusion, the present data indicate that phenolic compounds inhibit ACE activity in vitro and that the activity against ACE and the mode of action depend on the class (subclass) and the structure of the phenolic compound. We believe that these structure−function relationships can be useful for designing new ACE inhibitors based on phenolic compounds. Further strategies, such as encapsulation and chemical modification to increase the solubility and bioavailability, and, subsequently, formulate biologically active phenolic compounds that reach the target site, need to be evaluated.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figure 1 represents the sigmoid dose response curve of ACE inhibition by lisinopril. In Figure 2, the linear regression correlation between the observed and the predicted IC_{50} value for 10 different phenolic acids (compounds **2**−**7** and **9**−**12**) is demonstrated. Docking of lisinopril in the binding cavity of ACE is shown in Figure 3. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

ACE, angiotensin-converting enzyme; IC_{50}, value expressing the concentration of a compound inhibiting 50% of ACE activity; HHL, hippuryl-histidyl-leucine; BSA, bovine serum albumin; tACE, testicular ACE; MLR, multiple linear regression

■ REFERENCES

(1) Hartmann, R.; Meisel, H. Food-derived peptides with biological activity: From research to food applications. *Curr. Opin. Biotechnol.* **2007**, *18*, 163−169.

(2) Mittal, B. V.; Singh, A. K. Hypertension in the developing world: Challenges and opportunities. *Am. J. kidney Dis.* **2010**, *55*, 590−598.

(3) Lopez-Sendon, J.; Swedberg, K.; McMurray, J.; Tamargo, J.; Maggioni, A. P.; Dargie, H.; Tendera, M.; Waagstein, F.; Kjekshus, J.; Lechat, P.; Torp-Pedersen, C. Expert consensus document on angiotensin converting enzyme inhibitors in cardiovascular disease: The task force on ACE-inhibitors of the European Society of Cardiology. *Eur. Heart J.* **2004**, *25*, 1454−1470.

(4) Zhang, R. Z.; Xu, X. H.; Chen, T. B.; Li, L.; Rao, P. F. An assay for angiotensin-converting enzyme using capillary zone electrophoresis. *Anal. Biochem.* **2000**, *280*, 286−290.

(5) Imig, J. D. ACE inhibition and bradykinin-mediated renal vascular responses: EDHF involvement. *Hypertension* **2004**, *43*, 533−535.

(6) Antonios, T. F.; MacGregor, G. A. Angiotensin converting enzyme inhibitors in hypertension: potential problems. *J. Hypertens. (Suppl.)* **1995**, *13*, S11−S16.

(7) Israili, Z. H.; Hall, W. D. Cough and angioneurotic edema associated with angiotensin-converting enzyme inhibitor therapy. A review of the literature and pathophysiology. *Ann. Intern. Med.* **1992**, *117*, 234−242.

(8) Cinq-Mars, C. D.; Li-Chan, E. C. Optimizing angiotensin I-converting enzyme inhibitory activity of Pacific hake (*Merluccius productus*) fillet hydrolysate using response surface methodology and ultrafiltration. *J. Agric. Food Chem.* **2007**, *55*, 9380−9388.

(9) Vercruysse, L.; Van Camp, J.; Smagghe, G. ACE inhibitory peptides derived from enzymatic hydrolysates of animal muscle protein: a review. *J. Agric. Food Chem.* **2005**, *53*, 8106−8115.

(10) Somova, L. O.; Nadar, A.; Rammanan, P.; Shode, F. O. Cardiovascular, antihyperlipidemic and antioxidant effects of oleanolic

and ursolic acids in experimental hypertension. *Phytomedicine* **2003**, *10*, 115−121.
(11) Actis-Goretta, L.; Ottaviani, J. I.; Fraga, C. G. Inhibition of angiotensin converting enzyme activity by flavanol-rich foods. *J. Agric. Food Chem.* **2006**, *54*, 229−234.
(12) Dong, J.; Xu, X.; Liang, Y.; Head, R.; Bennett, L. Inhibition of angiotensin converting enzyme (ACE) activity by polyphenols from tea (*Camellia sinensis*) and links to processing method. *Food Funct.* **2011**, *2*, 310−319.
(13) Ojeda, D.; Jimenez-Ferrer, E.; Zamilpa, A.; Herrera-Arellano, A.; Tortoriello, J.; Alvarez, L. Inhibition of angiotensin convertin enzyme (ACE) activity by the anthocyanins delphinidin- and cyanidin-3-*O*-sambubiosides from *Hibiscus sabdariffa*. *J Ethnopharmacol.* **2010**, *127*, 7−10.
(14) Hackl, L. P.; Cuttle, G.; Dovichi, S. S.; Lima-Landman, M. T.; Nicolau, M. Inhibition of angiotesin-converting enzyme by quercetin alters the vascular response to brandykinin and angiotensin I. *Pharmacology* **2002**, *65*, 182−186.
(15) Alam, M. A.; Sernia, C.; Brown, L. Ferulic acid improves cardiovascular and kidney structure and function in hypertensive rats. *J. Cardiovasc. Pharmacol.* **2013**, *61*, 240−249.
(16) Thekkumkara, T.; Gumaste, U.; Snyder, R.; Karamyan, V. Tannic acid, an inhibitor for renal angiotensin type 1 receptor and hypertension in spontaneously hypertensive rats. *Endocr. Rev.* **2012**, *33*, SAT-248.
(17) Guerrero, L.; Castillo, J.; Quinones, M.; Garcia-Vallve, S.; Arola, L.; Pujadas, G.; Muguerza, B. Inhibition of angiotensin-converting enzyme activity by flavonoids: Structure-activity relationship studies. *PloS One* **2012**, *7*, e49493.
(18) Chang, B. W.; Chen, R. L.; Huang, I. J.; Chang, H. C. Assays for angiotensin converting enzyme inhibitory activity. *Anal. Biochem.* **2001**, *291*, 84−88.
(19) Vercruysse, L.; Smagghe, G.; Herregods, G.; Van Camp, J. ACE inhibitory activity in enzymatic hydrolysates of insect protein. *J. Agric. Food Chem.* **2005**, *53*, 5207−5211.
(20) Balasuriya, N.; Rupasinghe, H. P. V. Antihypertensive properties of flavonoid-rich apple peel extract. *Food Chem.* **2012**, *135*, 2320−2325.
(21) Tetko, I. V.; Gasteiger, J.; Todeschini, R.; Mauri, A.; Livingstone, D.; Ertl, P.; Palyulin, V.; Radchenko, E.; Zefirov, N. S.; Makarenko, A. S.; Tanchuk, V. Y.; Prokopenko, V. V. Virtual computational chemistry laboratory — Design and description. *J. Comput.-Aided Mol. Des.* **2005**, *19*, 453−463.
(22) Natesh, R.; Schwager, S. L.; Sturrock, E. D.; Acharya, K. R. Crystal structure of the human angiotensin-converting enzyme−lisinopril complex. *Nature* **2003**, *421*, 551−554.
(23) Baxter, N. J.; Lilley, T. H.; Haslam, E.; Williamson, M. P. Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. *Biochemistry* **1997**, *36*, 5566−5577.
(24) Dinnella, C.; Recchia, A.; Fia, G.; Bertuccioli, M.; Monteleone, E. Saliva characteristics and individual sensitivity to phenolic astringent stimuli. *Chem. Senses* **2009**, *34*, 295−304.
(25) Jobstl, E.; O'Connell, J.; Fairclough, J. P.; Williamson, M. P. Molecular model for astringency produced by polyphenol/protein interactions. *Biomacromolecules* **2004**, *5*, 942−949.
(26) Bacon, J. R.; Rhodes, M. J. Binding affinity of hydrolyzable tannins to parotid saliva and to proline-rich proteins derived from it. *J. Agric. Food Chem.* **2000**, *48*, 838−843.
(27) Kawamoto, H.; Nakatsubo, F.; Murakami, K. Quantitative-determination of tannin and protein in the precipitates by high-performance liquid-chromatography. *Phytochemistry* **1995**, *40*, 1503−1505.
(28) Liu, J. C.; Hsu, F. L.; Tsai, J. C.; Chan, P.; Liu, J. Y.; Thomas, G. N.; Tomlinson, B.; Lo, M. Y.; Lin, J. Y. Antihypertensive effects of tannins isolated from traditional Chinese herbs as non-specific inhibitors of angiontensin converting enzyme. *Life Sci.* **2003**, *73*, 1543−1555.
(29) Ottaviani, J. I.; Actis-Goretta, L.; Villordo, J. J.; Fraga, C. G. Procyanidin structure defines the extent and specificity of angiotensin I converting enzyme inhibition. *Biochimie* **2006**, *88*, 359−365.
(30) Wijesinghe, W. A.; Ko, S. C.; Jeon, Y. J. Effect of phlorotannins isolated from *Ecklonia cava* on angiotensin I-converting enzyme (ACE) inhibitory activity. *Nutr. Res. Pract.* **2011**, *5*, 93−100.
(31) Parellada, J.; Suarez, G.; Guinea, M. Inhibition of zinc metallopeptidases by flavonoids and related phenolic compounds: Structure-activity relationships. *J. Enzyme Inhib.* **1998**, *13*, 347−359.
(32) Ende, C.; Gebhardt, R. Inhibition of matrix metalloproteinase-2 and -9 activities by selected flavonoids. *Planta Med.* **2004**, *70*, 1006−1008.
(33) Geng, F.; He, Y.; Yang, L.; Wang, Z. A rapid assay for angiotensin-converting enzyme activity using ultra-performance liquid chromatography-mass spectrometry. *Biomed. Chromatogr.* **2010**, *24*, 312−317.
(34) Hollman, P. C. H.; Katan, M. B. Dietary flavonoids: Intake, health effects and bioavailability. *Food Chem. Toxicol.* **1999**, *37*, 937−942.
(35) Xu, Y. C.; Leung, S. W. S.; Yeung, D. K. Y.; Hu, L. H.; Chen, G. H.; Che, C. M.; Man, R. Y. K. Structure−activity relationships of flavonoids for vascular relaxation in porcine coronary artery. *Phytochemistry* **2007**, *68*, 1179−1188.
(36) Tsutsumi, Y.; Shimada, A.; Miyano, A.; Nishida, T.; Mitsunaga, T. In vitro screening of angiotensin I-converting enzyme inhibitors from Japanese cedar (*Cryptomeria japonica*). *J. Wood Sci.* **1998**, *44*, 463−468.
(37) Lapouge, C.; Dangleterre, L.; Cornard, J. P. Spectroscopic and theoretical studies of the Zn(II) chelation with hydroxyflavones. *J. Phys. Chem. A* **2006**, *110*, 12494−12500.
(38) Dangleterre, L.; Cornard, J. P.; Lapouge, C. Spectroscopic and theoretical investigation of the solvent effects on Al(III)−hydroxyflavone complexes. *Polyhedron* **2008**, *27*, 1581−1590.
(39) Wang, X. M.; Wu, S. S.; Xu, D. G.; Xie, D. G.; Guo, H. Inhibitor and substrate binding by angiotensin-converting enzyme: Quantum mechanical/molecular mechanical molecular dynamics studies. *J. Chem. Inf. Model.* **2011**, *51*, 1074−1082.
(40) Del Rio, D.; Rodriguez-Mateos, A.; Spencer, J. P. E.; Tognolini, M.; Borges, G.; Crozier, A. Dietary (poly)phenolics in human health: Structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxid. Redox Signaling* **2013**, *18*, 1818−1891.
(41) Kuhnau, J. The flavonoids. A class of semi-essential food components: Their role in human nutrition. *World Rev. Nutr. Diet.* **1976**, *24*, 117−191.
(42) Manach, C.; Scalbert, A.; Morand, C.; Remesy, C.; Jimenez, L. Polyphenols: Food sources and bioavailability. *Am. J. Clin. Nutr.* **2004**, *79*, 727−747.
(43) Bruneval, P.; Hinglais, N.; Alhenc-Gelas, F.; Tricottet, V.; Corvol, P.; Menard, J.; Camilleri, J. P.; Bariety, J. Angiotensin I converting enzyme in human intestine and kidney. Ultrastructural immunohistochemical localization. *Histochemistry* **1986**, *85*, 73−80.
(44) Duggan, K. A.; Mendelsohn, F. A.; Levens, N. R. Angiotensin receptors and angiotensin I-converting enzyme in rat intestine. *Am. J. Physiol.* **1989**, *257*, G504−G510.
(45) Erickson, R. H.; Suzuki, Y.; Sedlmayer, A.; Song, I. S.; Kim, Y. S. Rat intestinal angiotensin-converting enzyme: Purification, properties, expression, and function. *Am. J. Physiol.* **1992**, *263*, G466−G473.