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Synthesis and antioxidant activities of 3,5-dialkoxy-4-hydroxycinnamamides

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Abstract—A series of 3,5-dialkoxy-4-hydroxycinnamamides **6** and **7** was synthesized, and their antioxidant activity was assessed using the thiobarbituric acid reactive substance (TBARS) assay. Interestingly, cinnamamides with longer alkoxy groups on the C-3 and C-5 positions display enhanced inhibition, and most of the compounds in the series tested exhibit excellent lipid peroxidation inhibitory activities. Some cinamamides bearing hexyloxy or 2,6-di-*tert*-butyl-4-methyl phenol groups have submicromolar inhibitory activities.

Reactive oxygen species (ROS), including the hydroxyl radical, superoxide anion, hydrogen peroxide, and peroxynitric species are continuously generated in the process of respiration as natural byproducts of oxygen metabolism.[1] ROS play an important roles in biochemical processes of the immune system, cell differentiation, and internal signal transduction.[2] These species are sufficiently reactive to cause injury to cells through the destruction of components such as proteins, lipids, sugars and nucleotides.[3] Under normal circumstances, cells are able to defend against ROS damage through the use of enzymes such as superoxide dismutases and catalases.[4] Small molecule antioxidants such as ascorbic acid (vitamin C), uric acid, and glutathione also play important roles as cellular antioxidants.[5] Thus, under normal conditions ROS are maintained at constant levels in body by the antioxidant defense mechanism. However, imbalances between the formation and detoxification of ROS can result in significant damage to cells, a situation known as oxidative stress. Oxidative stress leads to the initiation and/or progression of various diseases, including for example cancer,[6] ischemia-reperfusion injury,[7] atherosclerosis,[8] cardiovascular diseases,[9] inflammation,[10] and neurodegenerative disorders[11] such as Alzheimer's and Parkinson's diseases. Because of this, there is a growing interest in the discovery of natural and unnatural antioxidants that attenuate oxidative stress and, as result, can serve as protective agents against these diseases.[12]

In a previous report,[13] we described the synthesis and antioxidant activities of a series of *N*-{3-(3,4-dimethylphenyl)propyl}-4-hydroxyphenylacetamides, substituted at C-3 positions with various alkoxy groups (**1**–**3**) and several *N*-{3-(3,4-dimethylphenyl)propyl}-4-hydroxycinnamamides (**4**, **5**). The results of this effort showed that, among the substances investigated, the 4-hydroxycinnamamides had potent antioxidant properties.

The structures of **1** and **2** are closely related to that of capsaicin, a vanilloid receptor agonist.[14] Previous studies with these amides showed that they are centrally acting agent with potent analgesic activities.[15] These findings led to current study, aimed at the development of new antioxidant as neuroprotective agents, which focus on novel 3,5-dialkoxy-4-hydroxycinnamamides **6** and **7**.

Keywords: Antioxidant; 4-Hydroxycinnamamide; Lipid peroxidation; Reactive oxygen species.
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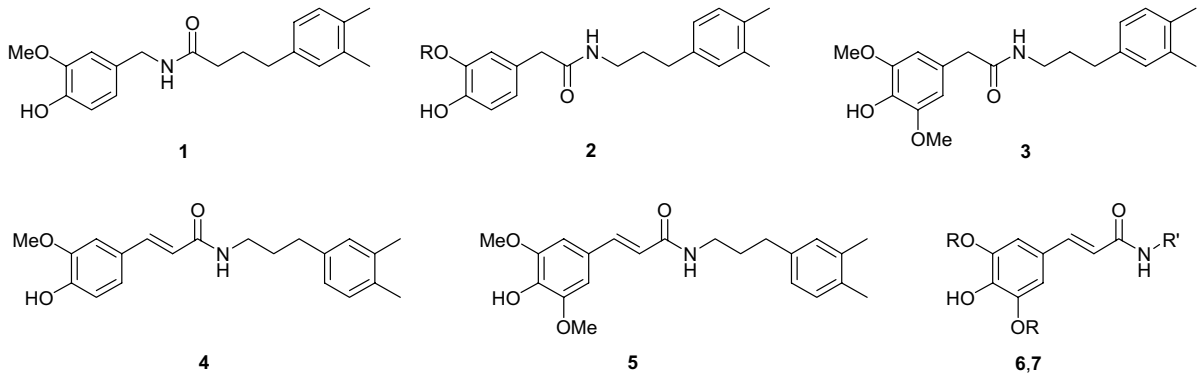
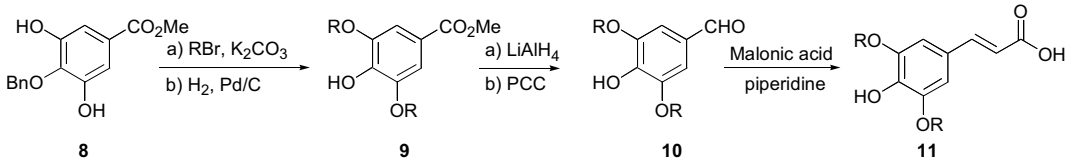
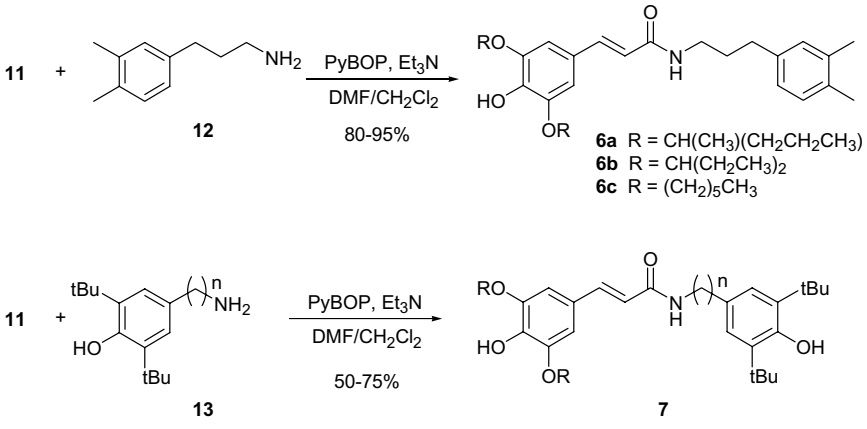


In order to prepare the new 3,5-dialkoxy-4-hydroxycinnamamides **6** and **7** as antioxidants, we needed 3,5-dialkoxy-4-hydroxycinnamic acids **11** with various alkoxy groups at the C-3 and C-5 ring positions. To the best of our knowledge, the synthesis of 3,5-dialkoxy-4-hydroxycinnamic acids **11** had not been reported until our recent report,[16] even though sinapic acid **11a** is commercially available.[17] The 3,5-dialkoxy-4-hydroxycinnamic acids **11** were generally prepared starting with methyl 4-benzyloxy-3,5-dihydroxybenzoate **8**[18] in 38–70% overall yields (Scheme 1).

Coupling of the acids **11** with 3-(3,4-dimethylphenyl)propyl amine **12** or **13** using (benzotriazole-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP)[19] and triethylamine in CH_2Cl_2 and DMF at 0 °C provided the cinnamamides **6** or **7** in 50–95% yields after flash column chromatography (Scheme 2). Initial attempts to perform these coupling reactions by using 1,3-dicyclohexycarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) gave the amides **6** in less than 50% yields. Amine **13**, which is required for the synthesis of **7**, was obtained from the benzyl bromide **15** that is

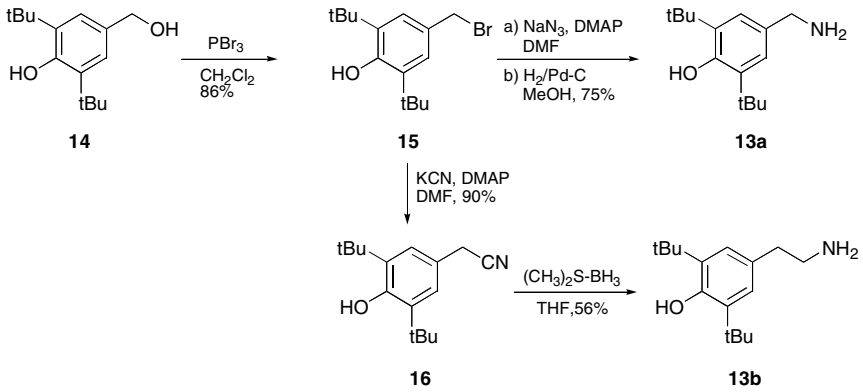


Scheme 1. Synthesis of 3,5-dialkoxy-4-hydroxycinnamic acids (PCC: pyridinium chlorochromate).



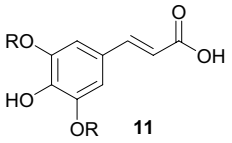
Compound	R	n	Compound	R	n
7a	CH_3	1	**7g**	$CH(CH_3)(CH_2CH_2CH_3)$	1
7b	CH_3	2	**7h**	$CH(CH_3)(CH_2CH_2CH_3)$	2
7c	$(CH_2)_2CH_3$	1	**7i**	$CH(CH_2CH_3)_2$	1
7d	$(CH_2)_2CH_3$	2	**7j**	$CH(CH_2CH_3)_2$	2
7e	$(CH_2)_2CH_3$	1	**7k**	$(CH_2)_5CH_3$	1
7f	$(CH_2)_2CH_3$	2	**7l**	$(CH_2)_5CH_3$	2

Scheme 2. Synthesis of 3,5-dialkoxy-4-hydroxycinnamamides **6**.



Scheme 3. Synthesis of 3,5-di-*tert*-butyl-4-hydroxyphenyalkyl amines **13**.

Table 1. Inhibition test of lipid peroxidation by TBARS assay



11

Compound	R	Inhibition of lipid peroxidation (IC_{50}, μM)
1		57.17
2a[a]	CH_3	25.90
2b[a]	$(CH_2)_3CH_3$	9.28
3[a]		11.77
4[a]		9.94
5[a]		3.41
11a	CH_3	4.77
11b	$(CH_2)_3CH_3$	3.68
11c	$CH(CH_3)(CH_2CH_2CH_3)$	4.84
11d	$CH(CH_2CH_3)_2$	2.21
11e	$(CH_2)_5CH_3$	2.24
α-Tocopherol		4.68
BHT		4.03

[a] See Ref. 13a.

obtained from the corresponding alcohol **14**. Reaction of **15** with NaN_3 followed by Pd/C reduction provided benzyl amine **13a**, and reaction of **15** with KCN followed by reduction with $(CH_3)_2S$–BH_3 gave phenylethyl amine **13b**, in moderate yields (Scheme 3).

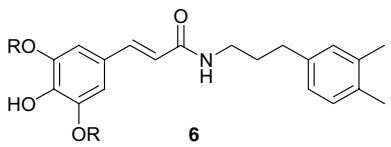
The design of 3,5-dialkoxy-4-hydroxycinnamamides **6** and **7** was based on the lipid peroxidation inhibitory activity data shown in Table 1. The results showed that (1) homovanillic amide **2a** (25.90 μM) was a better inhibition than **1** (57.17 μM), (2) 3,5-dialkoxy-4-hydroxyphenylacetamides **3** (11.77 μM) were more active than 3-alkoxy-4-hydroxyphenylacetamides **2** (25.90 μM for **2a**), and (3) cinnamamides **4** (9.94 μM) and **5** (3.41 μM) have higher potencies than phenylacetamides **2a** (25.90 μM) and **3** (11.77 μM). In addition, longer alkoxy substituents at the C-3 position of amides **2** were shown to lead to enhanced inhibition, although the potency was still lower than that of 2,6-di-*tert*-butyl-4-methylphenol (BHT) or α-tocopherol.[13] Based on these observed structure-activity relationships, we designed and prepared 3,5-dialkoxy-4-hydroxycinnamamides **6**. To evaluate the antioxidant properties of the newly synthesized amides, we examined the lipid peroxidation in a rat brain homogenate by using the thiobarbituric acid reactive substances (TBARS) assay[20] according to the method of Stocks.[21] We first examined antioxidant property of the cinamicacids **11** with alkoxy chains of various lengths (Table 1). Generally, longer alkoxy groups enhanced inhibition. Also, the order of inhibition potency was **11e** > **11b** > **11a**. The inhibitory potencies of all the cinnamic acids **11** were comparable with that of 2,6-di-*tert*-butyl-4-methylphenol (BHT) or α-tocopherol, a likely result of the stabilization of the phenyloxy radicals by the *ortho* substituted alkoxy groups.

The preliminary results encouraged an examination of the cinnamamides **6**. We observed that all three amides **6** were more potent inhibitors than the corresponding acids **11** (Table 2). It is clear that the 3,4-dimethylphenylpropyl amine moiety is responsible for this gain in inhibitory activity. Among the amides, **6c** with *n*-hexyloxy groups was the most potent compound toward inhibition of lipid peroxidation, and **6c** is more potent than **5**, a substance containing with methoxy substituents. Important information was gained from these results. Specifically, we learned that inhibitory activities are strongly influenced by longer, straight chain alkyoxy groups rather than shorter, branched alkoxy groups.

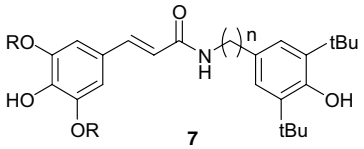
Since the amides **6** were found more potent than the acids **11**, we next focused on new cinnamamides **7** that are linked to with the well-known synthetic radical scavenger, BHT. As shown at Table 2, most of these amides **7** were very potent inhibitors. The higher activities of **7a**–**7j** over **6** are probably a consequence of the presence of BHT group in the amide. Generally the inhibition potency of **7** was not strongly affected by the alkoxy groups. The amide **7k** and **7l** which contain n-hexyoxy substituents were also investigated, but lower potencies were observed in for these substances as compared to other amides. Among these amides, **7c** and **7d** with propyloxy groups showed the most potent inhibition properties.

The results obtained for assessment of the antioxidant activities of amides **6** and **7** by using $ABTS^{\bullet+}$ and DPPH

Table 2. Inhibition of lipid peroxidation by the newly synthesized amides



6



7

Compound	R	*n*	Yield(%) from **11**	Inhibition of lipid peroxidation (IC_{50}, μM)
6a	$CH(CH_3)(CH_2CH_2CH_3)$		80	1.33
6b	$CH(CH_2CH_3)_2$		84	1.73
6c	$(CH_2)_5CH_3$		95	0.29
7a	CH_3	1	68	1.46
7b	CH_3	2	74	0.69
7c	$(CH_2)_2CH_3$	1	56	0.27
7d	$(CH_2)_2CH_3$	2	58	0.26
7e	$(CH_2)_3CH_3$	1	50	0.42
7f	$(CH_2)_3CH_3$	2	52	0.41
7g	$CH(CH_3)(CH_2CH_2CH_3)$	1	58	0.57
7h	$CH(CH_3)(CH_2CH_2CH_3)$	2	75	0.36
7i	$CH(CH_2CH_3)_2$	1	50	0.82
7j	$CH(CH_2CH_3)_2$	2	52	0.44
7k	$(CH_2)_5CH_3$	1	72	3.52
7l	$(CH_2)_5CH_3$	2	64	2.74
α-Tocopherol				4.68
BHT				4.03

Table 3. ABTS and DPPH radical scavenging activity of the amides **6**, **7**

Compound	$ABTS^{\bullet+}$ (IC_{50}, μM)	DPPH (IC_{50}, μM)
6a	26.3	68.4
6b	49%[a]	84.4
6c	28.5	77.7
7a	11.3	80.3
7b	15.6	69.0
7c	24%[a]	73.0
7d	37%[a]	114.1
7e	57%[a]	176.8
7f	—	—
7g	47%[a]	66.6
7h	64%[a]	85.1
7i	44%[a]	67.7
7j	—	55%[b]
7k	23.2	75%[b]
7l	27.4	75.6

[a] % inhibition at 150 μM.
[b] % inhibition at 300 μM.

assays[22] are given in Table 3. The 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) assay involves the long-lived ABTS cation radical, which is chemically produced by oxidation of the corresponding colorless sulfonic acid with potassium persulfate. The green-blue $ABTS^{\bullet+}$ has excellent spectral characteristics, is stable over a wide range of pH and is applicable to the study of both water-soluble and lipid-soluble antioxidants that convert $ABTS^{\bullet+}$ back to the initial sulfonic acid.[23] The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay measures the ability of antioxidants to donate a hydrogen atom in the conversion of the stable DPPH free radical to 1,1-diphenyl-2-picrylhydrazine.[24] The reaction is accompanied by a change in color from deep-violet to light-yellow and is monitored spectrophotometrically. In these tests, the amides **7a** and **7b** were found to be the most active compounds.

In summary, we have synthesized a series of 3,5-dialkoxy-4-hydroxycinnamamides **6** and **7** bearing 3,4-dimethyphenylpropyl amine and BHT moieties. Studies have shown that the amides exhibit excellent antioxidant activities, especially in their inhibition of lipid peroxidation.

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20. *TBARS:* Brain tissue from male Sprague–Dawley rats (about 10-week old) was obtained after decapitation and homogenated in ice-cold 10 mM Tris–HCl buffer (pH. 7.4) using a Teflon-glass homogenizer. The homogenate was centrifuged for 10 min at 1000*g*, and the supernatant was used in the test. Lipid peroxidation was stimulated in assays containing 250 μL rat brain homogenate by additions of 0.02 mM $FeCl_2$ and 0.25 mM ascorbic acid, and the mixture was incubated for 30 min at 37 °C. The reaction was stopped by the addition of 0.05 ml of 35% perchloric acid. After centrifugation for 10 min at 1000*g*, 200 μL of the resulting supernatant was added to 100 μL of an aqueous solution containing 0.5% TBA and reacted at 80 °C for 1 h. Then, the reaction mixture was cooled to room temperature, and its absorbance at 532 nm was measured. Typical TBARS formation in brain homogenate was calculated from the absorbance at 532 nm using 1,1,3,3-tetraethoxypropane as an external standard.
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23. *DPPH radical scavenging activity:* DPPH radical scavenging activity was determined by using the method of Brand-Williams et al. [25] with minor modifications. The solution of the sample (10 μL) in ethanol was added to 90 μL of a 0.15 mM DPPH radical in ethanol in a 96-well plate. The sample solution refers to the tested compounds and the reference antioxidants at various concentrations, as well as ethanol as a control. The solutions of the tested compounds had concentrations ranging from 3 to 1000 μg/ml, whereas the concentrations of the solutions of the reference compounds varied from 0.1 to 1000 μg/ml. The reaction leading to the scavenging of DPPH radical was complete within 10 min at 25 °C. The absorbance of the mixture was then measured at 517 nm using a microplate reader. The reduction of DPPH radical was expressed as percentage:

$$\text{Scavenged DPPH}(\%) = (1 - A_{\text{test}}/A_{\text{control}}) \times 100,$$

where A_{test} is the absorbance of a sample at a given concentration after 10 min. reaction time, and A_{control} is the absorbance recorded for 10 μL ethanol. The EC_{50} value is defined as the concentration of sample that causes 50% loss of the DPPH radical.
24. *ABTS cation radical scavenging activity:* The ABTS cation radical was produced by the reaction between 7.0 mM ABTS/water and 2.45 mM potassium persulfate for 12 h in the dark at room temperature. The ABTS solution was diluted with PBS until $A_{734} = 0.7$. The reaction was initiated by adding 190 μL of ABTS to 10 μL sample solution at 25 °C. The percentage of reduction of A_{734} was recorded and was plotted as a function of the sample's concentration.
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