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Novel 3,5-diaryl pyrazolines and pyrazole as low-density lipoprotein (LDL) oxidation inhibitor

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Abstract—Compounds **4a**–**j** and **5** were synthesized by cyclocondensation of **3a**–**j** and hydrazine and showed significant LDL-antioxidant activities in the TBARS assay, the lag time of conjugated diene production, the relative electrophoretic mobility (REM) of ox-LDL, the apoB-100 fragmentation, and the macrophage-mediated LDL oxidation. Among compounds **4a**–**j** and **5**, **4a** was found to be the most active compound as an inhibitor of LDL oxidation and **4a** ($IC_{50} = 0.1\,\mu M$) was 6-fold more potent than probucol ($IC_{50} = 0.6\,\mu M$) in the TBARS assay.

Since it has been reported that oxidized LDLs (ox-LDLs) play a key role in the early stages of atherosclerosis,[1] many antioxidants have been developed to exhibit the antiatherogenic activities by inhibiting the foam cell formation in animal model.[2] Among these antioxidants, natural, or synthetic antioxidants (vitamin E, probucol) are well known to lower lipid levels and coronary heart disease incidence.[3] However, both vitamin E and probucol as antioxidants have an untoward effect of lowering serum high-density lipoprotein (HDL)-cholesterol levels. By analysis of their molecular structures, we realized that these two antioxidants have a structural characteristic of hindered phenol as shown in Figure 1. It has been revealed that bulky substituents on phenol ring stabilize the phenoxy radical formed at the *ortho*-positions of the phenolic hydroxyl group.[4] In general, various heterocyclic compounds including pyrazole moiety have been reported to show potent biological activities.[5] Therefore, we introduced pyrazoline and pyrazole moieties between two phenol rings instead of the central disulfide carbon of probucol and bulky substituents at *ortho*-positions of each phenolic ring. This paper deals with the study of biological activity of **4a**–**j** and **5**. The antioxidant activity of LDL was monitored by various tools, such as conjugated diene formation, relative electrophoretic mobility (REM), fragmentation of apoB-100, and macrophage-mediated LDL oxidation.

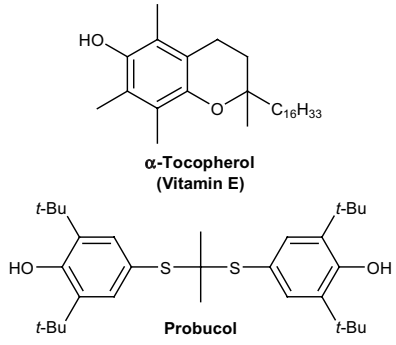

Figure 1. Natural and synthetic antioxidants.

Pyrazolines **4a**–**j** were prepared according to the methods shown in Scheme 1. Treatment of 1-(3,5-di-*tert*-butyl-4-hydroxyphenyl)ethanone (**1**) with 3,5-di- or 2,3,5-tri-substituted 4-hydroxybenzaldehydes **2a**–**j** gave α,β-unsaturated ketones **3a**–**j** by a typical acid-catalyzed aldol condensation in 28–86% yields, as shown in Scheme 1. The reaction of **3a**–**j** and hydrazine monohydrate afforded 1,3-pyrazolines **4a**–**j** via a one-pot addition–cyclocondensation process in 40–96% yields

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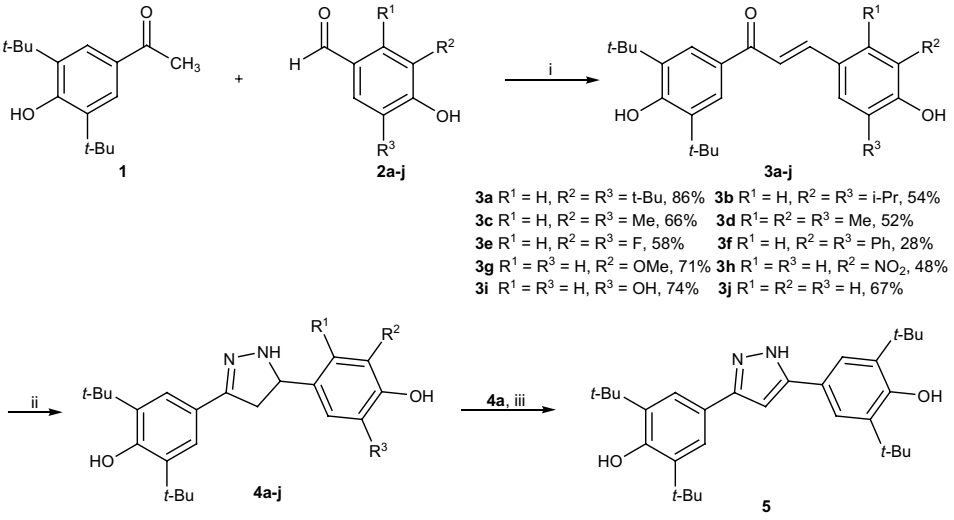
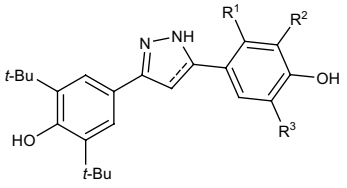


Scheme 1. Reagents and conditions: (i) H_2SO_4, MeOH, reflux; (ii) N_2H_4, EtOH, rt—reflux; (iii) MnO_2, CH_2Cl_2, rt.

(Table 1). The compound **4a** was oxidized by manganese dioxide to give the corresponding pyrazole **5** in good yield.[6] The structures of **4a**–**j** and **5** were determined by their spectroscopic analyses.[7]

Various substituted pyrazolines **4a**–**j** and pyrazole **5** were synthesized and evaluated. All were examined in vitro for their abilities to protect human LDL against Cu^{2+}-induced peroxidation.[8] The prescreening test as preliminary evaluation to select the best candidate is based on a malondialdehyde (MDA) dosage, and the results are expressed as IC_{50} (concentration inhibiting 50% of the Cu^{2+}-induced lipid peroxidation), determined by the thiobarbituric acid reactive substances (TBARS),[9] and expressed as a MDA equivalent. The ability of compounds **4a**–**j** and **5** to attenuate LDL oxidation was measured by measuring the amount of TBARS, as shown in Table 1. Effects of **4a**–**j** and **5** on production of TBARS were examined by incubating human LDL (120 μg/mL) in the presence of 10 μM $CuSO_4$ as an oxidation initiator for 4 h. Compound **4a** proved to be 6-fold more active than probucol (0.6 μM), with IC_{50} values of 0.1 μM. In addition, human plasma (final concn 38.8 mg/mL) was subjected to a radical reaction initiated by 400 μM of Cu^{2+} for 4 h at 37 °C in the presence or absence of **4a** or probucol.[10] The IC_{50} value (0.8 μM) of **4a** was significantly lower than probucol (2.5 μM) in the radical reaction of human plasma. The various substituted compounds, **4c**, **4e**, and **4g**–**j**, were less effective than **4a** but more effective than probucol to be a positive control. The relative efficacy of compounds **4b**, **4d**, and **4f** was low, but with similar LDL-antioxidant activity of probucol. In addition, the compound **4a** was converted to pyrazole **5** by oxidation using MnO_2 with expecting more antioxidant activity. However, the compound **5** having a conjugated system showed a similar activity as compared to the probucol. These results demonstrated that bulky di-*tert*-butyl groups contributed to more potent activity, which agrees with earlier work.[11] Also it has been demonstrated that 2,6-di-*tert*-butylphenol is more reactive toward radical source to afford 2,6-di-*tert*-butylphenoxy radical in inhibition of free radical reaction.[12] Furthermore, the steric and electronic factors of substituents to stabilize phenoxy radical formed from phenolic hydroxy group may influence antioxidant activities for human low-density lipoprotein.[4] Therefore, **4a** was selected for further studies on the development of a potent antioxidant, using the following tools, such as detection of

Table 1. Inhibition of copper-induced lipid peroxidation by pyrazolines **4a**–**j** and pyrazole **5**



Compd	R^1	R^2	R^3	Inhibition of lipid peroxidation	
				Yield (%)[a]	IC_{50} (μM)
4a	H	*t*-Bu	*t*-Bu	50	0.1
4b	H	*i*-Pr	*i*-Pr	50	0.7
4c	H	Me	Me	84	0.2
4d	Me	Me	Me	79	0.6
4e	H	F	F	95	0.2
4f	H	Ph	Ph	40	0.6
4g	H	H	OMe	96	0.2
4h	H	H	NO_2	96	0.3
4i	H	H	OH	65	0.3
4j	H	H	H	96	0.2
5	H	*t*-Bu	*t*-Bu	78[b]	0.7
P[c]	—	—	—	—	0.6

[a] Isolated yield from **3a**–**j**.
[b] Isolated yield from **4a**.
[c] Probucol was used as a reference antioxidant.

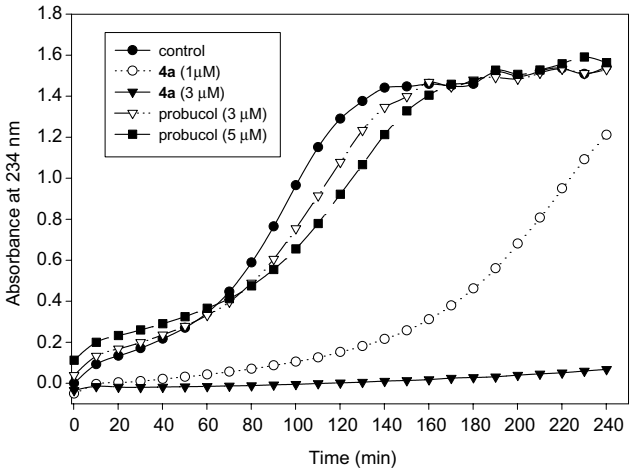


Figure 2. Effect of **4a** on the oxidation of LDL copper ion. LDL (100 μg/mL) in phosphate-buffered saline, at pH 7.4, was incubated with 5 μM $CuSO_4$ at 37 °C in the presence or absence of antioxidant, **4a**. Conjugated diene formation was measured by determining the absorbance at 234 nm every 10 min for 4 h. Probucol was used as reference antioxidant.

conjugated diene formation, REM of ox-LDL, fragmentation of apoB-100, and macrophage-mediated LDL oxidation.

The extent of lag time was interpreted as the antioxidant resistant capacity of LDL.[13] The formation of the conjugated diene during LDL oxidation represents the early peroxidation of the LDL. The LDL (100 μg/mL) was incubated with 5 μM $CuSO_4$ in the absence or presence of **4a**. The oxidation of LDL was determined by measuring conjugated diene formation at 234 nm for 240 min. The results show a typical effect of the compound **4a** (1.0 and 3.0 μM) on the prolonging of lag time, as shown in Figure 2. First, the LDL was incubated with $CuSO_4$ alone to have a lag time of 66 min. The addition of **4a** at 1.0 μM appeared to have a moderate effect on the lag time (163 min) delay, whereas the addition of 3.0 μM of **4a** did not detect the lag time because of too prolong. Whereas, probucol (3.0 and 5.0 μM) extended lag time 74 and 85 min, respectively. Thus, **4a** delayed LDL oxidation to a much greater extent than did probucol.

The effects of various concentration of **4a** on Cu^{2+}-mediated oxidation of LDL were determined by REM assay.[14] The 5 μM $CuSO_4$ incubated with LDL for 12 h was used to induce the oxidation of LDL. When treating with 20 and 10 μM of **4a**, the REM was reduced completely, whereas REM was lowered to 68% and 26% at 5 and 2 μM of **4a**, respectively, compared to that of oxidized LDL. On the other hand, the mobility of LDL in probucol used as a standard was reduced to 100 (20 μM), 84 (10 μM), 37% (5 μM), and 16% (2 μM), respectively, as shown in Figure 3.

The inhibition of the oxidative process of **4a** or probucol, as a positive control, was evaluated also by the study of the fragmentation of the apoB-100 through the electrophoretic analysis on 4% polyacrylamide gel in

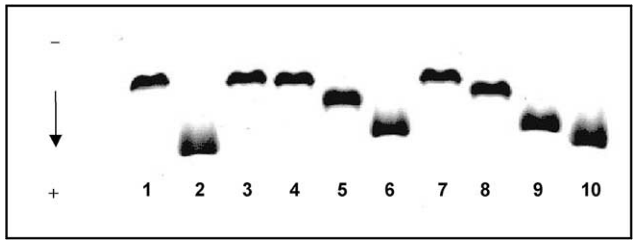


Figure 3. Antioxidant effect of **4a** on the Cu^{2+}-mediated oxidation and electrophoretic mobility of LDL. LDL (100 μg/mL in PBS) was incubated for 12 h at 37 °C with 5 μM $CuSO_4$. After incubation, approximately 3.0 μg of LDL protein was loaded onto 0.7% agarose gel for electrophoresis. The gel was dried and stained with Coomassie brilliant blue R-250. Lane 1: native LDL (absence of $CuSO_4$), Lane 2: ox-LDL, Lane 3: **4a** (20 μM), Lane 4: **4a** (10 μM), Lane 5: **4a** (5 μM), Lane 6: **4a** (2 μM), Lane 7: Probucol (20 μM), Lane 8: Probucol (10 μM), Lane 9: Probucol (5 μM), Lane 10: Probucol (2 μM). Probucol is positive control.

Table 2. Antioxidant effect of **4a** on the Cu^{2+}-mediated oxidation and apoB-100 fragmentation in LDL[a]

Compounds (μM)	Area[b](AU/mm)
Native LDL	18.95
Ox-LDL	0
4a (20)	9.97
4a (10)	9.26
4a (5)	5.63
Probucol (20)	9.22
Probucol (10)	4.73
Probucol (5)	0

[a] LDL (240 μg/mL in PBS) was incubated for 12 h at 37 °C with 5 μM $CuSO_4$ in the absence or presence of 5–20 μM of **4a** or probucol. After incubation, approximately 2.0 μg of LDL protein was applied to SDS-PAGE (4%). After the electrophoresis, the gel was stained with Coomassie Brilliant blue R250 and subjected to densitometric scanning by Bio Rad Model GS-800 with Bio Rad Quantity One-4.4.0 software.

[b] Areas of the peaks of the apoB-100 expressed as absorbance units per millimeter.

the presence of sodium dodecylsulfate (SDS-PAGE), because apoB-100 is a major component of the LDL.[15] As shown in Table 2, the densitometric values related to the areas of the peaks of the apoB-100[16] were expressed as absorbance units per millimeter for the compound **4a** and probucol at dose-dependent concentration ranging from 20 to 5 μM. The band of apoB-100 was observed on the native LDL (100 μg/mL in PBS), which was incubated without $CuSO_4$ for 12 h at 37 °C, but the band completely disappeared when the LDL was incubated with 5 μM $CuSO_4$. In the presence of 20, 10, and 5 μM of **4a**, the percent of remaining apoB-100 against intact apoB-100 of native LDL was 52.6%, 48.9%, and 29.7%, respectively. Whereas, in the presence of 20 and 10 μM of probucol, it was 48.7% and 25.0%, respectively, and the band of apoB-100 did not preserve in the presence of 5 μM probucol. As a result, **4a** was more potent than probucol in the protection of human LDL against copper-mediated oxidation.

Next, we were interested in antioxidant activity of **4a** in macrophage-mediated oxidation of LDL.[17] The cellular

Table 3. Effects of **4a** on macrophage-mediated LDL oxidation

Incubation conditions[a]	MDA nmol/mg LDL protein[b]
LDL+Cu^{2+}	40.15 ± 5.35
LDL+Cell+Cu^{2+} (control)	210.61 ± 10.72
LDL+Cell+Cu^{2+}+2.0 μM **4a**	43.64 ± 1.71*
LDL+Cell+Cu^{2+}+1.0 μM **4a**	74.09 ± 4.49*
LDL+Cell+Cu^{2+}+0.4 μM **4a**	153.79 ± 6.64*
LDL+Cell+Cu^{2+}+2 μM probucol	199.09 ± 10.71*
LDL+Cell+Cu^{2+}+1 μM probucol	209.09 ± 3.85*

*$P < 0.01$ versus control.

[a] LDL (120 μg/mL) was incubated for 24 h at 37 °C in serum-free RPMI 1640 medium with 2 μM of Cu^{2+} in 12-well plate containing macrophages, in the absence (control) or presence of increasing concentrations of the compound **4a** tested (0.4–2.0 μM).

[b] The extent of LDL oxidation was determined directly in the harvested medium using the TBARS assay. Data are shown as means ± SD ($n = 3$).

oxidative modification of LDL to a form recognized by the scavenger receptor requires the presence of transition metal ions in the medium.[18] And so, the LDL (120 μg/mL) was incubated for 24 h in serum-free RPMI 1640 medium with 2 μM $CuSO_4$ at 37 °C in a 12-well plate containing macrophages, in the presence or absence of **4a** or probucol. In macrophage-mediated LDL oxidation, the TBARS formation in the harvested medium was also inhibited by **4a** at a similar order of activity to that obtained in Cu^{2+}-induced LDL oxidation. The LDL oxidation (210.61 ± 10.72 MDA nmol/mg LDL protein) measured by TBARS production was five-fold higher in the presence of THP-1 macrophages compared with incubations in the absence of cells (40.15 ± 5.35 MDA nmol/mg LDL protein) (Table 3). This result coincides with the previous reports.[19] Therefore, antioxidant activities of **4a** and probucol were tested by macrophage-mediated LDL oxidation at dose-dependent concentration ranging from 2 to 0.4 μM. In the concentration of 2, 1, and 0.4 μM of **4a**, the content of ox-LDL was 43.64 ± 1.71, 74.09 ± 4.49, and 153.79 ± 6.64 MDA nmol/mg LDL protein, respectively. At the concentration of probucol (2 and 1 μM), the content of ox-LDL was 199.09 ± 10.71 and 209.09 ± 3.85 MDA nmol/mg LDL protein, respectively. As a result, antioxidant activity of **4a** was about five-fold higher than that of probucol in macrophage-mediated LDL oxidation (Table 3).

In this study, we demonstrated that 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)-5-(multi-substituted-4-hydroxyphenyl)-2-pyrazolines **4a**–**j** and pyrazole **5** were synthesized and evaluated for antioxidant activity. Among compounds **4a**–**j** and **5**, **4a** was found to be the most active compound as an inhibitor of LDL oxidation. These findings need further study to clarify the mechanism of antioxidant action of the pyrazolines and pyrazoles in LDL system.

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