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2,3-Dimethoxy-5-methyl-1,4-benzoquinones and 2-methyl-1,4-naphthoquinones: glycation inhibitors with lipid peroxidation activity

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Abstract—Anti-glycation activity of our anti-oxidant quinone library was measured and several 2,3-dimethoxy-5-methyl-1,4-benzoquinones and 2-methyl-1,4-naphthoquinones were identified as novel inhibitors of glycation, of which 2,3-dimethoxy-5-methyl-1,4-benzoquinones **13b** is the most potent glycation inhibitor with around 50 μM of the IC_{50} value.

1. Introduction

Nonenzymatic protein glycation initiated by the interaction between amino groups of protein and reducing sugars, such as glucose leads to chemical modification of proteins. The resulting reaction products; advanced glycation end products (AGEs) have been implicated in health complications associated with the normal aging process and the development of diabetes related complications.[1] It was already proposed that the design and discovery of inhibitors of the glycation cascade should offer a promising therapeutic approach for the prevention of diabetic or other pathogenic complications.[2] For examples, administration of amino compounds, such as alanine, can react with glucose and counteract the potentially deleterious consequences of hyperglycemia in diabetes.[3] Hydrazine reagent is introduced to trap the reactive carbonyls formed during the Maillard reaction, especially Amadori intermediates, thus impeding their conversion to AGEs.[4] As a similar approach, aminoguanidine is well known as an inhibitor of AGEs.[4,5]

It is also known that glycation generates reactive oxygen species, free radicals, reactive α-dicarbonyl species, and protein cross-links, causing age-, diabetes-, and smoking-related complications.[6] Several reports indicate the production of free radicals and highly reactive oxidants from glycated proteins under physiological conditions.[7] Free radicals are known to stimulate AGE production by autoxidation of sugars.[8] Oxidative stress has been linked to diabetic complications and diabetic atherogenesis.[9] α-Tocoperol is a potent anti-oxidant and is an anti-glycating substance in vitro.[10] More recently the combination of vitamins C and E more efficiently inhibited glycation and AGE than the single vitamins, and this is an evidence for the inhibition of glycation by anti-oxidants.[11]

In the continuing our efforts to search the possibility of anti-oxidants as therapeutic agents, we are interested in the development of anti-oxidants that have potent anti-glycation activity. In the screening of our anti-oxidant compound library to anti-glycation assay system, several 2,3-dimethoxy-5-methyl-1,4-benzoquinones and 2-methyl-1,4-naphthoquinones provided inhibitory activity of glycation even though some other phenol anti-oxidant compounds showed low anti-glycation activity.

The present paper deals with the synthesis and the biological evaluation of a series of 2,3-dimethoxy-5-methyl-1,4-benzoquinones substituted at the C-6 position with various groups and 2-methyl-1,4-naphthoquinones substituted at the C-3 position with various halogen groups.

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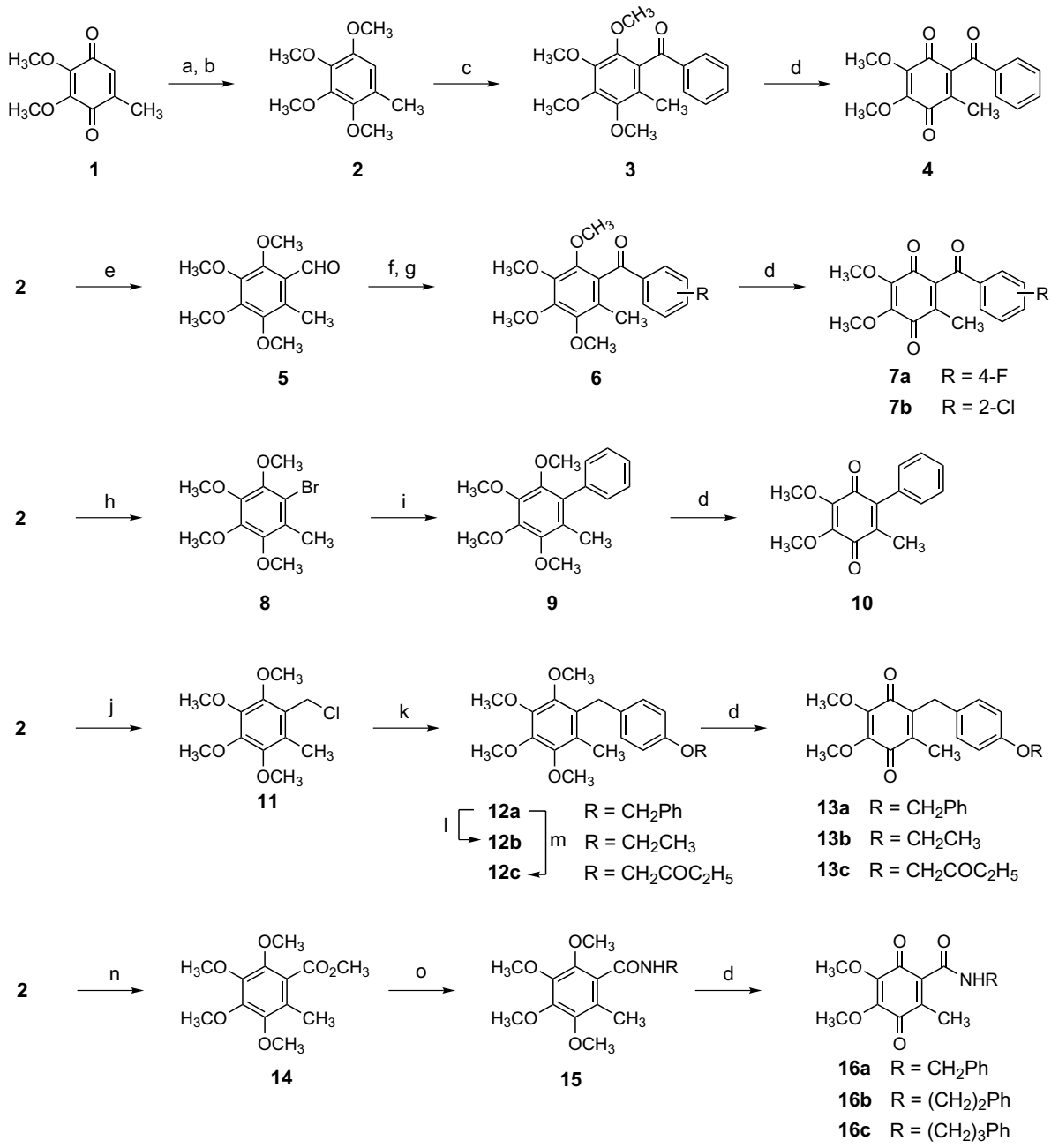
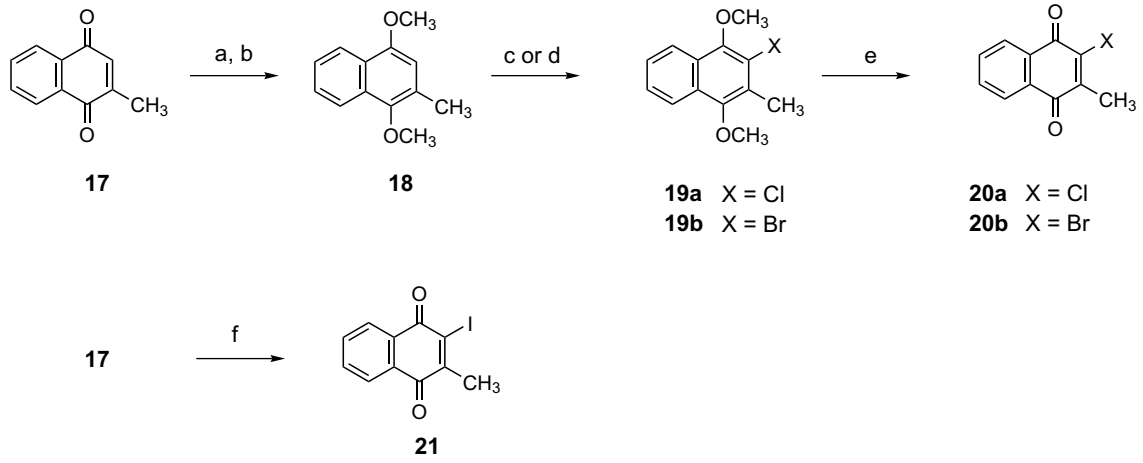
2. Chemistry

For the preparation of tetramethoxytoluene **2** as our initial starting material, the reduction of benzoquinone **1** was carried out under the condition of either the catalytic hydrogenation under high pressure (30–45 psi) or the reduction with $Na_2S_2O_4$, and then methylation was conducted with dimethylsulfide (Scheme 1). Various substituents were introduced to give fully substituted toluenes such as the substitution with acyl-, phenyl-, aralkyl-, or carbamide-group. For examples, acylation with benzoyl chloride provided benzophenone **3**, however acylations with halogen substituted benzoyl chlorides were not succeeded. We have overcome this problem by three consequent steps; formylation, addition of Grignard reagents, and oxidations to give **6**. Biaryl compound **9** was prepared by bromination of **2** followed by Suzuki coupling with phenyl boronic acid. Chloromethlyation of **2** followed by treatment with aryl lithium reagent provided **12a**, and the debenzylation under reduction condition and alkylations with alkyl halides gave **12b** and **12c**. Amide functional groups were formed from substitution of ester with amines to give **15**. Finally the conversion of the fully substituted tolu-



Scheme 1. Reagents: (a) H_2, Pd/C, MeOH; (b) $(CH_3O)_2SO_2$, NaOH/MeOH; (c) ArCOCl, $AlCl_3$, CH_2Cl_2; (d) CAN/H_2O–MeCN; (e) Cl_2CHOCH_3, $TiCl_4$, CH_2Cl_2; (f) ArMgBr, THF; (g) PCC, CH_2Cl_2; (h) NBS/CH_2Cl_2; (i) $PhB(OH)_2$, $Pd(PPh_3)_4$, Na_2CO_3, C_6H_6; (j) HCHO, HCl; (k) $LiC_6H_4OCH_2Ph$, THF; (l) H_2, Pd/C then $BrCH_2CH_3$, K_2CO_3; (m) H_2, Pd/C then $BrCH_2COCH_3$, K_2CO_3; (n) *n*-BuLi/THF then $ClCO_2CH_3$; (o) RNH_2, DCC, HOBT, CH_3CN.



Scheme 2. Reagents: (a) H_2, Pd/C, MeOH; (b) $(CH_3O)_2SO_2$, NaOH/MeOH; (c) $C_6H_5I(OAc)_2$, TBSCl, CH_2Cl_2 for **19a**; (d) NBS/CH_2Cl_2 for **19b**; (e) CAN/H_2O–MeCN; (f) $TMSN_3$, I_2/pyridine.

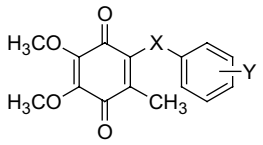
enes (**3**, **6**, **9**, **12**, **15**) was conducted with ceric ammonium nitrate (CAN) oxidation to give the derivatives of 2,3-dimethoxy-5-methyl-1,4-benzoquinone (**4**, **7**, **10**, **13**, **16**).

The naphthoquinones (**20**, **21**) are synthesized via known methods from 2-methyl-1,4-naphthoquinone **17** as shown in Scheme 2. Our initial material **18** which prepared from 2-methyl-1,4-naphthoquinone **1 7** was halogenated to give chlorine- and bromine-containing compounds **19a** and **19b**. Compound **19a** was obtained in high yield from **18** with phenyliododiacetate and trimethylsilyl chloride in CH_2Cl_2. Bromination of **18** with NBS in CH_2Cl_2 at rt for 0.5 h afforded **19b** in 95% yield. Following ceric ammonium nitrate (CAN) oxidation of **19** should proceed to naphthoquinone **20**. Iodine substituted 2-methyl-1,4-naphthoquinone **21** was prepared from treatment of **17** with trimethylsilyl azide and a mixture of iodine and pyridine in CH_2Cl_2 followed by CAN oxidation.[12]

3. Results and discussion

The evaluation of anti-oxidant property of 2,3-dimethoxy-5-methyl-1,4-benzoquinones and 2-methyl-1,4-naphthoquinones was examined by the effects on lipid peroxidation in rat brain homogenate by thiobarbituric acid reactive substances (TBARS) assay[13] according to the method of Stocks et al.,[14] as modified by Barrier et al.[15] The IC_{50} values for 2,3-dimethoxy-5-methyl-1,4-benzoquinones are shown in Table 1. The substituents of benzoquinones at the C-6 position are benzoyl (**4**, **7**), phenyl (**10**), benzyl (**13**), and *N*-aralkylamide (**16**), and the inhibitory activity was strongly influenced by the substituents. The inhibitory activity of lipid peroxidation was highest when the substituent is benzoyl (**4**, **7**) in this series of compounds, and the order is benzoyl > *N*-benzyl amido > phenyl > benzyl.[16] Therefore more electron withdrawing substituents increase the inhibition activity of lipid peroxidation. The mechanism of anti-oxidant activity of the quinone has been proposed to be by formation of reduced phenols, which can scavenge free radicals.[17] For 2-methyl-1,4-naphthoquinone compounds, the substituents at the C-3 position enhanced the inhibition of lipid peroxidation compared with the unsubstituted quinone **17** (Table 2). The potency is 10 times higher than that of the unsubstituted quinone **17** regardless of the kind of halogen atom. The naphthoquinones (**20**, **21**) are also more potent than α-tocopherol.

Table 1. Inhibition of lipid peroxidation by 2,3-dimethoxy-5-methyl-1,4-benzoquinones

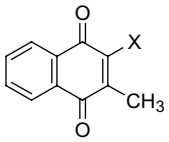


Compound	X	Y	Inhibition of lipid peroxidation (IC_{50}, μM)
4	C=O	H	11.84
7a	C=O	4-F	8.36
7b	C=O	2-F	15.30
10	—	H	39.43
13a	CH_2	4-OBn	64.17
13b	CH_2	4-OEt	57.70
13c	CH_2	4-OCH_2COEt	102.00
16a	$CONHCH_2$	H	31.91
16b	$CONH(CH_2)_2$	H	29.21
16c	$CONH(CH_2)_3$	H	16.25
α-Tocopherol			4.68

NA: no activity was observed in our test concentrations.

We then examined their anti-glycation activity. As the glycation reactions progressively develop Maillard fluorescence (λ_{ex} = 370 nm and λ_{em} = 440 nm) that is attributed to the AGE formation of heterocyclic aromatic structures, the anti-glycation activity was measured as the inhibition of the Maillard fluorescence formation. The anti-glycation activity for selected compounds are shown in Table 3.[18]

Table 2. Inhibition of lipid peroxidation by 2-methyl-1,4-naphthoquinones



Compound	X	Inhibition of lipid peroxidation (IC_{50}, μM)
17	H	12.24
20a	Cl	1.41
20b	Br	1.10
21	I	1.23
α-Tocopherol		4.68

Table 3. Anti-glycation activities of 2,3-dimethoxy-5-methyl-1,4-benzoquinones and 2-methyl-1,4-naphthoquinones

Compound	Inhibition of glycation (IC_{50}, μM)	Inhibition of BSA glycation (IC_{50}, μM)
4	120	269
10	85	218
13a	83	120
13b	36	74
20a	115	199
20b	98	144
21	93	125
α-Tocopherol	NA	NA
Trolox methyl ester	468	NA

NA: no activity was observed in our test concentrations (3.80 nM to 1.00 mM).

2,3-Dimethoxy-5-methyl-1,4-benzoquinones and 2-methyl-1,4-naphthoquinones decrease the formation of fluorescent AGEs significantly at micromolar concentration ranges. Phenyl group substituted benzoquinone **10** and benzyl group substituted benzoquinones (**13a**, **13b**) show strong activity. Therefore electron donating substituents may contribute to the anti-glycation activity. For the naphthoquinone compounds, all halogen atom substituted compounds at C-3 position (**20a**, **20b**, **21**) show anti-glycation activity. It is interesting that **13b**, which is rather weak anti-oxidant in this series, is the most potent anti-glycation agent in this series, whereas the reference anti-oxidants α-tocopherol and trolox methyl ester showed no and weak anti-glycation activity, respectively. Glycation consists of complex network reactions of which many are not well understood. Also, fluorescent AGEs are formed through multiple reaction pathways such that blocking multiple reaction steps may be more efficient rather than blocking a particular reaction. The mechanism of anti-glycation activity of the quinone is likely related to that of anti-oxidant activity; however, other mechanisms should also be considered as the correlation between the anti-oxidant potency and the anti-glycation potency is rather poor.

In summary, from the anti-glycation screening of our anti-oxidant compound library, we found several anti-oxidants 2,3-dimethoxy-5-methyl-1,4-benzoquinones and 2-methyl-1,4-naphthoquinones as novel inhibitors of glycation.

Acknowledgements

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13. Lipid peroxidation assays: To assess lipid peroxidation, thiobarbituric acid reactive substances (TBARS) assay was adopted in the present study. TBARS were assayed according to the method of Stocks et al. (1974), as modified by Barrier et al. (1998). In brief, a mixture containing 250 μL brain homogenate (5 mg protein/mL), 10 μL test compound and 20 μL assay buffer was pre-incubated at 37 °C for 20 min with shaking. Lipid peroxidation was stimulated 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. The IC_{50} value is determined as the concentration of malondialdehyde (MDA) to give 50% inhibition.

$$\text{Inhibition percentage } (\%) = [(A - B)/A] \times 100$$

A: nmol of MDA/mg protein (control); B: nmol of MDA/mg protein (test compound).

14. Stocks, J.; Gutteridge, J. M. C.; Sharp, R. J.; Dormandy, T. L. *Clin. Sci. Mol. Med.* **1974**, *47*, 215–222.
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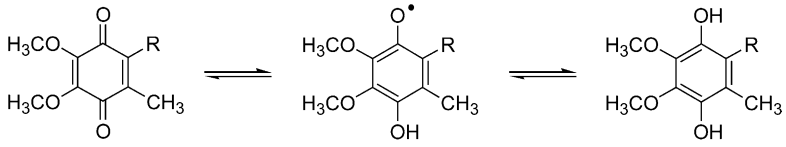
16. This order can be explained that the quinones substituted with more electron negative group (C=O) have higher reduction potential ($E_{1/2}(-)$) and they can be transferred to the hydroquinones more easily. The hydroquinones might be actual molecules, which inhibit the lipid peroxidation. Thermodynamic feasibility of electron transfer can be predicted by consideration of the redox potentials ($E_{1/2}(+)$, $E_{1/2}(-)$) of the donor and acceptor components as shown in Eq. 1.



Quinone Semequinone Hydroquinone

$$\Delta G_{SET} = E_{1/2}(+) - E_{1/2}(-) \quad (1)$$

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18. Glycation assay: The stock solutions of bovine serum albumin (BSA, 67 kDa) and D-ribose were prepared separately into PBS (Phosphate-buffered saline). All hydroxyquinone compounds were initially dissolved in DMSO and diluted by PBS to prepare the appropriate concentrations. Then, stock solutions of BSA, D-ribose and hydroxyquinone compounds were mixed in a 96-well plate and incubated for 5 days at 37 °C under mild shaking, in the dark. The final concentrations of BSA and D-ribose were 0.075 mM and 50 mM, respectively. The range of hydroxyquinone concentrations were 3.80 nM to 1.00 mM. After incubation, the Maillard fluorescence (λ_{ex} = 370 nm and λ_{em} = 440 nm) of each well was measured to determine the IC_{50} values of inhibition of glycation in Table 3 as described below. Then, aliquots of the sample solutions were applied to a size exclusion chromatographic column (Superrose 12PC 3.2/30, 100 mM phosphate buffer pH 7.4) to separate the proteins and the small molecules and to measure the Maillard fluorescence (λ_{ex} = 370 nm and λ_{em} = 440 nm) of the glycated BSA. The inhibitory activity of the hydroxyquinone analogs was calculated using the equation:

$$\text{Inhibition } (\%) = \{1 - (F - F_0)/(F_{100} - F_0)\} \times 100$$

where F_0 is the fluorescence of incubated BSA alone, F_{100} is the fluorescence of incubated BSA and D-ribose, F is the fluorescence of incubated BSA, D-ribose and hydroxyquinone analog. The inhibition of glycation and inhibition of BSA glycation were determined by using the fluorescence of the reaction solution and of the glycated BSA after the size exclusion chromatography, respectively. The inhibition (%) is plotted against logarithm of the concentration of hydroxyquinone analog. Sigmoidal curve fitting is applied to the data by using software 'Origin 6.0', and the IC_{50} value is determined as the concentration of hydroxyquinone analog to give 50% inhibition.