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Glutathione Conjugates of 2- or 6-Substituted 5,8-Dimethoxy-1,4-Naphthoquinone Derivatives: Formation and Structure

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Thirty-four glutathione conjugates of 5,8-dimethoxy-1,4-naphthoquinones (DMNQ) were synthesized and their structure was determined. The yield of GSH conjugate was dependent on size of alkyl group; the longer the size of alkyl group was, the lower was the yield. It was also found that the length of alkyl side chain influenced the chemical shift of quinonoid protons; the quinonoid protons of 2-glutathionyl DMNQ derivatives with R=H to propyl, 6.51-6.59 ppm vs. other ones with R=butyl to heptyl, 6.64-6.68 ppm. This was explained to be due to a folding effect of longer alkyl group. Glutathione (GSH) reacted with DMNQ derivative first to form a 1,4-adduct (2- or 3-glutathionyl-1,4-dihydroxy-5,8-dimethoxynaphthalenes) and then, the adduct was autooxidized to 2- or 3-glutathionyl-DMNQ derivatives. Moreover, GSH reduced DMNQ derivatives to their hydrogenated products. It was suggested that such an organic reaction might play an important role for a study of metabolism or toxicity of DMNQ derivatives in the living cells.

Key words : 5,8-Dimethoxy-1,4-naphthoquinone derivatives, Glutathione conjugates

INTRODUCTION

Glutathione (GSH) is an essential substance participating in phase II of drug metabolism. It is well known that GSH attacks a electrophilic site of drug structure or its metabolic intermediate. The quinones are good electrophiles which should be attacked by GSH in the liver, resulting in the formation of various reaction products. The reaction produces GSH-quinone con-jugates as main products and others such as the GSH-hydroquinone adducts, hydroquinones and polyglutathionylated quinones. The GSH-hydroquinones and hydro-quinones so produced are readily oxidized to release toxic oxygen radicals, which are mostly harmful to the cell. Both the electrophilicity and the formation of the harmful oxy-gen radicals have been interpreted as cause of cytotoxicity of quinones (Belisario et *al.*, 1994; Gant et *al.*, 1988)

In previous report (Baik and Song et *al.*, 1997), it was found that 2- or 6-(1-hydroxyalkyl)-5,8-dimethoxy-1,4-naphthoquinone derivatives showed a good antitumor activity. Recently, we have discovered that enhancement of the quinone electrophilicity led to the increase of antitumor activity, while steric hindrance of 2-substitutent lowered the activity. (You and Zheng et *al.*, 1998), where it was confirmed that formation rate of glutathione-naphthoquinone conjugates was good correlated with the reactivity and cytotoxic activity of the naphthoquinone derivatives. However, the details of synthetic pathways and the structure of the conjugates remained to be further discussed.

Thus, present study is targeting to provide an information on structure of the GSH-naphthoquinone conjugates and trace the proceeding of the reaction, which should be useful for metabolic study of naphthoquinone compounds and drug design thereof.

MATERIALS AND METHODS

Chemicals and instruments

All chemicals used were of reagent grade and commercially available. Sephadex LH-20 (Pharmacia, Sweden). TLC; RP-18 plate (Merck). NMR spectrometer; Bruker AC80 DRX-300. IR-spectrometer; Perkin-Elmer 780. Mass spectrometer; VG7070, VG Analyticals, UK.

General procedure for synthesis of the glutathione conjugates

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5,8-Dimethoxy-1,4-naphthoquinone derivative (0.5 mmol) was dissolved in methanol (3 ml), and to this solution was added 0.3 mmol of glutathione dissolved in 10 ml of potassium phosphate buffer (pH 7.4). After stirring for 10 h at 5°C, methanol was evaporated from the reaction mixture and then the remaining solution was extracted three times with ethyl acetate (5 ml). Evaporation of ethyl acetate extract left a dark brown mass, which was dissolved in 1 ml water and chromatographied on a Sephadex LH-20 column (1.5×35 cm), which was eluted with a mixture of methanol:water (1:1 in volume) as a mobile phase to separate main reaction product. The separation of the reaction products was pursued on a reverse phase silica gel plate (RP-18, F_{254},), developed in solvent system of water/methanol.

2-Glutathionyl-1,4-naphthoquinone (1): Yellowish brown mass. Yield: 78.1%, TLC (MeOH:H_2O=3:1): Rf=0.58; ^{1}H-NMR (D_2O, 300 MHz) δ 7.85 (s, 2H, H-5,8), 7.68 (s, 2H, H-6,7), 6.56 (s, 1H, H-3), 4.52 (Cystα-1H), 3.84 (Glyα-2H), 3.50 (Gluα-1H), 3.34-3.12, (Cystβ-2H), 2.32 (Gluγ-2H), 1.93 (Gluβ-2H).

2-Glutathionyl-5,8-dimethoxy-1,4-naphthoquinone (2): dark brown mass. Yield: 56.7%, TLC (MeOH:H_2O=2:1): Rf=0.72, m.p=189-191°C; ^{1}H-NMR (D_2O, 300 MHz) δ 7.20 (s, 1H, H-6), 7.18 (s, 1H, H-7), 6.23 (s, 1H, H-3), 3.97 (s, 6H, 2OCH_3), 4.76 (Cystα-1H), 4.08 (Glyα-2H), 3.92 (Gluα-1H), 3.35-3.12 (Cystβ-2H), 2.65 (Gluγ-2H), 2.25 (Gluβ-2H).

3-Glutathionyl-2-(1-hydroxymethyl)-5,8-dimethoxy-1,4-naphthoquinone (3): dark brown. Yield: 31.9%, TLC (MeOH:H_2O=2:1): Rf=0.76, m.p=192-194°C; ^{1}H-NMR (D_2O, 300 MHz) δ 7.39 (s, 2H, H-6,7), 4.76 (s, dd, 2H, H-1'), 4.63 (Cystα-1H), 3.88 (s, 6H), 3.85-3.64 (Glyα-2H, Gluα-1H), 3.60-3.42 (Cystβ-2H), 2.51-2.32 (Gluγ-2H), 2.16-2.01 (Gluβ-2H).

3-Glutathionyl-2-(1-hydroxyethyl)-5,8-dimethoxy-1,4-naphthoquinone (4): Yield: 43.2%, TLC (MeOH:H_2O= 2:1): Rf=0.72; ^{1}H-NMR (D_2O, 300 MHz) δ 7.54 (s, 2H, H-6,7), 5.33 (m, 1H, H-1'), 4.68 (Cystα-1H), 3.93 (s, 6H, 2OCH_3), 3.91-3.73 (Glyα-2H, Gluα-1H), 3.65-3.22 (Cystβ-2H), 2.65-2.40 (Gluγ-2H), 2.26-2.05 (Gluβ-2H), 1.55 (s, 3H, H-2).

3-Glutathionyl-2-(1-hydroxypropyl)-5,8-dimethoxy-1,4-naphthoquinone (5): Yield: 35.5%, TLC (MeOH:H_2O= 2:1): Rf=0.64; ^{1}H-NMR (D_2O, 300 MHz) δ 7.53 (s, 2H, H-6,7), 5.28 (m, 1H, H-1'), 4.67 (Cystα-1H), 3.93 (s, 6H, 2OCH_3), 3.88-3.75 (Glyα-2H, Gluα-1H), 3.63-3.20 (Cystβ-2H), 2.64-2.40 (Gluγ-2H), 2.25-2.03 (Gluβ-2H), 1.45-1.26 (t, 2H, H-2), 0.98 (t, J=6.0Hz, 3H, H-3).

3-Glutathionyl-2-(1-hydroxybutyl)-5,8-dimethoxy-1,4-naphthoquinone (6): Yield: 29.8%, TLC (MeOH:H_2O= 2:1): Rf=0.63; ^{1}H-NMR (D_2O, 300 MHz) δ 7.52 (s, 2H, H-6,7), 5.24 (m, 1H, H-1'), 4.68 (Cystα-1H), 3.94 (s, 6H, 2OCH_3), 3.86-3..63 (Glyα-2H, Glu-1H), 3.56-3.23 (Cystβ-2H), 2.62-2.39 (Gluγ-2H), 2.23-2.04 (Gluβ-2H), 1.48-1.22 (t, 4H, H-2,3), 0.95 (t, *J*=6.0Hz, 3H, H-4).

3-Glutathionyl-2-(1-hydroxypentyl)-5,8-dimethoxy-1,4-naphthoquinone (7): Yield: 28.1%, TLC (MeOH:H_2O =2:1): Rf=0.56; ^{1}H-NMR (D_2O, 300 MHz) δ 7.50 (s, 2H, H-6,7), 5.17 (m, 1H, H-1'), 4.68 (Cystα-1H), 3.93 (s, 6H, 2OCH_3), 3.85-3.50 (Glyα-2H, Glu-1H), 3.52-3.20 (Cystβ-2H), 2.61-2.41 (Gluγ-2H), 2.22-2.05 (Gluβ-2H), 1.50-1.20 (t, 6H, H-2,3,4), 0.93 (t, *J*=6.0 Hz, 3H, H-5).

3-Glutathionyl-2-(1-hydroxyhexyl)-5,8-dimethoxy-1,4-naphthoquinone (8): Yield: 21.7%, TLC (MeOH:H_2O =2:1): Rf=0.54; ^{1}H-NMR (D_2O, 300 MHz) δ 7.51 (s, 2H, H-6,7), 5.14 (m, 1H, H-1'), 4.69 (Cystα-1H), 3.95 (s, 6H, 2OCH_3), 3.84-3.76 (Glyα-2H, Gluα-1H), 3.51-3.19 (Cystβ-2H), 2.61-2.40 (Gluγ-2H), 2.20-2.01 (Gluβ-2H), 1.55-1.26 (t, 8H, H-2,3,4,5), 0.88 (t, *J*=6.0 Hz, 3H, H-6).

3-Glutathionyl-2-(1-hydroxyheptyl)-5,8-dimethoxy-1,4-naphthoquinone (9): Yield: 23.7%, TLC (MeOH:H_2O= 2:1): Rf=0.52; ^{1}H-NMR (D_2O, 300 MHz) δ 7.48 (s, 2H, H-6,7), 5.15 (m, 1H, H-1'), 4.68 (Cystα-1H), 3.93 (s, 6H, 2OCH_3), 3.82-3.60 (Glyα-2H, Gluα-1H), 3.50-3.18 (Cystβ-2H), 2.62-2.41 (Gluγ-2H), 2.21-2.03 (Gluβ-2H), 1.65(m, 2H, H-2), 1.55-1.10 (t, 8H, H-3,4,5,6), 0.86 (t, *J*=6.0 Hz, 3H, H-7).

3-Glutathionyl-2-(1-hydroxyoctyl)-5,8-dimethoxy-1,4-naphthoquinone (10): Yield: 22.1%, TLC (MeOH:H_2O= 2:1): Rf=0.52; ^{1}H-NMR (D_2O, 300 MHz) δ 7.48 (s, 2H, H-6,7), 5.13 (m, 1H, H-1'), 4.66 (Cystα-1H), 3.94 (s, 6H, 2OCH_3), 3.83-3.60 (Glyα-2H, Gluα-1H), 3.48-3.17 (Cystβ-2H), 2.63-2.43 (Gluγ-2H), 2.20-2.05 (Gluβ-2H), 1.63 (m, 2H, H-2), 1.55-1.10(t, 10H, H-3,4,5,6,7), 0.85 (t, *J*=6.0 Hz, 3H, H-8).

3-Glutathionyl-2-(1-hydroxy-4-isopentyl)-5,8-dimethoxy-1,4-naphthoquinone (11): Yield: 31.7%, TLC (MeOH: H_2O=2:1): Rf=0.48; ^{1}H-NMR (D_2O, 300 MHz) δ 7.36 (s, 2H, H-6,7), 5.07 (m, 1H, H-1'), 4.67 (Cystα-1H), 3.89 (s, 6H, 2OCH_3), 3.86-3.65 (Glyα-2H, Gluα-1H), 3.56-3.34 (Cystβ-2H), 2.65-2.45 (Gluγ-2H), 2.23-2.08 (Gluβ-2H), 1.70-1.15 (t, 5H, H-2,3,4), 0.82 (d, *J*=6.1 Hz, 6H, H-5,6).

2-Glutathionyl-6-(1-hydroxymethyl)-5,8-dimethoxy-1,4-naphthoquinone (12): Yield: 53.4%, TLC (MeOH:H_2O= 2:1): Rf=0.72, ^{1}H-NMR (D_2O, 300 MHz) δ 7.56 (s, 1H, H-7), 6.58 (s, 1H, H-3), 4.79 (dd, *J*=6.1 Hz, *J*=1.6 Hz, 2H, H-1), 3.95 (s, 3H), 3.75 (s, 3H), 4.72 (Cystα-1H), 3.98 (Glyα-2H), 3.81 (Gluα-1H), 3.62-3.24 (Cystβ-2H), 2.55 (Gluγ-2H), 2.16 (Gluβ-2H).

2-Glutathionyl-6-(1-hydroxyethyl)-5,8-dimethoxy-1,4-naphthoquinone (13): Yield: 62.4%, TLC (MeOH: H_2O= 2:1): Rf=0.70, ^{1}H-NMR (D_2O, 300 MHz) δ 7.57 (s, 1H, H-7), 6.51 (s, 1H, H-3), 5.27 (m, 1H, H-1'), 3.93 (s, 3H, OCH_3), 3.74 (s, 3H, OCH_3), 1.48 (m, 3H, H-2), 4.89 (Cystα-1H), 4.02 (Glyα-2H), 3.85 (Gluα-1H), 3.34-3.17 (Cystβ-2H), 2.59 (Gluγ-2H), 2.21 (Gluβ-2H).

2-Glutathionyl-6-(1-hydroxypropyl)-5,8-dimethoxy-1,4-naphthoquinone (14): Yield: 43.2%, TLC (MeOH:H_2O=

2:1): Rf=0.67; ^{1}H-NMR (D_2O, 300 MHz) δ 7.59 (s, 1H, H-7), 6.56 (s, 1H, H-3), 4.80 (m, 1H, H-1'), 3.90 (s, 3H, OCH_3), 3.72 (s, 3H, OCH_3), 1.83 (t, 2H, H-2), 0.76 (m, 3H, H-3), 4.55 (Cysα-1H), 3.79 (Glyα-2H), 3.63 (Gluα-1H), 3.12 (Cysβ-2H), 2.36 (Gluγ-2H), 1.98 (Gluβ-2H).

2-Glutathionyl-6-(1-hydroxybutyl)-5,8-dimethoxy-1,4-naphthoquinone (15): Yield: 36.4%, TLC (MeOH:H_2O= 2:1): Rf=0.64; ^{1}H-NMR (D_2O, 300 MHz) δ 7.60 (s, 1H, H-7), 6.59 (s, 1H, H-3), 4.92 (m, 1H, H-1'), 3.94 (s, 3H, OCH_3), 3.76 (s, 3H, OCH_3), 1.80-1.76 (m, 2H, H-2), 1.51-1.18 (m, 2H, H-3), 0.82 (t, *J*=6.0 Hz, 3H, H-4), 4.76 (Cysα-1H), 3.86 (Glyα-2H), 3.74 (Gluα-1H), 3.31-3.27 (Cysβ-2H), 2.40 (Gluγ-2H), 2.13 (Gluβ-2H).

2-Glutathionyl-6-(1-hydroxypentyl)-5,8-dimethoxy-1,4-naphthoquinone (16): Yield: 45.8%, TLC (MeOH:H_2O= 2:1): Rf=0.60; ^{1}H-NMR (D_2O, 300 MHz) δ 7.63 (s, 1H, H-7), 6.68 (s, 1H, H-3), 5.18 (m, 1H, H-1'), 4.02 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 1.78 (m, 2H, H-2), 1.45-1.40 (m, 4H, H-3,4), 0.95 (t, *J*=6.0 Hz, 3H, H-5), 4.83 (Cysα-1H), 4.00 (Glyα-2H), 3.86 (Gluα-1H), 3.29 (Cysβ-2H), 2.61 (Gluγ-2H), 2.23 (Gluβ-2H).

2-Glutathionyl-6-(1-hydroxyhexyl)-5,8-dimethoxy-1,4-naphthoquinone (17): Yield: 36.4%, TLC (MeOH:H_2O= 2:1): Rf=0.54; ^{1}H-NMR (D_2O, 300 MHz) δ 7.65 (s, 1H, H-7), 6.67 (s, 1H, H-3), 5.20 (m, 1H, H-1'), 4.04 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3), 1.80 (m, 2H, H-2), 1.65 (m, 2H, H-3), 1.50-1.44 (m, 4H, H-4,5), 0.98 (t, *J*=6.0 Hz, 3H, H-6), 4.85 (Cysα-1H), 3.98 (Glyα-2H), 3.82 (Gluα-1H), 3.32-3.27 (Cysβ-2H), 2.54 (Gluγ-2H), 2.20 (Gluβ-2H).

2-Glutathionyl-6-(1-hydroxyheptyl)-5,8-dimethoxy-1,4-naphthoquinone (18): Yield: 30.5%, TLC (MeOH:H_2O= 3:1): Rf=0.46; ^{1}H-NMR (D_2O, 300 MHz) δ 7.65 (s, 1H, H-7), 6.65 (s, 1H, H-3), 5.21 (m, 1H, H-1'), 4.05 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 1.86 (m, 2H, H-2), 1.68-1.58 (m, 4H, H-3,4), 1.52-1.45 (m, 4H, H-5,6), 0.99 (t, *J*=6.0 Hz, 3H, H-7), 4.87 (Cysα-1H), 3.99 (Glyα-2H), 3.85 (Gluα-1H), 3.35-3.31 (Cysβ-2H), 2.55 (Gluγ-2H), 2.26 (Gluβ-2H).

2-Glutathionyl-6-(1-hydroxyoctyl)-5,8-dimethoxy-1,4-naphthoquinone (19): Yield: 30.5%, TLC (MeOH:H_2O= 3:1): Rf=0.48; ^{1}H-NMR (D_2O, 300 MHz) 7.65 (s, 1H, H-7), 6.64 (s, 1H, H-3), 5.20 (m, 1H, H-1'), 4.04 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 1.86 (m, 2H, H-2), 1.68-1.58 (m, 4H, H-3,4), 1.52-1.45 (m, 6H, H-5,6,7), 0.98 (t, *J*=6.0 Hz, 3H, H-8), 4.86 (Cysα-1H), 3.98 (Glyα-2H), 3.85 (Gluα-1H), 3.34-3.30 (Cysβ-2H), 2.54 (Gluγ-2H), 2.25 (Gluβ-2H).

2-Glutathionyl-6-(1-hydroxy-4-isopentyl)-5,8-dimethoxy-1,4-naphthoquinone (20): Yield: 41.7%, TLC (MeOH: H_2O=2:1): Rf=0.55; ^{1}H-NMR (D_2O, 300 MHz) 7.62 (s, 1H, H-7), 6.65 (s, 1H, H-3), 5.22 (m, 1H, H-1'), 4.05 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 1.88 (m, 2H, H-2), 1.70 (m, 2H, H-3), 1.50 (m, 1H, H-4), 1.06 (d, *J*=6.1 Hz, 6H, H-5,6), 4.81 (Cysα-1H), 3.95 (Glyα-2H), 3.85 (Gluα-1H), 3.28 (Cysβ-2H), 2.62 (Gluγ-2H), 2.23 (Gluβ-2H).

2-Acetoxymethyl-3-glutathionyl-5,8-dimethoxy-1,4-naphthoquinone (21): 2-Acetoxymethyl-5,8-dimethoxy-1,4-naphthoquinone (0.20 mmol) was used as starting material. Other procedure was the same as the general procedure. Yield: 23%, TLC (MeOH:H_2O=2:1): Rf=0.71, m.p=196-198; ^{1}H-NMR (D_2O, 300 MHz) δ 7.41 (s, 2H, H-6,7), 5.25 (s, dd, H-1), 4.72 (Cysα-1H), 3.87 (s, 6H, $2OCH_3$), 3.90-3.76 (Glyα-2H, Gluα-1H), 3.40-3.60 (Cysβ-2H), 2.50 (Gluγ-2H), 2.12 (Gluβ-2H), 2.21(s, 3H, $COCH_3$).

2-(1-Acetoxyethyl)-3-glutathionyl-5,8-dimethoxy-1,4-naphthoquinone (22): Yield: 45.3%, TLC (MeOH:H_2O= 2:1): Rf=0.69; ^{1}H-NMR (D_2O, 300 MHz) δ 7.48 (s, 2H, H-6,7), 5.13 (m, 1H, H-1), 4.70 (Cysα-1H), 3.88 (s, 6H), 3.88-3.75 (Glyα-2H, Gluα-1H), 3.42-3.65 (Cysβ-2H), 2.32 (Gluγ-2H), 2.10(Gluβ-2H), 1.98 (s, 3H, $COCH_3$), 1.38 (s, 3H, H-2).

6-Acetoxymethyl-2-glutathionyl-5,8-dimethoxy-1,4-naphthoquinone (23): 6-Acetoxymethyl-5,8-dimethoxy-1,4-naphthoquinone was used as starting material. Others were the same as the general procedure. Yield: 42.8%, TLC (MeOH:H_2O=2:1): Rf=0.69; ^{1}H-NMR (D_2O, 300 MHz) δ 7.45 (s, 1H, H-7), 6.55 (s, 1H, H-3), 5.18 (s, 2H, H-2), 4.71 (Cysα-1H), 3.95 (s, 3H, OCH_3), 3.82 (Glyα-2H), 3.79 (s, 3H, OCH_3), 3.73 (Gluα-1H), 3.43 (Cysβ-2H), 2.51 (Gluγ-2H), 2.20 (s, 3H, $COCH_3$), 2.13 (Gluβ-2H).

6-(1-Acetoxyethyl)-2-glutathionyl-5,8-dimethoxy-1,4-naphthoquinone (24): Yield: 35.6%, TLC (MeOH:H_2O= 2:1): Rf=0.67; ^{1}H-NMR (D_2O, 300 MHz) δ 7.29 (s, 1H, H-7), 6.54 (s, 1H, H-3), 5.12 (s, 1H, H-1'), 4.50 (Cysα-1H), 3.81 (s, 3H, OCH_3), 3.76 (Glyα-2H), 3.63 (s, 3H, OCH_3), 3.45 (Gluα-1H), 3.26 (Cysβ-2H), 2.27 (Gluγ-2H), 1.95 (s,3H, $COCH_3$), 1.91 (Gluβ-2H), 1.35 (s, 3H, H-2).

2-Glutathionyl-6-acetyl-5,8-dimethoxy-1,4-naphthoquinone (25): 6-(1-Oxomethyl)-5,8-dimethoxy-1,4-naphthoquinone was used as starting material. Others were the same as those for 6-glutathionyl-2-(1-hydroxyethyl)-5,8-dimethoxy-1,4-naphthoquinone. Yield: 52.3%, TLC (MeOH:H_2O=3:1): Rf=0.69; ^{1}H-NMR (D_2O, 300 MHz) δ 7.33 (s, 1H, H-7), 6.50 (s, 1H, H-3), 3.80 (s, 3H, OCH_3), 3.61 (s, 3H, OCH_3), 4.67 (Cysα-H), 3.62 (Glyα-2H), 3.68 (Gluα-1H), 3.17-2.93 (Cysβ-2H), 2.52 (m, 3H, H-2), 2.38 (Gluγ-2H), 1.98 (Gluβ-2H).

2-Glutathionyl-6-propionyl-5,8-dimethoxy-1,4-naphthoquinone (26): Yield: 45.8%, TLC (MeOH:H_2O=3:1): Rf=0.68; ^{1}H-NMR (D_2O, 300 MHz) δ 7.33 (s, 1H, H-7), 6.48 (s, 1H, H-3), 3.79 (s, 3H, OCH_3), 3.58 (s, 3H, OCH_3), 2.55 (m, 2H, H-2), 0.97 (m, 3H, H-3), 4.63 (Cysα-1H), 3.69 (Glyα-2H), 3.58 (Gluα-1H), 3.16-2.85 (Cysβ-2H), 2.35 (Gluγ-2H), 1.97 (Gluβ-2H).

2-Glutathionyl-6-butanoyl-5,8-dimethoxy-1,4-naphthoquinone (27): Yield: 42.7%, TLC (MeOH:H_2O=3:1): Rf=0.65; ^{1}H-NMR (D_2O, 300 MHz) δ 7.34 (s, 1H, H-7),

.54 (s, 1H, H-3), 3.82 (s, 3H, OCH_3), 3.60 (s, 3H, OCH_3), 4.65 (Cystα-1H), 3.74 (Glyα-2H), 3.62 (Gluα-1H), .05-3.21 (Cystβ-2H), 2.87 (m, 2H, H-2), 2.38 (Gluγ-H), 1.99 (Gluβ-2H), 1.31-1.29 (m, 2H, H-3), 0.91(m, H, H-4).

2-Glutathionyl-6-pentanoyl-5,8-dimethoxy-1,4-naphthoquinone (28): Yield: 55.2%, TLC (MeOH:H_2O=3:1): f=0.62; ^{1}H-NMR (D_2O, 300 MHz) δ 7.35 (s, 1H, H-7), .60 (s, 1H, H-3), 3.84 (s, 3H, OCH_3), 3.63 (s, 3H, OCH_3), 4.67 (Cystα-1H), 3.79 (Glyα-2H), 3.67 (Gluα-1H), .25-3.4 (Cystβ-2H), 2.88 (m, 2H, H-2), 2.40 (Gluγ-2H), .01 (Gluβ-2H), 1.53-1.51 (m, 2H, H-3), 1.25-1.23 (m, H, H-4), 0.86 (m, 3H, H-5).

2-Glutathionyl-6-hexanoyl-5,8-dimethoxy-1,4-naphthoquinone (29): Yield: 41.7%, TLC (MeOH:H_2O=3:1): Rf=0.60; ^{1}H-NMR (D_2O, 300 MHz) 7.34 (s, 1H, H-7), 5.58 (s, 1H, H-3), 3.83 (s, 3H, OCH_3), 3.61 (s, 3H, OCH_3), 4.68 (Cystα-1H), 3.80 (Glyα-2H), 3.69 (Gluα-1H), 3.28-3.5 (Cystβ-2H), 2.90 (m, 2H, H-2), 2.42 (Gluγ-2H), 2.11 (Gluβ-2H), 1.61-1.59 (m, 2H, H-3), 1.21-1.20 (m, 4H, H-4,5), 0.88 (m, 3H, H-6).

2-Glutathionyl-6-heptanoyl-5,8-dimethoxy-1,4-naphthoquinone (30): Yield: 36.2%, TLC (MeOH:H_2O=3:1): Rf=0.56; ^{1}H-NMR (D_2O, 300 MHz) δ 7.36 (s, 1H,H-7), 6.62 (s, 1H, H-3), 3.85 (s, 3H, OCH_3), 3.62 (s, 3H, OCH_3), 4.67 (Cystα-1H), 3.79 (Glyα-2H), 3.67 (Gluα-1H), 3.45-3.24 (Cystβ-2H), 2.90 (m, 2H, H-2), 2.40 (Gluγ-2H), 2.01 (Gluβ-2H), 1.75-1.48 (m, 4H, H-3,4), 1.35-1.12 (m, 4H, H-5,6), 0.90 (m, 3H, H-7).

2-Glutathionyl-6-octanoyl-5,8-dimethoxy-1,4-naphthoquinone (31): Yield: 23.1%, TLC (MeOH:H_2O=3:1): Rf=0.53; ^{1}H-NMR (D2O, 300 MHz) δ 7.36 (s, 1H, H-7), 6.61 (s, 1H, H-3), 3.85 (s, 3H, OCH_3), 3.62 (s, 3H, OCH_3), 4.68 (Cystα-1H), 3.77 (Glyα-2H), 3.68 (Gluα-1H), 3.46-3.24 (Cystβ-2H), 2.90 (m, 2H, H-2), 2.41 (Gluγ-2H), 2.11 (Gluβ-2H), 1.76-1.45 (m, 4H, H-3,4), 1.40-1.10 (m, 6H, H-5,6,7), 0.91 (m, 3H, H-8).

2-Glutathionyl-6-nonanoyl-5,8-dimethoxy-1,4-naphthoquinone (32): Yield: 22.4%, TLC (MeOH:H_2O=3:1): Rf=0.50; ^{1}H-NMR (D_2O, 300 MHz) δ 7.36 (s, 1H, H-7), 6.62 (s, 1H, H-3), 3.82 (s, 3H, OCH_3), 3.63 (s, 3H, OCH_3), 4.67 (Cystα-1H), 3.80 (Glyα-2H), 3.66 (Gluα-1H), 3.46-3.23 (Cystβ-2H), 2.89 (m, 2H, H-2), 2.42 (Gluγ-2H), 2.10 (Gluβ-2H), 1.75-1.45 (m, 6H, H-3,4,5), 1.38-1.09 (m, 6H, H-6,7,8), 0.90 (m, 3H, H-9).

2,3-Diglutathionyl-5,8-dimethoxy-1,4-naphthoquinone (33): Molar ratio of 5,8-Dimethoxy-1,4-naphthoquinone (0.23m mol) to glutathione was 1:2. Other procedure was the same as that for synthesis of 2-gulutathionyl-DMNQ. Yield: 28%, TLC (MeOH:H_2O=2:1): Rf=0.76; ^{1}H-NMR (D_2O, 300 MHz) δ 7.49 (s, 2H, H-6,7), 3.94 (s, 6H, $2OCH_3$), 4.75 (Cystα-2H), 3.84-3.81 (Glyα-4H; Gluα-2H), 3.60-3.38 (Cystβ-4H), 2.55-2.47 (Gluγ-4H), 2.17-2.11 (Gluβ-4H).

1,4-Dihydroxy-5,8-dimethoxynapthoquinone (34): 5,8-Dimethoxy-1,4-naphthoquinone (1.9 g) was dissolved in chloroform (100 ml), and to this solution was added 100 ml of water solution saturated with sodium hydrosulfite ($Na_2S_2O_4$) under stirring. After 20 min, the chloroform phase was separated. The water solution was extracted two times with chloroform. The chloroform extract was dried over anhydrous sodium sulfate and brought to a dry mass. This was recrystallized from methanol.

Colorless crystal. Yield: 94%, IR (KBr); 3350, 3080, 2940, 1610 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 80 MHz) δ 9.08 (s, 2H, 2OH), 6.82 (s, 2H, H-6,7), 6.57 (s, 2H, H-2,3), 3.96 (s, 6H, OCH_3).

2-Glutathionyl-1,4-dihydroxy-5,8-dimethoxynaphthoquinone (35): Prepared according to the procedure for synthesis of **33**. .Dark brown mass. Yield: 25%, TLC (MeOH:H_2O=2:1): Rf=0.75; ^{1}H-NMR (D_2O, 300 MHz) 6.77 (s, 3H), 4.58 (Cystα-1H), 3.91 (Glyα-2H), 3.77 (Gluα-1H), 3.23, 2.94 (Cystβ-2H), 2.49 (Gluγ-2H), 2.12 (Gluβ-2H).

Reaction steps of glutathione with 5,8-dimethoxy-1,4-naphthoquinone

To 0.1 ml of 1 mmol naphthoquinone derivative in methanol was added 0.1 ml of 1 mmol GSH in 0.1 mol potassium phosphate buffer (pH=7.4) and the mixture was stirred at room temperature. The samples for analysis were taken at a time interval of 0, 1 and 1.5 min, and loaded on a silica gel plate (RP-18, F_{254}) which was developed in 50 % methanol.

Detection of hydrogen peroxide

Formation of H_2O_2 was measured according to the method of Hyslop and Sklar *et al.* (1984).

The above reaction mixture (0.2 ml), stirred for 1.5 min, was added to a mixture of 1 mmol sodium azide (0.2 ml) and 10 mmol *p*-hydroxyphenylacetic acid (0.2 ml) and 0.1 mg/ml horseradish peroxidase (0.2 ml) in 0.1 mol potassium phosphate buffer. The resulting solution was diluted to 2 ml with the same buffer solution. After stirring for 20 min at room temperature, the fluorescence intensity of the reaction mixture was determined by LS-3E Fluorescence spectrophotometer (Perkin-Elmer, England) in excitation and emission wavelengths of 334 and 425 nm, respectively.

Detection of oxidized GSH (GSSG)

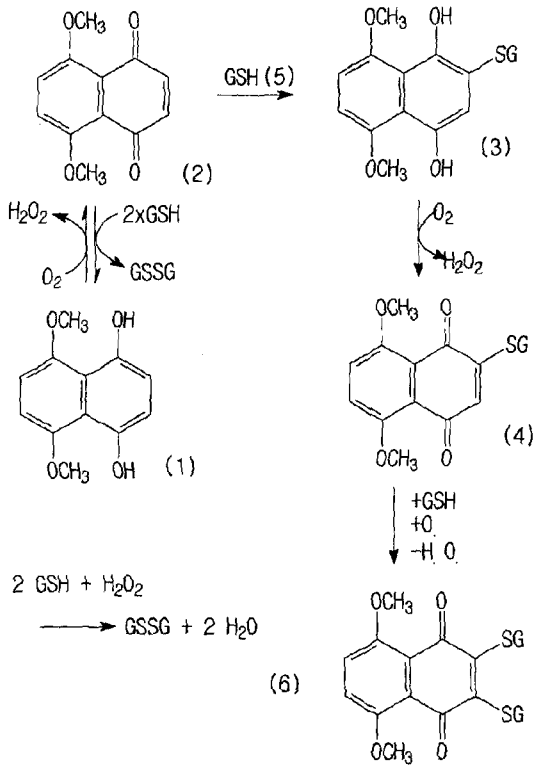
The level of GSSG was determined by the described method of Hissin et al. (1976). The reaction mixture (0.1 ml), stirred for 1.5 min, was added to 0.2 ml of 0.04 mol *N*-ethylmaleimide (NEM) in ethanol and the mixture was stirred for 30 min at room temperature. Then, 0.1 ml of 0.1% *o*-phthaldialdehyde (OPA) in methanol was added to the reaction mixture, and the mixture was diluted with

0.1 N NaOH to volume of 2 ml. After standing for 60 min at room temperature, the fluorescence intensity of the mixture was determined in excitation and emission wavelengths of 350 and 420 nm, respectively.

RESULTS AND DISCUSSION

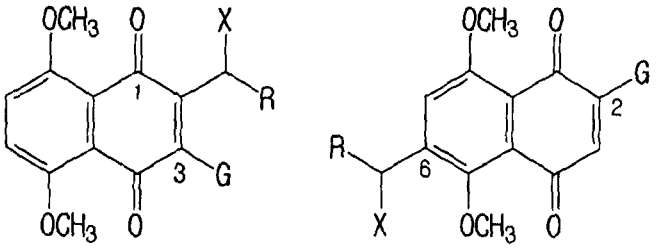
Synthesis of glutathione conjugates

2-Glutathionyl or 3-glutathionyl-(1-hydroxyalkyl)-5,8-dimethoxy-1,4-naphthoquinone derivatives were synthesized according to the procedure indicated in Scheme 1. In general, the yield of 2-glutathionylated products was higher than that of 3-glutathionyl products (Table I). As previously reported (You and Zheng *et al.*, 1998), it was evident that the sterically bulky alkyl group at C-2 position of 2-(1-hydroxyalkyl)-, 2-(1-acetoxyalkyl)- and 2-acylated DMNQ derivatives retarded the formation of the conjugates. However, the yield of 2-acyl-3-glutathionyl DMNQ derivatives (**24-32**) was, despite the presence of the steric hindrance in the starting material, higher than that of 6-acylated-2-glutathionyl DMNQ derivatives. This might be due to the enhanced electrophilicity in quinonoid moiety of 2-acylated DMNQ derivatives, the starting materials. Among the same series of compounds, the longer the size of the side chain is, the lower reaction yield was; **11**(R, heptyl; yield, 22%) vs. **4** (R, methyl; yield, 43%) and **24** (R, methyl; yield, 64%) vs. **32** (R, nonyl; yield, 32%).



Scheme 1. Reaction of glutathione with 5,8-dimethoxy-1,4-naphthoquinone number in parenthesis is the same as designated in Fig. 2.

Table I. Yields and ^{1}H-NMR data of glutathione conjugates



3-glutathionyl isomer **2-glutathionyl isomer**

No.	R	X	Yield (%)	NMR(ppm of aromatic protons)		
				H-3	H-6	H-7
1 (NQ)[1]	-	-	78.1	6.56	6.56	6.56
2 (DMNQ)[2]	-	-	56.7	6.23	7.20	7.18
3(3)	H	OH	31.9	7.39	7.39	7.39
4(3)	Methyl	OH	43.2	7.54	7.54	7.54
5(3)	Ethyl	OH	35.5	7.53	7.53	7.53
6(3)	Propyl	OH	29.8	7.52	7.52	7.52
7(3)	Butyl	OH	28.1	7.50	7.50	7.50
8(3)	Pentyl	OH	21.7	7.51	7.51	7.51
9(3)	Hexyl	OH	23.7	7.48	7.48	7.48
10(3)	Heptyl	OH	22.1	7.48	7.48	7.48
11(3)	Isobutyl	OH	31.7	7.36	7.36	7.36
12(2)	H	OH	53.4	6.58		7.56
13(2)	Methyl	OH	62.4	6.51		7.57
14(2)	Ethyl	OH	43.2	6.56		7.59
15(2)	Propyl	OH	36.4	6.59		7.60
16(2)	Butyl	OH	45.8	6.68		7.63
17(2)	Pentyl	OH	36.4	6.67		7.65
18(2)	Hexyl	OH	30.5	6.65		7.65
19(2)	Heptyl	OH	30.5	6.64		7.65
20(2)	Isobutyl	OH	41.7	6.65		7.62
21(3)	H	Acetoxy	22.9	7.41	7.41	7.41
22(3)	Methyl	Acetoxy	45	7.48	7.48	7.48
23(2)	H	Acetoxy	42.8	6.55		7.45
24(2)	Methyl	Acetoxy	35	6.54		7.29
25(2)	Methyl	Oxo	64.0	6.80		7.75
26(2)	Ethyl	Oxo	53	6.50		7.33
27(2)	Propyl	Oxo	45	6.48		7.33
28(2)	Butyl	Oxo	42	6.54		7.34
29(2)	Pentyl	Oxo	65	6.60		7.35
30(2)	Hexyl	Oxo	41	6.58		7.34
31(2)	Heptyl	Oxo	36	6.62		7.36
32(2)	Octyl	Oxo	23	6.61		7.36
33(2)	Nonyl	Oxo	32	6.62		7.36

(NQ)[1]; 1,4-naphthoquinone, (DMNQ)[2]; 5,8-dimethoxy-1,4-naphthoquinone, R; alkyl group, G; glutathionyl group, number in ( ); bond position of glutathionyl group.

Structure elucidation of the GSH-conjugates was carried out mainly using ^{1}H-NMR method. The presence of naphthoquinone protons in the structure of the conjugates is a keystone for interpretation of the spectra (Table I). For 1,4-dihydroxy-1.4-naphthoquine, it is well known that the benzenoid protons appeared in fields lower than the quinonoid protons, being apparently due to the anisotropic effect of benzene ring of the structure (Moore and Scheuer, 1966). For the glutathionyl-DMNQ derivatives, the same phenomena were observed; benzenoid protons, 7.21~7.63 ppm vs. quinonoid protons, 6.23~6.80 ppm as seen in ^{1}H-NMR spectrum of 2-glutathionyl-6-(1-hydroxyethyl)-DMNQ as an example (Fig. 1). The bond formation between GSH and DMNQ derivatives could be confirmed by disappearance of a proton in the range of the qui-nonoid protons. For example, the benzenoid and qui-nonoid protons of 5,8-dimethoxy-1,4-naphthoquinone (DMNQ) showed chemical shifts at 7.31 ppm (s, 2H) and 6.75 ppm (s. 2H), respectively, while two benzenoid protons of 2-glutathionyl DMNQ were observed at 7.20 ppm and 7.18 ppm, and one quinonoid proton at 6.23 ppm. For 2,3-diglutathionyl DMNQ the quinonoid protons disappeared in the spectrum.

The benzenoid protons of 3-glutathionyl-2-(1-hydroxyalkyl)-DMNQ and 2-glutathionyl-6-(1-hydroxyalkyl)-DMNQ derivatives appeared at 7.30~7.54 ppm and 7.56~7.66 ppm, respectively. The quinonoid protons of the latter compounds were shifted to a lower field probably due to paramagnetic effect of glutathione compared with those of 6-(1-hydroxyalkyl)-DMNQ derivatives(6.74~6.80 ppm) (Ahn and Baik *et al.*, 1995). The signals of the quinonoid protons at C-3 of 2-glutathionyl-6-(1-hydroxyalkyl)-DMNQ derivatives were shifted to higher fields (6.51~6.68 ppm). This higher shift could be explained by the +M effect of glutathionyl group, which may enforce the electron density of C-3. The proton signals at C-3 of 2-glutathionyl-6-(1-hydroxyalkyl)-DMNQ derivatives could be divided into two distinct shift groups; 6.51~6.59 ppm where R=H to propyl (**12~15**) and 6.64~6.68 ppm where R=butyl to heptyl (**16~17**). The lower shift of the proton signals, where R=butyl to heptyl, might be explained by a folding effect of the long alkyl chain (Leo and Hansch *et al.*, 1971), which could deform the C-S bond at C-2. This bond deformation would decrease the electron-enforcing effect of glutathionyl group on C-3. Though it remains to be studied further, it is expected that the folding effect have an implication to a bioactivity of 6-(1-hydroxyalkyl)-DMNQ derivatives.

Reaction mechanism of the conjugate formation

It was found that the main products from the reaction between glutathione and DMNQ derivatives were 2- or 3-glutathionyl-5,8-dimethoxy-1,4-naphthoiquinone derivatives, but not 1,4-aducts (1,4-dihydroxy-5,8-dimethoxy-naphthalene derivatives). It is a well-known phenomenon that 1,4-aducts was sensitive to an autooxidation in presence of oxygen. Because this reaction process could be important for a study on the metabolism of DMNQ derivatives, the reaction was examined in more detail. As a representative model, we have taken the reaction between glutathione and 5,8-dimethoxy-1,4-naphthoquinone. When 5,8-dimethoxy-1,4-naphthoquinone (0.1 mM) and glutathione (0.5 mM) in 0.1 mM phosphate buffer (pH 7.4) was stirred at 5°C, and the the reaction products were analysized on a reverse phase silica gel TLC plate

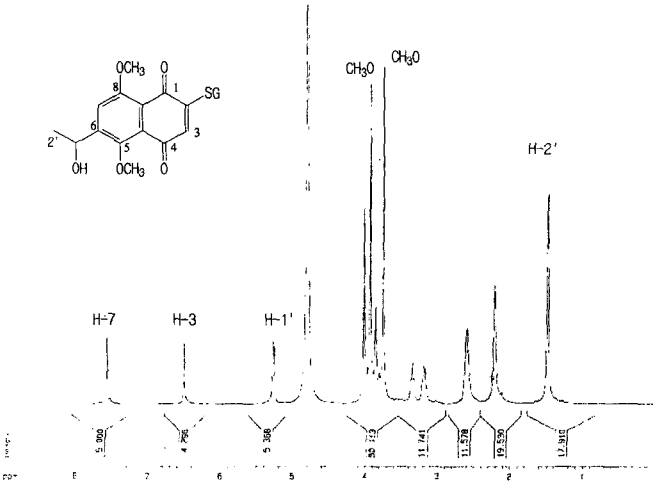


Fig. 1. ^{1}H-NMR spectrum of 2-glutathionyl-6-(1-hydroxyethyl)-5,8-dimethoxy-1,4-naphthoquinone. Protons of glutathionyl moiety were not marked.

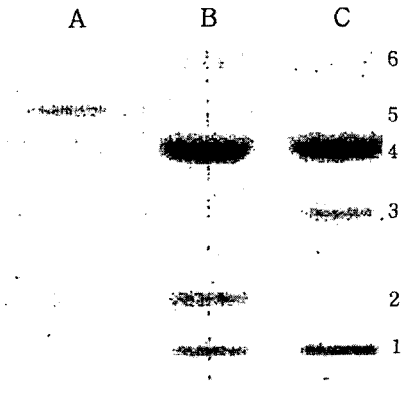


Fig. 2. TLC pattern of the reaction products between glutathione and 5,8-dimethoxy-1,4-naphthoquinone on RP-18 plate. Visualized with 0.2% ninhydrin and UV. **A**: immediately after mixing, **B**: 60 sec, **C**: 90 sec. **1**: 1,4-dihydroxy-5,8-dimethoxynaphthalene, **2**: 5,8-dimethoxy-1,4-naphthoquinone (DMNQ), **3**: 2-glutathionyl-1,4-dihydroxy-5,8-dimethoxy-naphthalene, **4**: 2-glutathionyl-DMNQ, 5: glutathione, 6: 2,3-digluta-thionyl-DMNQ.

(RP-18) in a time dependent manner, it was found that four new spots of 1,3,4 and 6 appeared on the TLC, while the amount of the starting materials, 2 (DMNQ) and 5 (GSH), decreased (Fig. 2). In TLC analysis, spots 4 and 6 were found to be identical to those of 2-glutathionyl-DMNQ and 2,3-diglutathionyl-DMNQ, re-spectively.

Next, when DMNQ and 2-Glutathionyl-DMNQ were treated with sodium hydrosulfite, it was found that the reduction products were observed to migrate to spot 1 and 3, respectively. From this observation it is evident that first, GSH forms an 1,4-adduct (1,4-dihydroxy-2-glutathionylnaphthalene), which then is autooxidized to 2-glutathionyl DMNQ, and, second, GSH reduces DMNQ to 1,4-dihydroxy-5,8-dimethoxynaphthalene. The autooxidation was confirmed by detection of hydrogen peroxide formed during the reaction. The steps of the reaction were summarized in Scheme 1.

Detection of GSSG in the reaction mixture also supports the reaction pathways. Meanwhile, reaction of GSH with DMNQ in equivalent molar ratio produced 2-glutathionyl-DMNQ, as a predominant product, with production of other byproducts being negligible.

It is highly probable that all of the products mentioned above may be produced in liver cells of animals administered with DMNQ derivatives, because GSH occurs more abundantly in liver cell compared with amount of DMNQ transported there.

ACKNOWLEDGEMENTS

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