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Synthesis of 3-Alkylthio-6-Allylthiopyridazine Derivatives and Their Antihepatocarcinoma Activity

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The allylthio group of allicin and other organosulfur compounds, isolated from garlic, is considered a pharmacophore, and a key structure component of the molecule, which affords biological activities. In the foregoing studies, various 3-alkoxy-6-allylthiopyridazine derivatives (K-compounds) were synthesized, and their biological activities tested in animals. As expected, the various derivatives showed good hepatoprotective activities on carbon tetrachloride-treated mice and aflatoxin B1-treated rats, and chemopreventive activities on hepatocarcinoma cells in rats. Other new pyridazine derivatives, with the oxygen atom at the 3-position of the 3-alkoxy-6-allylthiopyridazine displaced by sulfur (*S*), were synthesized, and their activities tested *in vitro*. Thio-K6, one of the sulfur-substituted compounds, showed better chemopreventive activity toward hepatocarcinoma cells.

Key words: Pyridazine, Allylthiopyridazine, Allicin, Organosulfur compounds, K-6, K-16, K-17, Thio-K Compound, Antihepatocarcinoma, Chemopreventive

INTRODUCTION

Allicin and other organosulfur compounds, isolated from garlic (*Allium sativum L.*), have allylthio groups in their chemical structures, and their biological activities have previously been scientifically elucidated (Wertheim, 1884; Semmler, 1892a, 1892b; Cavllito and Bailey, 1944a, 1944b, 1945; Block, 1985). The main biological activities include bactericidal, antifungal, antithrombotic, cholesterol-lowering, antineoplastic and hepatoprotective activities (Fenwick and Hanley, 1985; Kwon, 2003). The allylthio group is considered a pharmacophore, and a key structure component of the molecule, which affords biological activities.

In order to develop more effective drugs, where the defects of the unstable and bad-smell natural organosulfur compounds of garlic were removed, and the biological activities improved, a new structure containing the allylthio group was designed. A pyridazine was selected to provide a new heterocyclic ring, as previous studies on the pyridazine ring have been limited, especially in the field of medicinal chemistry. Thus, an allylthio group was introduced into the pyridazine nucleus, and a substituent, such as a halogen or alkoxy, was also introduced at the *p*-position of the allylthio group (Kwon, 1998, 2002a, 2002b; Lee, 2001). As expected, three 3-alkoxy-6-allylthiopyridazine derivatives (K-6, K-16 and K-17) showed especially good hepatoprotective activities on carbon tetrachloride-treated mice (Kwon, 1998, 1999, 2003; Shin, 2002) and aflatoxin B1-treated rats (Shin, 2003a, 2003b), and chemopreventive activities on hepatocarcinoma cells in rats (Jung, 2001; Lee, 2003). K6 especially, exhibited antitumor activities both *in vitro* and *in vivo* tumor regressions in nude mice transplanted with Hep-G2 cells (Chai, 2004).

Other new pyridazine derivatives, with the oxygen atom at the 3-position of the 3-alkoxy-6-allylthiopyridazine displaced by sulfur (S), were synthesized, and their activities tested. Thio-K6, one of the sulfur-substituted compounds, showed better chemopreventive activity on hepatocarcinoma cells. Other research results have suggested that the magnitude of the antimicrobial activity of diallyl polysulfides follows the order of the number of sulfur atoms in the molecule (O'Gara, 2000; Tsao, 2001a, 2001b). The number of sulfurs in garlic organosulfur compounds also seems to be an important factor in other biological as well as antibacterial activities.

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MATERIALS AND METHODS

3-Chloro-6-allylthiopyridazine (2)

1.15 g (0.05 mol) of metallic sodium was dissolved in 80

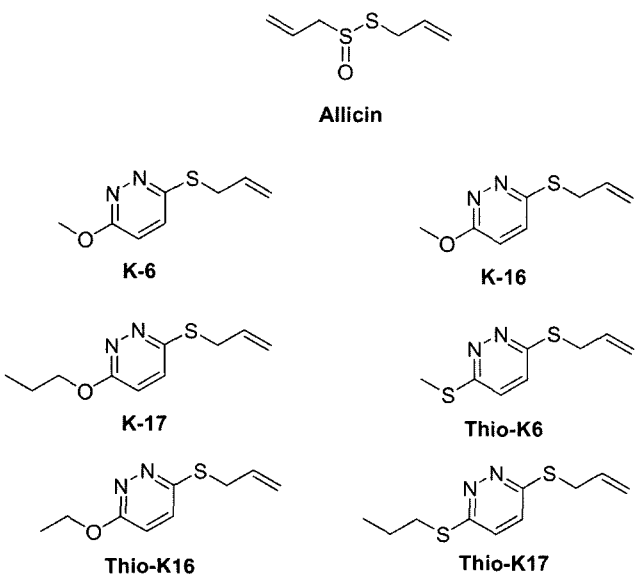


Fig. 1. Structure of allicin and allylthiopyridazine

mL of absolute methanol and then mixed with 4.2 mL (0.05 mol) of 2-propene-1-thiol. To this mixture was added 7.45 g (0.05 mol) of 3, 6-dichloropyridazine (**1**) (Kwon, 1999). The reaction solution was stirred at room temperature for 3 h, and then concentrated under reduced pressure to remove the methanol. 40 mL of ethyl acetate and 20 mL of water were added to the residue, with vigorous stirring. The organic phase was separated, washed twice with water, dried over anhydrous sodium sulfate and concentrated to a pale yellow crystalline product.

Data and spectral analyses of 3-chloro-6-allylthiopyridazine (2)

Yield: 9.08 g (97.3%), Formula $C_7H_7N_2SCl$ (M.W. 186.66), mp 68-70, TLC [*n*-hexane : ethyl acetate (2:1)] Rf 0.52, ^{1}H-NMR (DMSO-d_6) 7.76 (d, *J*=7.9 Hz, 2H, aromatic), 6.01-5.89 (m, 1H, CH=), 5.36 (d, *J*=7.9 Hz, 1H, =CH), 5.16 (d, *J*=10.2 Hz, 1H, =CH), 3.96 (d, *J*=6.6 Hz, 2H, SCH_2). ^{13}C-NMR (DMSO-d_6) 162.01, 153.74 (aromatic), 133.23 (=CH_2), 129.52, 128.53 (aromatic), 118.92 (CH=), 32.62 (SCH_2). FT-IR (NaCl) cm^{-1} 3060 (aromatic), 1564 (N=N), 736 (C-Cl). GC-MS: m/z 186.66 (M+) 171.1 (100.0), 173.0 (51.9), 73.1 (27.8), 118.1 (22.4), 153.1 (21.7).

General procedure for the synthesis of 3-alkylthio-6-allylthiopyridazine derivatives (Thio-K compounds)

0.01 mol of sodium hydroxide was dissolved in 12 mL of methanol and then mixed with 0.01 mol of thioalcohol (R-SH). To this mixture was added 0.01 mol of 3-chloro-6-allylthiopyridazine. The reaction solution was refluxed for 3 h, and then concentrated under reduced pressure to remove the methanol. 20 mL of ethyl acetate and 10 mL of water were added to the residue, with vigorous stirring. The organic phase was separated, washed twice with water, dried over anhydrous sodium sulfate and concentrated to obtain a yellow oil residue. The obtained residue was subjected to TLC, and two spots (Rf = 0.64 and 0.57) obtained. The compound with the Rf value of 0.64 was separated by silica gel column chromatography (eluent solvent: *n*-hexane/ethyl acetate = 10/1). The eluted fractions were concentrated to obtain a white crystalline solid.

Data and spectral analyses of 3-methylthio-6-allylthiopyridazine (Thio-K6)

Yield: 0.63 g (31.8%), Formula $C_8H_{10}N_2S_2$ (M.W. 198.30), mp 56-57, TLC [*n*-hexane:ethyl acetate (2:1)] Rf 0.64, ^{1}H-NMR (DMSO-d_6) 7.47 (d, *J*=8.0 Hz, 2H, aromatic), 5.99-5.88 (m, 1H, CH=), 5.32 (d, *J*=16.9 Hz, 1H, =CH), 5.13 (d, *J*=9.9 Hz, 1H, =CH), 3.92 (d, *J*=6.8 Hz, 2H, SCH_2), 2.59 (s, 3H, CH_3). ^{13}C-NMR (DMSO-d_6) 160.22, 158.75 (aromatic), 134.23 (=CH_2), 126.64, 126.28 (aromatic), 119.06 (CH=), 32.95 (SCH_2), 13.58 (CH_3). FT-IR (NaCl) cm^{-1} 3054 (aromatic), 1573 (N=N). GC-MS: m/z 186.66 (M+) 183.1 (100.0), 114.0 (39.1), 118.1 (25.1), 184.1 (17.9), 185.1 (16.7).

Data and spectral analyses of 3-ethylthio-6-allylthiopyridazine (Thio-K16)

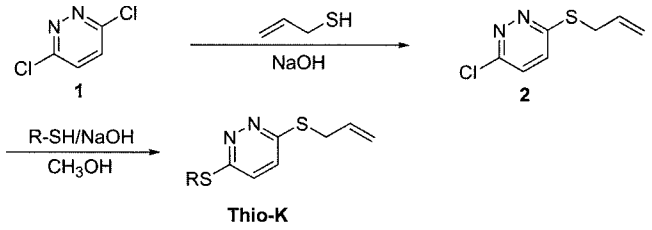
Yield: 3.0 g (70.6%), Formula $C_9H_{12}N_2S_2$ (M.W. 212.33), mp 47, TLC [*n*-hexane:ethyl acetate (2:1)] Rf 0.68, ^{1}H-NMR (DMSO-d_6) 7.46 (d, *J*=8.6 Hz, 2H, aromatic), 6.00-5.91 (m, 1H, CH=), 5.33 (d, *J*=17.7 Hz, 1H, =CH), 5.13 (d, *J*=10.0 Hz, 1H, =CH), 3.92 (d, *J*=6.8 Hz, 2H, SCH_2), 3.23 (q, *J*=7.3 Hz, 2H, CH_2) 1.33 (t, *J*=7.3 Hz 3H, CH_3). ^{13}C-NMR (DMSO-d_6) 159.09, 158.33 (aromatic), 133.72 (=CH_2), 126.24 (aromatic), 118.56 (CH=), 32.45 (SCH_2), 24.12 (CH_2), 14.66 (CH_3). FT-IR (NaCl) cm^{-1} 3052 (aromatic), 1571 (N=N). GC-MS: m/z 212.33 (M+) 197.1 (100.0), 114.0 (36.8), 151.1 (19.5), 118.1 (18.9), 198.1 (16.9).

Data and spectral analyses of 3-propylthio-6-allylthiopyridazine (Thio-K17)

Yield: 3.2 g (71.6%), Formula $C_{10}H_{14}N_2S_2$ (M.W. 226.35), mp 43-44, TLC [*n*-hexane:ethyl acetate (2:1)] Rf 0.67, ^{1}H-NMR (DMSO-d_6) 7.45 (d, *J*=7.9 Hz, 2H, aromatic), 6.00-5.89 (m, 1H, CH=), 5.33 (d, *J*=16.9 Hz, 1H, =CH), 5.13 (d, *J*=9.9 Hz, 1H, =CH), 3.92 (d, *J*=6.8 Hz, 2H, SCH_2), 3.20 (t, *J*=7.1 Hz, 2H, CH_2) 1.69 (q, *J*=7.2 Hz 2H, CH_2), 099 (t, *J*=7.3 Hz 3H, CH_3). ^{13}C-NMR (DMSO-d_6) 159.17, 158.32 (aromatic), 133.72 (=CH_2), 126.28, 126.19 (aromatic), 118.55 (CH=), 32.45 (SCH_2), 31.55 (CH_2), 22.43 (CH_2), 13.58 (CH_3). FT-IR (NaCl) cm^{-1} 3054 (aromatic), 1571 (N=N). GC-MS: m/z 226.35 (M+) 211.1 (100.0), 151.1 (25.4), 114.0 (21.2), 169.0 (19.1), 137.1 (18.2).

Cell line and culture conditions

SK-Hep-1 cells were purchased from the Korean Cell



Scheme 1. Synthesis of 3-alkylthio-6-allylthiopyridazine derivatives

Line Bank (Seoul, Korea), and were maintained at 37°C in a humidified atmosphere, with 95 and 5% air and CO_2, in DMEM medium supplemented with 10% fatal bovine serum and 1% penicillin-streptomycin.

MTT assay

Cells (5×10^4) were cultured in 96-well plate, and treated with the K- and sulfur-substituted K-compounds (Thio-K) for 48 h. MTT (3-(4, 5-dimethylthiazol- 2-yl) 2, 5-diphenyl tetrazolium bromide, 5 mg/mL) (Ferrari, 1990; van de Loosdrecht, 1994), purchased from Sigma Chemical Co. (St. Louis, MD), was added to the medium, and the cells further incubated for 4 h. After 100 μL of the supernatant had been replaced with the same volume of DMSO, the absorbance of each well was measured at 540 nm using a micro-ELISA reader (Molecular Devices, Sunnyvale, CA). The percentage cell survival was determined as the relative absorbance of treated *versus* untreated cells.

RESULTS

The thio-K6 compounds showed higher cytotoxicities than K6 in the SK-Hep-1 cells. In order to compare the chemopreventive effects of the K and their sulfur-substituted compounds (Thio-K) on hepatocarcinoma cells, the cytotoxicity of each compound was compared on the SK-Hep-1 hepatocellular carcinoma cells using the MTT assay. As shown in Fig. 2A, treatment of cells with 500 μg/mL of K6, K16 and K17 for 48 h markedly inhibited the viability. The cytotoxicity exerted by Thio-K6 was significantly higher than that by K6, whereas neither K16 nor K17 showed significant differences between the cytotoxicities of the K and Thio-K compounds. A dose response study was conducted to further confirm the cytotoxic activities of K6 and Thio-K6. Both compounds inhibited the viability of SK-Hep-1 cells, in dose-dependent manners. A significant difference between the cytotoxicity of K6 and Thio-K6 was observed only when the cells were treated with the highest concentration (500 μg/mL) of these compounds. The data showed that Thio-K6 exerted a higher cytotoxicity than K6 on the SK-Hep-1 cells, suggesting that the sulfur-substituted compound of K6 may possess better chemopreventive activity toward hepatocarcinoma cells.

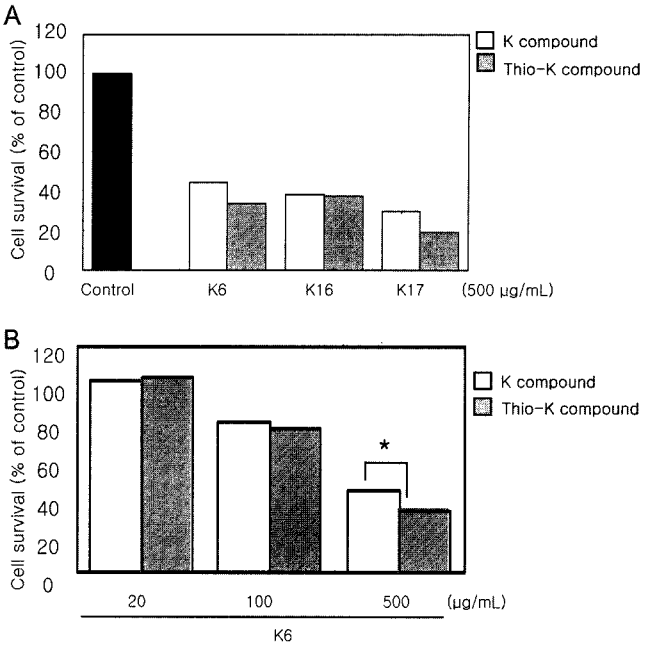


Fig. 2. The effects of the K (K6, K16 and K17) and Thio-K compounds (Thio-K6, Thio-K16 and Thio-K17) on the viability of SK-Hep-1 cells. MTT assay was performed on SK-Hep-1 cells treated with 500 μg/mL of the K and Thio-K compounds (A) or various concentrations of K6 and Thio-K6 (B) for 48 h. Values are the means±S.E. of triplicate experiments. As the errors were very small, they have not been shown in the graph. *, Statistically different at $p < 0.05$.

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