Antimutagenicity of 3-allyl-5-substituted 2-thiohydantoins derived from allyl isothiocyanate and amino acids in *Salmonella* assay

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Summary

Nine 3-allyl-5-substituted 2-thiohydantoins (ATH-amino acids) which were prepared from allyl isothiocyanate (AITC) and amino acids were studied for their antimutagenic activities against 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and 4-nitroquinoine 1-oxide (4-NQO) using the Ames assay. The assay against IQ was performed on *S. typhimurium* TA98 in the presence of a metabolic activation system (S9 mix) and that against 4-NQO was carried out on *S. typhimurium* TA100 in the absence of S9 mix. When ATH-amino acids except for that prepared from AITC and aspartic acid were simultaneously treated with the bacterial strain and IQ, an inhibition of IQ mutagenicity was observed. Also, all ATH-amino acids showed a suppressive effect on 4-NQO mutagenicity when the bacterial strain was incubated in the presence of both 4-NQO and ATH-amino acids. In contrast, little antimutagenic effect was observed when ATH-amino acids were added to the bacterial strains which has been pretreated with a mixture of IQ and S9 mix or only 4-NQO. These results suggest that ATH-amino acids are capable of acting as inhibitors of the S9 mix-mediated activation of IQ and/or as modulators of the direct-acting mutagen, 4-NQO.

Keywords: 2-thiohydantoin, allyl isothiocyanate, antimutagenicity, *Salmonella* assay, blocking agents

Introduction

Cruciferous vegetables contain glucosinolates that undergo enzymatic hydrolysis to form pungent compounds known as isothiocyanates. The pungent flavor of isothiocyanate plays an important role in our daily food habits. Allyl isothiocyanate (AITC) is the main pungent principle of horseradish, wasabi, and black mustard which are used as condiments. It is known that isothiocyanates, due to their electrophilicity, can easily react with nucleophilic reagents such as water, amines, and amino acids. As for the reaction products of AITC in an aqueous medi-

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(Shapiro et al., 1998; Getahun and Chung, 1999; Elfoul et al., 2001; Krul et al., 2002). These facts suggest that isothiocyanates in food can possibly react with amino acids to form 2-thiohydantoins during food processing or the cooking of isothiocyanate-containing food, and even in intestinal tract. Therefore, the biological activity of 2-thiohydantoins should be further studied.

Many reports have focused on the biological activity of 2-thiohydantoins. For example, 2-thiohydantoins can act as antitumor (Al-Obaid et al., 1996), antiviral (El-Barbary et al., 1994), and antifungal (Marton et al., 1993) agents. Gesler et al. (1961) examined the anticonvulsant activity of several ATH-amino acids. However, little information is available regarding antimutagenic and/or anticarcinogenic activities of 2-thiohydantoins being derivable from amino acids and naturally occurring isothiocyanates. We report here the antimutagenic activity of nine ATH-amino acids against IQ and 4-NQO on Salmonella typhimurium TA98 and S. typhimurium TA100 with or without the metabolic activation mixture (S9 mix).

Materials and Methods

Materials

AITC, 4-NQO and IQ were purchased from Wako Pure Chemical Inc. (Tokyo, Japan). Rat liver 9,000 x g supernatant (S9) fraction and cofactors were obtained from Oriental Yeast Co., Ltd (Tokyo, Japan). The cofactors dissolved in 9.0 mL of distilled water were filtered through a sterilized membrane filter (0.22 μm) and mixed with 1.0 mL of the S9 fraction. This was immediately used as S9 mix. One mL of the S9 mix contained MgCl₂ (8 μmol), KCl (33 μmol), glucose-6-phosphate (5 μmol), NADPH (4 μmol), NADH (4 μmol), and sodium phosphate buffer (pH 7.4, 100 μmol). Salmonella typhimurium TA98 (IFO 14193) was obtained from the Institute for Fermentation (Osaka, Japan) and S. typhimurium TA100 was provided by the National Institutes of Public Health (Tokyo, Japan).

Spectral analysis

Ultraviolet (UV) spectra were obtained in ethanol (EtOH) or methanol (MeOH) using a Hitachi 330 double-beam spectrophotometer. Infrared (IR) spectra were recorded on a Nicolet Nexus 670 FTIR spectrometer using the attenuated total reflection infrared (ATR) method. High-resolution (HR) and low-resolution (LR) electron impact mass spectral (EI-MS) analysis was performed using a JEOL AX-500 mass spectrometer at 70 eV. ¹H and ¹³C nuclear magnetic resonance (NMR) spectra were measured in acetone-d₆ or dimethyl sulfoxide (DMSO)-d₄ on a JEOL EX-400 nmr spectrometer using tetramethylsilane (TMS) as the internal standard.

Antimutagenicity assay

Antimutagenicity of ATH-amino acids against IQ was examined using S. typhimurium TA98 with S9 mix, and that against 4-NQO was investigated on S. typhimurium TA100 without S9 mix. The assays were carried out as described by Maron and Ames (1983) with modifications as follows.

A mixture of 0.1 mL of the test compound (0-500 μg) in DMSO, 0.1 mL of IQ (4 ng) or 4-NQO (0.1 μg) in DMSO, 0.1 mL of overnight-cultured bacterial suspension (1.0-2.5 x 10⁶ cells/mL), and 0.5 mL of S9 mix or 0.1 M phosphate buffer (pH 7.4) was incubated for 20 min at 37 °C. The mixture was then plated onto a Vogel Bonner E agar plate with 2 mL of top agar containing 0.05 mM of L-histidine and 0.05 mM biotin. After incubation at 37 °C for 48 h, revertant colonies were counted. In another experiment, the bacterial strains which had been incubated with the same amount of mutagen and/or S9 mix were twice rinsed with phosphate buffer in order to remove the mutagen, and were treated with the test compounds for a further 20 min. The same procedure as described above was followed. Spontaneous revertants were subtracted from the number of revertants in all plates exposed to the mutagen in the presence or absence of the test compounds. All assays were conducted twice using duplicate plates.

Preparation and structural confirmation of ATH-amino acids

ATH-amino acids were prepared from AITC and nine different amino acids (L-Gly, L-Ala, L-Val, L-Phe, L-Trp, L-Asp, L-Glu, and L-Lys) by means of our previous method (Takahashi et al., 1998), and their chemical structures (Fig. 1) were confirmed from the UV, IR, EI-MS, and NMR spectral data. Among the nine ATH-amino acids, the three (ATH-Phe, ATH-Trp, and ATH-Val) obtained by reaction of AITC and L-Phe, L-Trp, and L-Val showed spectral data identical to those described in our previous report (Takahashi et al., 1998). The data of other six ATH-amino acids are shown below.

3-Allyl-2-thiohydantoin (ATH-Gly) : UV λmax (EtOH) 264 nm; HR-EI-MS m/z (M⁺) calcd for C₇H₁₀N₂S 156.0357, found 156.0339; LR-EI-MS m/z (%) rel intems. 156 (M⁺, 94), 128 (100), 99 (64), 72 (79), 41 (67); IR vmax cm⁻¹ 3224 (NH), 1748 (C=O), 1517 (N=C=S), 1171 (C=S); ¹H NMR (acetone-d₆) δ (ppm) 8.94 (1H, s), 5.79-5.89 (1H, m), 5.10-5.19 (2H, m), 4.35-4.37 (2H, m), 4.18 (2H, d, J = 0.98 Hz); ¹³C NMR (acetone-d₆) δ (ppm) 185.8, 173.1, 133.1, 117.9, 49.6, 45.8.

3-Allyl-5-methyl-2-thiohydantoin (ATH-Ala) : UV λmax (EtOH) 267 nm; HR-EI-MS m/z (M⁺) calcd for C₉H₁₂N₂O₂S 170.0514, found 170.0513; LR-EI-MS m/z (%) rel intems. 170 (M⁺, 100), 155 (8), 141 (3), 129 (15), 100 (23), 72 (5), 41 (31); IR vmax cm⁻¹ 3168 (NH),
1746 (C=O), 1532 (N-C=S), 1169 (C=S); 1H NMR (DMSO-d$_6$) δ (ppm) 10.33 (1H, s), 5.74-5.84 (1H, m), 5.03-5.17 (2H, m), 4.32 (1H, q, J=6.8, 7.3 Hz), 4.25-4.28 (2H, m), 1.27 (3H, d, J=7.3 Hz); 13C NMR DMSO-d$_6$ δ (ppm) 181.9, 175.1, 131.8, 116.5, 54.4, 41.9, 16.1.

3-Allyl-5-isobutyl-2-thiohydantoin (ATH-Leu): UV λmax (MeOH) 267 nm; HR-ESI-MS m/z (M$^+$) calcd for C$_{19}$H$_{20}$O$_2$S, 271, found 271.0943; LR-ESI-MS m/z (% rel. inten.) 271 (M$^+$, 100), 179 (N-C=S), 1171 (C=S); 1H NMR (acetone-d$_6$) δ (ppm) 9.16 (1H, s), 5.80-5.89 (1H, m), 5.10-5.18 (2H, m), 4.34-4.37 (2H, m), 4.28-4.32 (1H, m), 1.89-2.02 (1H, m), 1.67-1.74 (1H, m), 1.58-1.65 (1H, m), 0.97 (3H, d, J=5.9 Hz), 0.96 (3H, d, J=6.8 Hz); 13C NMR (acetone-d$_6$) δ (ppm) 184.3, 175.3, 132.7, 117.5, 58.5, 43.3, 41.2, 25.3, 23.3, 22.0.

3-Allyl-2-thiohydantoinacetic acid (ATH-Asp): UV λmax (EtOH) 265 nm; HR-ESI-MS m/z (M$^+$) calcd for C$_{19}$H$_{19}$O$_3$NS, 214.0412, found 214.0394; LR-ESI-MS m/z 214 (M$^+$), 196, 186, 168, 153, 99, 57, 41; 1H NMR (DMSO-d$_6$) 3194 (NH), 1747 (C=O), 1703 (C=O), 1507 (N-C=S), 1169 (C=S); 1H NMR (acetone-d$_6$) δ (ppm) 8.97 (1H, s), 5.77-5.88 (1H, m), 5.08-5.24 (2H, m), 4.30-4.49 (2H, m), 4.52 (1H, dd, J=4.4, 6.4 Hz), 2.96 (1H, dd, J=4.4, 17.1 Hz), 2.81 (1H, dd, J=6.4, 17.1 Hz); 13C NMR (acetone-d$_6$) δ (ppm) 184.9, 174.2, 170.9, 132.7, 117.4, 56.3, 43.6, 35.3.

3-Allyl-2-thio-hydantoinpropionic acid (ATH-Glu): UV λmax (MeOH) 267 nm; HR-ESI-MS m/z (M$^+$) calcd for C$_{20}$H$_{21}$O$_3$NS, 288.0569, found 288.0566; LR-ESI-MS m/z (%, rel. inten.) 288 (M$^+$, 35), 210 (8), 182 (4), 168 (7), 99 (67), 56 (35), 41 (100); IR ν max cm$^{-1}$ 3184 (NH), 1746 (C=O), 1701 (C=O), 1525 (N-C=S), 1171 (C=S); 1H NMR (acetone-d$_6$) δ (ppm) 9.15 (1H, s), 5.80-5.89 (1H, m), 5.11-5.19 (2H, m), 4.35-4.39 (1H, m), 4.35-4.39 (2H, m), 1.91-2.04 (2H, m), 1.71-2.23 (1H, m), 1.97-2.08 (1H, m); 13C NMR (acetone-d$_6$) δ (ppm) 184.1, 174.7, 173.7, 132.6, 117.5, 58.9, 43.4, 37.6, 29.5, 27.5.

3-Allyl-2-thiohydantoin-5-spiro-2'-piperidine (ATH-Lys): UV λmax (EtOH) 271 nm; HR-ESI-MS m/z (M$^+$) calcd for C$_{20}$H$_{25}$O$_2$NS, 225.0936, found 225.0943; LR-ESI-MS m/z (% rel. inten.) 225 (M$^+$, 100), 210 (5), 182 (25), 169 (16), 141 (18), 99 (12), 56 (46), 41 (41); IR ν max cm$^{-1}$ 3301 (NH), 3089 (NH), 1719 (C=O), 1504 (N-C=S), 1114 (C=S); 1H NMR (DMSO-d$_6$) δ (ppm) 10.89 (1H, s), 5.73-5.83 (1H, m), 5.03-5.11 (2H, m), 4.29-4.33 (2H, m), 3.32 (2H, m), 3.19 (1H, s), 2.86 (2H, m), 1.71 (2H, m), 1.50 (2H, m); 13C NMR (DMSO-d$_6$) δ (ppm) 180.7, 173.8, 131.9, 116.3, 74.5, 41.8, 40.3, 32.3, 24.1, 19.3.
Effects of ATH-amino acids on the mutagenicity of IQ. *S. typhimurium* TA98 was simultaneously incubated with ATH-amino acids and IQ for 20 min at 37°C in the presence of S9 mix. Data are given as mean ± SD of two independent assays. The numbers of IQ-induced revertants and spontaneous revertants were 1881 and 17, respectively.

High Performance Liquid Chromatography of ATH-amino acids

High Performance Liquid Chromatography (HPLC) was carried out on a Hewlett-Packard series 1100 instrument equipped with a photodiode array detector. The detector was set at 270 nm. A Mightysil RP-18 column (150 × 3 mm I.D., Kanto Chemical Co., Ltd., Tokyo, Japan) was employed, and the mobile phase was composed of acetonitrile (solvent A) and 25 mM phosphate buffer (pH 6.6, solvent B). The flow rate was kept constant at 0.5 mL/min. The system was run with the following gradient program: 10% A isocratic for 3 min, then linearly increased to 60% A in 9 min, and held for 15 min.

Results

Effects of ATH-amino acids on the IQ mutagenicity

None of the ATH-amino acids examined showed mutagenic activity in *S. typhimurium* TA98 with or without S9 mix (data not shown). The antimutagenic activities against the IQ mutagenicity are shown in Fig. 2. When the bacterial strain was simultaneously treated with IQ and ATH-amino acids in the presence of S9 mix, ATH-Phe and ATH-Trp showed a dose-dependent inhibition of the mutagenicity of IQ, where about 85% of the mutagenicity was inhibited at a 125 µg/plate dose. ATH-Leu, ATH-Val, ATH-
Ala, ATH-Gly, and ATH-Glu also showed a dose-dependent inhibition by 34 to 83% at 500 μg/plate dose. ATH-Lys showed a slight inhibition (10%) at 500 μg/plate, and only ATH-Asp behaved as a non-antimutagen against IQ. In contrast, no antimutagenic activity was observed when ATH-Phe or ATH-Val was added to the bacterial strain which was pretreated with IQ and S9 mix (Fig. 4).

**Effects of ATH-amino acids on the 4-NQO mutagenicity**

None of the ATH-amino acids showed mutagenic activity in *S. typhimurium* TA100 with or without S9 mix (data not shown). The antimutagenic activities against the 4-NQO mutagenicity are shown in Fig. 3. When the bacterial strain was simultaneously incubated with 4-NQO and ATH-amino acids, all ATH-amino acids inhibited 4-NQO mutagenicity. ATH-Leu and ATH-Ala were the most effective in the suppression of 4-NQO mutagenicity, where 72-82% of the mutagenic activity was suppressed with a dose at 125 μg/plate. The mutagenicity-inhibition by ATH-Phe, ATH-Trp, ATH-Val, and ATH-Gly ranged from 45 to 65% at 125 μg/plate. Also, ATH-Glu, ATH-Asp, and ATH-Lys showed about 50% inhibition at 125 μg/plate. In contrast, when several ATH-amino acids were added to the bacterial strain which was pretreated with 4-NQO, no suppressive effect was observed (Fig. 4).
The relationship between antimitogenic activity and hydrophobic property of ATH-amino acids

Fig. 5a shows the relationship between the antimitogenic activity of ATH-amino acids on IQ mutagenicity and their retention time recorded by the ODS-HPLC. Their hydrophobic property is nearly reflected by the retention time on an ODS-column. Among ATH-amino acids obtained from AITC and such amino acids as Leu, Phe, Trp, Val, Ala, and Gly, their suppressive effects on the IQ mutagenicity appeared to depend upon the hydrophobicity of the molecules. In contrast, no relationship was shown between the antimitogenic activity against 4-NQO and retention time (Fig. 5b).

Discussion

The effects of nine ATH-amino acids on the mutagenic activity of IQ or 4-NQO were examined with or without a metabolic activation system, S9 mix. When S. typhimurium TA98 was simultaneously incubated with IQ (4 ng/plate) and ATH-amino acids in the presence of S9 mix, most of the ATH-amino acids showed an inhibitory effect on the mutagenicity of IQ. The antimitogenic activity of ATH-amino acids appeared to depend upon the amino acid employed for their preparation. Among the ATH-amino acids, those derived from aromatic amino acid (Phe and Trp) behaved as a strong inhibitors of the IQ mutagenicity, and those made with aliphatic amino acid (except for Leu) showed a moderate inhibition of the mutagenicity. ATH-amino acids derived from acidic amino acids (Glu, Asp) and basic amino acid (Lys) showed no or slight inhibition. These results suggested that the antimitogenic activity of ATH-amino acids against IQ depends upon the substituent at the 5-position of the 2-thiobisulphamide structure (Fig. 1). It is known that IQ can be converted to N-hydroxylated IQ by CYP1A2-mediated activation, which is responsible for the mutagenicity to S. typhimurium TA98 (Okamoto et al., 1981; Yamazoe et al., 1983). The rat liver S9 fraction used in the present study contains such an enzyme as CYP1A2. Because IQ mutagenicity was not influenced by ATH-amino acids when they were added after metabolic activation of IQ with S9 mix (Fig. 4), it was assumed that ATH-amino acids can inhibit the S9 mix-mediated activation of IQ.

As for the effect of ATH-amino acids on 4-NQO mutagenicity, all ATH-amino acids tested behaved as antimitogenic agents when the ATH-amino acids were added to S. typhimurium TA100 with 4-NQO (0.1 μg/plate) before incubation. The antimitogenic activity of ATH-amino acids against 4-NQO was different from that against IQ. ATH-Leu, ATH-Ala, and ATH-Phe strongly suppress the 4-NQO mutagenicity. Also, ATH-Glu, ATH-Asp, and ATH-Lys showed antimitogenic activity against 4-NQO. When

Fig. 4 Effects of ATH-amino acids on the mutagenicity of IQ (■) or 4-NQO (□). The bacterial strains which had been incubated with a mixture of IQ and S9 mix or only 4-NQO were twice rinsed with phosphate buffer in order to remove the mutagen, and were treated with ATH-amino acids for a further 20 min. Data are given as mean ± SD of two independent assays.
ATH-amino acids were added to the bacterial strain that had been preincubated with 4-NQO, no antimutagenic effect was observed (Fig. 4), suggesting that 4-NQO was possibly inactivated by ATH-amino acids by means of a certain chemical modulation.

From the above results, it was concluded that ATH-amino acids could act as desmutagens (Kada and Shimoi, 1987) or blocking agents (Wattenberg, 1983) in the mutation of both S. typhimurium TA98 and TA100 strains. From the relationship between the antimutagenic activity of ATH-amino acids and their retention time obtained from the ODS-HPLC, which should nearly reflect a hydrophobicity of ATH-amino acids, it appeared that their suppressive effects on the IQ mutagenicity depends on hydrophobic property of ATH-amino acids molecules. Feng et al. (2003) showed that the lipophilicity of some phenolic compounds correlated well with the inhibition of 7-ethoxyresorufin-O-deethylase (EROD) activity, which is specific for CYP1A. The same authors described that the inhibition of EROD activity correlated with the antimutagenicity of the phenolic compounds. In another study (Conaway et al., 1996), it was shown that inhibitory effect of arylalkyl isothiocyanates on EROD activity increased with increasing alkyl chain length up to C₆, and then decreased with greater alkyl chain length (C₇-C₁₀). These facts suggested that the hydrophobicity of ATH-amino acids molecules may be important in the enzymatic reaction. Kinetic analysis of the inhibition of S9 mix-mediated activation of IQ by ATH-amino acids is presently underway along with structural analysis of the reaction product(s) of ATH-amino acids and 4-NQO.

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