Note

Formation of Thioxopyrrolidines and Dithiocarbamates from 4-Methylthio-3-butetyl Isothiocyanates, the Pungent Principle of Radish, in Aqueous Media

Hiroki Matsuoka, Yoshinori Toda, Kenji Yanagi, Asaka Takahashi,† Koichi Yoneyama,,* and Yasushi Uda††

Laboratory of Food Chemistry, Department of Bioproductive Sciences, and * Weeds Science Center, Utsunomiya University, 350 Mine, Utsunomiya 321, Japan

Received May 15, 1997

Reaction products of 4-methylthio-3-butetyl isothiocyanate (MTBI), the radish pungent principle, in aqueous media were identified and their antimicrobial activities were examined. A rapid degradation of MTBI in aqueous media afforded a mixture of 3-[hydroxy]methylene-2-thioxopyrrolidine (1), (Z)-3-[methylthio]-methylene-2-thioxopyrrolidine (2), its (E)-isomer (3), methyl 4-methylthiobutylthioisothiocyanate (4), methyl (Z)-4-methylthio-3-butenylthiolicarbamate (5), and its (E)-isomer (6). The products 1, 2, and 3 were detected at all pHs examined, while 4, 5, and 6 were formed at pH over 6.0. The formation of 4 from 6 was accompanied by an oxidation of methanethiol released from MTBI in aqueous media. Antimicrobial activities of 2 and 3 against all microbes examined were much lower than that of 1, which had MICs ranging from 50 to 400 μg/mL. As for 4, 5, and 6, antifungal activity were comparable to that of 1, but little antibiotic activities were observed. The antimicrobial activities of the six products were considered to be far lower than that of MTBI.

Key words: radish pungent principle; thioxopyrrolidines; dithiocarbamates; antimicrobial activity

Cruciferous vegetables are known to generate isothiocyanates through enzymatic hydrolysis of the corresponding glucosinolates. Isothiocyanates are a class of compounds being highly reactive with amino, thiol or hydroxy group-containing compounds. However, a few studies have been done on the stability and reactivity of isothiocyanates in food systems. Kawakishi et al. reported decomposition of allyl isothiocyanate and its interactions with proteins. In our previous study, we found that the radish pungent principle, (E)-4-methylthio-3-butetyl isothiocyanate (MTBI), was unstable in aqueous media and readily converted to 3-[hydroxy]methylene-2-thioxopyrrolidine (1) accompanied by release of methanethiol. In addition, a tetrahydro-β-carboline compound was found to be formed from 1 and l-tryptophan during a salting process of radish roots. Subsequent investigations confirmed that 1 had a wide antimicrobial spectrum (Matsuoka et al. submitted for publication in Food Sci. Technol. Int. Tokyo). On the other hand, 3-[α-methoxy]-methylene-2-thioxopyrrolidine (raphitin) or raphanustin was isolated as a naturally occurring antihypertensive and anesthetic agent or as a light-induced growth inhibitor from methanol extract of radish roots or seedlings. Kosemura et al. subsequently reported that 3-[α-methoxy]-methylene-2-thioxopyrrolidine was formed as the major product from MTBI in 50% aqueous methanol, but no 1 was detected in the medium. In our preliminary study, however, MTBI was converted to 1 as a major product along with a smaller amount of 3-[α-methoxy]-methylene-2-thioxopyrrolidine in 20–60% methanol-containing aqueous media, but the main product formed in media containing over 60% methanol changed from 1 to 3-[α-methoxy]-methylene-2-thioxopyrrolidine (unpublished data). Furthermore, MTBI did not form 3-[α-methoxy]-methylene-2-thioxopyrrolidine in any media without methanom. Similarly, a synthetic analogue of MTBI, 4-methoxy-3-butetyl isothiocyanate was converted to 1 in a buffer, but the major products formed in methanol- or ethanol-containing media were 3-[α,α-dimethoxy]-methylene-2-thioxopyrrolidine or its 3-[α,α-ethoxy]-methylene derivative. Hence, products formed from MTBI were considered to depend on reaction media. Radish roots, the most abundantly produced vegetable in Japan, are eaten as a freshly grated or processed food. Therefore, reaction products of MTBI, in particular those formed in the presence of water, which have not been studied in detail, should be investigated along with their biological properties. In this report, we describe identification of the reaction products of MTBI in aqueous media and their antimicrobial activities. Their formation mechanisms are also discussed.

Reaction products were surveyed by ODS-HPLC analysis. The figure shows a typical chromatogram of the products obtained in aqueous medium at pH 7.0, in which 6 major peaks (relative ratios: P1, 46.2%; P2, 6.6%; P3, 17.0%; P4, 1.3%; P5, 7.7%; and P6, 21.0%) were detected. Among them, generation of peaks P1, P2, and P3 were observed at all pHs examined, but P4, P5, and P6 were detected in the media at pH over 6.0. No other products were found in any aqueous reaction media examined.

To obtain larger amounts of these 6 reaction products, MTBI was sonicated in the buffer at pH 3.0 for P1, and at pH 9.0 for P2–P6, because of their favorable formation at these pHs. Identification of these products was done by the 1H-NMR,

![Fig. A Typical HPLC Profile of the Reaction Products Generated from MTBI in the Aqueous Medium at pH 7.0](image-url)

P1–P6: the reaction products. Analytical conditions were: column, LiChrosorb RP-18 (4.6x250 mm), mobile phase, 30% acetonitrile in 25 mM phosphate buffer (pH 6.5); detector, photo-diode-array.

---

† Present address: Nagano Prefecture Junior College, Minato, Nagano 380, Japan.

†† To whom correspondence should be addressed.
The spectral data obtained for P1 were identical to those of P2 and P3 were identical to those of (Z)-3-(methylthio)methyleene-2-thioxopyrrolidine (2) and its (E)-isomer (3), respectively, which had been reported by Kosemura et al. as the minor products from MTBI in a 50% methanolic medium. The relative amount of 3 formed in the medium at pH 7.0 was about 2.5-fold higher than that of 2. The spectral data for P4 were: UV \( \lambda_{max} \) (EtOH) \( \mathrm{nm} \)-(e): 270 (9400), 253 (8900); IR \( \nu_{max} \) (KBr) cm\(^{-1}\): 3160 (NH), 2973 (SMe), 2919 (SMe), 1508 (NC=S), 1452, 1429, 1388, 1092, 1043, 955; HR-EIMS \( m/z \): found, 209.0355; calcd. for \( \text{C}_{13} \text{H}_{15} \text{NS}_{2} \), 209.0367; EI-MS \( m/z \): 209 (M\(^{+}\), 8), 176 (M\(^{+}\) - SH, 8), 162 (M\(^{+}\) - SMe, 100), 114 (16), 103 (30), 91 (28), 72 (24); \(^{1}H\)-NMR \( \delta \) (CDCl\(_3\)) \( 7.05 \) [7.69] (NH, s), 3.76 [3.47] (1H, t, \( J = 6.8 \) Hz, C1), 3.78 [3.48] (1H, t, \( J = 6.4 \) Hz, C1), 2.64 [2.68] (1H, s, C3'), 2.54 (2H, t, \( J = 7.1 \) Hz, C4), 2.01 (1H, s, C5), 1.79 (2H, quint, \( J = 7.1 \) Hz, C3), 1.68 (2H, dt, \( J = 7.1 \) and 6.4 Hz, C2), \(^{13}C\)-NMR \( \delta \) (CDCl\(_3\)) : 199.1 (C=O), 146.8 [159.1] (C1, CH\(_2\)), 33.8 [33.7] (C3, CH\(_2\)), 27.3 (C7, C2), 26.3 [26.2] (C4, CH\(_3\)), 18.2 [18.9] (C5, CH\(_3\)), 15.5 (C5, CH\(_3\)). From the data, P4 was identified as methyl 4-methylthiobutyldithiocarbamate (4). The NMR data in brackets were assigned to those of a conformer due to a restraint of free rotation around the N-C bond in CDCl\(_3\). This was supported by measuring \(^{1}H\)-NMR spectra at various temperatures in both CDCl\(_3\) (25–60°C) and DMSO-\(d_{6}\) (25–120°C), in which the signals attributed to the conformers fused into a single peak at 60°C in CDCl\(_3\), and at 90°C in DMSO-\(d_{6}\). On the basis of the signal intensities, 4 was estimated to be in an equilibrium between the conformers with a ratio of about 2:1 in CDCl\(_3\) at 25°C. On the other hand, both P5 and P6 had similar spectral features to those of 4, which were well coincident with those of methyl 4-methylthio-3-butenylidithiocarbamate. On the basis of a difference in the coupling constants of vinyl protons for (P5, 6.50, 1H, \( J = 9.3 \) and 7.1 Hz), (C3) and \( \delta 6.10 \) (1H, dt, \( J = 9.3 \) and 1.5 Hz, C4); for P6, \( \delta 5.35 \) (1H, dt, \( J = 15.1 \) and 6.8 Hz, C3) and \( \delta 6.15 \) (1H, d, \( J = 15.1 \) Hz, C4) recorded in DMSO-\(d_{6}\), P5 was identified as (Z)-isomer (5) of the dithiocarbonate and P6 as (E)-isomer (6). In the \(^{1}H\)-NMR data (CDCl\(_3\)) of 5 and 6, the signals assigned to C3'-CH\(_3\) for 5, \( \delta 2.62 \) [2.68] (3H, s); for 6, \( \delta 2.62 \) [2.68] (3H, s), C1-CH\(_2\) for 5, \( \delta 3.80 \) [3.51] (1H, t, \( J = 6.8 \) Hz) and \( \delta 3.82 \) [3.53] (1H, t, \( J = 6.4 \) Hz); for 6, \( \delta 3.77 \) [3.49] (1H, t, \( J = 6.8 \) Hz) and \( \delta 3.78 \) [3.50] (1H, t, \( J = 6.4 \) Hz) and NH (for 5, \( \delta 7.15 \) [7.75] (1H, s); for 6, \( \delta 7.30 \) [7.89] (1H, s)) began to fuse into a single peak at 50°C in CDCl\(_3\), and fused completely at 90°C in DMSO-\(d_{6}\), suggesting that both 5 and 6 were also in an equilibrium between their conformers. From their signal intensities, ratios of the conformers in CDCl\(_3\) at 25°C were estimated to be about 5:1 for 5 and about 3:1 for 6, respectively. It is well known that isothiocyanates like MTBI react with thiol compounds to give dithiocarbamates like 5 and 6. However, the formation of 4 was considered to be proceeded via a different mechanism, because GC-MS analysis of MTBI used in this study gave evidence that the amount of 4-methylthiobutyl isothiocyanate as an impurity of MTBI was negligible and not enough to form a detectable amount of 4. Since our previous study showed that methanethiol was evolved during formation of 1, a possibility that 4 was formed from 6 accompanied by an oxidation of methanethiol to dimethylsulfide was considered. Thus, we investigated to confirm or disprove this possibility. Table I shows that 4 was formed from 6 only in the presence of methanethiol. Formation of dimethylsulfide was also confirmed therein. These results clearly demonstrated that the formation of 4 from 6 proceeded by the addition of hydrogen which were evolved through oxidation of methanethiol to dimethylsulfide.

From the results described above, reaction pathways of MTBI in the presence of water were summarized as shown in the Scheme. In the aqueous media, the addition of water to MTBI preponderantly proceeded along with intermolecular cyclization to form 1. The products 2 and 3 were supposed to be formed by an intermolecular cyclization of MTBI, which should proceed
### Table II. Antimicrobial Activity of Thioxopyrroldines and Dithiocarbamates

<table>
<thead>
<tr>
<th>Microorganism (Molds)</th>
<th>MIC (µg/ml) of the products</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. fumigatus</td>
<td></td>
<td>200</td>
<td>&gt;800</td>
<td>800</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>C. coloecae</td>
<td></td>
<td>200</td>
<td>400</td>
<td>800</td>
<td>200</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>E. chevalieri</td>
<td></td>
<td>200</td>
<td>400</td>
<td>400</td>
<td>200</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>M. racemosus</td>
<td></td>
<td>400</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>200</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Microorganism (Yeasts)</th>
<th>MIC (µg/ml) of the products</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td></td>
<td>400</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>200</td>
</tr>
<tr>
<td>S. pombbe</td>
<td></td>
<td>200</td>
<td>400</td>
<td>800</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(Bacteria)</th>
<th>MIC (µg/ml) of the products</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. subtilis</td>
<td></td>
<td>50</td>
<td>400</td>
<td>400</td>
<td>&gt;400</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td>100</td>
<td>&gt;800</td>
<td>800</td>
<td>&gt;400</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td></td>
<td>200</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>&gt;400</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td></td>
<td>100</td>
<td>800</td>
<td>&gt;800</td>
<td>&gt;400</td>
<td>&gt;400</td>
<td>&gt;400</td>
</tr>
</tbody>
</table>

* a Maximum dose was 800 µg/ml for the thioxopyrroldines and 400 µg/ml for the dithiocarbamates.
* b The MICs were determined in glucose-peptone-broth at pH 5.0.
* c The MICs were determined in nutrient broth at pH 6.0.
* d Not examined.

in aqueous methanolic media. Methanethiol released in the process of the formation of 1 reacted with MTBI yielding the butenylthiocarbamates (5 and 6) and was also involved in the formation of the butylthiocarbamate (4). Consequently, MTBI, the pungent principle of radish, is highly reactive in food systems, and the thioxopyrroldines and dithiocarbamates should be formed in grated or processed radish.

Since MTBI and its reaction products identified in this study should occur in grated and processed radish, it is significant to investigate biological and physiological properties of the products. In this study, antimicrobial activities of the reaction products were examined. The results are summarized in Table II. Among the thioxopyrroldines, 1 showed an antimicrobial property against the microbes examined, where MICs ranged from 50 to 400 µg/ml. In contrast, both 2 and 3 exhibited fair weak growth-inhibition against all microbes tested. The results suggested that the hydrophilic hydroxy group of 1 was preferable for the antimicrobial activity. On the other hand, dithiocarbamates (4, 5, and 6) showed an antifungal activity comparable to that of 1, but little antibacterial activity was observed. It is known that dithiocarbamates have an antifungal activity. Since MTBI was unstable in a moistened medium, no available MICs against the microbes could be measured. However, according to our previous results, which were obtained by contact between MTBI and microbes in a tightly sealed gaseous phase, MTBI completely inhibited the growth of some fungi, such as Cladosporium coloecae and Alternaria helianthi at a dose of 7.5 µmol/plate (head space vapor; about 64 µl). In that case, the actual amount vaporized in the head space of the plate was estimated to be about 0.16% (1.9 µg) of the dose. Considering the result for MTBI, though it had been obtained in a different way from this study, antimicrobial activities of the six products were presumed to be far lower than that of MTBI.

### Experimental

**Instrumental analyses.** HPLC analysis was done on a Hitachi L-6200 instrument with a photo-diode array detector (254-400 nm) using a LiChrosorb RP-18 column (4.6 × 250 mm). Mobile phase was 30% acetonitrile in 25 mm phosphate buffer (pH 6.5). MS spectra were recorded at 70 eV on a JEOL AX 500 instrument with a capillary column (DB1, 0.25 mm i.d. × 25 m). H2(400 MHz) and 13C(100 MHz) NMR spectra were measured on a JEOL EX 400 instrument in DMSO-d6 or CDCl3 with TMS as an internal standard. IR (KBr disk) and UV (EtOH) spectra were obtained by a Horiba FT-200 infrared spectrometer and a Hitachi 330 spectrophotometer, respectively.

Survey of reaction products in the presence of water. MTBI (30 µmol, 4.8 mg) which had been obtained from radish seedlings by our previous methods, was sonicated in 3 ml of 0.1 M McIlvaine buffer (pH 3–9) for 5 h at about 40°C. The reaction mixtures were extracted with ethyl acetate, and the extracts were evaporated to dryness. Components therein were analyzed by the HPLC.

Isolation of the products generated in aqueous media. MTBI (1 mmol, 159 mg) was sonicated in 50 ml of McIlvaine buffer (pH 3.0 or 9.0) containing 0.1% triton X-100 for 5 h at ca. 40°C. The reaction mixture prepared at pH 3.0 was separated by our previous method, which gave 94 mg (73%) of 1 as a pale yellow powder. The reaction mixture at pH 9.0 was extracted with ethyl acetate, and the extract was first fractionated on an NH-silica gel column (12 × 200 mm). A mixture of 4-6 was eluted with 20% chloroform in n-hexane, and that of 2 and 3 was obtained by subsequently eluting the column with 40% chloroform in n-hexane. The products 2 and 3 were separated on an ODS column (20 × 300 mm) with 15% acetonitrile in water (6/1 mL/tube), in which 2 (fraction nos. 34–51) and 3 (fraction nos. 65–87) were isolated as pale yellow powders with yields 1.3 mg (0.8%) for 2 and 9.7 mg (61.5%) for 3, respectively. The products 4, 5, and 6 were purified on a silica gel column (12 × 250 mm) with 5% diethyl ether in n-hexane (10 mL/tube). Yields were: for 4 (fraction nos. 79–110), 4.9 mg (2.3%) as a pale yellow oil; for 5 (fraction nos. 34–44), 20.2 mg (9.8%) as a colorless oil; and for 6 (fraction nos. 51–69), 26.1 mg (12.6%) as a colorless oil.

Formation of 4 from 6 in the presence of methanethiol. The product 6 (97 µmol, 20 mg) dissolved in 50 mL of 40% aqueous methanethiol was sonicated for 90 min at pH 3.0 or 9.0, during which methanethiol (5.1 g) was introduced with a stream of nitrogen. After extraction of the reaction mixture with ethyl acetate, the organic layer was analyzed by HPLC for 4 and 6. Dimethylsulphide therein was measured by GC: a Shimadzu 6A MP GC with a dual-FID; column: 5% SP-1000 (3 mm x 2 m); column temp., 40°C; and nitrogen flow rate, 40 ml/min.

**Assay for antimicrobial activity.** Molds used were Aspergillus fumigatus IFO 7080, Cladosporium coloecae IFO 6698, Eurotium chevalieri IFO 4090, and Mucor racemosus IFO 5403, and yeasts examined were Candida albicans IFO 1061 and Schizosaccharomyces pombe IFO 0638. Bacterial strains used were Escherichia coli ATCC 11775, Salmonella typhimurium ATCC 13311, Bacillus subtilis ATCC 6633, and Staphylococcus epidermidis ATCC 14990. MIC was measured using 0–800 µg/ml of test compounds dissolved in a glucose–peptone broth (pH 5.0) at 25°C for the molds and yeasts, and in a nutrient broth (pH 6.0) at 37°C for the bacteria. MICs were taken after the incubation for 24 h for the bacteria, 72 h for the yeasts and 120 h for the molds. Inoculated scales were: 5 × 10⁶ colony forming unit (CFU)/ml for the molds and yeasts; and 1 × 10⁶ CFU/ml for the bacteria.

**Acknowledgment.** We are grateful to Dr. Y. Yamada, the Department of Education, Utsunomiya University, for his valuable suggestions in the analysis of the NMR data.

**References**