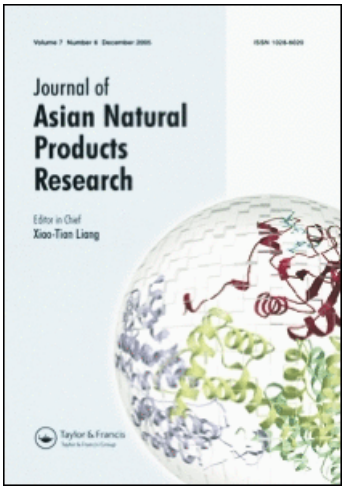

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ORIGINAL ARTICLE

Two novel sulfur compounds from the seeds of *Raphanus sativus* L.

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The seeds of *Raphanus sativus* L., known as Lai-fu-zi in traditional Chinese Medicine, are always roasted before clinical use for avoiding nausea. During an investigation of the chemical difference between roasted and pre-roasted products, two novel sulfur-containing compounds, which mainly existed in the pre-roasted products, were isolated. Their structures and absolute configurations were established by spectroscopic and X-ray diffraction analysis.

Keywords: *Raphanus sativus* L.; sulfur-containing compound; processing Chinese medicine

1. Introduction

The process of Chinese medicine is a traditional pharmaceutical technology and known as one of the unique characters of traditional Chinese medicine (TCM). Hopefully, the toxicity of the TCM is deduced, or the side effects are minimized, or even the functions are changed after post-harvest processing. Roasting is one of the processing methods which make materials dry, kill the endogenous enzyme, and so on. It can be used to treat the seeds of *Raphanus sativus* L., which are known as Lai-fu-zi in TCM. Lai-fu-zi has been used for the treatment of gastrointestinal diseases and as expectorants in China for thousands of years. The roasted products have been used more often than the raw products to avoid some side effects such as nausea. To explore the function of the roasting process on the seeds of *R. sativus*, the chemical constituents of the roasted and the pre-roasted products were investigated. As a result, two novel sulfur compounds **1** and **2**, which mainly existed in the pre-roasted products, were discovered (Figure 1). According to HPLC analysis, the content of compound **1** in the pre-roasted products was 0.30% and reduced to 0.044% after the roasting process. Compound **2** existed only in the pre-roasted products (0.042%). Herein, we report the isolation, structural elucidation, as well as the biological test of these two novel sulfur-containing compounds.

2. Results and discussion

Compound **1** was obtained as colorless needles and had a molecular formula of $C_6H_{11}NOS_3$ as established by HR-ESI-MS at *m/z* 210.0078 $[M+H]^+$, whose melting point was determined as 196–198°C and $[\alpha]_D^{20}$ was −109.8 (water). The ^1H NMR spectrum exhibited the resonances for one

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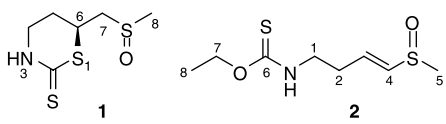

Figure 1. The structures of compounds **1** and **2**.

tertiary methyl (δ_H 2.62, s) and one downfield proton (δ_H 10.41, s, NH). The ^{13}C NMR and DEPT spectra displayed six carbons, including a thioketone group (δ_C 190.9, s), one methenyl, three methylenes and one methyl. The $^1H-^1H$ COSY and HMBC experiments did not give more information about the structure except for the fragment of $-CH_2-CH_2-CH-CH_2-$. The gross structure and the absolute stereochemistry of **1** were established by X-ray crystallographic analysis (Figure 2). Thus, **1** was determined as *S*-6-(methylsulfinyl)methyl-1,3-thiazinan-2-thione. The CCDC number of compound **1** is 742902.

Compound **2** was shown as one peak in HPLC analysis and isolated as colorless crystals, with mp 80–82°C and $[\alpha]_D^{20}$ −86.2 (EtOH). Its molecular formula of $C_8H_{15}NO_2S_2$ was determined by HR-ESI-MS data at *m/z* 222.0629 $[M+H]^+$. The NMR spectra of **2** recorded in $CDCl_3$ at room temperature gave two sets of signals (2.2:1). From the COSY spectrum, it was clear that the major compound showed signals of H-4 and H-3 at δ_H 6.43 and 6.47 with a *trans* coupling constant of 15.0 Hz. The minor signal was more second order and showed signals of H-4 and H-3 at δ_H

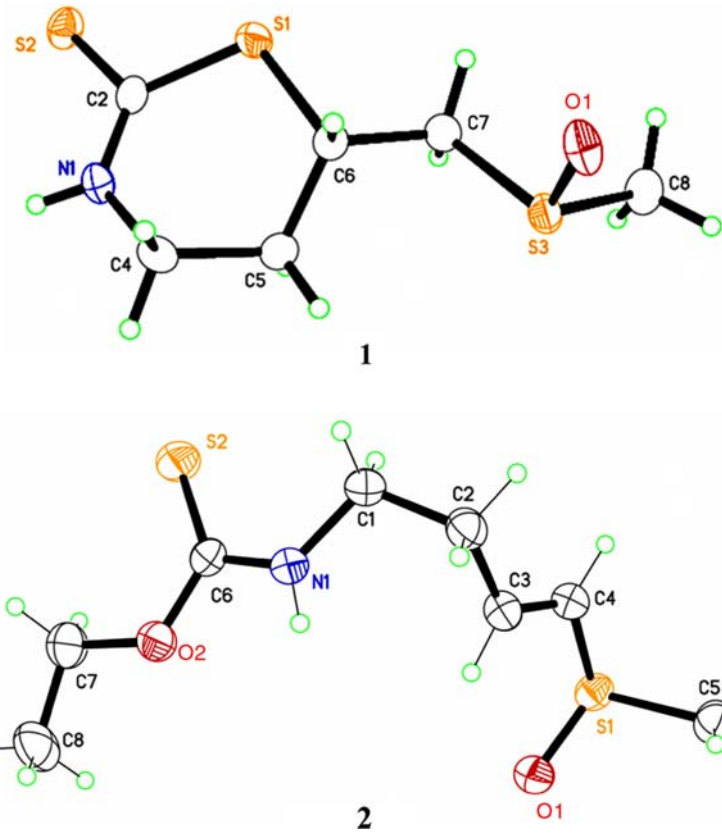

Figure 2. X-ray ORTEP drawings of compounds **1** and **2**.

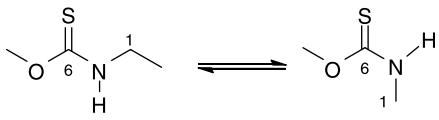

Figure 3. Isomerization of compound **2** in solvent.

6.40 and 6.43, also with an apparent coupling constant of 15.0 Hz. Thus, both isomers have *trans* double bonds. Inspection of the chemical shifts of the other signals indicated that the largest shift differences between the two compounds are experienced by H-1 and the NH protons, and not H-2. This implies that isomerization is caused by rotation about the thioamide bond, as shown in Figure 3. These were confirmed by a ^{1}H NMR experiment in DMSO-d_6 at different temperatures. The spectra recorded at 298 K showed two sets of signals (3:1) and the signals of H-4 were very clear [δ_H 6.63 (1H, d, $J = 15.1$ Hz) and 6.62 (1H, d, $J = 15.1$ Hz)]. When the temperature was raised to 343 K, there was almost only one signal of H-4 at δ_H 6.59 (1H, d, $J = 15.1$ Hz). Finally, the structure of **2** was established by X-ray crystallographic analysis (Figure 2). Therefore, **2** is identified as *O*-ethyl *N*-(*E*)-4-(methylsulfinyl)but-3-enylcarbamothioate. The CCDC number of compound **2** is 742907.

It is well known that the Brassicaceae plant contains a group of glucosinolates, which coexist with endogenous thioglucosidases called myrosinases, and can be hydrolyzed to a wide range of degradation products, such as isothiocyanates, nitriles, thiocyanates, epithionitriles, oxazolidinethiones, and so on [1–4]. Glucosinolate degradation products, especially isothiocyanates, have anticarcinogenic properties [5]. However, the presence of degradation products is not always beneficial [2]. In our study, neither of the two sulfur compounds showed cytotoxicity on human tumor cell lines (HL-60, Molt-4, A549, and Bel-7402, $IC_{50} > 100\,\mu M$) or antimicrobial activities (*Candida albicans*, *Aspergillus niger* and sake yeast, minimum inhibitory concentration (MIC) > 50 μg/ml; *Helicobacter pylori*, MIC > 200 μg/ml). These suggested that the roasting process might play a role in preventing glucosinolates degradation. Herein, we report the isolation and structural elucidation of the two novel sulfur compounds. A novel structure skeleton for glucosinolate degradation products is firstly reported.

3. Experimental

3.1 General experimental procedures

Melting points were determined on a BEIJING TECH XT-4 (Beijing Tech Instrument Co., Ltd, Beijing, China) apparatus and are uncorrected. Optical rotations were obtained on a GYROMAT-HP automatic process polarimeter (Anton Paar, Innsbruck, Austria). UV spectra were obtained on a Shimadzu UV-256FW spectrophotometer (Shimadzu Seisakusho Ltd, Kyoto, Japan). IR spectra were recorded on a Nicolet FT-IR Avatar 330 spectrophotometer (Thermo Nicolet Corporation, Madison, WI, USA). ^{1}H and ^{13}C NMR spectra were measured on a Varian INOVA 600 spectrometer (Varian Inc., Walnut Creek, CA, USA). ESI-MS and HR-ESI-MS spectra were performed on a Waters Q-TOF Ultima Global mass spectrometer (Waters Corp., Milford, MA, USA). HPLC analysis was performed using HPLC (Agilent 1100; Agilent Technologies, Palo Alto, CA, USA) system coupled to a photodiode array detector (DAD). The separation column (250 × 4.6 mm, L × i.d.) was prefilled with Luna 5μ C18 silica gel (Phenomenex, Torrance, CA, USA), and a linear gradient of 0.02% H_3PO_4 in H_2O and MeOH was used as the mobile phase. Silica gel (100–200, 200–300 mesh; Qingdao Marine Chemical Inc., Qingdao, China) and silica gel GF-254 (Qingdao Marine Chemical Inc.) were used for column chromatography and TLC, respectively.

3.2 Plant material

The seeds of *R. sativus* L. were purchased from Juancheng Lianghai TCM Pieces Co. Ltd (Heze, China) in 2006 and identified by Prof. Feng-Qin Zhou, Shandong University of Traditional Chinese Medicine. A specimen (No. 060513) is deposited in the School of Chinese Pharmacy, Shandong University of Traditional Chinese Medicine, Jinan.

3.3 Extraction and isolation

The seeds of *R. sativus* (2.5 kg) were repeatedly extracted with 15 liters of 60% EtOH three times after being soaked in water for 4 h. Then, the EtOH extract (340 g) was partitioned equally between water and EtOAc three times to obtain the EtOAc extract (65 g), which was followed by fractionation by VLC over silica gel eluting with CH_2Cl_2 with an increasing amount of MeOH. The second fraction (CH_2Cl_2–MeOH, 99:1, 4.1 g) was purified by Sephadex LH-20 (CH_2Cl_2–MeOH, 1:1) to give compound **2** (320 mg). Compound **1** (490 mg) was crystallized as colorless needles from the last fraction (MeOH, 1.9 g).

3.3.1 S-6-(methylsulfinyl)methyl-1,3-thiazinan-2-thione (*1*)

Colorless needles; mp 196–198°C; $[\alpha]_D^{20}$ −109.8 (water); UV (MeOH) λ_{max} (nm): 206, 239, 287; IR (KBr) ν_{max}: 3099, 3019, 2894, 1542, 1417, 1341, 1313, 1236, 1056, 1020, 975, 904, 806, 692, 637, 570 cm^{-1}. ^1H NMR (600 MHz, DMSO-d_6): δ 1.86 (1H, m, H-5a), 2.31 (1H, m, H-5b), 2.62 (3H, s, H-8), 2.94 (1H, dd, $J = 13.2$, 4.8 Hz, H-7a), 3.15 (1H, dd, $J = 13.2$, 9.3 Hz, H-7b), 3.34 (1H, m, H-4a), 3.45 (1H, m, H-4b), 3.76 (1H, m, H-6), 10.41 (1H, s, NH). ^{13}C NMR (125 MHz, DMSO-d_6): δ 25.6 (C-5), 38.4 (C-8), 38.5 (C-6), 42.2 (C-4), 57.3 (C-7), 190.9 (C-2). ESI-MS: *m/z* 210 $[M+H]^+$; positive HR-ESI-MS: *m/z* 210.0078 $[M+H]^+$ (calcd for $C_6H_{12}NOS_3$, 210.0081).

Crystal data for **1**: a suitable colorless crystal ($0.34 \times 0.20 \times 0.15\ mm^3$) was grown by slow evaporation of methanol at room temperature. Diffraction intensity data were acquired with a Bruker SMART APEX single-crystal X-ray diffractometer with graphite-monochromated Mo Kα radiation ($\lambda = 0.71073$ Å). The structure was solved by direct methods (SHELXS-97) and expanded using Fourier techniques (SHELXS-97). Orthorhombic, space group $P2_12_12_1$, $T = 298$ (2) K, $a = 8.3400$ (15) Å, $b = 8.7935$ (16) Å, $c = 13.041$ (2) Å, $V = 956.4$ (3) $Å^3$, $D_c = 1.454\ mg/m^3$, $Z = 4$, $F(000) = 440$, μ (Mo Kα) $= 0.721\ mm^{-1}$. A total of 5010 reflections were collected in the range $2.79° < \theta < 25.48°$, with 1779 independent reflections [R(int) = 0.0266], completeness to θ_{max} was 99.9%; psi-scan empirical absorption correction applied; full-matrix least-squares refinement on F^2, the number of data/restraints/parameters was 1779/0/101; Flank parameter −0.01 (12); goodness of fit on $F^2 = 1.052$; final R indices [$I > 2\sigma(I)$], $R_1 = 0.0370$, $wR_2 = 0.0855$; R indices (all data), $R_1 = 0.0390$, $wR_2 = 0.0865$; largest difference peak and hole, 0.355 and −0.188 e/$Å^3$.

3.3.2 O-ethyl N-(E)-4-(methylsulfinyl)but-3-enylcarbamothioate (*2*)

Colorless needles; mp 80–82°C; $[\alpha]_D^{20}$ −86.2 ($c = 0.1$, EtOH). ^1H NMR (600 MHz, $CDCl_3$): δ 1.31 (3H, t, $J = 7.1$ Hz, H-8), 2.61 (2H, dt, $J = 6.5$, 6.5 Hz, H-2), 2.63 (3H, s, H-5), 3.71 (2H, dt, $J = 6.5$, 6.5 Hz, H-1), 4.47 (2H, q, $J = 7.1$ Hz, H-7), 6.43 (1H, d, $J = 15.0$ Hz, H-4), 6.47 (1H, dt, $J = 15.0$, 6.5 Hz, H-3), 6.78 (1H, s, NH). ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.3 (C-8), 30.8 (C-2), 40.6 (C-5), 43.3 (C-1), 66.4 (C-7), 136.0 (C-3), 136.1 (C-4), 190.7

(C-6). Isomer: ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.38 (3H, t, $J = 7.1$ Hz, H-8), 2.50 (2H, dt, $J = 6.5$, 6.5 Hz, H-2), 2.63 (3H, s, H-5), 3.43 (2H, dt, $J = 6.5$, 6.5 Hz, H-1), 4.55 (2H, q, $J = 7.1$ Hz, H-7), 6.40 (1H, $J = 15.0$ Hz, H-4), 6.43 (1H, m, H-3), 7.29 (1H, br, s, NH). ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.3 (C-8), 31.7 (C-2), 40.6 (C-5), 41.4 (C-1), 67.9 (C-7), 135.3 (C-3), 136.5 (C-4), 190.0 (C-6). All the signals were assigned by ^{1}H–^{1}H COSY and HMBC experiments. ESI-MS: *m/z* 222 $[M+H]^+$; positive HR-ESI-MS: *m/z* 222.0629 $[M+H]^+$ (calcd for $C_8H_{16}NO_2S_2$, 222.0622).

Crystal data for **2**: a suitable colorless crystal ($0.43 \times 0.34 \times 0.15$ mm^3) was grown by slow evaporation of the ethyl acetate at room temperature. Diffraction intensity data were acquired with a Bruker SMART APEX single-crystal X-ray diffractometer with graphite-monochromated Mo Kα radiation ($\lambda = 0.71073$ Å). The structure was solved by direct methods (SHELXS-97) and expanded using Fourier techniques (SHELXS-97). Orthorhombic, space group, $P2_12_12_1$, $T = 298$ (2) K, $a = 7.8022$ (13) Å, $b = 8.6674$ (14) Å, $c = 16.728$ (3) Å, $V = 1131.3(3)$ Å^3, $D_c = 1.300$ mg/m^3, $Z = 4$, $F(000) = 472$, μ (Mo Kα) $= 0.442$ mm^{-1}. A total of 4974 reflections were collected in the range $2.44° < \theta < 25.23°$, with 1645 independent reflections [R(int) = 0.0187], completeness to θ_{max} was 100.0%; psi-scan empirical absorption correction applied; full-matrix least-squares refinement on F^2, the number of data/restraints/parameters was 1645/0/120; Flank parameter 0.02 (9); goodness of fit on $F^2 = 1.047$; final R indices $[I > 2\sigma(I)]$, $R_1 = 0.0283$, $wR_2 = 0.0726$; R indices (all data), $R_1 = 0.0299$, $wR_2 = 0.0734$; largest difference peak and hole, 0.158 and -0.128 e/Å^3.

3.4 Antitumor tests

Lung cancer cell line A549, hepatocellular carcinoma cell line Bel-7402, leukemia cell line HL-60, and Molt-4 were cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and maintained at 37°C in a humidified atmosphere of 5% CO_2. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed as described previously [6]. Cells (3000 cells) were seeded on 96-well microtiter plates in RPMI-1640 medium with 10% FBS and incubated overnight. Different doses of compounds **1** and **2** were then added to the cells, which were then cultured for another 72 h. The MTT reagent was added to the cell supernatant so as to give a final concentration of 0.5 mg/ml of MTT. After 3 h at 37°C, the cell culture medium was removed. Formazan crystals in adherent cells were dissolved in 200 μl DMSO and the absorbance of the formazan solution was measured at 570 nm.

3.5 Antimicrobial tests

The *in vitro anti-H. pylori* activities of compounds **1** and **2** were estimated by determining the MIC with agar dilution method according to the protocols described previously [6]. A series of agar plates were prepared with the base of Campylobacter selective agar (Merck, Darmstadt, Germany) containing 5% of fetal bovine serum. Then, various concentrations of twofold diluted test compounds were dispersed into the prepared agar plates. Cells of *H. pylori*, suspended in saline at the density of 10^8 cfu/ml, were added to the well-prepared agar plates and were incubated at 37°C for 72 h under an atmosphere of 5% O_2, 10% CO_2, and 85% N_2. Blank control and positive control were performed using the same conditions as described above, except that, in the case of blank controls, no compound was dispersed into the agar plates, while in the case of positive controls, various concentrations of twofold diluted metronidazole were dispersed. The

MIC value was defined as the lowest concentration of the compound for inhibiting the visible growth.

The *in vitro* antifungal activities against *C. albicans*, *A. niger*, and sake yeast were performed as described previously [7]. The fungi were incubated in Sabouraud dextrose broth at 37°C for 48 h with the respective compounds, and the positive control (Amphotericin B) was dissolved in DMSO. The blank controls of fungal cultures were incubated with limited DMSO under the same conditions.

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