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AMINO ACID PRECURSORS OF THE GARLIC-LIKE ODOUR IN *SCORODOCARPUS BORNEENSIS*

KIKUE KUBOTA,* HISAKO HIRAYAMA, YUKI SATO, AKIO KOBAYASHI and FUMIO SUGAWARA†

Laboratory of Food Chemistry, Ochanomizu University, 2-1-1 Ohtsuka, Bunkyo-ku, Tokyo 112-8610, Japan; † Department of Applied Biological Science, Science University of Tokyo, Noda, Chiba 278-0022, Japan

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Key Word Index—*Scorodocarpus borneensis*; Olacaceae; Malaysia; amino acid odour precursor; *S*-[(methylthio)methyl]-L-cysteine sulfoxide; *S*-[(methylthio)methyl]-L-cysteine; methyl methylthiomethyl disulfide; bis(methylthiomethyl) disulfide.

Abstract—Two new natural amino acids, (R_s)-3-[(methylthio)methylsulfinyl]-L-alanine and *S*-[(methylthio)methyl]-L-cysteine, were isolated from the fruit of *Scorodocarpus borneensis* which is known to have a garlic-like odour. C–S lyase-mediated enzymatic conversion showed that both amino acids play an important role in developing the main odorous components of methyl methylthiomethyl disulfide and bis(methylthiomethyl) disulfide. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Scorodocarpus borneensis Becc. is a tall tree belonging to the Olacaceae whose leaves, flowers and fruit develop a strong garlic-like odour, especially when they are crushed. We have reported in previous papers that methyl methylthiomethyl disulfide (I) and bis (methylthiomethyl) disulfide (II) were the main odour compounds and that (II) also strongly inhibited the growth of fungi [1]. Both compounds have a disulfide moiety in their structure which suggests that they were enzymatically formed in the same way as related compounds in *Allium* species [2]. The present paper describes the isolation and structural elucidation of the amino acid precursors of I and II from *S. borneensis*.

RESULTS AND DISCUSSION

A boiling water extract from the fruit pulp of *S. borneensis* was purified by column chromatography in an Amberlite IR 120-B (H form) column to give amino acid-rich fraction. Further purification by prep. HPLC afforded two sulfur-containing amino acids, compounds **1** and **2**, as a white powder in respective yields of ca 0.01% and 0.007% wet weight.

The HRFABMS data of **1** demonstrated the elemental composition of $C_5H_{11}NO_3S_2$, and IR data revealed the presence of sulfoxide (1028 cm^{-1}), carboxyl and amino groups (3400, 3020, 1605 cm^{-1}). The ^{1}H-NMR spectrum showed a methyl signal adjacent to sulfur at δ 2.30, two *dd* signals of the asymmetric methylene protons of $S(O)CH_2CH$ at δ 3.54 (J = 6.6, 13.7 Hz) and 3.29 (J = 7.0, 13.7 Hz), two *d* signals of $SCH_2S(O)$ at δ 3.99 (J = 13.9 Hz) and 4.20 (J = 14.6 Hz), and a triplet of the methyne proton at δ 4.23 (J = 7.0 Hz). ^{13}C-NMR signals assigned by DEPT and ^{13}C-^{1}H HETECORR experiments indicated four signals corresponding to each proton. These data collectively show that **1** had a $CH_3SCH_2S(O)CH_2CH$ moiety in the molecule. In addition, **1** gave the signal of a carboxyl group at δ173.9 by carbon spectrum. A comparison of these data with those in the literature showed the planar structure of **1** to be identical to that of 3-[(methylthio)methylsulfinyl]-L-alanine, CH_3S-$CH_2SOCH_2CH(NH_2)COOH$, which has been named marasmine [3]. γ-Glutamyl-marasmine (**3**), a natural dipeptide, which has been isolated from the Basidiomyceteous mushrooms, *Marasmius alliaceus*, *M. scorodonius* and *M. prasiosmus* by Gmelin *et al.*, are known for their garlic-like odour [3]. Broek *et al.* have determined the absolute configuration of the sulfoxide sulfur atom of **3** by total syntheses of both epimers for the S atom and by comparing the J values from ^{1}H-NMR data [4]. The signal pattern for $CHCH_2S(O)$ showed the AB part of the ABX spectrum and the (R_s) isomer displayed typical splitting by the coupling constants of J_{AX} = 8.6 Hz, J_{BX} = 5.3 Hz and J_{AB} = 13.6 Hz. On the other hand, the same study showed that the (S_s) isomer did not display a clear quartet line by the slightly different values of J_{AX} = 10.4 Hz, J_{BX} = 3.9 Hz and J_{AB} = 13.6 Hz. A

* Author to whom correspondence should be addressed.

similar difference in signals has been observed between each epimer of the sulfoxide of *S*-oxo-*S*-[(methylthio)methyl]-L-cysteinol or sparsomycin [5]. In the present study, the proton signals of $CHC\underline{H}_2S(O)$ for **1** exhibited eight clear lines with corresponding coupling constants; that is, the absolute configuration of the sulfoxide sulfur atom was determined as (*R*) and different from that of **3** (S_s). The stereostructure of the cysteine part will be described later.

HRFABMS data for compound **2** gave the elemental composition of $C_5H_{11}NO_2S_2$, and absorptions of both carboxyl and amino groups were observed by IR like those in **1**, but without a sulfoxide group. ^{1}H-NMR data showed a singlet signal of the methyl proton of CH_3S at δ 2.18, a triplet of the methylene protons of SCH_2S at δ 3.77, two *dd* signals of asymmetric methylene protons adjacent to the sulfur and α-carbon of amino acid at δ 3.15 and 3.24, and a *dd* signal of the α-proton of the cysteine moiety at δ 3.97. All signals in the ^{13}C NMR spectrum were assigned by ^{13}C-^{1}H HETECORR. Four signals corresponding to the protons supported the chemical structure, while the signal of a carboxyl group was also observed. These data enabled the planar structure of **2** to be determined as *S*-[(methylthio)methyl]-cysteine. To confirm this structure and the absolute configuration of the α-amino acid, the (D)- (**4**) and (L)-isomers (**5**) were respectively synthesized from D- or L-cystine and methylthiomethyl chloride. The NMR spectra of synthesized **4** and **5** coincided well with that of **2**. Since the enantiomers were not distinguishable from each other by NMR, the three kinds of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) derivatives from natural **2** and from synthesized **4** and **5**, were prepared. They were clearly separated by HPLC, and the cysteine moiety of **2** was confirmed to be L-cysteine from a comparison of the RR_t values.

Compounds **1** and **2** were the main components among some peaks detected by HPLC under the described conditions. The concentrations of **1** and **2** in the pulp of *S. borneensis* were calculated as ca 1.9 mg/g (dry wt.) and 1.2 mg/g (dry wt.) which are each almost the same level as that of *S*-methylcysteine sulfoxide in *Brassica* [6]. Considering that both amino acids were present at almost the same level and that *S. borneensis* is a higher plant, it is natural to think that the chirality of the cysteine moiety would be the same between compounds **1** and **2**. These facts enabled the chemical structures of **1** and **2** to be assigned as (R_s)-3-[(methylthio)methylsulfinyl]-L-alanine and *S*-[(methylthio)methyl]-L-cysteine, respectively, as shown in Fig. 1. Both of them were isolated first as natural amino acids, although the marasmine moiety has been found as γ-glutamate. It was noted that the chirality of the sulfoxide sulfur atom of **1** was different from that of γ-glutamyl-marasmine arising from the mushroom.

Gmelin *et al.* have shown that marasmine which was produced by γ-glutamyltranspeptidase from γ-glutamyl-marasmine, was split by a C–S lyase into pyruvic acid, ammonia and an unstable sulfur compound, which decomposed to form the odorous secondary products, compound II and CH_3S-$CH_2S(O_2)SCH_2SCH_3$(III) [3]. On the other hand, the present authors have reported in previous papers that I and II are the odour compounds of *S. borneensis* [1], and that compound III from the same plant is an antibiotic [7]. To determine the participation of these natural amino acids in the odour formation of *S. borneensis*, **1** and **2** were incubated in an enzymatic model system by using C–S lyase prepared from broccoli, and the volatiles formed were investigated. Pyruvate and compounds I and II were produced by the enzymatic reaction, with II being formed much more than I. The ratio of II:I was almost the same as that obtained from the fruit [1]. It was concluded from these observations, that both **1** and **2** play an important role in forming garlic-like odorous components with the following possible formation routes: (1) **1** is mainly converted to $CH_3SCH_2S(O)SCH_2SCH_3$ by the mediation of C–S lyase, this being followed by rapid decomposition into $CH_3SCH_2SSCH_2SCH_3$(II) and $CH_3SCH_2S(O_2)SCH_2SCH_3$(III) in the same way as that for γ-glutamyl-marasmine [3]; (2) CH_3SH is partly formed by C–S lyase from **1**, and an *SS*-exchange reaction with $CH_3SCH_2S(O)SCH_2SCH_3$ might take place to form $CH_3SCH_2SSCH_3$ (II), or CH_3SH and $CH_3SCH_2S(O)H$ might condense with dehydration to form I, although details of the reaction mechanism are not known at present; (3) **2** is split by C–S lyase into CH_3SCH_2SH (main) and CH_3SH (minor) with pyruvate, and they oxidatively condense with each other to form mainly II and partly I.

EXPERIMENTAL

Analytical conditions

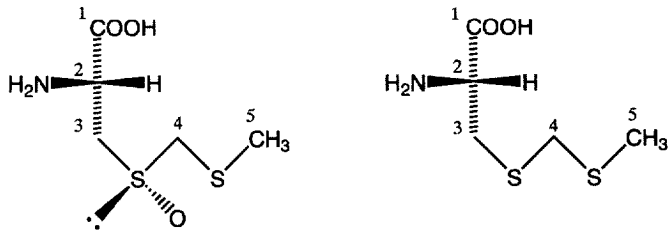
^{1}H-NMR and ^{13}C-NMR: 270 and 66.9 MHz, respectively, in D_2O with DSS as an int. standard, and MS data, were taken in the FAB+ mode by a direct inlet system with PEG as an int. standard.

Plant material

The fruit of *Scorodocarpus borneensis* was collected in Saba, Malaysia, in 1994. Some specimens were treated as soon as possible, and the remaining ones were stored in a freezer at $-50°$.

Extraction and isolation

After removing the outer shell of each fresh fruit, the pulp (320 g) was granulated in MeOH (500 ml); 2.1 l of boiling H_2O was immediately added, and the soln was refluxed for 20 min. The aq. extract was separated by centrifuging at 5° for 10 min at 3000 rpm; an equal vol. of MeOH was added, and the flocculent ppt. was filtered off. After evaporating the MeOH, the aq. layer was run through a column of



1 **2**

Fig. 1.

Amberlite IR 120 (H form) and, after washing with H_2O, amino acids were eluted with a 2% NH_4OH soln. The ninhydrin-positive fractions were collected, and compounds **1** (37.6 mg) and **2** (22.1 mg) were isolated by repeated semi-prep. HPLC. HPLC conditions: column, PEGASIL ODS 250 × 20 mm (Senshu Scientific Co., Tokyo); solvent, MeCN–H_2O–THF (15:85:1 or 0:100:1); detection, UV 210 nm.

Compound **1**. Powder, mp 162–166° (decomp.), $[\alpha]_D$ −26.7 (*c* 0.165, H_2O); HRFABMS *m/z* (obsvd. int.): 198.0286 [M+H, err. +2.7 mmu for $C_5H_{12}NO_3S_2$]. IR^{ν}_{max} cm^{-1}: 3400 (*br*), 3020 (*br*), 1605, 1415, 1028 (—SO—). ^{1}H-NMR δ: 2.30 (3H, *s*, H-5), 3.29 and 3.54 (1H each, *dd*, J = 7.3, 13.7 and J = 6.6, 13.7 Hz, H-3), 3.99 and 4.20 (1H each, *d*, J = 13.9 Hz, H-4), 4.23 (1H, *t*, J = 7.0 Hz, H-2). ^{13}C-NMR (^{13}C-^{1}H resonance) δ: 18.8 (*q*, C-5), 53.0 (*t*, C-3), 53.4 (*d*, C-2), 58.1 (*t*, C-4), 173.9 (*s*, C-1).

Compound **2**. Powder, mp 185–187° (decomp.), $[\alpha]_D$ +20.0 (*c* 0.06, H_2O); HRFABMS *m/z* (obsvd. int.): 182.0316 [M+H, err. +0.6 mmu for $C_5H_{12}NO_2S_2$]. IR^{ν}_{max} cm^{-1} (KBr): 3380 (*br*), 3000 (*br*), 1600 and 1580, 1415. ^{1}H-NMR δ: 2.18 (3H, *s*, H-5), 3.15 and 3.24 (1H each, *dd*, J = 6.8, 14.9 and J = 4.6, 14.9 Hz, H-3), 3.77 (2H, *t*, J = 13.9 Hz, H-4), 3.97 (1H, *dd*, J = 4.5, 7.0 Hz, H-2). ^{13}C-NMR δ: 16.4 (C-5), 34.0 (C-3), 39.7 (C-4), 56.2 (C-2), 175.5 (C-1)

Concentrations of compounds 1 and 2

The concs of **1** and **2** in the pulp of *S. borneensis* were analysed by HPLC. The solvent for the above mentioned ninhydrin-positive fraction from 100 g of pulp was evaporated and made up to 10 ml with H_2O. Isocratic separation with H_2O was accomplished with a PEGASIL ODS column (250 × 4.6 mm, Senshu Scientific Co.) and a UV detector (210 nm). The peak area was converted into mg/ml of **1** or **2** by using a calibration curve based on the amount of isolated **1** (0.8–2.4 mg/ml) or **2** (0.5–1.0 mg/ml). The moisture content was determined as 44.1% by drying at 105°.

Preparation of cystine lyase from broccoli

Cystine lyase (C–S lyase) was partially purified from broccoli bud, and its activity was confirmed by measuring the amount of pyruvate liberated from cystine according to the method of refs [8, 9].

Enzymatic reaction of compounds 1 and 2

A *tris*-HCl buffer soln (0.5 ml) of 1 or 2 (0.34 mg/ml at pH 8.5) was mixed with 0.1 ml of 0.1 mM pyridoxal 5′-phosphate, 0.35 ml of H_2O, and 0.05 ml of an enzyme soln. After incubating at 30° for 19 h, the liberated volatiles were extracted 3 times with 1 ml of ET_2O and analysed by GC-MS under the same conditions as those recorded in our previous report [1]. MS data and the retention indices by GC showed only $CH_3SCH_2SSCH_3$ and $CH_3SCH_2SSCH_2SCH_3$, the aroma components of *S. borneensis*, in the reaction mixture of both **1** and **2** at respective ratios of 1:4.5 and 1:10 as calculated from the GC peak area. A portion of the incubation mixture was used to investigate the formation of pyruvate. The formation of pyruvate corresponding to almost 10% of the substrate was detected in both **1** and **2**. The control reaction mixture, in which boiled C–S lyase was used, produced very little pyruvate and no volatiles.

Syntheses of S-[*(methylthio)methyl*]-D- *or* L-*cysteine* (***4*** *or* ***5***), *and their FDAA derivatives*

According to the synthesis method for *S*-[(benzylthio)methyl]-L-cysteine reported in the literature [10], [(methylthio)methyl]-L- or D-cysteine was produced with (methylthio)methyl chloride, L- or D-cystine and Na in liquid ammonia. Compounds **4** and **5** were obtained as white crystals (ca 75% yield based on the crude product), with subsequent recrystallization in H_2O–ET_2O, which had mp values of 192–197°C (decomp.) and 188–193°C (decomp.), respectively. Their MS, NMR and IR spectral data were in good agreement with those of **2**. FDAA derivatives of **2**, **4** and **5** were prepared by mixing 0.5 ml of an aq. soln (5 mM) of each sample with 1 M $NaHCO_3$ and a 1% FDAA (Marfey's reagent, Pierce, U.S.A.) acetone soln for 1 h at room temp. After adding a 2 M HCl soln, the Me_2CO was evaporated, and the product was dissolved in 0.1 ml of DMSO and 0.9 ml of MeOH. The retention times of the FDAA derivatives of **2**, **4** and **5** by HPLC (PEGASIL ODS

column, 25 × 4.6 mm, 65% MeOH–H_2O (0.05% CH_3COOH); UV, 340 nm) were 5.33, 6.53 and 5.37 min, respectively.

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