Studies on the Decomposition of Sinalbin

Part II. The Decomposition Products of Sinalbin and their Degradation Pathways

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Total degradation scheme of sinalbin has been clarified. Under physiological condition (pH 5~7), a major part of *p*-hydroxybenzyl isothiocyanate (I) (white mustard oil) was decomposed to *p*-hydroxybenzyl alcohol (II) and SCN⁻, and a minor part of I was degraded to di-(*p*-hydroxybenzyl)disulfide through an intermediate (III). Alkaline hydrolysis, UV, IR and mass spectra suggested that the III was *p*-hydroxybenzyl *p*-hydroxybenzylidithiocarbamate, C₁₅H₁₅NO₂S₂. The structure was confirmed also by synthesis.

Another decomposition product (V) of sinalbin was isolated from the reaction mixture of sinalbin and myrosinase at pH 3.0 and identified as *p*-hydroxybenzyl cyanide. Results showed that I, V and sulfur were simultaneously formed from sinalbin under acidic condition (pH 3~4).

In the previous paper¹⁾ we described that a major part of *p*-hydroxybenzyl isothiocyanate (I), enzymatically formed from sinalbin, was decomposed to *p*-hydroxybenzyl alcohol (II) and SCN⁻, and a minor part of I to di-(*p*-hydroxybenzyl) disulfide (IV) through an unknown intermediate (III).

On the other hand, under acidic condition (pH 3~4), it is well known that many kinds of mustard oil glucoside are hydrolyzed with myrosinase to alkyl isothiocyanate and alkyl cyanide.²⁾ Recently, 3-indolylacetonitril^{3,4)}

and 1-methoxy-3-indolylacetonitril⁵⁾ were identified from glucobrassicin (in cabbage and cauliflower) and neoglucobrassicin (in rutabaga) respectively as their decomposition product in an acidic medium.

The present study was undertaken to isolate and identify the unknown product III, and to see if a nitril derivative was also formed from sinalbin under an acidic condition in order to compare with its degradation pathway under physiological condition.

As shown in thin-layer chromatogram (Fig. 6), III was formed not only in the neutral medium, but in the acidic medium. Furthermore, III was so unstable in alkaline solution¹⁾ that isolation of III was attempted from the mustard paste buffered at pH 4.0 with Mc-

1) S. Kawakishi and K. Muramatsu, This Journal, **30**, 688 (1966).

2) M. G. Ettlinger, G. P. Daeto Jr., B. W. Harrison, T. J. Mabry and C. P. Thompson, *Pro. Nat. Acad. Sci. USA*, **47**, 1875 (1961).

3) R. Gmelin, M. Saarivirta and A. I. Virtanen, *Suomen Kemistilehti*, **B33**, 172 (1960).

4) R. Gmelin and A. I. Virtanen, *ibid.*, **B34**, 15 (1961).

5) R. Gmelin and A. I. Virtanen, *Acta Chem. Scand.*, **16**, 1378 (1962).

FIG. 1. UV Spectra of the Product III.
— isolated, --- synthesized

FIG. 2. IR Spectra of the Product III.
— isolated, --- synthesized

FIG. 3. Mass Spectra of the Product III.

Ilvaine buffer solution. The paste was extracted with ethanol, and the ethanol extracts, after concentration, were reextracted with ether. The ether solution was concentrated and submitted to column chromatography on silica gel. Fractions containing III were combined and purified by repeated column

chromatography, and III was obtained as a colorless plate, m.p. 152°C (decomp.). The yield of III was 180 mg from 800 g of defatted mustard powder. The molecular formula $C_{15}H_{15}NO_2S_2$ was assigned to this product from the data of elementary analyses and molecular weight determination (298 by Signer method and 305 by mass spectrometry). IR spectrum (Fig. 2) showed the presence of *p*-substituted phenol group (3400, 1611, 1603, 1236 and 838 cm^{-1}) and thioamide group, $-NH-C(S)-$, (3240, 1507, 1322 and 1096 cm^{-1}). Color reaction of thiocarbonyl group, $-C(S)-$, using iodine-azide solution was positive (with evolution of nitrogen gas). III was hydrolyzed with alkali at room temperature to give *p*-hydroxybenzylamine (VI), di-(*p*-hydroxybenzy) disulfide (IV) and *p*-hydroxybenzyl alcohol (II) (small amounts), and furthermore, hydrogen sulfide was developed from the alkaline hydrolyzate upon neutralization, which might be due to the secondary decomposition of carbonyl sulfide formed from III. *p*-Hydroxybenzylamine (VI) was also detected in the reaction mixture of sinalbin and myrosinase by paper chromatography. These data suggested that III contained *p*- $HOC_6H_4CH_2NHC(S)-$ and *p*- $HOC_6H_4CH_2S-$ groups. Mass spectrum (Fig. 3) also supported this presumption; M/e 76 ($C_6H_4^+$), 107 ($HOC_6H_4CH_2^+$), 122 ($HOC_6H_4CH_2NH^+$), 140 ($HOC_6H_4CH_2SH^+$) and 165 ($HOC_6H_4CH_2NCS^+$).

The chemical constitution of III was thus proposed to be *p*-hydroxybenzyl *p*-hydroxybenzylidithiocarbamate.

This dithiocarbamate was synthesized for identification of the product III. A. Rieche⁶⁾ described a general synthetic method of dithiocarbamate as follows:

6) A. Rieche, G. Hilgetag, D. Martin und I. Kreyzi, *Arch. Pharm.*, **296**, 310 (1963).

carbon disulfide in sodium hydroxide-methanol solution to give sodium *p*-hydroxybenzylthiocarbamate. The reaction between this dithiocarbamate and *p*-acetoxybenzyl chloride,⁸¹ afforded *p*-acetoxybenzyl *p*-hydroxybenzylthio-

8) R. Grice and L. B. Owen, *J. Chem. Soc.*, **1963**, 1947.

carbamate, which was hydrolyzed with acetic acid to give a mixture of *p*-hydroxybenzyl *p*-hydroxybenzylthiocarbamate and di-(*p*-hydroxybenzyl) disulfide. The former was isolated from the mixture by column chromatography on silica gel using chloroform-ether, m.p. 153°C (decomp.). The synthesized dithiocarbamate was identical with III in all respects, including UV spectrum (Fig. 1), IR spectrum (Fig. 2) and thin-layer chromatographic behaviours.

From these experiments, it was confirmed that the minor part of *p*-hydroxybenzyl isothiocyanate (I) was degraded to di-(*p*-hydroxybenzyl) disulfide (IV) through *p*-hydroxybenzyl *p*-hydroxybenzylthiocarbamate (III).

Next, the decomposition of sinalbin under various medium pH was studied.

The reaction mixtures of sinalbin and myrosinase buffered with McIlvaine buffer solution (pH 3, 4, 5, 6 and 7, respectively) were prepared and incubated at 37°C for 24 hrs. Thin-layer chromatogram of the ether extracts of each solution is shown in Fig. 6. Spots which were positive to a phenol test due to the presence II, III and IV were observed on each chromatogram. Another phenolic substance V was also observed on chromatograms obtained from the solutions of pH 3 and 4.

Effect of the reaction time on the formation of these products at pH 3.0 was examined by thin-layer chromatography (Fig. 7). II, III and IV did not appear at initial stage of the reaction, but later formed gradually. On the other hand, V was already formed at initial stage simultaneously with I, and the amount of V did not change in spite of a decrease of I. Therefore, it may be true that V was directly formed from sinalbin and not from I by its secondary decomposition. The results obtained by thin layer chromatography for the mustard paste were nearly identical with that in the case of the reaction mixtures.

Since *p*-hydroxybenzyl alcohol (II) was also a major product on the secondary decomposi-

FIG. 6. Thin-layer Chromatogram of the Decomposition Products of Sinalbin at Several pH.

Samples: ether extracts of the reaction mixtures of sinalbin and myrosinase (pH of medium, 3, 4, 5, 6 and 7, respectively) incubated at 37°C, 24 hrs.

Solvent: chloroform-ether (4:1).

Coloring reagent: diazotized sulfanilic acid and 10% sodium carbonate

FIG. 7. Thin-layer Chromatogram of the Decomposition Products of Sinalbin at pH 3.0 in Time Course.

Samples: ether extracts of the reaction mixtures of sinalbin and myrosinase at pH 3.0 incubated at 37°C for 2, 6, 10, 15, 24 and 48 hrs., respectively.

Solvent: chloroform-ether (4:1).

Coloring reagent: diazotized sulfanilic acid and 10% sodium carbonate

tion of I in acidic medium, the amount of SCN^- formed in the reaction mixture of sinalbin and myrosinase was determined in

FIG. 8. Formation of SCN^- from Sinalbin.

- Sinalbin and myrosinase at pH 3.0.
 --○-- Sinalbin and myrosinase at pH 7.0.
 —●— Mustard paste at pH 3.0.

The ordinate indicates the molar amount of SCN^- formed from 100 ml of the reaction mixture or 1 g of the mustard powder.

time course. The results obtained are shown in Fig. 8. The rate of SCN^- formation at pH 3.0 was slower than that in the neutral medium (pH 7.0), and this could be due to a low activity of myrosinase at pH 3.0.⁹⁾

After incubation for fifteen hours, during which I was mostly decomposed, the SCN^- formation did not occur any more. The amount of SCN^- formed in the acidic medium was about a half of that formed in the neutral one, corresponded to about fifty four and ninety percent of the added sinalbin, respectively. This fact suggested that some other product, not formed through I, should be produced from sinalbin.

The facts mentioned above clearly showed that a new product (V) was formed from sinalbin directly in acidic medium, and this compound (V) isolated from the reaction mixture by ether extraction, followed by column chromatography on silica gel. Recrystallization from water gave plates, m.p. 71°C. The elementary analyses of V supported $\text{C}_8\text{H}_7\text{NO}$ for its molecular formula. IR spectrum (Fig. 9) showed the presence of *p*-substituted phenol group (3380, 1613,

FIG. 9. IR Spectra of the Product V.

— isolated, --- synthesized

1600, 1503, 1213 and 813 cm^{-1}) and $-\text{C}\equiv\text{N}$ group (2280 cm^{-1}). From these results, V was assumed to be *p*-hydroxybenzyl cyanide, and the sample obtained from the natural source was identical in every respects (m.p., IR and chromatographic behaviour) with a synthetic one derived from *p*-hydroxybenzyl alcohol. Sulfur was also isolated as S_8 , m.p. 119°C from ether extracts of the reaction mixture. Therefore, it was demonstrated that sinalbin was decomposed under acidic condition as follows:

Total degradation scheme of sinalbin was summarized in Fig. 10. As shown in above experiments, *p*-hydroxybenzyl *p*-hydroxybenzylthiocarbamate (III) is so unstable that it is possible that the actual decomposition of III in mustard paste to di-(*p*-hydroxybenzyl) disulfide (IV), *p*-hydroxybenzylamine (VI) and carbonyl sulfide might proceed without any action of enzyme. However, it remains unsolved whether or not the formation of III from I (especially formation of the *p*-hydroxybenzyl—S linkage) is enzymatical process. With respects to this, A. I. Virtanen et al.¹⁰⁻¹¹⁾ reported interesting facts that benzyl isothiocyanate formed from glucotropaeolin in the seeds of *Lepidium sativum* was converted to benzyl thiocyanate (benzyl—S linkage) with some isomerase contained in the seeds.

10) A. I. Virtanen and M. Saarivirta, *Suomen Kemistilehti*, **B35**, 102, 428 (1962).

11) M. Saarivirta and A. I. Virtanen, *Acta Chem. Scand.*, **17**, S74 (1963).

9) Z. Nagashima and M. Uchiyama, *J. Agr. Chem. Soc. Japan*, **33**, 484 (1959).

FIG. 10. Total Degradation Scheme of Sinalbin.

Myrosinase used in our experiments was not such a pure preparation that it might be contaminated by such characteristic enzyme. There have been many studies^{3~5,12,13)} on the secondary decomposition of mustard oils, but no experiments have yet been made on the formation of disulfide from mustard oil through dithiocarbamate. D. A. Forss¹⁴⁾ demonstrated

that an essential oil obtained from *Coronopus didymus* a cruciferous weed, by steam distillation, contained benzyl cyanide and dibenzyl disulfide, but he did not describe on their precursors. It seems likely that the decomposition of isothiocyanate to disulfide through dithiocarbamate occurs in the nature besides the present study of white mustard.

EXPERIMENTAL

All melting points were uncorrected. UV spectra were measured in 95% ethanol with Hitachi-Perkin Elmer 139 and Hitachi EPS-2; IR spectra with JASCO IR-S and Hitachi EPI-2 in KBr disks. Mass spectra

12) E. B. Astwood, M. A. Greer and M. G. Ettlinger, *J. Biol. Chem.*, **181**, 121 (1949).

13) A. Kjaer and Bo W. Christensen, *Acta Chem. Scand.*, **13**, 1575 (1959).

14) D. A. Forss, *Australian J. Applied Sci.*, **2**, 396 (1951).

were measured with Hitachi Mass Spectrometer RMU-6D.

1. Isolation and Chemical Structure of the Product III

Isolation of III. Mustard paste, which was prepared with defatted powder of white mustard (800 g) and McIlvaine buffer, pH 4.0, (1200 ml), was incubated at 37°C for 48 hrs. To the paste, ethanol (3l) was added and the mixture was well agitated and kept at room temperature for overnight. After centrifugation of this mixture, the precipitate was repeatedly extracted with ethanol. The ethanol extracts were combined and concentrated under reduced pressure to remove ethanol. The concentrated solution was extracted with ether, and the ether extract was dried on anhydrous sodium sulfate and concentrated. The ether extract was chromatographed over silica gel using chloroform-ether (90:10 v/v) and fractionated every 50 ml. Each fraction was submitted to thin-layer chromatography and the fractions containing III were combined. On repeating the column chromatography, crude crystals III were obtained. After treatment with chloroform to remove resinous materials, recrystallization was carried several times from dilute ethanol to give colorless plate. M.p. 152°C, yield 180 mg. M.w. 305 by mass spectrometry (Fig. 3). *Anal.* Found: C 58.97, H 4.75, N 4.74, S 20.94. Calcd. for $C_{15}H_{15}NO_2S_2$: C 59.01, H 4.95, N 4.59, S 20.97% (m.w. calcd. 305). UV spectrum is shown in Fig. 1: λ_{max}^{EtOH} 229, 257 and 269 m μ ($\log \epsilon$ 4.40, 4.18 and 4.15). IR spectrum is shown in Fig. 2. Solubility: easily soluble in ether, acetone, ethanol and methanol; sparingly soluble in benzene, chloroform and water.

Alkaline hydrolysis of III. A solution of III (10 mg) in ether (5 ml) was shaken with three 2 ml portions of 1 N NaOH. The alkaline solution was neutralized to pH 6.0 with 5 N H_2SO_4 and extracted with ether. The water and ether layers were concentrated to a small volume respectively, and submitted to paper and thin-layer chromatography. *p*-Hydroxybenzylamine was detected in the water solution, and di-(*p*-hydroxybenzyl)disulfide and *p*-hydroxybenzyl alcohol were in the ether solution. During the neutralization of the alkaline solution, evolution of hydrogen sulfide was detected by lead acetate paper.

Synthesis of *p*-Hydroxybenzyl *p*-Hydroxybenzyl-dithiocarbamate

i) *p*-Hydroxybenzylamine hydrochloride. This

compound was synthesized from *p*-hydroxybenzaldehyde by the method described by A. Kjaer et al.⁷⁾

M.p. 197°C. *Anal.* Found: C 52.19, H 5.76, N 8.80. Calcd. for $C_7H_{10}NOCl$: C 52.67, H 6.31, N 8.77%.

ii) **Sodium *p*-hydroxybenzylidithiocarbamate.** To a mixture of the above amine hydrochloride (2 g) in methanol (60 ml) and sodium hydroxide (1.5 g) in water (10 ml), an ether solution of carbon disulfide (1 g) was added dropwise with stirring for 30 minutes and followed with stirring for additional 30 minutes. After concentration of the reaction mixture under reduced pressure below 30°C, large amount of cooled acetone was added to the concentrate to give white precipitates. The precipitates were collected and dissolved in methanol to remove insoluble matters (mostly sodium chloride) by filtration. The methanol solution was concentrated and cooled acetone was added again, and the precipitates were collected, washed with ether and dried. Sodium *p*-hydroxybenzylidithiocarbamate was obtained as hygroscopic powder (yield, 1.9 g).

iii) ***p*-Acetoxybenzyl chloride.** This compound was prepared from *p*-hydroxybenzyl alcohol by the method of R. Grice et al.⁸⁾

iv) ***p*-Acetoxybenzyl *p*-Hydroxybenzylidithiocarbamate.** To a stirred solution of sodium *p*-hydroxybenzylidithiocarbamate (1.9 g) in methanol, *p*-acetoxybenzyl chloride (2.8 g) in methanol was added. The stirring was continued further for one hour. The solution was concentrated and the residual oil was extracted with boiling benzene. The benzene extracts were concentrated to dryness. The residue was purified by column chromatography on silica gel using chloroform-ether, and *p*-acetoxybenzyl *p*-hydroxybenzylidithiocarbamate was obtained 1.23 g as an oil.

v) ***p*-Hydroxybenzyl *p*-Hydroxybenzylidithiocarbamate.** A solution of *p*-acetoxybenzyl *p*-hydroxybenzylidithiocarbamate (1.23 g) in methanol was acidified with acetic acid and left to stand at room temperature overnight. The solution was concentrated, and the residue was extracted with ether. Thin-layer chromatography showed that this ether extracts contained *p*-hydroxybenzyl *p*-hydroxybenzylidithiocarbamate, di-(*p*-hydroxybenzyl)disulfide and the starting material. The reaction mixture was chromatographed on silica gel using chloroform-ether and fractionated every 50 ml. The presence of *p*-hydroxybenzyl *p*-hydroxybenzylidithiocarbamate was tested by thin-

layer chromatography, and the fractions containing the dithiocarbamate were combined, concentrated and purified by repeated column chromatography on silica gel until it became chromatographically homogeneous on silica gel plate. After evaporation of the solvent, the residue was crystallized in refrigerator and recrystallized from dilute ethanol to give colorless plates of *p*-hydroxybenzyl *p*-hydroxybenzylthiocarbamate. Yield, 0.22 g m.p. 152°C (decomp.). *Anal.* Found: C 59.21, H 4.94, N 4.51, S 21.04. Calcd. for $C_{18}H_{15}NO_2S_2$: C 59.01, H 4.95, N 4.59 S 20.97%. The UV spectrum (Fig. 1) showed $\lambda_{\max}^{EtOH}$ 229, 257 and 269 $m\mu$ ($\log \epsilon$ 4.39, 4.18 and 4.14) and the IR spectrum is shown in Fig. 2.

2. Decomposition of Sinalbin in Acidic Condition

Sinalbin and myrosinase used in this experiments were prepared from mustard powder by the procedures described in the previous paper.¹⁾

Thin-layer Chromatography of Decomposition Products in Acidic Medium

i) **Effect of pH on sinalbin decomposition.** The reaction mixtures composed of 2.4×10^{-4} M sinalbin (25 ml), myrosinase solution (25 ml) and McIlvaine buffer solution (pH, 3, 4, 5, 6 and 7, respectively) (50 ml) were incubated at 37°C for 24 hrs., and then extracted with ether. The ether extracts were dried over anhydrous sodium sulfate and concentrated. The decomposition products in the extracts were detected by thin-layer chromatography on silica gel (solvent, chloroform-ether 4:1 v/v). Phenolic spots were developed by spraying diazotized sulfanilic acid solution, followed by 10% sodium carbonate solution. The chromatogram are shown in Fig. 6. Four spots were detected as the phenolic decomposition products in acidic medium.

ii) **Sinalbin decomposition in time course.** Six reaction mixtures of pH 3.0 were incubated at 37°C and extracted with ether one after another at 2, 6, 10, 15, 24 and 48 hrs., respectively. The ether extracts were subjected to thin-layer chromatography as mentioned above (Fig. 7).

Quantitative Analysis of Thiocyanate Ion in Reaction Mixtures

SCN^- formed in the reaction mixture was determined by the colorimetric method described in the

previous paper¹⁾ (Fig. 8).

Isolation of V

The reaction mixture, which consisted of sinalbin (3 g in 375 ml of water), myrosinase solution (375 ml) and McIlvaine buffer solution (750 ml, pH 3.0), was incubated at 37°C for 24 hrs. and extracted with ether. The ether solution was dried with anhydrous sodium sulfate and concentrated. Yellow substance was crystallized from the concentrate and recrystallized from *n*-hexane to give pale yellow prisms, m.p. 119°C. It was identified as S_8 by the mixed melting point with authentic S_8 . The concentrate was submitted to column chromatography on silica gel using chloroform-ether. Fractions containing V were combined and concentrated to give crystals. Recrystallization from water afforded plates of V, m.p. 71°C, yield 50 mg. *Anal.* Found: C 71.97, H 5.29, N 9.95. Calcd. for C_8H_7NO : C 72.16, H 5.30, N 10.52%.

Synthesis of *p*-Hydroxybenzyl Cyanide and Identification of the Product V

To a solution of sodium cyanide (5 g) in water (10 ml), *p*-acetoxybenzyl chloride (10 g) (cf. I-iii) in 95% ethanol (30 ml) was slowly added and the mixture was refluxed for 4 hrs. The reaction mixture was filtered to remove sodium chloride and concentrated. The concentrate was refluxed with 2 N hydrochloric acid in methanol (50 ml) for 2 hrs. and then evaporated to brown oil. The oil was dissolved in boiling benzene, cooled and crystallized. Recrystallization from water afforded plates, m.p. 72°C, yield 2.3 g. *Anal.* Found: C 72.33, H 5.24, N 10.49. Calcd. for C_8H_7NO : C 72.16, H 5.30, N 10.52%.

The mixed melting point with V was 71°C. The isolated and synthetic samples were identical in respect to IR spectra (Fig. 9) and thin-layer chromatographic behaviours.

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