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Original Article

Novel Metabolites of Orbencarb and Benthiocarb Herbicides in Soil, Plant and Rat Liver Microsomes: *S*-2-Chlorobenzyl *N*-Ethyl-*N*-vinylthiocarbamate and Its 4-Chloro Isomer

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The biotransformation of orbencarb (*S*-2-chlorobenzyl *N*,*N*-diethylthiocarbamate) in soils under upland conditions, shoots of soybean plant and rat liver microsomes, was studied to obtain a novel metabolite using [ring-U-^{14}C]orbencarb. *S*-2-Chlorobenzyl *N*-ethyl-*N*-vinylthiocarbamate in which two hydrogen atoms were removed from an *N*-ethyl moiety of orbencarb was found to be a novel metabolite in all cases and it was tentatively identified by two-dimensional thin-layer co-chromatography with the authentic compound. A similar type of novel metabolite was isolated from the soil treated with non-radioactive benthiocarb (4-chloro isomer of orbencarb) under upland conditions, and its chemical structure was confirmed as *S*-4-chlorobenzyl *N*-ethyl-*N*-vinylthiocarbamate by comparing it with the synthetic compound in GC retention time and GC-mass spectrum.

INTRODUCTION

Orbencarb (*S*-2-chlorobenzyl *N*,*N*-diethylthiocarbamate) is known to be highly effective against weeds in upland crops such as wheat and soybean, while benthiocarb, 4-chloro isomer of orbencarb, in paddy fields.

Biotransformation of both thiocarbamate herbicides has been studied extensively in mice,[1] plants,[2-4] soils,[5,6] and microorganisms,[7] and a large number of degradation products which might be formed by similar reaction from orbencarb or benthiocarb were identified.

This paper describes the synthesis and identification of *S*-2-chlorobenzyl *N*-ethyl-*N*-vinylthiocarbamate and its 4-chloro isomer which were found to be novel metabolites of orbencarb and benthiocarb.

MATERIALS AND METHODS

1. Chemicals

1.1 Orbencarb and benthiocarb

[Ring-U-^{14}C]orbencarb used was 1.20 mCi/mmol for the degradation study of soils[6] and 2.39 mCi/mmol for the metabolism studies of soybean plant[4] and rat liver microsomes. The radiochemical purity was more than 99% as determined by thin-layer chromatography (TLC).

Non-radioactive orbencarb and benthiocarb were supplied by Ihara Chemical Ind. Co., Ltd.

1.2 Synthesis of S-2-chlorobenzyl N-ethyl-N-vinylthiocarbamate (N-vinyl-orbencarb) and its 4-chloro isomer (N-vinyl-benthiocarb)

N-Vinyl-orbencarb and *N*-vinyl-benthiocarb were synthesized according to the following

steps:

Step 1 Synthesis of S-2-chlorobenzyl N-ethyl-N-β-hydroxyethylthiocarbamate (N-CH_2CH_2OH-orbencarb) and its 4-chloro isomer (N-CH_2CH_2OH-benthiocarb): A solution of 2-(ethylamino)-ethanol (8.9 g, 0.1 mol) and triethylamine (11.2 g, 0.11 mol) in benzene (10 ml) was cooled at 0–5°C, and carbonyl sulfide (6.0 g, 0.1 mol) was slowly bubbled for 2.5–3.5 hr. To this solution, 2- or 4-chlorobenzyl chloride (16.2 g, 0.1 mol) was added dropwise for 1.5 hr. The mixture was stirred overnight at room temperature and then water was added. The organic layer was separated, washed successively with dilute HCl and water, dried over Na_2SO_4 and concentrated. In the case of N-CH_2CH_2OH-orbencarb, the oily residue was distilled under reduced pressure (bp 175–178°C/0.05 mmHg). The distillate (N-CH_2CH_2OH-orbencarb fraction) or the concentrate (N-CH_2CH_2OH-benthiocarb fraction) was further purified by column chromatography on silica gel with a solvent mixture of *n*-hexane–ethyl acetate (3 : 1, v/v). N-CH_2CH_2OH-orbencarb; yield 40.0%; colorless liquid; n_D^{20} 1.5738; ^{1}H NMR $\delta_{TMS}^{CDCl_3}$ ppm: 1.18 (3 H, t, J=7 Hz, N-CH_2-C$\underline{H}_3$), 2.90 (1 H, bs, O$\underline{H}$), 3.42 (2 H, q, J=7 Hz, N-C$\underline{H}_2$-CH_3), 3.50 (2 H, t, J=7 Hz, N-C$\underline{H}_2$-CH_2OH), 3.71 (2 H, bs, N-CH_2-C$\underline{H}_2$OH), 4.27 (2 H, s, S-C$\underline{H}_2$), 7.0–7.6 (4 H, m, Ar-$\underline{H}$). N-CH_2CH_2OH-benthiocarb; yield 64.5%; colorless liquid; n_D^{20} 1.5729.

Step 2 Synthesis of S-2-chlorobenzyl N-ethyl-N-β-chloroethylthiocarbamate (N-CH_2CH_2Cl-orbencarb) and its 4-chloro isomer (N-CH_2CH_2Cl-benthiocarb): A solution of N-CH_2CH_2OH-orbencarb or N-CH_2CH_2OH-benthiocarb (27.5 g, 0.1 mol) in benzene (100 ml) was cooled in an ice bath and thionyl chloride (13.1 g, 0.11 mol) was added dropwise. After stirring for 2 hr at room temperature, the mixture was poured into cold water and extracted with ether. The ether layer was washed successively with 5% Na_2CO_3 and water, dried over Na_2SO_4 and concentrated. The yield of N-CH_2CH_2Cl-orbencarb was 87.0% and that of N-CH_2CH_2Cl-benthiocarb 85.3%.

Step 3 Synthesis of N-vinyl-orbencarb and N-vinyl-benthiocarb: The N-CH_2CH_2Cl-orbencarb or N-CH_2CH_2Cl-benthiocarb (14.7 g, 0.05 mol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 38.1 g, 0.25 mol) was stirred at 70°C for 8 hr, and then ether and water were added and shaken. The ether layer was washed successively with dilute HCl and water, dried over Na_2SO_4 and concentrated. The oily residue was purified by column chromatography on silica gel with a solvent mixture of *n*-hexane–ethyl acetate (4 : 1, v/v) and further purified by high performance liquid chromatography (HPLC). Preparative HPLC was conducted on a System 500 (Waters Associates) using a μ-Bondapack C_{18} column with methanol–water (7 : 3, v/v) as a mobile phase at a flow speed of 200 ml/min. N-Vinyl-orbencarb; yield 9.7%; yellow liquid; n_D^{20} 1.5852; ^{1}H NMR $\delta_{TMS}^{CDCl_3}$ ppm: 1.18 (3 H, t, J=7 Hz, N-CH_2-C$\underline{H}_3$), 3.66 (2 H, q, J=7 Hz, N-C$\underline{H}_2$-CH_3), 4.31 (2 H, s, S-C$\underline{H}_2$), 4.37 (1 H, dd, J=9 Hz, N-CH=C-*cis*-$\underline{H}$), 4.47 (1 H, dd, J=16 Hz, N-CH=C-*trans*-$\underline{H}$), *ca.* 7.0 (1 H, bs, N-C$\underline{H}$=CH_2), 7.0–7.6 (4 H, m, Ar-$\underline{H}$); MS *m/z*: no M^+, 130, 125 (base peak), 102, 89, 70. N-Vinyl-benthiocarb; yield 17.1%; colorless liquid; n_D^{20} 1.5860; ^{1}H NMR $\delta_{TMS}^{CDCl_3}$ ppm: 1.18 (3 H, t, J=7 Hz, N-CH_2-C$\underline{H}_3$), 3.65 (2 H, q, J=7 Hz, N-C$\underline{H}_2$-CH_3), 4.13 (2 H, s, S-C$\underline{H}_2$), 4.37 (1 H, dd, J=9 Hz, N-CH=C-*cis*-$\underline{H}$), 4.48 (1 H, dd, J=16 Hz, N-CH=C-*trans*-$\underline{H}$), *ca.* 7.0 (1 H, bs, N-C$\underline{H}$=CH_2), 7.25 (4 H, s, Ar-$\underline{H}$); MS *m/z*: no M^+, 130 (base peak), 125, 102, 89, 70.

The ^{1}H NMR spectra of N-vinyl-orbencarb and N-vinyl-benthiocarb showed the presence of N-ethyl-N-vinyl group. Such signals were identical to those of lysergic acid ethylvinylamide which was a metabolite of lysergic acid diethylamide (LSD) by soil microorganisms.[8]

The mass spectra of the vinyl thiocarbamates showed no parent ion at *m/z* 255 (^{35}Cl base) in which two hydrogen atoms were removed from orbencarb or benthiocarb.

1.3 Synthesis of S-4-chlorobenzyl pyrrolidine-1-carbothioate (N-tetramethylene-benthiocarb)

This compound was synthesized in the same procedure as described at Step 1 in section *1.2*, using pyrrolidine instead of 2-(ethylamino)-ethanol. It had: mp 57°C; ^{1}H NMR $\delta_{TMS}^{CDCl_3}$ ppm: 1.7–2.0 (4 H, bs, N-CH_2-C$\underline{H}_2$-C$\underline{H}_2$-CH_2-N), 3.1–3.6 (4 H, bs-m, N-C$\underline{H}_2$-CH_2-CH_2-C$\underline{H}_2$-N), 4.05 (2 H, s, S-C$\underline{H}_2$), 7.16 (4 H, s, Ar-$\underline{H}$); MS *m/z*: 255 (M^+, ^{35}Cl base), 125, 98 (base peak), 89, 55.

Table 1 TLC *Rf* values and GC retention times for authentic compounds.

Compounds	TLC *Rf* values[a]				GC retention times (min)[b]			
	A	B	C	D	Thermon-3000	PEGA	SE-30	OV-1
N-Vinyl-orbencarb	0.65	0.48	0.68	0.47	2.0	1.6	2.7	2.0
Orbencarb	0.55	0.44	0.66	0.36	3.0	1.6	2.7	2.2
N-Vinyl-benthiocarb	0.64	0.48	0.68	0.46	2.5	2.0	3.0	2.2
Benthiocarb	0.53	0.43	0.63	0.36	4.5	1.9	2.9	2.3
N-Tetramethylene-benthiocarb	0.53		0.64		11.1	8.2	7.1	8.9

[a] TLC solvent systems; A: ethyl acetate–benzene=1 : 9, v/v %, B: acetone–dichloromethane=1 : 99, C: acetone–dichloromethane=1 : 19, D: ethyl acetate–*n*-hexane=1 : 3.

[b] GC conditions; column length: 100 cm × 3 mm i.d., column oven temp.: 130°C for 2% Thermon-3000 on Chromosorb W AW-DMCS (Shimadzu), 140°C for 1% PEGA on Gaschrom Q, 150°C for 5% SE-30 on Chromosorb W AW and 160°C for 1% OV-1 on Gaschrom Q, carrier gas: N_2 45 ml/min, air 40 ml/min and hydrogen 40 ml/min.

1.4 Mass spectra of synthetic compounds

Mass fragmentation of the molecules was quite different among benthiocarb, *N*-tetramethylene-benthiocarb and *N*-vinyl-benthiocarb. The base peak appeared at *m/z* 100 corresponding to $CON(Et)_2$ for benthiocarb, *m/z* 98 corresponding to $CON(CH_2)_4$ for *N*-tetramethylene-benthiocarb and *m/z* 130 corresponding to $SCONEtCH{=}CH_2$ for *N*-vinyl-benthiocarb.

2. Instrumentation

For TLC, precoated silica gel chromatoplates $60F_{254}$ (Merck, thickness: 0.25 mm, size: 20×20 cm) were used with several solvent systems. Compounds on a chromatoplate after developing were detected under UV-ray (254 nm). GC was conducted on a Shimadzu GC-5A with a flame photometric detector in sulfur mode using 4 different columns. TLC *Rf* values and GC retention times for authentic compounds are shown in Table 1. The *N*-vinyl compounds gave higher *Rf* values than the parent compound. TLC could not distinguish *N*-tetramethylene-benthiocarb from benthiocarb, but GC could.

GC-MS was conducted on a Shimadzu LKB-9000B mass spectrometer using 1% PEGA on Gaschrom Q column (100 cm×3 mm i.d.) with electron impact (EI) on an ionization energy 70 eV. ^{1}H-NMR spectra were obtained by a JEOL FX-90 Fourier Transform NMR spectrometer, in $CDCl_3$ and TMS as an internal standard.

3. Metabolism Studies

3.1 Soil metabolism studies

The soil degradation studies of ^{14}C-orbencarb and non-radioactive benthiocarb followed the official procedure provided by the Environment Agency, Japan (1978). Nagano and Tsukuba volcanic ash soils were used for the tests. Their physico-chemical properties were reported previously.[6]

Fifty grams of each soil on a dry weight basis was placed in a 100 ml Erlenmeyer flask. The soil under upland moisture condition was prepared by adding distilled water to make 55% of the maximum water holding capacity and kept in a dark chamber at 28°C for pre-incubation. Seven days after, ^{14}C-orbencarb (240 μg, 1.12 μCi) in 1 ml acetone solution was added to each flask to prepare 4.8 ppm orbencarb and well mixed. In order to identify a novel metabolite of benthiocarb in soil under upland condition, non-radioactive benthiocarb (3.2 mg) in 2 ml acetone solution was added to 300 g of Nagano soil in a 500 ml Erlenmeyer flask to prepare 10.7 ppm benthiocarb. Soil samples treated with ^{14}C-orbencarb or non-radioactive benthiocarb were covered with aluminum foil and kept in a dark chamber at 28°C.

3.2 Plant metabolism

Radioactive orbencarb (172 μg, 1.6 μCi) was dissolved in 20 ml of distilled water to make

8.6 ppm orbencarb solution. The stems of soybean seedlings at second-true-leaf stage without the roots (5.3 g) were dipped in the solution and kept at 23–25°C for 90 hr.

3.3 In vitro study by rat liver microsomes with NADPH

Rat liver microsomes fraction was prepared from a male Wistar rat. The mixture containing the microsomes equivalent to 3.3 g fresh liver, 5.0 μmol NADPH and 52 μg (0.48 μCi) ^{14}C-orbencarb in 2 ml phosphate buffer (0.01 M, pH 7.4) was incubated at 37°C for 20 min.

4. Analysis

4.1 Analysis of radioactive metabolites

The soils treated with ^{14}C-orbencarb were analyzed after 5, 15, 30, 60, 90 and 150 days of incubation. Analysis for the radioactive metabolites followed the same method previously described.[4,6]

4.2 Analysis of N-vinyl-benthiocarb

The soil treated with non-radioactive benthiocarb was analyzed 15 days after treatment. The soil (300 g) was extracted with aqueous 75% methanol solution and the concentrate of the aqueous methanol extract was partitioned with dichloromethane in the same procedures previously described.[6] The concentrate of the dichloromethane extract was spotted on three silica gel chromatoplates as a band and developed with a solvent system A (Table 1). The regions corresponding to the *N*-vinyl-benthiocarb on the chromatoplates were extracted with acetone and further purified twice by TLC with solvent systems B and D (Table 1). Four microliters out of 0.2 ml acetone extract from the final purification was subjected to GC under the conditions shown in Table 1. *N*-Vinyl-benthiocarb was also identified by GC-MS as mentioned above and unchanged benthiocarb in the soil extract by GC under the conditions shown in Table 1.

RESULTS

1. Formation of N-Vinyl-derivatives from Orbencarb or Benthiocarb in Soils

Degradation products detected from Nagano or Tsukuba soil treated with ^{14}C-orbencarb numbered thirteen in the dichloromethane-soluble fraction and one in the water-soluble fraction, of which our previous paper have already reported.[6] A novel metabolite discussed in this study was detected in the dichloromethane-soluble fraction at a very low level. The metabolite in the Nagano soil accounted for 0.1% of the applied radioactivity 15 days after treatment and remained at the same level throughout the experiment for 150 days, while that in the Tsukuba soil only less than 0.1%. The *Rf* values of the metabolite separated by TLC with solvent systems A, B, C or D were higher than orbencarb's (Table 1). The metabolite was tentatively identified as *N*-vinyl-orbencarb by two-dimensional co-TLC using solvent systems A and B with the synthetic compound.

A similar metabolite detected from the Nagano soil treated with non-radioactive benthiocarb was purified by preparative TLC and submitted to both GC and GC-MS analysis. GC retention times of the metabolite using 4 different columns well agreed with those of the synthetic *N*-vinyl-benthiocarb (Table 1). As shown in Fig. 1, the mass spectrum of the metabolite derived from benthiocarb revealed no molecular ion peak but showed major frag-

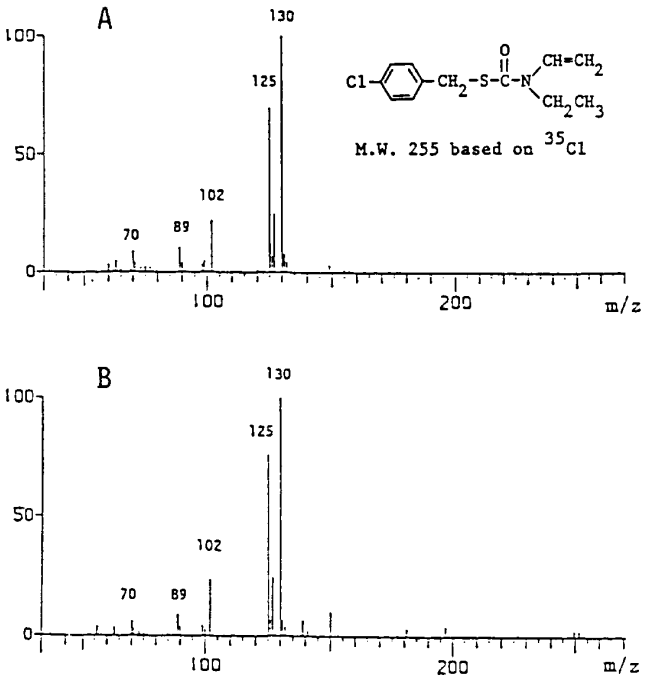

Fig. 1 Electron-impact mass spectra of *N*-vinyl-benthiocarb.
A: Synthetic compound, B: metabolite obtained from benthiocarb-treated soil.

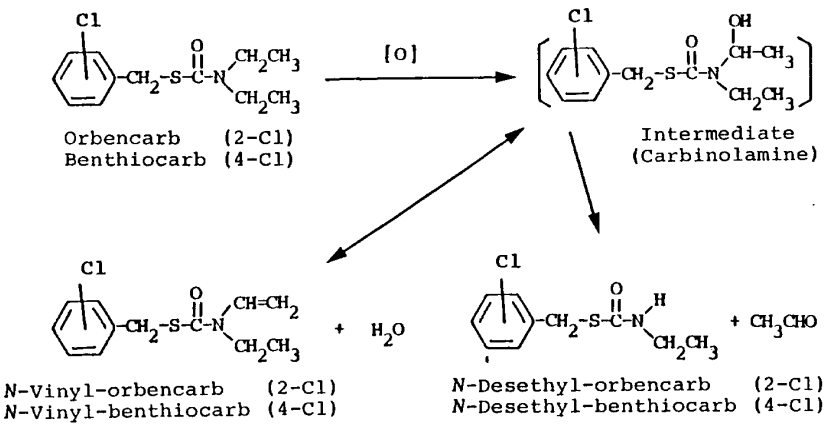

Fig. 2 Possible metabolic pathways from orbencarb and benthiocarb to *N*-vinyl and *N*-desethyl derivatives in soil, plant and rat liver microsomes.

ment ions at *m/z* 130 ($SCONEtCH{=}CH_2$), 127/125 ($CH_2C_6H_4Cl$, ^{37}Cl and ^{35}Cl base) and 102 ($SCONHCH{=}CH_2$). Since this was the fragment pattern of the synthetic *N*-vinyl-benthiocarb, it was concluded that the metabolite was *N*-vinyl-benthiocarb. *N*-Vinyl-benthiocarb accounted for only 0.02% of the applied benthiocarb at the time of 65% dissipation.

2. *Formation of N-Vinyl-orbencarb from Orbencarb in the Soybean Plant and Rat Liver Microsomes*

The possible biotransformation of orbencarb to *N*-vinyl-orbencarb was investigated using ^{14}C-orbencarb-treated soybean seedlings and an incubation mixture of ^{14}C-orbencarb and rat liver microsomes fraction. *N*-Vinyl-orbencarb was detected and identified by two-dimensional co-TLC with the authentic compound using solvent systems A and B. The results clearly indicated the formation of *N*-vinyl-orbencarb from orbencarb both in the soybean plant and rat liver microsomes. The metabolite in the soybean plant represented only 0.7% of the applied radioactivity 90 hr after treatment and that in the rat liver microsomes 2.8% during 20 min incubation.

DISCUSSION

As shown in Fig. 2, carbinolamine which is oxygenated to α-carbon atom against nitrogen may be a precursor for *N*-vinyl and *N*-desethyl analogs of orbencarb or benthiocarb. Loss of water from the carbinolamine intermediate should yield the *N*-vinyl compound, while loss of acetaldehyde the *N*-desethyl compound. The former reaction was previously observed in the metabolism study of lysergic acid diethylamide (LSD) in microorganisms[8,9] or rat liver microsomes.[10] The latter is a well-known biotransformation mechanism of pesticides having a *N,N*-diethylamide moiety such as orbencarb,[3,4,6] benthiocarb[1,2,5,7] and R-7465[11] (2-α-naphthoxy-*N,N*-diethyl-propionamide). In case of ^{14}C-orbencarb, the *N*-desethyl-orbencarb accounted for 1.4 and 8.5% of the applied radioactivity in the soybean plant and rat liver microsomes,[12] respectively, and these values were relatively high compared to those of *N*-vinyl-orbencarb as described before.

In 1980, Golovleva *et al.*[7] in USSR reported on the basis of mass spectrographic analysis that *N*-tetramethylene-benthiocarb was a metabolite derived from benthiocarb by microorganisms. The mass spectrum of *N*-tetramethylene-benthiocarb shown in their paper was quite similar to that of the synthesized *N*-vinyl-benthiocarb (Fig. 1). It is highly possible, therefore, that they concluded erroneously that the metabolite was *N*-tetramethylene-benthiocarb only based on that mass spectrum.

In the field of pesticide metabolism, there have been many reports on *N*-dealkylation of *N,N*-dialkylamide derivatives. As a novel biochemical reaction, we suggest a metabolic pathway through which the alkyl side chain of *N,N*-diethylamide group is transformed to *N*-ethyl-*N*-vinylamide group *via* carbinolamine intermediate.

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要　　約

除草剤 orbencarb と benthiocarb の土壌，植物およびラット肝ミクロゾームから検出される新代謝物：*S*-2-chlorobenzyl *N*-ethyl-*N*-vinylthiocarbamate とその 4-クロロ異性体

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[Ring-U-^{14}C] オルベンカルブを用い，畑条件土壌，ダイズ茎葉部およびラット肝ミクロゾーム画分におけるオルベンカルブの生物変換を調べた．オルベンカルブの *N*-ethyl 基から2水素原子が除かれた新代謝物がすべての試験において検出され，合成標品との2次元 Co-TLC により *S*-2-chlorobenzyl *N*-ethyl-*N*-vinylthiocarbamate と同定された．また，非放射性ベンチオカーブ（オルベンカルブの4-クロロ異性体）を処理した畑条件土壌からも *S*-4-chlorobenzyl *N*-ethyl-*N*-vinylthiocarbamate が単離され，その化学構造は合成標品の GC 保持時間および GC-マススペクトルとの比較照合によって確認された．