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Microbial Metabolism of N-Methylcarbamate Insecticide. IV.[1] New Metabolites of *o-sec*-Butylphenyl N-Methylcarbamate by *Cladosporium Cladosporioides*

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The metabolism of *o-sec*-butylphenyl N-methylcarbamate (BPMC) has been investigated using a fungus, *Cladosporium cladosporioides*. This fungus was isolated from the soil over which the drug had been sprayed. As the result, it has become apparent that BPMC is metabolized to phenols with hydroxylated side-chains and to ω-carboxylic acid.

o-sec-Butylphenyl N-methylcarbamate, BPMC (I), is one of the representative carbamate insecticides currently manufactured and used in Japan. Studies on the metabolism of N-methylcarbamate insecticides were mostly focused on phenolic substances.[3] In the preceding studies on the metabolic degradation of BPMC by microorganisms, a number of metabolites were obtained and particularly *Cladosporium* species, among microorganisms isolated from the soil previously sprayed with the insecticide, metabolized it to highly polar acid substances with a remarkable efficiency. The present study was undertaken to assess their chemical and physicochemical properties and to investigate phenolic metabolites in more detail, which were not theretofore detected.

After incubation for 11 days, broth cultures of *Cladosporium* species were acidified to pH 2.0 with hydrochloric acid and then extracted with ether, followed by further extraction of the ether layer with a saturated sodium bicarbonate solution. From the remaining ether phase previously shown to contain neutral and phenolic substances, three phenolic metabolites

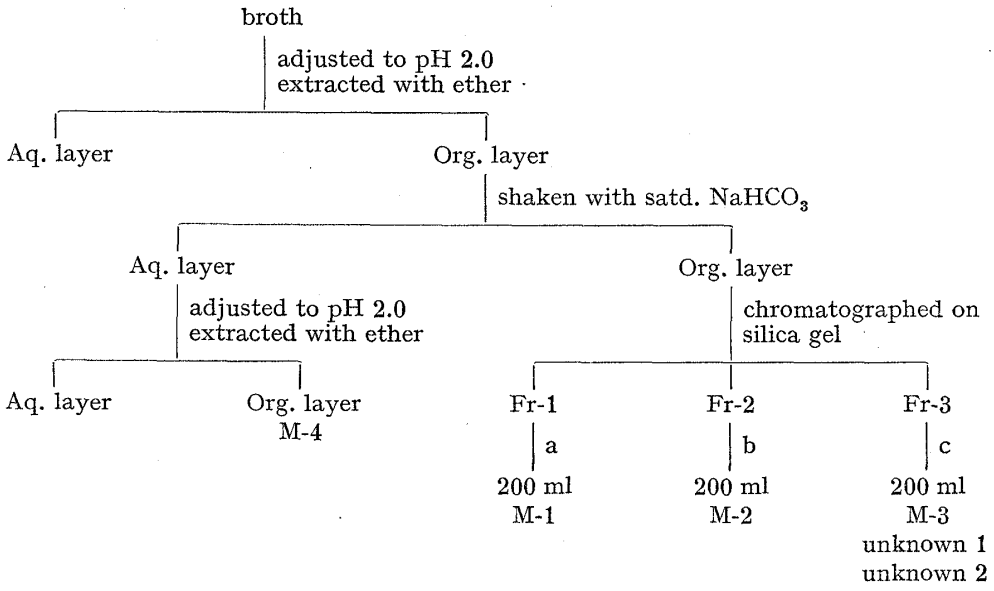

Chart 1. Isolation of the Metabolites

solvent systems of column chromatography, a: benzene, b: $CHCl_3$, c: 5% MeOH in $CHCl_3$

1) Part III: T. Suzuki and M. Takeda, *Chem. Pharm. Bull.* (Tokyo), **24**, 1983 (1976).
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were obtained by column chromatographic fractionation, followed by thin-layer chromatography (TLC) (Chart 1). The foregoing sodium bicarbonate extract was readjusted to pH 2.0 and extracted with ether to isolate carboxylic acid.

TABLE I. Thin-Layer Chromatography[a] of Authentic Compounds and Metabolites of BPMC

Authentic samples	Metabolites	*Rf* values in solvent system[b] of								Detection[c] of spots with	
		A	B	C	D	E	F	G	H	P	Q
II	M-1	0.52		0.53	0.51					+	
III	M-2	0.36		0.41	0.47					+	
IV	M-3	0.23	0.73	0.28	0.30					+	
VI	M-4					0.48	0.32	0.25	0.36		−
VI-methyl ester	M-4-methyl ester			0.26							+
				0.12[d]							
VII						0.48	0.28	0.29	0.39		−
VII-methyl ester				0.26							+

a) Silica gel HF_{254} was used unless otherwise stated.
b) Following solvent systems were used; (A) MeCN-benzene (1: 9, v/v), (B) ether–hexane (2: 1, v/v), (C) ether hexane (1: 1, v/v), (D) MeOH–$CHCl_3$ (5: 95, v/v), (E) $CHCl_3$–MeOH–AcOH (18: 1: 1, v/v/v), (F) $CHCl_3$–ether–AcOH (20: 10: 1, v/v/v), (G) benzene–MeOH–AcOH (45: 8:4, v/v/v) and (H) benzene–dioxane–AcOH (90: 25: 4, v/v/v).
c) Following color reactions were carried out. Phenolic metabolites were detected by spraying 1% aq. $FeCl_3$, followed by 1% (w/v) aq. $K_3Fe(CN)_6$ (P). Phenylcarbamate and phenyl N-methylcarbamate were detected by red-purple colors given in their reaction with 2% (w/v) ninhydrin in acetone containing 0.1% of collidine (w/v) at 100° (Q).
d) Aluminum oxide HF_{254} was used.

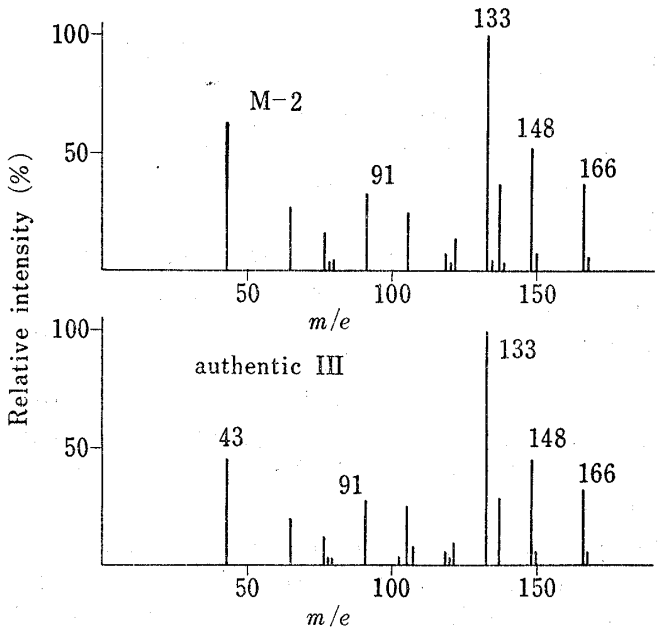

Fig. 1. Mass Spectra of M-2 and Authentic *o*-(1-Hydroxy-1-methylpropyl)phenol (III)

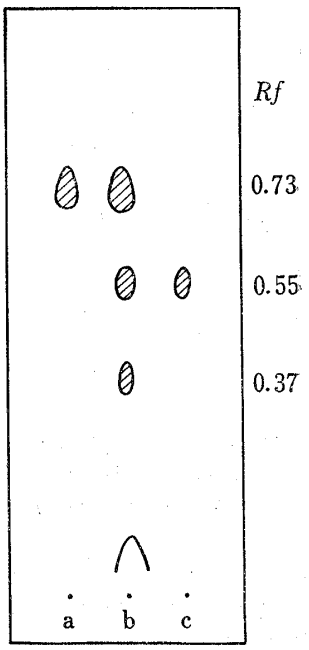

Fig. 2. Thin-Layer Chromatogram of the Metabolites and the Authentic Samples on Silica gel HF_{254}

a: authentic *o*-(2-hydroxy-1-methylpropyl)-phenol (IV)
b: Fr-3
c: authentic *o*-(3-hydroxy-1-methylpropyl)-phenol (V)
The plate was developed with solvent system B and sprayed with phenolic reagent. Shadowed spots were positive to phenolic reagent.

The structure of each metabolite thus obtained was determined by comparison of physicochemical properties, especially mass spectrum and gas chromatogram, with chemically synthesized standard preparations.

TLC of Fr-1 gave a spot (M-1) positive to phenolic reagents. Depend on the results of TLC with several solvent systems (Table I), M-1 was assigned as *o-sec*-butylphenol, which was reported as a metabolite by *Aspergillus niger* in the previous paper.[4] M-2 obtained from Fr-2 was positive to phenolic reagent and its structure was determined as *o*-(1-hydroxy-1-methylpropyl)phenol by the same behaviors on TLC as shown in Table I and the same fragment pattern in mass spectrum as shown in Fig. 1, to those of an authentic sample (III), respectively.

As shown in Fig. 2, Fr-3 gave three spots positive to phenolic reagent on a silica gel plate with solvent system B (*Rf* 0.73, 0.55 and 0.37, respectively). M-3 (*Rf* 0.73) seems to be a hydroxyalkylphenol which showed a molecular ion peak at a *m/e* value corresponding to $C_{10}H_{14}O_2$ by analysis with a high resolution mass spectrograph. Acetylation of M-3 led to formation of diacetate, $C_{14}H_{18}O_4$. From the NMR data of M-3-diacetate including the integral ratio of methyl groups of both acetoxyls, M-3 was noted to represent a mixture[5] of two compounds with different configuration of the hydroxyl group at the 2-position. Accordingly, M-3 was assinged to a mixture of *threo-* and *erythro-o*-(2-hydroxy-1-methylpropyl)phenol (*threo*: *erythro*, 3: 1). The material (*Rf* 0.55, unknown 1), corresponding to authentic *o*-(3-hydroxy-1-methylpropyl)phenol (V) on TLC, was acetylated by a conventional method and applied to gas chromatography. Any identities with V-acetate, however, were not obtained. The material (*Rf* 0.37, unknown 2) was so small in quantity that spectral behaviors and the chemical entities of the compound could not be further elucidated.

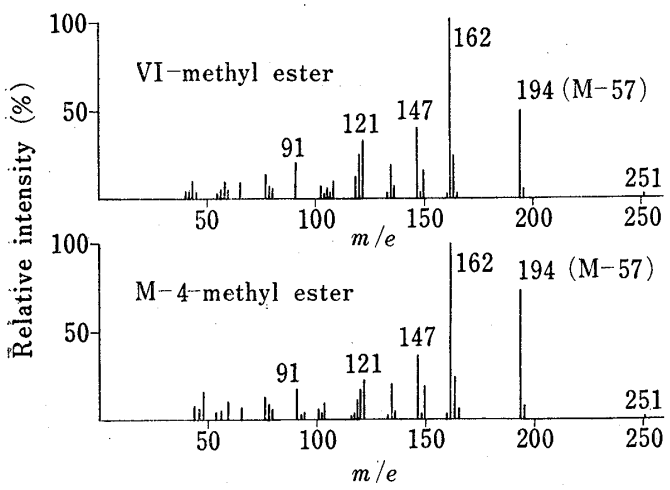

Fig. 3. Mass Spectra of M-4 Methyl Ester and Authentic *o*-(2-Methoxycarbonyl-1-methylethyl)phenyl N-Methylcarbamate (VI-methyl ester)

In the preceding paper,[1] *o*-(2-carboxy-1-methylethyl)phenyl N-methylcarbamate (VI) was suggested to form in the metabolism of ^{3}H-BPMC by *C. cladosporioides* or by other fungi species, but their chemical identity was not confirmed. Methyl ester of M-4 obtained from the $NaHCO_3$-soluble fraction proved to give a positive ninhydrin test and show a mass spectrum identical with that of compound VI-methyl ester (Fig. 3). However, since compounds VI-methyl ester and *o*-(1-methoxycarbonylpropyl)phenyl N-methylcarbamate (VII-methyl ester) display essentially the same *Rf* value on TLC, it remained unclear whether M-4 contained a small amount of compound VII-methyl ester as contaminant (Table I). Subsequent analysis by gas-liquid chromatography showed that there was no evidence of contamination of M-4 with compound VII-methyl ester (Table II).

By the present study it has become apparent that BPMC is metabolized to phenols, hitherto not detected, with hydroxylated side-chains and to ω-carboxylic acid. It cannot be concluded, nevertheless, from these experimental findings alone whether the hydrolysis of carbamoyl group precedes the hydroxylation of the side-chain or the reverse, in the microbial BPMC metabolism.

4) Part I: T. Suzuki and M. Takeda, *Chem. Pharm. Bull.* (Tokyo), **24**, 1967 (1976).
5) Part II: T. Suzuki and M. Takeda, *Chem. Pharm. Bull.* (Tokyo), **24**, 1976 (1976).

TABLE II. Gas-Liquid Chromatography of Metabolites and Authentic Samples

Authentic samples	Metabolites	Retention times	
		Condition I[a]	Condition II[b]
IV-acetate	M-3-acetate	5.1	3.0
VI-methyl ester	M-4-methyl ester	5.4	2.6
VII-methyl ester		3.3	2.0

a) packed with 5% Apiezon L Temp.: 140°
b) packed with 5% OV-1 Temp.: 120°

Experimental

Nuclear magnetic resonance spectra (NMR) were measured with a spectrometer Model 3H-60, Japan Electron Optics Lab., using tetramethylsilane (TMS) as an internal standard. Multiplicities of signals are represented as s (singlet), d (doublet), t (triplet), qt (quintet), m (multiplet). Mass spectra were taken with a JEOL-OlSG-2 mass spectrometer equipped with EI source, with ionizing energy at 75 eV.

Chemicals——*o-sec*-Butylphenol (II), *o*-(1-hydroxy-1-methylpropyl)phenol (III), a mixture of *threo*- and *erythro-o*-(2-hydroxy-1-methylpropyl)phenol (IV) (*threo*: *erythro*, 1.3: 1), IV-diacetate, *o*-(3-hydroxy-1-methylpropyl)phenol (V), *o*-(2-carboxy-1-methylethyl)phenyl N-methylcarbamate (VI), VI-methyl ester, *o*-(1-carboxypropyl)phenyl N-methylcarbamate (VII) and VII-methyl ester were prepared same as reported in the previous paper.[5]

Cultivation——*Cladosporium cladosporioides*[1] was used for the present study. Broth cultures were prepared and incubated with the same media and in the same manner as described previously[4] with the exception of addition level (10 mg of BPMC/300 ml of broth).

Fractionation of Metabolites——Extraction was achieved according to Chart 1. The $NaHCO_3$ extract was stored for the preparation of M-4. The ether extract was applied to silica gel column chromatography (20 g of silica gel) and the column was eluted successively with benzene (Fr-1), $CHCl_3$(Fr-2) and MeOH–$CHCl_3$ (5: 95, v/v) (Fr-3).

Isolation of M-1——The oily residue obtained from Fr-1 was subjected to preparative TLC with solvent system C and the spot (*Rf* 0.53) was extracted to give 4 mg of M-1, *o-sec*-butylphenol, as an oily material.

Isolation of M-2——Fr-2 was submitted to preparative TLC with solvent system A and the spot showing *Rf* 0.36 was extracted to give 1 mg of M-2, *o*-(1-hydroxy-1-methylpropyl) phenol, as a syrupy residue. Mass Spectrum *m/e*: 166 (M^+), 148, 137, 133 (base peak), 105, 91.

Isolation of M-3——The spot showing *Rf* 0.73 on TLC of Fr-3 with solvent system B was further purified by rechromatography with solvent system C to give 13 mg of M-3, a mixture of *threo*- and *erythro-o*-(2-hydroxy-1-methylpropyl)phenol, $[\alpha]_D^{20}$ +0.35° (c=1.0, $CHCl_3$). Mass Spectrum *m/e*: 166.1008 (M^+) (Calcd. for $C_{10}H_{14}O_2$, 166.0994). Acetate: M-3 (8 mg) was acetylated as usual to give 7 mg of M-3-acetate as a colorless oil, $[\alpha]_D^{24}$ +1.52° (c=0.5, $CHCl_3$). IR $\nu_{max}^{liq.\ film}$ cm^{-1}: 1765, 1736 (CO). NMR ($CDCl_3$) δ (*J*=Hz): 1.13 (3H, d, *J*=5.4, CH(OAc)C<u>H</u>$_3$), 1.23 (3H, d, *J*=6.0, PhCC<u>H</u>$_3$), 1.92 (3H, 77%, s, CHOCOC<u>H</u>$_3$), 2.07 (3H, 23%, s, CHOCOC<u>H</u>$_3$) 2.36 (3H, s, PhOCOC<u>H</u>$_3$), 3.26 (1H, qt, *J*=7.8, PhC<u>H</u>CH$_3$), 5.12 (1H, qt, *J*=7.2, C<u>H</u>OCOCH$_3$), 6.8—7.3 (4H, m, aromatic H). Mass Spectrum *m/e*: 250.1214 (M^+) (Calcd. for $C_{14}H_{18}O_4$, 250.1205), 190, 148, 133, 121, 91.

Isolation of M-4——The $NaHCO_3$ extract was acidified with dil. HCl to pH 2.0 and extracted with Et_2O to recover acidic substances (280 mg), which were submitted to preparative TLC with solvent system E to give 5 mg of crude M-4 as a syrupy residue. Methylation of this residue by MeOH–H_2SO_4 gave 2 mg of M-4 methyl ester, *o*-(2-methoxycarbonyl-1-methylethyl) phenyl N-methylcarbamate (authentic VI-methyl ester) as a colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3350 (NH), 1735, 1707 (CO). UV λ_{max}^{EtOH} nm: 262, 267. Mass Spectrum *m/e*: 251.1102 (M^+) (Calcd. for $C_{13}H_{17}O_4N$, 251.1109), 194 (M^+-57), 162, 147, 121.

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