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Microbial Metabolism of N-Methylcarbamate Insecticide. I. Metabolism of *o-sec*-Butylphenyl N-Methylcarbamate by *Aspergillus niger* van Tieghem[1)]

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In order to understand the metabolic fate of N-methylcarbamate in soil, the metabolism of *o-sec*-butylphenyl N-methylcarbamate (BPMC) (I) in a fungus, *Aspergillus niger* van Tieghem, was investigated. The following ten metabolites were detected in addition to unchanged BPMC; *o-sec*-butylphenol (M-1), *o-sec*-butylphenylcarbamate (M-2), *o*-(1-methylacetonyl)phenyl N-methylcarbamate (M-3), *o-sec*-butylphenyl N-hydroxymethylcarbamate (M-4), *o*-(1-hydroxy-1-methylpropyl)phenyl N-methylcarbamate (M-5), *threo-o*-(2-hydroxy-1-methylpropyl)phenyl N-methylcarbamate (M-6), *erythro-o*-(2-hydroxy-1-methylpropyl)phenyl N-methylcarbamate (M-7), *o*-(1-hydroxymethylpropyl)phenyl N-methylcarbamate (M-8), *o*-(3-hydroxy-1-methylpropyl)phenyl N-methylcarbamate (M-9) and one unidentified product (UK-1).

Both thin-layer chromatography (TLC) and gas-liquid chromatography (GLC) were used for detection and isolation of these metabolites, and the structures of these materials were characterized by ultraviolet, infrared, nuclear magnetic resonance and mass spectral analyses. Some major metabolites were identified by comparison of their chemical and physicochemical properties with those of synthesized materials.

The microbial degradation of pesticides is a problem that has received considerable attention in recent years. Among the common pesticides carbamate insecticides, unlike chlorine insecticides, are known to be readily biodegradable, so the total annual consumption of carbamates is gradually increasing every year. Therefore, the extensive investigation to determine their degradation products in an environment became necessary.

Several reviews are presented so far in regard to the biological transformation of N-methylcarbamate in animal,[3)] plants and insect.[4)] A few reports are also available on the metabolism of N-methylcarbamate by soil organisms,[5a-e)] in which it has been recognized that carbamates are readily metabolized at the carbamate ester linkage.

Environmental factors such as organic material level, moisture and temperature can effectively provide the alterations of microflora and microbial activity. Accordingly, the

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3) H.W. Dorough, *J. Agr. Food Chem.*, **18**, 1015 (1970).
4) R.J. Kuhr, *J. Agr. Food Chem.*, **18**, 1032 (1970).
5) *a*) S.-Y. Liu and J.-M. Bollag, *J. Agr. Food Chem.*, **19**, 487 (1971); *b*) B.V. Trucker and D.E. Pack, *ibid.*, **20**, 412 (1972); *c*) S.-Y. Liu and J.-M. Bollag, *Nature*, **236**, 177 (1972); *d*) H. Kazano, P.C. Kearney, and D.D. Kaufman, *J. Agr. Food Chem.*, **20**, 975 (1972); *e*) D.D. Kaufman, *ibid.*, **15**, 582 (1967).

studies on mode of metabolism by selected microbes may furnish informations as valuable reference for environmental toxicology.

The present investigation was attempted to elucidate the mechanisms of degradation of *o-sec*-butylphenyl N-methylcarbamate (BPMC) by *Aspergillus niger* (*A. niger*) van Tieghem, which has been used in Japan as a synthetic insecticide in order to protect rice plant against green rice leafhopper (*Tsumaguro yokobai*) and planthoppers (*Unka*).

Material and Method

Microorganisms and Cultivation——*A. niger*, isolated from soil of Tokyo metropolitan area, was used for this study. To 300 ml of cultivation medium which consisted of glucose (50 g), $NaNO_3$ (2 g), KH_2PO_4 (1 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), KCl (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), $ZnSO_4 \cdot 7H_2O$ (0.01 g), $CuSO_4 \cdot 5H_2O$ (0.005 g), Malt extract (0.5 g) and Yeast extract (0.2 g) in 1 liter of H_2O (final pH 4.62), was added an acetone solution (200 μl) containing 20 mg of *o-sec*-butylphenyl N-methylcarbamate (I) and spore of *A. niger*. Cultures were achieved in stationary flasks at 25° for 10 days, and the fungal mats were removed from the broth by decantation.

Extraction and Fractionation of Metabolites——The broth (18 liters) was treated as shown in Chart 1.

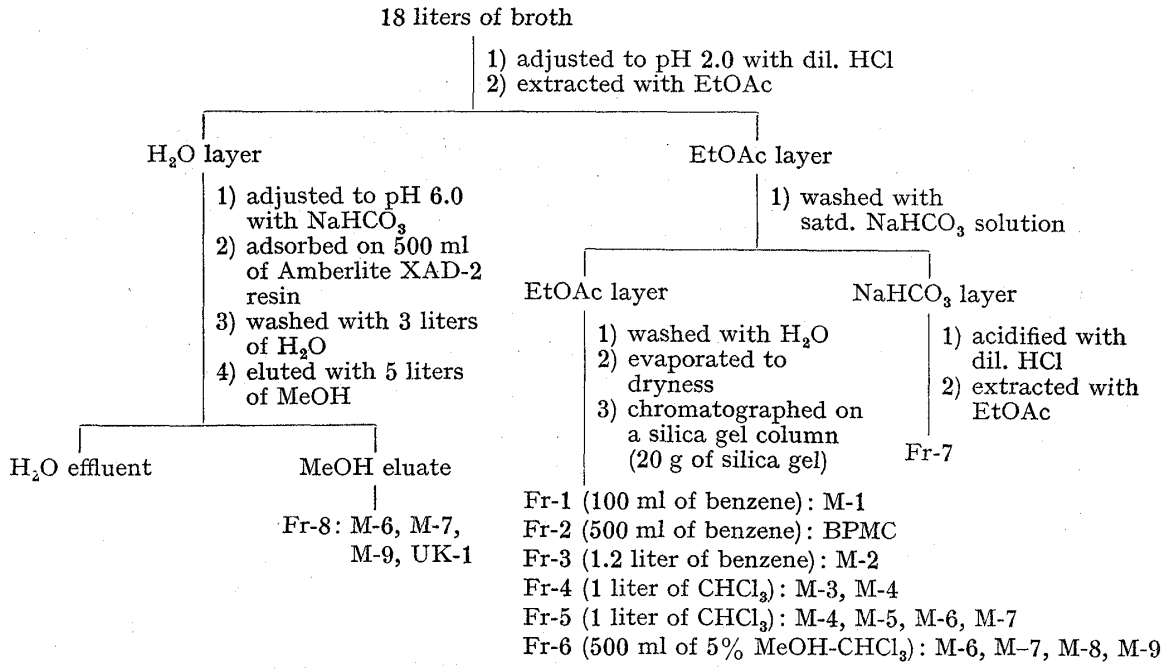

Chart 1. Procedure for the Extraction and Fractionation of Metabolites of BPMC

Acetylation of Metabolites——The hydroxyl derivatives obtained from the fractions such as Fr-5, Fr-6, and Fr-7, which were unsuitable for further purification and gas chromatographic analysis, were acetylated as described below. A mixture of Ac_2O (5 ml), pyridine (2 drops) and a sample was allowed to stand for 24 hr at room temperature and the solvent was then evaporated to dryness *in vacuo*. H_2O (5 ml) was added to the residue and the solution was extracted 3 times with EtOAc (30 ml each). The extracts were washed once with satd. $NaHCO_3$ (20 ml) and twice with H_2O (10 ml each), and the solvent was then evaporated to dryness *in vacuo* after dried over anhyd. Na_2SO_4.

Thin-Layer Chromatography (TLC)——TLC was carried out according to the following method. Plate: Silica gel HF_{254} or aluminum oxide HF_{254} (E. Merck) was coated on plates in 250 or 500 μ thickness. The former was used for qualitative studies and the latter for isolation and purification of metabolites. Developing solvent systems for TLC were described in the legend of Table I. Detection of spots on the chromatogram was carried out by three different procedures; *i. e.* exposure to I_2 vapor (L), ultraviolet (UV) irradiation (253 nm) (M), and spraying with chromogenic reagents.

Color Reactions——Phenolic metabolites were detected by spraying 1% (w/v) $FeCl_3$ solution, followed by 1% (w/v) $K_3Fe(CN)_6$ solution[6] (reagent-N). Carbamate esters and phenols were detected by spraying with 1% KOH solution followed by 1 N HCl and then reagent-N (reagent-O). Phenylcarbamate and phenyl N-

6) G.M. Barton, R.S. Evans, and J.A.F. Gardner, *Nature*, **170**, 249 (1972).

methylcarbamate were detected by red-purple colors given in their reactions with 2% (w/v) ninhydrin in acetone containing 0.1% (w/v) of collidine at 100° (reagent-P).[7] With 2,4-dinitrophenylhydrazine, ketone gave a yellow-orange color (reagent-Q). Chromotropic acid[8] (0.2% (w/v) in 12 N H_2SO_4 at 100°) gave purple colors with N-hydroxymethyl derivatives (reagent-R).

Gas-Liquid Chromatography (GLC)——A Shimadzu gas chromatograph (GC-3AF) was used throughout the present study. The column (glass column, 3 mm × 150 cm) was packed with 5% Apiezon L on Chromosorb G (60—80 meshes). The temperature of inlet and column oven was conditioned to 120—160°. Nitrogen was used as a carrier gas (0.8 kg/cm²).

Authentic Sample——Syntheses of the following samples will be described in the forthcoming paper[9] in this series: *o-sec*-Butylphenyl N-methylcarbamate (BPMC) (I), *o-sec*-butylphenol(II), *o-sec*-butylphenylcarbamate (III), *o*-(1-hydroxy-1-methylpropyl)phenyl N-methylcarbamate (IV), a mixture of *threo*- and *erythro*-*o*-(2-hydroxy-1-methylpropyl)phenyl N-methylcarbamate (V: *threo*/*erythro* 2.0; VI: *threo*/*erythro* 1.3), V-acetate, VI-acetate, *o*-(3-hydroxy-1-methylpropyl)phenyl N-methylcarbamate (VII), VII-acetate, *o*-(1-acetoxymethylpropyl)phenyl N-methylcarbamate (VIII-acetate), 3-methyl-2H-1,3-benzoxazine-2,4 (3H)-dione (IX), *o*-(2-carboxy-1-methylethyl)phenyl N-methylcarbamate (X), X-methyl ester, a mixture of *threo*- and *erythro*-*o*-(2-hydroxy-1-methylpropyl)phenol (XI: *threo*/*erythro* 0.20; XII: *threo*/*erythro* 1.3), XI-acetate and XII-diacetate.

Spectral Measurements——Instruments and conditions were depicted in the legend of each Table.

Result

As shown in Chart 1, solvent extraction and subsequent chromatography gave eight fractions, which were submitted to TLC to obtain individual metabolites. The 5% MeOH–$CHCl_3$ extracts from TLC bands corresponding to a metabolite or metabolites mixture, were evaporated under reduced pressure and applied to spectroscopy.

Separation of Metablites

M-1 (8 mg), BPMC (820 mg) and M-2 (3 mg) were purified by TLC of Fr-1, Fr-2 and Fr-3, respectively, on Silica gel HF_{254}, with solvent system A for the former two and with solvent system F followed by solvent system G for the latter.

M-3 (8 mg) and M-4 (12 mg) were separated by the preparative TLC of Fr-4 on Silica gel HF_{254} with solvent system A, followed by TLC on aluminum oxide with solvent system F for the former and recrystallization from ether–hexane for the latter, respectively.

M-4 (25 mg), M-5 (12 mg) and M-6 (230 mg as acetate) in Fr-5 were separated by preparative TLC on Silica gel HF_{254} with solvent system F, followed by recrystallization from ether–hexane for the first and acetylation for the last, respectively.

M-7 (45 mg as acetate), M-8 (2 mg as acetate) and M-9 (14 mg as acetate) in Fr-6 were separated by preparative TLC on Silica gel HF_{254} with solvent system G with four times repetition, followed by recrystallization from benzene–hexane for the first and rechromatography on aluminum oxide HF_{254} with solvent system E for the latter two, respectively.

A mixture of M-6 and M-7 (55 mg), M-9 (5 mg) and UK-1 (trace) in Fr-8 were separated by TLC on Silica gel HF_{254} with solvent system C followed by rechromatography with solvent system E after acetylation.

Fr-7 contained various kinds of natural product, so that separation and purification were very difficult. In addition to this fact, the proper method of detection on the plate had not been found out, so further experiments were not performed on this fraction.

Identification of Metabolite

M-3——M-3 on TLC was visualized as yellow by reagent-Q or violet by reagent-P and positive to reagent-O or negative to reagent-N, indicating that M-3 was a carbamate having a ketone function. Nuclear magnetic resonance (NMR) spectrum of M-3 exhibited proton signals as follows: δ ppm 1.34 (3H, doublet (d), J=7.0, $C_6H_5CHC\underline{H}_3$), 2.01 (3H, singlet (s),

7) E.G. Gemrich, *J. Agr. Food Chem.*, **15**, 617 (1967).
8) R.L. Metcalf, T.R. Fukuto, C. Collins, K. Borck, S. Abdel Aziz, and C.C. Cassil, *J. Agr. Food Chem.*, **16**, 300 (1968).
9) T. Suzuki and M. Takeda, *Chem. Pharm. Bull.* (Tokyo), **24**, 1976 (1976).

Table I. Summary of Metabolites of BPMC detected by TLC

Authentic samples	Metabolites	*Rf* values in solvent system[a] of							Detection[b] of spots with						
		A	B	C	D	E	F	G	L	M	N	O	P	Q	R
II	M-1	0.54	0.56	—	—	—	0.97	—	+	+	+	+	—	—	—
I	BPMC	0.46	0.42	—	—	—	0.97	—	+	+	—	+	+ (v)		
III	M-2	0.33	0.36	—	—	—	0.71	—	+	+	—	+	+ (y)		
	M-3	0.29	0.28	—	—	0.29	0.59	—	+	+	—	+	+ (v)	+ (y)	—
	M-4	0.16	0.22	—	—	—	0.50	—	+	+	—	+	+ (y)	—	+ (p)
IV	M-5	0.22	0.21	—	—	—	0.43	—	+	+	—	+	+ (v)	—	—
V, VI	M-6, M-7	0.14	0.13	0.91	0.73	—	0.27	— —	+	+	—	+	+ (v)	—	—
V-, VI-acetate	M-6-, M-7-acetate	—	—	—	—	—	—	0.55[c]	+	+	—	+	+ (v)	—	—
VIII	M-8	—	—	—	—	—	0.29	—							
VIII-acetate	M-8-acetate	—	—	—	—	0.18[c,d]	—	0.44[c]	+	+	—	+	+ (v)	—	—
VII	M-9	0.14	0.13	—	—	—	0.29	—							
VII-acetate	M-9-acetate	—	—	—	—	0.14[c,d]	—	0.44[c]	+	+	—	+	+ (v)	—	—
	UK-1	—	—	—	—	0.07	—	0.34[c]			+		+ (v)		

a) Following solvents systems were used: (A) MeCN–benzene (1: 5, v/v), (B) hexane–ether (1: 3, v/v), (C) iso-PrOH–$CHCl_3$–MeOH–H_2O (37: 37: 19: 2, v/v/v/v), (D) iso-PrOH–$CHCl_3$–MeOH–10N NH_4OH (37: 37: 19: 7, v/v/v/v), (E) MeCN–benzene (1: 9, v/v), (F) MeCN–benzene (1: 9, v/v), followed by ether–hexane (1: 1, v/v) (four times) and (G) hexane–ether (1: 1, v/v).
b) Following abbreviations were used; yellow (y), violet (v) and pink (p).
c) Plates were four times developed with the same solvent system to the same distance.
d) Aluminum oxide HF_{254} was used.

$COCH_3$), 2.88 (3H, d, J=5.0, $NHC\underline{H}_3$), 3.80 (1H, quartet (q), J=7.0, $C_6H_5C\underline{H}$), 5.00 (1H, broad signal, NH), 7.1—7.3 (4H, multiplet (m), aromatic H). On the other hand, M-3 showed carbonyl absorptions at 1710 and 1738 cm^{-1}, and the peak *m/e* 221 (Calcd. for $C_{12}H_{15}O_3N$: 221.1052, Found, 221.1060) in the infrared spectra (IR) and high resolution mass spectra, respectively. Accordingly, the structure of M-3 was assumed to be *o*-(1-methylacetonyl)-phenyl N-methylcarbamate.

M-4——M-4, $[\alpha]_D^{26}$ +0.26° (c=1.86, $CHCl_3$), mp 82—83°, on TLC was visualized as yellow by reagent-P or pink by reagent-R and positive to reagent-O or negative to reagent-N, suggesting that M-4 was a N-hydroxymethylcarbamate. NMR spectrum of M-4 exhibited proton signals as follows: δ ppm 0.80 (3H, triplet (t), J=6.5, $CH_2C\underline{H}_3$), 1.12 (3H, d, J=7.0, C_6H_5-$CHC\underline{H}_3$), 1.57 (2H, quintet (qt), J=7.0, $C\underline{H}_2CH_3$), 2.82 (1H, sextet (sx), J=6.9, $C_6H_5C\underline{H}$), 4.70 (2H, d, J=7.0, $C\underline{H}_2OH$), 5.98 (1H, broad signal, NH), 6.8—7.2 (4H, m, aromatic H). On the other hand, M-4 showed a carbonyl absorption at 1714 cm^{-1} and the parent peak *m/e* 223 (Calcd. for $C_{12}H_{17}O_3N$: 223.1208, Found, 223.1213) in the IR and high resolution mass spectra, respectively. Therefore the structure of M-4 was assigned to *o-sec*-butylphenyl N-hydroxymethylcarbamate.

M-6——M-6-acetate, a colorless oil, $[\alpha]_D^{26}$ −4.3° (c=0.81, $CHCl_3$), on TLC was visualized as violet by reagent-P and positive to reagent-O or negative to reagent-N, revealing that M-6 was a hydroxycarbamate. The molecular formula of M-6-acetate was established as $C_{14}H_{19}O_4N$ from the molecular ion (M^+) in a high resolution mass spectrum (Calcd. 265.1314, Found, 265.1342). The IR spectrum showed an absorption band due to a carbamate and a acetate at 1745 cm^{-1}. NMR spectrum of M-6-acetate shown in Fig. 1a exhibited proton signals as follows: δ ppm 1.10 and 1.07 (3H, two doublets, J=7.0 and 6.5, $CH(OAc)C\underline{H}_3$), 1.23 (3H, d,

J=7.2, $C_6H_5CHC\underline{H}_3$), 1.94 (3H, 87%, s, $OCOC\underline{H}_3$), 2.84 (3H, d, J=5.0, $NHC\underline{H}_3$), 3.29 (1H, qt, J=7.5, $C_6H_5C\underline{H}$), 5.18 (1H, qt, CHOCO), 5.51 (1H, broad signal, NH), 7.1 (4H, m, aromatic H). In addition to these signals, the other minor signal was observed at 2.04 ppm (3H, 13%, s, $OCOC\underline{H}_3$), revealing that the obtained material was contaminated with one having a similar structure. On the basis of these facts, the structure of M-6 was assigned to *o*-(2-hydroxy-1-methylpropyl)phenyl N-methylcarbamate. Hydrolysis of M-6-acetate (20 mg) with MeOH–KOH, followed by acetylation gave 13 mg of the diacetate, whose molecular formula was established as $C_{14}H_{18}O_2$ based on the mass spectroscopy, exhibiting the signals due to an aliphatic acetoxyl at 1.92 ppm (88%) and 2.07 ppm (12%), respectively, in the NMR spectrum.

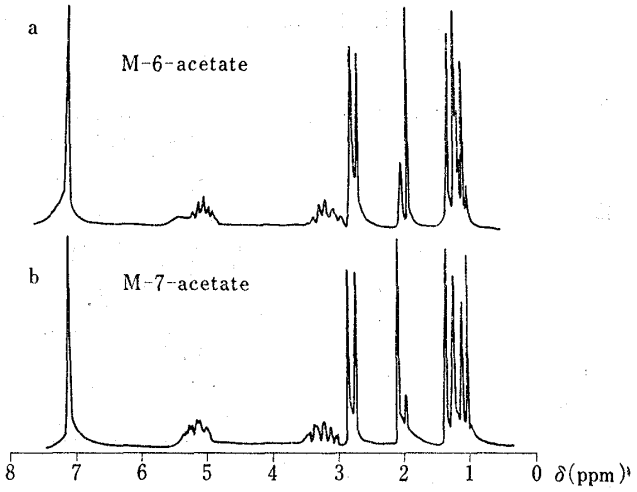

Fig. 1. NMR Spectra of M-6-and M-7-Acetates

M-7——M-7-acetate, needles, mp 89—91°, showed the similar spectra with those of M-6-acetate in the IR and mass. The molecular formula of M-7-acetate was established as $C_{14}H_{19}O_4N$ from the molecular ion (M^+) in a high resolution mass spectrum (Calcd. 265.1314, Found, 265.1342). NMR spectrum of M-7-acetate shown in Fig. 1b exhibited proton signals as follows: δ ppm 1.10 and 1.07 (3H, two doublets, J=7.0 and 6.5, $CH(OAc)C\underline{H}_3$), 1.23 (3H, d, J=7.0, $C_6H_5CHC\underline{H}_3$). 2.04 (3H, 85%, s, $OCOC\underline{H}_3$), 2.90 (3H, d, J=5.1, $NHC\underline{H}_3$), 3.13 (1H, qt, J=6.5, $C_6H_5C\underline{H}$), 5.17 (1H, qt, J=5.3, CHOCO), 5.20 (1H, broad signal, NH), 7.3 (4H, m, aromatic H). On the basis of these spectral findings the structure of M-7 was assumed to be *o*-(2-hydroxy-1-methylpropyl)phenyl N-methylcarbamate. The NMR spectra of M-7-acetate was close similar to that of M-6-acetate but remarkably different in the chemical shift of acetoxyl group, that is, M-6-acetate showed an acetoxyl signal at 1.94 ppm while M-7-acetate exhibited it at 2.04 ppm. These results led us to the conclusion that M-6-acetate and M-7-acetate were stereomers different in the orientation of the introduced hydroxyl groups. And also it is probable that the small signal at 1.94 ppm (15%) in Fig. 1b is owing to a small amount of contaminated M-6-acetate, while the signal at 2.04 ppm in Fig. 1a is attributable to contaminated M-7-acetate.

Treatment of a mixture (46 mg, *Rf* 0.27), which was obtained by direct TLC (Silica gel HF_{254}, solvent system F) of the crude extracts from broth without any chromatographic

Table II. Summary of Metabolites of BPMC detected by GLC

Authentic samples	Metabolites	Retention times (min) under the column temperature of		
		120°	140°	160°
II	M-1	2.3	1.2	—
I	BPMC	—	4.9	—
III	M-2	2.3[a], 10.6	1.2[a], 4.7	—
	M-3	—	8.0	—
V-, VI-acetate	M-6-, M-7-acetate	—	—	5.7
VIII-acetate	M-8-acetate	—	—	7.9
VII-acetate	M-9-acetate	—	—	10.3

a) In gas chromatographic column, this sample was considered to be partly degradated into its component, *i.e.* phenylcarbamate to phenol.

TABLE III. Nuclear Magnetic Resonance of Reference Compounds and Metabolites

Authentic samples	Metabolites	NMR[a] assignment δ (ppm)						
		$CH_2\underline{CH_3}$	ϕ-$CHC\underline{H_3}$	$C\underline{H_2}CH_3$	ϕ-CH	$NHC\underline{H_3}$	NH	Other signals
II	M-1	0.86 (3H, t, J=6.8)	1.22 (3H, d, J=7.5)	1.66 (2H, qt, J=6.9)	2.95 (1H, sx, J=6.8)			
I	BPMC[b]	0.83 (3H, t, J=6.8)	1.20 (3H, d, J=7.5)	1.56 (2H, qt, J=7.5)	2.5—3.1 (1H, sx, J=6.8)	2.83 (3H, d, J=4.8)	4.98 (1H, br signal)	
III	M-2	0.84 (3H, t, J=6.9)	1.20 (3H, d, J=7.1)	1.62 (2H, qt. J=7.2)	2.90 (1H, sx, J=6.9)			NH_2, 5.00 (2H, br signal)
IV	M-5	0.79 (3H, t, J=7.2)	1.56 (3H, s)	1.87 (2H, q, J=7.0)		2.88 (3H, d, J=4.8)	4.97 (1H, br signal)	
VIII-acetate	M-8-acetate	0.81 (3H, t, J=7.0)		1.65 (2H, unresolv.)	3.23 (1H, qt, J=6.9)	2.90 (3H, d, J=5.0)	5.10 (1H, br signal)	CH_2O, 4.18 (2H, d, J=6.5)
VII-acetate	M-9-acetate		1.24 (3H, d, J=6.8)	1.96 (2H, q, J=8.2)	2.8—3.1 (1H, m, J=7.2)	2.90 (3H, d, J=4.9)	5.10 (1H, br signal)	CH_2O, 5.02 (2H, t, J=6.8)
IX	M-9a					3.48 (3H, s)		
X	M-9b		1.29 (3H, d, J=7.0)	1.4—1.7 (2H, unresolv.)	3.51 (1H, q, J=7.3)	2.84 (3H, d, J=4.6)	5.24 (1H, br signal)	CH_2CO, 1.4—1.7 (2H, unresolv.)

a) NMR spectra were measured in $CDCl_3$ solution with a spectrometer Model 3H-60, Japan Electron Optics Lab., using TMS as an internal standard. Multiplicities of signals are represented as a (singlet), d (doublet), t (triplet), q (quartet), qt (quintet), sx (sextet), m (multiplet), br signal (broad signal) and unresolv. (unresolved signal).
b) This sample was recovered from broth.

TABLE IV. Summary[a] of IR and Mass Spectral Data of Metabolites

Metabolites	Authentic compounds	IR cm^{-1}	Mass		
			Calcd. for [M^+]		Found
M-1	II	3600(OH)[b]	$C_{10}H_{14}O$;	150.1045	150.1032
M-2	III	3430(NH_2) 1745(CO)[c]	$C_{11}H_{13}O_2N$;	193.1098	193.1090
M-5	IV	3600(OH) 3350(NH) 1730(CO)[b]	$C_{12}H_{17}O_3N$;	223.1208	223.1214
M-8-acetate	VIII-acetate	1743(CO)[d]	$C_{14}H_{19}O_4N$;	265.1314	265.1359
M-9-acetate	VII-acetate	1738(CO)[d]	$C_{14}H_{19}O_4N$;	265.1314	265.1351
M-9a	IX	1763(CO) 1687(CO)[c]			
M-9b	X	3450(NH), 3000—2400 (COOH), 1736, 1708(CO)[b]			

a) Infrared (IR) spectra were measured with a spectrometer, Model DS-402 G, Japan Spectroscopic Co., Ltd. Mass spectra were taken with a JEOL JMS-OISG-2 mass spectrometer with the ionizing energy at 75 eV, equipped with EI source.
b) $CHCl_3$ soln c) KBr tab d) liq. film

purification and thought to be corresponding to M-6, M-7, M-8, or M-9 on TLC, with chromium trioxide gave, from neutral fraction, a syrupy residue, which was submitted to TLC on Silica gel HF_{254} with solvent system E. One spot (M-6a, *Rf* 0.29, 30 mg) was identified as (+) *o*-(1-methylacetonyl)phenyl N-methylcarbamate by comparison with M-3. ORD (c=0.142, MeOH) $[\alpha]^{24}$ (nm): −279° (277) (trough), +271° (313) (peak), +73° (350). Another spot (*Rf* 0.51) was extracted and evaporation of the solvent gave 2 mg of M-9a as colorless needles, mp 148—149°, which was characterized as 3-methyl-2H-1,3-benzoxazine-2,4 (3H)-dione (IX) by comparison with reference compound. On the other hand, aqueous $NaHCO_3$ washings gave 3 mg of M-9b as an oil, which was assigned to *o*-(2-carboxy-1-methylethyl)phenyl N-methylcarbamate by comparison with reference compound (X).

UK-1——UK-1, on TLC was visualized as violet by reagent-P, which was too small in quantity for examination of some spectral behaviors, so its structure was not further elucidated.

Other Metabolites——The identification of other metabolites was accomplished by co-chromatography and by the comparison of IR, mass and NMR spectra with those of synthesized reference compounds. As shown in Table I and II, M-1, BPMC, M-2, M-5, M-8-acetate and M-9-acetate were indistinguishable from II, I, III, IV, VIII-acetate and VII-acetate, respectively, on TLC and GLC. The NMR and IR spectra were also same as those of authentic compounds as shown in Table III and IV. In addition to these results, M-9-acetate showed a weak specific optical rotation, $[\alpha]_D^{26}$ +1.2 (c=1.7, MeOH).

Discussion

The metabolic fate of BPMC in *A. niger* van Tieghem was summerized in Chart 2, based on the metabolites obtained in the present study. Hydroxylation of alkyl side-chain seemed to be the most important process of transformation of the carbamate by *A. niger*.

The complete separation of a mixture of M-6 and M-7 into their component was tried by TLC and GLC but not succeeded. Based on the results of NMR, mass and IR spectroscopy, however, it was concluded that these two metabolites were *threo* and *erythro* isomers. Further-

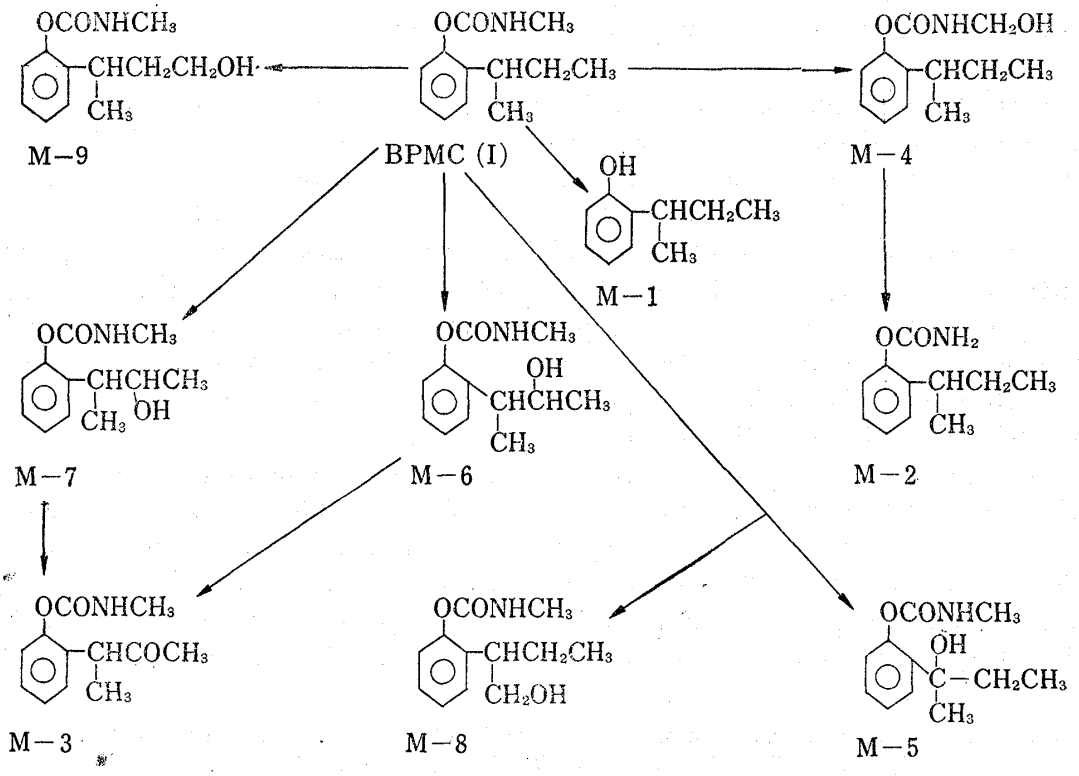

Chart 2. Proposed Pathways of Microbial Degradation of BPMC

more determination of the configuration was attempted by comparison of their NMR spectra with those of the synthetic diacetates.

It had been clarified that *threo-o*-(2-hydroxy-1-methylpropyl)phenol diacetate showed the signal due to an aliphatic acetoxyl group at 1.92 ppm whereas *erythro* isomer exhibited that at 2.07 ppm.[9] On the other hand, M-6-acetate and M-7-acetate showed the signals assignable to an aliphatic acetoxyl group at 1.94 and 2.04 ppm, respectively. Accordingly, it will be expected that M-6 is a *threo* and M-7 is an *erythro* isomer, respectively. This assignment was also supported by following chemical conversion. That is, the diacetate (M-6b) derived from M-6 (1.94 ppm, 87%; 2.04 ppm, 13%) showed the signals due to an aliphatic acetoxyl group at 1.92 ppm (88%) and 2.07 ppm (12%), respectively. Therefore, the signal at 1.94 ppm of M-6-acetate was correlated to that at 1.92 ppm of the diacetate (M-6b) while the signal at 2.04 ppm due to the minor product contained as an impurity (M-7-acetate), was correlated to one at 2.07 ppm of the diacetate (M-6b). On the basis of the above described reason, it seems reasonable to assess M-6 as a *threo* and M-7 as an *erythro* isomer, respectively.

Next, the stereospecific metabolism is now discussed. M-4, M-6, M-9 and M-6a showed the specific optical rotations. Especially, M-6a, which was chemically yielded by oxidation of an about 6: 1 mixture of M-6 and M-7, and identified to be M-3, exhibited a positive Cotton effect in the optical rotatory dispersion curve. Moscowitz, *et al.*[10] have previously pointed out in relation to the determination of absolute configuration of 1-methylacetonylbenzene that the open chain α-phenyl ketone may exist in two possible conformational equilibria for each enantiomer (*R*) and (*S*), respectively, and that (*S*)-isomer show a positive and (*R*)-isomer a negative Cotton curve, respectively. When the concept cited above may be extended to M-6a, the absolute configuration of M-6a and M-6 with respect to their common carbon atom, namely the 1-position, should be assumed to be (*S*) configuration. Accordingly, the absolute configuration of M-6 with regard to another asymmetric center, the 2-position, should be assigned to be (*S*) configuration since the configuration of M-6 with respect to the 1- and 2-position had already been clarified as *threo*. Unfortunately, optical rotatory dispersion (ORD) measurement of oxidation product of M-7 was not attempted because of that minor quantity, so the absolute configuration was not determined.

These results led us to the assumption that one enantiomer of BPMC may be more selectively transformed than the other.

Oxidation of a mixture of M-6 and M-7 gave a small amount of 3-methyl-2H-1,3-benzoxazine-2,4 (3H)-dione (M-9a) besides M-6a. However, it remains unsolved what is a precursor of M-9a. In spite of our exhaustive efforts, any other metabolites could not be found out in this fraction except M-6, M-7, M-8 and M-9. Accordingly, it would be suggested that by oxidation of a part of M-6a or by other degradative pathways M-9a was artificially produced.[9]

Furthermore, one hydrolyzed metabolite (M-1) was obtained. Meanwhile, Baggi, *et al.*[11] reported that a *pseudomonas* utilized 2-phenylbutane as sole carbon and energy source. In this case, 2-phenylbutane was converted to 2-(2,3-dihydro-2,3-dihydroxyphenyl)butane and then subjected to ring fission. Also in the case of BPMC, after cleavage of the carbamoyl linkage, such a degradation pathways may be present.

The ring-hydroxylated product, *i.e.* main metabolites of aromatic compounds, as in the case of carbaryl,[5a,c] were not found. In metabolism of 3,5-dimethylphenyl N-methylcarbamate by *A. niger*, however, a ring-hydroxylated metabolite was obtained.[12] This phenomenon may be due to the substrate specificities of the enzymes.

In the present experiment, *o-sec*-butylphenyl N-hydroxymethylcarbamate and *o-sec*-butylphenyl carbamate were also isolated from the broth. This demonstrates not only the

10) A. Moscowitz, K. Mislow, M.A.W. Glass, and C. Djerassi, *J. Am. Chem. Soc.*, **84**, 1945 (1962).
11) G. Baggi, D. Catelani, E. Gall, and V. Treccani, *Biochem. J.*, **126**, 1092 (1972).
12) Unpublished data.

possibilities that the degradations of N-methylcarbamate are, as postulated by Kaufman,[5e] initiated by direct attack of carbamoyl linkage also proceed *via* the pathway by which N-methylcarbamates are demethylated after oxidation of N-methyl group, followed by fission of carbamoyl linkage.

One unknown metabolite (UK-1) detected by TLC was so small in quantitity that the chemical entities could not be clarified further.

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