Fatty Acid Components of the Defensive Substances in Acid-Secreting Carabid Beetles¹

Katsuo KANEHISA

*Laboratory of Applied Entomology, Institute for Agricultural and Biological Sciences,
Okayama University, Kurashiki 710, Japan*

Kazuyoshi KAWAZU

Department of Agricultural Chemistry, Okayama University, Okayama 700, Japan

(Received March 10, 1982)

Fatty acid components of the defensive secretion in carabid beetles were identified by gas chromatography and mass spectrometry. Major components so far detected were divided into six groups: (1) formic acid, (2) methacrylic and tiglic acids, (3) methacrylic and α -ethylacrylic acids, (4) 2-Me-propanoic, 2-Me-butanoic and 3-Me-butanoic acids, (5) methacrylic, crotonic, senecioic and tiglic acids, (6) methacrylic, tiglic and 2-hexenoic acids. Each group of major components was accompanied by common minor acid components. There was a large difference in acid quantity but little difference in quality. Their relationships with each other are discussed on the basis of presumed synthetic pathways.

INTRODUCTION

It was previously reported that carabid beetles secreted fatty acids, *m*-cresol and benzoquinones, as defensive substances. These substances were produced by the synthetic lobes of each characteristic shape in the pygidial parts, and there were clear relationships between the substances and beetle taxonomy. Many beetles had the same substances at the genus level and some beetles had species-specific substances (KANEHISA and MURASE, 1977, KANEHISA and SHIRAGA, 1978). However, some problems remained to be solved; one was the precise identification of fatty acids having similar retention times on gas chromatography, and another was the elucidation of the minor components. Recently synthetic pathways of fatty acids in insect defensive secretion have been presented as metabolic pathways from branched amino acids (Benn et al., 1973, Duffy, 1976, Blum, 1978). These pathways were useful for presumption of the minor components. This report describes the identification of fatty acids as determined by gas chromatography and mass spectrometry.

¹ This work was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (No. 56360005, 1981).

MATERIALS AND METHODS

Carabid beetles. About 200 species were collected in the Chugoku district, mostly in Okayama Prefecture.

Analytical specimens. Defensive substances were extracted in the following three ways: 1) by immersing the beetle in diethyl ether for a few minutes just after its capture, 2) by immersing it in diethyl ether at -20°C from its capture till the sample preparation, 3) by diethyl ether extraction of the homogenated abdominal parts in which synthetic and reservoir organs were located. These ether solutions were fractionated to acid and neutral components. For the identification of unsaturated acids, samples were routinely subjected to catalytic hydrogenation over Adams catalyst (KANEHISA and MURASE, 1977).

Gas chromatography. Gas chromatography was conducted on a Hitachi 163 gas chromatograph equipped with an FID and nitrogen as carrier gas, and a Hitachi 063 gas chromatograph equipped with a TCD and helium as carrier gas. The following 2-m long and 3-mm in diameter glass columns were used: 1) 25% FAL-M on Chromosorb W, 2) 10% FON on Celite 545 A, 3) 10% PEG-20M on Chromosorb W (AW-DMCS), and 4) 10% and 15% FFAP on Chromosorb W (AW).

Gas chromatography—mass spectrometry. Mass spectra were obtained on a JEOL JMS-D 300 mass spectrometer combined with a JGC-20K gas chromatograph (glass column, 1-m $\times$ 2-mm) which was on-lined with a mass data analysis system, JMA-2000. Peaks, which were resolved on a 15% FFAP (Neosorb 60/80 mesh) column at a temperature programmed from 90° – 150°C at a rate of $3^{\circ}\text{C}/\text{min}$ and with a 25 ml/min helium flow, were scanned at 70 eV from m/z 25 to m/z 250 (ion source 200°C).

RESULTS

Most of the acid components in the carabid defensive secretions were separated and identified by gas chromatography. The identification of the main unsaturated acids were verified by hydrogenation.

Retention times of the authentic samples are shown in Table 1. FAL-M was the best column for separation of methacrylic, 2-Me-butanoic and 3-Me-butanoic acids, which showed similar retention times on the FON, PEG-20M and FFAP columns. FAL-M was also the best column for separation of crotonic, α -ethylacrylic, angelic and senecioic acids, which nevertheless showed successive retention times on the other three columns. But FON, PEG-20M and FFAP columns were superior to FAL-M for separation between formic and acetic acids.

For precise identification, GC/MS analysis (15% FFAP column) was performed, and the characteristic ions with relative intensity in parentheses are given in Table 2. The mass spectra of beetle samples are shown in Table 3. Coexistence of 2-Me-butanoic acid and 3-Me-butanoic acid in *Pterostichus sulcitaris* and *Patrobis flavipes* was revealed by the relative intensity of ions at m/z 74, 60, 57 and 39. But *Oodes vicarius* secreted almost 2-Me-butanoic acid and a trace of 3-Me-butanoic acid, confirmed by the predominance of m/z 74 and 57. Existence of crotonic, α -ethylacrylic and senecioic acids in specific species were also confirmed (Table 2 and 3). Mass spectral ions of beetle samples, especially α -ethylacrylic acid and senecioic acid, showed

Table 1. Retention times (min) of authentic samples of fatty acids
(constant temperature, carrier gas flow rate; 30 ml/min)

Acid	(after hydrogenation)	FALM-25% 130°C	FON-10% 150°C	PEG20M-10% 150°C	FFAP-10% 150°C
Acrylic	(Propanoic)	6.0	8.0	10.5	8.0
Methacrylic	(2-Me-Propanoic)	9.0	9.8	13.4	9.8
3-Butenoic	(Butanoic)	9.6	12.4	16.6	13.2
Crotonic	(")	13.2	14.0	19.0	14.8
	(2-Butenoic)				
α -Et-acrylic	(2-MeButanoic)	14.8	14.8	20.4	15.8
	(2-Et-2-Propenoic)				
Angelic	(")	15.8	15.0	21.0	16.0
	(cis-2-Me-2-Butenoic)				
Tiglic	(")	22.0	17.2	25.8	20.8
	(trans-2-Me-2-Butenoic)				
Senecioic	(3-Me-Butanoic)	16.8	15.4	22.2	16.4
	(3-Me-2-Butenoic)				
4-Pentenoic	(Pentanoic)	14.6	16.0	22.6	17.8
2-Pentenoic	(")	24.6	20.3	29.2	23.4
3-Hexenoic	(Hexanoic)	28.0	27.4	39.2	31.8
2-Hexenoic	(")	34.0	30.4	43.6	35.8
Formic		3.0	5.0	6.6	4.8
Acetic		3.2	4.0	4.8	3.8
Propanoic		4.6	5.6	7.0	5.4
2-Me-Propanoic		6.4	6.4	8.0	5.8
Butanoic		7.2	8.0	10.4	7.8
2-Me-Butanoic		10.2	9.6	12.6	9.4
3-Me-Butanoic		9.8	9.6	12.6	9.0
Pentanoic		13.4	13.0	17.0	12.4
2-Et-Butanoic		15.8	14.0	18.8	14.3
2-Me-Pentanoic		17.2	15.2	19.0	15.0
3-Me-Pentanoic		17.6	15.4	20.8	16.0
4-Me-Pentanoic		20.0	16.0	22.4	17.4
Hexanoic		24.4	18.6	26.8	21.4

some variations; these were caused by sampling fractions having little contamination of successive substantial peaks. In clearly separated peaks, i.e. 2-Me-propanoic, tiglic and 2-hexenoic acids, or in very high peaks having another trace substance, i.e. methacrylic acid, the mass spectra in beetle samples and authentic samples was almost the same.

About 200 species of carabid beetles were divided into six groups according to their main acid components, as shown in Fig. 1.

(1) Formic acid secreting species: Beetles of Agonini, Harpalini, Licinini, Lebiini, Dryptini and Zuphini species secreted formic acid comprising more than 95 percent of the acid component, in addition to small amounts of methacrylic, tiglic and acetic acids. This type of beetle also secreted different kinds of neutral compounds, such as 2-tridecanone or some esters, in amounts large than other types described below.

(2) Methacrylic and tiglic acid secreting species: Many Carabini, Patrobini,

Table 2. Mass spectra of authentic samples of fatty acids (15% FFAP, 70 eV)

	MW	M/Z (% Base peak)
Formic	46	46(100), 29(98), 45(76), 44(18),
Acetic	60	43(100), 45(90), 60(51), 42(16), 29(10),
2-Me-Propanoic	88	43(100), 41(60), 73(36), 27(30), 39(22), 45(16),
Butanoic	88	60(100), 73(52), 41(36), 42(31), 28(25), 39(22),
2-Me-Butanoic	102	74(100), 57(64), 29(51), 41(50), 97(23), 27(21),
3-Me-Butanoic	102	60(100), 41(32), 43(26), 39(17), 87(15), 27(14),
Pentanoic	102	60(100), 43(40), 41(37), 87(34), 45(31), 74(26),
Hexanoic	116	60(100), 73(47), 41(31), 29(18), 39(17), 87(15),
Methacrylic	86	41(100), 39(77), 86(62), 40(20), 38(16), 45(15),
Crotonic	86	39(100), 41(98), 86(91), 69(34), 68(32), 40(25),
3-Butenoic	86	41(100), 39(83), 42(40), 86(36), 45(30), 58(25),
2-Pentenoic	100	55(100), 100(85), 39(55), 54(37), 27(61), 29(52), 82(28), 83(24), 85(4),
Angelic	100	55(100), 100(73), 39(70), 54(39), 27(48), 29(29), 82(27), 85(26), 83(14),
Tiglic	100	55(100), 100(78), 39(51), 54(32), 29(43), 27(40), 85(27), 82(19), 83(14),
(α -Et-Acrylic)	100	Beetle samples
<i>Carabus procerulus</i>		55(100), 39(96), 100(82), 41(59), 82(42), 85(38), 27(37), 29(35), 83(9),
<i>Damaster blaptoides</i>		55(100), 39(72), 100(68), 41(40), 82(39), 27(34), 85(32), 29(32), 83(8),
Senecioic	100	39(100), 100(90), 55(50), 41(43), 82(50), 83(47), 27(41), 85(40), 29(36),
2-Hexenoic	114	73(100), 42(66), 41(60), 39(50), 18(28), 68(24),

Table 3. Mass spectra of beetle samples (15% FFAP, 70 eV)

2-Me-Butanoic acid and 3-Me-Butanoic acid	
<i>Oodes vicarius</i>	74(100), 41(99), 57(60), 29(56), 39(35), 27(33), 87(25), 45(22), 60(16),
<i>Pterostichus sulstarsis</i>	41(100), 60(85), 74(78), 43(43), 39(39), 29(38), 87(35), 57(34), 27(31),
<i>Patrobis flavipes</i>	60(100), 74(62), 43(43), 29(38), 87(29), 27(30), 41(27), 57(25), 39(22),
<i>Bradytus ampliatus</i>	41(100), 60(54), 39(52), 74(41), 43(38), 27(35), 45(30), 29(28), 87(25),
Crotonic acid	
<i>Patrobis flavipes</i>	39(100), 41(93), 69(30), 68(27), 40(25), 86(21), 45(20), 27(19), 42(14),
<i>Scarites terricola</i>	39(100), 41(80), 86(32), 40(24), 45(23), 38(21), 68(21), 55(11), 69(11),
<i>Bradytus ampliatus</i>	39(100), 41(90), 86(46), 69(24), 68(23), 45(22), 38(22), 37(16), 40(5),
Senecioic acid	
<i>Oodes vicarius</i>	39(100), 100(80), 41(54), 82(49), 83(40), 27(45), 55(43), 29(37), 85(36),
<i>Patrobis flavipes</i>	39(100), 100(54), 41(42), 27(41), 55(38), 82(38), 83(35), 29(34), 85(28),
<i>Scarites vicarius</i>	39(100), 41(45), 100(45), 82(44), 83(38), 55(38), 27(37), 85(27), 54(21),
<i>Curtonotus giganteus</i>	39(100), 100(98), 82(66), 41(55), 83(53), 43(44), 55(35), 27(33), 54(28),

Bembidiini, Pterostichini and Amarini secreted these two acids in a quantity more than 98 percent of their acid components. Methacrylic acid was always secreted in higher amounts. This type of beetle secreted also small amounts of acetic, 2-Me-propanoic and 2-Me butanoic acids, the latter two being saturated acids of methacrylic and tiglic acids. Very small amounts of senecioic acid and 3-Me-butanoic acid, (a saturated acid of senecioic acid), and sometimes crotonic and α -ethylacrylic acids were also detected in trace amounts.

(3) Methacrylic and α -ethylacrylic acids secreting species: Only three Carabini beetles, *Hemicarabus tuberculatus*, *Carabus procerulus* and *Damaster blaptoides*, secreted

these two acids in more than a 98 percent quantity of the acid components. α -Ethylacrylic acid was regarded as a substitute of tiglic acid in type (2); constituents of minor acids and their amounts were similar to type (2).

(4) 2-Me-propanoic, 2-Me-butanoic and 3-Me-butanoic acids secreting species: *Pterostichus sulcitaris* of Pterostichini was a typical species, and secreted more than 95 percent of these three acids in the acid components. *Craspedonotus tibialis* of Broscini and *Oodes vicarius* of Oodini were also of this type, but these beetles secreted higher amounts of senecioic acid and lower amounts of 3-Me-butanoic acid than *P. sulcitaris*. These beetles are classified as saturated acid preceding species.

(5) Methacrylic, crotonic, senecioic and tiglic acid secreting species: All of the four Scaritini species tested and *Patrobis flavipes* of Patrobini secreted these four acids in a quantity more than 98 percent of their acid components. Methacrylic acid was secreted in the highest amount, and each of the other three acids was one-third methacrylic acid in amount. This type of beetle also secreted saturated acids of the above unsaturated acids as minor components.

(6) 2-Hexenoic, methacrylic and tiglic acid secreting species: This type was remarkable in *Bradytus ampliatus* of Amarini, which secreted 2-hexenoic acid in an amount similar to tiglic acid. Except for 2-hexenoic acid, other constituents and amounts were almost the same as type (2). *Bradytus simplicidens* also secreted a small amount of 2-hexenoic acid, but other Amarini, *Amara chalcites*, *A. chalcophaea* and *Curtonotus giganteus* secreted only a trace or, more often, no detectable amount of 2-hexenoic acid.

DISCUSSION

In the previous report (KANEHISA and MURASE, 1977), acid-secreting carabid beetles were divided into three groups according to their acids: (1) formic acid, (2) methacrylic, tiglic and/or α -ethylacrylic acids, (3) more than three acids. This clas-

Fig. 1. Gas chromatograms of acid components, FAL-M 25%, 130°C, 30 ml/min.

(1) *Harpalus capito*, (2) *Carabus yaconinus*, (3) *Carabus procerulus*, (4) *Pterostichus sulcitaris*, (5) *Patrobis flavipes*, (6) *Bradytus ampliatus*. a : acetic acid, c : crotonic acid, e : α -ethylacrylic acid, f : formic acid, he : 2-hexenoic acid, ib : 2-methylpropanoic acid, m : methacrylic acid, mb : 2-methylbutanoic acid with 3-methylbutanoic acid, s : senecioic acid, t : tiglic acid.

sification was made based only on the major acid components, because minor components often could not be detected with TCD gas chromatography. These acids were in part or whole detected by EISNER and MEINWALD (1966), MOORE and WALLBANK (1968), BENN et al. (1973), SCOTT et al. (1975), OOSHIMA et al. (1975), and MOORE (1979). In this report, they are classified into the six groups shown in Fig. 1 according to their acid components, including minor components, with the aid of sensitive FID gas chromatography. This classification is more reasonable, because it was arrived at with consideration of synthetic pathways and minor components.

Recently synthetic pathways of fatty acids detected in carabid secretions have been presented as metabolic pathways of special amino acids (BENN et al., 1973, DUFFY, 1976). Similar pathways were also presumed to be in *sk* mutant of the silkworm (IGUCHI and YOSHITAKE, 1978). They are shown as follows.

Crotonic acid was described as one of the lysine metabolic products in microorganisms and vertebrates (NODA et al., 1976).

Most of the detected fatty acids in this experiment are understood to be synthesized products from the above amino acids in the synthetic lobes, and they are stored in reservoirs until secreted by stimulation, such as are caused by the attacks of predators. Each carabid beetle has its own synthetic system, which is almost identical at the genus level and sometimes at the subfamily level, and is species-specific in some species.

Methacrylic acid and 2-Me-propanoic acid, derived from valine, were always secreted in the highest amounts. Tiglic acid, α -ethylacrylic and 2-Me-butanoic acids from isoleucine were the second acid component in quantity. Senecioic and 3-Me-butanoic acids from leucine were the third component, and higher amounts of crotonic or 2-hexenoic acids were seldom detected and only in limited species.

Unsaturated fatty acids were more predominant than saturated fatty acids in many carabid beetles; the reverse was observed in limited species. Intraspecific variation of different localities, seasons or individuals was observed mainly as a variation in amount between saturated and unsaturated acids. Enzyme systems concerning synthetic pathways will change individually even at a low level.

Quite a few carabid beetles secreted formic acid, which is known in many ants and is thought to come from serine (BLUM, 1978). This type of beetle secreted very small amounts of methacrylic and tiglic acids, so it is presumed to be quite different from the branched amino acid utilizing groups of types (2) to (6). Indeed, a high content of neutral compounds, such as 2-tridecanone or some esters with formic acid were also a characteristic of this type. Acetic acid was usually found in many tissues, such as haemolymph. The small amounts of acetic acid detected in the defensive systems may be due to this property.

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