[Chem. Pharm. Bull. 25(7)1497—1508(1977)]

UDC 547.466.1.02.08 : 543.51.06

Mass Spectral Differentiation of α- and γ-Linkages in Glutamyl Oligopeptides and Its Application for Structure Elucidation of naturally Occurring Peptides[1,2]

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(Received August 13, 1976)

In order to establish a simple and rapid method for the differentiation of α- and γ-glutamyl linkages, mass spectra of a series of α- and γ-glutamyl oligopeptides including di-, tri-, and tetrapeptides were examined and the following results were obtained.

1) Generally the spectra of the α- and γ-isomers were distinguishable in several fragment peaks; particularly, ions c and h arising from the cleavage of glutamyl C^{α}–CO bond (Chart 2) are characteristic to the α-isomers, while ions e and g due to the ring formation of glutamyl chain (Chart 3) appeared intensely in the γ-isomers. The peak intensity ratios of these ions, c/g and h/e, which were large for the α- and small for the γ-isomers, were especially useful for the assignment of the glutamyl linkages.

2) As a result the whole structures of the α- and γ-glutamyl peptides (up to around tetrapeptide) were easily determined from the mass spectra of their Z or Dec derivatives.

3) All of the four structural isomers of glutamyllysine were easily distinguished from their mass spectra.

4) The method presented here was successfully applied to determine the full structures of some natural glutamyl peptides such as glutathione and cross-linking peptides found in certain peptidoglycans and proteins.

Keywords——mass spectra; α- and γ-glutamyl peptides; isomer differentiation; cross-linking peptides; glutamyllysine isomers; glutathione; amino protecting groups; peak-intensity ratios

Recently reports on di- and tripeptides possessing γ-glutamyl linkage increased in number, discussing their natural origins, metabolisms, and physiological functions.[4–11] Such a situation therefore urgently requires the rapid and unequivocal method for determination of the nature of the glutamyl linkages in the peptide samples. Of course, the standard methods generally used for sequence analysis are useless for this special purpose. Several

1) A part of this paper was presented at the 12th Symposium on Peptide Chemistry, Kyoto (Japan), November, 1974.
2) The abbreviated designations of amino acids, peptides, and their derivatives used are those from *J. Biol. Chem.*, **247**, 977 (1972). α-Amino acid symbols denote the L configuration, unless otherwise stated. Abu=γ-aminobutyric acid, Acp=ε-aminocaproic acid, Dec=decanoyl, Dec(OH)=3-hydroxydecanoyl, EOC=ethoxycarbonyl, iBOC=isobutyloxycarbonyl.
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4) J.J. Corrigan, *Science*, **164**, 142 (1969).
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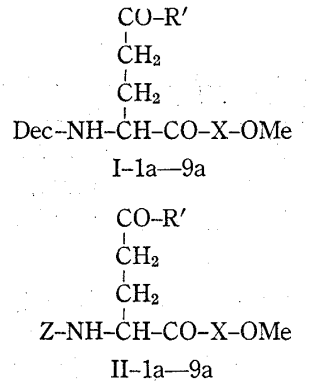
proposals such as tritium labeling,[12] infrared (IR), nuclear magnetic resonance (NMR), and paper, ion exchange, and gas-liquid chromatographies[13–15] for distinguishing pairs of α- and γ-glutamyl peptides still have disadvantages: some of them require large quantity of pure materials or authentic samples for references, and some require much of time. Since peptide samples from natural sources are usually available in only minute amounts, one of the most preferable choices for the structural elucidation may be a mass spectrometric method which will give the informations of amino acid sequence and of nature of the glutamyl linkage simultaneously by use of an only microgram quantity of the samples.

There are two discussions on mass spectral behaviour of the α- and γ- glutamyl peptides. Kiryushkin, *et al.*[16] described that characteristic fragmentation of the γ-isomer is the loss of water from the M^+ or from fragment ions containing the glutamyl residue. This is, however, only true for the peptides of low volatility. Since the loss of water from the glutamyl moiety is a pyrrolytic process occurring only at above *ca.* 200°, most of small peptides with high volatility do not give these peaks with sufficient intensities owing to rapid decrease of total ion volume at the higher temperature. In fact, all of the γ-glutamyl peptides we have examined showed these peaks with only negligible intensities. Heijenoort, *et al.*[17] have proposed that the α-isomer would be characterized from the very intense aldimine fragment (*c*) (Chart 1), while the corresponding peak was absent in the spectra of the γ-isomers. We have observed, however, that there is not such a difference between the isomeric pairs of some α- and γ-glutamyl di- or tripeptides (*cf.* I-, and II-12a,b in Table IV, and II-, III-14a,b, and III-, IV-14′a,b in Table VI), indicating that their proposal is not always applicable.

We present here our results of the mass spectral differentiation of a series of the α- and γ-glutamyl oligopeptides in detail.

The samples prepared include di- to tetrapeptides, most of which have glutamyl residue at the N-terminus and some at the middle. The Dec- and Z-groups were mainly employed as the amino protecting group. All the derivatives had sufficient volatility for mass spectra. The fragmentations of the protecting groups under electron impact are well-known.[18,19]



	X	R′
I-, II-1a, b	Ala	OMe
I-, II-2a, b[20]	Val	OMe
I-, II-3a, b[20]	Leu	OMe
I-, II-4a, b	Phe	OMe
I-, II-5a, b	Pro	OMe
I-, II-6a, b	β-Ala	OMe
I-, II-7a, b	Abu	OMe
I-, II-8a, b	Acp	OMe
I-, II-9a, b	Ala	NH_2

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17) J. van Heijenoort, E. Bricas, B.C. Das, E. Lederer, and W.A. Wohlstenholme, *Tetrahedron*, **23**, 3403 (1976).
18) *a*) R.T. Aplin, J.H. Jones, and B. Lieberek, *J. Chem. Soc.*, **1968**, 1011; *b*) S. Nagai, Y. Shinosaki, K. Okada, and M. Hiramoto, The 93rd Annual Meeting of Pharmaceutical Society of Japan, Tokyo, April, 1973.
19) *a*) E. Bricas, J. van Heijenoort, M. Barber, W.A. Wolstenholme, B.C. Das, and E. Lederer, *Biochemistry*, **4**, 2254 (1965); *b*) M.M. Shemyakin, Yu.A. Ovchinnikov, A.A. Lipkin, Y.B. Shvetsuv. N.S. Wulfson, B.V. Rosinov, V.N. Bachrarev, and V.M. Burikov, *Nature* (London), **211**, 361 (1966).

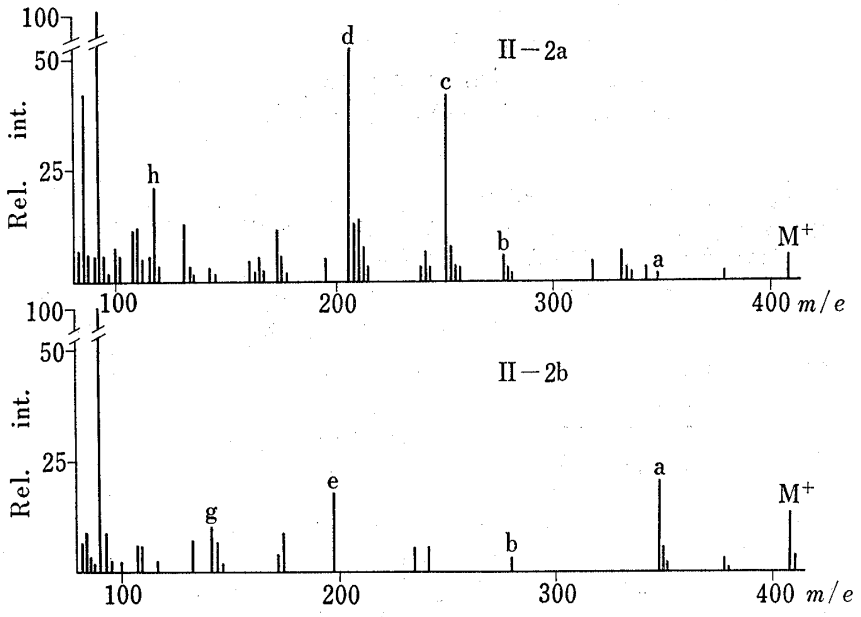


Fig. 1. Mass Spectra of Z-Glu(OMe)-Val-OMe (II-2a) and Z-Glu(Val-OMe)-OMe (II-2b)

The regions above *m/e* 80 are indicated.

TABLE I. Relative Abundances and Intensity Ratios of Selected Ions in Mass Spectra of Dec-α- and γ-Glutamyl Amino Acid Methyl Esters (I-la,b—8a,b) and Dec-Glutaminylalanine Methyl Ester (I-9a) and Its Isomer (I-9b)

Compd.	Mol. wt.	Relative abundances of ions[a,b]							Peak intensity ratio	
		M+	a	c	e	g	h	i	c/g	h/e
I-1a	400	2	0.5	100	2	1	100	7	100	50
I-1b	400	36	73	2	100	85	3	83	<0.1	<0.1
I-1a[c]	400	1	—	25	1	—	100	2	>25	100
I-1b[c]	400	4	11	1	100	87	4	21	0.1	0.1
I-2a	428	4	1	100	7	1	55	7	100	8
I-2b	428	9	58	2	81	100	8	37	<0.1	0.1
I-3a	442	1	—	32	1	4	73	1	8	73
I-3b	442	25	99	11	100	96	5	46	0.1	0.1
I-4a	476	13	—	56	1	1	100	2	56	100
I-4b	476	19	7	1	37	100	4	11	<0.1	0.1
I-4a[c]	476	2	—	17	1	1	68	0.5	17	68
I-4b[c]	476	13	1	1	19	100	5	4	<0.1	0.2
I-5a	426	6	2	28	1	15	100	4	2	100
I-5b	426	3	3	1	100	72	2	3	0.1	<0.1
I-6a	400	2	—	58	2	—	100	5	>58	50
I-6b	400	4	3	1	60	13	2	12	<0.1	<0.1
I-7a	414	4	—	79	1	4	100	5	19	100
I-7b	414	6	3	44	60	86	63	12	0.5	1
I-8a	442	8	—	100	1	9	73	6	11	73
I-8b	442	5	3	2	18	100	17	7	<0.1	0.9
I-9a	385	—	—	22	5	1	63	1	22	13
I-9b	385	7	5	9	100	26	3	4	0.3	<0.1

a) For I-1a, b—8a, b, a, [M-59]; c, *m/e* 270; e, [M-230], except for I-5a, b in which [M-256] was used[23]; g, *m/e* 144; h, *m/e* 116; i, [M-112]. For I-9a, b, a, [M-44]; c, *m/e* 255; e, [M-215]; g, *m/e* 129; h, *m/e* 101; i, [M-112].

b) Base peaks; *m/e* 84 in I-3a and I-7b (g-COOMe-H) and in I-9a (g-$CONH_2$-H); *m/e* 162 in I-4a[c] (ph-CH=CH-CO_2H); *m/e* 256 in I-6b (M-C_8H_{17}-MeOH).

c) Determined with a Hitachi RMU-6D mass spectrometer at 200°.

Mass Spectra of Dipeptides

The mass spectra of Z-α- and γ-glutamyl valine dimethyl esters (II-2a,b)[20] are presented in Fig. 1 as examples, and those of the isomeric pairs of other dipeptides (I-1a,b—9a,b, II-1a,b and II-3a,b—9a,b) are summarized in Tables I and II.

Table II. Relative Abundances and Intensity Ratios of Selected Ions in Mass Spectra of Z-α- and γ-Glutamyl Amino Acid Methyl Esters (II-1a,b—8a,b) and Z-Glutaminylalanine Methyl Ester (II-9a) and Its Isomer (II-9b)[a]

Compd.	Mol. wt.	Relative abundances of ions[b,c]							Peak intensity ratio		
		M+	a	c	d	e	g	h	d/e	c/g	h/e
II-1a	380	6	1	17	20	2	—	6	10	>17	3
II-1b	380	16	13	—	—	47	18	—	0	0	0
II-1a[d]	380	2	1	40	34	1	—	6	34	>40	6
II-1b[d]	380	9	15	0.5	—	31	3	1	0	0.2	<0.1
II-3a	422	8	2	63	83	1	8	28	83	8	28
II-3b	422	8	20	0.3	1	36	10	—	<0.1	<0.1	0
II-4a	456	2	—	19	19	0.5	1	5	38	19	10
II-4b	456	36	6	2	2	73	23	2	<0.1	<0.1	<0.1
II-4a[d]	456	1	—	3	4	1	2	3	4	2	3
II-4b[d]	456	4	2	—	—	5	12	1	0	0	0.2
II-5a	406	6	1	11	17	100[e]	0.3	6	0.2	33	<0.1
II-5b	406	33	38	—	1	100	1	1	<0.1	0	<0.1
II-6a	380	12	—	95	95	1	—	22	95	>95	22
II-6b	380	25	18	—	—	100	6	5	0	0	<0.1
II-7a	394	4	—	25	45	—	4	28	>45	6	28
II-7b	394	20	8	—	—	27	6	28	0	0	1
II-8a	422	6	—	79	89	0.3	3	31	30	26	103
II-8b	422	2	1	6	—	3	7	—	0	0.9	0
II-9a	365	6	—	27	4	—	—	9	>4	>27	>9
II-9b	365	6	32	—	—	42	1	1	0	0	0.1

a) Spectra of II-2a, b are shown in Fig. 1.
b) For II-1a, b—8a, b, a, [M-59]; c, *m/e* 250; d, *m/e* 206; e, [M-210], except for II-5a, b in which [M-236] was used; g, *m/e* 144; h, *m/e* 116. For II-9a, b, a, [M-44]; c, *m/e* 236; d, *m/e* 191; e, [M-195]; g, *m/e* 129; h, *m/e* 101.
c) *m/e* 91 (tropyrium) was the base peak in all cases except in II-5a, b.
d) Determined with a Hitachi RMU-6D mass spectrometer at 200—260°.
e) The exceptionally high abundance seemed due to an alternative fragment arising from loss of a benzyl alcohol from ion b.[18a]

Most of the compounds gave, in addition to molecular ions, ions due to cleavages of the peptide and ester bonds[21] (ions b and c, and [M-OMe]+) allowing to assign readily the total amino acid sequences. The mass spectra of the α- and γ-isomers exhibited several fragmentations characteristic of each isomer: Four sets of ions c, e, g, and h, have exhibited the most significant difference between the two isomers, as seen from Tables I and II. The γ-isomers gave [M-59]+(ion a) appreciably, while the corresponding ion was absent or very weak in the spectra of the α-isomers and instead ion c appeared intensely. Both ions a and c are attributed to fission of C^{α}-CO bond in the glutamyl moiety. In the α-isomers the fission gives rise to the aldimine fragment (c) and in the γ-isomers [M-COOMe]+ is formed (Chart 1). The formation of the latter was supported by the fact that the ion due to [M-$CONH_2$]+ was observed in the spectra of the corresponding amides (I- and II-9b).

20) The samples used in this study contained D-amino acid derivatives which were prepared for the purpose of synthetic study of some peptide antibiotics. It is well-known that the configuration of amino acid scarcely affects on the fragmentation pattern of peptides.[21]
21) *a*) E. Lederer, *Pure Appl. Chem.*, **17**, 489 (1968); *b*) M.M. Shemyakin, *ibid.*, **17**, 313 (1968).

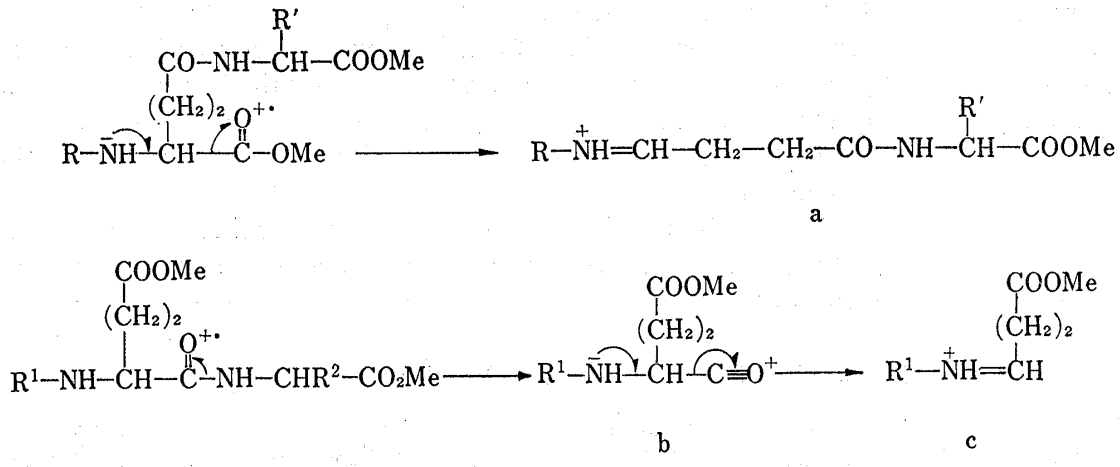


Chart 1

Table III. Results of Accurate Mass Measurements of Some Important Ions of Methyl Esters of Dec-Glutamyl- and Dec-Glutaminyl Amino Acids

Compd.	*m/e*	Measured mass	Assigned formula	Difference from Calcd. mass(mmU)
I-1a	116	116.068	$C_5H_{10}NO_2$	−3
I-1b	144	144.070	$C_6H_{10}NO_3$	+4
	170	170.082	$C_8H_{12}NO_3$	0
I-4a	116	116.078	$C_5H_{10}NO_2$	+2
I-4b	144	144.068	$C_6H_{10}NO_3$	+2
I-9a	101	101.070	$C_4H_9N_2O$	−1
I-9b	170	170.082	$C_8H_{12}NO_3$	+4
	129	129.066	$C_5H_9N_2O_2$	0

One of the intense peaks, *m/e* 116 (h), was unique to the α-isomers and was independent of the nature of the amino protecting group used. The composition of this ion, $C_5H_{10}NO_2$, determined by accurate mass measurements (Table III) suggested the structure (h) in Chart 2, which was supported by the fact that the ion was shifted to *m/e* 101 in the corresponding amides (I- and II-9a) (Tables I—III). This is evidently due to the elimination of the N-acyl group from the aldimine ion c.[22] The abundant ions e and g were characteristic of γ-glutamyl isomers, either of them being employed as the base peak in most cases.[23] In contrast, these peaks were very weak in the α-isomers. Ion e appeared depending on the kind of the second amino acid in the molecule but was unaffected by the nature of the amino protecting group used. The peak g appeared at the constant position (*m/e* 144) throughout all the glutamyl peptides examined. High-resolution mass spectra of compound I-1b indicated empirical compositions

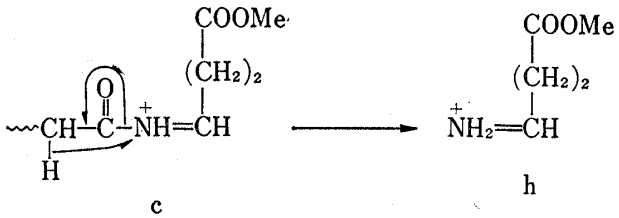


Chart 2

22) *cf.* The deacylation of aldimine ions has been suggested with other glutamic acid derivatives [G.L. Nelsestuen and T.H. Zytokovics, *J. Biol. Chem.*, **249**, 6349 (1974); E. Lerch and M. Hesse, *Helv. Chim. Acta*, **57**, 1584 (1974)].

23) In the spectrum of the proline derivative (I-5b), peak corresponding to the ion e was observed at [M-256], probably owing to its tendency to undergo further loss of ethylene moiety from the heterocyclic ring.[19b,21b]

of the peaks e and g to be $C_8H_{12}NO_3$ and $C_6H_{10}NO_3$, respectively (Table III). Evidences for the structures of these ions were obtained by the following facts: (i) The ion g in the amides, I-9b and II-9b, was found at *m/e* 129, which was 15 mass units lower than that in the corresponding methyl esters, I-1b and II-1b, but the ion e was observed unchanged in the both compounds (Table I or II). (ii) The ion e in the γ-series shifted to *m/e* 241 in the tripeptides (I- and II-10b) compared to *m/e* 170 of the dipeptides (I- and II-1b) (Table IV), while the ion g appeared invariably at *m/e* 144. The structures elucidated from the above evidences and their probable formation pathways, which include the ring formation of glutamyl chain, are shown in Chart 3.

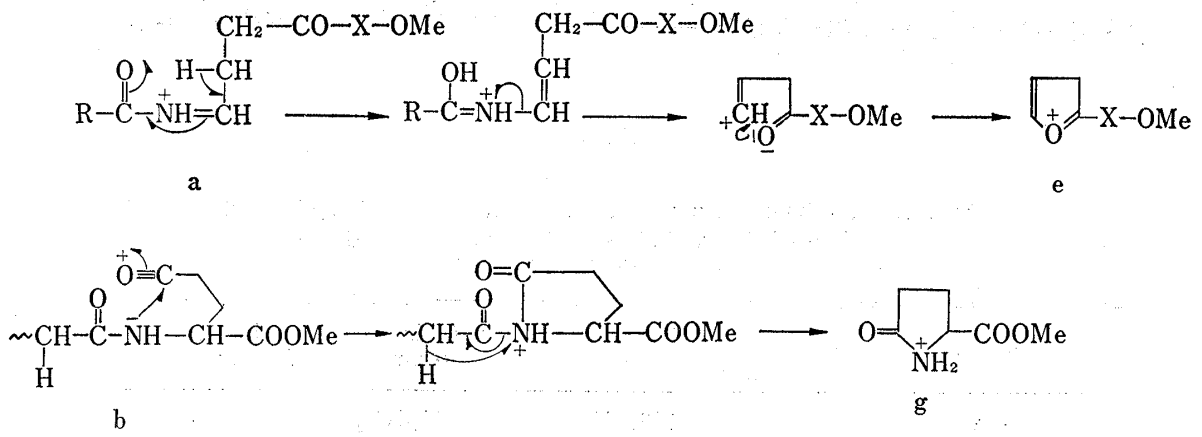


Chart 3

Intense peaks, ion i ([M-112]) and ion d at *m/e* 206, were also useful in characterizing the types of the glutamyl peptide bond (Tables I and II); the former ion was characteristic of the γ-isomers resulted from a McLafferty rearrangement of the Dec group[17] and the latter was highly specific to the Z-derivatives of the α-isomers, d formed from ion c by expulsion of CO_2 with concomitant migration of a benzyl group[18a] (Chart 4).

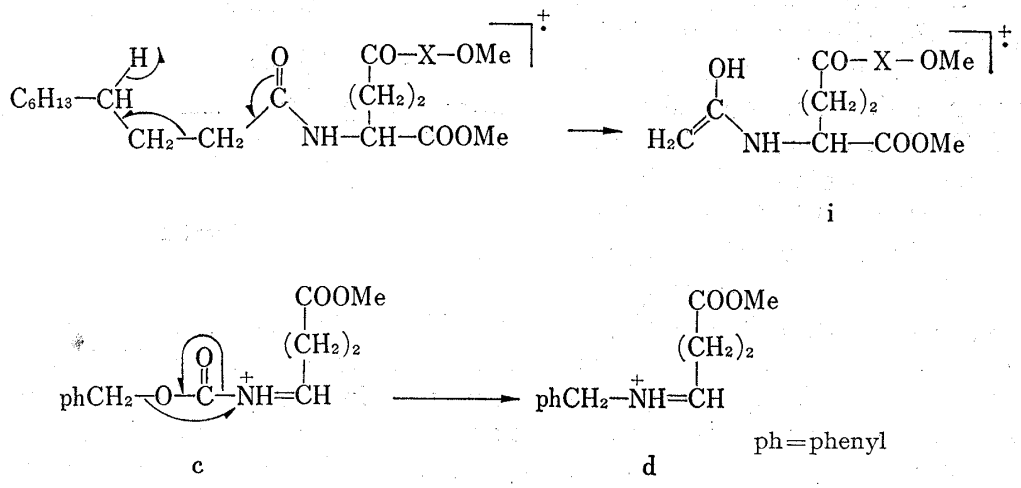
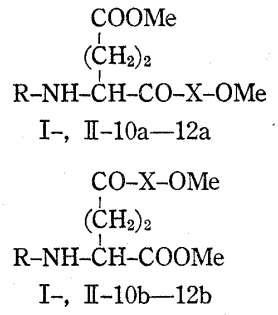


Chart 4

The relative intensities (expressed as percentages against a base peak) of the characteristic fragment peaks described above were affected considerably by the nature of connected amino acid, by the size of peptide (see later), and, to a lesser extent, by the type of mass spectrometer employed (see Tables I, II, and IV). Therefore, it is recommendable for the isomer differentiation to adopt the intensity ratios of the characteristic peaks than to use the relative peak intensity alone. In the last two or three columns of Tables I and II there

TABLE IV. Relative Abundances and Intensity Ratios of Selected Ions in Mass Spectra of N-Acylated Methyl Esters of Isomeric Pairs of Glutamyl Tri- and Tetrapeptides (I-10a,b—12a,b, and II-10a,b—12a,b)



	R	X
I-10a, b	Dec	Ala_2
II-10a, b	Z	Ala_2
I-11a, b	Dec-Ala	Ala
II-11a, b	Z-Ala	Ala
I-12a, b	Dec	Ala_3
II-12a, b	Z	Ala_3

Compd.	Mol. wt.	Relative abundances of ions[a,b]							Peak intensity ratio	
		M+	a	c	e	g	h	others	c/g	h/e
I-10a	471	—	0.1	14	1	1	100	1(i)	14	100
I-10b	471	12	6	1	38	100	3	16(i)	<0.1	0.1
I-10a[c]	471	1	0	18	1	2	100	1(i)	9	100
I-10b[c]	471	2	1	1	21	100	4	4(i)	<0.1	0.2
I-11a	471	0.6	0.2	3	1	0.2	100	2(j)	15	100
I-11b	471	6	10	0.4	29	13	2	100(j)	<0.1	0.1
I-12a	542	—	—	2	2	0.2	12	0(i)	10	6
I-12b	542	—	—	1	2	15	2	0.4(i)	<0.1	1
II-10a	451	—	1	27	5	1	22	44(d)	27	4
II-10b	451	27	9	2	100	32	3	1(d)	<0.1	<0.1
II-10a[c]	451	—	0	3	9	1	5	5(d)	3	0.6
II-10b[c]	451	3	2	0	28	39	1	0(d)	0	<0.1
II-11a	451	6	1	39	1	1	82	15(j)	39	82
II-11b	451	5	7	0.3	33	14	2	100(j)	<0.1	<0.1
II-12a	522	1	—	2	6	2	6	5(d)	1	1
II-12b	522	2	—	2	5	6	—	8(d)	0.3	0

a) a, [M-59]; c, [M-CO-X-OMe], *i.e.*, *m/e* 270 in I-10a, b, and I-12a, b, *m/e* 250 in II-10a, b and II-12a, b, *m/e* 341 in I-11a, b, *m/e* 321 in II-11a, b; d, *m/e* 206; e, [M-R-NH_2-COOMe], *i.e.*, *m/e* 241 in I- and II-10a, b, *m/e* 170 in I- and II-11a, b, *m/e* 312 in I- and II-12a, b; g, *m/e* 144; h, *m/e* 116; i, [M-112]; j, *m/e* 273.
b) Base peaks: *m/e* 91 (tropyrium) in the all Z-derivatives; *m/e* 84 (g-COOMe-H) in I-12a, b.
c) Determined with a Hitachi RMU 6D mass spectrometer at 200—220°.

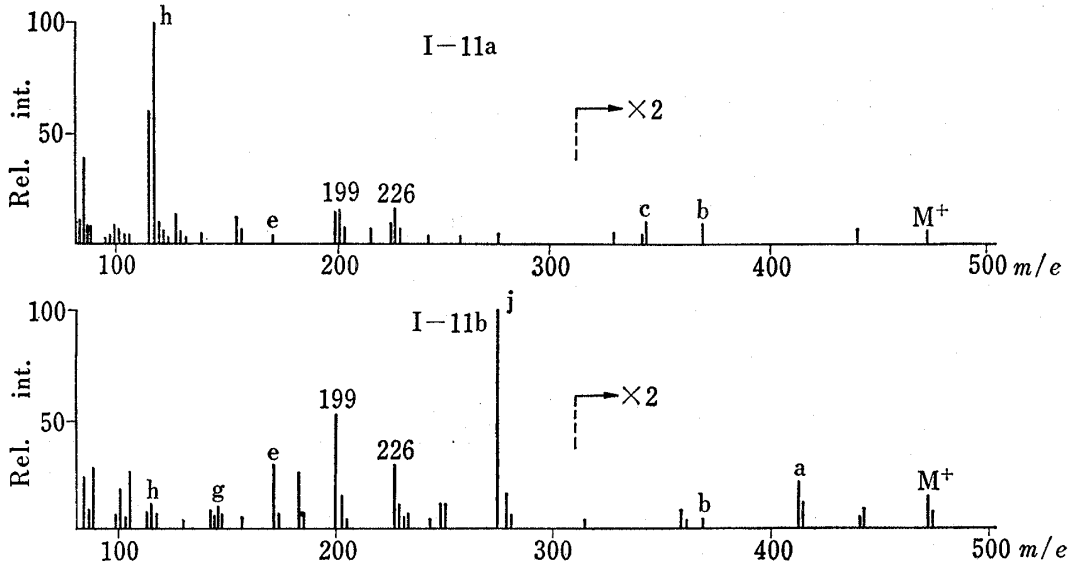


Fig. 2. Mass Spectra of Dec-Ala-Glu(OMe)-Ala-OMe (I-11a) and Dec-Ala-Glu-(Ala-OMe)-OMe (I-11b)

The regions above *m/e* 80 are indicated.

are given the peak intensity ratios, which allowed to unequivocally distinguish the two isomeric series of glutamyl dipeptides.

Mass Spectra of Tri- and Tetrapeptides

Mass spectra of isomeric pairs of N-acyl glutamyl tri- and tetrapeptides (I- and II-10a,b —12a,b) are given partly in Table IV, and the spectra of N-Dec-alanyl-α-glutamyl alanine dimethyl ester (I-11a) and its γ-isomer (I-11b) are illustrated in full in Fig. 2. The tripeptides (I-10a,b) gave the fragment ions, a, e, and i at *m/e* 412, 241, and 359, respectively, the values being 71 mass units higher than those of the corresponding respective ions in the dipeptides (I-1a,b), while each of the ions c, d, g, and h was observed unchanged in the di- and tripeptides (I-1a,b and I-11a,b). These results were in accord with the evidence described in the dipeptides series. The spectra of the tripeptides (I- and II-11a,b) having glutamyl residue in the middle of the molecule indicated that, as in the case of the isomers (I- and II-10a,b) with the same residue at the N-terminus, ions c, e, g, and h were useful in distinguishing the α-(I- or II-11a) and γ-isomers (I- or II-11b) of this kind of peptide, in which ion c appeared at *m/e* 341 (I-11a) or at *m/e* 321 (II-11a) and ion e at *m/e* 170. Further the γ-isomer (I- or II-11b) gave an abundant ion *m/e* 273(j) which was employed as the base peak, while this peak was very weak in the α-isomer (I- or II-11a). This ion may be $\overset{+}{O}\equiv C$–Glu(Ala-OMe)-OMe resulting from the splitting of the C^{α}-CO bond of the N-terminal acyl alanine part, although the reason why the ion arises predominantly from the γ-isomers still remains equivocal. In the mass spectra of the isomeric pair of the tetrapeptides, I-12a,b or II-12a,b, there was no significant difference in the intensities of ions a, c, and e, however, it was still possible to correlate the type of glutamyl linkage with the ratios of the peak intensities c/g and h/g, as shown in Table IV.

Next, the mass spectra of the N-Dec methyl ester derivatives (I-13a,b) of the branched glutamyl tripeptides, Glu(Gly)-Glu and Glu(Glu)-Gly, have been examined, the latter being found in a cross-linking part in the peptidoglycan of *Arthrobacter Strain* J 39.[24] Each compound (Fig. 3) showed molecular ion at *m/e* 529, together with two intense peaks (b and c)

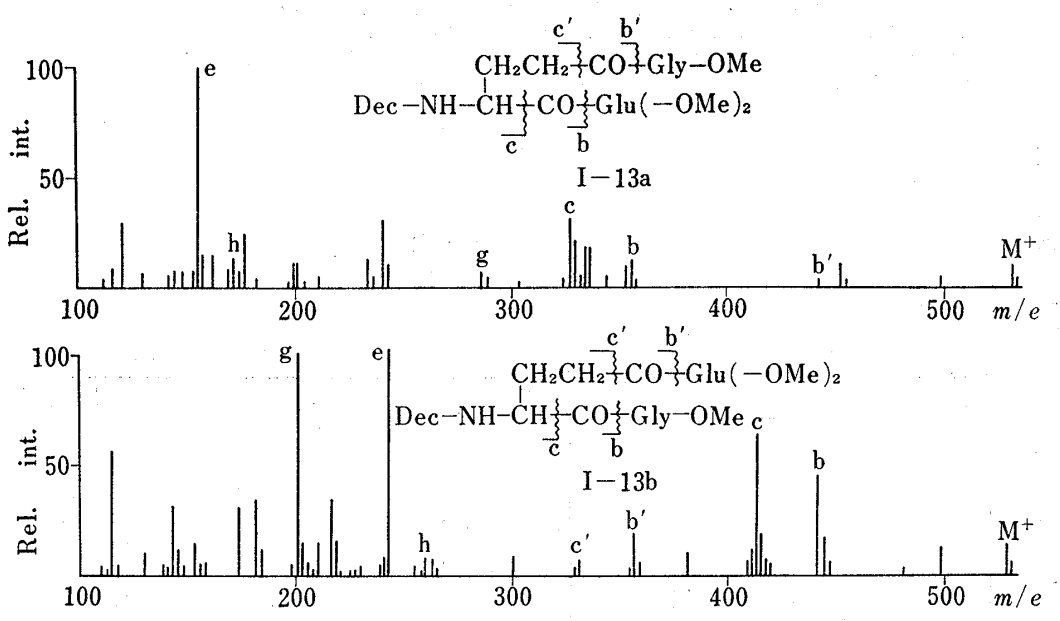
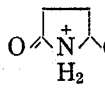


Fig. 3. Mass Spectra of Dec-Glu(Gly-OMe)-Glu(-OMe)$_2$ (I-13a) and Dec-Glu-[Glu(-OMe)$_2$]-Gly-OMe (I-13b)

The regions above *m/e* 100 are indicated.

24) B. Cziharz, K.H. Schleifer, and O. Kandler, *Biochemistry*, **10**, 3574 (1971).

due to the cleavage of the α-peptide bond, *i.e.*, *m/e* 355 and 327 (for I-13a) and *m/e* 441 and 413 (for I-13 b), whereas peaks b′ and c′ (Fig. 3) due to the isopeptide bond cleavage, *i.e.*, *m/e* 441 and 413 (for I-13a) and *m/e* 355 and 327 (for I-13b), were very weak. Of particular importance was the presence of a very intense peak at *m/e* 156 for I-13a or *m/e* 242 for I-13b, attributable to the ion e, which easily differentiates the isomeric branched peptides to one another. On the other hand, rather high intensity of the peak at *m/e* 287 (for I-13a) or *m/e* 201 (for I-13b) corresponding to ion g is useful in assignment of the tripeptide structures, since the residue X will be easily assigned from its mass number (Chart 5).



m/e 287: X=Glu(OMe)
m/e 201: X=Gly

Chart 5

Comparison of Different Amino Protecting Groups for Use in Mass Spectral Analysis

One of the most important considerations for successful sequencing of peptide by mass spectrometric method is the choice of an amino protecting group.[25] Therefore several amino protecting groups including Ac, Dec(OH), EOC, and iBOC were examined using the isomeric pair of glutamylalanine as model compound. These protecting groups were chosen for the ease of derivative formation with a small quantity of sample and for the favorable volatility of the resulting derivatives.

A few μ moles of the peptide were acylated with the relevant active ester or anhydride, followed by esterification with diazomethane or methanolic HCl. The product was introduced directly into the mass spectrometer without further purification. The main peaks in the mass spectra of the N-acylated dipeptides (III-, IV-, V-, and VI-1a,b) are listed in Table V. Comparison of the spectra with those of the corresponding Dec- or Z-derivatives (see Tables I- and II) indicated that there is no great difference in fundamental fragmentation. Unexpectedly both of the α-glutamyl compounds with urethan substituents, V-1a and VI-1a,

Table V. Relative Abundances and Intensity Ratios of Selected Ions in the Mass Spectra of N-Acyl α- and γ-Glutamylalanine Dimethyl Esters (III-, IV-, V-, and VI-1a,b)

R-Glu-Ala-OMe
III-, IV-, V-, VI-1a

R-Glu-OMe
III-, IV-, V-, VI-1b

III: R=Ac, IV: R=Dec(OH), V: R=EOC, VI: R=iBOC

Compd.	Mol. wt.	Relative abundances of ions[a,b]						Peak intensity ratio	
		M+	a	c	e	g	h	c/g	h/e
III-1a	288	5	2	75	1	1	100	75	100
III-1b	288	6	96	9	100	68	1	0.1	<0.1
IV-1a	416	2	0	57	43	1	100	57	2
IV-1b	416	11	22	0	100	100	4	0	<0.1
V-1a	318	—	0.8	61	5	22	10	3	2
V-1b	318	5	53	2	100	13	1	0.2	<0.1
VI-1a	346	1	2	37	0.4	5	14	7	35
VI-1b	346	6	28	0.4	100	51	1	<0.1	<0.1

a) a, [M-59]; c, [M-130]; e, *m/e* 170; g, *m/e* 144; h, *m/e* 116.
b) *m/e* 84 (g-COOMe-H) was the base peak in the cases of V- and VI-1a.

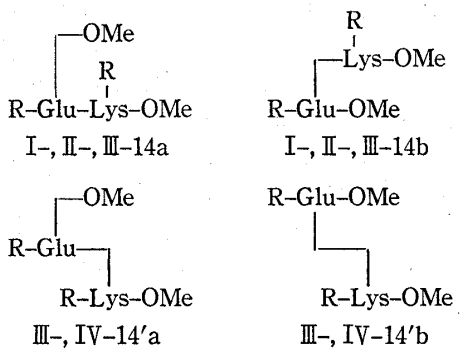
25) *a*) R.T. Aplin, I. Eland, and J.H. Jones, *Org. Mass Spectrom.*, **2**, 795 (1969); *b*) K. Okada, S. Nagai, T. Uyehara, and M. Hiramoto, *Tetrahedron*, **30**, 1175 (1974).

gave only very weak ion d. Further, it was found that ion g is useless for the isomer differentiation of the EOC compound. These results suggest that acyl groups such as Dec, Ac, and Dec(OH) might be preferable to the carboalkoxy groups as amino protecting group for the mass spectrometric analysis of the glutamyl peptides.

Mass Spectra of the Positional Isomers of Glutamyllysine

To test the applicability of the mass spectrometric method to structure analysis of another kind of biologically important peptides, we have examined four kinds of the positional isomers of glutamyllysine, *i.e.*, α-(α-glutamyl)-lysine, α-(γ-glutamyl)-lysine, ε-(α-glutamyl)-lysine, and ε-(γ-glutamyl)-lysine. The latter two are known as forming cross-linking portion of bacterial peptidoglycans and of proteins including the stabilized fibrin, hair, and seimal vesicle.[26] Table VI shows the mass spectra of their diacyl dimethyl ester derivatives. Compounds I-, II-, and III-14b with (γ→α) link were distinguishable from the isomers with (α→α) link (I-, II-, and III-14a) by the intense ions e and g. On the other hand, the (α→α) isomers gave gener-

Table VI. Relative Abundances of Selected Ions in Mass Spectra of N-Acylated Dimethyl Esters of Glutamyllysine Isomers (I-, II-, III-14a,b and III-, IV-14′a,b)



I: R=Dec, II: R=Z, III: R=Ac, IV: R=EOC

Compd.	Mol. wt.	Relative abundances of ions[a,b]						
		M+	a′	b	c	e	g	h
I-14a	611	20	2	27	17	—	4	14
II-14a	571	1	—	2	4	0.2	1	5
III-14a	387	1	2	23	13	—	12	32
I-14b	611	12	7	19	4	21	26	4
II-14b	571	5	—	4	3	10	9	3
III-14b	387	4	6	35	27	30	100	1
III-14′a	387	5	82	13	17	1	16	25
IV-14′a	447	4	13	2	92	5	5	25
III-14′b	387	12	25	9	22	24	22	30
IV-14′b	447	7	24	8	81	19	13	23

a) a′, [M-91] (for explanation, see the text); b, [M-Lys(R)-OMe], *i.e.*, *m/e* 298 in I-14a, b, *m/e* 278 in II-14a, b, *m/e* 186 in III-13a, b and III-14′a, b, *m/e* 216 in IV-14′a, b; c, [b-CO]; e, [M-R-NH_2-COOMe], except for II-14a, b where [M-R-NH_2-COOMe-$C_6H_5CH_2O$] was employed, *i.e.*, *m/e* 381 in I-14a, b, *m/e* 254 in II-14a, b, *m/e* 269 in III-14a, b and III-14′a, b, *m/e* 300 in IV-14′a, b; g, *m/e* 144; h, *m/e* 116.

b) *m/e* 84 was the base peak in all cases, except in I-14a and II-14a, b where *m/e* 155 (Dec) and *m/e* 91 (tropyrium) were used as base peak, respectively.

26) J.M. Glmysen, *Bacterial Rev.*, **32**, 425 (1968); D. Jarvis and J.L. Strominger, *Biochemistry*, **6**, 2591 (1967); T. Takagi and S. Iwanaga, *J. Biochem.*, **69**, 699 (1971); H.W.J. Harding and G.E. Rogers, *Biochemistry*, **10**, 624 (1971); H.G. Williams-Ashman, A.C. Notides, S.S. Paball, and L. Lorand, *Proc. Natl. Acad. Sci. U.S.*, **69**, 2322 (1972).

ally more abundant ion h than the ($\gamma\rightarrow\alpha$) isomers, while the aldimine peak (ion c) was not useful in differentiating these isomers. The mass spectrum of ε-(α-glutamyl)-lysine was essentially the same as that of ε-(γ-glutamyl)-lysine, except that the latter gave the ion e of fairly higher intensity (III- or IV-14'b).

Table VII shows the peak intensity ratios, which are useful in differentiation among the all glutamyllysine isomers. The ratio, c/g, was remarkably small in the isomer with ($\gamma\rightarrow\alpha$) linkage, while that of h/e decreased in the following order: ($\alpha\rightarrow\alpha$)>($\alpha\rightarrow\varepsilon$)>($\gamma\rightarrow\varepsilon$)>($\gamma\rightarrow\alpha$). Thus from these two ratios of the peak intensity, all kinds of the glutamyllysine isomers, except one pair *i.e.*, ($\alpha\rightarrow\alpha$) and ($\alpha\rightarrow\varepsilon$), were discernible from each other. Fortunately, the ($\alpha\rightarrow\varepsilon$) isomer as well as the ($\gamma\rightarrow\varepsilon$) one showed loss of (MeO+MeOCO) from the molecular ions giving abundant [M–91] ions[27] (ion a'), whereas the corresponding ion was weak or absent in the spectra of the ($\alpha\rightarrow\alpha$) and ($\gamma\rightarrow\alpha$) isomers (Table VI). It is, therefore, possible to differentiate the isomers, ($\alpha\rightarrow\alpha$) and ($\alpha\rightarrow\varepsilon$), by comparing the peak intensity ratio a'/b (Table VII).

Table VII. Ranges of the Ratio of the Peak Intensities used for Differentiation among Glutamyllysine Isomers[a]

Compd.	Type of peptide link[b]	c/g	h/e	a'/b
I-,II-,III-14a	($\alpha\rightarrow\alpha$)	1—4	25—32	0—0.1
I-,II-,III-14b	($\gamma\rightarrow\alpha$)	0.2—0.3	<0.1—0.3	0—0.4
III-,IV-14'a	($\alpha\rightarrow\varepsilon$)	1—18	5—25	6—6.5
III-,IV-14'b	($\gamma\rightarrow\varepsilon$)	1—6	1.2—1.3	2.8—3

a) All ratios were calculated from the values in Table VI.
b) For example, ($\gamma\rightarrow\varepsilon$) represents a linkage between the γ-carboxyl group of glutamic acid and the ε-amino group of lysine.

We have then applied the mass spectrometric method to reduced glutathione. The spectrum obtained after acetylation followed by esterification is shown in Fig. 4. Although the spectrum was somewhat complex, owing to the presence of several peaks arising from the cleavage of the side chain of the cysteine residue, it still gave sufficient diagnostic ions which made capable to assign the full structure; ion a (*m/e* 360), [M-AcSH] (*m/e* 344), abundant ion g (*m/e* 144), and relatively intense peak (*m/e* 211) which might be probably due to loss of Ac-S-CH_3 from the ion e (M-$AcNH_2$-COOMe), in addition to M^+ (*m/e* 419). In con-

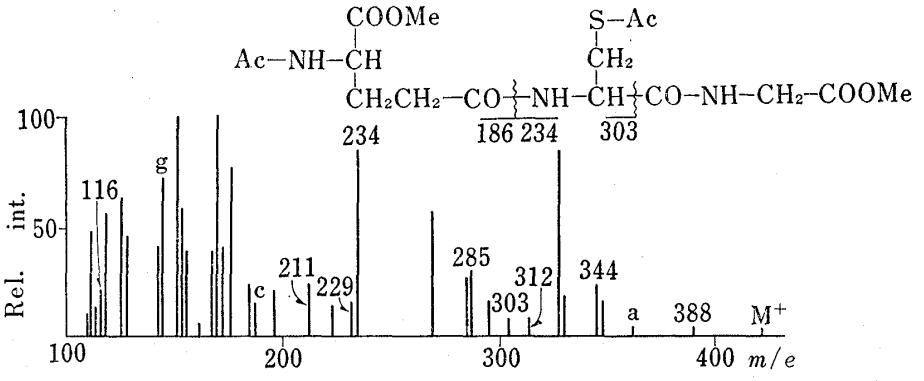


Fig. 4. Mass Spectrum of Acetylated Glutathione Methyl Ester
The regions above *m/e* 100 are indicated.

27) The detailed structure of this fragment ion and the precise mechanism for its formation are not elucidated at this stage.

trast, the ions c and h were of low abundance. The ion intensity ratios c/g, h/e, and h/g amount to 0.1, 0.8, and 0.3, respectively, indicating the compound to be a γ-glutamyl peptide.

The method we presented here was therefore shown to be useful for distinguishing the positional isomers of glutamyl di-, tri-, and tetrapeptides. An attempted extension of this method to glutamyl peptides with high molecular weight (penta-, hexa-, heptapeptide, *etc.*) was unsuccessful, because the isomer discrimination ions markedly declined with increase of the chain length of peptides. For overcoming this limitation we have examined chemical ionization mass spectra, the results of which will be described elsewhere,[28] though they were found to be not so effective for the purpose.

Experimental

Unless otherwise stated, mass spectra were determined using a Nippon Denshi Model JMS-01SG mass spectrometer with the direct inlet system operating at 75 eV; the heating temperature varied between 90° and 200°. Accurate mass measurements were carried out on the same spectrometer by the previously described method.[25b]

The compounds, I-1a,b—14a,b and II-1a,b—14a,b, were synthesized as described in the previous paper.[29] ε-(α-Glutamyl)-lysine was prepared by the method of Caldwell, *et al.*[30] and ε-(γ-glutamyl)-lysine was kindly provided by Dr. S. Iwanaga, Institute for Protein Research, Osaka University. Glutathione (reduced) was purchased from Kohjin Co., Ltd., Tokyo.

N-Acylation and Esterification of Free Peptides in a Small Scale——N–EOC and N–iBOC derivatives were obtained by treating peptide sample (0.5—1 mg) in an alkaline aqueous solution with diethylpyrocarbonate and isobutylchloroformate, respectively, according to the published procedures.[31,32] Other N-acylations were carried out by the method previously described.[25b,33] The resulting N-acylpeptides were esterified by treating with an excess of ethereal CH_2N_2 or with 0.1N methanolic HCl at 20° for 12 h.

Acknowledgement The authors are indebted to Miss H. Hyuga, Kanazawa University, and Prof. M. Kondo, Osaka University, for mass spectral measurements. Thanks are also due to Dr. S. Iwanaga, Osaka University, for a gift of ε-(γ-glutamyl)-lysine.

28) Part of the results were preliminarily presented at the 9th Symposium of Organic and Biomedical Mass Spectrometry, Sendai, Oct., 1974.
29) K. Okada, M. Kawase, Y. Takeuchi, and S. Nagai, *Yakugaku Zasshi*, **96**, 1038 (1976).
30) J.B. Caldwell, L.A. Holt, and B. Milligan, *Aust. J. Chem.*, **24**, 435 (1971).
31) P.B. Loder and E.P. Abraham, *Biochem. J.*, **123**, 471 (1971).
32) M.A. Paz, A. Bernath, E. Henson, O.O. Blumenfeld, and P.M. Gallop, *Anal. Biochem.*, **36**, 527 (1970).
33) K. Okada, T. Uyehara, M. Hiramoto, H. Kato, and T. Suzuki, *Chem. Pharm. Bull.* (Tokyo), **21**, 2217 (1973).