  Bull. Chem. Soc. Jpn., 58,1903—1912 (1985)

Gas Chromatography-Mass Spectrometry of Trimethylsilylated Imino Derivatives of Alanine

Katsuhiro KAWASHIRO,* Shiro MORIMOTO, and Hideyuki YOSHIDA
Department of Chemical Engineering, Faculty of Engineering, Tokushima University, Minamijosanjima, Tokushima 770
(Received December 4, 1984)

Trimethylsilylation of seven imino derivatives of alanine with *N,O*-bis(trimethylsilyl)trifluoroacetamide in acetonitrile was studied. The imino derivatives include 2,2′-iminodipropionitrile (**1**), 2,2′-iminodipropionamide (**2**), 2,2′-iminodipropionic acid (**3**), 2-(1-cyanoethylamino)propionamide (**4**), 2-(1-cyanoethylamino)-propionic acid (**5**), 2-(1-carbamoylethylamino)propionic acid (**6**), and 3,5-dimethyl-2,6-piperazinedione (**7**). The reaction products were identified by gas chromatography-mass spectrometry. Under the present reaction conditions (at 100 °C for 30 min), hydrogen atoms of carboxyl and carbamoyl groups and an imide hydrogen were readily replaced by the trimethylsilyl (TMS) group, but imino hydrogens were not replaced because of the steric hindrance of *N*-substituent group. For the carbamoyl group, only one hydrogen was replaced. Consequently, **1** was not trimethylsilylated. Furthermore, no definite trimethylsilylation products were obtained for **4**. Upon electron impact at 70 eV, **2**, **3**, and **5**—**7** gave rather simple spectra with molecular (M^+) and M −15 (loss of CH_3) ions characteristic of TMS derivatives. A fragment, M−117 (loss of COOTMS) or M −116 (loss of CONHTMS), was very prominent for **2**, **3**, **5**, and **6**. Other important ions such as *m/z* 70 were noted. Preparations and properties of some of the imino derivatives are also described.

In recent years, gas chromatography-mass spectrometry (GC-MS) has become a useful method for unequivocal identification of nonvolatile compounds such as amino acids and related compounds after their conversion into suitable volatile derivatives. Of numbers of such volatile derivatives,[1] the trimethylsilyl (TMS) derivative has received great interest because of its simple preparation and both good gas chromatographic and mass spectral properties.

In our previous paper[2] the presence of certain imino derivatives of alanine (Ala) in the polymerization product of 2-aminopropionitrile (2-APN) was suggested by ion-exchange chromatography. Ion-exchange retention data, however, are generally not sufficient to identify unequivocally amino acids and related compounds present in complex reaction mixtures. This has led us to study the GC-MS of the imino derivatives of Ala as shown in Fig. 1, after conversion into their TMS derivatives. The main reason for choosing the TMS derivative is that TMS groups can be readily introduced into functional groups such as carboxyl, hydroxyl, amino, carbamoyl groups, *etc.* with an easily available reagent such as *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). Because of the absence of an acid or a base no hydrolysis takes place during trimethylsilylation; this makes it possible to distinguish, for example, between acid and amide groups present in the molecules. In this respect the TMS derivative has an advantage over the *N*-trifluoroacetyl alkyl ester derivative, preparations of which generally involve an esterification catalyzed by HCl.

Leimer *et al.*[3] have reported a comprehensive study of the complete mass spectra (electron impact at 70 eV), and the general fragmentation pathways for TMS derivatives of protein amino acids. The GC-MS of TMS derivatives of nonprotein amino acids such as

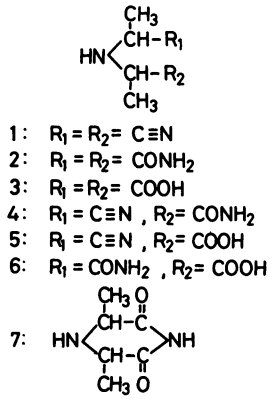


Fig. 1. Imino derivatives of Ala.

N-substituted amino acids, however, has far less been studied. In the previous paper,[4] we have reported GC-MS of TMS derivatives of some iminodicarboxylic acids including 2,2′-iminodipropionic acid (**3**).

This paper describes trimethylsilylation of imino derivatives of Ala as shown in Fig. 1 with BSTFA, and mass spectra of the resultant TMS derivatives upon electron impact at 70 eV. The imino derivatives include 2,2′-iminodipropionitrile (**1**), 2,2′-iminodipropionamide (**2**), (**3**), 2-(1-cyanoethylamino)propionamide (**4**), 2-(1-cyanoethylamino)propionic acid (**5**), 2-(1-carbamoylethylamino)propionic acid (**6**), and 3,5-dimethyl-2,6-piperazinedione (**7**). The structures of **1**—**7** are shown in Fig. 1. Preparations, ion-exchange chromatography, and lability in aqueous solutions are also described for **1**—**7**.

Results and Discussion

Preparations and Properties of 1—7. Of **1**—**7**, symmetrical compounds such as **1**—**3** were easily pre-

pared according to the method of Dubsky.[5] Compound **4** was prepared for the first time in a 40% yield in the following way:[6]

$$CH_3CHO\cdot NaHSO_3 + H_2NCH(CH_3)CONH_2 + NaCN \xrightarrow{0\,^\circ C} \mathbf{4}$$

However, preparations of unsymmetrical compounds such as **5** and **6**, were rather difficult.[7]

Karrer *et al.*[8] have reported preparation of **5**, which involved partial hydrolysis of **1** *via* an imidate derivative. In the present study, a product of mp 188—189 °C was obtained according to their method. Although the results of elemental analysis suggested the formation of **5**, spectral data were not consistent with the structure of **5**. IR spectrum showed no C≡N absorption near at 2250 cm^{-1}, but two strong absorptions due to $\nu_{C=O}$ were observed at 1680 and 1710 cm^{-1}, characteristic positions of an imide structure. The ^1H NMR spectrum of the product is rather simple, and equivalence of both two methyl (δ=1.20) and methine (3.45) protons suggests a cyclized imide structure as shown in Fig. 2. On the basis of these spectral data, the product was determined to be not **5**, but its isomer **7**. Repeated preparations gave the same product. Although the mechanism of the formation of **7** from **1** is not well understood, this reaction may be speculated as shown in Scheme 1.[9] A longer elution time of the product on ion-exchange chromatography

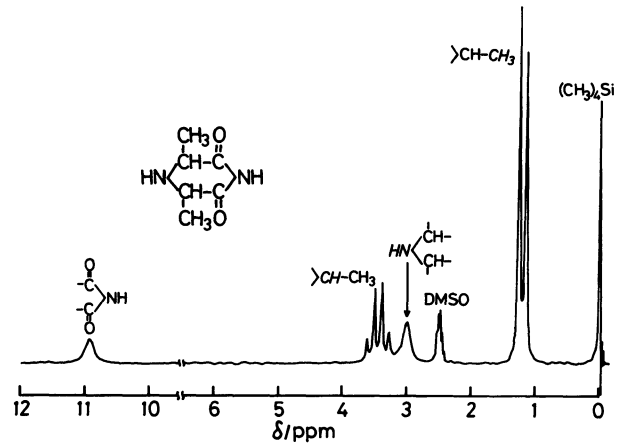
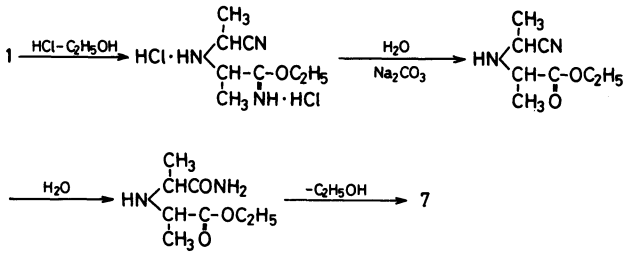
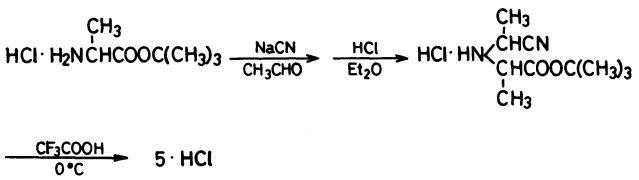


Fig. 2. ^1H NMR spectrum of the product obtained from **1** (DMSO-d_6).



Scheme 1. Possible route for the formation of **7** from **1**.



Scheme 2. Synthetic route for **5**.

(158 min, Table 1) revealed that it was of a basic nature rather than of an acidic nature. This is an additional evidence for the imide structure.

Compound **5** was successfully synthesized by the Strecker reaction and subsequent removal of the *t*-butyl group as shown in Scheme 2. An intermediate, *t*-butyl 2-(1-cyanoethylamino)propionate, was isolated as its hydrochloride (mp 120—122 °C), which was characterized by ^1H NMR spectrum and elemental analysis. Addition of CF_3COOH (TFA) to it gave a very hygroscopic hydrochloride of **5** with removal of the *t*-butyl group.[10] On ion-exchange chromatography, the product exhibited a single peak with a very short elution time (57 min, Table 1); the elution behavior was consistent with an acidic nature of **5**. After conversion into the TMS derivative, the product was further characterized by GC-MS which revealed the presence of both M^+ (*m/z* 214) and M−15 (*m/z* 199) ions corresponding to the mono-TMS derivative (Table 3).

Although two methods[5,11] have been reported for the preparation of **6**, they have individual disadvantages.[12] In the present study, **6** was prepared by partial hydrolysis of **2**. Compound **2** was found to be hydrolyzed to give **3** *via* an intermediate **6** in a hot dilute aqueous solution. The yield of **6** reached a maximum after 1 d at 100 °C, when starting compound **2** disappeared almost completely. After separation by ion-exchange chromatography, **6** was obtained as the monohydrate.

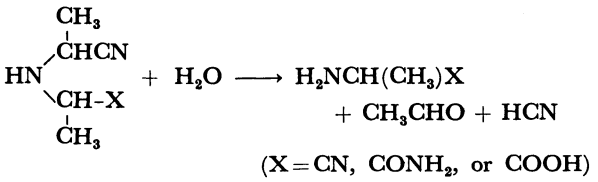
Table 1 gives elution times and color constants of **1**—**7** except **3** on ion-exchange chromatography using an amino acid analyzer. Compound **3** is known to be ninhydrin-negative.[8,13,14] Data of some related compounds such as Ala, alaninamide (Ala-NH_2), and 2-APN are also included. With buffer system A (pH 3.25—pH 5.28, buffer change of 30 min), elution orders of imino derivatives of Ala were similar to those of imino derivatives of glycine (Gly) reported previously.[6] With buffer system B (pH 3.25 alone), elution of basic components was retarded significantly, and **7** was found not to be eluted as a peak. The elution order of 2-APN and Ala-NH_2 reversed with the two buffer systems. As shown in Table 1,[15] **2a** and **6a** exhibited a single peak. On the other hand, the separation between diastereomers was observed for both **2b** and **6b**. It is noted that the peaks of **2a** and **6a** corresponded to the second peaks of **2b** and **6b**, respectively.

Table 1. Elution times and color constants of imino derivatives of Ala and related compounds on ion-exchange chromatography[a)]

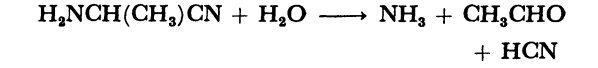
Imino derivative	Elution time/min[b,c)] A	B	Relative color constant[d)]
1	54		1.03
5	57		0.65
6	60, 66		0.095
Ala	98		1.00
4[e)]	112, 131		0.63
7	158	—	0.17
2	206, 240	294, 363	0.70
2-APN	221	390	1.05
NH_3	249	348	0.96
Ala-NH_2	276	379	1.05

a) Column: 0.25ϕ×50 cm (Aminex A-4). Conditions of chromatography are shown in the Experimental section. b) Buffer systems A and B refer to pH 3.25—5.28 buffer with buffer change of 30 min, and to pH 3.25 buffer alone, respectively. c) In the cases of **2** and **6**, the separation between diastereomers was observed for **2b** and **6b**, whereas **2a** and **6a** gave a single peak. The peaks of **2a** and **6a** corresponded to the second peaks of **2b** and **6b**, respectively. d) Color constant of Ala: 124.9 μmol^{-1}. In the cases of **2** and **6**, color constants were calculated for **2a** and **6a**, respectively. e) The separation between diastereomers was observed.

Lability of α-amino nitriles in dilute aqueous solutions is well known.[6,16,17)] Figure 3 shows the time course of decomposition of **1**, **4**, and **5** in both H_2O and the buffer solutions of pH 3.25 at room temperature. Lability increased in the order of **4**, **1**, and **5** in both solutions and the decomposition was more rapid in the buffers (pH 3.25) than in aqueous solutions. α-Aminonitriles **1**, **4**, and **5** decomposed with the formation of 2-APN, Ala-NH_2, and Ala, respectively. Consequently, the decomposition may be explained in terms of the reverse Strecker reaction as follows:[6)]



On decomposition, **4** and **5** gave almost stoichiometric amounts of Ala-NH_2 and Ala, respectively, and no other ninhydrin-positive products were found. In contrast, a significant amount of NH_3 was released in the case of **1** as shown Fig. 4. The formation of NH_3 can be explained in terms of the decomposition of the unstable intermediate, 2-APN, as follows:



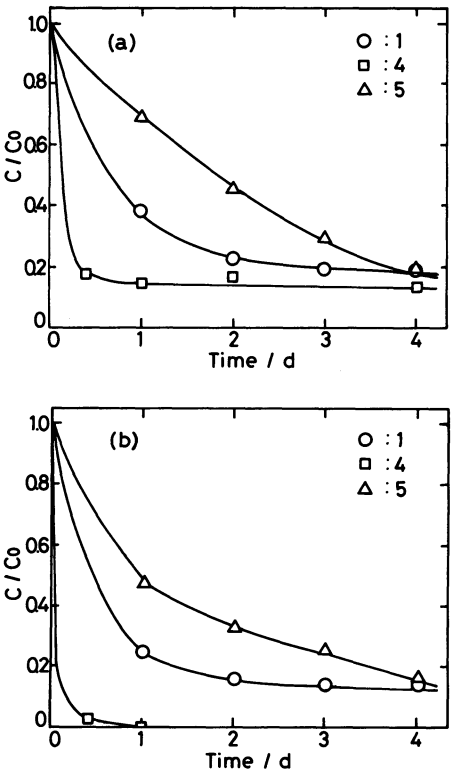


Fig. 3. The time course of decomposition of **1**, **4**, and **5** in aqueous solutions at room temperature. Initial concentration (C_0) is 1 mM (1 M = 1 mol dm^{-3}) for **4** and **5**, but 0.5 mM for **1**. a: H_2O. b: Citrate buffer solution of pH 3.25.

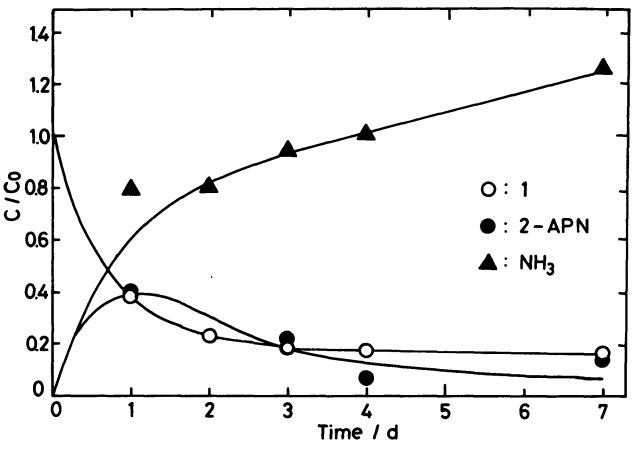


Fig. 4. The time course of decomposition of **1** in aqueous solution at room temperature. C_0: 0.5 mM.

There were some differences in decomposition of **1**, **4**, and **5**. As shown in Fig. 3a, **5** decomposed irreversibly according to the pseude first-order kinetics,[18)] whereas **1** and **4** appeared to reach equilibria after 2 and 1 d, respectively. In the buffer solution of pH 3.25, **4** decomposed completely after 1 d (Fig. 3b); this fact indicates that the equilibrium was shifted greatly to the decomposition-product side with a decrease in

pH. In aqueous solutions, α-amino nitriles **1**, **4**, and **5** were found to be more labile than the corresponding imino derivatives of Gly reported previously.[6] Therefore, when these α-amino nitriles are to be analyzed with an amino acid analyzer, freshly prepared aqueous sample solutions should be charged on the analyzer as promptly as possible.

Trimethylsilylation and Gas Chromatography. When trimethylsilylation was performed in BSTFA–CH_3CN (1:1, v/v)[19] at 100 °C for 30 min, completely clear solutions were obtained for **1**—**7**. This observation suggests the formation of the corresponding TMS derivatives. In general, active hydrogens such as NH (imino), COOH, $CONH_2$, and NH (imide), were expected to be replaced by TMS groups. The GC–MS of the derivatized products revealed that among the active hydrogens, the imino NH could not be trimethylsilylated under the present conditions because of the steric hindrance of the *N*-substituent group.[4] Consequently, **1** having only imino NH as an active hydrogen was not trimethylsilylated. Nevertheless, free **1** was eluted most rapidly on GC because of its high volatility (Table 2). In contrast, some GC peaks were observed for **4**, but any definite trimethylsilylation products could not be identified, suggesting the decomposition of **4** during derivatization.

Table 2 gives retention times of trimethylsilylated imino derivatives of Ala on GC and the number of TMS groups introduced into them. A fused silica capillary column (OV-101 FS-WCOT, 0.35 mmφ×25 m) had advantages over a packed column (3 mmφ×1 m, 1.5% OV-101 on Chromosorb G HP) in terms of the good separation and the absence of decomposition of TMS derivatives. As shown in Table 2, separation between diastereomers of **3** was rather poor as compared with those of **2** and **6**. However, almost base line separation between the diastereomers was achieved when an SE-30 fused silica capillary column (FS-WCOT, 0.35 mmφ×25 m) was used.

Table 2. Retention times of trimethylsilylated imino derivatives of Ala on GC[a]

Parent compound	Number of TMS group	Retention time/min[b]
1	0	6.6
2	2	23.7, 24.7
3	2	16.8, 17.0
4[c]	—	—
5	1	11.1
6	2	20.1, 20.9
7	1	14.6

a) Initial temp: 100 °C. Initial hold: 5 min. Program rate: 5 °C/min. Final temp: 250 °C. Injector temp: 250 °C. Column: OV-101 fused silica capillary (0.35 mmφ × 25 m). Retention time of phenanthrene: 25.9 min. b) In the cases of **2**, **3**, and **6**, the separation between diastereomers was observed for **2b**, **3b**, and **6b**, whereas **2a**, **3a**, and **6a** gave a single peak. The peak of **3a** corresponded to the first peak of **3b**. On the other hand, the peaks of **2a** and **6a** corresponded to the second peaks of **2b** and **6b**, respectively. c) No definite trimethylsilylation products were detected.

There was a difference in the elution behavior between **3a** and **3b**. Two GC peaks were eluted for **3b**, indicating separation between the diastereomers, which were proved on the basis of mass spectral data. On the other hand, a single peak was observed for **3a**, which corresponded to the first peak of **3b**. From these findings, it is evident that **3b** is a mixture of the diastereomers whereas **3a** is a racemate (*R,R*-+*S,S*-) or meso-form (*R,S*-). Similarly, two peaks were eluted for **2b**, while a single peak was observed for **2a**. It is noted, however, that the peak of **2a** corresponded to the second peak of **2b**. The reversed elution order of the diastereomers may be attributable to the nature of the carbamoyl groups, because it seems reasonable to assume no inversion of the two asymmetric centers during the preparation of **2a** from **3a**. Similar elution behavior of diastereomers was also found for **6a** and **6b**. For **2** and **6**, their elution behavior was well consistent with that on ion-exchange chromatography (Tables 1 and 2). Karrer and Appenzeller have reported that 2,2′-iminodipropionic acid (mp 233 °C) prepared by acid hydrolysis of **1**, was not a racemate, but a meso-form or a mixture of these two forms, by comparing its melting point with those of authentic samples.[13,20] The fact that **6a** prepared from **3a** as the starting material exhibited a single GC peak in the present study,[21] is not consistent with the results obtained by Karrer and Appenzeller. If **3a** is a meso-form, **6a** must become a mixture of the diastereomers. This discrepancy needs to be investigated further.

Figure 5 shows the time course of trimethylsilylation of **2b** at 100 °C, as a typical example. The peak area ratio of the di-TMS derivative to an internal standard (phenanthrene) becomes almost constant after 20 min, and its conversion into a more highly

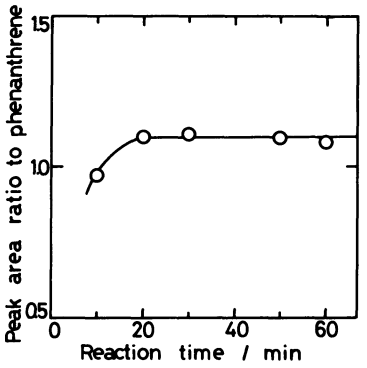


Fig. 5. The time course of trimethylsilylation of **2b** at 100 °C (**2b**: 50 μmol, BSTFA: 0.25 ml, CH_3CN: 0.25 ml, phenanthrene: 3.0 mg).

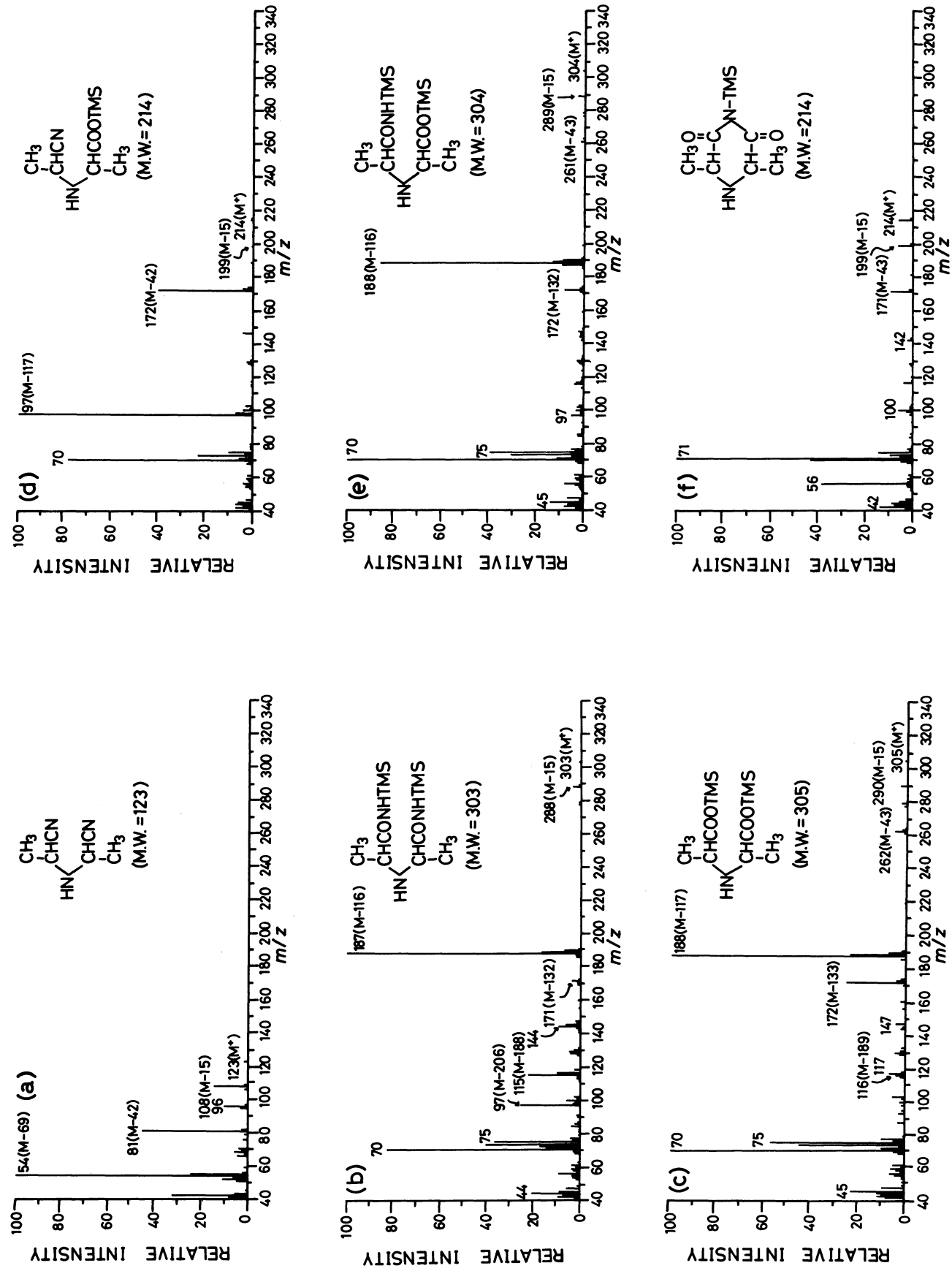


Fig. 6. Mass spectra of trimethylsilylated imino derivatives of Ala. a: **1**, b: **2**, c: **3**, d: **5**, e: **6**, and f: **7**.

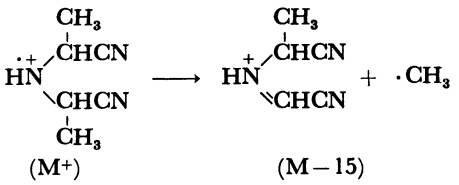
trimethylsilylated derivative was not observed under the present conditions. Compound **7** having an imide NH also exhibited similar behavior on derivatization. Trimethylsilylation of **3b** was found to be more facile than **2b**. Therefore, derivatization of the functional groups present in the imino derivatives of Ala (except the imino NH) may be taken as completed under the present conditions (at 100 °C for 30 min).

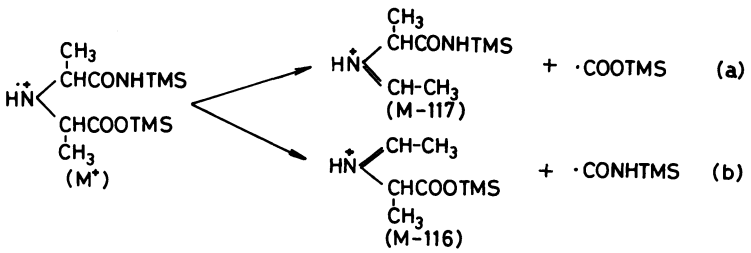
Mass Spectra. Figures 6a—f present mass spectra of trimethylsilylated products for **1**—**3** and **5**—**7**. No TMS groups were introduced into **1**, as described above. Some characteristic ions are listed in Table 3. Both M^+ and M−15 ions are present in all the spectra.

For **2**, **3**, and **5** which have either a carboxyl or carbamoyl group, the most important ions arise from the M^+ by the loss of a COOTMS or CONHTMS group (α-cleavage to the imino nitrogen) with charge retention on the amine fragments. That the carbon-carbon bond adjacent to the nitrogen is preferentially cleaved is well known for amino acids and related compounds.[3,4] Thus both **3** and **5**, and **2** are characterized by the prominent M−117 (COOTMS) and M−116 (CONHTMS) ions, respectively. These fragments resulted from the α-cleavage constitute base peaks. The predominance of the fragments can be explained in terms of their stabilization due to resonance. There are two possibilities of α-cleavage for **6**, which has both carboxyl and carbamoyl groups (Scheme 3). As shown in Table 3, the M−116 ion is about 10 times abundant than the M−117 ion. This fact indicates that the CONHTMS group is more preferentially split off from the M^+ than the COOTMS. The elementary compositions of both the M−116 and M−117 ions were confirmed by hgh-resolution mass measurement.

For **5**, there are also two possibilities of α-cleavage, the loss of COOTMS and CN, with charge retention on the amine fragment. As shown in Fig. 6d, the M−117 (COOTMS) ion at m/z 97 constitutes a base peak, whereas the M−26 (CN) ion at m/z 188 has a relative intensity of less than 1%. This fact indicates that the cleavage of the carbon-carbon bond adjacent to the cyano group is very difficult. The increased stability of the bond compared with those adjacent to electron-withdrawing functional groups such as carbonyl, has been explained by McLafferty.[22]

As is expected, for **1** having two cyano groups no cleavage of the carbon-carbon bond adjacent to the cyano group, is observed. However, instead of the cleavage, the following fragmentation becomes significant, which accounts for the presence of a rather intense M−15 ion (rel. intensity, 14.2%) for **1** without





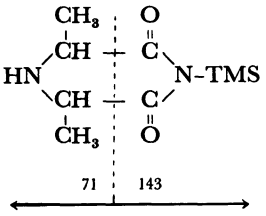
Scheme 3. Two possible α-cleavages for **6**.

TABLE 3. CHARACTERISTIC IONS OF TRIMETHYLSILYLATED IMINO DERIVATIVES OF Ala

Parent compound	Number of TMS group	m/z[a]						
		M^+	M−15	M−42	M−43	M−116	M−117	Others
1	0	123 (1.3)	108 (14.2)	81 (45.0)	—	—	—	54 (100)
2	2	303 (0.4)	288 (3.4)	261 (0.3)	—	187 (100)	186 (0.4)	115[M−118], 97[M−206], 70 (22.0) (25.2) (82.3)
3	2	305 (1.3)	290 (3.0)	—	262 (5.0)	189 (23.2)	188 (100)	172[M−133], 116[M−189], 70 (25.0) (3.1) (99.3)
5	1	214 (0.7)	199 (0.3)	172 (30.0)	—	98 (7.4)	97 (100)	70 (70.2)
6	2	304 (0.3)	289 (2.4)	—	261 (1.1)	188 (86.7)	187 (9.1)	172[M−132], 97[M−207], 70 (8.2) (4.8) (100)
7	1	214 (6.2)	199 (6.1)	—	171 (9.5)	98 (0.5)	97 (1.1)	71, 70 (100) (43.8)

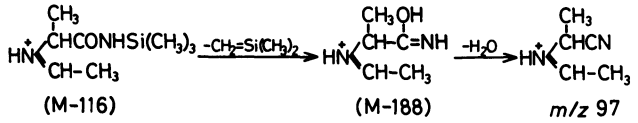
a) Values in parentheses indicate relative intensity.

TMS groups. A base peak at *m/z* 54 corresponds to M−69 (NHCH(CH$_3$)CN), indicating that the N-C bond between the imino nitrogen and methine carbon is cleaved with charge retention on the 1-cyanoethyl fragment. Two ions characteristic of the nitrile, the M−27 (HCN) at *m/z* 96 and M−42 (CH$_3$+HCN) at *m/z* 81, are observed. The latter ion also appears at *m/z* 172 for **5**, indicating the presence of a -CH(CH$_3$)-CN group in the molecule. For **7**, the M$^+$ ion is rather intense because of its cyclic structure. An ion at *m/z* 71 constitutes a base peak, which may result from the following cleavage:

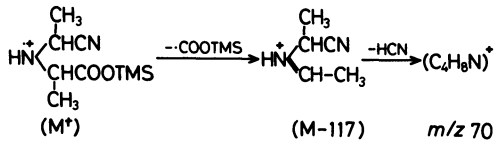


As is expected, the 70 eV spectrum of **3** becomes slightly complicated as compared with the corresponding 20 eV spectrum reported previously,[4] although similar characteristic ions are present. In the 70 eV spectrum, ions in the low mass region such as *m/z* 70, 73, and 75, become significant, whereas the M+1 ion which was larger than the M$^+$ in the 20 eV spectrum is not seen.

As shown in Fig. 6c, **3** having two carboxyl groups gives characteristic ions such as M−43 (CH$_3$+CO), M−133 (CH$_3$+CO+(CH$_3$)$_3$SiOH), and M−189 (COOTMS+CH$_2$=Si(CH$_3$)$_2$). Analogous characteristic ions are observed for **2**, which has two carbamoyl groups (Fig. 6b). Ions at *m/z* 261 and 171 were determined to be M−42 (CH$_3$+CNH) and M−132 (CH$_3$+CNH+(CH$_3$)$_3$SiOH), respectively. Although an ion at *m/z* 170 corresponds to M−131 (CH$_3$+CNH+(CH$_3$)$_3$SiNH$_2$), this is far less significant than the M−132 ion. A prominent ion at *m/z* 115 was determined to be M−188 (CONHTMS+CH$_2$=Si(CH$_3$)$_2$). This fact indicates that the ion results from the α-cleavage fragment (M−116) by the loss of a neutral species, CH$_2$=Si(CH$_3$)$_2$. The elementary composition of an ion at *m/z* 97 was determined to be C$_5$H$_9$N$_2$, which corresponds to M−206 (CONHTMS+CH$_2$=Si(CH$_3$)$_2$+H$_2$O). Consequently, this ion arises most likely from the M−188 ion by the loss of H$_2$O. The fragmentation may be depicted as shown in Scheme 4.



Scheme 4. Proposed fragmentation to the *m/z* 97 for **2**.



Scheme 5. Proposed fragmentation to the *m/z* 70 for **5**.

As is expected, the ions characteristic of both **2** and **3** (except M−42 and M−133) are observed for **6**, which has both carboxyl and carbamoyl groups. In this case, ions at *m/z* 116 and 115 were determined to be M−188 (CONHTMS+CH$_2$=Si(CH$_3$)$_2$) and M−189 (COOTMS+CH$_2$=Si(CH$_3$)$_2$), respectively. The ion *m/z* 97 corresponds to M−207 (COOTMS+CH$_2$=Si(CH$_3$)$_2$+H$_2$O) and, therefore, results from the M−189 by the loss of H$_2$O. Most of the characteristic ions described above were confirmed by high-resolution mass measurement.

In the low mass region, a very prominent ion at *m/z* 70 appears in most of the spectra (except **1**); this is a base peak for **6**. Generally, this unusual ion is not found for the TMS derivatives of common amino acids. High-resolution mass measurement revealed that it had an elementary composition of C$_4$H$_8$N without a silicon atom. Since the *m/z* 70 ion corresponds to M−144 (COOTMS+HCN) for **5**, its formation may be depicted as shown in Scheme 5. Similarly, for **2** and **3**, the *m/z* 70 ion arises most likely from the *m/z* 97 (M−206) and *m/z* 116 (M−189) by the loss of neutral species, HCN and HCOOH, respectively. For **6**, both the two fragmentations are possible for the formation of the *m/z* 70 ion.

In the previous paper,[4] tri-TMS derivative of **3** exhibited an abundant ion at *m/z* 70. In the present study, under forced derivatization conditions, **6** was found to give a small amount of the tri-TMS derivative along with the di-TMS derivative. The tri-TMS derivative also gave a prominent *m/z* 70 ion upon electron impact.[23] These observations indicate that imino derivatives of Ala (except **1**) generally exhibit a very prominent ion at *m/z* 70, whether their imino NHs are trimethylsilylated or not. Consequently, the presence of this ion can be used as a method of detecting them by GC-MS.

Experimental

All the melting points were determined with a Yanagimoto micro melting point apparatus and were not corrected. ^{1}H NMR spectra were measured with a Hitachi R-24B spectrometer using tetramethylsilane or sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard. IR spectra were taken with a Hitachi 285 infrared spectrophotometer. Ala and 2-bromopropionic acid used as starting materials were of (*RS*)-form.

2,2'-Iminodipropionitrile (1). Compound **1** was prepared by the method of Dubsky.[5] Yield, 13%; mp, 65—

66 °C (lit,[5] 68 °C). Found: C, 58.29; H, 7.31; N, 34.14%. Calcd for $C_6H_9N_3$: C, 58.52; H, 7.37; N, 34.12%.

2,2'-Iminodipropionic Acid (3). ***3a***: Compound **1** was hydrolyzed with 6 M† HCl (at 100 °C for 6 h)[5] and then the product was isolated as a zinc salt to remove Ala produced by a side reaction. The free acid was obtained by decomposition of the salt with H_2S in aq solution. Yield, 25%; mp (dec), 239—241 °C (lit,[5] 233—235 °C). Found: C, 44.81; H, 6.96; N, 8.68%. Calcd for $C_6H_{11}NO_4$: C, 44.72; H, 6.88; N, 8.69%.

3b: Compound **3b** was prepared by the reaction of Ala and 2-bromopropionic acid in the presence of NaOH.[14] Yield 32%; mp (dec), 256—258 °C (lit,[14] 250—252 °C). Found: C, 44.56; H, 6.62; N, 8.48%.

2,2'-Iminodipropionamide (2). Compounds **2a** and **2b** were prepared from **3a** and **3b**, respectively, by the method of Dubsky,[5] which involves esterification with methanol and subsequent ammonolysis.

2a: Yield 20%; mp, 133—136 °C (lit,[5] 127 °C). Found: C, 45.11; H, 8.41; N, 26.09%. Calcd for $C_6H_{13}N_3O_2$: C, 45.27; H, 8.23; N, 26.40%.

2b: Yield 41%; mp 135—138 °C. Found: C, 45.01; H, 8.45; N, 26.56%.

2-(1-Cyanoethylamino)propionamide (4). A mixture of acetaldehyde-sodium hydrogensulfite[24] (6.67 g) and H_2O (5 ml) was cooled below 0 °C with stirring, to which was added dropwise a solution of NaOH (1.2 g, 30 mmol) and Ala-NH_2·AcOH[25] (4.45 g, 30 mmol) dissolved in a small amount of H_2O. After the addition was complete, the mixture was stirred for 2h at 0 °C and further for 30 min at room temperature. A solution of NaCN (1.60 g, 33 mmol) in a minimum amount of H_2O was added to it under cooling. After being left overnight at room temperature, the solution was extracted with ethyl acetate (AcOEt) (40 ml×4) and the extract was dried (Na_2SO_4), filtered and then evaporated to give a pale yellowish oil, which solidified on the addition of diethyl ether (Et_2O). Recrystallization was performed from AcOEt-petroleum ether; yield 1.70 g (40%); mp 92—95 °C; IR (KBr) 3410 (NH), 2210 (C≡N), and 1670 cm^{-1} (amide I); ^1H NMR (DMSO-d_6) δ=1.18 (3H, d, J=7.0 Hz, CH_3), 1.47 (3H, d, J=7.0 Hz, CH_3), 2.80—3.92 (2H, m, 2CH), 3.30 (1H, s, NH), and 7.01, 7.27 (2H, br, $CONH_2$). Found: C, 50.91; H, 8.10; N, 29.80%. Calcd for $C_6H_{11}N_3O$: C, 51.05; H, 7.85; N, 29.76%.

As an alternative approach, ammonolysis of methyl 2-(1-cyanoethylamino)propionate was attempted, which was prepared from acetaldehyde-sodium hydrogensulfite, methyl alaninate, and NaCN in a similar manner. However, the product was found to contain a considerable amount of a by-product (Ala-NH_2), which could not be removed by repeated recrystallizations.

2-(1-Cyanoethylamino)propionic Acid (5). *t-Butyl 2-(1-Cyanoethylamino)propionate Hydrochloride*: *t*-Butyl alaninate hydrochloride (mp 143—145 °C; lit,[26] 139—140.5 °C) was prepared by the reaction of *N*-benzyloxycarbonyl-Ala[27] and 2-methylpropene in the presence of H_2SO_4, and subsequent hydrogenation[10] and introduction of dry HCl gas. A mixture of *t*-butyl alaninate hydrochloride (1.0 g, 5.5 mmol), acetaldehyde (0.43 g, 9.8 mmol), and Et_2O (20 ml) was cooled below 0 °C with stirring, to which was added a solution of NaCN (0.49 g, 10 mmol) in a small amount of H_2O. After the addition was complete, stirring was continued for 4 h. The Et_2O layer was separated and then the aq layer was extracted with Et_2O (50 ml×3). The combined Et_2O layer was dried (Na_2SO_4) and filtered. Introduction of dry HCl gas into the Et_2O layer caused crystallization of the hydrochloride, which was recrystallized from ethanol-Et_2O; yield 0.60 g (46%); mp 120—122 °C; ^1H NMR (DMSO-d_6) δ=1.47 (9H, s, $C(CH_3)_3$), 1.54 (3H, d, J=7.1 Hz, CH_3CHCO), 1.65 (3H, d, J=7.1 Hz, CH_3CHCN), 3.93 (1H, q, J=7.1 Hz, CHCO), 4.57 (1H, q, J=7.1 Hz, CHCN), and 7.58 (2H, br, NH_2^+). Found: C, 51.08; H, 8.25; N, 11.94%. Calcd for $C_{10}H_{19}N_2O_2Cl$: C, 51.17; H, 8.16; N, 11.93%.

Removal of the t-Butyl Group: The removal of the *t*-butyl group was carried out with TFA, giving a very hygroscopic product (**5**·HCl). A typical example was as follows. *t*-Butyl 2-(1-cyanoethylamino)propionate·HCl (50 μmol) was dissolved in TFA (0.5 ml) below 0 °C. After being kept for 10 min, the solution was allowed to stand at room temperature for 1 h. Evaporation of the TFA in vacuum gave the product. When a portion of an aq solution of the product was analyzed with the amino acid analyzer, only one peak was eluted (elution time, 57 min; Table 1). The product was also trimethylsilylated with BSTFA-CH_3CN (1:1, v/v) (at 100 °C for 30 min) for GC-MS. GC of the derivatized solution exhibited only one peak with a retention time of 3.1 min (column, 3 mmϕ× 1 m packed with 1.5% OV-101 on Chromosorb G HP; temperature, 100 °C). Its mass spectrum contained both M^+ (m/z 214) and M−15 (m/z 199) ions, indicating the formation of a mono-TMS derivative (mol wt=214) (Table 3).

2-(1-Carbamoylethylamino)propionic Acid (6). Compounds **6a** and **6b** were obtained as monohydrates by partial hydrolysis of **2a** and **2b**, respectively.

6a: A solution of **2a** (0.25 g, 1.6 mmol) in H_2O (45 ml) was heated on a sand bath at 100 °C for 24 h. After evaporation, the residue was extracted with hot methanol (15 ml) to remove **3a**. The methanol was evaporated and then the residue was dissolved in H_2O (3 ml). The solution was charged on a column (2.0 ϕ×5 cm) packed with Amberlite CG-120 (H form). After the column was washed with H_2O, the product was eluted with 1% aq pyridine; yield 0.11 g (38%); mp (dec) 208—209 °C (lit,[5] 210 °C (as **6**·1.5H_2O)). Found: C, 40.68; H, 8.00; N, 15.39%. Calcd for $C_6H_{12}N_2O_3$·H_2O: C, 40.44; H, 7.92; N, 15.72%.

6b: Yield 42%; mp (dec) 196—199 °C; ^1H NMR (TFA) δ=1.87 (6H, d, J=7.2 Hz, $2CH_3$), 4.14—4.73 (2H, m, 2CH), 7.34 and 7.44 (2H, br, $CONH_2$), and 7.82 (2H, br, NH_2^+). Found: C, 40.54; H, 7.61; N, 15.69%.

3,5-Dimethyl-2,6-piperazinedione (7). Into a solution of **1** (1.0 g, 8.1 mmol) in anhydrous ethanol (20 ml) was introduced dry HCl gas under cooling until first-formed white precipitates dissolved completely. The introduction of HCl was continued further for 30 min. The solution was stored in a refrigerator overnight, and then filtered into a flask containing Et_2O (200 ml). The resultant hygroscopic precipitates were collected by filtration, washed with Et_2O, and then dried in vacuum, which were dissolved in H_2O (5 ml). After the addition of Na_2CO_3 (0.86 g, 8.1 mmol), the solution was extracted with Et_2O (30 ml×10). The extract was dried (Na_2SO_4), filtered and then evaporated to give crystals which were recrystallized from AcOEt-petroleum ether; yield 0.21 g (18%); mp 188—189 °C (lit,[5]

† 1 M=1 mol dm^{-3}.

186 °C); IR (KBr) 3250 (imino NH), and 1710 and 1680 cm^{-1} (imide C=O); ^1H NMR (DMSO-d_6) δ=1.20 (6H, d, J=7.2 Hz, $2CH_3$), 3.00 (1H, br, imino NH), 3.45 (2H, q, J=7.2 Hz, 2CH), and 10.92 (1H, br, imide NH). Found: C, 50.42; H, 7.06; N, 19.63%. Calcd for $C_6H_{10}N_2O_2$: C, 50.69; N, 7.09; N, 19.71%.

It is well known that a C≡N group can be easily converted into an amide in hydrobromic acid-acetic acid (HBr-AcOH).[6,17] Treatment of this product with 25% HBr-AcOH (Protein Research Foundation), however, resulted merely in the formation of HBr salt of **7**, which was confirmed by elemental analysis and ^1H NMR spectrum. This is an additional evidence for the absence of a C≡N group in the product.

Ion-exchange Chromatography. Ion-exchange chromatography was carried out with a Sibata amino acid analyzer AA-600 under the following conditions: Column 0.25 ϕ×50 cm packed with Aminex A-4 (Bio-Rad); flow rate of an eluent, 6 ml/h; flow rate of a ninhydrin solution, 3 ml/h; jacket temp, 30 °C. Absorbance at 570 nm was measured by continuous photometry. Sodium citrate buffers of pH 3.25 (0.2 M Na^+) and 5.28 (0.35 M Na^+) were used as eluents (Wako Pure Chemical Industries, Ltd.). Each of imino derivatives of Ala and related compounds was chromatographed separately and its color constant was calculated according to the common H×W method. Ala-NH_2 and 2-APN were prepared as Ala-NH_2·AcOH[25] and 2-APN·1/2 H_2SO_4,[28] respectively, according to the reported methods.

Decomposition of 1, 4, and 5 in Aqueous Media. Both H_2O and pH 3.25 citrate buffer solutions were prepared for **1**, **4**, and **5**. Compound **5**·HCl was obtained from a known amount of *t*-butyl 2-(1-cyanoethylamino)propionate hydrochloride by the treatment of TFA. The concentration of the solutions was 1 mM for **4** and **5**, but 0.5 mM for **1**. The solutions were allowed to stand at room temperature. Portions (0.1 ml) of the solutions were analyzed with the amino acid analyzer (buffer system A) at appropriate time intervals. The reason why the concentration of 0.5 mM was chosen for **1** is that the calibration curve of **1** does not follow Beer's law at a concentration higher than this. Decomposition products were identified by comparing elution times with those of the authentic samples. The other imino derivatives of Ala were stable under the conditions.

Trimethylsilylation. About 25 μmol of each imino derivative was weighed into a 1 ml screw vial. Then, 0.25 ml of BSTFA (Nakarai Chemicals, Ltd.) and 0.25 ml of CH_3CN (Wako Pure Chemical Industries, Ltd., S grade reagent) were added to the vial. It was closed tight with a silicone cap and then heated at 100 °C for 30 min in a Reacti-Therm (Pierce Chemical Company) or in a constant-temperature air bath (Yamato Drying-Oven, DX-58). For the experiments of the time course of trimethylsilylation, the latter was used because of its constant temperature. The derivatized solutions were usually clear, 0.5—1 μl portions of which were injected into a GC column. For GC-MS, about 50 μmol of each imino derivative was trimethylsilylated with 0.4 ml of BSTFA and 0.4 ml of CH_3CN.

GC. GC was carried out with a Hitachi 163 gas chromatograph equipped with a flame ionization detector. The conditions were as follows: Column, 3 mmϕ×1 m (glass) packed with 1.5% OV-101 on Chromosorb G HP (100/120 mesh) (Shimadzu); injector temp, 230 °C. Flow rates of carrier gas (N_2), H_2, and air were 30, 35, and 920 ml/min, respectively. The column oven was operated isothermally at 100 °C for 5 min after injection and then programmed to be heated up to 230 °C at a rate of 5 °C/min. A fused silica capillary column (FS-WCOT, 0.35 mmϕ×25 m, OV-101 or SE-30) (Gasukuro Kogyo Co. Ltd.) was also used instead of the packed column under the following conditions: Flow rate of carrier gas (N_2). 1 ml/min; split ratio, 1:50. Peak area ratios were calculated by a Chromatogram Processor 7000B (System Instrument Corporation).

GC-MS. Mass spectra were obtained with a Varian MAT 312 mass spectrometer which was connected with a Varian 3700 gas chromatograph. Each volatile derivative was chromatographed on a glass column (1.6 mmϕ×1 m) packed with 3% OV-101 on Gas Chrom Q (80/100 mesh) by the use of He as the carrier gas (flow rate, 15 ml/min). The column oven was operated isothermally at 70 °C for 5 min after injection (injector temp, 230 °C) and then programmed to be heated up to 230 °C. The GC-MS combination was operated under the following conditions: Separator temp, 240 °C; energy of electron, 70 eV; accelerating voltage, 3.0 kV; ion source temp, 300 °C. The spectrometer was continuously scanned.

High-Resolution Mass Measurement. High-resolution mass spectra (70 ev) of selected samples were obtained with a JEOL JMS-D 300 mass spectrometer by the use of perfluorokerosene as an internal standard.

We wish to thank Mr. Hideo Mori, Otsuka Pharmaceuticals Co. Ltd., Tokushima Factory, for obtaining low-resolution mass spectra by GC-MS.

References

1) P. Hušek and K. Macek, *J. Chromatogr.*, **113**, 139 (1975).

2) S. Morimoto, K. Kawashiro, H. Yoshida, and K. Sugiura, "The polymerization products of α-aminopropionitrile. The component separation using cation-exchange resin" in "Origin of Life," ed by Y. Wolman, D. Reidel Publishing Company, Dordrecht, Holland (1981), pp. 247—254.

3) K. R. Leimer, R. H. Rice, and C. W. Gehrke, *J. Chromatogr.*, **141**, 355 (1977).

4) K. Kawashiro, S. Morimoto, and H. Yoshida, *Bull. Chem. Soc. Jpn.*, **57**, 2871 (1984).

5) J. V. Dubsky, *Ber.*, **49**, 1045 (1916).

6) K. Kawashiro, S. Morimoto, and H. Yoshida, *Bull. Chem. Soc. Jpn.*, **56**, 792 (1983).

7) In the previous paper (Ref. 6), 2-(cyanomethylamino)acetic acid and 2-(carbamoylmethylamino)acetic acid were prepared by deprotection of *N*-(*p*-methoxybenzyloxycarbonyl)(Z(OMe))-2-(cyanomethylamino)acetic acid with TFA and HBr-AcOH, respectively. The *N*-protected 2-(cyanomethylamino)acetic acid was obtained by introduction of Z(OMe) to the imino nitrogen of ethyl 2-(cyanomethyl)-acetate with *S*-(*p*-methoxybenzyloxycarbonyl)-4,6-dimethyl-2-pyrimidinethiol followed by saponification with aq NaOH. In the present study, attempted preparations of **5** and **6** according to this method were unsuccessful, because introduction of Z(OMe) to the imino nitrogen of ethyl 2-(1-cyanoethylamino)propionate did not take place under the same conditions. This is due to the steric hindrance of the

N-substituent group.

8) P. Karrer, H. Koenig, and R. Legler, *Helv. Chim. Acta*, **24**, 861 (1941).

9) P. G. Pietta, P. Cavallo, and G. R. Marshall, *J. Org. Chem.*, **36**, 3966 (1971). A base-catalyzed elimination of an alcohol leading to a cyclic imide formation is known for *N*-protected asparagine and glutamine esters.

10) G. W. Anderson and F. M. Callahan, *J. Am. Chem. Soc.*, **82**, 3359 (1960).

11) G. Ciamician and P. Silber, *Ber.*, **39**, 3942 (1906).

12) The method of Dubsky (Ref. 5) is difficult to isolate **6** from a reaction mixture and that of Ciamician and Silber (Ref. 11) is very time-consuming.

13) P. Karrer and R. Appenzeller, *Helv. Chim. Acta*, **25**, 595 (1942).

14) K. Kawashiro, S. Morimoto, and H. Yoshida, *Bull. Chem. Soc. Jpn.*, **57**, 1097 (1984).

15) Compound **3** was obtained by the following two methods. Compounds **3a** and **3b** were prepared by acid hydrolysis of **1** and by reaction of Ala and 2-bromopropionic acid (Ref. 14), respectively. Compounds **2** and **6** prepared from **3a** as the starting material were designated as **2a** and **6a**, respectively. Similarly, **2** and **6** from **3b** were designated as **2b** and **6b**, respectively.

16) R. E. Moser and C. N. Matthews, *Experientia*, **24**, 658 (1968).

17) C. Ressler, G. R. Nagarajan, M. Kirisawa, and D. V. Kashelikar, *J. Org. Chem.*, **36**, 3960 (1971).

18) Pseude first-order rate constants at room temperature were found to be 4.17×10^{-1} and 5.31×10^{-1} d^{-1} for H_2O and pH 3.25 buffer solution, respectively.

19) C. W. Gehrke, H. Nakamoto, and R. W. Zumwalt, *J. Chromatogr.*, **45**, 24 (1969).

20) Karrer *et al.* (Ref. 8) once assigned this acid to a racemate on the basis of its degradation with kidney (*R*)-amino acid oxydase.

21) Strictly speaking, GC analysis for **3a** revealed the presence of a small amount (about 3%) of the diastereomers corresponding to the second peak of **3b**. Similarly, **6a** contained about 5% of the diastereomers corresponding to the first peak of **6b**.

22) F. W. McLafferty, *Anal. Chem.*, **34**, 26 (1962).

23) MS (70 eV) *m/z* (rel. intensity) 361($M-CH_3$, 3), 260 (M−CONHTMS, 100), 259 (M−COOTMS, 2), 188 (M−CONHTMS−CH_2=$Si(CH_3)_2$, 2), 147 (8), 75 (11), 73 (7), and 70 (31).

24) H. Scheibler and H. Neef, *Ber.*, **59**, 1500 (1926).

25) K. Kawashiro, H. Yoshida, and S. Morimoto, *Bull. Chem. Soc. Jpn.*, **50**, 2956 (1977).

26) E. Taschner, A. Chimiak, B. Bator, and T. Sokolowska, *Justus Liebigs Ann. Chem.*, **646**, 134 (1961).

27) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932).

28) K. Kawashiro, S. Morimoto, H. Yoshida, and K. Sugiura, *Origins Life*, **8**, 347 (1977).