# Separation and Sequence of Dipeptides Using Gas Chromatography and Mass Spectrometry of Their Trimethylsilylated Derivatives†

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ABSTRACT: Dipeptides containing all 20 common amino acids in the amino and carboxyl positions were trimethylsilylated under a variety of conditions. The dipeptides (about 200 of the possible 400) were synthesized by conventional means or derived from polypeptides digested with dipeptidyl aminopeptidase. Derivatives were prepared in high yield by reaction with *N,O*-bis(trimethylsilyl)trifluoroacetamide in acetonitrile. Reaction products were identified by mass spectrometry. Numerous stationary phases and supports were compared in the gas chromatographic analysis of the derivatives. Best results were obtained with a 0.2 × 60 cm column packed with Chromosorb 750 coated with a 1% solution of OV-1. All $Me_3Si$ dipeptides except those containing arginine eluted in less than 30 min when the column was temperature programmed from 100 to 280 °C. The typical mass spectrum of trimethylsilylated dipeptides contains two significant ions used for identification: the ion derived from the amino terminal residue by $\beta$ cleavage of the central CH–CO bond and the molecular ion minus a methyl group. Other useful ions also have been noted. A sufficient variety of samples have been examined to establish predictable fragmentations which should allow the identification of all 400 possible dipeptides.

An interesting method for determining the amino acid sequence of polypeptides (Callahan et al., 1970) involves their degradation into dipeptide fragments with dipeptidyl aminopeptidase (DAP)[1] (McDonald et al., 1969, 1972). The released dipeptides may be correctly aligned by their time of release or, more commonly, by repeating the degradation of the native peptide after removal of the amino-terminal amino acid by the Edman method. Dipeptides have been analyzed by ion-exchange and paper chromatography (Callahan et al., 1970); however, these procedures are cumbersome and so time consuming as to limit the rate of polypeptide sequencing. Several laboratories, as well as ours (Krutzsch & Pisano, 1975, 1977), have considered gas chromatography (GC) or combined gas chromatography-mass spectrometry (GC–MS) for dipeptide analysis because of the speed, sensitivity, and ease of identification attainable with these techniques. Dipeptides have been analyzed as their *N*-perfluoroalkylacylamide methyl esters (Weygand et al., 1960, 1961, 1963; Ovchinikov & Kiryushkin, 1972; Caprioli et al., 1973; Caprioli & Seifert, 1975; Young & Desiderio, 1976), *N*-acetylacetonyl methyl esters (Schier et al., 1974; Frank et al., 1977), *N*-ethylacetoacetatyl methyl esters (Schier et al., 1976), and, following reduction, as their pertrimethylsilyl ($Me_3Si$) amino alcohols (Kelley et al., 1975; Nau et al., 1975).

Dipeptides have been trimethylsilylated with hexamethyldisilazane (Birkofer et al., 1962), diethylaminotrimethylsilane (Ruhlman et al., 1966) and *N,O*-bis(trimethylsilyl)acetamide chlorotrimethylsilane (Baker et al., 1969). As was previously noted with amino acids (Gehrke et al., 1969) we have found that dipeptides may be easily pertrimethylsilylated in high yield with *N,O*-bis(trimethylsilyltrifluoroacetamide) (BSTFA),[1] and the derivatives generally have good chromatographic properties and, contrary to a previous report (Baker et al., 1969), easily interpretable mass spectra which provide vital sequence data. Dipeptides containing arginine are not sufficiently volatile for gas chromatography. However, they may be analyzed by direct introduction into the mass spectrometer.

## Materials and Methods

Dipeptides were purchased from Sigma, Vega-Fox, and ICN Pharmaceutical, Inc., synthesized by established procedures, or derived from polypeptides by DAP digestion. Dipeptides used for determining relative GC responses, with the exception of $\alpha$-Aba-Ala, Met-Ala, Gln-Gln, Ala-His, and His-Lys, were synthesized by hydrogenation of their *N*-carbobenzoxybenzyl esters, which were either purchased from Vega-Fox or synthesized by carbodiimide coupling of the suitably protected amino acids. Prior to hydrogenation, all compounds were recrystallized from ethyl acetate–hexane and, if necessary, from ethanol–water. Purity was checked by thin-layer chromatography. The following were obtained commercially: spectral grade acetonitrile, anhydrous methanol, toluene, and chloroform (Burdick and Jackson Co.); Reacti-Vials with Teflon-lined screw caps (Pierce Chemical Co.); dichlorodimethylsilane, 80–100 mesh Chromosorb 750, OV-1, and other supports and liquid phases for GC (Supelco Co. or Pierce Chemical Co.). Derivatized dipeptides were analyzed with a Nuclear-Chicago Model 5000 gas chromatograph or a LKB Model 9000 gas chromatograph–mass spectrometer. The ionizing voltage of the mass spectrometer was set at 20 eV for the total ion plot; spectra were scanned at 70 eV with an ionizing current of 60 $\mu$A.

*Silylation of Dipeptides.* To the dry sample (25–100 nmol) in a 1-mL Reacti-Vial cleaned with $HNO_3$–$H_2SO_4$ (1:4) is added 0.1 mL each of BSTFA and dry acetonitrile. If the sample contains <25 nmol of peptide, a 0.4-mL vial and 25 $\mu$L of each reagent is used. The vials are than tightly capped with Teflon-lined screw caps and heated for 10 min at 140 °C with occasional vortex mixing.

*Gas Chromatography of $Me_3Si$ Dipeptides.* The technique involves short columns, low loading of stationary phase, and rigorous masking of active sites. Procedures for cleaning the column, solid support, and glass wool, masking active sites and coating the support with stationary phase are adaptations of

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[1] Abbreviations used: DAP, dipeptidyl aminopeptidase; GC, gas chromatography; MS, mass spectrometry; GC–MS, gas chromatography-mass spectrometry; $Me_3Si$, trimethylsilyl; BSTFA, *N,O*-bis(trimethylsilyltrifluoroacetamide).

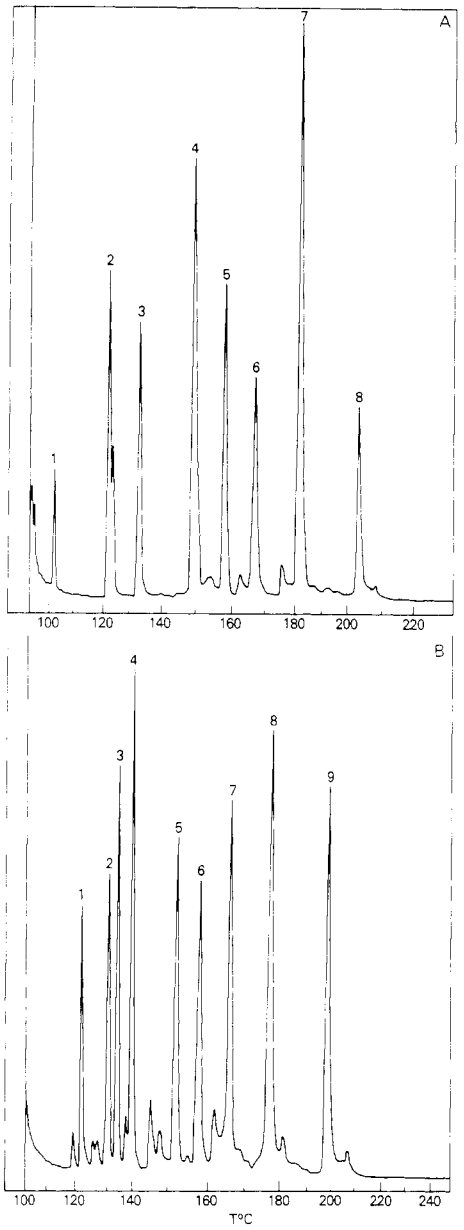



FIGURE 1: Chromatogram of typical pertrimethylsilylated dipeptides. Extraneous peaks are largely due to sample and reagent impurities. Temperature was raised 10 °C/min. (Panel A) Gly-Ala (1), Ala-Gly (2), Ala-Ala (3), Ala-Asp (4), Ala-Phe (5), Ala-Gln (6), Ala-Tyr (7), Gln-Phe (8). (Panel B) Ala-Ala (1), Val-Ala (2), Ala-Leu (3), Ser-Ala (4), Met-Ala (5), Asn-Ala (6), Lys-Ala (7), Ala-His (8), Trp-Ala (9). About 5 nmol of each peptide was injected.

TABLE I: Relative GC Molar Responses of 5 nmol of Some $Me_3Si$ Dipeptides.

| $Me_3Si$ dipeptide | approx. elution temp (°C) | rel response[a] | $Me_3Si$ dipeptide | approx. elution temp (°C) | rel response[a] |
|---|---|---|---|---|---|
| Gly-Ala | 105 | 0.91 | Ala-Phe | 160 | 1.1 |
| Ala-Gly | 125 | 0.90 | Glu-Ala | 160 | 1.1 |
| Ala-Ala | 125 | 0.99 | Asn-Ala | 160 | 1.1 |
| Aba-Ala[b] | 130 | 0.95 | Gln-Ala | 165 | 1.1 |
| Asp-Ala | 135 | 0.90 | Ala-Gln | 165 | 1.2 |
| Val-Ala | 135 | 1.1 | Lys-Ala | 170 | 1.1 |
| Ala-Leu | 135 | 1.0 | Ala-His | 180 | 0.98 |
| Ile-Ala | 135 | 1.1 | Ala-Tyr | 185 | 1.1 |
| Pro-Ala | 140 | 1.0 | Trp-Ala | 205 | 1.1 |
| Ser-Ala | 140 | 1.1 | Gln-Phe | 205 | 1.0 |
| Thr-Ala | 145 | 1.0 | Gln-Gln | 215 | 0.84 |
| Ala-Asp | 150 | 1.1 | Asn-Trp | 230 | 0.84 |
| Met-Ala | 155 | 1.0 | His-Lys | 230 | 0.85 |

[a] Peak areas (from the total ion monitor of the mass spectrometer) are relative to that of $Me_3Si$-Met-Ala set at 1.0. [b] Aba, α-L-aminobutyric acid.

a previously published procedure (Sweeley et al., 1959). The support, approximately 25 g of 80–100 mesh Chromosorb 750, is soaked overnight in $HNO_3$–HCl (3:1) and then washed (by suspension and decantation) with concentrated HCl until the washes are colorless. Acid is removed by washing the support to neutrality with deionized water. It is then allowed to stand for 5 min in 5% $Na_2CO_3$ and again washed with water to neutrality. Any material not settled in 5 min is decanted. The support is dried overnight at 110 °C and silanized overnight at room temperature by allowing it to stand in a 20% (v/v) solution of dichlorodimethylsilane in toluene. To ensure reagent penetration, the support is degassed with an aspirator or by house vacuum after the reagent is added. The silanized support is rinsed several times with toluene. Care should be taken to keep the support covered with toluene. This prevents reaction of remaining -Si-Cl groups with atmospheric moisture. Replacement of -Cl with methoxy groups is effected by allowing the support to stand for 10 min in anhydrous methanol. The deactivated support is finally washed several times with toluene and dried by heating at 110 °C. Glass GC columns and glass wool plugs are treated with 1:4 $HNO_3$–$H_2SO_4$, washed in $HNO_3$–HCl, silanized with dichlorodimethylsilane, and finally rinsed with anhydrous methanol. The filtration technique is used to coat the support with stationary phase. An OV-1 column (nominally 1%) is prepared by adding 1 g of support to 20 mL of chloroform containing 0.2 g of OV-1. The mixture is degassed by gently swirling under reduced pressure and filtered on a sintered glass funnel. Suction is applied for about 15 min, and then the damp support is thoroughly dried by heating for 5 min at 110 °C. The section of silanized column used in the LKB GC-MS which fits into the column oven is 0.2 cm × 60 cm; the section which fits into the flash heater is 0.3 × 15 cm. The column is filled up to the 0.3-cm section using suction and gentle tapping. Columns 0.2 cm throughout presumably would be equally satisfactory. Column packing is held in place at both ends with silanized glass wool. Silicone rubber septums are soaked overnight in chloroform to remove low molecular weight polysiloxanes and then air dried. Columns are conditioned by heating for 48 h at 290 °C with a 30 mL/min helium flow rate. Periodically, 10 μL of BSTFA is injected to silanize any active sites which were exposed during filling or conditioning. New or reused columns require one or two injections of sample before reproducible results are obtained. Samples (usually 2–10 μL, 0.2–100 nmol) are analyzed with the flash heater set at 280 °C, the helium flow at 30 mL/min and the initial column temperature at 100 °C. After injection of the sample, the column temperature is raised to 280 °C at the rate of 10 °C/min.

*Mass Spectrometry.* Spectra were scanned at 70 eV with a LKB 9000 mass spectrometer. The gas chromatogram was taken from the total ion monitor with the source set at 20 eV ionizing voltage and 60 μA ionizing current.

*Conversion of Arginine to Ornithine.* Dipeptides which contain arginine are not sufficiently volatile for GC analysis. However, they may be chromatographed after conversion of arginine to ornithine. This was performed by a modification of the method of Nau et al. (1975). To the dry sample in a Reacti-Vial is added 50 μL of freshly distilled hydrazine hy-

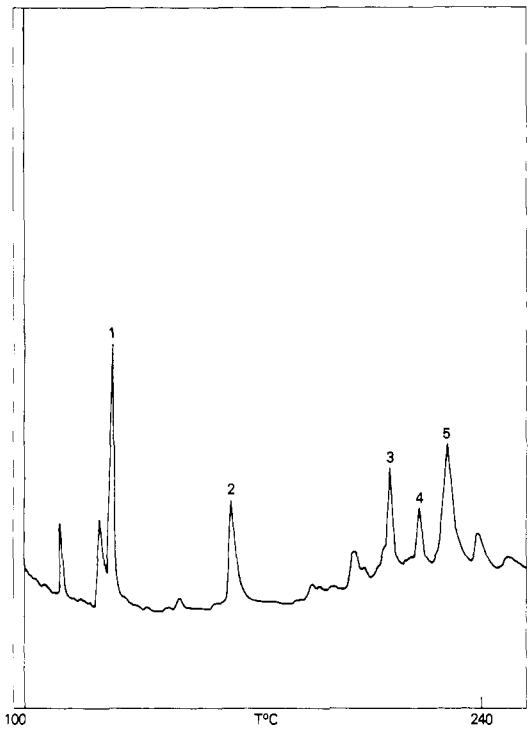



FIGURE 2: Gas chromatogram of approximately 0.5 nmol each of pertrimethylsilylated Ser-Ala (1), Ala-Gln (2), Gln-Phe (3), Gln-Gln (4), Asn-Trp, and His-Lys (5). Dipeptides 2-5 are representative of the most troublesome dipeptides. Extraneous peaks are largely due to sample and reagent impurities.

drate, 50 μL of distilled water, and 5 μL of glacial acetic acid. The solution is heated at 95 °C for 30 min with occasional vortex mixing and then evaporated to dryness in a $N_2$ stream. The converted peptide is dissolved in 200 μL of distilled water and freeze-dried to remove traces of reagents.

*Analysis of Arginine-Containing Dipeptides by Direct Introduction into the Mass Spectrometer.* An alternative method for analyzing arginine-containing dipeptides is to introduce the $Me_3Si$ dipeptide directly into the mass spectrometer. Approximately 10–40 μL of sample is added to the probe cup and, if necessary, evaporated in a dry nitrogen stream to about 10 μL. A silylated glass wool plug is placed over the sample and the probe is introduced into the ion source with the external temperature set at room temperature. The temperature is raised approximately 10 °C/min to 150 °C. Most of the $Me_3Si$ dipeptides which chromatograph volatilize before the source temperature reaches approximately 80 °C, when the arginine-containing derivatives of interest begin to appear.

## Results

*Trimethylsilylation of Dipeptides.* *N,O*-Bis(trimethylsilyl)trifluoroacetamide was a more effective trimethylsilylating agent than *N,O*-bis(trimethylsilyl)acetamide, trimethylsilylimidazole, or chlorotrimethylsilane. Reaction temperature was varied from 25 to 160 °C, reaction time from 5 min to 24 h and the reagent/solvent ratios from 1:0 to 1:3 BSTFA/$CH_3CN$. Maximum GC peak heights were obtained by reaction of dipeptides for 10 min at 140 °C in 1:1 BSTFA/$CH_3CN$. $Me_3Si$ dipeptides are generally stable for at least 1 month when the reaction mixtures are stored in tightly capped vials. Silicone rubber caps not lined with Teflon cause numerous extraneous GC peaks and are not recommended.

*Gas Chromatography.* Numerous stationary phases and supports were compared in the gas chromatographic analysis. The stationary phases tested were OV-1, OV-17, OV-25, OV-210, SE-30, XE-60, DC-560, and Dexsil-300. The supports were 80–100 and 100–120 mesh Chromosorb W, Gas Chrom Q, and Chromosorb 750. They were coated with solutions of stationary phases which ranged from 0.5 to 5% (w/v). Using the least volatile and most troublesome $Me_3Si$ dipeptides as test compounds, including His-Gln, His-Lys, His-Tyr, His-Phe, Asn-Trp, Gln-Trp, Trp-Gln, Gln-Phe, and Gln-Gln, the highest peaks were observed with columns containing 80–100 mesh Chromosorb 750 coated with a 1% solution of OV-1. A supplementary 1% Dexil-300 column was useful because it had a different selectivity. However, the peaks tended to be smaller.

TABLE II: Loss in Responses of $Me_3Si$ Dipeptides When the Sample Size Is Decreased.

| nmol injected | $Me_3Si$-Ser-Ala (%) | $Me_3Si$-Gln-Phe (%) | $Me_3Si$-His-Lys (%) |
|---|---|---|---|
| 10.0 | 100 | 100 | 100 |
| 5.0 | 100 | 100 | 100 |
| 3.0 | 87 | 89 | 68 |
| 1.0 | 58 | 57 | 25 |
| 0.5 | 55 | 44 | 11 |
| 0.1[a] | 36 | 28 | 5 |

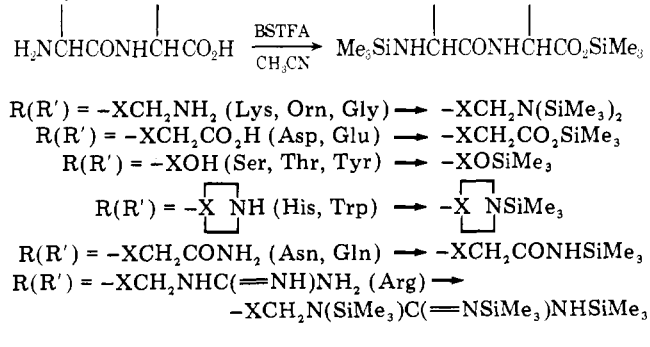
[a] Below 0.1 nmol most $Me_3Si$ dipeptides often give undetectable responses.

All $Me_3Si$ dipeptides synthesized were chromatographed on the 1% OV-1 column. $Me_3Si$ dipeptides containing only aliphatic, aromatic, hydroxylic, carboxylic, and amino side chains always gave excellent responses (Figure 1 and Table I). Dipeptides containing Gln, Asn, and His are least likely to respond well (Figure 2). Under the best circumstances, the limit of detection of such dipeptides is 0.1 nmol (Table II).

Arginine-containing dipeptides do not elute from the GC column. They may be analyzed by either direct introduction into the mass spectrometer (probe analysis) or by GC–MS after alteration of the arginine residue. Most of our analyses have been done by probe analysis. In preliminary experiments we have found that conversion of arginine to ornithine with hydrazine is preferable to converting the guanidinium group to the dimethylpyridinium group with acetylacetone. The latter method tended to give lower yields and more background, which was especially evident with enzymatic digests.

Analysis of about 200 $Me_3Si$ dipeptides containing the 20 common amino acids in both positions revealed hydrogen–$Me_3Si$ exchange with the following functional groups: amino, carboxyl, hydroxyl, imidazole NH, indole NH, primary amide, and guanido. One proton of the α-amino and primary amido groups, both protons on the ε-amino groups of lysine, the δ-amino group of ornithine, the α-amino group of glycine, and three protons of the guanido group were replaced (see Chart

CHART I



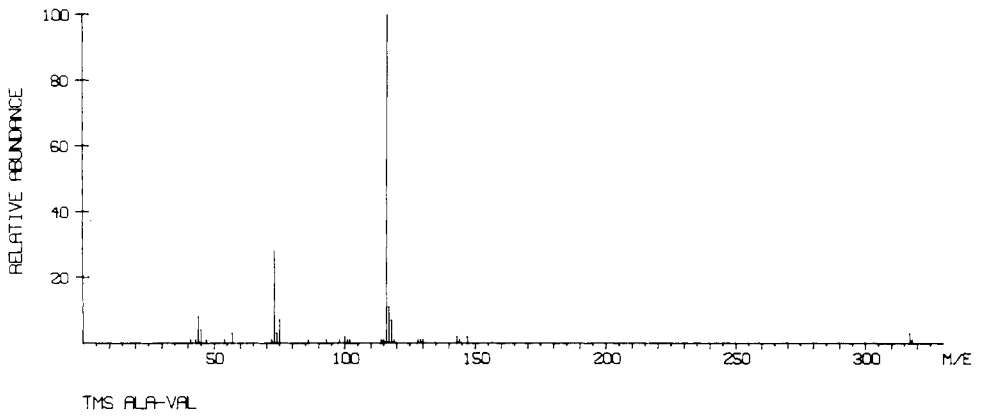



FIGURE 3: Mass spectrum of $Me_3Si$-Ala-Val; ion at *m/e* 73 is $[Me_3Si]^+$, ion at *m/e* 116 is $[Me_3Si{-}NH{=}CHCH_3]^+$ ($\beta$ cleavage), and ion at *m/e* 317 is $[M - 15]^+$.

TABLE III: Ions Used to Identify $Me_3Si$ Dipeptides.

| amino acid | mass of $\beta$-cleavage ion when amino acid is amino-terminal | mass to be added to $\beta$-cleavage ion when amino acid is carboxy-terminal[b] | other significant ions |
|---|---|---|---|
| Gly[c] | 174 | 159 | |
| Ala | 116 | 173 | |
| Aba[a] | 130 | 187 | |
| Pro | 142 | 199 | |
| Val | 144 | 201 | |
| Leu | 158 | 215 | 43 > 41 |
| Ile | 158 | 215 | 43 ≤ 41 |
| Met | 176 | 233 | |
| Phe[d] | 192 | 249 | 91 |
| Ser | 204 | 261 | |
| Thr | 218 | 275 | |
| Asn | 231 | 288 | |
| Asp | none[e] | 289 | |
| Gln[f] | 245 | 302 | |
| Glu | 246 | 303 | |
| His | 254 | 311 | 154 |
| $CySCH_2CONH_2$ | 277 | 334 | |
| $CySCH_2CO_2H$ | 278 | 335 | |
| Tyr[d] | 280 | 337 | 179 |
| Orn[g] | 303 | 360 | |
| Trp[d,g] | 303 | 360 | 202 |
| Lys[g] | 317 | 374 | |
| Arg[g,h] | 142,[c] 417 (s) | 474 | |
| $CyS(CH_2)_2NH_2$ | 345 | 392 | |

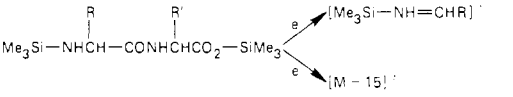
[a] Aba, $\alpha$-L-aminobutyric acid. [b] These masses are calculated and not observed in the spectrum. They are used to calculate the mass of $[M - 15]^+$. [c] Diketopiperazines formed with Gly-Ala, Gly-Arg, Gly-Gln, Gly-Glu, Gly-Gly, Gly-Met, and Gly-Lys. [d] Values may be 72 amu lower when an old GC column is used. [e] Amino-terminal Asp cyclizes to the five-membered imide. Add 142 to the carboxy-terminal value to calculate mass of $[M - 15]^+$. [f] Values may be 89 amu lower when an old GC column is used. [g] Exhibits $M^+$ as well as $[M - 15]^+$. [h] Add 417 to carboxy-terminal value to calculate mass of $[M - 15]^+$.

I). The hydrogen of the NH group of the peptide bond is not replaced except under forcing conditions with sterically unhindered dipeptides such as Ala-Ala.

*Typical Fragmentations of $Me_3Si$ Dipeptides.* Significant ions representative of each amino acid when it is amino and carboxy-terminal are given in Table III. The trimethylsilylated dipeptides that have been examined to obtain these data and the data cited above are listed in Table IV.

In general, the molecular ion of $Me_3Si$ dipeptides is absent from their mass spectra. A silyl methyl group (15 amu) is lost on electron impact, and molecular weight is calculated from $[M - 15]^+$. This ion is present, with varying intensity, in the mass spectra of all $Me_3Si$ dipeptides. There are, however, several cases where both $[M - 15]^+$ and $M^+$ are detected. The molecular ion is seen with trimethylsilylated dipeptides which contain Arg, Lys, Orn, or Trp in either the amino or carboxy position and with all Asp-X and certain Gly-X dipeptides which form cyclic imides and diketopiperazines, respectively, during trimethylsilylation (see below). Since $M^+$ is always accompanied by $[M - 15]^+$, no confusion arises in deciding whether a given peak is $M^+$ or $[M - 15]^+$.

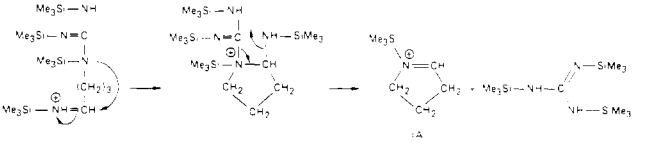
The sequence of the dipeptide is established by the ion generated by $\beta$-cleavage of the central CH-CO bond:




The charge is retained on the amino-terminal fragment and this ion is usually the most abundant ion in the spectrum, as shown in the spectrum for $Me_3Si$-Ala-Val (Figure 3). The $\beta$-cleavage ion is at *m/e* 116 and $[M - 15]^+$ at *m/e* 317, the highest mass ion in the spectrum.

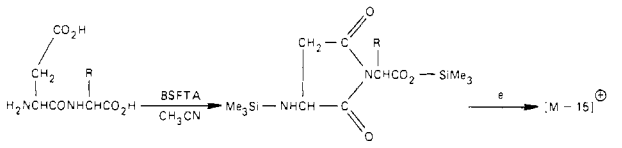
Dipeptides containing Leu and Ile in either the amino or carboxyl terminal position are distinguished by comparing the heights of the ions at *m/e* 41 and 43. When Ile is present, the fragment at *m/e* 41 is larger.

*Exceptions to the Typical Fragmentations.* (1) Amino-Terminal Arginine. Dipeptides containing amino-terminal Arg lose the $(Me_3)_3Si$-guanido group from the $\beta$-cleavage ion, yielding instead the cyclic ion A. The masses of $M^+$ and $[M - 15]^+$ are not similarly affected:



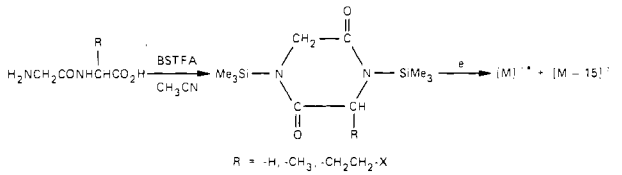

When the guanido group is converted to the aminodimethylpyrimidine moiety with acetylacetone, trimethylsilylamine is lost instead. Dipeptides containing amino-terminal Orn also exhibit a significant ion (A) in addition to the expected $\beta$-cleavage ion.

(2) Amino-Terminal Aspartic Acid. Dipeptides containing amino-terminal Asp formed cyclic imides during synthesis of the $Me_3Si$ derivative and, of course, the $\beta$-cleavage ion is absent:



The mass spectra of $Me_3Si$-Asp-Ala (Figure 4) illustrate this phenomena. Note the intense $[M - 15]^+$ at *m/e* 315.

(3) Amino-Terminal Glycine. Certain dipeptides containing amino-terminal Gly (and Pro-Pro) form diketopiperazines during trimethylsilylation, and, again, the $\beta$-cleavage ion is not seen. Tests of 19 of the 20 common Gly-X dipeptides (Gly-CySR not tested) revealed that cyclization only occurs when X is $\alpha$-Aba, Ala, Arg, Gln, Glu, Gly, Met, or Lys. Both protons on the ring amide nitrogens are exchanged:



This can be seen in the spectra of $Me_3Si$-Gly-Gly ($M^+$ at *m/e* 258, $[M - 15]^+$ at *m/e* 243, Figure 5). The high intensities of $M^+$ and $[M - 15]^+$ are characteristic for diketopiperazines.

(4) Amino-Terminal Glutamine. $Me_3Si$-Gln-X dipeptides sometimes cyclize during chromatography to the pyroglutamyl derivative. When this occurs, both $[M - 15]^+$ and the $\beta$-cleavage ion are reduced by 89 amu (loss of $Me_3Si$-$NH_2$).

(5) Amino-Terminal Phenylalanine, Tyrosine, and Tryptophan. The $\alpha$-amino $Me_3Si$ group of dipeptides containing amino- or carboxy-terminal Phe, Tyr, or Trp may be replaced by a proton during chromatography, especially when old GC columns are used. As with amino-terminal Gln, this is a variable occurrence, and the relative labilities are Trp > Tyr > Phe. When this happens, both the $\beta$-cleavage ion and $[M - 15]^+$ (and $M^+$ with Trp) are reduced by 72 amu.

(6) Other Significant Ions. Trimethylsilylated dipeptides containing His, Phe, Trp, or Tyr also give ions corresponding to $[ArCH_2]^+$. When these residues are at the amino-terminal position, with the exception of His, a rather intense ion corresponding to $(M - ArCH_2)^+$ is also observed. In an analogous fashion, dipeptides with amino terminal CySR, Ser, and Thr exhibit a rather intense ion resulting from loss of $RSCH_2\cdot$, $Me_3Si$-$OCH_2\cdot$, and $Me_3Si$-$OCH(CH_3)\cdot$, respectively, from their molecular ions. In the spectrum of $Me_3Si$-Phe-Ala (Figure 6), the peak at *m/e* 289, which is absent in that of Ala-Phe, is due to $[M - C_6H_5CH_2]^+$; the peak at *m/e* 91 is $C_6H_5CH_2^+$; the $\beta$-cleavage ion is at *m/e* 192 and $[M - 15]^+$ at *m/e* 365.

TABLE IV: Dipeptides Identified.

| | | | | |
|---|---|---|---|---|
| Aba-Arg[a-c] | CM | His-Ala | Orn-Ala | Thr-Ala |
| Gly[b] | CyS-Asn | Asp[b] | Asp | Asp[b] |
| His[b] | CM | Gln | Leu | AE |
| Orn[b] | CyS[b] | Leu[b] | Tyr | CyS[b] |
| Phe[b] | Ser[b] | Lys[a] | | His[b] |
| Ser[b] | Thr[b] | Phe[a] | Phe-Ala | Leu[b] |
| Trp[b] | | Ser[b] | Arg[a,b] | Lys[b] |
| | Gln-Ala | Tyr[b] | Asn | Met[b] |
| Ala-Ala | Asn | | Asp[b] | Phe[b] |
| Arg[a] | CM | Ile-Ala | Gln | Ser[b] |
| Asn | CyS[b] | Arg[a] | Gly[b] | Thr[b] |
| Asp | Gln | Asp[b] | Ile[b] | Tyr[b] |
| Gln | Gly | CM | Leu[b] | Val[c] |
| Glu | His[b] | CyS[b] | Orn | |
| Gly | Ile[b] | Gly[b] | Phe | Trp-Gly[b] |
| His | Leu[b] | Ile | Pro | Gln[a] |
| Ile | Phe | Leu | Ser[b] | Leu[b] |
| Leu | | Orn | Thr[b] | Met[b] |
| Lys | Glu-Ala | Ser[b] | Tyr[b] | Trp |
| Met | Arg[a,b] | Val[b] | Val[b] | |
| Orn | Asn[b] | | | Tyr-Ala |
| Phe | Asp[b] | Leu-Ala | Pro-Ala | AE |
| Pro | Gln[b] | Gln[b] | Gly | CyS[b] |
| Ser | Glu | Glu[b] | Phe | CM |
| Thr | His[b] | Ile | Pro | CyS[b] |
| Trp | Lys | Leu[b] | | Gln[b] |
| Tyr | Orn | Met[b] | Ser-Ala | Glu[b] |
| Val | | Pro[b] | Arg[a,b] | Leu[b] |
| | Gly-Aba[c] | Thr[b] | Asn[b] | Ser[b] |
| Asn-Arg[a,b] | Ala | Tyr | Gln[b] | Thr[b] |
| Asn[b] | Arg[a] | Val[b] | Gly[b] | Tyr[b] |
| Gln[b] | Asn | | His[b] | |
| Phe[b] | Asp | Lys-Ala | Ile[b] | Val-Ala |
| Thr[b] | Gln | Asp | Leu[b] | Asn[b] |
| Tyr[b] | Glu | Lys | Met[b] | Gln[b] |
| Val[b] | Gly | Phe[b] | Phe[b] | Glu[b] |
| | His | Pro[b] | Pro[b] | Gly[b] |
| Asp-Ala | Ile[b] | | Ser[b] | Lys[b] |
| Arg[a,b] | Leu[b] | Met-Ala | Thr[b] | Ser[b] |
| Glu[b] | Lys[b] | Asn[b] | Tyr[b] | Tyr |
| Ile[b] | Met | Asp[b] | Val[b] | |
| Lys[b] | Orn | Glu[b] | | |
| Orn[b] | Phe | Lys | | |
| Phe[b] | Pro[b] | Ser[b] | | |
| Ser[b] | Ser[b] | | | |
| Tyr[b] | Thr[b] | | | |
| Val[b] | Trp | | | |
| | Tyr | | | |
| | Val[b] | | | |

[a] Sample introduced via the probe instead of through the GC column. [b] Dipeptide was obtained from dipeptidyl aminopeptidase digestion of polypeptide of known sequence. [c] Aba, $\alpha$-L-aminobutyric acid.

## Discussion

The GC analysis of dipeptides was undertaken to facilitate their identification by mass spectrometry (MS) since identification of the 400 possible dipeptides could not be made with GC alone. The major purpose of GC is to separate reagents, by-products, and other components in the mixture which could complicate the MS analysis. Resolution of mixtures of dipeptide derivatives is not necessary because it is possible to identify mixtures of dipeptides in GC peaks.

Several volatile derivatives of dipeptides have been employed for GC–MS analysis, but they do not appear to be as suitable as the $Me_3Si$ derivatives. Preparation of the *N*-perfluoroacylamide methyl esters (Weygand et al., 1960; Ovchinikov & Kiryushkin, 1972; Caprioli et al., 1973; Caprioli & Seifert, 1975; Young & Desiderio, 1976) involves several manipulations and about 2 h of time. Unfortunately, identification of Asn and Gln is not always possible. The *N*-acetylacetonyl (Schier et al., 1974; Frank et al., 1977) and *N*-ethylacetoacetatyl methyl esters (Schier et al., 1976) are almost as cumbersome to prepare and their GC characteristics have not been fully explored. Reduction of the dipeptides with $LiAlH_4$ followed by trimethylsilyation (Kelley et al., 1975; Nau et al., 1975) offers no advantage over the other derivative and appears to require the largest amount of sample. The single step trimethylsilylation of the native dipeptide is most attractive because $Me_3Si$ derivatives are prepared in high yield in 10 min. Furthermore, they have excellent GC–MS properties.

The trimethylsilyl proton exchanges observed by reacting dipeptides with BSTFA in acetonitrile are similar to those previously reported for amino acids (Gehrke et al., 1969) and

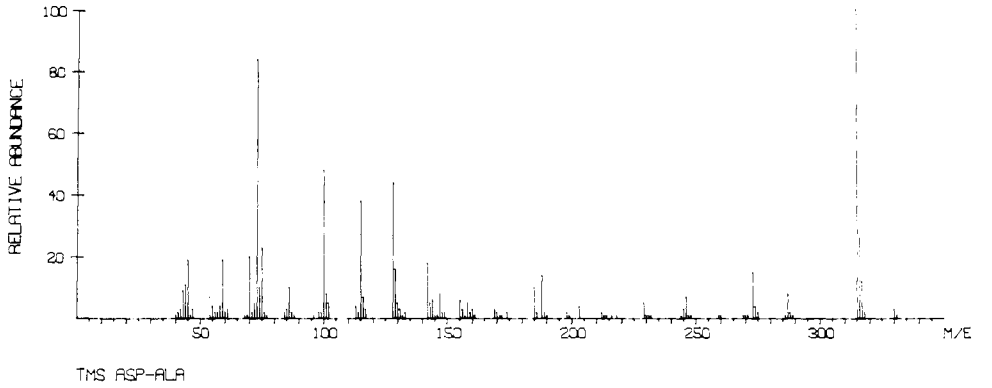



FIGURE 4: Mass spectrum of $Me_3Si$-Asp-Ala; ion at *m/e* 315 is $[M - 15]^+$ for cyclic imide.

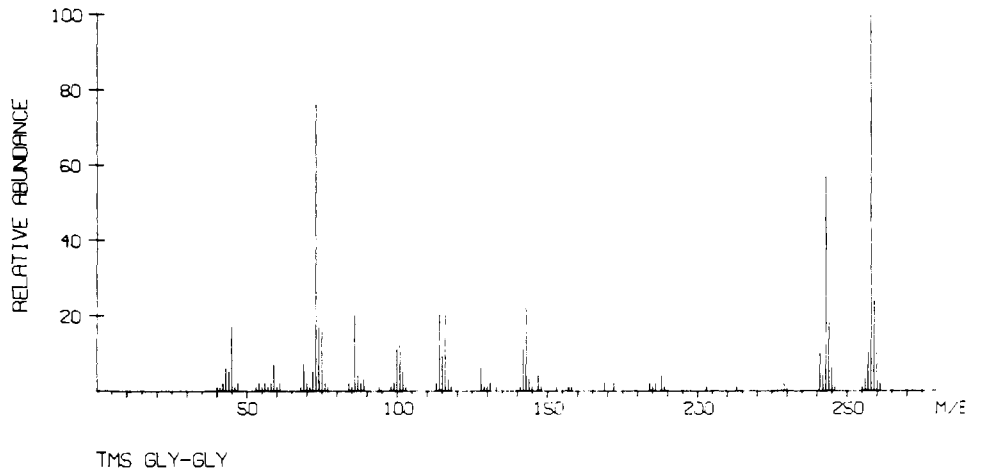



FIGURE 5: Mass spectrum of $Me_3Si$-Gly-Gly diketopiperazine; ion at *m/e* 243 is $[M - 15]^+$ for diketopiperazine, and ion at *m/e* 258 is $M^+$.

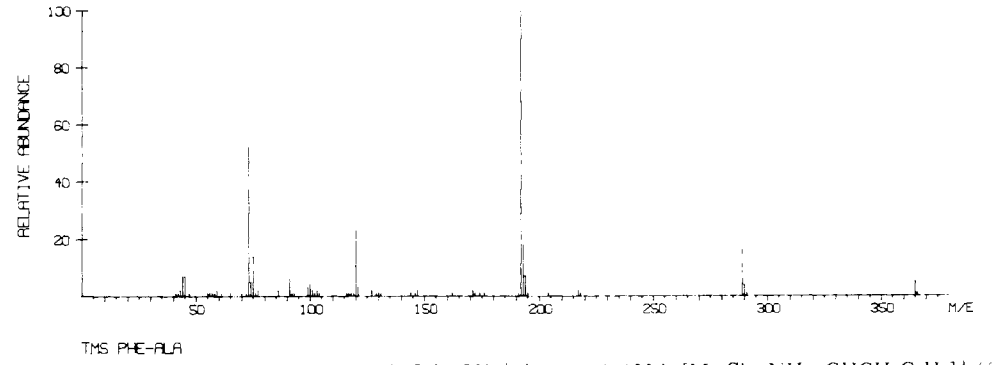



FIGURE 6: Mass spectrum for $Me_3Si$-Phe-Ala; ion at *m/e* 91 is $C_6H_5CH_2^+$, ion at *m/e* 192 is $[Me_3Si{-}NH{=}CHCH_2C_6H_5]^+$ ($\beta$ cleavage), ion at *m/e* 289 is $[M - C_6H_5CH_2]^+$, and ion at *m/e* 365 is $[M - 15]^+$.

dipeptides reacted with diethylaminotrimethylsilane (Ruhlman et al., 1966). In some cases trimethylsilylations are governed by steric factors. For example, both protons of the amino group of glycine (and the $\epsilon$-amino group of lysine) are exchanged but only one of the protons is exchanged in Ala-X and other dipeptides. Also, Ala-X dipeptides do not form the diketopiperazines observed with the corresponding Gly-X dipeptides when the $\beta$ position of the side chain of X is either a methyl or methylene group (ornithine excepted). In contrast, all Asp-X dipeptides tested formed the five-membered cyclic imide. Asn-X, Glu-X, and Gln-X dipeptides do not cyclize.

Columns used for the analysis of standard $Me_3Si$ dipeptides last several months but may begin to deteriorate in a few weeks when used daily for the analysis of enzymatic digests. Column performance is best determined with the least volatile and most polar derivatives. A significant reduction in the peak heights of $Me_3Si$ dipeptides containing Asn, Gln, and His is usually a sign of a failing column which should be replaced. We have not as yet prepared a column which gave the same peak heights for polar and nonpolar derivatives compared at the 1-nmol level. However, a low response for the polar derivatives (presumably due to adsorption) usually is not a serious problem until samples smaller than 1 nmol are analyzed. The limit of their detection is about 0.1 nmol (Table II). Encouraging preliminary results have been obtained recently with LKB bonded phase capillary columns. The new columns are at-

tractive for dipeptide analysis because they offer the potential for less adsorption (due to the absence of solid support), better resolution, and greater sensitivity.

The data gathered from the mass spectra of approximately 200 dipeptides (Table IV), and summarized in Table III, should permit recognition of all 400 possible dipeptides. The loss of $CH_3$ by $Me_3Si$ dipeptides on electron impact to form $[M - 15]^+$ is typical of trimethylsilylated compounds (Beynon et al., 1968). The fact that some trimethylsilylated compounds also exhibit $M^+$ can probably be attributed to stabilization of the molecular ion by electron donation (Arg, Lys, Orn, Trp) or by ring formation (diketopiperazines or cyclic imides). The $\beta$ cleavage of the central CH–CO bond, which yields the ion used to determine the dipeptide sequence, has also been reported for other dipeptide derivatives. The relative intensity of the peak from this ion varied between derivatives and was less for *N*-perfluoroacylamide methyl ester derivatives (Stenhagen, 1961; Ovchinikov & Kiryushkin, 1972; Caprioli et al., 1973; Caprioli & Seifert, 1975; Young & Desiderio, 1976), and greater for either the trimethylsilyl derivative reported here or the *N*-ethylacetoacetatyl (Schier et al., 1974, 1976) and *N*-acetylacetonyl (Frank et al., 1977) methyl ester derivatives. The differences in relative intensity are probably due to the differences in electron-withdrawing power exerted on the $\alpha$-amino group. The $\beta$-cleavage ion of reduced mass that occurs with $Me_3Si$ dipeptides containing amino-terminal Arg appears to be a special case in this series of compounds. This loss is probably facilitated by internal assistance from the $\alpha$-$Me_3Si$ amino group via formation of a five-membered ring and by the stable departing $Me_3Si$ guanidine moiety.

The $[ArCH_2]^+$ species that appear in mass spectra of dipeptides containing His, Phe, Trp, and Tyr also are observed in the mass spectra of other compounds containing these functional groups (Beynon et al., 1968). Although not usually necessary for identification of dipeptides, the presence of one of these ions helps confirm it. However, distinguishing between Orn and Trp is one case where this type of ion is necessary. Because both residues fortuitously have the same mass after trimethylsilylation, the presence or absence of an ion at *m/e* 202 for $[ArCH_2]^+$ of Trp allows a choice to be made. Trimethylsilylated dipeptides containing Phe, Trp, or Tyr, as well as CySR, Ser, or Thr exhibited abundant ions of the type $[Me_3SiNH{=}CHCONHCHRCO_2SiMe_3]^+$. The lack of a similar side chain loss upon electron impact when these residues were carboxy terminal may be due to a greater degree of stabilization of the positively charged $\alpha$-nitrogen atom imparted by attachment of $Me_3Si$ instead of an $\alpha$-aminoacyl group. The relative selectivity of this phenomenon reflects the need for an $\alpha$-stabilizing group on the $R–CH_2$ fragment that is split off, such as an aromatic ring (Phe, Trp, Tyr) or a sulfur or oxygen atom (CySR, Ser, Thr). These types of ions, when they occur, help confirm both the presence and position of these residues in the dipeptide that were deduced first from the value of $[M - 15]^+$ and the $\beta$-cleavage ion. The GC–MS analysis of dipeptides generated from the digestion of polypeptides with DAPs will be presented in a forthcoming communication.

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