Decomposition Products of L-Aspartyl-L-phenylalanine Methyl Ester and Their Identification by Gas–Liquid Chromatography

Samples of L-aspartyl-L-phenylalanine methyl ester (APM) and APM hydrochloride were stored in aqueous solutions and their degradation products were identified by glc and mass spectroscopy as their trimethylsilyl derivatives. Although both compounds furnished the same decomposition pattern, their degree of decomposition differed considerably. It is believed that the low pH of the APM hydrochloride solution is the primary reason for its instability.

It has been shown that L-aspartyl-L-phenylalanine methyl ester (APM), a new dipeptide sweetener, has a limited stability in aqueous systems (Mazur and Craig, 1971; Mazur and Beck, 1973). These authors claim that APM solutions are most stable in the narrow pH range of 3–5 and at low temperature. As the temperature and pH increase (pH >5), the stability of APM decreases. It is believed that the major product of the decomposition of APM is 3-carboxymethyl-6-benzyl-2,5-diketopiperazine. This compound is a product of APM demethoxylation and cyclization and is formed primarily at neutral and basic conditions. Since 3-carboxymethyl-6-benzyl-2,5-diketopiperazine is not sweet, the result of this conversion is a loss of sweetness.

Because APM has limited solubility in aqueous solutions, APM salts with greater solubility were recently prepared (Haas and Berg, 1973a,b). One salt, APM hydrochloride, dissolves about eight times faster and forms more concentrated solutions than unmodified APM. Our objective was to determine the relative stabilities of the APM in solution as compared to its salt, and experiments were undertaken to identify the degradation products.

EXPERIMENTAL SECTION

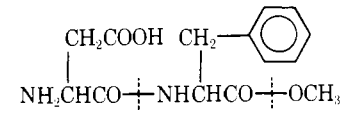
Materials and Methods. L-Aspartyl-L-phenylalanine methyl ester was a product from Ajinomoto Co., Japan (Lot =56637). Its purity was confirmed by tlc. L-Aspartyl-L-phenylalanine was from Mann Research Lab., 3-carboxymethyl-6-benzyl-2,5-diketopiperazine was obtained from G. D. Searle & Co., L-aspartic acid and L-phenylalanine were from California Foundation for Biochemical Research, and L-phenylalanine methyl ester hydrochloride was from Aldrich Chemical Co., Inc.

All samples were dried before derivatization and then trimethylsilylated with BSA-*N,O*-bis(trimethylsilyl)acetamide from Pierce Chemical Corp. A 60% solution of BSA in DMF was used for derivatization (Klebe *et al.*, 1966). The derivatization was achieved by heating the samples at 70° for 2.5 hr. The trimethylsilyl ethers were then analyzed by gas–liquid chromatography (glc) using a Perkin-Elmer Model 900 gas chromatograph with dual column flame ionization detectors. The column used was a 6 ft × ⅛ in. stainless steel, packed with 15% OV-101 on 80–100 mesh supelcoport. The injection port and manifold temperatures were 230°. The initial column temperature was 90°. It was held at this temperature for 16 min and then programmed at 6°/min to a final temperature of 215°. The final hold was 20 min.

Mass spectra were obtained using a Perkin-Elmer Model 990 gas chromatograph interfaced through a glass jet enricher to a Dupont 21-491 mass spectrometer. Spectra were obtained at 70 eV, a source temperature of 200°, and scan speeds of 4 sec/decade.

RESULTS AND DISCUSSION

We have observed that APM (1), and particularly its hydrochloride, partially decompose when stored in water for a prolonged time at an elevated temperature. Besides 3-carboxymethyl-6-benzyl-2,5-diketopiperazine (2) and L-aspartyl-L-phenylalanine, L-aspartic acid, L-phenylalanine, and L-phenylalanine methyl ester were also produced.



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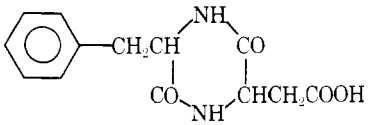


2

L-Aspartic acid and L-phenylalanine methyl ester are the decomposition products resulting in the splitting of APM's peptide linkage. L-Phenylalanine is formed by demethoxylation of L-phenylalanine methyl ester.

L-Aspartyl-L-phenylalanine (AP) is the product of APM's demethoxylation and 3-carboxymethyl-6-benzyl-2,5-diketopiperazine is the product of APM's cyclization with the release of methanol.

These products can be separated by tlc (Brenner and Niederwieser, 1960) which usually requires the use of two different spraying agents for visualization (ninhydrin and sulfuric acid). We were also able to separate all of them by glc as their TMS derivatives.

Figure 1 shows the separation of a standard mixture containing APM and its five degradation products in equal weight ratios.

Both amino acids (L-aspartic acid and L-phenylalanine), L-phenylalanine methyl ester, and 3-carboxymethyl-6-benzyl-2,5-diketopiperazine displayed single peaks corresponding to their trimethylsilyl derivatives (peaks 1, 2, 3, and 8).

Each dipeptide—APM and its demethoxylated form L-aspartyl-L-phenylalanine (AP)—furnished one major and one minor peak. The presence of two or more peaks for TMS derivatives of different amino acids, such as ω-amino acids, α,ω-diamino acids, or asparagine, has been reported (Bergström and Gürtler, 1971; Gehrke and Leimer, 1971).

Based on mass spectral analysis, the second peak of APM (peak 6), which is the major peak, was designated the dehydrated mono-TMS derivative of APM. The spectrum of this peak had a molecular ion at m/e 348 which corresponds to the mono-TMS derivative of APM without 1 mol of water. Because the reaction for the primary amino group (ninhydrin) was negative, it was reasoned that the TMS group was attached to the amino group of APM and the free carboxyl could then participate in dehydration (probably with the hydrogen of the peptide linkage). The intense ions at m/e 333 (mol wt − CH_3) and m/e 73 and 75 showed the presence of the trimethylsilyl derivative.

The mass spectrum of the first peak of APM (peak 4), which is the minor peak, contained a molecular ion at

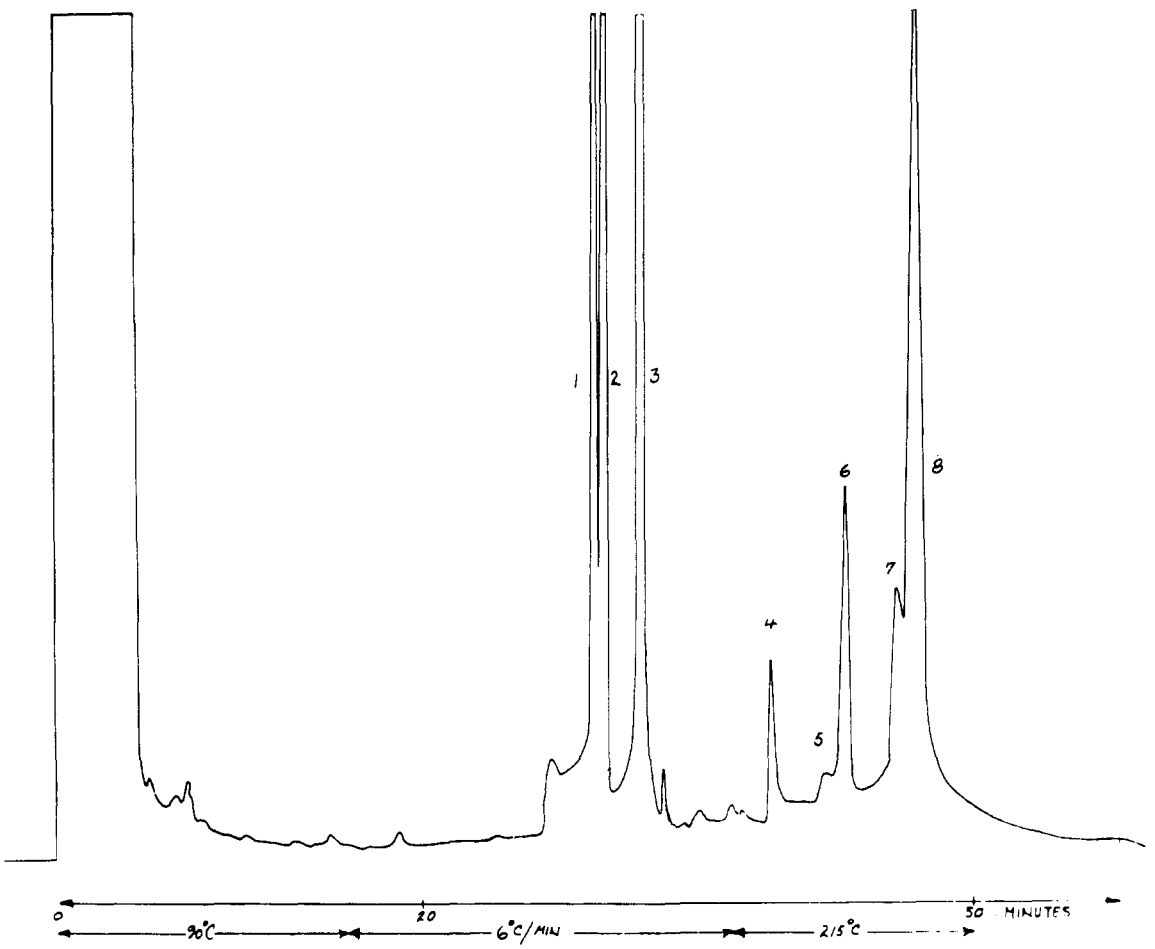


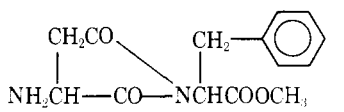
Figure 1. Separation of the standard mixture of L-phenylalanine methyl ester (peak 1), L-aspartic acid (2), L-phenylalanine (3), L-aspartyl-L-phenylalanine methyl ester (4, 6), L-aspartyl-L-phenylalanine (5, 7), and 3-carboxymethyl-6-benzyl-2,5-diketopiperazine (8) in equal weight ratios.

m/e 276; this corresponds to the dehydrated form of APM. The spectrum did not indicate the presence of a TMS group.

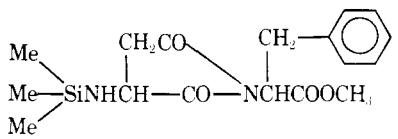
The first peak of L-aspartyl-L-phenylalanine (AP) (peak 5) is a very small peak and it is hardly noticeable on all chromatograms. Its mass spectrum showed the strong ions at *m/e* 334, 221, 220, 75, and 73. This suggests that we are dealing here with a dehydrated form of the mono-TMS derivative of AP.

The major peak of AP (peak 7) displayed ions at *m/e* 406, 221, 220, 75, and 73 which indicates the presence of a dehydrated form of the di-TMS derivative of AP. The presence of ions at *m/e* 205, 220, and 221 indicated the cleavage of the CH–NH bond.

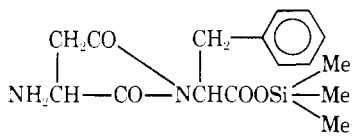
The possible structures of peaks 4, 5, 6, and 7 are shown.



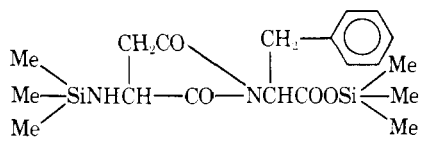
peak 4



peak 6



peak 5



peak 7

It is speculated that the TMS derivatives of APM and AP dehydrate during the high-temperature glc separation and a small amount of them hydrolyze (loss of TMS group). The products of the hydrolysis are a dehydrated form of APM and a dehydrated mono-TMS derivative of AP represented on the chromatogram by minor peaks 4 and 5.

Figure 2 shows the analysis of APM hydrochloride solution. The sample of APM hydrochloride was prepared by neutralization of the free amino group of APM with the stoichiometric amount of 0.1 *N* hydrochloric acid and subsequent freeze drying of the solution. A 1% solution of this compound in deionized water was prepared and stored in a closed erlenmeyer flask at 37° for 2 months. The pH of the solution was 2.2. The separation pattern on the chromatogram demonstrates considerable decomposition of APM and the formation of all expected degradation products. The presence of L-phenylalanine methyl ester was confirmed by mass spectroscopy. The mass spectrum of this compound contained ions at *m/e* 236 (mol wt – CH_3), 160, 192, 161, 89, 91, 208, 100, 130, 105, 145, 176,

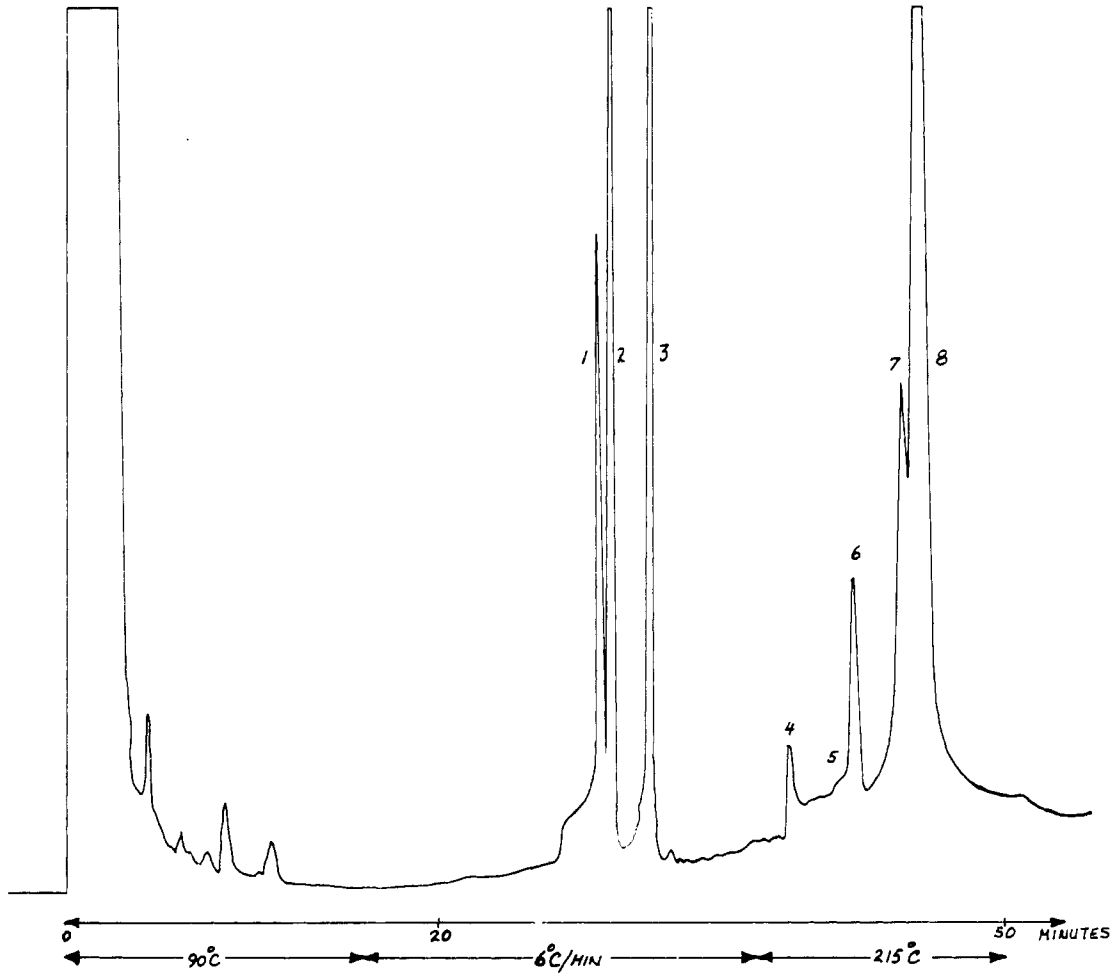


Figure 2. Analysis of APM hydrochloride solution stored at 37° for 2 months; pH 2.2.

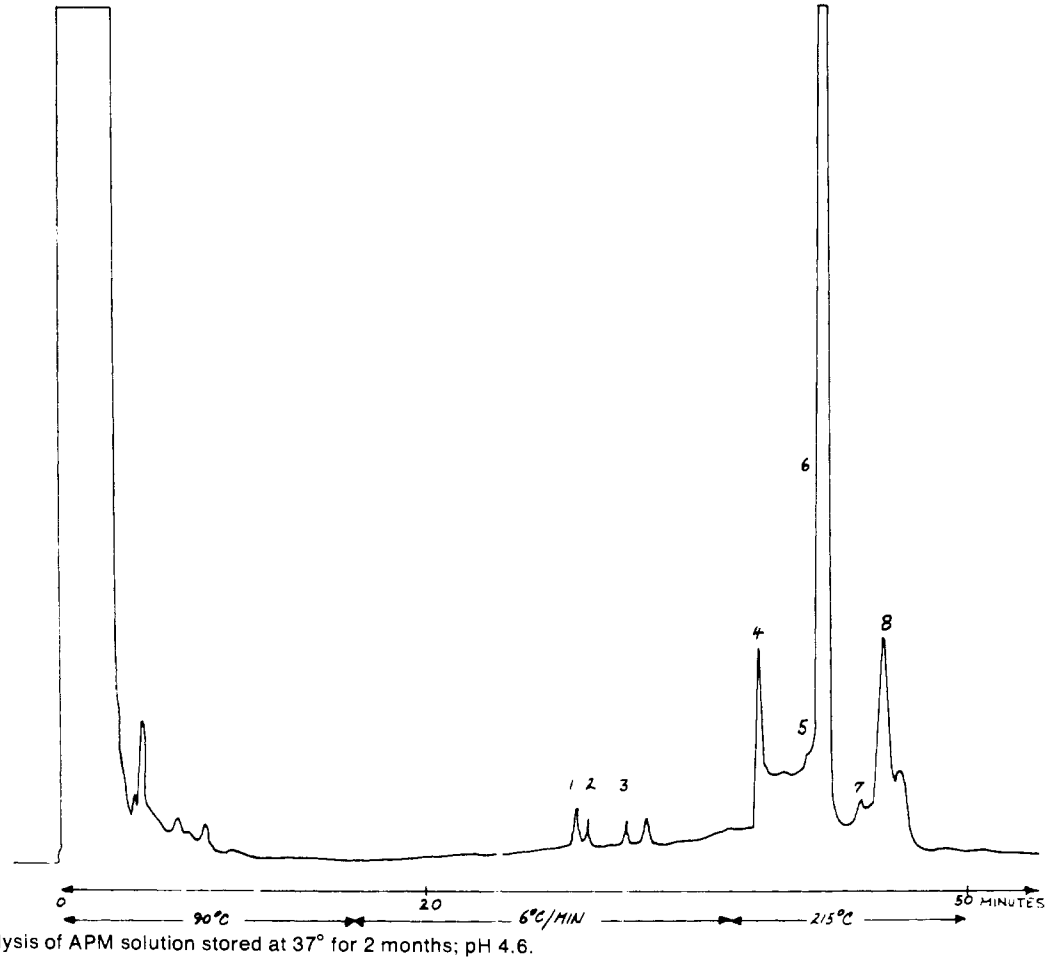


Figure 3. Analysis of APM solution stored at 37° for 2 months; pH 4.6.

and 120 which matched with the mass spectrum of the L-phenylalanine methyl ester standard.

Figure 3 shows the decomposition pattern of APM stored at the same conditions as the APM hydrochloride. The pH of the 1% solution of APM was 4.6. Though APM was degraded to a much lower degree than APM hydrochloride, the presence of the same degradation products was detected.

The glc analysis of APM and its degradation products may be a useful tool for the investigation of APM stability in different systems.

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Germacrene D in Douglas Fir Young Needles

A sesquiterpene hydrocarbon which exhibits a transitory existence in the young needles of Douglas fir, *Pseudotsuga menziesii* (Mirb.) Franco, has been identified by spectroscopic and gas chromatographic techniques as germacrene D.

In an earlier investigation of the changes in composition of volatile terpenes in Douglas fir needles during maturation (Maarse and Kepner, 1970) we observed the transitory existence of a sesquiterpene hydrocarbon (peak 25) in relatively large amounts in the young needles. This sesquiterpene reached a maximum concentration approximately 10 days after emergence of the new growth in the spring and then rapidly decreased in concentration as the needles matured. We also isolated the same component from the new needle growth of Douglas fir seedlings grown in the Aboretum of Schovenhorst at Putten, The Netherlands. This communication presents evidence identifying this sesquiterpene hydrocarbon as germacrene D.

EXPERIMENTAL SECTION

Material. Approximately 2 kg of Douglas fir new growth (tips about 2 cm in length) was collected from young Douglas fir trees located in the Pacific Coast Range west of Ukiah, Calif. The new tips were macerated in a blender and steam distilled at atmospheric pressure, the distillate extracted with *n*-pentane, and the pentane removed on a rotary evaporator at 0° to give 6.5 ml of oil. The oil was stored in a brown bottle at 0° under nitrogen until used for glc separations.

Gas Chromatography. Isolation of the sesquiterpene hydrocarbon was by preparative glc on a 10 ft × 0.16 in. i.d. glass column packed with 5% Carbowax 20M on 60–70 mesh Chromosorb G DMCS in a Hewlett Packard 7620A chromatograph with FID and a 15:1 exit splitter; oven temperature isothermal at 130° for 15 min, programmed at 2°/min to 160° then at 30°/min to 200°; injection port and detector 200°; He flow 60 ml/min. Final purification was by rechromatography on the above column and on a 10 ft × 0.16 in. i.d. glass column packed with 5% SE 30 + Igepal C0880 (5% w/w) on 60–70 mesh Chromosorb G DMCS. Analytical separations and Kovats Index determinations were on 500 ft × 0.03 in. i.d. stainless steel columns coated with Carbowax 20M + Igepal C0880 (5% w/w) or SF 96(50) + Igepal C0880 (5% w/w) in an F&M Model 810 gas chromatograph with FID.

Instrumental Analyses. Infrared analyses were taken between sodium chloride plates in a Beckman IR8 spectrophotometer using a beam condensor. Mass spectra were determined on a Consolidated Electrodynamics Corporation Model 201 spectrograph or on a Varian Mat CH4 mass spectrometer. Ultraviolet analyses were carried out on a Cary 14 Ultraviolet and Visible spectrometer in spectroquality *n*-hexane. Irradiation of the sesquiterpene hydrocarbon was carried out for 2 hr in a quartz nmr tube using spectroquality *n*-hexane as solvent with a Hanovia 350-W medium pressure mercury-arc lamp.

RESULTS AND DISCUSSION

The germacrene D (peak 25; Maarse and Kepner, 1970) isolated in this study gave infrared and mass spectra and Kovats' indices completely consistent with the data of Maarse and van Os (1973) and with the infrared and ultraviolet spectral data of Yoshihara *et al.* (1969) for germacrene D. Photoisomerization of germacrene D from Douglas fir gave mainly β-bourbonene (Yoshihara *et al.*, 1969), identified by its infrared spectrum (Wenninger *et al.*, 1967) and Kovats' indices (Maarse and van Os, 1973), and trace amounts of β-copaene and β-ylangene (Kovats' indices 1626 and 1609 on Carbowax 20M, and 1445 and 1433 on SF 96(50), respectively).

Yoshihara *et al.* (1969) reported the hydrogenation of germacrene D to give germacrane, identified by the infrared spectrum. Catalytic hydrogenation of the germacrene D from Douglas fir oil using platinum oxide (Adam's catalyst) at atmospheric pressure and 0° gave a complex mixture of products which separated into 20 peaks on SF 96(50). No evidence could be observed from mass spectra determination for any of the germacrane isomers in the products. Hydrogenation of germacrene D from Douglas fir oil in the gas chromatograph injection port over Pd at 190° (Kepner and Maarse, 1972; Maarse, 1974) gave a mixture of products (eight peaks on an SF 96(50) capillary column) identical with the products obtained by similar hydrogenation of germacrene D isolated from Origanum oil. Mass spectral evidence (Maarse, 1974)