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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF ASPARTAME DIASTEREOMERIC DECOMPOSITION PRODUCTS

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SUMMARY

The diastereomeric dipeptides and diketopiperazines generated from the decomposition and epimerization of L-aspartyl-L-phenylalanine methyl ester (aspartame) have been separated by reversed-phase high-performance liquid chromatography using UV detection (215 nm). Six diastereomeric products are identified and a scheme is proposed for their formation.

INTRODUCTION

The separation and quantification of diastereomeric dipeptides and their cyclic (diketopiperazine) analogues has wide application in many fields of chemistry. A few examples include: biochemical mechanisms for peptide uptake and utilization[1]; kinetic analysis of amino acid racemization in peptides isolated from fossils[2,3]; biogeochemical studies of peptide decomposition[4]; and theoretical studies of peptide conformation[5,6].

We chose L-α-aspartyl-L-phenylalanine methyl ester as an inexpensive, easily obtainable compound for the study of diketopiperazine formation, racemization, and hydrolysis in dipeptides. In aqueous solution, aspartame undergoes ester hydrolysis and cyclization producing the corresponding dipeptide and diketopiperazine (DKP)[7–9]. The rapid rate of epimerization generally observed in DKPs[10–12] implies that various diastereomeric compounds may be amongst the decomposition products[8]. Fig. 1 gives a proposed decomposition scheme showing the various possible products.

Since its discovery as a sugar substitute in 1969, various methods for monitoring the concentrations of aspartame and some of its decomposition products have been developed. These have utilized gas chromatography[7] and high-performance liquid chromatography (HPLC)[9,13,14] but none of these investigations has detected and recognized the full array of diastereomeric products which could result from aspartame decomposition and racemization. This paper describes a simple HPLC separation of aspartame and its various diastereomeric decomposition products, de-

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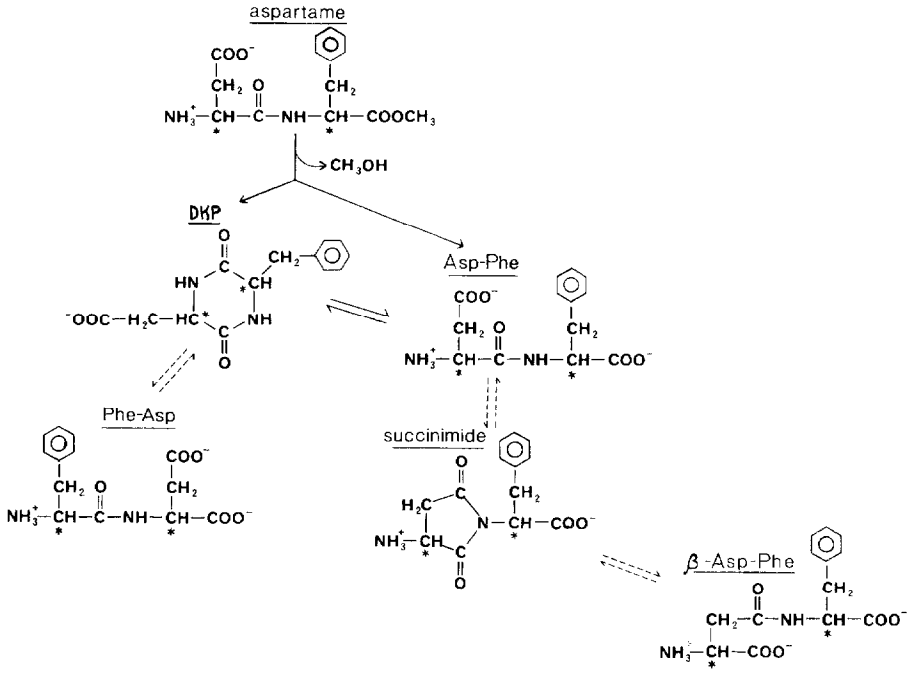

Fig. 1. Proposed decomposition scheme of aspartame showing the various possible products. The symbol * denotes chiral centers so that each of these products may undergo epimerization producing four stereoisomers. Solid arrows represent known decomposition reactions (7–9). Dashed arrows represent possible dipeptide and DKP pathways (11, 17) which may occur during aspartame decomposition.

veloped in the course of a study of cyclization and epimerization in a dipeptide–DKP system.

EXPERIMENTAL

Standard and sample preparation

Aspartame was obtained from Sigma, L-phenylalanine (L-Phe) from Calbiochem, L-aspartyl-L-phenylalanine (L-Asp-L-Phe) from Vega and L-phenylalanyl-L-aspartic acid (L-Phe-L-Asp) from Bachem. Standards were dissolved in the HPLC mobile phase (0.05 *M* KH_2PO_4, pH 3.15). In order to minimize errors due to decomposition during sample preparation, care was taken to expose all standards in a series dilution to the same length of time at room temperature and to freeze them until analysis.

In general DKP formation and amino acid racemization proceed most rapidly[10–12] at basic pH. For this reason an array of aspartame decomposition and epimerization products was obtained by heating a 0.01 *M* aspartame solution in pH 8.8 or 9.8 borate buffer at 100°C for several hours. These samples were then acidified to pH 3–4 and diluted in HPLC mobile phase for analysis. All samples were filtered through glass microfibre filters before HPLC analysis.

High-performance liquid chromatography

An Altex Model 310 gradient liquid chromatograph equipped with an Altex Model 155 variable-wavelength UV detector was employed. The eluent was monitored at 215 nm. Data was collected simultaneously on a strip chart recorder for peak height measurements and a DEC PDP-1103 computer for peak area integration. Analyses were carried out using a 5-μm C_{18} Alltech Econosphere (250 mm $\times$ 4.5 mm) column.

The solvent was Fisher brand HPLC grade acetonitrile. The mobile phase was 0.05 *M* KH_2PO_4 titrated to pH 3.15 with H_3PO_4 and filtered through a 0.45-μm Millipore filter before use. Doubly distilled water was used in buffer and sample preparations.

RESULTS AND DISCUSSION

Some representative chromatograms showing the separation of aspartame decomposition products and their diastereomers are presented in Fig. 2. The unshaded peaks were identified based on the retention times of appropriate standards. Standards of the D,L-dipeptides are not easily obtained. To avoid the expense and time

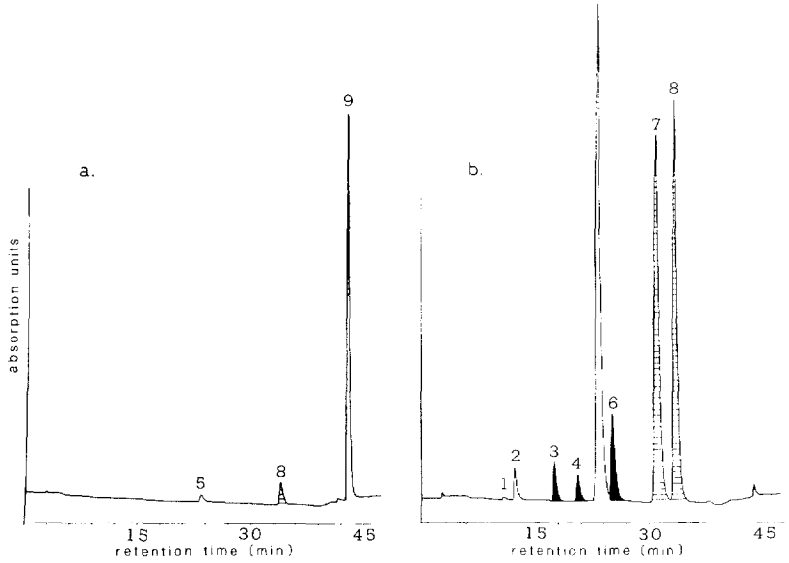

Fig. 2. HPLC chromatograms of aspartame and its decomposition products. (a) Aspartame dissolved in pH 9.8 borate buffer and exposed to room temperature only long enough for mixing and sample manipulation. An amount of 10 nmol of aspartame was injected (based on the original concentration of aspartame). (b) Aspartame dissolved in pH 8.8 borate buffer and heated at 100°C for 5.67 h. An amount of 50 nmol of aspartame was injected (based on the original concentration of aspartame). The column solvent and mobile phase are described under Experimental; the flow-rate was 1.0 ml/min. The gradient is a linear decrease in mobile phase from 94% at 0 min (time of injection) to 93% in 23 min. At 24 min it drops to 90% in 2 min; there is a hold at 90% until 36 min when there is a decrease to 75% in 3 min. Peak identification: 1 = Phe, 2 = LL- and DD-Phe-Asp, 3 = D,L-Phe-Asp, 4 = D,L-β-Asp-Phe or LL- and DD-β-Asp-Phe, 5 = LL- and DD-Asp-Phe, 6 = D,L-Asp-Phe, 7 = D,L-DKP, 8 = LL- and DD-DKP, 9 = aspartame.

involved in their synthesis, the shaded peaks in Fig. 2 were identified using indirect methods. In order to identify the two diastereomeric DKPs a heated sample of aspartame was acidified and applied to a cation-exchange resin (Bio-Rad AG50W-X8) in the hydrogen form[11]. The water wash was collected and analyzed on the HPLC system. Only the two crosshatched peaks (7 and 8) in Fig. 2 were detected. The ammmonia wash was also analyzed and found to contain peaks 1–6. The DD- + LL- and D,L-DKP (D-Asp-L-Phe DKP + L-Asp-D-Phe DKP) assignments were made from a time series of heated aspartame samples; the peak assigned as LL appeared rapidly, while the D,L peak appeared more slowly. In order to confirm these assignments several samples from the time series were hydrolyzed and the D/L ratios of Asp and phe determined. Samples containing only the LL-DKP would be expected to have D/L ratios of zero, whereas samples containing D,L-DKP would be expected to have non-zero D/L ratios. The D/L ratios were determined by making the *o*-phthaldialdehyde N-acetyl-L-cysteine (OPA-NAC) derivatives and analyzing them on HPLC with fluorometric detection as described by Aswad[15]. The phenylalanine OPA-NAC diastereomers do not separate in the HPLC buffer system of Aswad. These were separated using an optically active copper chelate complex [1:2 Cu(II)–L-proline] in the mobile phase[16]. Samples containing primarily peak 8 (LL-DKP), with low proportions of peak 7 and other unidentified peaks, had low D/L ratios (<0.1); those containing near equal proportions of peaks 7 and 8 had high D/L ratios (>0.7).

The L,D-Asp-Phe (L-Asp-D-Phe + D-Asp-L-Phe) and L,D-Phe-Asp (L-Phe-D-Asp + D-Phe-L-Asp) peaks were assigned in the following manner. A mixture of L-Asp-L-Phe and L-Asp-D-Phe was synthesized from L-Asp-β-benzyl ester N,carboxyanhydride (L-Asp-β-benzyl NCA) (Fox Chemical) and racemic Phe (Sigma). The procedure used was a modification of that used by Tou and Vineyard[17] for the synthesis of aspartame from L-aspartyl-β-methyl ester N-carboxyanhydride and L-Phe. Their procedure was followed through hydrolysis of the β-ester, at which time the product mixture was diluted with HPLC mobile phase and analyzed. Peaks 4, 5 (L-Asp-L-Phe) and 6 were identified in the mixture. At low concentrations, or with slight gradient variations, a fourth peak (peak A) was resolved from peak 5. All four products were present in comparable amounts. Dipeptide syntheses using Asp-β-benzyl NCA yield mixtures of α- and β-dipeptides[19]. This is consistent with the results of our synthesis wherein two diastereomers of Asp-Phe and of β-Asp-Phe were produced. No L-Phe-L-Asp was present in the reaction mixture so it was clear that no D-Phe-L-Asp could be present. Thus peak 3, being the only unidentified peptide peak not present in the reaction mixture, was assigned as D,L-Phe-Asp.

The assignment of peak 3 as D,L-Phe-Asp is supported by heating experiments with aspartame and L-Phe-L-Asp. When aspartame is heated peak 3 appears, along with L-Phe-L-Asp, only after there has been extensive D,L-DKP formation as would be expected from the reaction path shown in Fig. 1. When L-Phe-L-Asp is heated at pH 3.4 peak 3 appears more rapidly than D,L-DKP and Asp-Phe, as would be expected from direct epimerization of the L-Phe-L-Asp. Furthermore, with extended heating of aspartame the ratio of peak 3 to L-Phe-L-Asp quickly obtains and then maintains a ratio near 1.1. This behaviour is that expected of two diastereomers approaching and maintaining equilibrium. Peak 6 was assigned as D,L-Asp-Phe based on its production in the synthesis described above, as well as on heating experiments with aspartame. When aspartame is heated at neutral and basic pH L-Asp-L-Phe is

the most rapidly and extensively formed of the non-cyclic peptides[18]. It is formed from hydrolysis of the LL-DKP and, to a lesser extent, from hydrolysis of aspartame. Peak 6 is the next most rapidly formed of the dipeptides, as would be expected for D,L-Asp-Phe from hydrolysis of D,L-DKP and epimerization of L-Asp-L-Phe. Again, as expected for the two diastereomers, peak 6 increases steadily as L-Asp-L-Phe decreases to an equilibrium ratio of about 1.1. These interconversions are discussed in detail, and the heating experiments described in ref. 18. An example of data from these experiments which lead to the above conclusions is given in Table I.

TABLE I

EXAMPLE OF DATA FROM HEATING EXPERIMENTS WITH ASPARTAME AND L-Phe-L-Asp WHICH SUPPORTS ASSIGNMENT OF PEAKS 3 AND 6

Aspartame or L-Phe-L-Asp were heated under the given conditions for various lengths of time[18]. Values given are percents of total starting concentrations. Concentrations of the LL-peptides were determined from the correlation curves described in the text; concentrations of peaks 3 and 6 were calculated using the correlation curves for the proposed diastereomers.

Heating time (h)	*LL- + DD-DKP*	*D,L-DKP*	*LL- + DD-Phe-Asp*	*Peak 3*	*LL- + DD-Asp-Phe*	*Peak 6*
Starting material: aspartame (pH 8.8, 100°C)						
1.23	39.1	24.4	0.4	0.2	34.1	1.0
3.20	35.4	31.4	1.0	0.9	26.9	2.7
5.67	29.5	27.0	1.9	2.4	31.6	5.6
23.89	22.0	21.7	5.2	5.9	25.1	13.7
44.95	16.3	17.6	7.2	8.2	22.8	17.1
94.32	12.0	11.2	9.9	9.8	20.0	17.9
Starting material: L-Phe-L-Asp (pH 3.4, 100°C)						
1.42	24.3	0.1	67.9	2.0	0.4	0.0
5.48	44.5	0.9	32.5	3.9	0.4	0.0
9.28	52.0	2.2	15.8	3.8	0.4	0.0
23.45	55.6	6.6	2.5	2.4	0.5	0.0

Peak 4 is a relatively minor decomposition product even after extensive heating (always $<6\%$ of the starting material). Another minor peak (always $<4\%$) is hidden by peak 5 in Fig. 2 but has been resolved using a different gradient program or when the concentration of peak 5 is low, *e.g.* after excessive heating. This peak was identified as peak A from the peptide synthesis. Peaks 4 and A are tentatively assigned as the two diastereomers of β-Asp-Phe based on their production in the peptide synthesis. In heating experiments with aspartame at various pH values[18] the rate of appearance of peaks 4 and A increased with increasing pH. These two minor decomposition products may have formed via the succinimide intermediate[20] as shown in Fig. 1.

Calibration curves for the standards were derived from series dilutions ranging from $1 \cdot 10^{-5}$ *M* to $1 \cdot 10^{-3}$ *M*. Peak areas and peak heights were plotted against concentrations. Correlation coefficients (r) from least squares regressions for these curves are: L-Phe, $r = 0.9970$; L-Phe-L-Asp, $r = 0.9948$; L-Asp-L-Phe, $r = 0.9990$; aspartame, $r = 0.9967$. Difficulties in precise quantification arise from the fast con-

version of the dipeptide standards to the DKP. These difficulties can be circumvented by taking care that all samples in a series dilution are exposed to room temperature for equal lengths of time and minimizing this time. The DKP formed can then be accounted for in the correlation calculations. This was done for the curves derived in this study. Because formation of the DKP is much slower at low pH it is also important to dissolve samples and standards in a low pH buffer (< 5). An important aspect of the separation described here is the low pH of the HPLC mobile phase; this slows any dipeptide–DKP conversions which might otherwise take place during the analysis.

The dipeptide separation was found to be very sensitive to the salt concentration in the mobile phase and to slight gradient changes. It was imperative to acidify basic samples before analysis. The slow gradient at the beginning of the program was found to be necessary for separation of the various peptides. The decreases in mobile phase to 90% and later to 75% facilitate the elution of the DKPs and aspartame in a reasonable period of time with sharp peak shapes. Using a given Econosphere column at constant temperature, retention times showed excellent reproducibility (< 0.2 min variability). Separations were not reproducible on different Econosphere columns using the same gradient program. However, slight variations in the gradient or buffer concentration (*e.g.* to 0.0125 *M*, pH 3.2 mobile phase; initial gradient 94% to 90% in 30 min) produced separations similar to those shown here for the three different Econosphere columns tested. The sensitivity of this separation to operating conditions and column changes is to be expected, given the necessity of beginning with an extremely gradual gradient.

This study illustrates the successful use of reversed-phase HPLC to separate a complex mixture of diastereomeric dipeptides. The separation described here is, of course, specific to the dipeptide being studied which contains both hydrophylic (Asp) and hydrophobic (Phe) moieties; different dipeptide systems will probably require buffer and gradient modifications. Besides the broad range of applications in theoretical organic chemistry, biochemistry and geochemistry, the separation described here may be useful in ongoing studies of aspartame decomposition in foods and beverages[9,13]. Coelution of substances such as caffeine and coloring may present problems in such studies. The degree of interference from these substances in our system remains to be determined.

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REFERENCES

1 D. M. Matthews, in L. D. Stegink and L. J. Jr. Filer (Editors), *Aspartame: Physiology and Biochemistry*, Marcel Dekker, New York, 1984, p. 29.
2 S. M. Steinberg, P. M. Masters and J. L. Bada, *Bioorg. Chem.*, 2 (1984) 349.
3 N. Kriausakul and R. M. Mitterer, *Geochem. Cosmochim. Acta*, 44 (1980) 753.
4 S. M. Steinberg and J. L. Bada, *J. Org. Chem.*, 48 (1983) 2295.
5 P. Gund and D. F. Veber, *J. Am. Chem. Soc.*, 101 (1979) 1885.
6 K. A. Kopple and D. H. Marr, *J. Am. Chem. Soc.*, 89 (1967) 6193.

7 I. Furda, P. D. Malizia, M. G. Kolor and P. J. Vernieri, *J. Agric. Food Chem.*, 23 (1975) 340.
8 M. F. Boehm and J. L. Bada, *Proc. Natl. Acad. Sci. U.S.A.*, 81 (1984) 5263.
9 W. Tsang, M. A. Clarke and F. W. Parrish, *J. Agric. Food Chem.*, 33 (1985) 734.
10 A. Neuberger, *Adv. Protein Chem.*, 4 (1948) 298.
11 S. Steinberg and J. L. Bada, *Science (Washington, D.C.)*, 213 (1981) 544.
12 P. Gund and D. Veber, *J. Am. Chem. Soc.*, 107 (1979) 1885.
13 R. Cross and B. Cunico, *Liq. Chromatogr. HPLC Mag.*, 2 (1984) 678.
14 G. Verzella and A. Mangia, *J. Chromatogr.*, 346 (1985) 417.
15 D. W. Aswad, *Anal. Biochem.*, 137 (1984) 405.
16 E. Gil-Av, A. Tishbee and P. E. Hare, *J. Am. Chem. Soc.*, 102 (1980) 5115.
17 J. S. Tou and B. D. Vineyard, *J. Org.Chem.*, 50 (1985) 4982.
18 S. M. Gaines and J. L. Bada, submitted for publication.
19 R. Hirschmann, H. Schwam, R. G. Strachan, E. F. Schoenewaldt, H. Barkemeyer, S. M. Miller, J. B. Conn, V. Garsky, D. F. Veber and R. G. Denkewalter, *J. Am. Chem. Soc.*, 93 (1971) 2746.
20 E. D. Murray and S. Clarke, *J. Biol. Chem.*, 259 (1984) 10722.