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Analytical Methods Section

HPLC analysis of a backbone compound, β-benzyl-*N*-carbobenzoxy-L-aspartyl-D-alanine, of intense peptide sweeteners belonging to L-aspartyl-D-alanine amides

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A simple and reliable HPLC method for determining a backbone compound, β-benzyl-*N*-carbobenzoxy-L-aspartyl-D-alanine, from intense peptide sweeteners belonging to L-aspartyl-D-alanine amides is reported. The backbone compounds synthesized showed high enough purity to be used as standards. The most effective eluent composition and flow rate for the HPLC assay of the backbone compound were CH_3CN : 0·2 M triethylamine–H_3PO_4 (pH 3) in 1 : 1 ratio and 0·8 ml min^{-1}, respectively. The HPLC method established here was found to have excellent reproducibility and favourable recovery for the backbone compound. When analysed by this method, the optimum reaction time between the silylated D-alanine and mixed anhydride in the synthesis of the backbone compound was found to be 80 min.

INTRODUCTION

Refined sugar is currently the principal nutritive sweetener. However, it can cause dental decay and is suspected of contributing to obesity and adult diseases such as diabetes mellitus and hypertension (Glinsmann *et al.*, 1986; Finer, 1989).

So, many non-nutritive sweeteners have been developed in order to meet an increasing demand for sugar substitutes. Aspartame, which is one of these non-nutritive sweeteners and has been the foremost alternative to sugar, poses many problems including low stability at extreme temperature and in the region of neutral pH, low solubility, sensitivity to enzyme digestion and possible side-effects such as phenylketonuria and methanol accumulation (Furda *et al.*, 1975; Scherz *et al.*, 1983; Homler, 1988).

To resolve these problems, many dipeptide analogues based on aspartame have been devised. Among them, the peptide sweeteners belonging to L-aspartyl-D-alanine amides were found to be the most successful substitutes for aspartame (Brennan & Hendrick, 1983*a*; Janusz, 1989). Alitame [L-aspartyl-D-alanine *N*-(2,2,4,4 tetramethyl-3-thietanyl)amide] which was developed by Pfizer Inc. (New York, NY) and is 2000-fold sweeter than sugar is under regulatory review and expected to be commercialized in the near future. According to the data accumulated up till now, alitame is safe and able to resolve many problems encountered with aspartame (Freeman, 1989).

In many cases, L-aspartyl-D-alanine amides have been synthesized chemically through mixed anhydride processes (Brennan & Hendrick, 1983*a,b*; Bodanszky & Bodanszky, 1984). β-Benzyl-*N*-carbobenzoxy(Cbz)-L-aspartyl-D-alanine has been reported to be a backbone compound of L-aspartyl-D-alanine amides (Brennan & Hendrick, 1983*a*).

Recently the chemical synthesis of intense peptide sweeteners belonging to L-aspartyl-D-alanine amides was studied in our laboratory. In this instance the development of an assay method for the backbone compounds was found to be a prerequisite for the optimization of the synthetic procedures. Based on this necessity, this paper reports a simple and reliable method for determining the backbone compound using HPLC.

MATERIALS AND METHODS

Reagents

β-Benzyl-*N*-Cbz-L-aspartic acid, D-alanine and CD_3OD were purchased from Sigma Chemical Co. (St Louis, MO). Solvents for chromatography were of HPLC grade. The other reagents were products of Aldrich Chemical Co. (Milwaukee, WI).

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Synthesis and identification of the standard

The standard backbone compound was synthesized through a mixed anhydride process with β-benzyl-*N*-Cbz-L-aspartic acid and D-alanine as described by Brennan & Hendrick, (1983*a*). D-Alanine (56 mmol) was silylated with *N*,*N*-dimethylformamide (DMF) (100 ml) and trimethylchlorosilane (8·43 ml). β-Benzyl-*N*-Cbz-L-aspartic acid (50 mmol) was separately mixed with DMF (110 ml), tetrahydrofuran (110 ml), triethylamine (17·11 ml) and ethylchloroformate (6·55 ml). The resulting solution was cooled to −15°C. The silylated D-alanine was added dropwise to the solution of β-benzyl-*N*-Cbz-L-aspartic acid mixed anhydride. The mixture was stirred at −10°C for 1 h, 0·2 M HCl added until the pH of the mixture was below 3·5 and the resulting mixture extracted with chloroform (3 × 50 ml). The chloroform extracts were combined, washed several times with dilute HCl and dehydrated over anhydrous $MgSO_4$. The solvent was evaporated *in vacuo* and the backbone compound was obtained after recrystallization from ethylacetate.

The backbone compound thus synthesized was analysed for melting point (mp), R_f value on TLC, amino acid composition and product purity. Amino acid composition was determined with a LKB 4151 Alpha Plus amino acid analyser (LKB Biochrom Ltd, Cambridge, UK) as follows. A sample of the material (1·3 mg) was completely hydrolysed with 6 M HCl (1 ml) under N_2 at 110°C for 24 h. Distilled water (5 ml) was added to the hydrolysate and the mixture was evaporated *in vacuo* at 85°C. This process was repeated until no HCl could be detected. The final hydrolysate was dissolved in the starting buffer (0·2 M sodium citrate buffer, pH 2·2), filtered through a 0·2 μm membrane filter (Gelman Co.) and used for amino acid analysis.

Then, the backbone compound was further identified by instrumental analyses. Elemental analysis was carried out with a 2A1108 elemental analyser of Carlo Erba Co. (Milan, Italy) at the combustion temperature of 1000°C. Fast atom bombardment (FAB) mass spectrometric analysis was performed with a VG70VSEQ mass spectrometer (VG Elemental Co., Manchester, UK) using glycerol as matrix. NMR spectroscopic analysis was done with an AMX/500 NMR spectrometer (Bruker Instrument Corporation, Rheinstetten, Germany). In this instance, the sample for NMR spectroscopic analysis was prepared as follows. The substance, dissolved in CD_3OD, was passed through a Pasteur pipette containing defatted cotton filter into an NMR tube (Wilmad Co., Buena, NJ). A few drops of tetramethylsilane were added before NMR analysis. The final sample concentrations for ^1H- and ^{13}C-NMR were 16 and 6 mg ml^{-1}, respectively.

Sample preparation

The synthesis of the backbone compound described above was tried on one-fifth scale. After adding the silylated D-alanine to the solution of the mixed anhydride, a portion of the solution (1 ml) was taken into a test tube after 5, 10, 20, 40, 60, 80 and 120 min of reaction and mixed with 0·2 M HCl (1 ml) and chloroform (1 ml). Swirled gently, the upper layer was removed and the remaining chloroform layer dehydrated with a small amount of anhydrous $MgSO_4$. A portion of the chloroform fraction (0·4 ml) was taken into another test tube. The solvent was evaporated *in vacuo* and the HPLC eluent was added (1 ml). Then, the solution was passed through a 0·2 μm membrane filter (Gelman Co.) and diluted 11-fold before analysis.

Analytical conditions

An HPLC system (Waters Co., Milford, MA) equipped with a μBondapak C_{18} column was used. From the preliminary screening (Kim *et al.*, 1991), the most adequate eluent system for the separation of the backbone compound was CH_3CN/0·2 M triethylamine–H_3PO_4 (pH 3). The UV absorption of the substance was detected at 220 nm (absorbance units full scale range 0·4) with a 1000S diode-arrayed detector (Applied Biosystems, Foster City, CA) and peak area calculated with a Data Module 745 integrator (Waters Co.). The injection volume was 2 μl.

The HPLC method was compared to the TLC scanner method (Kim *et al.*, 1991) with respect to reproducibility and recovery. A CS-920 TLC scanner (Shimadzu Co., Japan) was used for the TLC assay and the predetermined analytical conditions for the TLC assay were as follows: plate, 20 cm × 20 cm glass plate; stationary phase, Kiesel gel 60G; mobile phase, ethyl acetate hexane acetic acid (9:9:2, v/v/v); visualization 5M, H_2SO_4; scanning wavelength, 450 nm; spotting, 5 μl.

Coefficient of variation (CV) which is expressed as SD/mean × 100 (%) was used as a measure of reproducibility of the assay method. For this purpose, the silylated D-alanine was reacted with the mixed anhydride for 1 h. Then, a portion of the solution (1 ml) was treated as described above. Thirteen measurements were carried out on the same sample. A recovery test was done by adding the standard backbone compound during sample preparation and determining the recovered amount as follows. The silylated D-alanine was reacted with the mixed anhydride for 1 h. Then, a portion of the solution (1 ml) was mixed with the material (4 mg) and treated as described above. Measurement was done in triplicate.

RESULTS AND DISCUSSION

The characteristics of the synthesized standard backbone compound are shown in Table 1. The product purity was 98·7% and a single peak was found on HPLC (data not shown). The results of elemental analysis and FABMS indicated that the observed molecular composition and mass are in agreement with those of the authentic compound. The presence of D-alanine was

Table 1. Characteristics of the synthesized standard β-benzyl-*N*-Cbz-L-aspartyl-D-alanine

mp (°C)	161–163
R_f[a]	0·48
Amino acid composition: shows L-aspartic acid and D-alanine peaks	
Elemental analysis (%)	
—Calcd: C, 61·7; H, 5·6; N, 6·5	
—Found: C, 61·3; H, 5·5; N, 6·4	
FABMS *m/z*: 429 (M+1, 13[b])	
NMR spectroscopy	
—^{1}H-NMR (CD_3OD) ppm (δ): 1·54 (d, 3H, J=7·2 Hz); 4·54 (m[c], 1H, J=8·9 Hz); 8·09 (s, 1H)	
—^{13}C-NMR (CD_3OD) ppm (δ): 18·01; 53·22; 172·92	
Purity (%)[d]	98·7

[a] TLC (silica gel; mobile phase, ethyl acetate hexane acetic acid (9:9:2, v/v/v); H_2SO_4 spray).
[b] Relative abundance (%).
[c] Quartet signal.
[d] Calculated from HPLC peak area.

confirmed by NMR. In ^{1}H-NMR, the doublet, quartet and broad singlet signals of methyl, Cα and amino protons in D-alanine were found at 1·54, 4·54 and 8·09 ppm, respectively. In ^{13}C-NMR, the signals of methyl, Cα and carboxylate carbons in D-alanine were found at 18·01, 53·22 and 172·92 ppm. Based on these data, the backbone compound synthesized here was assumed to be identical to β-benzyl-*N*-Cbz-L-aspartyl-D-alanine and used as the standard sample in the further experiments.

In order to be able to follow the synthesis of the backbone compound, the separation of the reactants from the product was achieved by varying the eluent composition and flow rate. In this instance, the eluent composition [ratios of CH_3CN:0·2 M triethylamine–H_3PO_4 (pH 3)] were 8:2, 7:3, 6:4, 5:5 and 4:6. The tested flow rates were 0·4, 0·6, 0·8, 1·0 and 1·2 ml min^{-1}. The retention time (RT) of β-benzyl-*N*-Cbz-L-aspartic acid and the backbone compound at the flow rate of 0·8 ml min^{-1} became longer by decreasing the amount of CH_3CN. These compounds were separated when the proportion of CH_3CN was below 50%. Taking separation power and time into consideration, CH_3CN:0·2 M triethylamine–H_3PO_4 (pH 3) in 1:1 ratio and 0·8 ml min^{-1} were selected as the most effective eluent composition and flow rate.

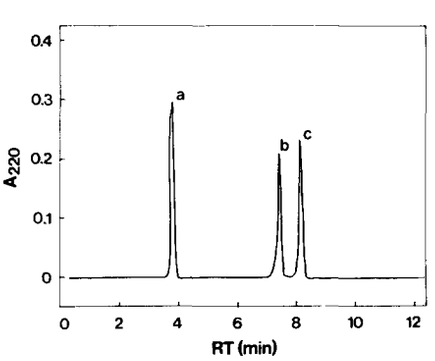

Fig. 1. HPLC chromatograms of the reactants and product for the synthesis of β-benzyl-*N*-Cbz-L-aspartyl-D-alanine. a, D-Alanine trimethylsilyl ester and mixed anhydride; b, β-benzyl-*N*-Cbz-L-aspartyl-D-alanine; c, β-benzyl-*N*-Cbz-L-aspartic acid.

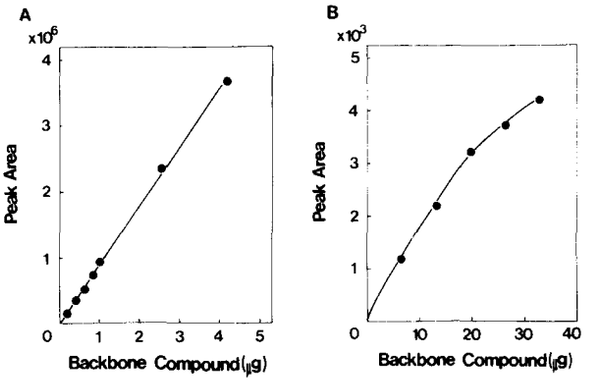

Fig. 2. Standard curves for β-benzyl-*N*-Cbz-L-aspartyl-D-alanine assay. (A), HPLC assay; (B), TLC assay.

The HPLC chromatogram using the above eluent composition and flow rate is shown in Fig. 1. The RT of the backbone compound and β-benzyl-*N*-Cbz-L-aspartic acid were found to be 7·43 and 8·18 min respectively, whereas, D-alanine trimethylsilyl ester and the mixed anhydride, two direct reactants, were eluted at the same RT of 3·81 min.

Figure 2 shows the standard curves of the backbone compound prepared by an external standard method. As shown in Fig. 2, the HPLC method was much more sensitive than the TLC method.

The CV values of the HPLC and TLC methods were 4·22 and 12·45% respectively. This fact meant that the HPLC method shows excellent reproducibility compared to the TLC method (Kim *et al.*, 1983). The recoveries of the backbone compound in the HPLC and TLC methods were 92·0 ± 1·5 and 80·4 ± 1·8% respectively. As expected, the HPLC method shows higher recovery of the substance compared to the TLC method.

Based on the reproducibility and recovery of analysis, the HPLC method was presumed to be superior to the TLC method in measuring the backbone compound. So, the reaction time between the silylated D-alanine and mixed anhydride in the synthesis of the backbone compound was optimized by this method (Fig. 3). The backbone compound was synthesized

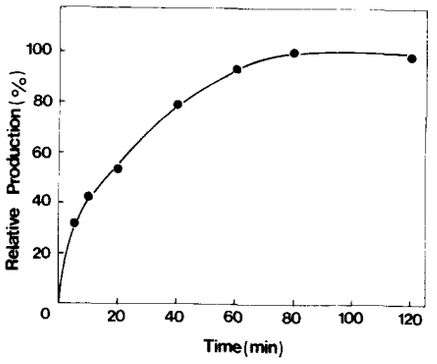

Fig. 3. Time course for the production of β-benzyl-*N*-Cbz-L-aspartyl-D-alanine. The silylated D-alanine was reacted with the mixed anhydride for 5, 10, 20, 40, 60, 80 and 120 min. Then, a portion of the solution (1 ml) was treated as described in the Materials and Methods. Measurement was done in triplicate.

rapidly during the initial phase of the reaction. After 20 min of the reaction, the relative production of the substance was approximately 55% of its final value. In prolonged reaction, the rate of formation of the backbone compound gradually decreased, synthesis of the substance effectively stopping after 80 min of the reaction. Eighty minutes was therefore used as an optimum reaction time.

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