Structure, Dynamics, and Stability of β-Cyclodextrin Inclusion Complexes of Aspartame and Neotame

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Studies of the high-intensity sweetener aspartame show that its stability is significantly enhanced in the presence of β-cyclodextrin (β-CyD). At a 5:1 β-CyD/aspartame molar ratio, the stability of aspartame is 42% greater in 4 mM phosphate buffer (pH 3.1) compared to solutions prepared without β-CyD. Solution-state ^{1}H NMR experiments demonstrate the formation of 1:1 β-CyD/aspartame complexes, stabilized by the interaction of the phenyl-ring protons of aspartame with the H3 and H5 protons of β-CyD. Inclusion complex formation clearly accounts for the observed stability enhancement of aspartame in solution. The formation of inclusion complexes in solution is also demonstrated for β-CyD and neotame, a structural derivative of aspartame containing an N-substituted 3,3-dimethylbutyl group. These complexes are stabilized by the interaction of β-CyD with both phenyl-ring and dimethylbutyl protons. Solid-state NMR experiments provide additional characterization, clearly demonstrating the formation of inclusion complexes in lyophilized solids prepared from solutions of β-CyD and either aspartame or neotame.

Keywords: *Aspartame; neotame; sweeteners; β-cyclodextrin; inclusion complexes; enhanced stability; NMR characterization*

INTRODUCTION

Aspartame (α-L-aspartyl-L-phenylalanine 1-methyl ester) is the world's most widely used high-intensity sweetener due to its broad application as a sweetening ingredient in foods and beverages. Neotame [*N*-[*N*-(3,3-dimethylbutyl)-α-L-aspartyl]-L-phenylalanine-1-methyl ester] is a new high-intensity sweetener that is currently being developed by the NutraSweet Co. On a weight basis, the sweetness potency of neotame is ∼40 times greater than that of aspartame and ∼8000 times that of sugar. Neotame has a clean, sweet taste profile and has recently been submitted to the U.S. Food and Drug Administration for approval as a new sweetener in a number of food categories. In addition to its higher sweetness potency, neotame has physicochemical properties that differ from those of aspartame and other high-intensity sweeteners (*1*), including its relatively low melting point (82 °C) and its greater solubility in ethanol.

Aspartame and, more recently, neotame have drawn attention from researchers interested in elucidating the structure−function relationship of high-intensity sweeteners (*2*−*5*). The three-dimensional structure of neotame, determined by single-crystal X-ray analysis, demonstrates that the compound exists in an *L-shaped* conformation, which fits the proposed model for sweetness of aspartyl-based dipeptide compounds. Neotame's conformation in the solid state demonstrates well-oriented hydrophobic and hydrophilic regions, which likely account for the high sweetness intensity of this dipeptide.

Although aspartame and neotame can be used in a wide variety of foods and beverages, sweetener stability remains an issue in many of these products. Extending shelf life by improving the stability of these sweeteners in different food products, in particular, diet beverages, would have significant economic impact. Factors such as pH, temperature, and time influence the stability of both sweeteners. Compared to aspartame, neotame has a similar stability profile in the pH range 3.0−5.5 but greater stability in the pH range 5.5−8.0. At higher pH, intramolecular cyclization to form the corresponding diketopiperizine derivative is the predominant mechanism of aspartame degradation. However, because of its structure, neotame cannot cyclize to form a diketopiperizine derivative. At lower pH values (pH 3.0−3.5) typical of beverage formulations, the predominant degradation pathway for both sweeteners is ester hydrolysis to form the aspartyl−phenylalanine derivative.

The ability of β-cyclodextrin (β-CyD) to form inclusion complexes with a wide variety of guest molecules is well-known (*6*, *7*), and the formation of inclusion complexes with aspartame has been demonstrated (*8*−*10*). For aspartame, studies have indicated the phenylalanine ring is positioned in the β-CyD cavity with the methyl ester exposed outward, away from the complex. Although it has been postulated that the enhanced stability of aspartame in β-CyD inclusion complexes results from a decrease in methyl ester hydrolysis (*10*), the prevailing structural model does not intuitively support this mechanism of stability enhancement because the methyl ester is not bound within the complex. Also,

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there are no published reports that show how the rates of formation of aspartame degradation products are affected when aspartame is present in a β-CyD inclusion complex at conditions typical of beverage formulations.

NMR spectroscopy, a powerful tool for the characterization of molecular structure and dynamics, has been used to characterize cyclodextrins and cyclodextrin inclusion complexes. Schneider recently published a comprehensive review of NMR in cyclodextrins (*11*). One-dimensional solution-state ^{1}H and ^{13}C NMR can provide information about structure and hydrogen bonding in cyclodextrins and substituted cyclodextrins, whereas more detailed information about their conformations is available from a variety of two-dimensional NMR experiments, including NOESY and ROESY. Monitoring changes in ^{1}H or ^{13}C chemical shifts as the composition of these complexes is varied can elucidate the stoichiometry of inclusion complexes and the dynamics of their formation. NMR relaxation measurements provide important insights into the dynamics of cyclodextrins and the changes induced during complex formation. Solid-state NMR spectroscopy can be used to obtain valuable information about solid inclusion complexes (*12*), including verification of complex formation (*13*) and stability and measurement of glycosidic conformations (*14*).

In this paper, we report the results of a study comparing the degradation of aspartame and the concomitant formation of its two main degradation products, diketopiperizine and α-L-aspartyl-L-phenylalanine, in 4 mM buffer and a simulated cola beverage prepared with and without β-cyclodextrin. We then characterize β-CyD/aspartame and β-CyD/neotame inclusion complexes, in both solution and the solid state, using a variety of NMR methods. The results of the study help us to understand, in greater detail, the properties of β-CyD inclusion complexes with these sweeteners.

MATERIALS AND METHODS

Materials. α-L-Aspartyl-L-phenylalanine 1-methyl ester, α-L-aspartyl-L-phenylalanine, *cis*-3,6-dioxo-5-(phenylmethyl)-2-*S*-piperazineacetic acid, and *N*-(*N*-3,3-dimethylbutyl)-α-L-aspartyl-L-phenylalanine methyl ester were used as certified reference standards provided by the NutraSweet Co. (Mt. Prospect, IL). Phosphoric acid (85%) and caffeine were obtained from Fisher Chemical (Pittsburgh, PA). Sodium 1-heptanesulfonate was from Regis Technologies, Inc. (Morton Grove, IL) and sodium benzoate from Kalama Chemical, Inc. (Seattle, WA). Sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$) and acetonitrile were from Mallinckrodt Specialty Chemical (Paris, KY). Double-strength caramel coloring, cola flavor, and cola acid were obtained from Universal Flavors (Indianapolis, IN). β-Cyclodextrin was obtained from American Maize (Hammond, IN), and 3,3-dimethylbutylamine (HCl) was obtained from Sigma Aldrich (St. Louis, MO). All materials were used without further purification.

Preparation of Cola Beverage. The cola beverage was designed to simulate the ingredient composition of commercially available diet cola beverages containing aspartame. Cola syrup was first prepared by mixing corresponding amounts of caffeine, sodium benzoate, cola acid, cola flavor, and deionized water. The cola beverage was prepared by diluting this syrup with deionized water and adjusting with dilute phosphoric acid or dilute NaOH to a final pH of 3.10 ± 0.02. The approximate concentrations of ingredients in the final cola beverage were as follows: caramel coloring, 1400 ppm; sodium benzoate, 250 ppm; caffeine, 200 ppm; and phosphoric acid, 4 mM.

Aspartame Stability Study. *Aspartame Stability Measurements.* Stability measurements of aspartame were carried out in 4 mM phosphate buffer and the cola beverage at 35 °C, with and without the addition of β-cyclodextrin. Solutions containing β-cyclodextrin were prepared by the addition, after all other ingredients had been added, of β-cyclodextrin at a molar ratio of 5:1 β-CyD/aspartame. Samples were prepared with an initial aspartame concentration of 535 ± 5 ppm, and the final pH was adjusted to 3.10 ± 0.02 using either dilute NaOH or phosphoric acid. Samples were stored in storage chambers controlled to ±2 °C. The concentrations of aspartame, diketopiperizine, and α-L-aspartyl-L-phenylalanine were determined at 0, 5, 15, and 25 days. Measurements of pH were made at each time point, and the pH did not deviate by more than ±0.1.

Quantification of Aspartame and Aspartame Degradation Products. The concentrations of aspartame, diketopiperizine, and α-aspartyl-L-phenylalanine were determined using a reversed-phase HPLC method. An HP1090 liquid chromatography system with UV detection set at 210 nm was used for all quantitation measurements. Separation was achieved using a Beckman Ultrasphere octyl 5 μm, 25 cm × 4.6 mm column (Fullerton, CA) maintained at 40 °C using an isocratic mobile phase flow rate of 1.5 mL/min. The mobile phase was prepared by mixing acetonitrile (15%, v/v) with a buffer solution consisting of 0.18 M NaH_2PO_4 and 0.02 M heptanesulfonate. The final pH of the mobile phase was adjusted to 2.50 ± 0.05 with phosphoric acid. Prior to use, the mobile phase was filtered using a 0.22 μm nylon membrane filter (MSI Inc., Westboro, MA). All samples were assayed using duplicate injections without dilution. The HPLC acquisition time was 40 min, and typical retention times were 3.8, 9.9, and 30.3 min for diketopiperizine, α-aspartyl-L-phenylalanine, and aspartame, respectively. Quantification of each compound was achieved by generating a three-point standard curve over the expected concentration range using certified reference standards.

Rate Constant k and Half-Life Determinations. All sample aliquots were analyzed for aspartame, diketopiperizine, and α-aspartylphenylalanine at time zero and each subsequent time point. The percentage remaining ($A_t/A_0 \times 100$, where A_0 and A_t are analytically determined amounts of aspartame at time zero and storage time t, respectively) versus storage time (t) was plotted and the first-order rate expression ($A_t/A_0 = e^{-kt}$) applied to generate a first-order decay equation. The rate constant k was then determined. The half-life, defined as the time needed for the compound to degrade to 50% of the initial concentration, was calculated from the first-order rate expression $t_{1/2} = 0.693/k$.

Preparation of Solid β-CyD Inclusion Complexes. A solution of β-cyclodextrin was first prepared by adding 3 mmol of β-cyclodextrin in 42.5 mL of H_2O. Four separate solutions of aspartame were prepared by adding 3, 1.2, 0.6, and 0.3 mmol of aspartame in 7.5 mL of water. These amounts of aspartame corresponded to final β-CyD/aspartame molar ratios of 1:1, 2.5:1, 5:1, and 10:1, respectively. The aspartame and β-CyD solutions were then mixed and heated at 50 °C until completely dissolved. The resulting clear solutions were cooled to room temperature and lyophilized. Four β-CyD/neotame complexes were prepared by using the same molar amounts as those used in preparing the β-CyD/aspartame complexes; however, for each neotame sample preparation, neotame were first dissolved in a solution of 80:20 water/ethanol and then added to the β-CyD solution. After mixing, each solution was heated at 50 °C until completely dissolved, cooled to room temperature, and lyophilized.

NMR Studies. *Solid-State NMR.* Cross-polarization magic-angle spinning (CPMAS) ^{13}C NMR spectra were collected on a Monsanto-built spectrometer operating at a proton resonance frequency of 127.0 MHz. Samples were spun at the magic angle with respect to the magnetic field in a double-bearing rotor system at a rate of 3 kHz. CPMAS ^{13}C NMR spectra were obtained at 31.9 MHz following 2-ms matched, 50-kHz ^{1}H−^{13}C cross-polarization contacts. High-power proton dipolar decoupling [H_1(H) = 65−75 kHz] was used during data acquisition. In spectra of uncomplexed aspartame and neotame, residual spinning sidebands were suppressed using the total suppression of sidebands (TOSS) method (*15*).

Proton Rotating-Frame Relaxation. Proton rotating-frame relaxation times, $T_{1\rho}$(H), were determined from the decay of carbon signal as a function of $^1H-^{13}C$ contact time, τ, in a series of CPMAS experiments (*16*). $T_{1\rho}$(H) was measured in this way to take advantage of the spectral resolution of the ^{13}C NMR experiment. The value $\langle T_{1\rho}(H)\rangle$ was calculated from a straight-line fit of log(carbon signal intensity) versus τ, where τ varies from 2 to 12 ms.

Solution-State NMR. NMR data were obtained on a Varian Unity 500 MHz spectrometer at 30 °C with a spectral width of 4800 Hz, 2.3-s acquisition time, and recycle delays of 4−6 s. Solution samples of β-cyclodextrin containing either aspartame or neotame were prepared accordingly in D_2O. All spectra were referenced to HOD at 4.70 ppm. The pD of a 15 mM 1:1 molar mixture of aspartame and β-CyD was 4.65. For a 15 mM 1:1 molar mixture of neotame and β-CyD, the pD was 5.55. Proton T_1 values, T_1(H), were determined using a standard inversion−recovery ($t-180-\tau-90-$acquire) pulse sequence, with $t = 8-12$ s. Eleven τ values were used in the analysis. Proton T_2 experiments utilized a $t-90_x-(\Delta-180_y-\Delta)_n-$acquire sequence, with $t = 8-12$ s and $\Delta = 0.3$ s. Eleven spin−lock times were used in the analysis. T_1 and T_2 values were calculated from exponential regression analysis of the recovery curves using routines supplied by Varian. Phase-sensitive two-dimensional NMR data were obtained with spectral widths of 4800 Hz in both dimensions, 2048 points in the *F*2 dimension and 256 points in *F*1. The ROE data were obtained using the pulse sequence of Shaka (*17*) with a mixing time of 300 ms and a recycle time of 2.2 s.

The association constant, K_a, was evaluated by following the chemical-shift changes upon dilution of a 15 mM 1:1 mixture of β-CyD/sweetener. The data were analyzed by a nonlinear least-squares fit of the right-hand-side of eq 1 (*18*) with ($\delta_b - \delta_f$) and K_a as parameters.

$$(\delta_{obs} - \delta_f)[L]_0 = (\delta_b - \delta_f)\{[S]_0 + [L]_0 + K_a^{-1} - \sqrt{([S]_0 + [L]_0 + K_a^{-1})^2 - 4[S]_0[L]_0}\}/2 \quad (1)$$

The method of continuous variation was used to determine complex stoichiometry and to estimate K_a. Mixtures of β-cyclodextrin and sweetener were prepared from 16 mM stock solutions of each in a manner such that $[\text{sweetener}]_0 + [\beta\text{-CyD}]_0 = 16$ mM. The chemical shift changes were measured as a function of the molar ratio, x, defined as $[L]_0/([S]_0 + [L]_0)$. The data were analyzed by a nonlinear least-squares fit of the right-hand side of eq 2 with ($\delta_b - \delta_f$) and K_a as parameters.

$$(\delta_{obs} - \delta_f)[L]_0 = (\delta_b - \delta_f)\{[S]_0 + [L]_0 + K_a^{-1} - \sqrt{([S]_0 + [L]_0 + K_a^{-1})^2 - 4([S]_0[L]_0)^2(x - x^2)}\}/2 \quad (2)$$

[Equation 2 was derived from eq 1 by noting that $[L]_0 = x([S]_0 + [L]_0)$ and $[S]_0 = (1 - x)([S]_0 + [L]_0)$.]

RESULTS AND DISCUSSION

Aspartame Stability Study. As described in the Introduction, the two predominant pathways of aspartame degradation that occur at the pH of diet beverages (pH 3.0−3.5) are the formation of diketopiperizine by intramolecular cyclization and the formation of α-L-aspartyl-L-phenylalanine, the result of acid hydrolysis of the methyl ester. Table 1 shows data that illustrate the enhancement of stability when aspartame is in the presence of β-cyclodextrin. Because the initial concentration of aspartame is virtually identical in each solution, the aspartame degradation in conjunction with the formation of diketopiperizine and α-L-aspartyl-L-phenylalanine over time can be directly compared to assess the stability enhancement provided by β-cyclodextrin. As seen in Table 1, the degradation of aspartame is slower in solutions containing β-cyclodextrin.

Table 1. Degradation of Aspartame and Formation of α-L-Aspartyl-L-phenylalanine and Diketopiperizine from Aspartame and Aspartame/β-Cyclodextrin in 4 mM Phosphate Buffer and Cola Beverage

	concn (ppm) on day			
	0	5	15	25
4 mM phosphate buffer				
aspartame degradation				
aspartame control	534.9	508.7	460.0	416.2
aspartame/β-CyD	535.5	516.8	481.4	448.7
α-L-aspartyl-L-phenylalanine formation				
aspartame control	2.5	10.4	18.5	27.3
aspartame/β-CyD	0.5	6.4	11.9	17.7
diketopiperizine formation				
aspartame control	1.7	10.1	20.8	35.8
aspartame/β-CyD	1.3	7.0	14.5	25.2
cola beverage				
aspartame degradation				
aspartame control	537.0	508.5	456.4	409.2
aspartame/β-CyD	537.2	515.7	474.9	437.3
α-L-aspartyl-L-phenylalanine formation				
aspartame control	2.4	10.4	18.4	28.0
aspartame/β-CyD	0.5	7.1	13.0	19.8
diketopiperizine formation				
aspartame control	0.9	8.4	17.9	32.3
aspartame/β-CyD	0.9	6.0	13.4	24.1

The calculated half-life of aspartame in 4 mM phosphate buffer containing aspartame was 69 days, while the half-life of aspartame in 4 mM phosphate buffer containing β-cyclodextrin was 98 days. Thus, the presence of β-cyclodextrin at a 5:1 molar ratio to aspartame yields a 42% improvement in aspartame stability. The stability enhancement was less in the cola beverage but still significant. In this case, the half-life of aspartame was 64 days, whereas the half-life with β-cyclodextrin present was 84 days. This represents a 31% enhancement of aspartame stability. The smaller stability enhancement in the cola beverage can be attributed to the other ingredients present in the beverage such as flavors and benzoate, which likely compete with aspartame for inclusion within the β-CyD cavity. The results clearly show, however, that the stability of aspartame can be significantly improved at pH 3.1 and at conditions typical of diet cola formulations.

The resulting concentrations of both diketopiperizine and α-L-aspartyl-L-phenylalanine formed over time in both 4 mM phosphate and cola beverage directly correspond to the rate of aspartame degradation in the two solutions prepared with and without β-cyclodextrin. It is interesting to note that the concentration differences of the two degradation products demonstrate that the rate of cyclization and ester hydrolysis reactions are both reduced in the presence of β-cyclodextrin. On the basis of the prevailing model (*8*−*10*) that β-CyD/aspartame complexation occurs via the inclusion of the phenyl ring within the β-CyD cavity, it is expected that a decrease in the rate of formation of diketopiperizine should take place, because formation of the inclusion complex in this manner would prohibit intramolecular cyclization. However, the model cannot entirely account for the concomitant decrease of α-L-aspartyl-L-phenylalanine formation because the methyl ester group is expected to still be exposed to the solution environment. Thus, additional elucidation of how the formation of the complex between β-cyclodextrin and aspartame takes place in solution is necessary.

NMR Characterization. *Solid-State NMR.* Our first objective was to determine the existence of the β-CyD/aspartame and β-CyD/neotame complexes in the solid

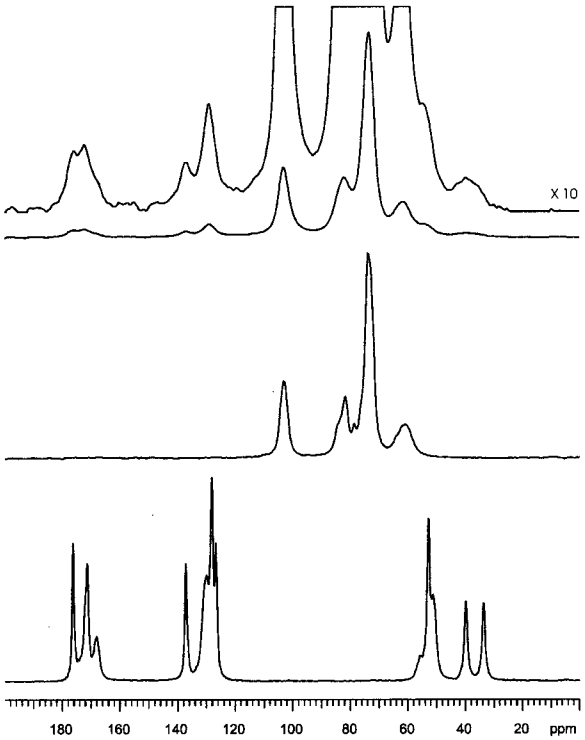

Figure 1. CPMAS ^{13}C NMR spectra at 31.9 MHz of aspartame (bottom), β-cyclodextrin (middle), and a 2.5:1 β-CyD/aspartame inclusion complex (top). Spectra were collected following 2-ms matched, 50-kHz $^{1}H-^{13}C$ cross-polarization contacts, with high-power proton decoupling [$\gamma B_2(H)/2\pi = 65$ kHz] and magic-angle spinning at 3 kHz. Residual spinning sidebands in the spectrum of aspartame were suppressed using the TOSS method (*15*).

state. Aspartame is well-known to crystallize in different polymorphic forms, and solid-state ^{13}C NMR data are sensitive to the crystal structure differences. Munson et al. (*19*) have studied aspartame polymorphs in detail using stable isotope labeling and solid-state NMR detection. Figure 1 (bottom) shows the solid-state ^{13}C NMR spectrum of aspartame, form I. Major resonances in this spectrum correspond well with aspartame's solution-state ^{13}C NMR spectrum and can be assigned to carboxyl (δ_c 165−180), aromatic (δ_c 125−140), methoxy/α-carbon (δ_c 48−60), and methylene (δ_c 30−45) carbons, respectively.

Figure 1 (middle) shows the solid-state ^{13}C NMR spectrum of β-cyclodextrin. The four major signals observed in the β-CyD spectrum are assigned to the different sugar carbons in the glucose subunits of the cyclodextrin as follows: C_6 (δ_c 60); C_2, C_3, C_5 (δ_c 72); C_4 (δ_c 80), and anomeric C_1 (δ_c 101). Figure 1 (top) shows the solid-state NMR spectrum of a 2.5:1 β-CyD/aspartame inclusion complex. Well-resolved aspartame resonances are clearly seen in the vertical expansion of this figure. The line widths of these aspartame signals are considerably greater than in the spectra of pure aspartame, suggesting aspartame molecules are in a less ordered, more amorphous environment, an observation consistent with the formation of a true inclusion complex.

To investigate the interaction of β-cyclodextrin and aspartame further, proton rotating-frame relaxation experiments, $T_{1\rho}(H)$, were carried out on a series of β-CyD/aspartame complexes ranging in stoichiometry from 5:1 to 1:1. $T_{1\rho}(H)$ is sensitive to kilohertz-regime motions and has been used extensively to characterize

Table 2. Solid-State $T_{1\rho}(H)$ for Aspartame, β-Cyclodextrin, and Aspartame/β-Cyclodextrin Complexes

	$\langle T_{1\rho}(H)\rangle$, ms				
	β-CyD resonances				aspartame
sample	60 ppm	73 ppm	81 ppm	102 ppm	128 ppm
aspartame					
crystalline					95
processed					45
β-CyD					
as received	7.8	7.4	7.5	7.4	
processed	6.5	5.7	6.0	6.1	
β-CyD/aspartame					
5:1	4.5	4.5	4.4	4.3	4.5
2.5:1	4.1	4.0	4.1	4.1	3.8
1:1	3.8	3.9	3.7	3.9	4.1

motions in polymers and to provide information about domain formation and phase separation in solids (*20*−*23*). In solids, efficient proton−proton communication among protons that are proximate in space, a process referred to as *spin diffusion*, causes them to behave as a single spin reservoir, characterized by a single, averaged, $\langle T_{1\rho}(H)\rangle$. This averaging has been used to assess the quality and degree of mixing of a wide variety of polymer blends. In contrast, the measurement of two or more different $\langle T_{1\rho}(H)\rangle$ values in a solid sample indicates the presence of distinct proton spin reservoirs that are separated from one another in space. As described under Materials and Methods, $\langle T_{1\rho}(H)\rangle$ is measured by monitoring the decay of the ^{13}C signal as a function of the $^{13}C-^{1}H$ contact time (τ) in a series of CPMAS experiments. Table 2 summarizes $T_{1\rho}(H)$ results for samples of β-cyclodextrin, aspartame, and the series of β-CyD/aspartame inclusion complexes.

Aspartame is a small, crystalline molecule anticipated to have relatively little low-frequency, cooperative molecular motion. The $\langle T_{1\rho}(H)\rangle$ for pure aspartame (line 1), which is nearly 100 ms, reflects the near absence of these motions. In contrast, $\langle T_{1\rho}(H)\rangle$ for β-cyclodextrin (line 3), an oligomeric molecule, is more than an order of magnitude shorter, reflecting efficient and cooperative polymer chain motions. $T_{1\rho}(H)$ results for three different β-CyD/aspartame complexes are reported on lines 5−7 of Table 2. These data show a dramatic shortening of $\langle T_{1\rho}(H)\rangle$ for aspartame in the inclusion complexes. The near equality of $\langle T_{1\rho}(H)\rangle$ as measured through the aspartame and β-CyD carbon signals reflects efficient *spin diffusion* between the protons on these two species. Further support for this conclusion comes from the $T_{1\rho}(H)$ measurements shown on lines 2 and 4 of Table 2, for which the measurements were carried out on samples of aspartame and β-cyclodextrin, processed as per the inclusion complex but in the absence of one another. For aspartame, $\langle T_{1\rho}(H)\rangle$ is approximately half the value for native, crystalline aspartame, reflecting changes in the aspartame dynamics induced by the processing conditions (e.g., changes in total aspartame crystallinity or crystal structure). Small differences in $\langle T_{1\rho}(H)\rangle$ for β-cyclodextrin signal small changes in molecular dynamics for the sugar rings and are probably due to minor changes in conformation or water content induced by processing. Still, there is a difference of ∼8 times in the value of $\langle T_{1\rho}(H)\rangle$ between aspartame and β-cyclodextrin that is erased by *spin diffusion* in β-CyD/aspartame inclusion complexes. This result is consistent with the formation of β-CyD/aspartame complexes and contrary to what would be expected for a cocrystallized mixture of these two solids. The relaxation curves for

Table 3. Solid-State $T_{1\rho}$(H) for Neotame and Neotame/ β-Cyclodextrin Complexes

	⟨$T_{1\rho}$(H)⟩, ms				
	β-CyD resonances				neotame
sample	60 ppm	73 ppm	81 ppm	102 ppm	29 ppm
neotame					
crystalline					21
processed					12
β-CyD/neotame					
5:1	6.1	4.8	5.8	5.1	5.9
2.5:1	6.8	5.6	6.1	6.0	8.8
1:1	6.7	6.1	6.4	6.2	7.9

the aspartame signals in these complexes (data not shown) all display single-exponential decay, indicating there is only a single population of aspartame molecules. If any of the samples were mixtures of inclusion complex and uncomplexed aspartame, we would have observed biexponential decay of the carbon signal.

Neotame can also crystallize in multiple polymorphic forms. CPMAS ^{13}C NMR spectra of neotame (data not shown) are consistent with the results reported in a recent NMR study of neotame solid-state structures by Munson (*24*). Table 3 shows results of similar experiments conducted on samples of neotame and β-CyD/neotame inclusion complexes. $T_{1\rho}$(H) data for β-CyD/neotame inclusion complexes are consistent with those observed for the aspartame complexes. ⟨$T_{1\rho}$(H)⟩ for crystalline neotame is ∼20 ms and shortens to 12−15 ms in neotame processed as in the preparation of inclusion complexes. A further shortening of ⟨$T_{1\rho}$(H)⟩ is observed for neotame in the β-CyD/neotame inclusion complexes, where *spin diffusion* again causes the values measured for β-cyclodextrin and neotame to approach a common value. Again, as with the aspartame complexes, the relaxation curves for neotame are single exponential, reflecting a single population of neotame molecules in the complexes.

Solution-State NMR. Figure 2 (top) shows the solution-state ^{1}H NMR spectrum of aspartame, and Figure 3 displays expansions of the ^{1}H NMR spectra of aspartame, β-cyclodextrin, and a mixture of β-cyclodextrin and aspartame. Upon complexation, upfield shifts are observed for the H3 and H5 protons of β-cyclodextrin and downfield shifts for the Asp αH and βH and for Phe βH protons. Each proton yields only a single set of resonances, and the spectral shifts indicate the existence of a β-CyD/aspartame complex in fast exchange with free β-cyclodextrin and aspartame. The upfield shifts of the H3 and H5 protons, which are located within the β-CyD cavity, are due to shielding effects induced by aromatic ring-current anisotropy of the phenylalanine and indicate the phenyl ring is included in the β-CyD cavity. The proton shifts of the aspartame α and β protons induced by β-cyclodextrin may be due predominately to changes in the microenvironment of these protons or to conformational changes of the peptide backbone upon complexation, because the shielding tensors of the alicyclic β-CyD skeleton are weak.

Although changes in chemical shifts provide unambiguous evidence for complex formation, ROE experiments provide a powerful approach for determining the geometry of β-CyD complexes. ROE data, presented in Figure 4, show that the site of interaction is the phenylalanyl ring. Intermolecular ROE cross-peaks are observed between both the H3,5 and H2,6 protons of the phenyl ring and the H3 and H5 protons of β-cyclodextrin. In addition, weaker ROEs are seen between the

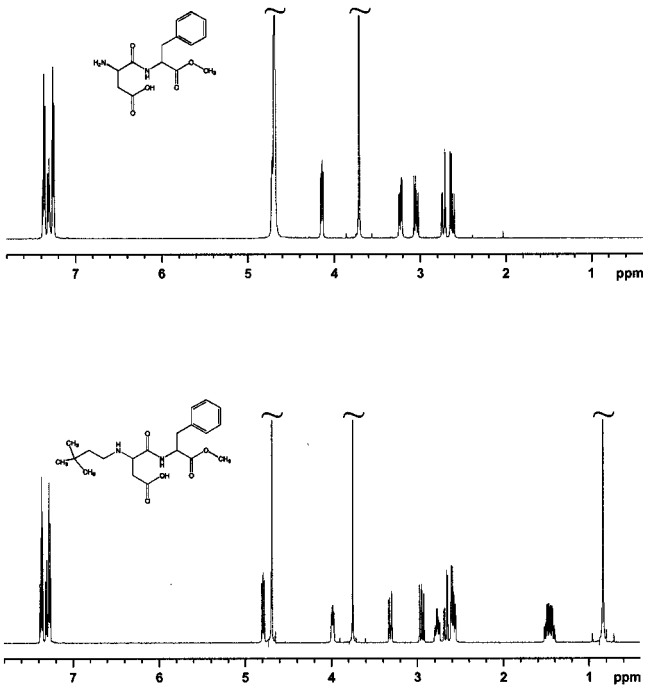

Figure 2. High-resolution ^{1}H NMR spectra of 15 mM solutions, in D_2O, of aspartame (top) and neotame (bottom): (top) ^{1}H spectrum of aspartame in D_2O (assignments: phenyl $H_{3,5}$, δ 7.37, t, 2H, J = 7.5 Hz; phenyl H_4, δ 7.31, t, 1H, J = 7.5 Hz; phenyl $H_{2,6}$, δ 7.26, d, 2H, J = 7.5 Hz; Phe αH, δ 4.72, q, ^3J = 5.8 Hz, ^3J = 8.9 Hz; Asp αH, δ 4.14, q, 1H, ^3J = 5.2 Hz, ^3J = 8.1 Hz, −OCH_3, δ 3.71, s, 3H; Phe βH2, δ 3.23, q, 1H, ^2J = 14.1 Hz, ^3J = 5.8 Hz; Phe βH1, δ 3.04, 1H, q, ^2J = 14.1, ^3J = 8.9 Hz; Asp βH2, 2.73, 1H,q, ^2J = 17.4 Hz, ^3J = 5.2 Hz; Asp βH1, δ 2.63, q, 1H, ^2J = 17.4 Hz, ^3J = 8.1 Hz); (bottom) ^{1}H spectrum of neotame in D_2O [assignments: phenyl $H_{3,5}$, δ 7.37, t, 2H, J = 7.6 Hz; phenyl H_4, δ 7.30, t, 1H, J = 7.6 Hz; phenyl $H_{2,6}$, δ 7.27, d, 2H, J = 7.6 Hz; Phe αH, δ 4.79, q, 1H, ^3J = 4.8 Hz, ^3J = 10.4 Hz; Asp αH, δ 3.98, q, 1H, ^3J = 4.9 Hz, ^3J = 8.7 Hz, −OCH_3, δ 3.75, s, 3H; Phe βH2, δ 3.31, q, 1H, ^2J = 14.0 Hz, ^3J = 4.8 Hz; Phe βH1, δ 2.92, 1H, q, ^2J = 14.0, ^3J = 10.4 Hz; Asp βH2, 2.67, 1H, q, ^2J = 17.4 Hz, ^3J = 4.9 Hz; Asp βH1, δ 2.58, q, ^2J = 17.4 Hz, ^3J = 8.7 Hz; dmb αH2, δ 2.77, m, 1H, ^2J = 11.9 Hz, ^3J = 11.9 Hz, ^3J = 5.6 Hz; dmb αH1, δ 2.58, m, ^2J = 11.9 Hz, ^3J = 11.9 Hz, ^3J = 5.2 Hz; dmb βH, δ 1.46, m, 2H; dmb $(CH_3)_3$, δ 0.84, s, 9H].

phenylalanyl βHs and the H3 protons of β-cyclodextrin and may indicate deep penetration of the ring into the β-CyD cavity. Because the only intermolecular ROEs observed are between the phenyl ring and β-cyclodextrin, the NMR data clearly suggest a 1:1 stoichiometry for the aspartame/β-CyD inclusion complex.

The 1:1 stoichiometry was verified by the method of continuous variation. The data (not shown) were symmetrical with a maximum at a molar fraction of 0.5. Figure 5 shows the effect of diluting an equimolar solution of β-cyclodextrin and aspartame on the ^{1}H NMR spectra of the solution. Nonlinear regression analysis of the observed shifts for the αH and βH protons of the aspartyl residue, the βH protons of the phenylalanyl residue, and the H3 and H5 of β-cyclodextrin, based upon a single-site binding model, yielded an association constant, K_a, of 149.2 ± 19.7 M^{-1}. T_1(H) and T_2(H) values were measured for aspartame, β-cyclodextrin, and an equimolar mixture of aspartame and β-cyclodextrin. In general, both relaxation times were shorter for aspartame in the mixture compared to those for the pure compound, whereas little change was seen in the relaxation times of the β-CyD protons. For example, T_1(H) for Asp αH decreased from 2.20 s for free aspar-

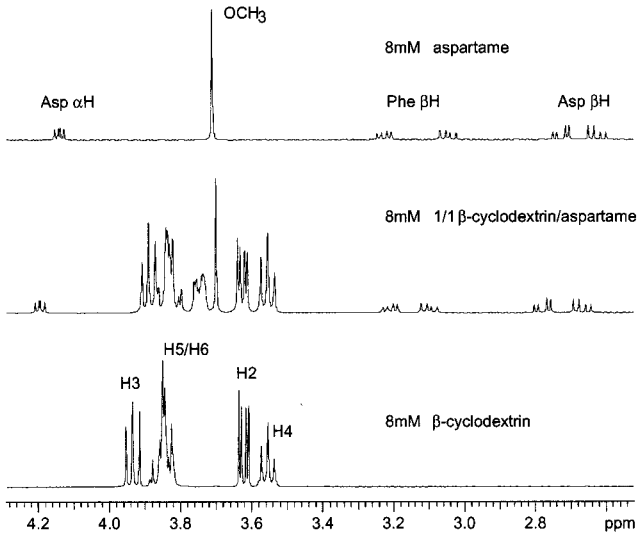

Figure 3. Solution-state ^{1}H NMR spectra of pure aspartame (top), pure β-cyclodextrin (bottom), and an 8 mM equimolar mixture of aspartame and β-CyD (middle).

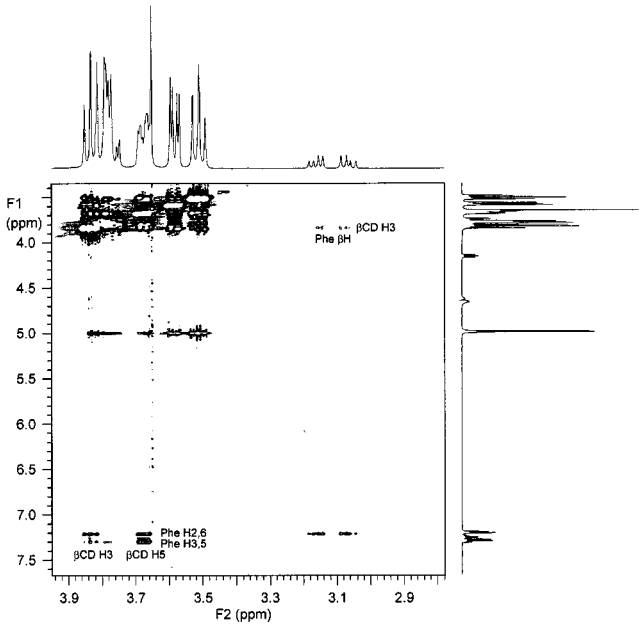

Figure 4. Expanded ROE spectrum of a mixture of 16 mM aspartame and 4 mM β-cyclodextrin. Annotated cross-peaks indicate intermolecular interactions within the aspartame/β-CyD complex. ROE data were obtained with a 300-ms mixing time.

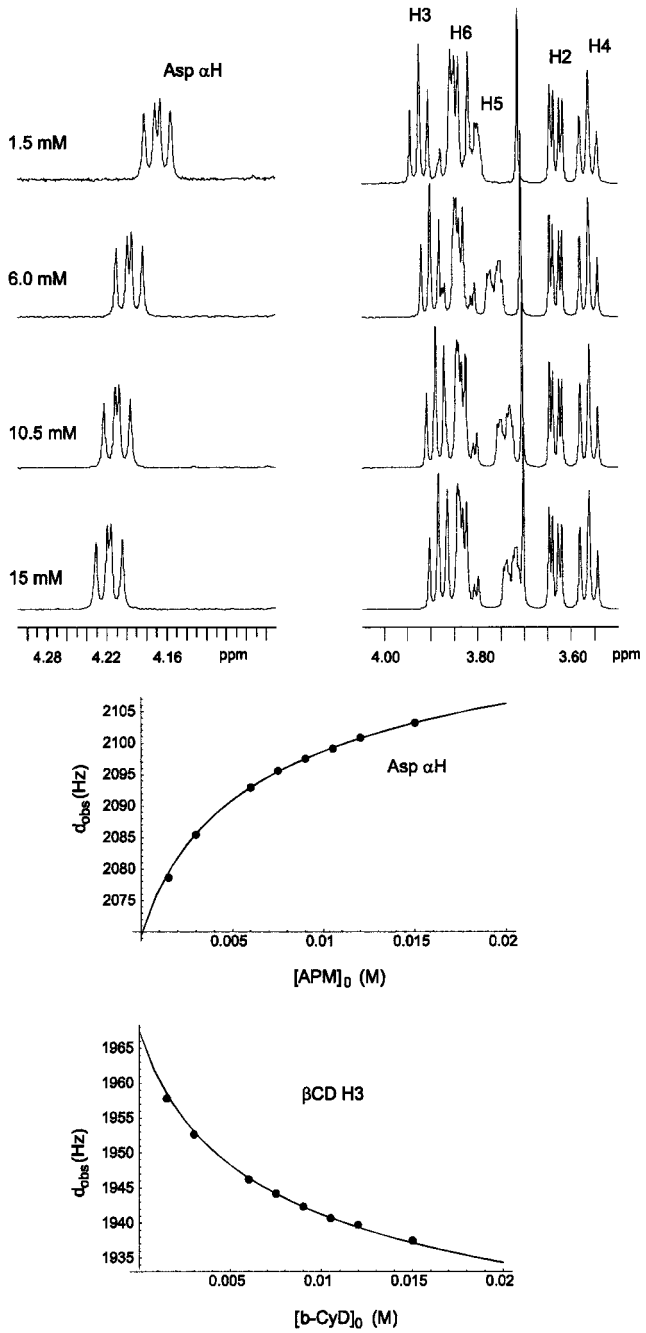

Figure 5. Solution-state ^{1}H NMR spectra and observed line positions as a function of dilution for an equimolar mixture of aspartame and β-cyclodextrin (initial, total concentration = 15 mM).

tame to 1.17 s upon complex formation; T_2(H) decreased from 1.17 to 0.86 s. Decreases in T_1(H) and T_2(H) are consistent with an increase in the average rotational correlation time of aspartame due to formation of an inclusion complex in solution. Because the measured T_1 and T_2 values are a mole-fraction-weighted average of the respective free and bound relaxation times, complex formation will have little effect on the relaxation values for β-cyclodextrin. In addition to chemical shifts and relaxation times, changes in the vicinal coupling constants were observed upon formation of the inclusion complex that reflect changes in the relative rotamer populations about the torsional angle χ_1. Upon complex formation, a decrease in the t^2g^3 ($\chi_1 = -60°$) and an increase in the g^2t^3 ($\chi_1 = 180°$) conformer populations were observed for both the aspartyl and phenylalanyl side chains, with the changes being greater for phenylalanine. For both residues, a smaller decrease was seen for the g^2g^3 ($\chi_1 = 60°$) conformer. These changes are similar to those observed by Takahashi (*9*).

Figure 2 (bottom) shows the ^{1}H NMR spectrum of neotame. Dilution of an equimolar solution of β-CyD/neotame (data not shown) led to similar chemical shift changes as observed for β-CyD/aspartame solutions. Again, signals from H3 and H5 of β-cyclodextrin shifted upfield at higher degrees of association, indicating formation of inclusion complexes in fast exchange with free β-cyclodextrin and neotame. The ROE spectrum shown in Figure 6 indicates that, in addition to deep penetration of the phenyl ring into the wide end of the β-CyD cavity, the 3,3-dimethylbutyl (dmb) group on the opposite end of the neotame molecule also interacts with β-cyclodextrin. ROE cross-peaks are observed between

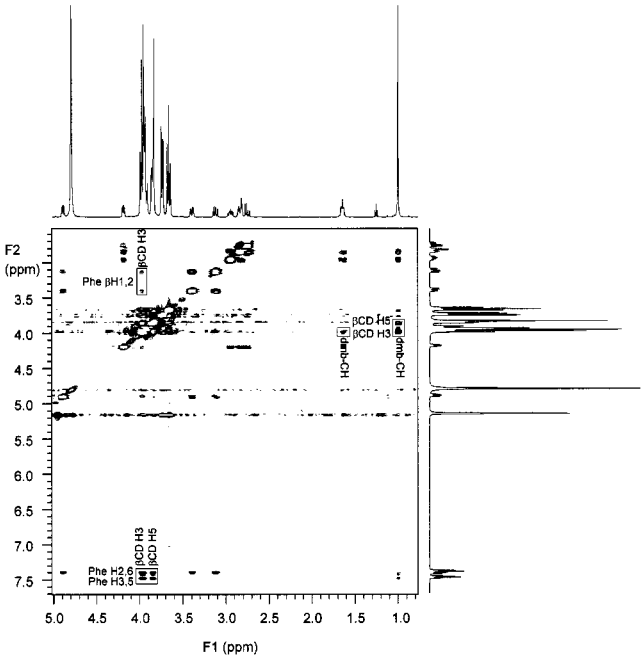

Figure 6. ROE spectrum of a 15 mM equimolar mixture of neotame and β-cyclodextrin. Cross-peaks within the boxed regions indicate intermolecular interactions within the complex. ROE data were obtained with a 300-ms mixing time.

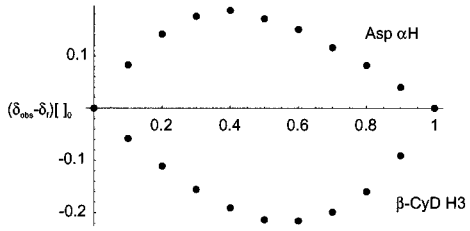

Figure 7. Continuous variation plot of neotame/β-cyclodextrin, showing the observed chemical shifts of Asp αH (APM) and H3 (β-CyD) as a function of mole fraction.

the dmb methyl protons and H3/H5 of β-cyclodextrin and between the β-methylene protons of the dmb group and H3 of β-cyclodextrin. Although the ROE data indicate that neotame has two sites of interaction with β-cyclodextrin, it could not be conclusively determined whether a ternary 2:1 β-CyD/neotame complex or two 1:1 binary complexes existed. The average stoichiometry of the complex was determined by the method of continuous variation, and the results are shown in Figure 7. The asymmetrical shapes of the curves, with maxima at molar fraction values other than 0.5, indicate a non-1:1 stoichiometry, suggesting that a fraction of the inclusion complexes exist as a 2:1 β-CyD/neotame complex. The measured T_1 and T_2 values for free neotame and β-cyclodextrin, and for an equimolar mixture of neotame and β-cyclodextrin, are consistent with complex formation in that a decrease in the values was observed upon complex formation. Changes were also observed in the relative rotamer populations upon complex formation. As for the aspartame/β-CyD complex, a decrease in the t^2g^3 conformer and an increase in the g^2t^3 conformer populations were seen for the phenylalanyl residue, although little change was observed for the aspartyl residue.

The interaction of 3,3-dimethylbutylamine **(**HCl), a model for neotame's dmb group, with β-cyclodextrin was also studied by the method of continuous variation. Upon complex formation with β-cyclodextrin, upfield shifts were observed for H3 and H5 of β-cyclodextrin, and downfield shifts were seen for the methyl and β-CH_2 of dimethylbutylamine. The continuous variation plot was symmetrical, with a maximum at a molar fraction of 0.5, indicating a 1:1 inclusion complex. The K_a, as determined from these data, was $\sim$270 M^{-1}. The T_1 values for the dmb protons were shorter in the complex compared to free dmb: α-CH_2, 1.42 s (complex), 3.10 s (free); β-CH_2, 1.37 s (complex), 3.14 s (free); CH_3, 1.09 s (complex), 2.35 s (free).

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Supporting Information Available: Tables of T_1(H), T_2(H), vicinal coupling constants, and rotamer populations for aspartame, neotame, and their respective inclusion complexes with β-cyclodextrin (*25*). This material is available free of charge via the Internet at http://pubs.acs.org.

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