Conformational Analysis of the Dipeptide Sweetener Alitame and Two Stereoisomers by Proton NMR, Computer Simulations, and X-ray Crystallography

Robert D. Feinstein,[†] Alexander Polinsky,[†] Alastair J. Douglas,[†] C. Magnus G. F. Beijer,[†] Raj K. Chadha,[†] Ettore Benedetti,[‡] and Murray Goodman*,[†]

Contribution from the Department of Chemistry, B-043, University of California, San Diego, La Jolla, California 92093, and Department of Chemistry, University of Naples, 80134 Naples, Italy. *Received October 17, 1990*

Abstract: The conformational preferences of the dipeptide sweetener alitame (L-aspartyl-D-alanine 2,2,4,4-tetramethylthietanylamide) and the related L,L and D,D stereoisomers of alitame were investigated by using high field proton NMR and computer simulations. In addition, the crystal structure of the L,D stereoisomer (alitame) was determined with a final R index of 0.079. The preferred conformations in DMSO of the intensely sweet L,D stereoisomer (alitame) can be described as possessing an "L shape", with the A–H and B containing aspartyl moiety as the stem of the L and the hydrophobic four-membered thietane ring as the base of the L, coplanar with but nearly perpendicular to the zwitterionic ring. These conformations were in agreement with those observed in the crystal structure, with only one backbone dihedral angle of the dipeptide differing somewhat (D-Ala ψ). For the L,L stereoisomer of alitame in solution, the thietane ring system projects beyond the plane of the zwitterionic ring, behind the stem of the "L" shaped peptide backbone. In contrast, the D,D stereoisomer of alitame shows considerable projection of the thietane ring system in a direction opposite of the enantiomeric L,L stereoisomer (i.e., in front of the L). The tastes of these molecules (i.e., the L,D, L,L, and D,D stereoisomers; sweet, bitter, and tasteless, respectively) are correctly predicted by our model for sweet taste developed with previous aspartyl-based peptide sweeteners. This work supports the notion that, in the case of aspartyl-based taste ligands, conformations in solution and in the crystal are closely related to each other.

Introduction

A stereoisomeric approach to the investigation of the molecular basis of taste was carried out with use of high field NMR techniques and flexible geometry energy minimizations to examine the intensely sweet dipeptide derivative alitame[1] (L-aspartyl-D-alanine 2,2,4,4-tetramethylthietanylamide) and the related bitter L,L and tasteless D,D stereoisomers of alitame. The conformations of the L,D stereoisomer were then determined by X-ray crystallography and compared to the conformations of the molecule determined in solution and in vacuo. Previous work based on L-aspartylalanine and retroalanine tetramethylcyclopentanyl amides[2,3] resulted in a proposed model describing the molecular features required for sweet taste. The inclusion of alitame into the family of peptide-based sweeteners studied thus far serves to strengthen our model.

Investigations into the molecular basis of sweet taste resulted in a model in which the essential components of the glucophore were shown to involve an electronegative atom (B), a polarized system (A–H), and a third hydrophobic moiety (X).[4-6] The aspartyl peptide sweeteners contain the putative A–H and B elements in the zwitterionic N-terminal aspartyl residue as the NH_3^+ and β-carboxyl groups. Their distances from one another when participating in the formation of a six-membered zwitterionic ring are in accordance with A–H to B distances found in many non-peptide sweet-tasting molecules (2.5–4.0 Å). The model developed from our work on retro-inverso and dipeptide amides incorporates this zwitterionic ring.[2,7] The overall conformations of the various sweet-tasting analogues can be described as possessing an L shape, with the A–H and B containing aspartyl moiety as the stem of the L and a hydrophobic moiety X as the base of the L, coplanar with but nearly perpendicular to the zwitterionic ring. The model fits the X-ray diffraction structure of the dipeptide sweetener aspartame (X = phenyl ring) with only a minor modification of the conformation (a 40° rotation about ϕ_{Phe}).[8] Similarly, the recently reported structures of the cocrystallized N-(L-aspartyl-N'-[(2,2,5,5-tetramethylcyclopentanyl)carbonyl]-(R and S)-1,1-diaminoethane dipeptide sweeteners exhibit this L shape after modification about the torsion corresponding to $\phi_{residue2}$.[9] Bitter-tasting analogues show characteristic conformations with significant extension of the X moiety onto the $-z$ axis. Tasteless analogues show extension of the X moiety onto the $+z$ axis. Our work involving retro-inverso and dipeptide amides incorporated a 2,2,5,5-tetramethylcyclopentanyl (TMCP) group as the X group. Alitame contains a nearly planar 2,2,4,4-tetramethylthietane ring (TAN) as the X group, providing a contrasting steric effect and degree of polarizability to the five-membered TMCP group (Figure 1).

With the previously studied retro-inverso and dipeptide amide sweeteners incorporating the TMCP moiety, NMR investigation showed the predominant aspartyl rotamer to be gauche$^-$. In assessing the contribution of various theoretically derived minimum energy conformers to the observed conformations in solution, nongauche$^-$ conformers were not considered. The existence of the gauche$^-$ zwitterionic ring in an N-terminal aspartyl residue of the L chirality prevents the β-carboxylic acid group from interacting significantly with the amides of the second and third residues in a di- or tripeptide. While this is consistent with experimental observations of previously studied sweeteners, there is no theoretical justification for excluding all nongauche$^-$ conformations. This is especially the case if one wishes to compare the relative energies of all possible minimum energy conformations that may be contributing to a dynamic equilibrium. For this reason, in this work there was no a priori assumption of a gauche$^-$ aspartyl residue. Thus, we indicated gauche$^+$ and trans conformers as possible contributing minima.

* To whom correspondence should be addressed.
† University of California, San Diego.
‡ University of Naples.

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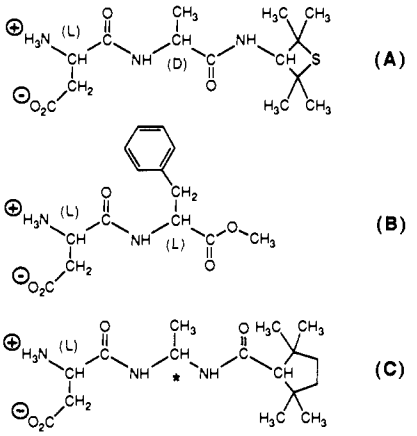

Figure 1. The structure of (A) L-aspartyl-D-alanine 2,2,4,4-tetramethylthietanylamide (alitame), (B) aspartame, and (C) *N*-(L-aspartyl)-*N'*-[(2,2,5,5-tetramethylcyclopentanyl)carbonyl]-1,1-diaminoethane.

Experimental Section

The conformational preferences of alitame and the L,L stereoisomer of alitame were investigated by using ^{1}H NMR and computer simulations (using DISCOVER).[10-14] (In exploring the conformational preferences of the L,L and D,D enantiomers, preliminary energy minimizations and NMR chemical shift comparison revealed that the two molecules show mirror-image behavior. Hence, only the bitter L,L stereoisomer was subjected to further conformational analysis, with the results appropriately modified for the tasteless D,D species.)

Computer Simulations. Computer analysis of the two stereoisomers consisted of an extensive search for minimum energy conformations, including the global minimum energy conformations for both stereoisomers. This was accomplished in a stepwise fashion, starting with smaller model compounds (described below) and working toward the alitame structure. All minimizations were performed with a quasi Newton–Raphson minimization until the maximum derivative was less than 0.001 kcal/mol Å. Morse bond stretching functions were used, and all calculations included full cross terms. The default dielectric constant of 1.0 was used for all calculations in vacuo.

Flexible geometry energy minimizations for the model compound Asp-NH-Me were carried out. The dihedral angles ϕ_{Asp}, ω_{Asp}, $\chi_{2(Asp)}$, and $\phi_{N\text{-}Me}$ were each set to an initial value of 180°. Initial conformations were then generated by varying ψ_{Asp} and $\chi_{1(Asp)}$ in increments of 30°, resulting in 144 initial conformations. Each conformer was then minimized with a forcing potential of 100 kcal/rad^2 about ψ_{Asp} and $\chi_{1(Asp)}$ to prevent rotation. From the resulting 144 minimized structures the 12 lowest in energy (from −58.321 to −54.203 kcal/mol) were subjected to unconstrained minimization, resulting in two minimum energy conformations. The lowest energy conformer was gauche$^+$ with dihedral angles of $\chi_{1(Asp)} = 57.7°$, $\chi_{2(Asp)} = -67.5°$, and $\psi_{Asp} = -104.6°$. The remaining minimum energy conformer, 0.920 kcal/mol higher in energy, was gauche$^-$ with dihedral angles of $\chi_{1(Asp)} = -63.8°$, $\chi_{2(Asp)} = 64.3°$, and $\psi_{Asp} = 159.5°$.

Flexible geometry energy minimizations of the model compound Ac-2,2,4,4-tetramethylthietane (Ac-TAN) were initiated from the starting geometry of a MM2 derived structure. The Ac-TAN model compound was subjected to further minimizations where the dihedral angle ϕ_{TAN} was varied by increments of 10°. After unconstrained minimizations, two minima were found corresponding to $\phi_{TAN} = 38.7°$ and −38.7°, where ϕ_{TAN} is defined as C′–N–Cα–Hα.

Determination of various local minima and the global minima for L,D-alitame and its L,L stereoisomer was achieved as follows. Given the two minima found for both model compounds Asp-NH-Me and Ac-TAN, four possible combinations exist. For each combination, a set of initial conformations was generated by varying ϕ_{Ala} and ψ_{Ala} by 30° increments, resulting in 144 initial conformations for each set. Each conformer was then minimized with a forcing potential of 100 kcal/rad^2 about ϕ_{Ala} and ψ_{Ala} to prevent rotation. From the resulting four sets of 144 minimized structures the 12 lowest in energy from each set (48 in total, from −31.145 to −35.825 kcal/mol) were subjected to unconstrained minimization.

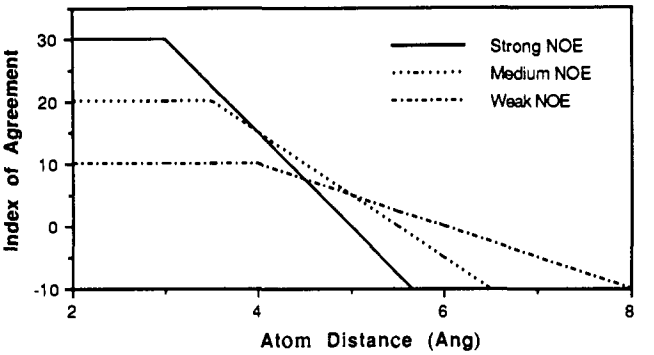

Figure 2. The index of agreement (IA) scale devised for assessing the agreement between calculated conformations and observed NOEs. Atom distances are determined as the minimum distance between sets of protons for which an NOE was observed.

Proton NMR Spectroscopy. Proton NMR spectra were obtained on a General Electric GN-500 spectrometer operating at 500 MHz. All experiments were carried out at a concentration of 4.0 mg/mL in DMSO-d_6 (MSD Isotopes). The L,D and L,L stereoisomers were pure by ^{1}H NMR. With the exception of the variable-temperature experiments, all spectra were recorded at 30 °C. One-dimensional NOEs were obtained with a variable length of decoupling (0.5–4 s) during a 10-s fixed delay time. Two-dimensional NOEs were obtained by using the rotating frame experiment originally proposed by Bothner-By (CAMELSPIN, more commonly referred to as ROSEY).[15] Linear buildup rates were observed in spectra carried out in the absorptive mode with mixing times of 50–400 ms with a relatively weak spin-locking field of 2 kHz, so as to suppress Hartmann–Hahn transfer.[16] The experiments consisted of 128 t_1 values with 48 scans of 1K data points. Gaussian multiplication and zero filling in t_1 were applied to result in a 1K × 1K data set.

The populations of the rotamers about the Cα–Cβ bond (χ_1) were estimated from the observed coupling constants ($J_{\alpha\beta_1}$ and $J_{\alpha\beta_2}$) of the Asp residue by using the expressions

$$f(t) = (J_{\alpha\beta_2} - J_G)/(J_T - J_G)$$

$$f(g^-) = (J_{\alpha\beta_1} - J_G)/(J_T - J_G)$$

$$f(g^+) = 1 - [f(t) + f(g^-)]$$

where $f(t)$, $f(g^-)$, and $f(g^+)$ are the fractions of the individual conformers trans, gauche$^-$, and gauche$^+$, respectively.[17] Following Pachler,[18] values of $J_T = 13.56$ and $J_G = 2.60$ Hz were used for the trans and gauche couplings, respectively.

In order to assess the agreement between the experimentally observed NOEs and the various theoretically determined minimum energy conformations in a more quantiative manner, a simple point system was devised. The NOEs were assigned as strong, medium, or weak relative to one another. For each minimum energy conformation, the shortest distances between atom sets corresponding to observed NOEs were calculated. Indices of agreement (IA) were assigned in a cumulative fashion for all NOEs. The IA assigned to a conformation for any given NOE were determined by

$$IA = P_{max} \quad (\text{for } r_{calculated} < r_{optimal})$$

$$IA = (P_{max})(1 - 0.5(r_{calculated} - r_{optimal})) \quad (\text{for } r_{calculated} \geq r_{optimal})$$

where P_{max} and $r_{optimal}$ equal 30, 20, or 10 and 3.0, 3.5, or 4.0 Å for a strong, medium, or weak NOE, respectively. This is illustrated in Figure 2. As a consequence, conformations with even one severely inappropriate distance corresponding to an NOE were penalized significantly. The cumulative index of agreement for each coordinate set was then multiplied by the fraction of NOEs it satisfied. Satisfaction was defined as containing an atom pair corresponding to an NOE at a distance no

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Table I. Minimum Energy Conformations of L-Aspartyl-D-alanine 2,2,4,4-Tetramethylthietanylamide

no.	ΔE_{total}[a] (kcal/mol)	torsion angles (deg) ψ_{Asp}	$\chi_{1(Asp)}$	ϕ_{Ala}	ψ_{Ala}	ϕ_{TAN}[b]	lett. code D-Ala[c]	NOE index
LD1	0.000	−73	59	89	42	−30	A*	100
LD2	5.051	−116	59	79	−79	43	C*	97
LD3	5.562	151	−63	78	−87	42	C*	90
LD4	5.622	−118	60	80	76	−34	C*	82
LD5	6.111	151	−63	80	−84	−33	C*	63
LD6	8.444	164	−65	80	53	−43	A*	88
LD7	8.732	164	−65	82	55	39	A*	54

[a] Relative to LD1. [b] TAN represents 2,2,4,4-tetramethylthietane for which torsion angle ϕ_{TAN} is defined as CO–N–C_α–H. [c] See Zimmerman et al.

Table II. Minimum Energy Conformations of L-Aspartyl-L-alanine 2,2,4,4-Tetramethylthietanylamide

no.	ΔE_{total}[a] (kcal/mol)	torsion angles (deg) ψ_{Asp}	$\chi_{1(Asp)}$	ϕ_{Ala}	ψ_{Ala}	ϕ_{TAN}[b]	lett. code Ala[c]	NOE index
LL1	0.000	−129	62	−77	−42	40	A	74
LL2	1.669	−126	62	−81	−43	−44	A	64
LL3	6.723	−80	55	−77	83	−45	C	90
LL4	7.288	−79	55	−78	82	32	C	78
LL5	7.927	154	−63	−81	79	−43	C	100
LL6	8.417	−78	54	−71	152	41	F	37
LL7	8.872	154	−63	−82	76	29	C	99
LL8	9.503	162	−65	−72	−44	43	A	91

[a] Relative to LL1. [b] TAN represents 2,2,4,4-tetramethylthietane, for which torsion angle ϕ_{TAN} is defined as CO–N–C_α–H. [c] See: Zimmerman et al.

greater than 1 Å further than the optimal distance. These distances were based on the observed intensity of the NOE signal relative to that betwen the two β-protons of Asp, incorporating the $(1/r^6)$ dependence of NOE intensity to interproton distance. Because the L,D stereoisomer exhibited more NOEs than the L,L stereoisomer (9 vs 14, respectively), the IA's were then normalized to allow interisomer comparison. After all quantitative NOE comparisons were completed, each minimum energy conformation was assessed further based on its agreement with other NMR data (i.e., J coupling constants and observed hydrogen bonding).

X-ray Structure Determination. Colorless, plate-like crystals of the L,D stereoisomer (alitame) were grown from isopropyl alcohol containing trace amounts of water by slow evaporation. Determination of cell constants, crystal system and symmetry, and data collection were carried out on a Siemens R3m/V four-circle diffractometer with Mo Kα radiation, by using a highly oriented graphite crystal in the range 4.0–45.0° of 2θ. A 2θ–θ scan type with variable speed (2.50–5.00 deg min^{-1}) and a scan range of 0.6° plus Kα separation was selected. Background counts were taken at the beginning and at the end of the scan. Three standard reflections were monitored every 200 reflections to detect crystal decay; no significant changes in their intensities was observed. The h,k,l ranges were 0 to 7, −12 to 12, and −25 to 25, respectively. A total of 5247 reflections were measured, 4574 of which had $F > 4.0\sigma(F)$ and thus were considered "observed".

The structure was determined by direct methods with the SHELXTL PLUS program (on a MicroVAX II computer). Full-matrix least-squares refinement minimizing the quantities $\sum w(F_o - F_c)^2$ and $1/w = \sigma^2(F) + 0.0005F^2$ was used. Sulfur, oxygen, and nitrogen atoms were refined anisotropically, while carbon atoms were refined isotropically. All hydrogen atoms were included in the final cycles of refinement with fixed thermal parameters of 0.08 Å^2. The total number of parameters refined was 550.

Results

Computer Simulations. For L,D-alitame, the 48 lowest energy structures converged to seven unique minima, as shown in Table I in order of increasing total energy. Interestingly, three of the four lowest energy final conformations were gauche$^+$ (LD1, LD2, and LD4). For the L,L stereoisomer of alitame, eight unique minima emerged, as shown in Table II. Similarly, five of the six lowest energy final conformations were gauche$^+$ (LL1–LL4 and LL6).

Proton NMR Spectroscopy. All resonances for both the L,L and L,D stereoisomer were assigned. The four methyl groups on the thietane ring could not be resolved individually. Nuclear Overhauser effects observed for alitame and its L,L stereoisomer are presented in Table III. Proton NMR amide temperature coefficients and coupling constants for the L,D and L,L stereoisomers of alitame are presented in Table IV. Experimentally, the NMR investigation of the L,D stereoisomer revealed that the predominant conformer about Aspχ_1 was gauche$^-$ (59%), with the remaining gauche$^+$ and trans nearly equal (22% and 19%, respectively). The NMR investigation of the L,L stereoisomer, likewise, revealed that the predominant conformer about Aspχ_1 was gauche$^-$ (≈61%), with the remaining gauche$^+$ and trans comprising 24% and 15%, respectively. The amide temperature coefficients for both amides (Ala/D-Ala and TAN) in both stereoisomers are too great to support the existence of a hydrogen bond involving either amide proton.

Table III. Nuclear Overhauser Effects for Alitame (L-Asp-D-Ala-2,2,4,4-Tetramethylthietanylamide) and Its L,L-Stereoisomer

Asp H_α	Asp H_β	D-Ala NH	D-Ala H_α	D-Ala H_β	(2,2,4,4)tetramethylthietane amide NH	(2,2,4,4)tetramethylthietane amide H_α	(2,2,4,4)tetramethylthietane amide H_γ	
M	W	0	M	0	S	S		(2,2,4,4)tetramethylthietane amide H_γ
W	M	M	0	W	S		S	(2,2,4,4)tetramethylthietane amide H_α
0	0	W	S	W		M	S	(2,2,4,4)tetramethylthietane amide NH
W	W	0	S		W	0	0	Ala H_β
W	0	0		S	S	0	M	Ala H_α
M	0		0	0	W	M	0	Ala NH
S		M	0	0	0	0	W	Asp H_β
	S	M	W	0	0	0	M	Asp H_α
H_α	H_β	NH	H_α	H_β	NH	H_α	H_γ	
Asp		L-Ala			(2,2,4,4)tetramethylthietane amide			

[a] S = strong, M = medium, W = weak, 0 = none.

Table IV. ^{1}H NMR Temperature Coefficients and Coupling Constants for the Stereoisomers of Alitame

	L,D stereoisomer	L,L stereoisomer
$NH_{Ala}\Delta\delta/\Delta T$	−6.7 ± 1.0 ppb/K	−8.0 ± 1.0 ppb/K
$NH_{TAN}\Delta\delta/\Delta T$[a]	−6.2 ± 1.0 ppb/K	−5.3 ± 1.0 ppb/K
$J_{Asp\alpha\beta_1}$	9.11 Hz	9.25 Hz
$J_{Asp\alpha\beta_2}$	4.66 Hz	4.25 Hz
$J_{Asp\beta_1\beta_2}$	−16.18 Hz	−16.00 Hz

[a] TAN = 2,2,4,4-tetramethylthietanylamide.

X-ray Structure Determination. The L,D stereoisomer (alitame) crystallized in the triclinic $P1$ space group with four independent molecules in the asymmetric unit along with ten cocrystallized water molecules. The final R index and goodness of fit were 0.079 and 2.90, respectively, with largest and mean Δ/σ of 0.114 and 0.001. Crystallographic data are reported in Table V. A list of relevant torsion angles for the four dipeptide units is given in Table VI. Final atomic parameters for all atoms including hydrogens and thermal parameters, bond distances, bond angles, torsion angles, and structure factor tables have been deposited and are available from the Cambridge Crystallographic Data Center.

The molecular packing within the asymmetric unit is depicted in Figure 3. The conformations adopted by the four independent alitame molecules allow for the formation of intermolecular hydrogen bonds. Every nitrogen-bound proton is observed to act

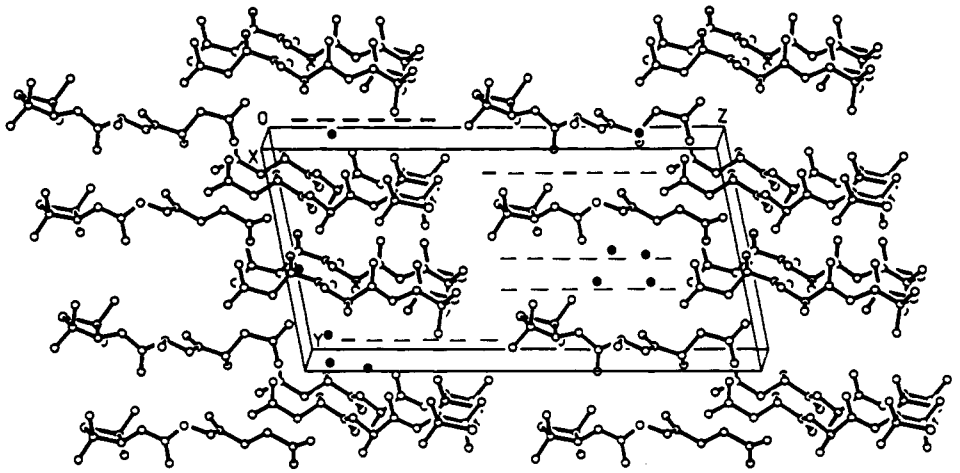

Figure 3. The molecular packing of alitame. Ten independent water molecules are indicated as crossed circles. The dotted lines indicate molecules of alitame that have been deleted to show clearly the positions of the water molecules.

Table V. Summary of Crystallographic Structure Determination for L-Aspartyl-D-alanine 2,2,4,4-Tetramethylthietanylamide

empirical formula	$C_{14}H_{25}N_3O_4S \cdot 2.5H_2O$
formula weight	376.5 g/mol
color, habit	colorless, plate-like
crystal size	0.11 × 0.42 × 0.69 mm
crystal system	triclinic
space group	$P\bar{1}$ [no. 1, C_i^1]
unit cell dimensions	
a =	7.258 (2) Å
b =	11.818 (2) Å
c =	23.823 (4) Å
α =	79.30 (2)°
β =	88.20 (2)°
γ =	85.54 (2)°
volume	2001.5 (7) Å^3
Z	4
density (calcd)	1.249 amu
density (exptl)	1.25 M g^{-3}

as a donor, as are the protons from the ten cocrystallized water molecules. Both the carboxylate and carbonyl oxygens from the aspartyl and alanyl residues and oxygens from all ten of the water molecules act as acceptors. The molecular packing perpendicular to the z axis consists of a double layer of alitame molecules in which the four independent dipeptide units (and those related by translation along the x and y axes) pack together through electrostatic interactions involving the facing zwitterionic moieties and water molecules. This double layer presents an inner core of hydrophilic groups which are involved with water molecules in the hydrogen-bonding scheme. Distally, the double layers pack together along the z direction by hydrophobic interactions and van der Waals forces between facing thietane rings and their methyl substituents at positions 1 and 3.

The conformation of the L-aspartyl residue in the four independent molecules is highly conserved, with no torsion varying by more than 20° (Table VI). This conformation of the aspartyl residue is very similar to that observed recently in the crystal structures of the two retro-inverso diastereomers of *N*-(L-aspartyl)-N′-[(2,2,5,5-tetramethylcyclopentanyl)carbonyl]-1,1-diaminoethane.[9] The observed rotamer about the Aspχ_1 dihedral angle is gauche$^-$. The C_α–C_β bond of the aspartyl residue is nearly coplanar with the carboxylate side chain, with the two χ_2 dihedral angles close to 0° or 180°. The terminal amino group and the aspartyl side-chain carboxylate in all four molecules are in the zwitterionic form. This is supported by the observation that all C_γ–O_δ distances are intermediate between those of a single and double C–O bond, typical of carboxylate ions. Further, the resulting NH_3^+ cation is spatially very close to the COO^- anion, with an intramolecular hydrogen bond between one of the cations protons and the nearest carboxylate oxygen (average distance of 2.81 Å with an N–H—O_δ angle of 126°). Molecules are also

Table VI. Selected Torsion Angles for Each of the Four Molecules in the Unit Cell of Crystalline L-Aspartyl-D-alanine 2,2,4,4-Tetramethylthietanylamide

torsion	molecule A	molecule B	molecule C	molecule D
Aspψ	158.5	144.1	153.3	167.4
Aspω	170.5	170.0	167.0	−174.2
Aspχ_1	−63.6	−66.8	−64.0	−63.7
Aspχ_2^1	−175.5	−172.0	−165.1	−168.4
Aspχ_2^2	5.9	8.1	15.5	8.7
D-Alaϕ	126.0	66.6	70.2	74.1
D-Alaψ	−158.4	−151.9	−151.1	−158.1
D-Alaω	−172.1	175.4	171.2	171.2
TAN[a]ϕ	−36.0	23.0	21.0	28.0

[a] TAN represents 2,2,4,4-tetramethylthietane for which torsion angle ϕ_{TAN} is defined as CO–N–C_α–H.

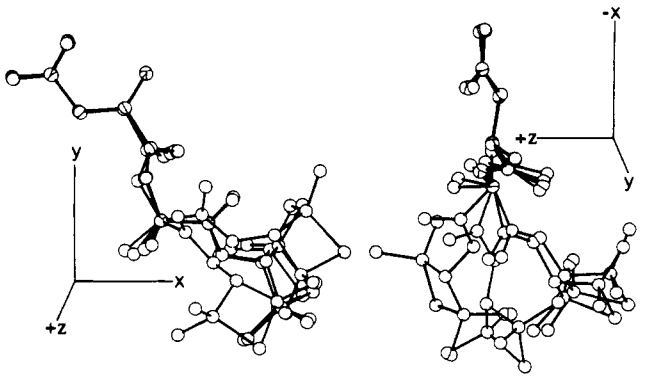

Figure 4. The superposition of the four independent alitame molecules observed in the crystal. Axes are shown to illustrate the L shape in the xy plane (left) and the z axis component (right).

involved in a complex intermolecular hydrogen-bonding scheme involving many of the ten cocrystallized water molecules (described above).

The backbone torsion ϕ of the D-alanyl residue shows the greatest variability, with molecule 1 differing from the other three by approximately 60° (Table VI). In a concerted fashion, the ϕ torsion of the TAN moiety is negative for molecule 1 only, differing by approximately 60° from values observed in molecules 2, 3, and 4. The effect of torsional variability on the overall geometry of the alitame molecules is best illustrated in the superposition shown in Figure 4.

Discussion

In solution, molecules with the size and constraints of alitame exist as an equilibrium mixture of conformations. The possible *accessible* (as opposed to *preferred*) conformations for any given molecule are mainly determined by van der Waals interactions. These steric interactions can be reliably determined by using any

Table VII. Selected Energy Components of L,D and L,L Stereoisomer Minima (kcal/mol)

no.	total	bond	θ	φ	repulsion	dispersion	Coulombic
LD1	-41.405	6.871	33.234	9.410	78.031	-58.371	-103.849
LD2	-36.353	6.691	33.771	8.286	76.058	-55.582	-98.347
LD3	-35.843	6.472	31.494	7.782	71.793	-52.629	-92.374
LD4	-35.783	6.625	33.993	8.033	75.383	-54.156	-98.442
LD5	-35.294	6.404	31.719	7.567	71.041	-51.000	-92.631
LD6	-32.961	6.534	32.033	7.765	70.638	-50.809	-90.941
LD7	-32.673	6.471	32.181	7.629	69.977	-49.696	-91.013
LL1	-43.642	6.884	33.435	9.102	80.102	-60.807	-105.468
LL2	-41.973	6.874	33.690	9.144	79.033	-58.732	-105.054
LL3	-36.919	6.685	33.088	8.738	75.762	-56.106	-98.013
LL4	-36.354	6.588	33.293	8.558	74.749	-54.168	-98.253
LL5	-35.715	6.554	31.594	7.947	72.619	-53.546	-92.559
LL6	-35.225	6.483	32.641	8.723	73.037	-52.951	-95.938
LL7	-34.770	6.482	31.819	7.683	71.680	-51.463	-92.649
LL8	-34.140	6.619	32.234	7.654	71.403	-52.668	-91.093

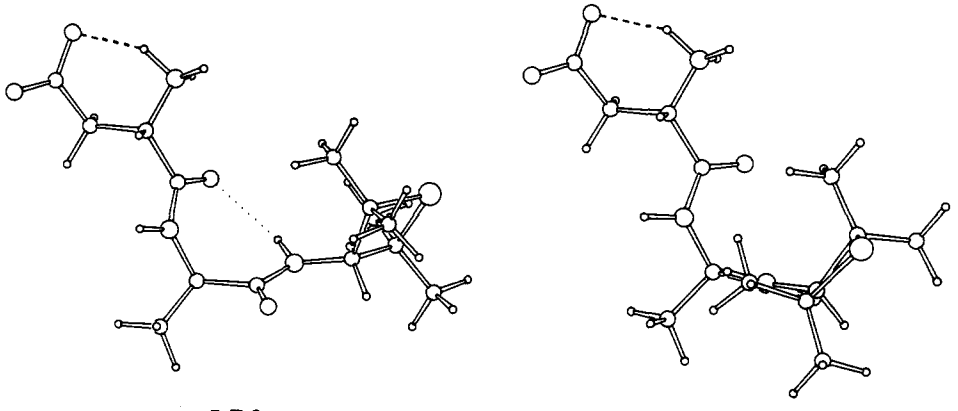

Figure 5. The preferred conformations, as determined by maximal agreement between theoretically derived structures and observed NOE distance constraints, of L-aspartyl-D-alanine 2,2,4,4-tetramethylthietanylamide (alitame): LD3 and LD6. (See text for details.)

one of the many existing energy force fields. The conformational analysis carried out for alitame (and its stereoisomers) resulted in the identification of virtually all possible minimum energy conformations. These together represent favorable positions within the larger accessible space of the molecule. However, because the force field is only an approximation (for reasons discussed below), we cannot rely on the relative energies of these conformations. It is for this reason that the experimental data are necessary, for they allow us to select preferred conformations from a larger set of accessible conformations.

Given a dipeptide such as alitame, our model proposed for sweet taste suggests that the preferred conformations about the central residue of a sweet tasting compound should maximize the L shape of the peptide backbone while allowing the zwitterionic ring and hydrophobic moiety to remain coplanar. In addition, given an *xy* plane defined by the zwitterionic ring, the bitter-tasting component of a dipeptide sweetener is enhanced when significant extension of the X moiety occurs along the perpendicular $-z$ axis. With such constraints the central residue of a sweet-tasting dipeptide derivative such as alitame is limited to two major conformational regions, the α-helical A (or A*) region ($\phi = -40$ to $-110°$ and $\psi = -10$ to $-90°$ for L amino acids, $\phi^* = 10$ to $110°$ and $\psi^* = 10$ to $90°$ for D amino acids) or the C_{7eq} C (or C*) region ($\phi = -40$ to $-110°$ and $\psi = 50$ to $130°$ for L amino acids, $\phi^* = 10$ to $110°$ and $\psi^* = -50$ to $-130°$ for D amino acids).[19]

For both the L,D-alitame and its L,L stereoisomer, the theoretically determined nongauche$^-$ conformers can be considered negligible, based on NMR investigation of their conformations in solution. In addition, the four unique conformations of the L,D stereoisomer observed in the crystal state all exhibit a gauche$^-$ rotation about the aspartyl side chain. However, the theoretical calculations contained in this work included gauche$^-$, gauche$^+$, and trans rotamers of the aspartyl residue as possible contributing minima, and, as a result, it was observed that of the 15 minima found, the 6 lowest in energy were gauche$^+$. The calculated energies of these gauche$^+$ structures are as much as 9.5 kcal/mol lower than the gauche$^-$ structures. One possible explantation for this inconsistency becomes apparent when the components of the total energy of each conformer are examined. Upon closer inspection it appears that the electrostatic interactions are exaggerated (Table VII). In order to maximize the magnitude of the favorable charge interactions (e.g., hydrogen bond formation), the molecule is forced to fold in on itself in a sterically and geometrically unfavorable way. In fact, every gauche$^+$ conformer (LD1, LD2, LD4, LL1–LL4, and LL6; Tables I, II, and VII) exhibits an unusually large favorable Coulombic component in their total energy, at the expense of other energy terms. Every one of these conformers shows putative hydrogen bonds between the β-carboxylic acid of the aspartyl moiety and one or both backbone amides. Experimentally, the temperature coefficients for the amide protons of both the Ala (or D-Ala) and TAN residues (Table IV) are beyond the range expected for an amide involved in an intramolecular hydrogen bond (>5.0 ppb/K). Because of this exaggeration of the electrostatics, many of the lowest energy conformers show unusually compact structures. As a consequence, many of these conformers show reasonable if not exceptional agreement with the NOE data. In an effort to optimize the methodology underlying the conformational analysis of aspartyl-based sweeteners, the effect of systematically varying the dielectric constant in the calculations is currently under study.

All of the conformational minima for alitame (LD1–LD7, Table I) fall into either the A* or C* region for the D-Ala residue. However, only four of the conformers (LD3, LD5, LD6, and LD7) are consistent with the observation that the molecule exists primarily as the gauche$^-$ rotamer about the Asp side chain. Of these

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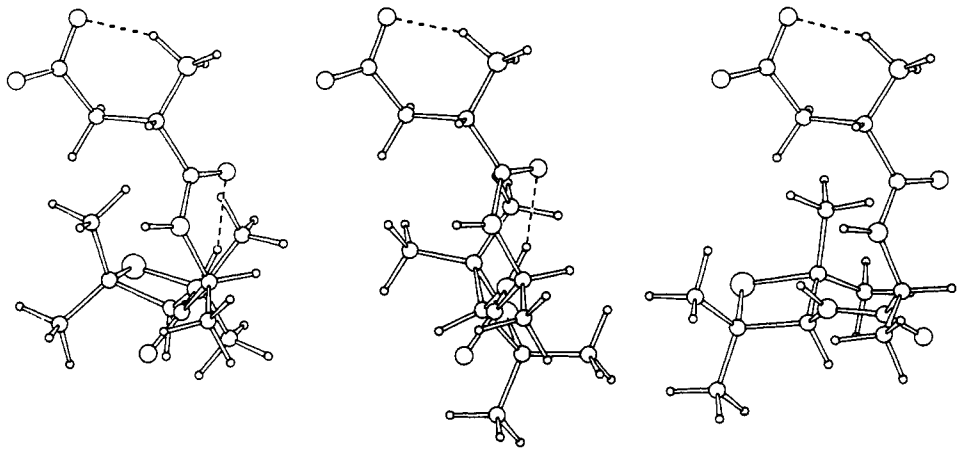

Figure 6. The preferred conformations, as determined by maximal agreement between theoretically derived structures and observed NOE distance constraints, of L-aspartyl-L-alanine 2,2,4,4-tetramethylthientanylamide: LL5, LL7, and LL8. (See text for details.)

four, LD3 and LD6 agree best with the NOE data (Figure 5). Although LD3 is almost three kcal/mol lower in energy than LD6, the calculated energy includes a favorable contribution from a putative hydrogen bond involving the TAN amide. The temperature coefficient (Table IV) for this amide is too high to support the existence of such a hydrogen bond. (It should be noted that for linear molecules of this size amide temperature coefficients are not always reliable indicators for hydrogen bonding.) The conformer LD6 lacks this unsubstantiated hydrogen bond, explaining in part the higher observed energy of this conformation. The conformer LD3 is clearly best in its agreement with the crystal data, with only a 40° modification about $\psi_{D\text{-}Ala}$ necessary to match the structure to three of the four observed crystalline conformations. None of the theoretically determined minimum energy conformations agree well with the one remaining crystalline conformation (molecule A, Table VI). In light of all available experimental evidence, LD3 can be considered the predominant preferred conformation for the L,D stereoisomer. In the cases of LD3 the plane of the four-membered thietane ring is very close to perpendicular to the plane of the zwitterionic ring and forms an L shape with essentially no projection of the backbone along the z axis, consistent with what has been reported previously for sweeteners incorporating the 2,2,5,5-tetramethylcyclopentanyl (TMCP) moiety. The two conformers LD3 and LD6 differ only in the two torsions ψ_{Ala} and ϕ_{TAN}. The net effect of an interconversion between the two is to flip the plane of the Ala–TAN amide bond from one side of the xy plane to the other. As a consequence of such a concerted change in torsions, the TAN moiety in the conformer LD6 is displaced and slightly reoriented toward the $+z$ axis. This, however, presents only a minor projection along the $+z$ axis. The LD6 conformer would still be predicted to elicit a sweet taste.

For the bitter L,L stereoisomer of alitame, all of the minimum energy conformations except one fall within either the A or C regions for the alanine residue. However, only conformations LL5, LL7, and LL8 are in agreement with NMR data concerning the existence of their aspartyl side chain in the gauche$^-$ conformation. Further, these three conformers are clearly superior to all except LL3 in terms of their agreement with experimentally observed NOE data (Table II). Although these conformers are in the expected region of ϕ/ψ space to elicit a sweet taste, closer inspection of the orientation of the two ring systems reveals conformations associated with a bitter taste (Figure 6).[19] The preferred conformations LL5, LL7, and LL8 all show marked extension of the thietane moiety along the $-z$ axis. Our model would predict these conformations to be associated with a bitter taste. It should be noted that the conformers LL5 and LL7 differ only by the dihedral angle ϕ_{TAN} and for that reason show similarity in their agreement to the NOE data. The difference in calculated energy, however, favors LL5 by nearly 1 kcal/mol. Both LL5 and LL7 predict an unsubstantiated hydrogen bond involving the TAN amide proton, while LL8 does not.

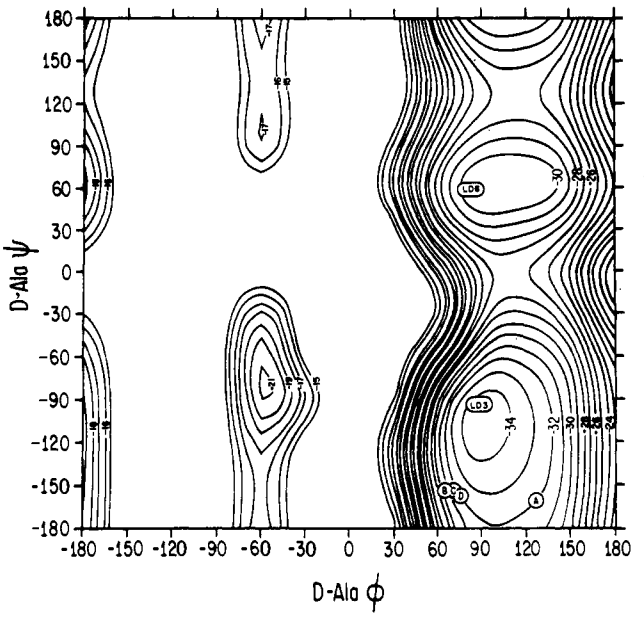

Figure 7. The $\phi_{D\text{-}Ala}/\psi_{D\text{-}Ala}$ energy map of alitame, where $\chi_{1(Asp)}$, $\chi_{2(Asp)}$, ψ_{Asp}, and ϕ_{TAN} were restrained to −63.8°, 64.3°, 159.5°, and 38.7°, respectively. The minimum energy conformation resulting from this region of space is denoted as LD3. Although differing at ϕ_{TAN}, LD6 is denoted as well. The four independent conformations of alitame observed in the crystal are denoted A–D.

The preferred conformations of the D,D stereoisomer of alitame are the mirror images of their enantiomers; LL5, LL7, and LL8. Thus, the D,D stereoisomer shows marked extension of the thietane moiety along the $+z$ axis. These conformations, for the reasons discussed above for the L,L stereoisomer, are associated with a lack of taste according to our model and previous sweeteners studied.

The investigation of the L,D stereoisomer (alitame) provided a unique opportunity for the integration of studies by NMR in solution, X-ray crystallography, and theoretical calculations. This combined approach is especially powerful, as in this work where the two experimental methods readily compliment one another toward an unambiguous conformational determination. The complimentary nature of all three methods is best illustrated in Figure 7, where the $\phi_{D\text{-}Ala}/\psi_{D\text{-}Ala}$ energy map for the conformation, where $\chi_{1(Asp)}$, $\chi_{2(Asp)}$, ψ_{Asp}, and ϕ_{TAN} were restrained to −63.8°, 64.3°, 159.5°, and 38.7°, respectively, is illustrated. The minimum energy conformation resulting from this sampling of conformational space was LD3, with $\phi_{D\text{-}Ala}$ and $\psi_{D\text{-}Ala}$ of 78° and −87°, respectively. Although the differences in the observed $\psi_{D\text{-}Ala}$ torsion

of the crystalline conformations and LD3 appear relative large (i.e., approximately 60°), it is evident that all five conformations reside within a broad shallow potential energy well. Quantitatively, these conformations in the crystal would be expected to differ from LD3 by less than 3 kcal/mol. In effect, this work supports the notion that for aspartyl-based taste ligands conformations in solution and in the crystal are closely related to each other.

Conclusions

For the sweet L,D stereoisomer (alitame) the minimum energy conformation supported most strongly by the experimental and theoretical data taken together is LD3. On the basis of our existing model, this conformer would be predicted to be sweet. For the bitter L,L stereoisomer of alitame, the conformations that clearly fit the NMR data best are LL5, LL7, and LL8. All three of these conformers would be predicted to be bitter. For the tasteless D,D stereoisomer of alitame, the conformations that clearly fit the NMR data best are the mirror images of LL5, LL7, and LL8. All three of these conformers would be predicted to be tasteless. Each of these minimum energy conformations can be considered a "snapshot" of the preferred conformations of the species in solution. Given the flexibility and kinetic energy of molecules in solution it is best to consider all of the above conformers as contributing species in a dynamic structure. Our model correctly predicts the taste properties of each stereoisomer's contributing conformers. In addition, the average of the minimum energy structures for each isomer retains the conformational characteristics consistent with its observed taste, as predicted by our model.

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Supplementary Material Available: Tables of atomic coordinates and isotropic coefficients, bond lengths and angles, H-atom coordinates and isotropic coefficients, anisotropic coefficients, and solution and refinement data, experimental data, and a figure consisting of the alitame structure (11 pages); table of observed and calculated structure factors (19 pages). Ordering information is given on any current masthead page.

A New Photolysis Intermediate in Artificial and Native Visual Pigments

C. E. Randall,[1a,d] J. W. Lewis,[1a] S. J. Hug,[1a] S. C. Björling,[1a] I. Eisner-Shanas,[1b] N. Friedman,[1b] M. Ottolenghi,[1c] M. Sheves,*[,1b] and D. S. Kliger*[,1a]

Contribution from the Department of Chemistry, University of California, Santa Cruz, Santa Cruz, California 95064, Department of Organic Chemistry, Weizmann Institute of Science, Rehovot 76 100, Israel, and Department of Physical Chemistry, The Hebrew University of Jerusalem, Jerusalem 91 904, Israel. *Received August 13, 1990. Revised Manuscript Received December 31, 1990*

Abstract: Nanosecond time-resolved and continuous illumination, low-temperature, spectroscopic studies reveal a new photolysis intermediate in a wide variety of artificial visual pigments as well as in native rhodopsin. This new intermediate, BSI, has a blue-shifted spectrum relative to the pigments as well as to their batho and lumi intermediates. At room temperature BSI is formed subsequent to batho and approaches an equilibrium with batho before decaying to the lumi intermediate. Chromophore modifications, which modify the β-ionone ring, eliminate conjugation between the ring and the polyene chain, add bulky groups to the C_4 position on the ring, or remove the 13-methyl group all yield time-resolved spectra which lead to the general scheme

$$\text{rho} \xrightarrow{h\nu} \text{batho} \rightleftarrows \text{BSI} \rightarrow \text{lumi...}$$

The same mechanism is shown to be valid in isorhodopsin and in the native bovine pigment rhodopsin. Chromophore modifications described above affect the batho ⇄ BSI equilibrium as well as the kinetics of approach to equilibrium but have little effect on the spectra of the intermediates or on the rate of the BSI to lumi transition. Implications for the nature of the BSI intermediate are discussed. Though BSI has a spectrum blue-shifted from that of batho, BSI is higher in enthalpy. It is proposed that this apparent conflict may be due to the fact that the photon energy, initially stored in chromophore–protein interactions, is transmitted to the protein during the batho-to-BSI transition. If energy at the BSI stage is still stored in the chromophore, models simply relating energy storage to bathochromic shifts must be ruled out.

Introduction

The complex series of reactions leading to visual transduction in vertebrates is initiated by light absorption of the visual pigment rhodopsin (rho). Rho consists of an 11-*cis*-retinal chromophore (Figure 1, I) bound via a protonated Schiff's base to the ε-amino group of lysine 296 in the protein opsin. It is now widely accepted that bathorhodopsin (batho-rho), the first photointermediate which can be trapped at low (liquid nitrogen) temperatures, results from a cis to trans photoisomerization of the 11,12-double bond.[2-10]

(1) (a) University of California, Santa Cruz. (b) Weizmann Institute of Science. (c) The Hebrew University of Jerusalem. (d) Currently at The Laboratory for Atmospheric and Space Physics, University of Colorado, Boulder, CO.

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