Pure Appl. Chem., Vol. 74, No. 7, pp. 1235–1242, 2002.

Structure–taste relationships of the sweet protein monellin*

Masanori Kohmura‡, Toshimi Mizukoshi, Noriki Nio, Ei-ichiro Suzuki, and Yasuo Ariyoshi

Central Research Laboratories, Ajinomoto Co., Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki, 210-8681 Japan

Abstract: Structure–taste relationship studies were carried out by chemically synthesizing monellin and its analogs. Replacement of the Asp^{B7} by L-2-aminobutyric acid, Gly and D-Asp resulted in complete loss of sweetness. Replacement of Ile^{B6} and Ile^{B8} by different amino acids resulted in a significant decrease of sweetness, or complete loss of sweetness. Comparison of short- and long-range nuclear Overhauser effects (NOEs) and chemical shifts between monellin and [Abu^{B7}]monellin showed no marked differences except for the region of the Asp^{B7}. Thus, the complete loss of sweetness in [Abu^{B7}]monellin is caused by the lack of free β-carboxyl group in the Asp^{B7} and not by a result of major disruption in the overall 3-dimensional structure. These results suggested that the free β-carboxyl group of the Asp^{B7} would possibly bind to the receptor site through ionic bonding and trigger the sensation of intense sweet taste, and Ile^{B6} and/or Ile^{B8} would be involved in the hydrophobic interaction with the receptor site. Selectively labeled monellin was synthesized by the solid-phase method by incorporating ^{15}N-labeled amino acids into 10 key residues including Asp^{B7}. Relaxation analysis shows that Asp^{B7} is the most flexible of these 10 residues. The flexibility of the active site may be important for receptor binding.

Five sweet proteins and two taste-modifying proteins have been reported: monellin, thaumatin, pentadin, mabinlin, brazzein, miraculin, and curculin [1–9]. Miraculin is not sweet by itself, but modifies sour taste to sweet taste. Curculin is sweet as itself, but also has an activity similar to that of miraculin. Thaumatin, single-chain monellin, miraculin, curculin, and mabinlin have been expressed in microorganisms such as yeast, *Aspergillus oryzae*, and *E. coli* [10–17]. Expressed thaumatin, single-chain monellin, and mabinlin were folded into their native, sweet conformations. We could also prepare monellin, brazzein, and mabinlin by solid-phase chemical synthesis, since we were interested in developing a convenient method of synthesis of sweet proteins for structure-function studies [18–20]. At the beginning of our research, we chose monellin as the first candidate since it was the smallest protein, for which the primary and tertiary structures were determined. Monellin is a sweet protein isolated from the fruit of an African plant, *Dioscoreophyllum cumminsii*. It consists of two noncovalently associated polypeptide chains, A chain with 44 amino acid residues and B chain with 50 residues (Fig. 1) [21]. The crystal structure has been determined at 2.75 Å (Fig. 2) [22]. Synthetic monellin is 4000 times as sweet as sucrose on a weight basis. The individual A and B chains are not sweet, and the native conformation is essential for the sweet taste.

**Pure Appl. Chem.* Vol. 74, No. 7, 2002. A special topic issue on the science of sweeteners.
‡Corresponding author

(F)REIKGYEYQLYVYASDKLFRADISEDYKTRGRKLLRFNGPVPPP
A chain (44 residues)
(T)GEWEII**D**IGPFTQNLGKFAVDEENKIGQYGRLTFNKVIRPCMKKTIYEEN
B chain (50 residues)

Fig. 1 Primary structure of monellin.

Fig. 2 Stereo diagram of the α-carbon trace of monellin.

SYNTHETIC STUDIES

Delineation of the active site of monellin was carried out by analog studies by chemically synthesizing monellin and its analogs [23]. Peptides were synthesized by automated peptide synthesizer and purified by preparative reversed-phase high-performance liquid chromatography (HPLC) and hydrophobic interaction chromatography (HIC). The purity of each peptide was confirmed by HPLC and electrospray ionization mass spectrometry, and by a quantitative amino acid analysis. Almost all of the analogs of synthetic A and B chains were spontaneously folded by simple mixing of two chains, and resulting monellin analogs were purified by HIC. The purity of each protein was confirmed by HIC and by a quantitative amino acid analysis. However, the [D-Tyr^{13}] A chain did not associate with the B chain, and [Ahx^{B36,43,44}]monellin could not be purified, since it was unstable under HIC condition. [D-Ala^{B6}]monellin was purified by HIC at 10 ºC, since it was unstable at room temperature. [Asn^{A22}]monellin and [Ser^{B41}]monellin were slightly unstable under HIC condition.

The sweetness was organoleptically evaluated by a panel of five people, at concentrations near the threshold to avoid confusion arising from the persistent lingering sweet taste of monellin. Thus, the sweetness was evaluated by matching a threshold concentration of the synthetic monellin with that 0.6 % (w/v) of sucrose. The synthetic monellin was 4000 times sweeter than sucrose in comparison to a 0.6 % (w/v) sucrose solution.

It is impossible to predict the active site of a sweet protein with certainty. The most promising approach to determine the active site would be replacement of the amino acid residues at possible active sites. If replacement of a certain amino acid residue in monellin removes the sweet taste, and yet the protein retains its native conformation, the position replaced should be related to the active site. Through the experience of examination of various sweet peptides, we considered that the free β-carboxyl group of certain Asp residue in monellin would possibly bind to the receptor site through ionic bonding and trigger the sensation of an intense sweet taste [24]. Monellin contains five Asp residues at positions A16, A22, A26, B7, and B21. At first, we replaced these Asp residues with L-Asn. Table 1 summarizes the structure and sweetness of these monellin analogs. Among them, the sweetness potency of [Asn^{B7}]monellin (**7**) was significantly decreased, while that of other analogs are slightly increased or decreased (see proteins **2**, **5**, **6**, **12**). In the X-ray crystal structure (3MON), all of the side-chains of these Asp residues are exposed to the solvent. The low potency of protein **7** indicates that the Asp^{B7} may be involved in binding with the receptor site. In order to clarify this, we replaced this residue by L-α-aminobutyric acid (Abu), which has neither an amide nor a free β-carboxyl group, L-Glu, Gly, and

Table 1 Replacement of Asp residues in monellin and sweetness of analogs.

No.	Protein	Sweetness potency[a]
1	monellin	4000
2	[Asn^{A16}]monellin	7500
3	[Abu^{A16}]monellin	8000
4	[D-Asp^{A16}]monellin	8000
5	[Asn^{A22}]monellin	(slightly unstable) 750
6	[Asn^{A26}]monellin	5500
7	[Asn^{B7}]monellin	20
8	[Abu^{B7}]monellin	0
9	[Glu^{B7}]monellin	10
10	[Gly^{B7}]monellin	0
11	[D-Asp^{B7}]monellin	0
12	[Asn^{B21}]monellin	7000

[a]Times as potent as sucrose (weight basis, 0.6 % sucrose = 1). Zero indicates that the protein was devoid of any sweetness and tasteless when tasted as a lyophilized powder.

D-Asp. [Glu^{B7}]monellin (**9**) was ten times sweeter than sucrose, while the other analogs were devoid of any sweetness (see proteins **8**, **10**, **11**). The circular dichroism (CD) spectra of these analogs were virtually the same as that of monellin (data not shown). These results suggest that the Asp^{B7} may be the active site of monellin. The sweetness potency of [Asn^{A22}]monellin (**5**) was fivefold decreased, and this protein was slightly unstable under HIC condition. In the crystal structure, the side-chain of Asp^{A22} is close to the side-chain of Arg^{A36}. Somoza et al. have reported that replacement of this Arg by Glu in single-chain monellin resulted in a decrease in sweetness of two orders of magnitude [22]. Thus, this region may be also important for eliciting a sweet taste, and decreasing of potency may be due to conformational change of this region.

It is frequently said that potently sweet compounds possess a hydrophobic site, which participates in the binding to the receptor through a hydrophobic interaction. In aspartyl peptide sweeteners, both the amino acids adjacent to the Asp residue are restricted in shape, size, stereochemistry, and so on. If the active site of monellin shares the same receptor site with aspartyl peptide sweeteners, both the amino acids adjacent to Asp^{B7} may be highly restricted. The replacement of Ile^{B6} or Ile^{B8} by different amino acids resulted in a decrease in sweetness of two orders of magnitude, or complete loss of sweetness (Table 2, see proteins **14–20**). This suggests that Ile^{B6} and/or Ile^{B8} would be involved in the hydrophobic interaction with the receptor site. However, in the crystal structure, the side-chain of the Ile^{B6} is located to the opposite side of the Asp^{B7} and half-buried to the inside of the molecule, while that of the Ile^{B8} is at the same side of the Asp^{B7} and exposed to the solvent (Fig. 3). [D-Ala^{B6}]monellin (**16**) was unstable, thus another explanation for decreasing of potency of Ile^{B6} replaced analogs may be due to the conformational change of this region. Following the foregoing substitution, Ile^{B5} and Ile^{B9} were replaced. The replacement of Ile^{B5} by Ala slightly decreased the sweetness potency (see protein **13**). On the other hand, the replacement of Gly^{B9} by Ala or D-Ala resulted in complete loss of sweetness (see proteins **21** and **22**). Gly has no functional side-chains, so it may not be the active site. The Gly^{B9} are located at the beginning of the α-helix, and the backbone structure of these residues is sterically constrained. A possible explanation for the complete loss of sweetness is that the introduced methyl group of Ala may induce conformational change around this region, or interfere with the receptor binding.

Monellin contains nine Lys residues. The replacement of these residues by L-2-aminohexanoic acid (Ahx), which lacks the ε-amino group of Lys, slightly reduced the sweetness potency (see proteins **23–31**). And the simultaneous replacement of two or three Lys residues significantly reduced the sweetness potency (see proteins **32** and **33**). This result suggests that basic residues may also be important

Table 2 Replacement of adjacent amino acid to Asp^{B7} in monellin and sweetness of analogs.

No.	Protein	Sweetness potency[a]
13	[Ala^{B5}]monellin	1500
14	[Gly^{B6}]monellin	10
15	[Ala^{B6}]monellin	100
16	[D-Ala^{B6}]monellin	(unstable) 0
17	[Gly^{B8}]monellin	20
18	[Ala^{B8}]monellin	90
19	[D-Ala^{B8}]monellin	0
20	[Phe^{B8}]monellin	10
21	[Ala^{B9}]monellin	0
22	[D-Ala^{B9}]monellin	0

[a]Times as potent as sucrose (weight basis, 0.6 % sucrose = 1). Zero indicates that the protein was devoid of any sweetness, and tasteless when tasted as a lyophilized powder.

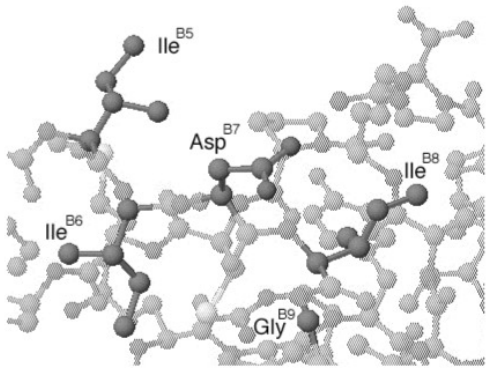

Fig. 3 Crystal structure of amino acid residues around the Asp^{B7} of monellin.

for eliciting a sweet taste. Kim et al. and Tancredi et al. have proposed that the Asp^{A16} and Tyr^{A13} are involved in the recognition of the putative sweet receptor, because the distance between the side-chains of these residues is comparable to the distance between the side-chains of Asp and Phe resides of aspartame [25,26]. However, the replacement of these residues by different amino acids did not remove the sweetness (see proteins **2–4**, **34**, and **35**) and proteins **2**, **3**, and **4** were twice as sweet as monellin. The Asp^{A16} is located in the loop between the third and fourth β-strand, and exposed to the solvent. This result suggests that these residues are not sweetness-determinant sites, but the Asp^{A16} may be involved in the interaction with the receptor. Assadi-Porter et al. have reported that des-$pGlu^1$-[Ala^{31}]brazzein was twice as sweet as des-$pGlu^1$-brazzein. In the 3-dimensional structure of brazzein determined by NMR, the His^{31} is located in the loop between the first α-helix and second β-strand, and exposed to the solvent [27]. Van del Wel has reported that chemical modification of carboxyl group of thaumatin by amide group resulted in increase of sweetness [28]. These results suggest that some charged residues on the surface of the protein would be involved in the interaction with the receptor. It has been reported that the Cys^{B41} play an important role in sweet taste by chemical modification studies [29]. However, the replacement of this residue by Ser did not remove the sweetness (see protein **36**), and in the crystal structure, the side-chain of Cys^{B41} is completely buried to the inside of the molecule. These data sug-

gest that the Cys^{B41} do not participate in receptor binding. D-Monellin consists of all D-amino acids except Gly, and is the mirror image of the L-monellin [30]. D-Monellin was devoid of any sweetness.

Table 3 Structure and sweetness of monellin analogs.

No.	Protein	Sweetness potency[a]
23	[Ahx^{A4}]monellin	1500
24	[Ahx^{A17}]monellin	1000
25	[Ahx^{A28}]monellin	1750
26	[Ahx^{A33}]monellin	1750
27	[Ahx^{B17}]monellin	1000
28	[Ahx^{B25}]monellin	1250
29	[Ahx^{B36}]monellin	750
30	[Ahx^{B43}]monellin	500
31	[Ahx^{B44}]monellin	500
32	[$Ahx^{B43, B44}$]monellin	50
33	[$Ahx^{B36, B43, B44}$]monellin	unstable (0)
34	[Gly^{A13}]monellin	3500
35	[Phe^{A13}]monellin	3500
36	[D-Tyr^{13}] A chain + B chain	not fold
37	[Ser^{B41}]monellin	(slightly unstable) 2000
38	D-monellin	0

[a]Times as potent as sucrose (weight basis, 0.6 % sucrose = 1). Zero indicates that the protein was devoid of any sweetness, and tasteless when tasted as a lyophilized powder.

NMR studies

Replacement of the Asp^{B7} by Abu resulted in complete loss of sweetness. In order to show that there are no differences in the 3-dimensional structure between monellin and the [Abu^{B7}]monellin (**8**), and the complete loss of sweetness is caused by a lack of β-carboxyl group, we carried out NMR studies [31]. Signal assignments of proteins **1** and **8** were carried out by 2-dimensional nuclear Overhauser enhancement spectroscopy (NOESY), total correlation spectroscopy (TOCSY), and double quantum filtered correlation spectroscopy (DQF-COSY) except for the Pro residues and some terminal residues, based on the method of sequence-specific resonance assignment [32]. The information on chemical shifts, NOEs, and amide-proton exchange rate are compared for each residue of the both proteins. There were chemical shift changes of less than 0.2 ppm between proteins **1** and **8**, with the exception of the α-proton at the position of B6, B7, and B8, the amide proton at B6 and B37 (Fig. 4).

The chemical shifts of Ile^{B6} and Ile^{B8} can be rationalized as the direct influence of the amino acid replacement at those adjacent B7. However, the up-field chemical shift of the amide proton at B37 upon the replacement suggests a loss of indirect interaction of Val^{B37} with the carboxylate oxygen at B7. According to the X-ray crystal structure, Arg^{B39} was found to be a probable candidate of the transit residue for this indirect interaction (Fig. 5).

The sequential NOEs provide a basis for identifying several secondary structures in solution. Secondary structures of proteins **1** and **8** show almost the same pattern except for the region around Asp^{B7}, and observed NOEs around this region were very poor (data not shown). Comparison of short- and long-range NOEs and chemical shifts between proteins **1** and **8** showed no marked differences except for this region. These results indicate that the replacement of Asp^{B7} with Abu induces no overall structural changes and the lack of the β-carboxyl group of Abu^{B7} is responsible for the loss of sweetness. Therefore, we concluded that the Asp^{B7} is the active site of monellin. We assume the active site

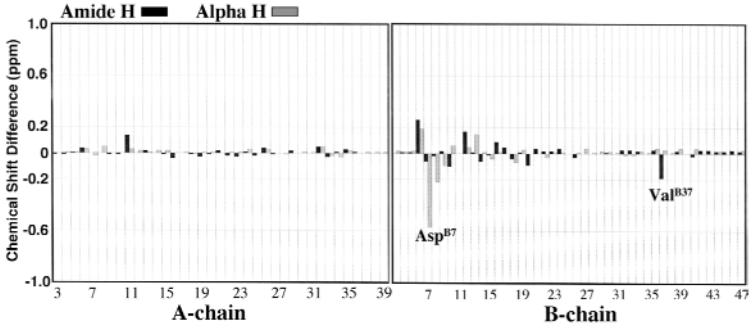

Fig. 4 Chemical shift differences of NH and αH between monellin and [Abu^{B7}]monellin.

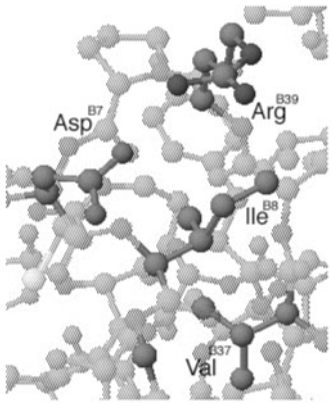

Fig. 5 Crystal structure of amino acid residues around the active site of monellin.

of monellin shares the same receptor site with aspartyl peptide sweeteners. Recently, Xiaodong Li et al. have reported that human taste-specific G protein-coupled heterodimeric receptors T1R2/T1R3 recognized diverse natural and synthetic sweeteners including monellin and aspartame [33]. These results support our assumption.

In general, internal flexibility of a protein is important for its biological activity. Poor NOEs around the active-site Asp^{B7} indicated the conformational flexibility of this region. Therefore, we expected to confirm that this residue has higher internal mobility by relaxation analysis. We synthesized selectively ^{15}N-labeled monellin using the solid-phase method by incorporating ^{15}N-labeled amino acids into 10 key residues including Asp^{B7} (Fig. 6). The relaxation parameters including T_1, T_2, $\{^1H\}$-^{15}N NOE of ^{15}N-labeled residues of monellin were measured, and data were analyzed by a model free approach to determine generalized order parameters (S^2) [34,35]. It was found that the value of S^2 of the Asp^{B7} is lower than the other nine residues. This result indicated that the ^{15}N backbone of the Asp^{B7} has relatively higher flexibility. This flexibility may be important when a part of the large molecule binds to the receptor and induces an active conformation.

We can assume that the free β-carboxyl group of Asp^{B7} of monellin is the hydrogen bond acceptor B, and the side-chain of Ile^{B8} is the hydrophobic site X. In the crystal structure, the side-chain of Arg^{B39} is close to the Asp^{B7} (Fig. 5). Therefore, this guanidino group is a candidate for the hydrogen bond donor AH, although there is no data about replacement of this residue. In the crystal structure, the distances between AH, B, and X groups were approximately 3.5 Å for AH-X, 3.8 Å for AH-B, and

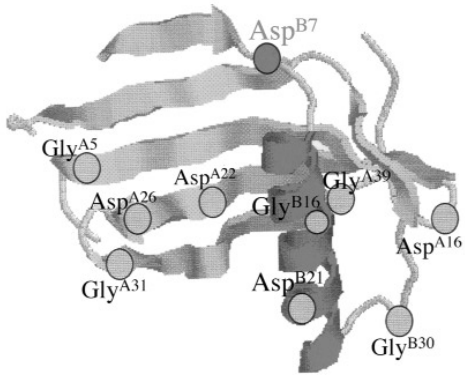

Fig. 6 Position of selectively ^{15}N-labeled residues on monellin.

4.9 Å for B-X. These values are close to those of Kier's model [36,37]. However, the result of the relaxation analysis indicated that these residues might be flexible in solution. Thus, it is also possible that the side-chains of Ile^{B6} and/or Ile^{B8} act as hydrophobic sites. These flexible active site residues may fit with other models, such as Goodman's "L-shape" model [38] and Tinti–Nofre model [39]. Structure study on the complex of the sweet receptor and sweetener is necessary to clarify this hypothesis.

ACKNOWLEDGMENTS

We thank Prof. S.-H. Kim of U.C. Berkeley for providing the atomic coordinates of monellin (3MON), and Dr. S. Tate and Prof. M. Kainosho of Tokyo Metropolitan University for providing programs of relaxation parameter analyses.

We thank Dr. K. Hirayama, Ms. T. Akashi, Ms. M. Furuya, Ms. U. Niitsu, and Ms. R. Yuji for measuring ESI-mass spectra, and Mr. S. Ozawa, Ms. Y. Amada, Ms. M. Fuchigami, and Ms. M. Ikuta for amino acid analyses

REFERENCES

1. J. A. Morris and R. H. Cagan. *Biochim. Biophys. Acta* **261**, 114–122 (1972).
2. H. van der Wel. *FEBS Lett*. **21**, 88–90 (1972).
3. H. van der Wel and K. Loeve. *Eur. J. Biochem*. **31**, 221–225 (1972).
4. H. van der Wel, G. Larson, A. Hladik, C. M. Hladik, G. Hellekant, D. Glaser. *Chem. Senses* **14**, 75–79 (1989).
5. Z. Hu and M. He. *Yunnan Zhiwu Yanjiu* **5**, 207–212 (1983); *Chem. Abst*. **99**, 85103c (1992).
6. D. Ming and G. Hellekant. *FEBS Lett*. **355**, 106–108 (1994).
7. K. Kurihara and L. M. Beidler. *Science* **161**, 1241–1243 (1968).
8. J. N. Brouwer, H. van der Wel, A. Francke, G. J. Henning. *Nature* **220**, 373–374 (1968).
9. H. Yamashita, S. Theerasilp, T. Aiuchi, K. Nakaya, Y. Nakamura, Y. Kurihara. *J. Biol. Chem*. **265**, 15770–15775 (1990).
10. J.-H. Lee, J. L. Weickmann, R. K. Koduri, P. Ghosh-Dastidar, K. Saito, L. C. Blair, T. Date, J. S. Lai, S. M. Hollenberg, R. L. Kendall. *Biochemistry* **27**, 5101–5107 (1988).
11. Y. T. Hahm and C. A. Batt. *Agric. Biol. Chem*. **54**, 2513–2520 (1990).
12. A. McPherson and J. Weickmann. *J. Biomol. Struc. Dynam*. **7**, 1053–1060 (1990).
13. S.-H. Kim, C.-H. Kang, R. Kim, J. M. Cho, Y.-B. Lee, T.-K. Lee. *Protein Eng.* **2**, 571–575 (1989).

14. K. Kondo, Y. Miura, H. Sone, K. Kobayashi, H. Iijima. *Nature Biotech*. **15**, 453–457 (1997).
15. Y. Masuda, S. Nirasawa, K. Nakaya, Y. Kurihara. *Gene* **161**, 175 (1995).
16. S. Nirasawa, T. Nishino, M. Katahira, S. Uesugi, Z. Hu, Y. Kurihara. *Eur. J. Biochem*. **223**, 989 (1994).
17. S. Nirasawa, Y. Masuda, K. Nakaya, Y. Kurihara. *Gene* **181**, 225 (1996).
18. M. Kohmura, N. Nio, Y. Ariyoshi. *Agric. Biol. Chem*. **55**, 539–545 (1991).
19. H. Izawa, M. Ota, M. Kohmura, Y. Ariyoshi. *Biopolymers* **39**, 95–101 (1996).
20. M. Kohmura and Y. Ariyoshi. *Biopolymers* **46**, 215–223 (1998).
21. M. Kohmura, N. Nio, Y. Ariyoshi. *Agric. Biol. Chem*. **54**, 2219–2224 (1990).
22. J. R. Somoza, F. Jiang, L. Tong, C.-H. Kang, J. M. Cho, S.-H. Kim. *J. Mol. Biol*. **234**, 390–404 (1993).
23. M. Kohmura, N. Nio, Y. Ariyoshi. *Biosci. Biotech. Biochem*. **58**, 1522–1524 (1994).
24. Y. Ariyoshi, M. Kohmura, Y. Hasegawa, M. Ota, N. Nio. In *Sweeteners*, D. E. Walters, F. T. Orthoefer, G. E. DuBois (Eds.), pp. 41–56, American Chemical Society, Washington, DC (1991).
25. S.-H. Kim, C.-H. Kang, J. M. Cho. In *Sweeteners*, D. E. Walters, F. T. Orthoefer, G. E. DuBois (Eds.), pp. 28–40, American Chemical Society, Washington, DC (1991).
26. T. Tancredi, H. Iijima, G. Saviano, P. Amodeo, P. A. Temussi. *FEBS Lett.* **310**, 27–30 (1992).
27. F. M. Assadi-Porter, D. J. Aceti, J. L. Markley. *Arch. Biochem. Biophys*. **376**, 259–265 (2000).
28. H. van der Wel. *The Quality of Food and Beverages*, G. Charalambous and G. Inglett (Eds.), pp. 113–118, Academic Press, New York (1981).
29. R. H. Cagan and J. A. Morris. *Proc. Soc. Expt. Biol. Med*. **152**, 635–640 (1976).
30. L.-W. Hung, Y. Ariyoshi, M. Kohmura, S.-H. Kim. *Acta. Crystallog. Sect. D* **54**, 494–500 (1998).
31. T. Mizukoshi, M. Kohmura, E.-I. Suzuki, Y. Ariyoshi. *FEBS Lett.* **413**, 409–416 (1997).
32. M. Billetter, W. Braum, K. Wuthrich. *J. Mol. Biol*. **155**, 321–346 (1982).
33. X. Li, L. Staszewski, H. Xu, K. Durick, M. Zoller, E. Adler. *Proc. Natl. Acad. Sci*. **99**, 94692–94696 (2002).
34. L. E. Kay, D. A. Torchia, A. Bax. *Biochemistry* **28**, 8972–8979 (1989).
35. M. J. Stone, W. J. FairBrother, A. G. Palmer III, J. Reizer, M. H. Saier Jr., P. E. Wright. *Biochemistry* **31**, 4394–4406 (1992).
36. L. B. Kier. *J. Pharm. Sci*. **61**, 1394–1397 (1972).
37. H. D. Holtje and L. B. Kier. *J. Pharm. Sci*. **63**, 1722–1725 (1974).
38. T. Yamazaki, E. Benedetti, D. Kent, M. Goodman. *Angew. Chem., Int. Ed. Engl*. **33**, 1437–1451 (1994).
39. J. M. Tinti and C. Nofre. In *Sweeteners*, D. E. Walters, F. T. Orthoefer, G. E. DuBois (Eds.), pp. 206–223, American Chemical Society, Washington, DC (1991).