

Sweeteners

Discovery, Molecular Design, and Chemoreception

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Foreword

THE ACS SYMPOSIUM SERIES was founded in 1974 to provide a medium for publishing symposia quickly in book form. The format of the Series parallels that of the continuing **ADVANCES IN CHEMISTRY SERIES** except that, in order to save time, the papers are not typeset, but are reproduced as they are submitted by the authors in camera-ready form. Papers are reviewed under the supervision of the editors with the assistance of the Advisory Board and are selected to maintain the integrity of the symposia. Both reviews and reports of research are acceptable, because symposia may embrace both types of presentation. However, verbatim reproductions of previously published papers are not accepted.

Preface

PEOPLE SEEM TO HAVE an almost insatiable desire for sweet taste, but it is the hedonic delight of sweet-taste sensation that is being sought rather than the calories. Therefore, there will continue to be a strong demand for noncaloric sweeteners that simulate the properties of sucrose. The noncaloric sweeteners known today fall short in one way or another of delivering the full sucrose-taste experience in all types of sweet food products. Thus, the search continues. This book and the symposium from which it was derived were conceived to update biochemists, chemists, food scientists, and sensory scientists on progress in the topics discussed.

Much has already been written about the structure–activity relationships of sweeteners, the biochemical mechanism of sweetener action, and rational molecular design of biologically active compounds, including sweeteners. In this book, the topics have been selected to bring the reader up to date with some of the most significant recent work in each of these areas. Although a compilation of chapters written by many authors cannot give a fully comprehensive treatment of any one of these topics, it will bring the reader to the cutting edge of sweetener and sweet-taste mechanism research.

Sweetener discovery has been an accidental process. Saccharin, cyclamate, and aspartame lead the list. The accidental discovery process will likely lead to still other sweet-tasting compounds. However, it is now possible to design new structures with high-potency sweetness. Gaining an understanding of the mechanism of sweet taste sensation is a fundamental problem currently being probed in a focused manner in many laboratories. Knowledge gained as a result of these efforts may find application in therapeutic approaches to taste-deficit disorders. It should also have application to the rational design of novel sweet-tasting compounds.

The chapters in this book fall into three major sections. The first covers structure–taste studies of peptides, proteins, and other natural products, as well as many synthetic sweeteners. Included in this section is the discovery of sucrononic acid, the most potently sweet compound reported to date. The second section describes experimental and computational approaches to the study of sweet-tasting compounds and their presumed receptor sites. Molecular modeling now plays an important role in sweetener design, and these chapters will be of interest to readers concerned with rational design of bioactive compounds. The third major section deals with biochemical, physiological, and psychophysical aspects of

sweet-taste chemoreception. Methods that have been used with success in studying drug, neurotransmitter, and hormone receptors are now being applied to sweet-taste receptors in an effort to understand how sweeteners are recognized.

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Chapter 1

The Rational Discovery of Sweeteners

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This chapter reviews the history of sweetener discovery (often serendipitous) and outlines directed approaches to sweetener discovery. Mechanisms of sweet taste transduction are being investigated at the cellular and molecular level, and computer-aided molecular design is being successfully applied to the discovery of new sweet entities.

How have sweeteners been discovered in the past?

Recent work in a number of laboratories has been directed to the discovery of sweeteners and the understanding of sweet taste chemoreception. The discovery of sweeteners is not very different from the discovery of other substances which interact with receptors to produce a biological response; serendipity, screening, and systematic study have all played important roles. In order to discuss the rational discovery of new sweeteners, it is useful to consider how sweeteners have previously been discovered.

Accidental Discovery. Many of the sweeteners currently in use were discovered unintentionally. Roberts, in a book entitled *Serendipity (1)*, describes the accidental discoveries of saccharin (1879), cyclamate (1937), and aspartame (1965). Saccharin was found to taste sweet when Constantin Fahlberg, working at Johns Hopkins University, accidentally spilled some of the substance onto his hand. Michael Sveda, a graduate student at the University of Illinois, noticed the sweet taste of cyclamate on a cigarette which he had temporarily set down on his laboratory bench. Jim Schlatter was working on the synthesis of gastric peptides at G.D. Searle Co. when he accidentally got some aspartame on his hand; later, as he licked his finger to pick up a piece of weighing paper, he noticed a sweet taste. The sweet taste of acesulfame was also

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discovered accidentally by a chemist (Karl Clauss) picking up a weighing paper (2), although the sweetness of acesulfame is perhaps not surprising in retrospect due to its structural similarity to saccharin. Selinger (3) reports that the sweet taste of sucralose was discovered when a foreign student at Queen Elizabeth College (S.P. Phadnis) misunderstood a request to test a compound as a request to taste the compound.

Discovery by Screening. Numerous compounds have been found to taste sweet through an inadvertent screening process: before the advent of spectroscopy and other sophisticated analytical methods, new chemical entities were often characterized by their taste. Piutti found in this way that D-asparagine tastes sweet, while L-asparagine is tasteless (4). In the chemical literature of the 1800's and early 1900's, comments on the tastes of newly synthesized substances are common. Hundreds of chemical structures and their reported tastes were compiled by Cohn (5) and, later, by Moncrieff (6).

Another kind of screening process is the tasting of plant materials. Numerous plants are known to have sweet tastes, and chemists have often isolated and identified the sweet-tasting components of these plants. Among the sweet-tasting compounds which come from natural sources are sugars (sucrose, fructose, glucose), amino acids (alanine, glycine), terpenoid glycosides (glycyrrhizic acid, osladin, stevioside, baiyunoside), and proteins (thaumatin, monellin, mabinlin, pentadin).

A rather unique case of sweetener discovery was the "rediscovery" of hernandulcin by Compadre and coworkers (7). Through a study of the reports of 16th century Spanish explorers, a reference was found to a sweet-tasting plant used by the Aztec people. The plant was identified as *Lippia dulcis*, and the sweet-tasting component was identified as a sesquiterpene and named "hernandulcin" after Francisco Hernández who described the plant in the 1500's.

Discovery by Structure-Activity and Modeling Studies. Still other sweeteners have been produced by studies of structure-activity relationships (SAR) of known sweeteners. Sweet-tasting compounds have produced a rich collection of structure-taste relationships because of the diversity of structural types which are known to have sweet taste. Examples of sweet-tasting compounds include low molecular weight carbohydrates (sucrose, fructose, glucose, glycerol, sorbitol, mannitol, lactitol), aminoacyl sugars (methyl 2,3-di-O-(L-alanyl)- α -D-glucopyranoside), amino acids (glycine, alanine, many D-amino acids), peptides (aspartame and over a thousand analogs), proteins (thaumatin, monellin, pentadin), terpenoids (stevioside, glycyrrhizic acid, hernandulcin), chlorinated hydrocarbons (chloroform), halogenated sugars (sucralose), N-sulfonyl amides (saccharin, acesulfame), sulfamates

(cyclamate), polyketides (neohesperidin dihydrochalcone, phyllo dulcin), anilines (P-4000), and ureas (suosan, dulcin). These structure-taste relationships could easily fill a separate volume. There are many excellent reviews to which the interested reader is referred (8-11).

Certainly the most extensive structure-taste studies of sweeteners were triggered by the discovery of aspartame (12). Over a thousand analogs have been prepared and tasted. Structural modifications of aspartame have led to the discovery of alitame (13, 14), the highly potent aspartyl-aminomalonyl-diester (15), aspartyl-anilides (16), aspartyl-gem-diaminoalkanes (17), a series of highly branched dipeptide derivatives (18), and the aspartyl-phenylglycine derivatives (19, 20).

An important aspect of structure-taste studies has been the development of models to rationalize the relationship between chemical structure and taste. These have progressed from simple structural fragment compilations (Cohn, 3; Moncrieff, 4; Oertly and Myers, 21) to a two-point "AH-B" model (Shallenberger and Acree, 22) to a three-point "AH-B-X" model encompassing a dispersion or hydrophobic binding site (Kier, 23). These were followed by a number of three-dimensional models. Shallenberger and coworkers (24) added a "steric barrier" to their model to account for the difference in taste between D- and L-amino acids. Brussel and coworkers (25) and Fujino and coworkers (26) proposed models for the size and shape of the hydrophobic site based on structure-potency considerations. Receptor site models have been developed on the basis of superimposition of several related structures (27-32). Several research groups have combined NMR spectroscopy and conformational energy calculations to derive three-dimensional models (33-35). Hopfinger and coworkers proposed a model based on calculated hydrogen bond energies (36, 37). Quantitative structure-activity relationships (QSAR) have been used to correlate physical, electronic, and steric features of sweeteners with their sweetness potencies (38-42). To date, such correlations have succeeded only for structurally homologous series of compounds; they have not been readily generalized across structural classes.

How can we rationally discover new sweeteners?

While serendipity has played a role in the discovery of many successful sweeteners, it is not a very viable strategy on which to build a research and development program; a more reasoned approach is needed. There are at least three rational approaches to the discovery of new sweeteners: (1) we can elucidate in detail the cellular and molecular mechanism(s) of sweet taste chemoreception and design agents to interact with these systems; (2) we can develop structure-activity relationships and models based on computational, spectroscopic, and synthetic studies of

analogues of known sweet-tasting compounds; (3) we can carry out screening studies of large numbers of compounds in search of new leads. The following sections will examine each of these approaches in turn.

Mechanism(s) of Sweet Taste Chemoreception. Although sweet taste chemoreception is usually assumed to be mediated by some sort of proteinaceous receptor, such a receptor has never been isolated and characterized. It is important to define a few terms: the *papillae* are the visible "bumps" on the tongue; *taste buds* are clusters of cells located mainly in the fungiform, circumvallate, and foliate papillae of the tongue (but also on the epiglottis, on the soft palate, in the pharynx, and in the upper one-third of the esophagus); the *taste cells* are the individual elongated cells which make up the taste bud. Figure 1 illustrates the locations and cross-sectional views of the four types of papillae. It also shows the location of taste buds within the papillae. Each taste bud is made up of 40-50 elongated cells which are of epithelial origin (this is in contrast to olfactory receptor cells, which are of neural origin). At the top of the taste bud (Figure 2) is the taste pore, the region where the taste bud cells are exposed to taste-active materials in saliva. From the taste cells there are microvilli which extend into the taste pore. It is believed that chemoreception takes place on these microvilli, which are about 2 microns long and 0.2 microns wide. Taste bud cells form tight junctions just below the taste pore, so that taste-active substances appear to be confined to the taste pore area and probably do not directly contact the nerve fibers at the basal end of the cell (43). The taste nerves begin as small unmyelinated fibers (50-200 nm in diameter). A single fiber may branch and attach to several neighboring taste cells. One fiber may also innervate as many as four taste buds, and each bud is served by two or three nerve fibers. Nerve fibers from the front two-thirds of the tongue and from the soft palate travel through the chorda tympani nerve, then travel to the brain in the facial (VII cranial) nerve. A second group of sensory nerve fibers, from the back one-third of the tongue, travels through the glossopharyngeal (IX cranial) nerve. A third group, from taste buds in the epiglottis and pharynx, joins the vagus (X cranial) nerve. All three groups run into the nucleus of the solitary tract in the medulla which, in turn, connects to the thalamus. The thalamic taste area, in turn, connects to the frontal operculum and anterior insula of the cortex in primates (44). This insular-opercular cortex is considered the primary gustatory cortex; it has further connections to the orbitofrontal cortex and the amygdala. Faurion (45) and Yamamoto (46) have reviewed studies of taste quality coding in the nerve signals and information processing in the central nervous system.

Are there (as is usually assumed) specific proteinaceous receptors which detect the presence of sweet-tasting compounds? Hiji provided evidence that this is the case (47): treatment of the

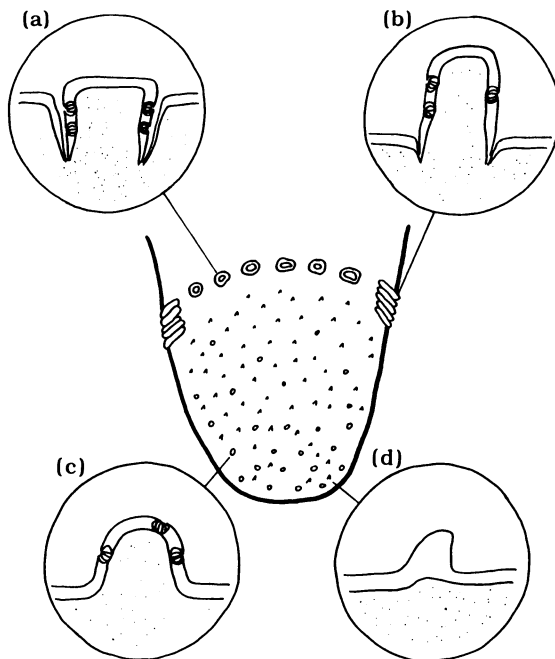


Figure 1. Schematic drawing of a human tongue, showing four types of papillae: (a) circumvallate papilla; (b) foliate papilla; (c) fungiform papilla; (d) filiform papilla. Expanded views show cross-sections through the papillae, illustrating locations of taste buds in the circumvallate, foliate, and fungiform papillae (filiform papillae do not have taste buds).

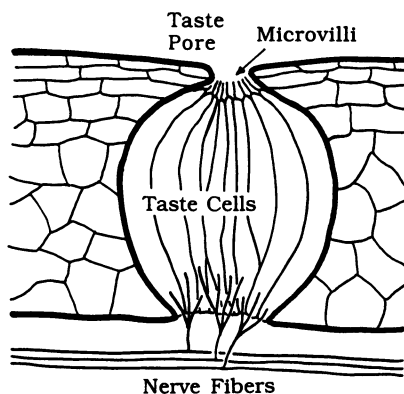


Figure 2. Cross-section through a taste bud, showing the microvilli, taste pore, taste cells, and nerve fibers.

tongue with specific proteases selectively abolished the response to sweet taste but not to other tastes. Several attempts have been made to identify and isolate such protein receptors (for a review, see ref. 48). In most cases, affinities have been very low, non-specific binding has been high, and no protein isolated has been shown to be a taste receptor. A major hindrance to sweet receptor binding studies and sweet receptor isolation has been the lack of high-affinity ligands. Ligands with binding constants of 10^{-9} M or lower would be desirable for such studies. If the binding constant is much higher than this, non-specific binding to other tissue components becomes a serious problem. Most sugars taste sweet at tenth-molar (10^{-1} M) concentrations. Aspartame is typically used at millimolar (10^{-3} M) concentrations. Even the most potent aspartic derivatives taste sweet only at micromolar (10^{-6} M) concentrations. Thaumatin, which tastes sweet at a concentration of a few hundred nanomolar (10^{-7} M), is likely to exhibit substantial non-specific binding to such negatively charged membrane components as phospholipids because of its high isoelectric point. The high-potency sweeteners recently described by Tinti and Nofre (49) and photoaffinity-labeling analogs derived from them (50) are the most promising chemical tools seen to date for the study of sweet taste receptors.

Is there a single type of receptor for sweet taste, or are there multiple types? Evidence for multiple receptors includes (a) the structural diversity of compounds which taste sweet; (b) single nerve fiber electrophysiology results; (c) cross-adaptation experiments; (d) synergy in sweetener mixtures. On the other hand, recent work with inhibitors (51, 52) and with photoaffinity labeling ligands (49) argue strongly for a single transduction mechanism. Faurion and coworkers (53) and Bartoshuk (54) have provided comprehensive overviews of this debate.

Does sweet taste transduction utilize a G-protein/second messenger system as many other receptor systems do? There is now evidence that this may be the case. Striem and coworkers (55) found that sugars stimulate adenylate cyclase in a concentration-dependent manner in membranes from the sensory epithelium of rat tongues (but not in membranes from non-sensory or muscle tissue). Adenylate cyclase activity was found to be dependent on the presence of guanine nucleotides, indicating a role for G-proteins. Saccharin also increased adenylate cyclase activity, while aspartame and neohesperidin dihydrochalcone did not, consistent with the fact that rats taste saccharin as sweet, but not aspartame or neohesperidin dihydrochalcone. A still-unresolved observation in this study was the stimulation of adenylate cyclase by saccharin in muscle tissue membranes. Indirect evidence for G-protein involvement in humans comes from a series of papers in the literature. It has been shown that pseudo-hypoparathyroidism (type I) is the result of a defective G-protein (56, 57). Previously, Henkin and Shallenberger described two

patients with pseudohypoparathyroidism who were unable to recognize sugars or cyclamate as sweet, although their perception of salty, sour, and bitter tastes were normal (58).

There are several reasons why sweet taste chemoreception is so poorly understood. One problem has been the lack of good biochemical tools for studying receptors and receptor-binding (see above). Quantitative receptor-binding studies and receptor isolation have not yet been accomplished. In other biological receptor systems, high-affinity antagonists have often played an important part; no such substances are known for the sweet taste receptor(s). In the case of sweet taste chemoreception, if the antagonist has any taste at all, it is useless for *in vivo* studies because sweetness inhibition could be attributed to centrally mediated mixture suppression as well as to competition for a receptor site. Quantitative concentration-response data has been relatively scarce because of the need for trained human sensory panels (59). There are no perfect animal models for sweet taste chemoreception in humans. Species differences in sweet taste perception are common for sweeteners other than sugars (60,61). Even if electrophysiological recording shows that a compound is taste-active in an animal, extensive behavioral studies are required to show whether the taste is sucrose-like (Hellekant, G.; Walters, D.E., manuscript submitted for publication). Taste cells are difficult to work with in cell culture. It is hard to separate them from surrounding epithelial cells, and they rapidly de-differentiate and lose their polarity in culture, so that there is no apical end and there are no microvilli. This may result from lack of a nerve cell-derived trophic factor; *in vivo*, if the nerve fiber supplying a taste bud is cut, the taste bud cells die and are not replaced until the nerve is regenerated.

Our understanding of taste chemoreception at the cellular and molecular levels is currently extremely limited, especially when compared to what we know about other kinds of receptors (e.g., cholinergic, adrenergic, many hormones). Recent studies such as those of Lancet (62) and Simon (63) are beginning to open up this field, however. The availability of high-potency small molecule sweeteners and photoaffinity-labeling analogs derived from them should also facilitate progress.

Development of Structure-Taste Relationships and Models.

Numerous papers in this volume show the continuing utilization of structure-activity studies and modeling efforts in the discovery of new sweeteners. Perhaps the most encouraging result to date has been Tinti and Nofre's discovery of extremely high-potency guanidine-acetic acids, starting from modeling and structure-activity studies of peptides and ureas (49,64). In the absence of an isolated and characterized taste receptor, agonist models have been extremely useful in the design of new sweeteners. They have progressed from very qualitative generalizations to detailed three-

dimensional descriptions, in some cases incorporating both steric and electronic features (63-67). Models are now being used not only to rationalize existing structure-activity results, but to predict the taste of new analogs, to design new analogs of old compounds, to design new lead series, and to design sweet taste inhibitors (51, 68).

Screening Approaches to Sweetener Discovery. Plants which are known or found to taste sweet continue to be a source of new sweet substances. Kinghorn's chapter in this book (69) describes recent studies in this area.

Large-scale screening of organic chemicals would seem to be an unlikely approach for new sweetener discovery, given the lack of good animal models for human sweet taste and the medical/legal issues involved in having humans taste new chemical entities. However, advances in biochemistry, molecular biology, and immunology may make possible *in vitro* screening of large numbers of compounds. High-affinity ligands may lead to radiolabeled ligand binding assays for receptor affinity in taste epithelial tissue. If a receptor can be cloned and expressed, assays could be carried out in tissue cultures.

Antibodies raised against high-potency sweeteners could also serve as high-throughput screening systems for the identification of new lead compounds. Kim and coworkers have demonstrated cross-reactivity between anti-thaumatococcus antibodies and monellin, and between anti-monellin antibodies and thaumatococcus (70). Antibody binding would have to occur at the sweet taste determinant site of the protein in order for this approach to be successful.

Conclusion

The chapters which follow examine much of the most recent research into sweet taste chemoreception and sweetener discovery. The first section deals with *Sweetener Discovery and Structure-Taste Studies*. Included here are recent discoveries in several structural classes, both natural and synthetic, as well as results of structural modifications on sweet taste. The second section, *Sweetener and Sweet Taste Receptor Modeling*, is composed of a number of computational investigations of sweeteners and proposed models for the sweet taste receptor site(s). The third section describes studies of *Mechanisms of Sweet Taste Chemoreception*. This includes biochemical studies as well as psychophysical evaluations of sweetener taste qualities. The final section is composed of two chapters. S.W. Gunner writes on regulatory aspects of new sweetener development (which ultimately have tremendous impact on the success of new sweeteners in the marketplace). Robert Mazur, who was involved in the discovery and development of aspartame, concludes the book with some comments on the future of sweetener discovery.

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Chapter 2

New Highly Sweet Compounds from Natural Sources

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A number of terpenoids, flavonoids, and proteins have been discovered as highly sweet plant constituents. Candidate sweet plants for laboratory study may be chosen on the basis of inquiries in the field or after careful review of the available literature. Several new plant-derived sweeteners, comprising terpenoids (hernandulcin and abrusosides A-D), steroidal saponins (polypodosides A and B), and a group of sweet dihydroflavonols, will be discussed.

The nearly 50 highly sweet naturally occurring compounds so far discovered represent about 15 structural classes of organic compounds. Such compounds, which are at least 50-100 times sweeter than sucrose, and thus exclude the monosaccharide, disaccharide, and polyol classes of bulk sweeteners, are mainly terpenoids, flavonoids, and proteins. Thus far, all of the known highly sweet compounds have been obtained as constituents of green plants, rather than species of microorganisms, insects, or marine plants and animals. A number of recent reviews have detailed the occurrence, structures, physicochemical characteristics, safety studies, and sensory properties of potently sweet substances from natural sources (1-3).

Several highly sweet plant-derived substances are commercially utilized as sweetening, flavoring, or taste-modifying agents in one or more countries. For example, extracts of *Stevia rebaudiana* (Bertoni) Bertoni containing the diterpene glycoside, stevioside, and extracts of *Glycyrrhiza glabra* L. containing the triterpene glycoside, glycyrrhizin, both occupy a major share of the "high-intensity" sweetener market in Japan (3,4). In addition, products

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made from three additional plants are currently employed as sucrose substitutes in Japan, namely, *Thaumatococcus daniellii* (Bennett) Benth. (containing the sweet protein, thaumatin), *Hydrangea macrophylla* Seringe var. *thunbergii* (Siebold) Makino (containing the sweet dihydroisocoumarin, phylodulcin), and *Thladiantha grosvenorii* (Swingle) C. Jeffrey (formerly *Momordica grosvenorii* Swingle) (3,4). Products made from *S. rebaudiana* are also used in Brazil, Paraguay, South Korea and the People's Republic of China (5). Thaumatin is an approved sweetener in Australia and the United Kingdom in addition to Japan, and is currently being reviewed for this purpose in other countries. However, it is now apparent that thaumatin will have wider application as a flavor enhancer, for which purpose it has been accorded GRAS (Generally Regarded as Safe) status in the United States (6).

For our laboratory program on the discovery and evaluation of natural sweeteners, a crucial aspect of the work has been the judicious selection of sweet-tasting plants for investigation. In the remainder of this chapter, details will be presented of the approaches we have taken toward the selection of sweet plants, as well as the specialized phytochemical procedures developed for the isolation of natural sweeteners, and the types of novel sweet compounds that have consequently been obtained.

Selection of Sweet-Tasting Plants

It has been found on numerous occasions that plants representing restricted taxonomic groups often biosynthesize similar classes of chemical components. However, little evidence has so far been obtained that the sweet-tasting constituents of a given plant will occur in other species of the same genus. For example, when over 100 leaf herbarium specimens in the genus *Stevia* were examined organoleptically and phytochemically for the occurrence of sweet-tasting *ent*-kaurene glycosides such as stevioside, these compounds were detected in only one species other than *S. rebaudiana* (7,8). Similarly, rubusoside, another sweet *ent*-kaurene glycoside, was found to occur in only one of more than 40 species in the genus *Rubus* that were investigated phytochemically (9). By the same token, little overlap is apparent in the occurrence of structurally similar sweet-tasting plant constituents at the plant family level, although sweet oleanane- and cucurbitane-type triterpene glycosides have been observed in several genera of the families Fabaceae and Cucurbitaceae, respectively (3). A breakdown of highly sweet natural products classified in five major chemical types, in terms of the position of the families of the plants of compound origin in Dahlgren's classification of angiosperm (flowering plant) superorders, is presented in Figure 1. Thus, no pattern seems to exist which would enable one to predict the occurrence of sweet compounds on a taxonomic basis. In contrast, we have been able to identify candidate sweet-tasting plants on the

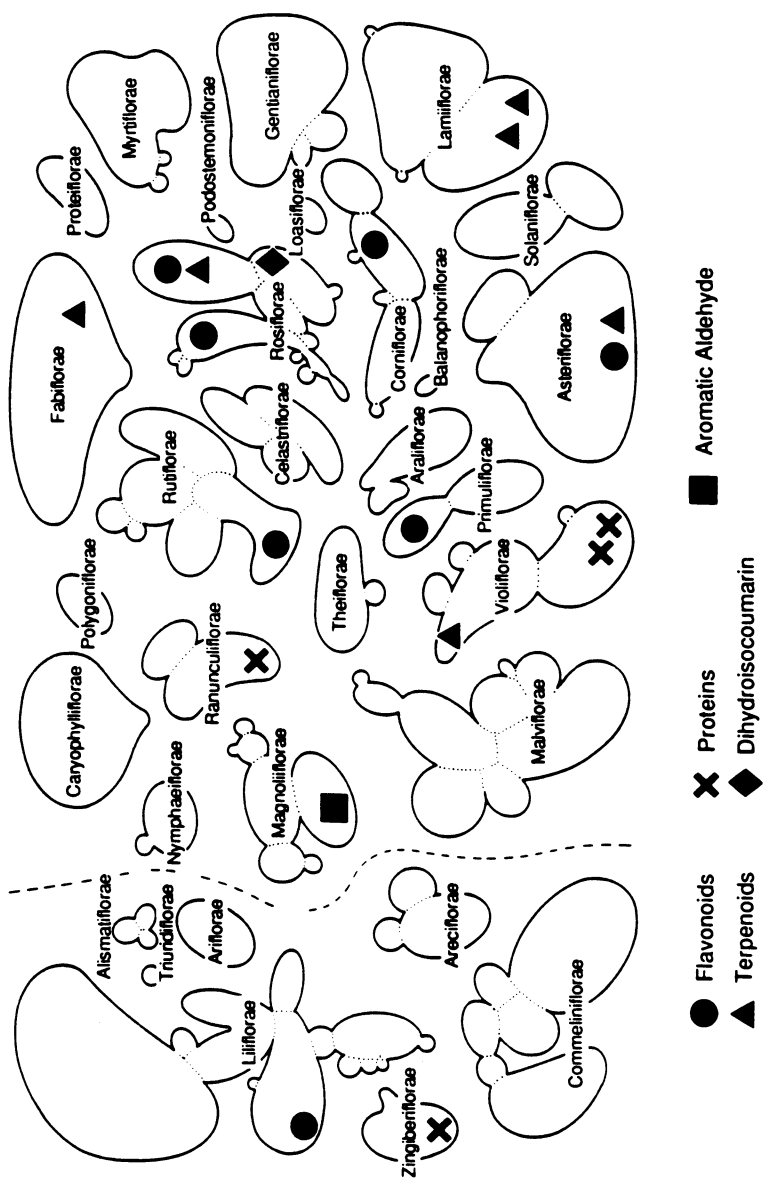


Figure 1. Distribution of known classes of highly sweet compounds in the plant kingdom, arranged according to Dahlgren's superorders.

basis of ethnobotanical observations in the field or by careful analysis of published botanical literature. In this manner, a systematic approach may be taken to the discovery of additional novel naturally occurring sweet substances.

Field Investigations. The most direct way of obtaining leads for sweet-tasting plants is to make inquiries in the field. It is apparent that unless a given plant part tastes as intensely sweet as sugar cane (*Saccharum officinale* L.), then it will probably not become widely known as sweet-tasting among indigenous peoples. Plants with such an overtly sweet taste that have found their way into the literature for this reason include *Stevia rebaudiana*, *Thaumatococcus daniellii*, and *Thladiantha grosvenorii*, as has been described by us earlier (1). However, plants that possess slight or moderate sweetness will probably not attract general attention, but may be noted by ethnobotanists and others, and then be appropriately documented. Alternatively, the slightly sweet taste of a particular species may be known to local members of the populace, such as vendors of medicinal plants. For example, we have investigated *Tessaria dodoneifolia* (Hook. & Arn.) Cabrera, which was purchased in a medicinal plants market in Asuncion, Paraguay, where it was sold as a native remedy under the Guarani name of "kaá hê-é" (sweet herb). Subsequent laboratory investigation afforded the first naturally occurring highly sweet flavonoid, namely, (+)-dihydroquercetin 3-acetate, and the first known member of the dihydroflavonol class of sweeteners (10).

Literature Sources. Literature information on medicinal plants is widely disseminated, and may be found in old herbals, botanical articles, and even material written for a largely non-scientific readership. Examples of how each of these can be utilized advantageously will be presented in turn. First, in our earlier work, the Mexican ethnobotanical literature was searched in order to uncover records of plants with a sweet taste. While examining a monograph entitled *Natural History of New Spain*, written between 1570 and 1576 by Francisco Hernández, a plant was described which was known to the Aztecs by the Nahuatl name *Tzonpelic xihuitl*. Based on the description provided by Hernández, and after the analysis of other literature, this sweet plant was identified as *Lippia dulcis* Trev., and a novel sesquiterpene sweetener, hernandulcin, was isolated (1,11,12). Second, in a botanical report of a field trip to West Africa to collect sweet plants, Inglett and May refer to the fact that the leaves of *Abrus precatorius* L. are equivalent in sweetness potency to sucrose (13). When we followed up on this lead, four novel cucurbitane-type triterpene glycosides, abrusosides A-D, were isolated and characterized (14,15). Finally, in following up on a lead that appeared in an article intended for a specialized lay readership (16), the previously unreported sweet constituent of *Myrrhis odorata* Scop. (sweet cicely), a plant used to

sweeten conserves and tart dishes, was established as the common phenylpropanoid, *trans*-anethole (17).

Another approach to the selection of candidate sweet plants from the literature that we have taken is to examine *Index Kewensis*, which is a repository of all of the Latin binomials of seed plants that have been published to date. This exercise was performed because it was reasoned that botanists have often recorded the sweet-tasting characteristics of a plant as its specific epithet when first described. Examples of epithets which could refer to a sweet taste are *saccharum* (Gk., *saccharon*, sugar), *saccharifera* (sugar-bearing), *dulcis* or *dulcificum* (L., sweet), and *glycyrrhiza* (Gk., *glykos*, *glyckeros*, sweet). Several sweet-tasting plants are indeed listed in this manner in *Index Kewensis*, such as *Acer saccharum* Marsh. (sugar maple); the previously mentioned *Lippia dulcis*; *Periandra dulcis* Mart. (the source of the sweet compounds, perianthrins I-IV) (1,3); and *Richardella dulcifica* (Schumach. & Thonning) Baehni [formerly *Synsapalum dulcificum* (Schumach. & Thonning) DC.], the source of the sweetness inducer, miraculin (1,3). Although not all sweet-tasting plants have a specific epithet that refers to their sweet-taste, a list of about 150 plants with a specific epithet suggestive of a sweet taste was drawn up for future investigations in the field and in the laboratory (18).

Phytochemical Aspects

We have found that methanol-water (4:1) is a good general solvent for plant secondary metabolites, and is typically employed for the initial extraction of plants collected for our program on sweet natural products. The dried 80% methanol-soluble extract of a given plant under investigation is taken up in methanol-water (1:1), and then successively partitioned into petroleum ether, ethyl acetate, and butanol, in order to afford extracts of various polarities (petroleum ether, ethyl acetate, butanol, and aqueous methanol) (19). The concentration of the sweetness of the plant under investigation in one of these solvents affords a useful preliminary notion of the type of natural sweetener present. For example, the petroleum ether extract might well contain sweet terpenoids or phenylpropanoids, the ethyl acetate extract sweet flavonoids, the butanol extract sweet di- and triterpenoid glycosides, and the aqueous methanol extract very polar sweet glycosides. On the rare occasions when the sweetness of a plant part is due to the presence of sweet proteins, extraction would normally have to be conducted using water (20).

A crucial step in the isolation of sweet compounds from plants is the assessment of the sweetness of extracts and chromatographically fractionated materials by one or more human volunteers (21). Usually, there is no anecdotal or literature evidence which will indicate if a plant found to be sweet in the field will also contain toxic substances. Therefore, to protect against

this possibility, we have made it a practice in our laboratory to subject one or more of the initial extracts of each plant acquisition to safety testing (depending upon the quantities of plant material available), prior to determining the presence or absence of sweetness by tasting. These materials are subjected both to an acute toxicity evaluation using mice, with administration by oral intubation up to a dose of 2 g/kg (22), as well as a forward mutation assay using *Salmonella typhimurium* strain TM677, in the absence and presence of a metabolic activator (23). In addition, all pure sweet compounds that are assessed by a human taste panel, must also be shown to be innocuous using these safety procedures. It has been our occasional experience that certain plant extracts are mutagenic. In such cases, these leads have not been pursued.

In order to proceed as expeditiously as possible, it is useful to rapidly assess the chemical type of sweet constituent(s) of a given sweet-tasting plant under consideration. Not all of the plants found to be sweet in the field will contain highly sweet constituents; in practice, this is the exception rather than the rule. We have found that two other major classes of sweet compounds may account for the perceived sweetness of a plant acquisition, namely, sugars and polyols, and sweet phenylpropanoids. In the former case, sugars and polyols will partition preferentially into methanol-water (1:1), or occasionally butanol, and may be concentrated by passage through a charcoal column and rapidly identified using gas chromatography/mass spectrometry (19). We have compared the saccharide and polyol concentration levels of several sweet-tasting plants whose sweetness is entirely due to sugars and polyols, to the fruits of *Thladiantha grosvenorii*, which contain both significant levels of sugars and polyols and over 1% w/v of an intensely sweet substance (mogroside V). In this way, it has been concluded that unless the saccharide and/or polyol content of a plant part is well over 5% w/w, then it is unlikely to exhibit an overtly sweet taste except if a highly sweet compound is also present (19). In addition, we have also found that sweet-tasting phenylpropanoids may occur in such high concentrations so as to confer a recognisably sweet taste to the plant part under consideration. For example, it was determined that *trans*-cinnamaldehyde was responsible for the sweet taste of *Cinnamomum osmophleoum* Kanehira leaves, where it occurred in a yield of 1.03% w/w (24). Similarly, a further phenylpropanoid, *trans*-anethole, was determined as the constituent responsible for the sweetness of five plants in which it proved to be the major volatile oil constituent (17). The observation that common phenylpropanoids can impart a sweet taste to a plant part is of considerable significance from the point-of-view of selecting candidate sweet plants for laboratory study. In order to avoid the expense of collecting large amounts of plant material that turn out to be sweet only because of the presence of high concentration levels of known phenylpropanoids, it is to be recommended that a petroleum ether extract or a steam

distillate of each acquisition be subjected to analysis by gas chromatography/mass spectrometry, so that these compounds can rapidly be detected and quantitated (17).

Use of Gerbils to Monitor the Sweetness of Plant Extracts. Since the practice of subjecting plant extracts to both acute toxicity tests in mice and bacterial mutagenicity evaluation is both time-consuming and expensive, we have investigated an alternative approach using gerbils (25). Thus, extracts of three plants known to be sweet-tasting owing to the presence of significant quantities of either diterpene or triterpene glycosides were investigated using Mongolian gerbil electrophysiological and conditioned taste aversion procedures (25). The electrophysiological method, employing the stimulation of the gerbil's intact chorda tympani nerve, has been utilized previously for the evaluation of sweet monosaccharides, disaccharides, polyols, and many naturally occurring and synthetic intensely sweet substances (26). In the behavioral conditioned aversion test, the degree of similarity of taste to sucrose is determined by the amount of experimental fluids consumed by gerbils trained to avoid sweet, salty, bitter, and sour taste qualities (27). The data obtained on the sweet plant extracts showed that good correlations were observed between the results of the gerbil experiments and the presence or absence of highly sweet substances (25). Experiments are underway on the evaluation of purified highly sweet natural products in these gerbil bioassays.

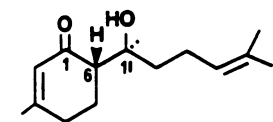
Novel Highly Sweet Natural Products Obtained in this Laboratory

Terpenoids. As mentioned earlier, the sweet sesquiterpenoid, hernandulcin (Figure 2, 1) was obtained from the plant, *Lippia dulcis*. This novel compound, which was named in honor of Francisco Hernández, was found to be a minor constituent of the volatile oil of the aerial parts of the plant, and its structure was determined after the performance of high-resolution mass spectrometry and two-dimensional NMR experiments. Racemic hernandulcin was synthesized in about 50% yield by directed-aldol condensation using 3-methyl-2-cyclohexen-1-one and 6-methyl-5-hepten-2-one as starting materials. The reaction occurred in a predictable stereoselective manner, with larger amounts of 1 being produced than the epimeric compound, the non-sweet epihernandulcin (Figure 2, 2). Synthetic hernandulcin was not acutely toxic for mice, and was not active as a bacterial mutagen at the doses tested. In a preliminary sensory test using a human taste panel, natural (+)-hernandulcin was perceived to be about 1,000 times sweeter than sucrose on a molar basis, although it was also found to exhibit undesirable hedonic effects, a fact which will no doubt reduce the prospects of this compound being developed commercially (11).

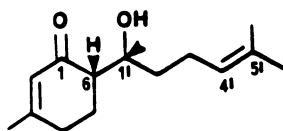
Several derivatives of hernandulcin have been made by directed-aldol condensation from appropriate starting ketones, in an effort to obtain compounds with improved hedonic qualities (28,29). Although none of these compounds turned out to be sweet, information has been gathered on the role of the various functionalities of hernandulcin in the mediation of sweetness. Thus, it is apparent that the C-1' hydroxyl and the C-1 carbonyl groups, respectively, correspond to Shallenberger's AH and B units. When the preferred conformations of (6S,1'S)- (natural) hernandulcin (**1**) and (6S,1'R)-epihernandulcin (**2**) were determined by molecular mechanics calculations, it was observed that epihernandulcin exhibits a lack of linearity compared with hernandulcin. Thus, even though these two compounds possess the same AH,B units, the bulky non-polar side-chain of compound **2** appears to restrict this non-sweet molecule from adequately fitting the sweet-taste receptor site(s) (28,29). The synthesis of various non-sweet derivatives of hernandulcin has also permitted the conclusion to be made that the C-4', C-5' double bond of hernandulcin is necessary for the exhibition of sweetness by this compound (29).

While the seeds of *Abrus precatorius* are toxic, the roots and leaves do not appear to be particularly poisonous, and have a long history of human internal consumption, particularly as licorice substitutes (15). According to even recent literature, the sweetness of *A. precatorius* leaves is due to the presence of high levels of the oleanane-type triterpene glycoside, glycyrrhizin (30). However, in our hands, glycyrrhizin was found to be absent from extracts of *A. precatorius* leaves collected in Florida, and four novel sweet cycloartane-type triterpene glycosides, abrusosides A-D (Figure 3, **3-6**), were obtained after chromatographic fractionation. The common aglycone of these compounds was obtained after acid hydrolysis, and characterized as (20S,22S)-3 β ,22-dihydroxy-9,19-cyclolanost-24-ene-26,29-dioic acid lactone, using the ¹H-¹H-COSY, ¹H-¹³C-HETCOR and selective INEPT NMR techniques. The structure and stereochemistry of this aglycone was confirmed by X-ray crystallography of its methyl ester derivative (14). The position of the sugar attachment and the linkages of the various saccharide moieties in compounds **3-6** were confirmed by application of the selective INEPT NMR technique on the intact heterosides (15).

Abrusosides A-D were found to be nonmutagenic and not acutely toxic for mice in preliminary safety tests. The water-soluble ammonium salts of these compounds exhibited sweetness potencies of between 30 and 100 times that of sucrose, with abrusoside D (Figure 3, **6**), the most abundant of these sweeteners in the plant, being rated by a small human taste panel as about 75x sweeter than 2% w/v aqueous sucrose solution. These compounds are equivalent in sweetness potency to glycyrrhizin, and offer the potential advantages over the latter substance in being easier to

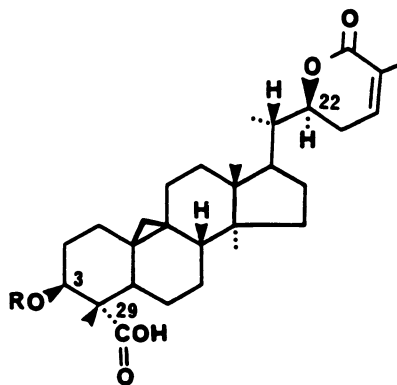


1



2

Figure 2. Structure of hernandulcin and epihernandulcin.



R

3 β -D-glc4 β -D-glcA-6-CH₃²- β -D-glc5 β -D-glc²- β -D-glc6 β -D-glcA²- β -D-glc

Figure 3. Structures of abrusosides A-D.

produce by cultivation (in being produced from the leaves of the plant rather than the roots), and in not possessing an unhindered β -unsaturated carbonyl group in their aglycone (15). It is the 11-oxo-12,13-dehydro- group of glycyrrhizin that is responsible for its undesirable adrenocorticomimetic effects that lead to edema and hypertension (31). Since it is likely that the abrusoside sweeteners will not produce the same toxic effects as glycyrrhizin, attempts are now being made to commercialize these compounds as a replacement for glycyrrhizin in the sweetening of foods, beverages, and medicines.

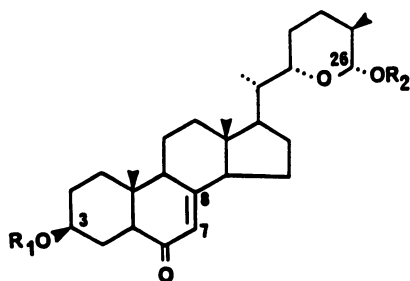
Steroidal Saponins. The rhizomes of the North American species, *Polypodium glycyrrhiza* D.C. Eaton, exhibit a bittersweet taste, and have a history of use as a foodstuff by humans. We became interested in this species initially as a potential source of osladin (Figure 4, 7), a steroidal saponin isolated in 1971 by Czechoslovakian workers from *Polypodium vulgare* L. (32). Osladin has been rated as up to 3,000 times sweeter than sucrose, making it one of the sweetest natural products known (1,3). Fractionation work on *P. glycyrrhiza* did not yield osladin, but the closely related compound, polypodoside A (Figure 4, 8) was isolated as a novel sweet-tasting principle instead. The structure of polypodoside A was determined after spectroscopic and hydrolytic experiments, and this substance was found to be a glycoside of the aglycone, polypodogenin, a compound of known stereochemistry (33). On biogenetic grounds, it is reasonable to suppose that polypodoside A is the $\Delta^{7,8}$ -derivative of osladin, although the configuration of the C-26 rhamnose unit of the latter compound has never been determined (33). Polypodoside A proved to be both nonmutagenic and not acutely toxic for mice at the doses tested, and was rated as possessing 600x the sweetness intensity of a 6% w/v aqueous sucrose solution (33). However, the compound exhibited a licorice-like off taste, and a lingering aftertaste. Polypodoside A does not seem to have good prospects for commercialization, since, in addition to its relatively unpleasant hedonic characteristics, it is rather insoluble in water, and contains an α,β -unsaturated lactone functionality in its aglycone, which could conceivably produce undesirable biological effects (33).

Two structural analogs of polypodoside A were also isolated from *P. glycyrrhiza* rhizomes, namely, polypodosides B and C (Figure 4, 9, 10). Polypodoside B was sweet-tasting, and apparently less intensely sweet than polypodoside A, although it was not evaluated by a taste panel because of the paucity of compound isolated from the plant. Polypodoside C, the 3"-O-methyl derivative of polypodoside B, was devoid of any sweet taste (34). Since it is known that the monodesmosidic polypodogenin glycoside, polypodosaponin (Figure 4, 11) is not a sweet-tasting compound (35), it may be concluded that polypodogenin heterosides must be bisdesmosidic in order to produce a sweet taste, with saccharide

substitution occurring at both the C-3 and the C-26 positions. However, even among such compounds, minor structural differences among the sugar units can markedly affect the sweet taste (34).

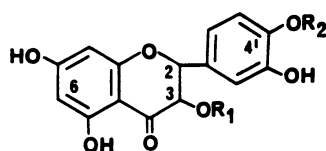
Dihydroflavanols. As mentioned earlier in this chapter, the sweet-tasting plant, *Tessaria dodoneifolia*, was obtained from a medicinal plants market in Paraguay. The plant was cultivated at the University of Illinois Pharmacognosy Field Station until sufficient amounts of the young shoots of the plant were available for isolation work. The sweetness was traced to the known dihydroflavonol, (+)-dihydroquercetin 3-acetate (Figure 5, **12**), which was extracted into the ethyl acetate extract during work-up. Although this compound was not novel, its sweet taste had never been recognized before. After the usual preliminary safety tests, compound **12** was rated as being about 80x sweeter than sucrose, when 3% ethanol-water solutions of each compound were matched in sweetness intensity (10). The absolute stereochemistry of **12** at C-2 and C-3 was determined as 2*R*,3*R*, by conversion to the pentacetate derivative of the commercially available compound, (+)-dihydroquercetin (taxifolin) (Figure 5, **13**). It is interesting to note that neoastilbin (Figure 5, **14**), another sweet dihydroflavonol constituent that occurs in the Chinese medicinal plant, *Englehardtia chrysolepis* Hance, has 2*S*,3*S*- stereochemistry, whereas its 2*R*,3*R*-analog, astilbin (Figure 5, **15**) is not sweet at all (36).

Compound **12** underwent slow spontaneous oxidation in neutral and basic media, and it was reasoned that the introduction of a 4'-methoxy group in ring B would create a more stable compound, since free-radical formation at this position would be inhibited. Furthermore, by analogy with the dihydrochalcone and dihydroisocoumarin classes of sweetening agents, it was also thought that the sweetness potency of **12** could be substantially enhanced by the introduction of a methyl ether group. Thus, when racemic dihydroquercetin 3-acetate 4'-methyl ether was synthesized, this non-toxic novel compound was found to be 400x sweeter than sucrose, and to exhibit a pleasant taste with no bitterness, albeit with a somewhat slow onset of sweetness. Preliminary structure-sweetness studies among the analogs of **12** have shown that the 3-acetate group is not essential for sweetness, since racemic dihydroquercetin 4'-methyl ether (Figure 5, **16**) is still sweet, being about one-tenth of the sweetness potency of compound **15** (10). In more recent work, a number of 6-methoxylated analogs of **12** have been isolated from *Hymenoxys turneri* K. Parker, and shown to be less sweet than compound **12** itself (37).



	R ₁	R ₂
7	β -D-glc ² - α -L-rha	rha (7,8-dihydro)
8	β -D-glc ² - α -L-rha	α -L-rha
9	β -D-glc	α -L-rha
10	β -D-glc	α -L-rha-3-Me
11	β -D-glc ² - α -L-rha	H

Figure 4. Structures of sweet and non-sweet steroidal saponins.



	R ₁	R ₂
12	Ac	H (2R,3R)
13	H	H (2R,3R)
14	α -L-rha	H (2S,3S)
15	α -L-rha	H (2R,3R)
16	Ac	Me
17	H	Me

Figure 5. Structures of sweet and non-sweet dihydroflavonol derivatives.

Summary and Conclusions

It seems likely that there will be a continued need for new noncaloric and noncariogenic sucrose substitutes in the future. Many natural, highly sweet substances, even with less than ideal properties, could find use in speciality markets. Opportunities also seem to exist in the Japanese market for the introduction of additional novel natural product sweeteners, where there is an extensive current use of these substances. It has been shown in this chapter that, as a result of a concerted effort with good botanical practices and the availability of modern phytochemical equipment, significant progress can be made in the discovery of many novel sweet substances for a modest capital investment. Plant-derived sweeteners are not only potentially useful as commercially exploitable compounds directly, but can also serve as lead molecules for synthetic optimization and sweetener computer design. These compounds could well have an increased role in the future in physiological experiments designed to further understand the nature of the sweetness receptor(s).

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Chapter 3

Sweet Proteins

Biochemical Studies and Genetic Engineering

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Monellin and thaumatin are two potently sweet natural proteins extracted from African berries. Despite their taste similarities they have no apparent similarities of significance in amino acid sequences or in three-dimensional backbone structures. Biochemical and structural studies suggest the potential sites for the sweet taste receptor binding. We have also redesigned monellin by genetic engineering to increase its thermal stability and renaturability.

Two unusual proteins, discovered in African berries, possess the interesting property of having a very high specificity for the sweet receptors, registering sweet taste at a concentration of 10^{-8} M. These proteins, monellin (1,2) and thaumatin (3), are approximately 100,000 times as sweet as sugar on a molar basis and several thousand times as sweet on a weight basis. Thaumatin is a single chain protein of 207 residues, and monellin consists of two peptide chains, the A chain of 45 and B chain of 50 amino acid residues (4,5). Neither contains carbohydrates or modified amino acids. Two aspects of these proteins are addressed in this paper, one dealing with the recognition site of the proteins by sweet taste receptor, and the other, improvement of practical properties of monellin as a natural sweetener.

Several interesting observations have been made about the two proteins: native conformations are essential for the sweet taste (6-8); although both proteins are intensely sweet, there are no statistically significant sequence similarities (9); despite the absence of sequence similarity, antibodies raised against thaumatin

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cross-react with and compete for monellin as well as many other sweet compounds (10,11) but not for chemically modified non-sweet monellin (10), and similar cross-reactivity was also found for antibodies raised against monellin (12); antibody-protein complexes lose their ability to elicit sweet taste (unpublished results); the cross-adaptation of monellin and thaumatin in human taste experiments (13) and electrophysiological experiments (14) suggests that they are recognized by the same receptor. Two questions of interest are: (a) How does an antibody recognize two proteins with no significant sequence similarities, and (b) what is the chemical basis for sweet receptor recognition? From a practical view point sweet proteins such as monellin and thaumatin have some important advantages over artificial sweeteners. Among these advantages are the following: They are low in calories due to their high potency. They are safe, natural, and neither introduce non-natural metabolites into the body nor perturb the balance of the amino acid pool (decomposition of the protein results in a natural distribution of all amino acids). This is not the case with amino acid sweeteners or dipeptide sweeteners, which, when hydrolyzed, may elevate specific amino acid pools and could cause adverse effects. It is relatively simple to clone the genes for the proteins and mass produce the proteins for practical use. It is relatively easy to generate a large number of variants with different amino acid substitutions, deletions and insertions at various positions in order to search for more desirable taste as well as physical properties. The genes can be introduced into edible fruits and plants as well as many microorganisms used in the food and beverage industry. The proteins or their variants can be used as reagents for isolating sweet taste receptors or receptor genes. However there are some disadvantages as well. Taste profiles of these proteins are different from sugars, and therefore their utility may be not as general as sugar. Heat stability of the proteins is usually not as good as that of low molecular weight sweeteners. When monellin is heated, the two peptide chains separate, and the sweet taste is lost. Cooling does not renature the protein well, and therefore the sweet taste does not return. Thaumatin, on the other hand, has eight disulfide bonds, and is a single chain protein of 207 amino acid residues (9,15). This protein is much more thermally stable than monellin, but once denatured, it does not renature well either. From the practical stand point of using these proteins as food additives, renaturability is just as important and perhaps more important, as thermal stability. We have chosen to redesign monellin to improve its renaturability by genetic engineering techniques because it is the smaller of the two proteins, has no disulfide bonds and has a very simple folding motif--one α -helix and one β -sheet.

Potential Sweet Determinant Site

Since there is no *in vitro* assay for sweet taste activity and no taste receptor molecule has yet been isolated, there has been interest in understanding immunological cross-reactivity as a possible clue for the location of the receptor binding site. This is based on the assumptions that antibody and receptor binding sites are likely to be exposed, and that one may be a subset of the other, as well as the observation that the antibody-protein complex loses its ability to elicit a sweet taste. Despite the absence of amino acid sequence similarity, it is possible that the two proteins may have a common surface region exposing a few key side chains or functional groups in similar stereospecific arrangements. To explore this possibility, the crystal structures of thaumatin (16) and monellin (17) have been determined at 3 Å resolution. The backbone structures of both proteins are shown in Figure 1. These structures show clearly that there is no overall similarity of significance at the level of backbone structures except for the β -sheet structural motif, which is common in many proteins.

Although there are no statistically significant sequence similarities, Iyengar et al (9) noticed that there are five pairs of homologous tripeptide sequences: residues B28-30, B6-8, A23-25, A30-32 and A22-24 in monellin (labeled 1 to 5 in Figure 1a) have the same sequences as residues 94-96, 100-102, 101-103, 118-120 and 128-130 (labeled 1 to 5 in Figure 1b) in thaumatin, respectively. Although the tripeptide similarities are not statistically significant, the possibility exists that a joint site made of two or more of these short homologous regions may act as an antigenic determinant. This possibility can be ruled out based on the examination of both crystal structures: regions 2 and 5 of thaumatin are in β -sheet and loop conformation respectively, but the corresponding regions 2 and 5 of monellin are in loop and β -sheet conformation, respectively. Out of the remaining three regions, 1, 3, and 4, region 3 can be ruled out as only one residue out of three in this region of monellin is exposed. The final two regions, 1 and 4, cannot be the joint common antigenic site because the separation between them is 26 Å in monellin and 18 Å in thaumatin.

It is clear that we have to examine the distribution of side chains and functional groups on the three-dimensional surface, rather than amino acid sequence, in search for the similarities. At present no method exists to compare the residues of the entire surfaces of two proteins. To simplify this task, we have identified small peptides in each protein that are recognized by respective cross-reacting antibodies (unpublished results). Comparison of the crystal structures of these regions reveals that a loop within a cross-reacting peptide of one protein has the same side chains of two non-contiguous residues in a conformation similar to those of

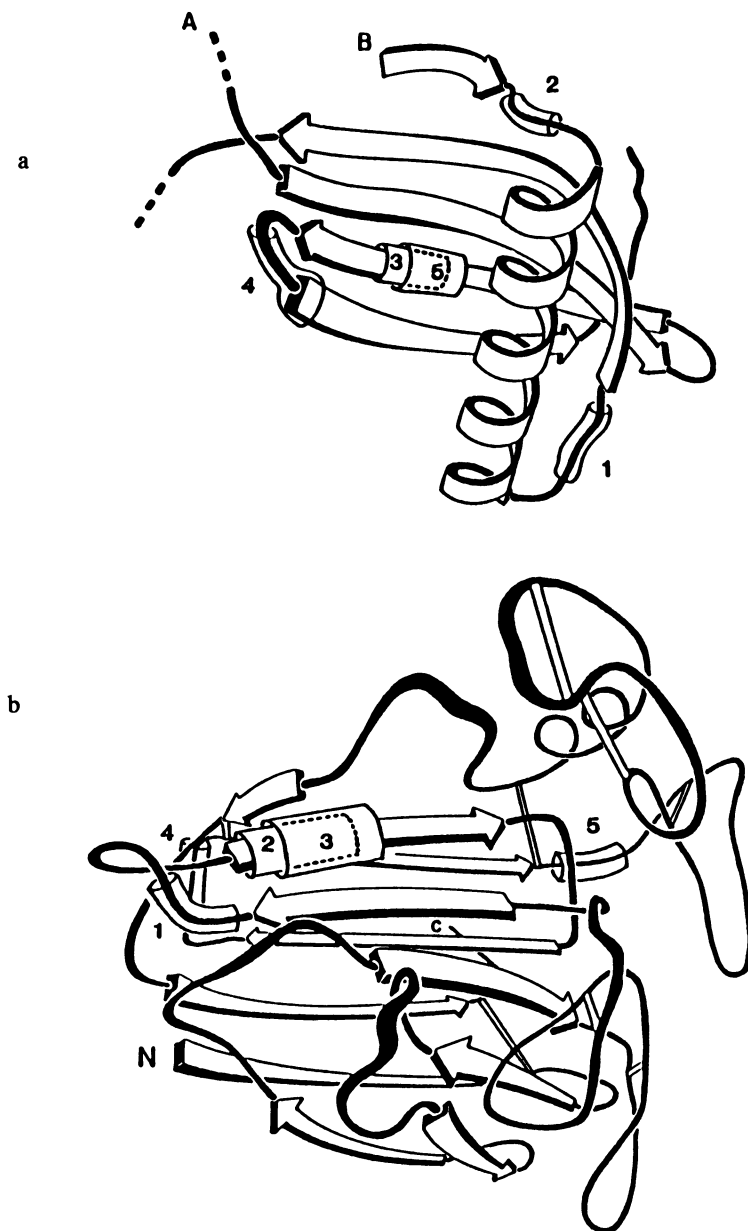


Figure 1. Backbone structures of monellin (top) and thaumatin (bottom). The tube sections indicate the regions with tripeptide sequence homologies between the two proteins. The homologous tripeptides of each pair are identified by the same number in both structures. The dashed lines for the top figure represent disordered regions of the monellin molecule. (Reproduced with permission from ref. 17. Copyright 1987 Macmillan.)

the other protein. This suggests that the regions may be a part of the common antigenic sites in the two proteins (see below).

Antibody Cross-reactivity between Monellin and Thaumatin

Antisera raised in rabbits against thaumatin cross-reacted with monellin as shown by Hough and Edwardson (11) and van der Wel and Bel (10). In a reciprocal experiment we have found that antibodies raised against monellin also cross-reacted with thaumatin (12). The ability of the one antiserum to recognize the other sweet tasting protein was determined in two ways: by an enzyme-linked immunoabsorbant (ELISA) assay and by Western immunoblotting. To identify the regions of the proteins that cross-react immunologically, both proteins were cleaved with TPCK-trypsin and the resultant peptides were purified by reverse phase HPLC. Each peptide was immuno-blotted with respective cross-reacting antibody. The location of the two cross-reacting peptides from each protein in the crystal structures are shown in Figure 2. The first thaumatin peptide is a part of the bottom β -sheet of thaumatin and the second is the highly exposed and protruding "hair-pin" structure. The first and second monellin peptides (Mo-12 and Mo-17 in Figure 2 respectively) that cross-react are a part of a β -sheet with a short "hairpin" at the end and a β -strand followed by a short α -helix respectively. Comparison of these peptides reveals a surprising similarity between the tip of the hairpin loop of the first thaumatin peptide and that of the first monellin peptide: the two most exposed side chains of the thaumatin hairpin loop are tyrosine 57 and aspartate 59, which are exposed in a similar way to tyrosine A14 and aspartate A17 of the monellin peptide (Figure 3a), although the backbone conformations are different and the monellin peptide has two intervening amino acids rather than one in the thaumatin peptide. Interestingly, the separation between aspartate and the aromatic sidechain of tyrosine is comparable to the separation between the aspartate and phenylalanine side chains of the sweet dipeptide aspartame (Figure 3b), although the backbone polarity of aspartame runs in the opposite direction. This observation suggests that the hairpin loops of monellin including the amino acid sequence of YASD (A14-A17) and of thaumatin including that of YFD (57-59) may be a part of the recognition sites for the cross-reacting antibodies. They may also be a part of the sweet receptor recognition site. This is based on the argument that antibodies raised against monellin or thaumatin also compete for aspartame and that the relative location of the aspartate and the phenylalanine of aspartame can be similarly superimposed to the aspartate and the tyrosine of the most exposed hairpin loops in both proteins (Figure 3).

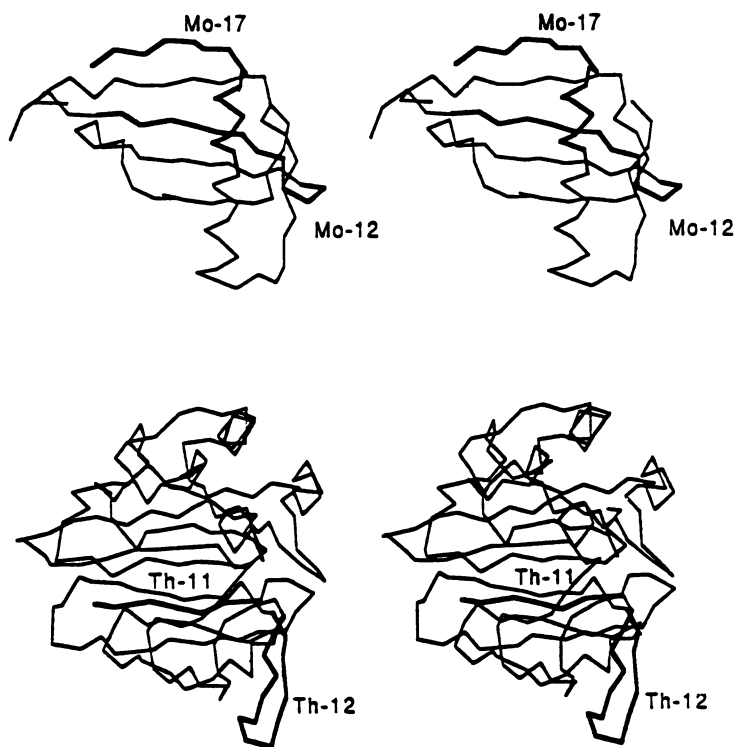


Figure 2. Stereoviews showing the location of the tryptic peptides that cross-react to heterologous antibodies are indicated by thicker lines in the crystal structures of monellin (top) and thaumatin (bottom).

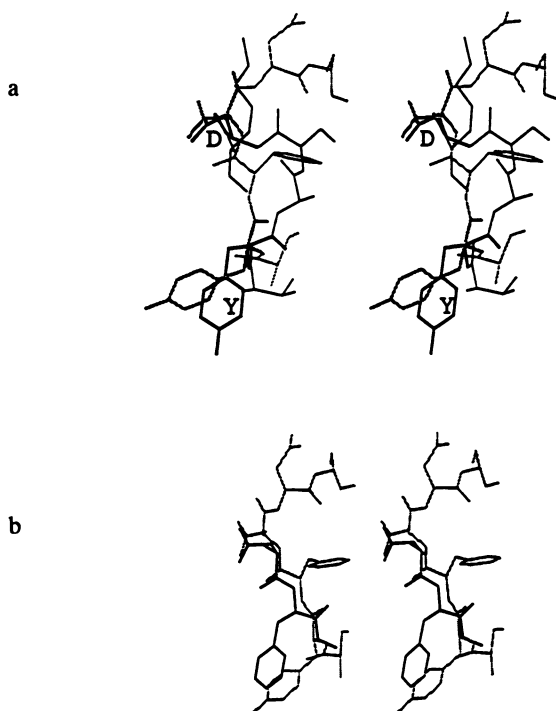


Figure 3. (a) Stereoview showing relative location and orientation of tyr 57 (Y) and asp 59 (D) in thaumatin (dotted lines) and tyr A14 and asp A17 of monellin crystal structures (continuous lines); (b) Stereoview showing the superposition of aspartame (thick lines) and thaumatin residues 57–59 (thin lines). In both figures α – β bonds of side chains are varied to maximize the overlaps.

Redesigning Monellin to Increase Thermal Stability and Renaturability

Using the information from the crystal structure of natural monellin (17) and genetic engineering techniques we fused the two chains of monellin into a single chain using several different linkers copied and "transplanted" from the same molecule (18). The linkers used were YENEREIK, YYASDKLK, YASDKL, EDYKTRGR, and EDYKTR. One of the newly designed proteins containing the first linker is as sweet as the natural one, is more stable upon temperature or pH changes and renatures easily even after brief heating to 100 °C at low pH. This fused monellin (SP1) is easier to express in *E. coli* or yeast and to purify in large quantities. Furthermore, various properties of the protein can now be altered and studied using site-directed mutagenesis.

Design of single chain monellin

The crystal structure of monellin has suggested a very natural and obvious way of fusing the two peptides of monellin. Figure 4 shows the topological structure based on the backbone structure of the monellin molecule as determined by X-ray diffraction studies at 3 Å resolution (17). The structure shows that the carboxy-terminal β -strand of the B-chain forms an antiparallel β -sheet with the amino-terminal β -strand of the A-chain. We can clearly see that isoleucine-46 of the B-chain is in register and hydrogen-bonded to glycine-6 of the A-chain. The most conservative design has a junction eight residues long. It contains the same amino acid sequence as natural monellin except that it lacks the first residue, phenylalanine, of the A-chain. Thus a new peptide bond formed is between Glu-50 of the B-chain and Arg-2 of the A-chain (see Figure 2), resulting in a single chain 94 residues long (SP1). A synthetic gene coding for the fused monellin design was cloned and expressed in *E. coli*. The expressed protein was as sweet as the natural one. We subsequently cloned the synthetic fused monellin gene into a yeast expression vector, which gave a high yield of fused monellin.

Characterization of Single-Chain Monellin

All single chain monellins we have constructed are sweet. Of these, SP1 is the sweetest, and characterization and taste assay were done primarily on it. The *E. coli* expressed single-chain monellin molecule SP1 has the apparent molecular weight corresponding to the sum of the two chains (A and B) of natural monellin upon analysis by electrophoresis on an SDS gel (Figure 5a). It binds specifically to the antibody raised against natural monellin (Figure 5b). N-terminal sequence indicated that the first residue, methionine, was cleaved off in the final protein. Compared to sucrose solutions of different concentrations, the intensity of

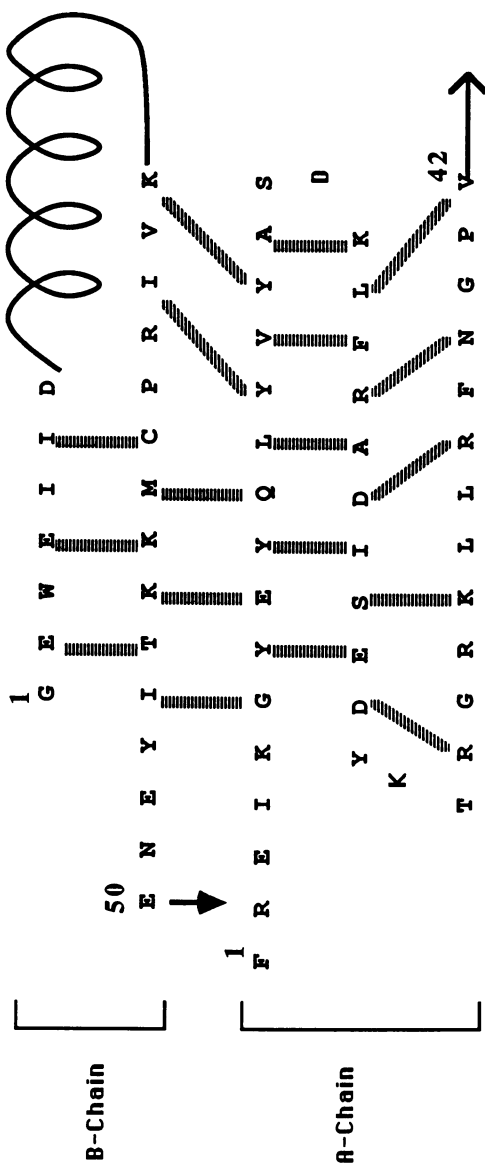


Figure 4. Topological structure and the registration of residues in the hydrogen-bonding scheme of the β -sheet in the monellin crystal structure. Hydrogen bonds are indicated by vertical bars. The covalent link between E(B50) and R(A2) is indicated by a diagonal bar. The horizontal arrow at the end of V42 indicates the presence of more residues.

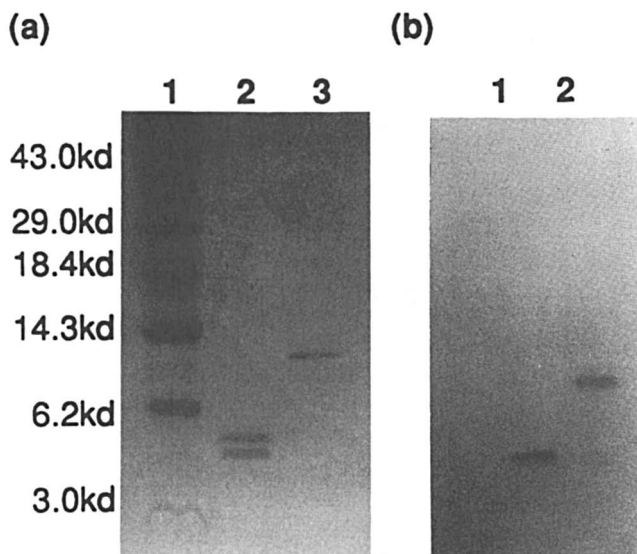


Figure 5. (a) Polyacrylamide slab gel electrophoresis of monellin and fused monellin, SP1. Lane 1, marker proteins: insulin (3kd), bovine trypsin inhibitor (6.2 kd), lysozyme (14.3 kd), β -lactoglobulin (18.4 kd), carbonic anhydrase (29 kd), ovalbumin (43 kd); lane 2, natural monellin (3 mg) which is composed of A (45 residues) and B chain (50 residues); lane 3, fused monellin SP1 (2 mg) which is composed of 94 residues. (b) Western blot using antibody raised against natural monellin: lane 1, natural monellin band corresponding to the B chain; lane 2, fused monellin SP1. (Reproduced with permission from ref. 18. Copyright 1989.)

sweetness of fused monellin is just as potent as the natural one. Circular dichroism spectra at room temperature (Figure 6) of the fused monellin SP1 are practically identical to that of natural monellin at pH 7, and stable throughout the entire pH range between 2 and 10. Natural monellin, on the other hand, changes its conformation at the extreme ends of the pH range. The thermal stability of the fused monellin is improved over that of natural monellin at all pH ranges tested as judged by the T_m measurements as well as by the taste assay (Table 1A), where slightly above minimal concentration (necessary to perceive sweet sensation) of protein solutions were tasted after heating at various pH values. Furthermore renaturability has also drastically improved, especially at very low pH. When natural monellin is heated to 50 °C at pH 2 (T_m is below 40 °C at pH 2) and allowed to cool down to room temperature, it loses its sweet taste. However, when the fused monellin SP1 is heated as high as 100 °C at the same pH (T_m is about 44 °C at pH 2) for several minutes and allowed to cool down to room temperature, it recovers its sweet

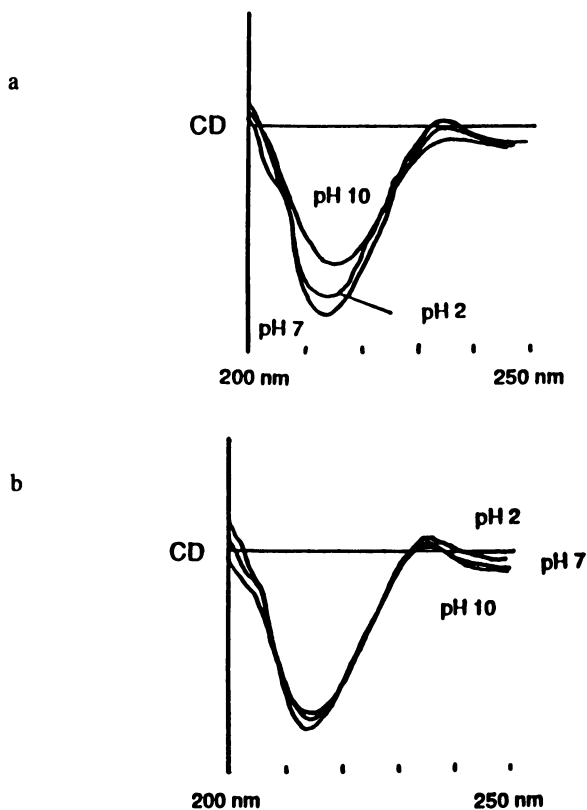


Figure 6. Circular dichroism spectra of (a) natural monellin at pH = 2, 7, 10; and (b) single-chain monellin, SP1, at pH = 2, 7, 10. (Reproduced with permission from ref. 18. Copyright 1989.)

taste almost completely (Table 1B). Reasons for the increase in T_m and renaturability of SP1 may be due to the fact that the disordered C-terminus of the B chain and the N-terminus of the A chain of the natural monellin, which could facilitate the denaturation of the native protein upon heating by "fraying", were joined in SP1, and that the renaturation of the SP1 may be favored over the natural counterpart due to the entropic effect of folding of a single chain over two-chain proteins. It is possible that thermal stability can be improved even more by removal of residues that are known to destabilize proteins. We have recently crystallized SP1 protein and preliminary X-ray studies indicate the structure is practically identical to the natural one except in the linker region.

The fact that several redesigned single chain monellins with different linker sequences taste sweet implies that the sweet determinant is not located in the linker region of the molecule. This is consistent with the observation discussed earlier that the potential common antigenic site and sweet determinant is located on the opposite side of the molecule from the linker region.

Table I. Heat stability of natural monellin (M) and fused monellin SP1 at pH 2, 4, and 6 (a) when tasted at the given temperature; (b) when tasted at room temperature. (+) = sweet; (-) = not sweet; nd = not determined.

(a) Taste at given temperature

Temp (°C)	pH 2		pH 4		pH 6	
	M	SP1	M	SP1	M	SP1
40	+	+	+	+	+	+
50	-	+	+	+	+	+
60	-	-	+	+	+	+
70	-	-	-	+	+	+
80	nd	nd	-	+	+	+
90	nd	nd	nd	+	(+)	(+)
100	nd	nd	nd	(+)	-	(+)

(b) Taste at room temperature

Temp (°C)	pH 2		pH 4		pH 6	
	M	SP1	M	SP1	M	SP1
40	+	+	+	+	+	+
50	-	+	+	+	+	+
60	-	+	+	+	+	+
70	-	+	+	+	+	+
80	nd	+	+	+	+	+
90	nd	+	+	+	+	+
100	nd	+	+	+	+	+

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Chapter 4

Sweet Peptides and Proteins

Synthetic Studies

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Preliminary results of a two-pronged approach to the delineation of the mode of sweetener-receptor binding are reported. First, the results of an extensive program based on N- and C-terminal extension of potentially sweet dipeptides are described. It can be concluded that the receptor has a small space that can accommodate an additional small D-amino acid residue at the site facing the N-terminus of sweet aspartyl dipeptides. It can also be concluded that there probably exists a spatial barrier at the site facing the C-terminal part of the sweet peptides. Second, the primary structure of the sweet protein monellin was unambiguously determined by a combination of solid phase peptide synthesis and comparative tryptic digest mapping of synthetic and natural products. It is proposed that knowledge of monellin structure will allow design of novel peptide sweeteners and will aid in determining the mode of sweetener-receptor binding.

In an attempt to deduce the mode of interaction between sweet-tasting peptides and their receptor, we have been engaged in a study of peptide and protein sweetener analogues. This review summarizes our progress in this program.

Peptide Sweetener Studies

In earlier work (1), the relative potencies of the aspartyl dipeptide esters 1-11 given in Table I were rationalized. In this work, the Fischer projection formulae "A" and "B" of Figure 1 were employed. It was found that the most potent dipeptide esters uniformly are of the "A" type while the inactive analogues are of the "B" type of molecular topography. This work clearly showed that activity did

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not depend on the L- or D-configuration of the second amino acid ester but rather on the size and shape of the amino acid ester carboalkoxy and sidechain substituents. When R^1 and R^2 are sufficiently dissimilar in size, the sweetness potency is very high.

On the basis of Structure-Activity-Relationships (SAR) and molecular mechanics calculations on sweet-tasting aspartyl dipeptide analogues, the mode of interaction between sweet peptides and the receptor has been hypothesized (2-7). These receptor models suggest that, if space remains in the dipeptide ester receptor binding site at the C- or N-terminus of sweet aspartyl dipeptides, then peptides extended at the C- or N-terminus of the sweet peptides may taste sweet. Thus, we undertook a study of the C- and N-terminal extension products of sweet-tasting peptides.

C-Terminal Extension of Sweet Aspartyl Peptides

Extension at the C-Terminus of Aspartyl Dipeptide Esters (Compounds **12-28** in Table II). In order for aspartyl tripeptide esters to be sweet, the second amino acid must have the D-configuration and a small, compact alkyl sidechain. In addition, an L-configuration for the third amino acid is required in order to achieve any significant sweetness potency. Thus, the general structure for sweet aspartyl tripeptide esters can be drawn as in Figure 2, in which R^1 and R^3 are hydrogen atoms, and R^2 and R^4 are small alkyl groups. As suggested by a comparison of the activities of tripeptide esters **12-16** with that of **18** or **20**, the small alkyl groups (R^2 and R^4) may participate in hydrophobic interactions with the receptor thus causing increase in their sweetness potencies.

Extension at the C-Terminus of Aspartyl Tripeptide Esters (Compounds **29-42** in Table II). Tetrapeptides **30-38** were predicted to be weakly sweet, since the C-terminal extension of aspartyl dipeptides significantly decreased their sweetness potencies as described above, although the second and third amino acids have the D- and L-configurations, respectively. As expected, the sweetness potencies of these aspartyl tetrapeptides were extremely low or devoid of sweetness.. The sweetness was accompanied by a bitter or astringent taste.

Extension at the C-Terminus of Aspartyl Tetrapeptides (Compounds **43-49** in Table II). The pentapeptides (i.e., **43-49**) were predicted not to be sweet, since the trend observed with aspartyl tripeptides **12-29** and tetrapeptides **30-42** is a significant decrease in sweetness potency with elongation of the peptide chain. As expected, all the pentapeptides are essentially tasteless or faintly bitter.

Table II. Sweetness Potencies of C-Terminus Elongation Peptides

	Compound	Sweetness Potency ^a	Ref.
12	L-Asp-Gly-Gly-OMe	0	13
13	L-Asp-Gly-L-Ala-OMe	2	13
14	L-Asp-Gly-D-Ala-OMe	1	13
15	L-Asp-Gly-L-Val-OMe	-	13
16	L-Asp-D-Ala-Gly-OMe	3	13
17	L-Asp-D-Val-Gly-OMe	0	13
18	L-Asp-D-Ala-L-Ala-OMe	50	13
19	L-Asp-D-Ala-D-Ala-OMe	5	13
20	L-Asp-D-Ala-L-Val-OMe	50	13
21	L-Asp-D-Ala-L-Phe-OMe	-	13
22	L-Asp-D-Val-L-Ala-OMe	25	13
23	L-Asp-D-Val-D-Ala-OMe	4	13
24	L-Asp-D-Val-L-Val-OMe	30	13
25	L-Asp-D-Leu-L-Ala-OMe	-	13
26	L-Asp-L-Ala-L-Ala-OMe	-	13
27	L-Asp-L-Ala-L-Val-OMe	0	13
28	L-Asp-L-Val-L-Val-OMe	+/- (4)	13
29	L-Asp-Gly-Gly-Gly-OMe	0	14
30	L-Asp-D-Ala-L-Ala-L-Ala-OMe	0.5	14
31	L-Asp-D-Ala-L-Ala-L-Val-OMe	-	14
32	L-Asp-D-Ala-L-Ala-L-Leu-OMe	-	14
33	L-Asp-D-Ala-L-Val-L-Ala-OMe	2	14
34	L-Asp-D-Ala-L-Val-L-Val-OMe	-	14
35	L-Asp-D-Val-L-Ala-L-Ala-OMe	+	14
36	L-Asp-D-Val-L-Ala-L-Val-OMe	-	14
37	L-Asp-D-Val-L-Val-L-Ala-OMe	+/- (5)	14
38	L-Asp-D-Val-L-Val-L-Val-OMe	-	14
39	L-Asp-L-Ala-L-Ala-L-Ala-OMe	-	14
40	L-Asp-L-Ala-L-Val-L-Ala-OMe	0	14
41	L-Asp-L-Val-L-Ala-L-Ala-OMe	0	14
42	L-Asp-L-Val-L-Val-L-Ala-OMe	0	14
43	L-Asp-D-Ala-L-Ala-L-Ala-L-Ala-OMe	0	15
44	L-Asp-D-Ala-L-Ala-L-Ala-L-Val-L-Ala-OMe	0	15
45	L-Asp-D-Ala-L-Val-L-Ala-L-Ala-OMe	-	15
46	L-Asp-D-Ala-L-Val-L-Val-L-Ala-OMe	0	15
47	L-Asp-D-Ala-L-Val-L-Ala-L-Leu-OMe	0	15
48	L-Asp-D-Val-L-Ala-L-Val-L-Ala-OMe	0	15
49	L-Asp-L-Ala-L-Val-L-Val-L-Ala-OMe	0	15

^a Times as potent as sucrose (weight basis, 0.6% sucrose = 1).
+, faintly sweet; -, bitter; 0, tasteless; +/-, bitter-sweet, the number in parentheses is the sweetness value.

The results described above suggest to us that the receptor site may exist in the form of a deep pocket with critical binding sites deep inside. Thus, as an example for the case of aspartyl tripeptide esters, we propose that the mode of interaction with the receptor may be as is illustrated in Figure 3. In the case of the sweet-tasting dipeptide ester aspartame (L-Asp-L-Phe-OMe), the methyl ester group corresponds to R² and will interact with the receptor through a hydrophobic interaction, and the benzyl moiety corresponding to -CONHCR³(R⁴)COOR⁵ will interact with the receptor at the complement of R⁴ through a hydrophobic interaction. It is apparent that these hydrophobic interactions enhance sweetness potency. It is our view that increases in the length of the peptide chain cause increased difficulty in fitting the proposed deep receptor pocket. If true, the concomitant decrease in binding affinity to the receptor would result in diminished sweetness potency. In conclusion, it is clear that the receptor can accommodate an additional small L-amino acid ester at the site facing the C-terminus of sweet aspartyl dipeptides as shown in Figure 3.

We hypothesize that there exists a spatial barrier at the site facing the C-terminus of the sweet-tasting peptides. This proposal is based on the observation that, while most of the aspartyl dipeptide analogues described are potently sweet, potency diminishes sharply on elongation at the C-terminus, finally reaching zero for the cases of the pentapeptides. This spatial barrier is the same as the spatial wall suggested by Iwamura (5), based on a quantitative analysis of the SAR of an extensive series of sweet-tasting dipeptides.

N-Terminal Extension of Sweet Aspartyl and Aminomalonyl Peptides (16)

Extension at the Free α -Amino Group of Sweet Aspartyl Dipeptides (Compounds **50-59** in Table III). It was found that extension of sweet aspartyl dipeptide esters by adding a small D-amino acid residue generally gave sweet compounds, although this alteration also significantly decreased sweetness potency. The D-configuration of a newly introduced amino acid appears to be essential for activity as can be seen by comparison of diastereoisomeric pairs **50/51** and **58/59**. Further extension at the N-terminus of the extended sweet tripeptides **50** and **58** resulted in the nonsweet compounds **60** and **61**, respectively.

Extension at the Free α -Amino Group of Sweet Aspartyl Tripeptides (Compounds **62-65** in Table III). The N-terminal extension of the sweet aspartyl tripeptides **18** and **24** gave the faintly sweet compounds **62** and **63** and the nonsweet compounds **64** and **65**. This may be explained by a poor fit of the tetrapeptides into the proposed narrow receptor pocket.

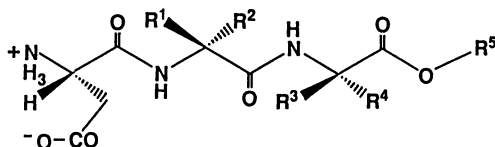


Figure 2. Sweet aspartyl tripeptide esters. $R^1 = R^3 = \text{H}$; $R^2 = R^4 = \text{small alkyl group}$.

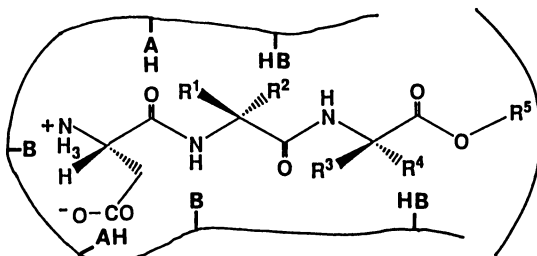


Figure 3. Schematic drawing of the mode of interaction between the sweet aspartyl tripeptides and the receptor. $R^1 = R^3 = \text{H}$; $R^2 = R^4 = R^5 = \text{small alkyl group}$; AH, Proton donor; B, Proton acceptor; HB, Hydrophobic binding site.

Table III. Sweetness Potencies of N-Terminus Elongation Peptides

	Compound	Sweetness Potency ^a	Ref.
50	D-Ala-L-Asp-D-Ala-OMe	12	16
51	L-Ala-L-Asp-D-Ala-OMe	+	
52	D-Val-L-Asp-D-Ala-OMe	+	16
53	D-Ala-L-Asp-D-Ala-OPr	30	
54	D-Pro-L-Asp-D-Ala-OPr	12	16
55	D-Ala-L-Asp-D-Val-OMe	1.5	
56	D-Val-L-Asp-D-Val-OMe	0	
57	D-Pro-L-Asp-D-Val-OMe	0	16
58	D-Ala-L-Asp-L-Phe-OMe	170	16
59	L-Ala-L-Asp-L-Phe-OMe	1	
60	L-Ala-D-Ala-L-Asp-D-Ala-OMe	Δ	16
61	L-Ala-D-Ala-L-Asp-L-Phe-OMe	-	16
62	D-Ala-L-Asp-D-Ala-L-Ala-OMe	+	16
63	D-Ala-L-Asp-D-Val-L-Val-OMe	1	
64	Gly-L-Asp-D-Val-L-Val-OMe	0	16
65	D-Val-L-Asp-D-Val-L-Val-OMe	0	
66	D-Ala-DL-Ama-D-Ala-OMe	-	16
67	D-Ala-DL-Ama-L-Phe-OMe	-	16
68	L-Ala-DL-Ama-L-Phe-OMe	-	
69	L-Asp(-Gly-OH)-L-Phe-OMe	0	16
70	L-Asp(-D-Ala-OH)-L-Phe-OMe	0	16
71	L-Asp(-L-Ala-OH)-L-Phe-OMe	0	16

^a Times as potent as sucrose (weight basis, 0.6% sucrose = 1). +, faintly sweet (<1); Δ, astringent; 0, tasteless; -, bitter.

Extension at the Free α -Amino Group of Sweet Aminomalonyl Dipeptides (Compounds **66-68** in Table III).

The N-terminal extension of the sweet aminomalonyl dipeptides **10** and **11** led to a complete loss of the sweet taste (cf. **66-68**). This result is surprising in view of the close structural similarity of **66-68** to the tripeptides **50**, **58** and **59**. The complete absence of sweetness in compounds **66**, **67** and **68** may be a consequence of distortion of an essential A-H/B binding system (A-H: NH_3^+ , B: COO^-) which has been proposed to be of critical dimensions (17). Slight changes in the distance between the A-H and B moieties may have rendered the peptides inaccessible to the narrow receptor pocket.

Extension at the Free β -Carboxyl Group of a Sweet Aspartyl Dipeptide (Compounds **69-71** in Table III). The peptides **69-71** which are extended at the β -carboxyl group of compound **8** are devoid of sweetness. As already described, in order for the tripeptide esters (Figure 2) to be sweet, small alkyl groups (R^2 and R^4) must be placed below the projection plane when the backbone chain of the peptide is projected on the plane of the paper. Exemplary is L-Asp-D-Ala-L-Ala-OMe (**18**, $\text{R}^1 = \text{R}^3 = \text{H}$, $\text{R}^2 = \text{R}^4 = \text{R}^5 = \text{Me}$) which is 50 times as potent as sucrose, whereas L-Asp-D-Ala-D-Ala-OMe (**19**, $\text{R}^1 = \text{R}^4 = \text{H}$, $\text{R}^2 = \text{R}^3 = \text{R}^5 = \text{Me}$) is only 5 times as potent as sucrose, and L-Asp-L-Ala-L-Ala-OMe (**26**, $\text{R}^2 = \text{R}^3 = \text{H}$, $\text{R}^1 = \text{R}^4 = \text{R}^5 = \text{Me}$) is bitter. This SAR suggests that a hydrophobic area involving interactions which enhance sweetness potency may reside below the plane.

In the above modifications (Table III), in order for the peptides to be sweet, the first amino acid must be a small D-amino acid, and the third amino acid must fit the model for the sweet-tasting aspartyl dipeptide esters illustrated in Figure 1. Thus, the general structure for the sweet peptides can be drawn as Figure 4, in which small alkyl groups (R^2 and R^3) should be placed below the plane when the backbone chain of the peptides is projected on the plane of the paper. Exemplary of this requirement is D-Ala-L-Asp-L-Phe-OMe (**58**, $\text{R}^1 = \text{H}$; $\text{R}^2 = \text{Me}$; $\text{R}^3 = \text{COOMe}$; $\text{R}^4 = \text{CH}_2\text{C}_6\text{H}_5$) which is 170 times as potent as sucrose, whereas L-Ala-L-Asp-L-Phe-OMe (**59**, $\text{R}^1 = \text{Me}$; $\text{R}^2 = \text{H}$; $\text{R}^3 = \text{COOMe}$; $\text{R}^4 = \text{CH}_2\text{C}_6\text{H}_5$) is only equivalent to sucrose in potency.

From the results described above, it is suggested that the receptor site for sweet peptides may have a small residual space at a site facing the N-terminus. This restricted space can accommodate an additional small D-amino acid residue. The receptor peptide-binding site appears to be so spatially restricted that very subtle changes of structure strongly affect binding. Thus, only a limited number of peptides such as aspartyl di- and tripeptide analogues, aminomalonyl dipeptide esters, and the above-

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mentioned D-AA-L-Asp-AA-OMe can fit into the receptor and elicit a sweet taste.

Protein Sweetener Studies

Synthesis of the Sweet Protein Monellin (18). In attempts to deduce the mode of interaction between the sweet-tasting peptides and the receptor, a number of groups have carried out calculations and made spectroscopic measurements in order to determine the conformational energy minima of aspartame under the assumption that the "active" conformation is one of the low energy conformers. The efforts by Temussi and co-workers (19), van der Heijden and co-workers (20) and Iwamura (5) are illustrative. However, it is not possible to determine the active conformation of the sweet-tasting peptides by these methods since peptides can assume a multitude of low energy conformations in water. We, therefore, began efforts to employ sweet proteins as tools for elucidating the mode of peptide and/or protein sweetener/receptor interaction, since proteins are generally of much reduced conformational mobility. Inherent in this approach are two assumptions. First, it is assumed that the protein and peptide sweeteners bind with equivalent, if not identical, functional groups to the same locus on the same sweetener receptor. Second, we assume that knowledge of the three-dimensional structures of the protein sweeteners will allow unambiguous identification of these equivalent groups. As the first step to study the SAR of sweet proteins, we undertook the synthesis and crystallization of the sweet protein monellin.

Monellin has been isolated from the fruit of the West African plant, *Dioscoreophyllum cumminsii* (Stapf) Diels, by Morris and Cagan (21) and by van der Wel (22). It consists of two noncovalently associated polypeptide chains, the A-chain of 44 amino acid residues and the B-chain of 50 residues. Monellin is approximately 3000 times (21) as potent as sucrose on a weight basis, while neither of the individual A- or B-chain subunits is sweet (23). This indicates that the native conformation of monellin is important for the sweet taste. Two different primary structures have been reported for each of the A- and B-chains (23-25) as shown in Figure 5. Thus, there are four possible combinations for constructing the monellin structure. The discrepancies lie in positions 22, 25 and 26 of the A-chain, and 49 and 50 of the B-chain. We first synthesized the structure proposed by Frank and Zuber (24).

The A- and B-chains were synthesized by the stepwise solid-phase method using Fmoc protection (26). The peptide synthesis was performed manually with a semi-automated peptide synthesizer, Labortec SP 640 (for the A-chain), and with a manual shaker in a reaction vessel (for the B-chain). A preliminary synthesis of the B-chain revealed that the sulfur atom of the Met residue was susceptible to autoxidation to form the corresponding

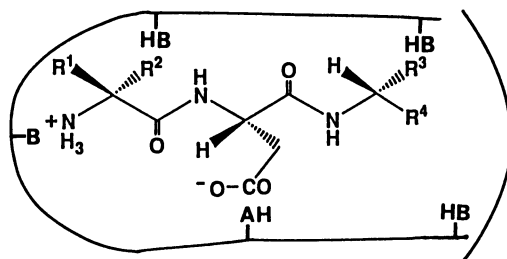


Figure 4. Schematic drawing of the mode of interaction between the sweet peptides and the receptor. $R^1 = H$; $R^2 = R^3 =$ small hydrophobic group; R^3 and R^4 are hydrophobic groups where $R^3 \leq R^4$ in size; AH, Proton donor; B, Proton acceptor; HB, Hydrophobic binding site.

(a) REIKGYEYQLYVYASDKLFRADISEDYKTRGRKLLRFNGPVPPP
 (b) REIKGYEYQLYVYASDKLFRANISQNYKTRGRKLLRFBGPVPPP

A chain

(a) GEWEIIDIGPFTQNLGKFAVDEENKIGQYGRLLTFNKVIRPCMKKTIIYENE
 (c) GEWEIIDIGPFTQNLGKFAVDEENKIGQYGRLLTFNKVIRPCMKKTIIYEE

B chain

Figure 5. Primary structures reported for monellin. For a, b and c, see references 22, 23, and 21, respectively.

sulfoxide. Contamination of the synthetic B-chain by the sulfoxide form complicated the HPLC chromatogram. Therefore, the B-chain was synthesized with Met in the sulfoxide form.

The protection and deprotection schemes for the stepwise solid-phase synthesis of monellin are shown in Figure 6. The peptide was assembled on a p-alkoxybenzyl alcohol resin (27). Elongation of the peptide chain was carried out by the DCC/HOBt method (28) in CH_2Cl_2 -DMF. The Fmoc group was removed with 50% or 20% piperidine in DMF. The coupling reaction and deprotection of the Fmoc group were monitored by the Kaiser test (29). When the deprotection was insufficient, the treatment with piperidine was repeated until a clear positive Kaiser test was obtained. When the coupling was incomplete, the reaction was repeated until a negative Kaiser test was obtained. When conversion was incomplete even following repeated coupling, the symmetrical anhydride method was used. If no further improvement could be made by these procedures, the "capping" procedure with acetic anhydride-pyridine was applied to eliminate the formation of deletion peptides.

Treatment of Fmoc-Pro-Pro-OCH₂-C₆H₄-OCH₂-resin with various concentrations of piperidine in DMF resulted in elimination of the dipeptide from the resin support. Therefore, Fmoc-Pro-Pro-OCH₂-C₆H₄-OCH₂-resin was synthesized by coupling Fmoc-Pro-Pro-OH with H-Pro-OCH₂-C₆H₄-OCH₂-resin.

In the synthesis of the A-chain, monitoring by the Kaiser test indicated that, for most residues, repeated coupling was necessary for quantitative incorporation, and for some residues, a second deprotection was necessary. After the last coupling step, the peptide-resin was treated with piperidine and then with CH_2Cl_2 -anisole-thiophenol-TFA, and the resulting peptide was further treated with thiophenol-thioanisole-TFA, and then purified by preparative HPLC to give pure A-chain in an overall yield of 14.1% based on the amine content of the starting amino acid resin.

In the synthesis of the B-chain, either the symmetrical anhydride method or the DCC/HOBt method in NMP was used for amino acid residues for which coupling had been found to be difficult in a preliminary synthesis of the B-chain. After the last coupling step, the peptide-resin was treated with CH_2Cl_2 -anisole-m-cresol-1,2-ethanedithiol-TFA, and the resulting peptide was further treated with thioanisole-m-cresol-1,2-ethanedithiol-TFA, and then with TMSBr (30). The crude B-chain was purified by HPLC to give the pure B-chain in an overall yield of 5.6% based on the amine content of the starting amino acid resin.

The purity of each peptide was confirmed by analytical HPLC, FAB-MS, quantitative amino acid analysis following hydrolysis in constant-boiling HCl containing 1% phenol at 110°C for 24 and 96 hr, and sequence analysis by automatic Edman degradation. HPLC analysis of each peptide gave satisfactory results. FAB mass spectrometric analysis of the A-chain showed a protonated

Fmoc-Arg(Mtr)-Glu(OBu^t)-Ile-Lys(Boc)-Gly-Tyr(Bu^t)-
 Glu(OBu^t)-Tyr(Bu^t)-Gln(Mbh)-Leu-Tyr(Bu^t)-Val-Tyr(Bu^t)-
 Ala-Ser(Bu^t)-Asp(OBu^t)-Lys(Boc)-Leu-Phe-Arg(Mtr)-Ala-
 Asp(OBu^t)-Ile-Ser(Bu^t)-Glu(OBu^t)-Asp(OBu^t)-Tyr(Bu^t)-
 Lys(Boc)-Thr(Bu^t)-Arg(Mtr)-Gly-Arg(Mtr)-Lys(Boc)-Leu-
 Leu-Arg(Mtr)-Phe-Asn(Mbh)-Gly-Pro-Val-Pro-Pro-Pro-OCH₂-
 C₆H₄-OCH₂-resin

1. CH₂Cl₂-Anisole-Thiophenol-TFA
2. thiophenol-thioanisole-TFA
3. HPLC purification

A chain (overall yield, 14.1%)

Boc-Gly-Glu(OBu^t)-Trp-Glu(OBu^t)-Ile-Ile-Asp(OBu^t)-Ile-
 Gly-Pro-Phe-Thr(Bu^t)-Gln(Mbh)-Asn(Mbh)-Leu-Gly-Lys(Boc)-
 Phe-Ala-Val-Asp(OBu^t)-Glu(OBu^t)-Glu(OBu^t)-Asn(Mbh)-
 Lys(Boc)-Ile-Gly-Gln(Mbh)-Tyr(Bu^t)-Gly-Arg(Mtr)-Leu-
 Thr(Bu^t)-Phe-Asn(Mbh)-Lys(Boc)-Val-Ile-Arg(Mtr)-Pro-
 Cys(MBzl)-Met⁰-Lys(Boc)-Lys(Boc)-Thr(Bu^t)-Ile-Tyr(Bu^t)-
 Glu(OBu^t)-Asn(Mbh)-Glu(OBu^t)-OCH₂-C₆H₄-OCH₂-resin

1. CH₂Cl₂-anisole-m-cresol-1,2-ethanedithiol-TFA
2. thioanisole-m-cresol-1,2-ethanedithiol-TFA-TMSBr
3. HPLC purification

B chain (overall yield, 5.6%)

Figure 6. Protection and deprotection schemes for the solid-phase synthesis of monellin.

monoisotopic molecular ion at m/z 5248.8 relative to a calculated value of m/z 5248.8. Similarly, the observed value for the B-chain was m/z 5832.1 vs. a calculated value of m/z 5832.0. Amino acid composition of each peptide gave the expected values. Sequencing of the synthetic A- and B-chains was carried out on an automated sequencer, using the intact peptides and their tryptic peptides, and the results fully supported the expected sequences.

We observed that the individual synthetic A- and B-chain subunits were not sweet when each individual substance was tasted as a powder. Combination of the A and B-chains, and subsequent HPLC purification gave monellin in a yield of 53.9%. Its amino acid analysis gave a satisfactory result. Thus, given the comparable sweetness potency of this synthetic monellin with the natural monellin described above, it appears that the natural sweet conformation was almost completely formed. The synthetic monellin was noted to exhibit a distinct, lingering sweet taste about 4000 times more potent than sucrose on a weight basis (130,000 times/molar basis) relative to 0.6% sucrose as reference.

Interestingly, we noted that, after tasting a 0.6% sucrose solution, the sweetness intensity of a solution of synthetic monellin was significantly enhanced. Moreover, after rinsing the tongue two times with a highly diluted subthreshold tasteless solution (0.075 mg/L) of synthetic monellin, the solution itself tasted sweet. Under these conditions, we estimated synthetic monellin to exhibit a potency of 8000 times that of sucrose (0.6% sucrose reference). A similar phenomenon has been observed for thaumatin, another sweet protein (31).

Crystallization of the Synthetic Monellin. Crystallization was carried out by a combination of the methods described by Tomlinson and Kim (32), and by Wlodawer and Hodgson (33), using the "hanging-drop" vapor diffusion method, in which the synthetic monellin was dissolved in a 14% (w/w) solution of polyethylene glycol and phosphate buffer, and equilibrated with a 28% (w/w) solution of polyethylene glycol and phosphate buffer at 4°C. Crystals obtained by this method are shown in Figure 7.

Comparison of the Synthetic Product with Natural Monellin. It is known that approximately 10% of the A-chain of natural monellin carries an extra Phe residue at the N-terminus (24). This peptide is termed Phe-A-chain. Thus, a comparison of the synthetic product with natural monellin was performed after separating these peptide chains. Separation of the A-, Phe-A- and B-chains of natural monellin was readily accomplished by HPLC. Recombination of the separated A-chain (44 residues) with the separated B-chain gave "natural monellin". The synthetic monellin was identical to the reconstituted monellin by HPLC, but not by tryptic mapping. Therefore, a comparison was made between the individual chains. The synthetic A-chain was identical to the natural A-chain (44

residues) by tryptic mapping, but the synthetic B-chain was not. These results indicate that the reported structure for the B-chain differs from that of the natural B-chain. Therefore, we then determined the primary structure of natural monellin.

Determination of the Primary Structure of Natural Monellin (34).

The sequence of monellin was determined by a combination of mass spectrometry (FAB and ESI), and automatic Edman degradation. The results are described briefly. A sample of monellin was obtained through the courtesy of Dr. J.G. Brand of Monell Chemical Senses Center. Sequencing was performed after separating the A-chain, Phe-A-chain and B-chain. The analytical HPLC of monellin revealed that approximately 10% of the A-chain carried an extra Phe residue at the N-terminus as described by Frank and Zuber (24).

Residues Arg¹ to Phe³⁷ of the A-chain were determined by automatic sequencing of the intact A-chain. The rest of the sequence was determined by automatic sequencing of a tryptic peptide after tryptic digestion of the A-chain. The complete amino acid sequence of the A-chain was determined to be as shown in Figure 8. This sequence is identical to that proposed by Frank and Zuber (24) and is also supported by our synthetic study described above (18).

Similarly, the separated B-chain was subjected to an automatic Edman degradation. By this method, Gly, Glu and Thr residues were identified as the N-terminal amino acids. This indicated that the B-chain was a mixture of three peptides, which we consider to be formed by enzyme action on the native protein. ESI mass spectrometric analysis of the mixture gave the measured mean molecular weights of 5834.3, 5777.1 and 5935.1, corresponding to calculated values of 5834.7, 5777.6 and 5935.8 for the respective intact B-chain subunit, and the des-Gly¹-B- and Thr-B-chain subunits. The ratio of these three components was semi-quantitatively estimated from the relative intensities of the peaks to be 100 : 43 : 33, respectively. Attempts to separate the modified peptides from the intact B-chain subunit by HPLC and by FSCE were unsuccessful. Therefore, the sequence was determined by automatic sequencing of tryptic peptides, after tryptic digestion of the mixture. The complete amino acid sequence of the B-chain was determined to be as shown in Figure 9. This sequence is identical to that reported by Bohak and Li (23).

We have synthesized monellin, the structure of which was determined as is described here, as well as analogues. The results of this effort will be comprehensively described elsewhere. Upon solution of the crystal structure of monellin and its analogues, we expect that the synthesis of oligopeptides as described in the first part of this review will lead to delineation of the receptor binding site of monellin. In addition, we expect these studies to lead to the

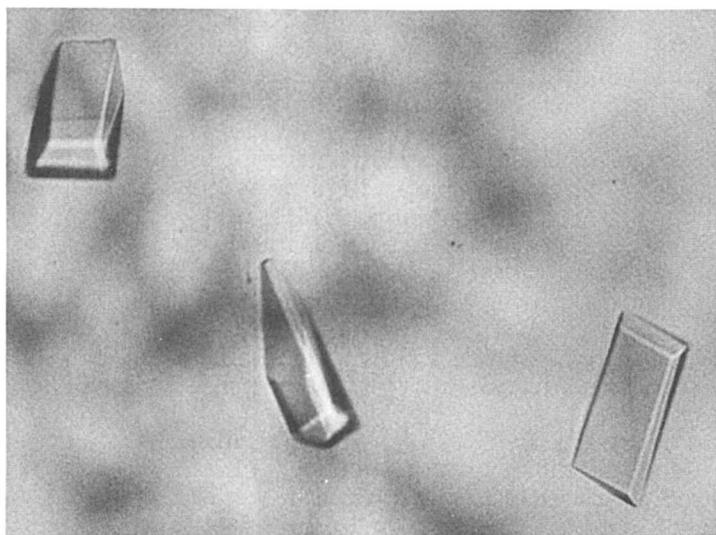


Figure 7. Crystals of synthetic monellin.

(H-Phe)¹-Arg-Glu-Ile-Lys⁵-Gly-Tyr-Glu-Tyr-Gln-Leu-Tyr-Val-
 Tyr-Ala-Ser-Asp-Lys-Leu-Phe-Arg-Ala-Asp-Ile-Ser-Glu-Asp-
 Tyr-Lys-Thr³⁰-Arg-Gly-Arg-Lys-Leu-Leu-Arg-Phe-Asn-Gly-Pro-
 Val-Pro-Pro-Pro-OH⁴⁴

Figure 8. The complete amino acid sequence of the A-chain of monellin, approximately 10% of which carries an extra Phe residue at the N-terminus as described by Frank and Zuber (22).

(H-Thr)¹-Gly-Glu-Trp-Glu-Ile⁵-Ile-Asp-Ile-Gly-Pro-Phe-Thr-
 Gln-Asn-Leu-Gly-Lys-Phe-Ala-Val-Asp-Glu-Glu-Asn-Lys-Ile-
 Gly-Gln-Tyr³⁰-Gly-Arg-Leu-Thr-Phe-Asn-Lys-Val-Ile-Arg-Pro-
 Cys-Met-Lys-Lys-Thr⁴⁵-Ile-Tyr-Glu-Glu-Asn-OH⁵⁰

Figure 9. The complete amino acid sequence of the B-chain of monellin, approximately 19% of which carries an extra Thr residue at the N-terminus, and approximately 24% of which lacks the N-terminal Gly residue.

design of novel sweeteners and the elucidation of the mode of interaction between sweet compounds and their receptor(s).

Acknowledgments. We thank Dr. J.G. Brand of Monell Chemical Senses Center for sending us the sample of monellin, Dr. S. Ikeda of Kokusan Chemical Works Ltd. for providing us with Fmoc-Pro-Pro-OH, Mr. M. Kaise and Mr. S. Fukushima of Applied Biosystems Japan for FSCE, Dr. B. N. Green of VG Masslab Ltd. and Dr. A. Shibata of Jasco International for measurements of ESI mass spectra, Dr. K. Hirayama, Ms. S. Akashi and Ms. M. Furuya for measurements of FAB mass spectra, Mr. S. Ozawa, Mr. T. Seino and Ms. M. Fuchiagami for amino acid analyses, and Mr. K. Iijima for elemental analyses.

Abbreviations follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature in *Eur. J. Biochem.* **1984**, *138*, 9-37. Other abbreviations used: OPPr, propyloxy; Ama, aminomalonyl; HPLC, high-performance liquid chromatography; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulfonyl; MBzl, p-methoxybenzyl; Mbh, 4,4'-dimethoxybenzhydryl; DMF, N,N-dimethylformamide; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid; TMSBr, trimethylsilyl bromide; FAB-MS, fast atom bombardment mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; FSCE, free solution capillary electrophoresis.

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Chapter 5

Development and Uses of Alitame

A Novel Dipeptide Amide Sweetener

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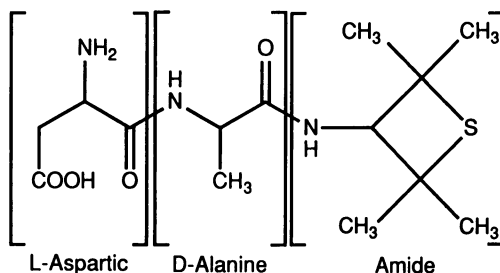
Following the accidental discovery of the potent sweet taste of L-aspartyl-L-phenylalanine methyl ester (aspartame) (1), peptide sweeteners became the subject of intense research. This active area has recently become the subject of an excellent review (2). During the 1970's an intensive, systematic program to develop high-potency sweeteners with increased stability (relative to aspartame) in food systems was carried out at Pfizer Central Research. In 1979 this effort culminated in the synthesis of alitame, and a U.S. patent covering branched amides of aspartyl D-amino acid dipeptides was granted in 1983. Following completion of an extensive battery of evaluations demonstrating the safety of alitame, a Food Additive Petition was filed with the U.S. Food and Drug Administration (FDA) in mid- 1986.

Alitame is a high potency dipeptide sweetener discovered and developed at Pfizer Central Research. The structure is shown in Figure 1, which emphasizes the component parts of the molecule. It consists of an N-terminal aspartic dipeptide amide, with alanine as the second amino acid. A key to the high sweetness potency of this molecule is the terminal amide of a novel amine (2,2,4,4-tetramethylthietanyl amine).

The molecule is similar to aspartame in that it is an aspartic acid-containing dipeptide. However, there are important structural differences which give alitame functionally improved properties over those of aspartame. Most notable is the terminal amide of alitame versus the methyl ester of aspartame, which provides improved hydrolytic stability to alitame.

¹Address correspondence to this author.

Structure:



Development:

Discovered: 1979 Pfizer Central Research
 Patented: 1983 (U.S. 4,411,925)
 FDA Filing: 1986 Food Additive Petition

Figure 1. Structure and development of alitame.

Key Features

As shown in Table I, Alitame has an exceptionally high sweetness potency - over 2000 times that of sugar (based on concentration ratios of iso-sweet aqueous solutions of alitame and 10% sucrose). Thus, one pound of alitame has the sweetening power of one ton of sugar. Further, alitame possesses more than 10 times the sweetness power of an equal weight of aspartame. Alitame provides a clean, sucrose-like sweetness with no aftertaste.

The hydrolytic and thermal stabilities of alitame both represent improvements over those of aspartame, permitting the use of alitame in food applications not previously possible with dipeptide sweeteners. The stability of alitame will be discussed in more detail subsequently.

Extensive testing in Pfizer's Drug Safety Evaluation Department has demonstrated the safety of alitame at levels of more than 200 times the estimated mean chronic daily intake of 0.34 mg/kg body weight. This intake estimate assumes that alitame is the only sweetener used in all 16 food categories requested in the U.S. Food Additive Petition. FDA review is in progress.

Table I. Key features of alitame

1.	High Sweetness Potency	
	Acesulfame K	130 x sucrose
	Aspartame	180 x
	Saccharin	300 x
	Alitame	2000 x
2.	Clean Sweetness--No Aftertaste	
3.	Improved Stability (vs. Aspartame)	
4.	Safe (FDA Review Underway)	

Discovery Approach

Our discovery approach is outlined in Figure 2. Based on the published literature (1), it was apparent that a dipeptide N-terminus of L-aspartic acid was required to achieve high sweetness potency. Additionally, it was proposed by the research team that both sterically small and large hydrophobic moieties were required on the second amino acid with a specific spatial orientation.

The large moiety was designed as an amide, known to be more hydrolytically stable than the methyl ester moiety in the aspartame molecule. The small group was set as alkyl, thus entering the D-amino acid series to maintain correct spatial configuration. With a constant amide structure, it was found that incorporation of D-alanine as the second amino acid yielded the highest sweetness potency of the various amino acids tried. Compounds derived from corresponding L-amino acids were found not to be sweet.

Having established the L-aspartyl-D-alanyl amide structure as a desired base, a wide range of amines were incorporated as terminal amides. Over 150 different amide end groups were evaluated for sweetness potency in this family of dipeptides. Sweetness potencies were found to be highly dependent on the N- amide moiety. The structural possibilities for the amines were investigated systematically and have been categorized according to structural features, such as ring size, branching and polarity.

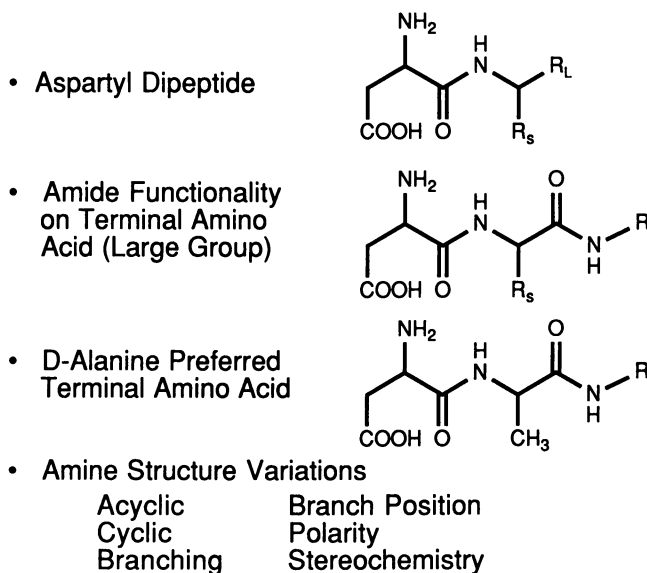


Figure 2. Discovery approach.

Structure Optimization

Some of the key structure/sweetness correlations that emerged from this study of amines are briefly reviewed and illustrated in Figures 3-5. It should be noted that all sweetness potencies are relative to 10% aqueous sucrose, and are not threshold values.

Comparison of the five- and six-membered cyclic amines with their alpha-methyl branched analogs revealed no strong dependence on ring size, but clearly showed that bulky alpha-branching in small rings can lead to high sweetness potencies. Carbocyclic rings of size larger than six or smaller than four resulted in reduced sweetness potencies.

In several cases a dependence of sweetness potency on stereochemical configuration of the amine was observed. In one case (2-amino-3,3-dimethylbutane) the dipeptide prepared from one enantiomer was sweet, and the other was devoid of sweetness. In two other cases (1-cyclopropyl-2,2-dimethyl-propylamine and the fenchylamine isomers) amine stereochemistry had only a limited effect on sweetness potency. In the 2,6-dimethylcyclohexylamine system preliminary results suggested a dependence of sweetness potency on stereochemistry, but this was not pursued.

An important discovery was the great sensitivity of sweetness potency to amine branching, as exemplified by a series of aliphatic amine end groups. Increasing steric bulk, either from trimethyl or cyclopropyl substitution at the alpha-carbon, was found to be required for high sweetness potency in the aliphatic series. Where only one branch contained a bulky substituent, sweetness potency was greatly reduced.

Finally, an additional structural feature was investigated. Introduction of heteroatoms led to the discovery of the thietane amine moiety as an important element in achieving extremely high sweetness potency in dipeptides. While the introduction of sulfur into a four-membered ring amine led to alitame, oxidation of the sulfur atom decreased the observed sweetness potency as polarity of the resultant dipeptide amides increased. A similar effect was found on introduction of an oxygen substituent onto the 3-position of 2,2,4,4-tetramethyl-1-aminocyclobutane.

It should be noted that alitame does not possess the highest sweetness potency of this series. Of the four diastereomers formed with 2,2,4-trimethyl-3-aminothietane, the most potent shows a potency of 3900 times that of sucrose. For various reasons, including synthetic accessibility, however, alitame was chosen for development.

Properties

Alitame is a crystalline, odorless, non-hygroscopic solid. It has high water solubility, 13% at its isoelectric point of pH 5.6, and much

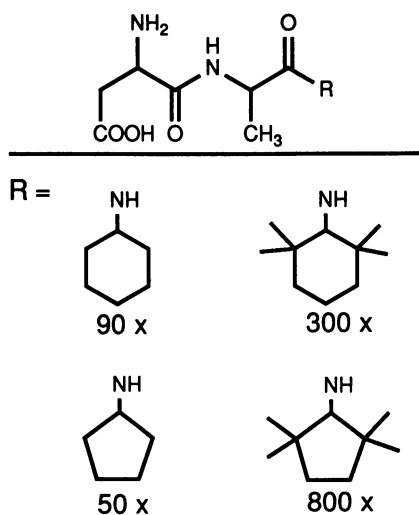


Figure 3. Structure optimization: ring size and stereochemistry.

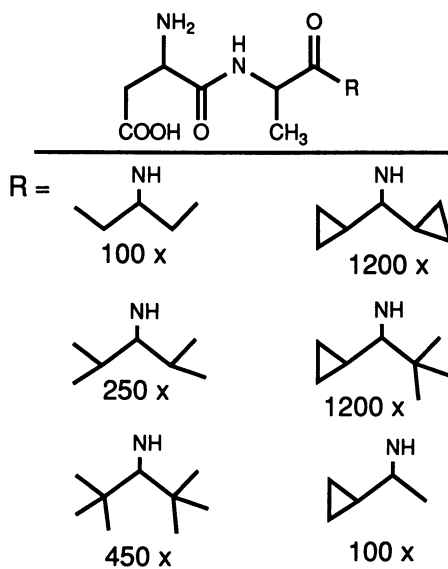


Figure 4. Structure optimization: branching.

higher at lower or higher pH. At pH 3 or pH 8 the solubility is in excess of 40% at room temperature. This high solubility, in contrast to the limited water solubility shown by other dipeptide sweeteners, is useful for preparing concentrated solutions for food formulation work. The solubility of alitame in several solvents is presented in Table II.

Although alitame is significantly more stable than aspartame in both aqueous solutions and under heat processing conditions, it will gradually hydrolyze on prolonged aqueous exposure. The degradation pathway for alitame is much simpler than that of aspartame. The aspartic end unit undergoes a slow α - β rearrangement such as occurs with aspartame (and is indeed common to all aspartyl dipeptides (3). Both the α and β forms slowly hydrolyze to yield aspartic acid and alanine amide. The two pathways are shown in Figure 6. These decomposition products are safe and tasteless at levels generated in foods. No cyclization to diketopiperazine, a problem with aspartame, is found with alitame. The alanine amide is stable to hydrolysis in solutions that have undergone >90% alitame decomposition.

The pH stability profiles for alitame and aspartame, in aqueous buffer solutions, are compared in Figure 7. The aspartame data is taken from the published literature (4). In the pH 2-4 range normally encountered with carbonated beverages, alitame is 2-3 times more stable than is aspartame. At pH greater than 5, the relative stability of alitame increases dramatically due to the absence of diketopiperazine formation and ester hydrolysis decomposition pathways.

Aqueous stability at 100 °C in the typical pH range of 7 to 8 encountered in baked goods shows alitame half life in hours or days, compared to aspartame half lives of minutes or seconds (Figure 8). In actual baking systems, Maillard reactions between the aspartic unit and carbohydrates can occur with either alitame or aspartame.

Metabolism

The metabolic fate of alitame has been studied by means of ^{14}C -labeled material in three animal species and man. In all species, principal metabolism involves cleavage of aspartic acid, which enters the amino acid pool. The remaining alanine amide moiety is excreted directly or as a glucuronide, and a minor fraction is oxidized to the corresponding sulfoxides and sulfone. From 5 to 20% of an administered alitame dose is excreted in the feces as unchanged alitame and alanine amide. No cleavage of the alanine amide linkage or rupture of the thietane ring is observed. A schematic outline of the alitame metabolism is presented in Figure 9.

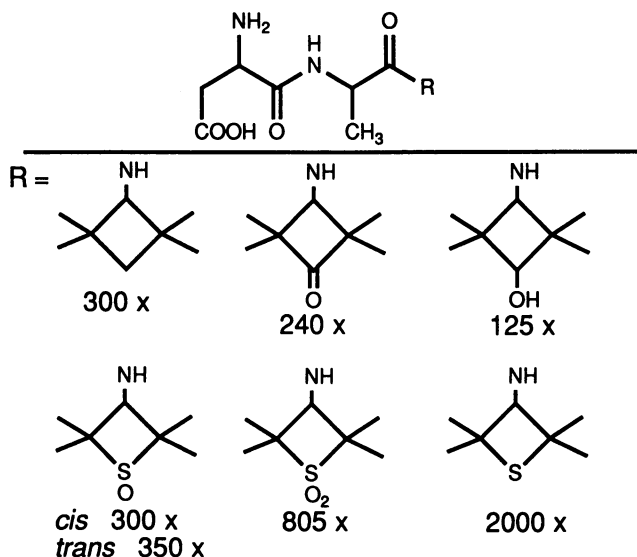


Figure 5. Structure optimization: heteroatoms.

Table II. Solubility of alitame

Solvent	Solubility (% w/v), 25 °C
Water	13.1 (isoelectric pH 5.6)
Methanol	41.9
Ethanol	61.0
Propylene Glycol	> 40
Chloroform	0.02
n-Heptane	0.001

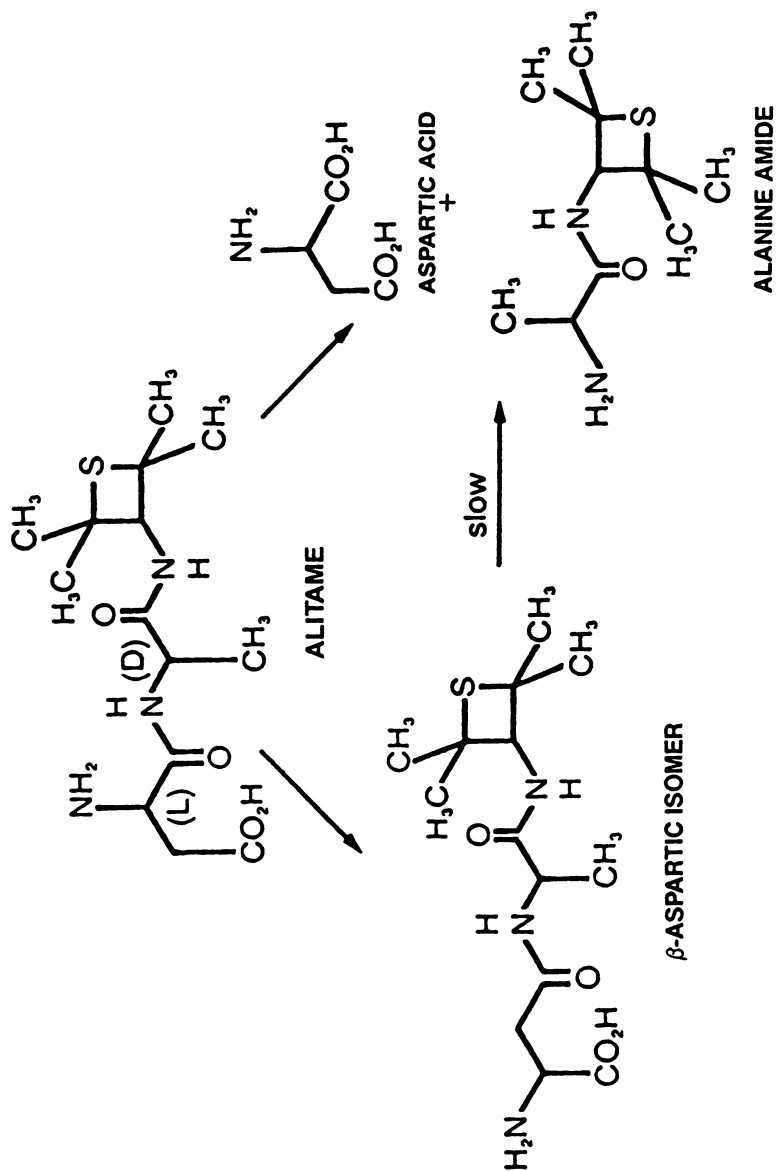


Figure 6. Main degradation pathways of alitame.

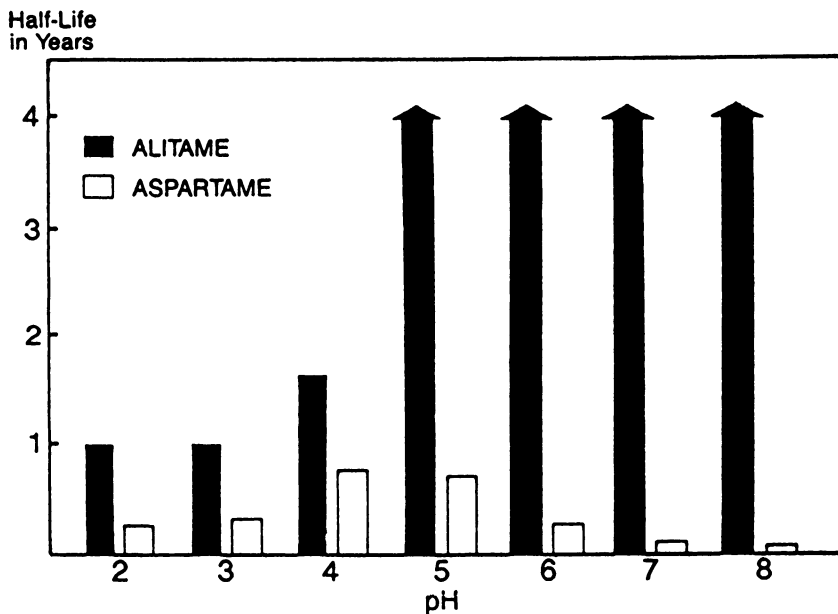


Figure 7. Stability of alitame in buffer solutions at 23 °C.

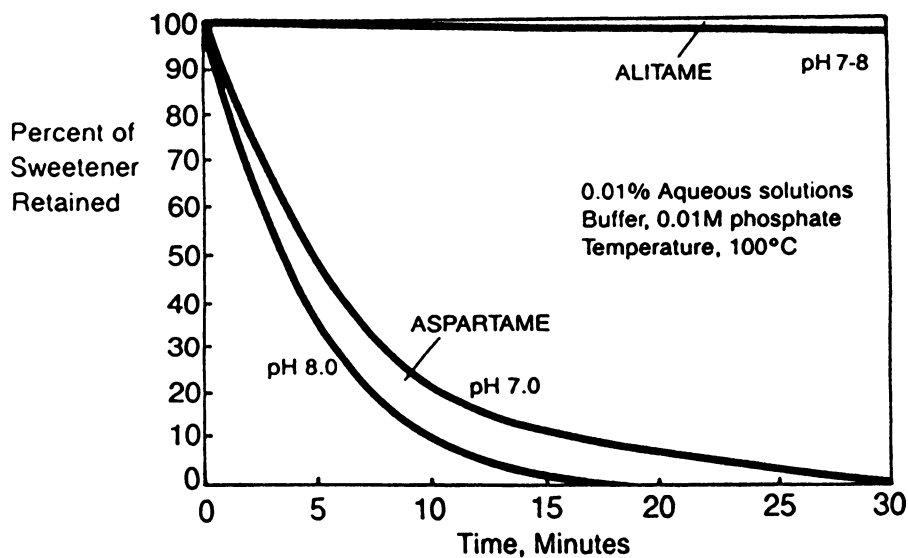


Figure 8. Thermal stability of alitame.

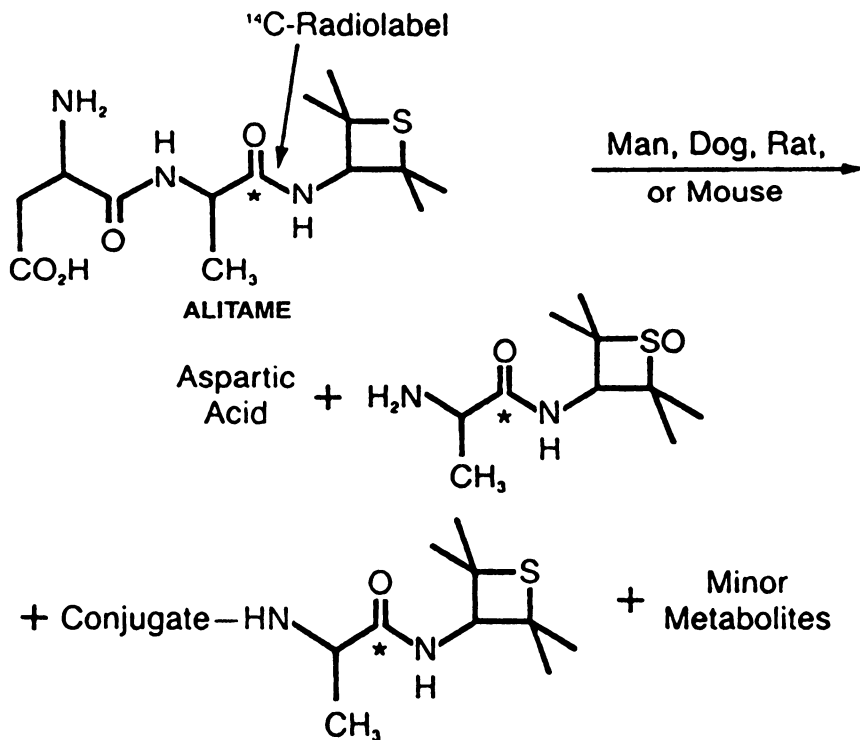


Figure 9. Metabolism of alitame.

Table III. Alitame food applications: food categories for which model recipes are now available.

Iced tea mix
Gelation dessert mix
Frosting mix
Pudding mix
Dry beverage mixes
Frozen dairy products
Yogurt
Tabletop sweetener
Table syrups
Baked goods
• Yellow cake mix
• Chocolate cake mix
• Sponge cake mix
• Vanilla cookie
• Butter cookie
Hard candy
Milk modifiers

Food Uses

Approval of alitame for use in sixteen food categories has been requested. Model recipes are now available for a number of these and are listed in Table III. The Food Additive Petition was filed with the FDA in August, 1986, and is currently undergoing review. To date, submissions have also been made in nine countries outside of the U.S. Additional filings are in progress.

The food categories include virtually all those employing a sweetener, with the exception of breads and alcoholic beverages. Included are foods which to date could not be prepared successfully with high potency sweeteners, such as baked goods and hard candies.

Summary

Alitame is a new dipeptide sweetener with a potency of 2000 times that of sucrose and with excellent taste qualities. A key feature of this sweetener is its stability both to hydrolytic and thermal degradation. Broad utility in food types is projected. Alitame should permit, for the first time, the preparation of high-quality candies and baked goods with reduced calories. Extensive testing has demonstrated its safety at greater than 300 times the projected daily intake. FDA review is in progress.

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Chapter 6

Sucralose

How To Make Sugar Sweeter

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The sweetness of sucrose can be increased dramatically by selective halogenation. A class of sucrose derivatives was discovered in which up to four of the available hydroxyl groups were replaced by halogens. This transformation resulted in enhancement of sweetness by up to several thousand times. The pattern of derivatisation needed to achieve high sweetness intensity was found to be very stereospecific as many of the compounds in the series are essentially tasteless. A rationalisation of the structural requirements for high sweetness intensity of sugar derivatives has been proposed. The sweetener sucralose emerged from this research programme following an assessment of many key attributes including sweetness quality and stability to the processing and storage conditions employed by the food industry.

Sucrose provides an excellent starting point for the study of sweetness. Not only is sucrose the gold standard for sweetness quality but it also possesses eight functional positions suitable for derivatisation. These comprise three primary and five secondary hydroxyl groups as shown in Figure 1.

During the 1960's and early 1970's sucrose and other carbohydrates were derivatised and tasted while elucidating the structural requirements for sweetness (1). This work provided insight into the hydroxyl groups responsible for the sweetness of sucrose, but it was far from comprehensive. None of the compounds evaluated in this period was sweeter than sucrose. For example, Lindley, Birch and Khan (2) reported on a series of partially methylated sucrose derivatives, as shown in Table I. Methylation of sucrose at the 6- or 6'-positions appeared to have little or no effect on the sweetness of

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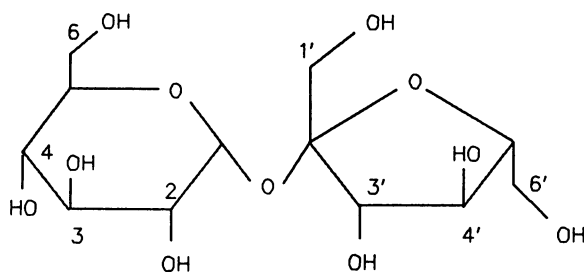


Figure 1. Sucrose

the molecule, while methylation at the 4- position appeared to reduce the sweetness.

Table I. Sweetness of methylated sucrose derivatives

Compound	Sweetness
Sucrose	Very Sweet
4- <i>O</i> -Methylsucrose	Sweet
6'- <i>O</i> -Methylsucrose	Very Sweet
6,6'-Di- <i>O</i> -methylsucrose	Very Sweet
4,6'-Di- <i>O</i> -methylsucrose	Sweet
4,6-Di- <i>O</i> -methylsucrose	Sweet
1',6'-Di- <i>O</i> -methylsucrose	Sweet

Source: Adapted from ref 2.

From this group of compounds they concluded that the hydroxyl group on the 4- position of the sucrose molecule was particularly important for its sweetness.

Lindley and Birch (3) also evaluated the sweetness of a selection of methyl ethers of methyl α -*D*-glucopyranoside, and α,α -trehalose as shown in Table II.

Table II. Sweetness of methyl glucopyranoside and trehalose derivatives

Compound	Sweetness
Methyl α - <i>D</i> -glucopyranoside	
2- <i>O</i> -methyl	Sweet
3- <i>O</i> -methyl	Sweet
4- <i>O</i> -methyl	Sweet
6- <i>O</i> -methyl	Sweet
2,3-di- <i>O</i> -methyl	Bitter
3,4-di- <i>O</i> -methyl	Bitter
4,6-di- <i>O</i> -methyl	Bitter
α,α -Trehalose	
2,2'-di- <i>O</i> -methyl	Sweet
3,3'-di- <i>O</i> -methyl	Sweet
4,4'-di- <i>O</i> -methyl	Sweet
6,6'-di- <i>O</i> -methyl	Sweet
2,3,2',3'-tetra- <i>O</i> -methyl	Bitter
4,6,4',6'-tetra- <i>O</i> -methyl	Bitter

Source: Adapted from ref. 3.

Monomethylation of methyl glucopyranoside did not significantly alter the sweetness of the parent compound, but introduction of two methyl groups eliminated the sweetness and caused the compounds to taste bitter, possibly due to an increase in lipophilicity of the molecule (4). A similar pattern was seen with

the trehalose derivatives where mono-methylation of each of the hexopyranoside rings caused little change in the sweetness whereas dimethylation of the hexopyranoside units resulted in a transformation from sweet to bitter taste.

Discovery of Sweetness Enhancement

During a collaborative research programme between Tate & Lyle and Prof. Hough's group at Queen Elizabeth College at The University of London in 1976, it was found that selective chlorination of certain hydroxyl groups in the sugar molecule could cause a dramatic increase in sweetness (5). This was remarkable in view of the fact that previous evaluation of many chlorinated carbohydrates, including derivatives of methyl α -D-glucopyranoside, α,α -trehalose, maltose, and lactose, had demonstrated such compounds to be extremely bitter (4).

The first compound tasted in the sucrose series contained four chlorine atoms located at the 4,6,1', and 6'- positions which was produced by selective chlorination of sucrose using sulphuryl chloride in the three-step process shown in Figure 2a and 2b (5,6). Treatment of sucrose with sulphuryl chloride in pyridine at low temperature, -30 °C, gave the 4,6,6'-trichloro-derivative. This was treated with mesitylenesulphonyl chloride at -5 °C for 6 days to selectively derivatise the 1'- position, which was then converted to the chloride by treatment with lithium chloride in dimethylformamide at 140 °C for 18 hours. The resulting product, 4,6,1',6'-tetrachlorogalactosucrose, had a sweetness roughly 200 times that of sugar (1). This was the first carbohydrate derivative observed to be significantly sweeter than the parent molecule. The term *galactosucrose* was first proposed by Hough *et al* in 1975 to denote a sucrose derivative which had undergone inversion of configuration at the 4- position, converting the glucopyranosyl ring into a galactopyranosyl unit (7).

Investigation of Structure Activity Relationships

Following this observation, a series of halogenated sucrose derivatives were synthesised and tasted (8). 1'-Chlorosucrose was prepared using the sequence shown in Figure 3a and 3b. The 1'- and 2- positions were first blocked by treatment with dimethoxydiphenylsilane in dimethyl formamide which produced the 1',2-diphenylsilylene derivative in a yield of about 20% (9). Acetylation of the remaining six hydroxyl groups was followed by cleavage of the silylene group using boiling aqueous acetic acid. Tritylation of the remaining primary hydroxyl was achieved using trityl chloride in pyridine and acetylation of the remaining secondary group gave 1'-tritylsucrose hepta-acetate. Detritylation was achieved using hydrogen bromide in glacial acetic acid. This was followed by chlorination using sulphuryl chloride in pyridine

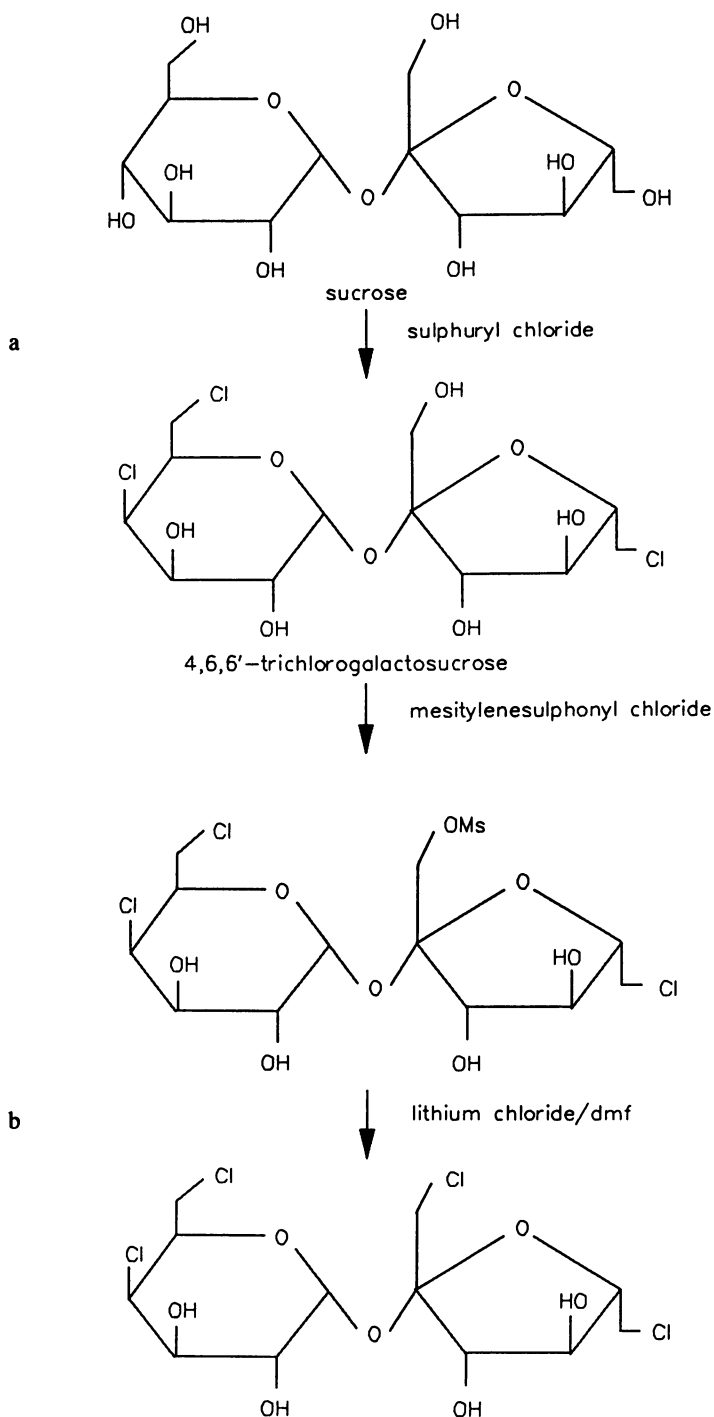
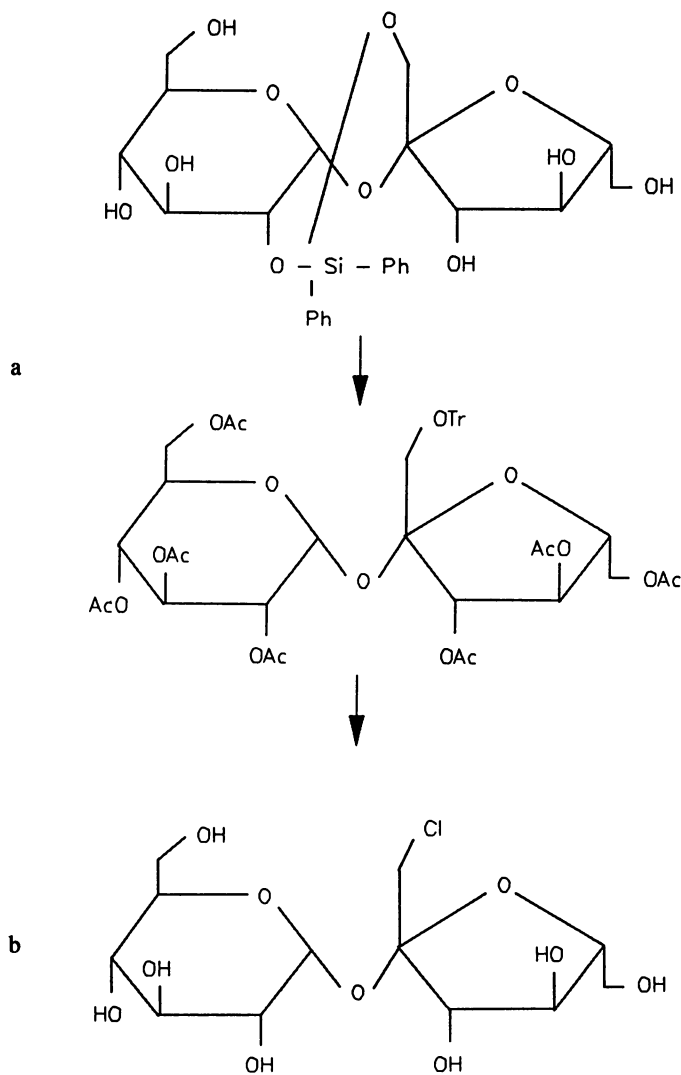


Figure 2. 4,6,1',6'-Tetrachlorogalactosucrose

**Figure 3. 1'-Chlorosucrose**

followed by lithium chloride in dimethylformamide. Finally de-esterification using sodium methoxide in methanol gave the 1'-chlorosucrose which was found to be 20 times sweeter than sugar (10).

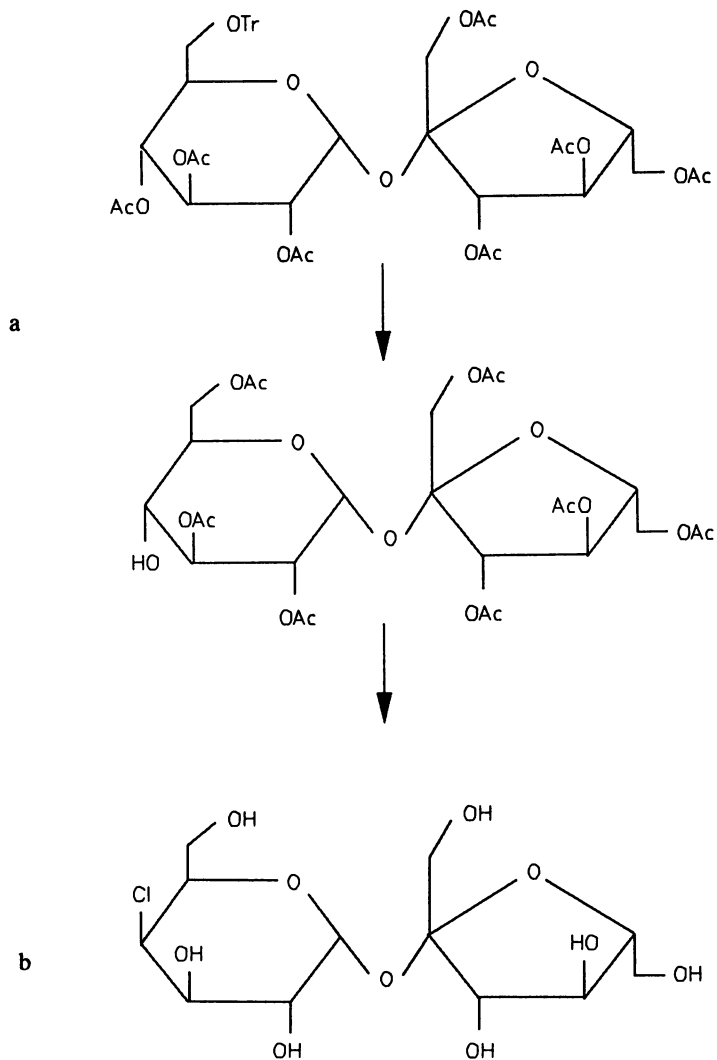
4-Chlorogalactosucrose was prepared using the sequence described in Figure 4a and 4b. Partial tritylation of sucrose using trityl chloride in pyridine at room temperature resulted in a mixture containing 6-tritylsucrose that could be isolated by silica gel column chromatography in low yield. Acetylation of 6-tritylsucrose followed by de-tritylation using boiling aqueous acetic acid resulted in a migration of the acetyl group from the 4 to the 6 position. Chlorination using sulphuryl chloride in pyridine followed by de-esterification produced the desired 4-chlorogalactosucrose which was found to be 5 times sweeter than sugar (10).

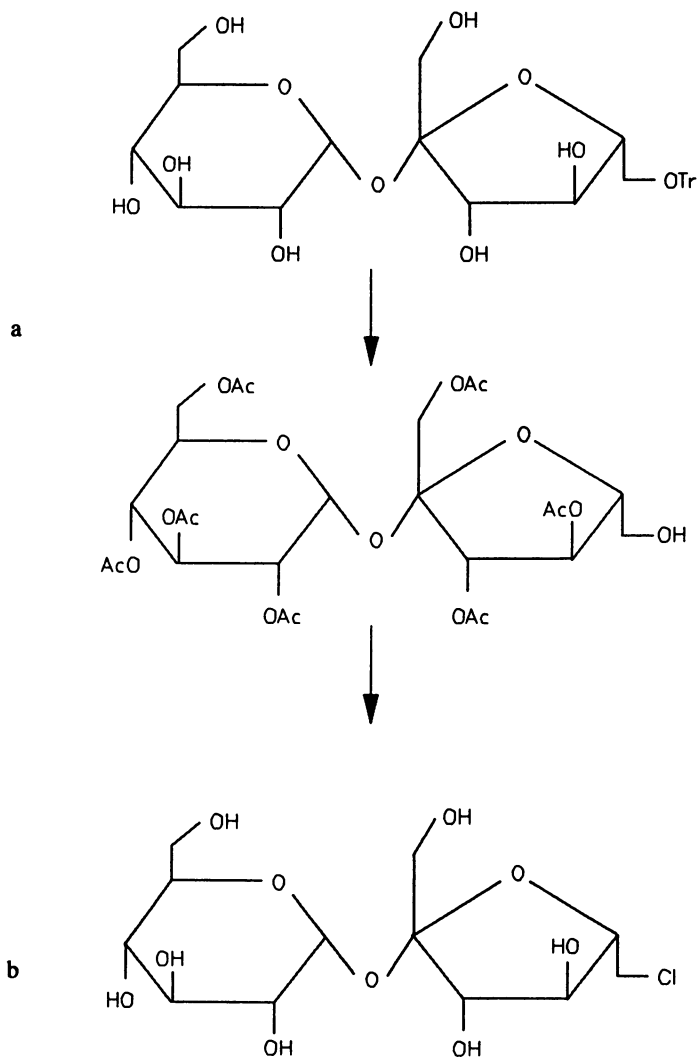
6'-Chlorosucrose was produced by a sequence which involved selective tritylation of the 6'-position of sucrose using trityl chloride in pyridine at room temperature followed by acetylation with acetic anhydride as shown in Figure 5a and 5b. De-tritylation was achieved using hydrogen bromide in glacial acetic acid. The resulting sucrose hepta-acetate was chlorinated using sulphuryl chloride in pyridine and the acetates removed with sodium methoxide in methanol to give 6'-chlorosucrose. This compound was found to be 20 times sweeter than sugar (8).

1',6'-Dichlorosucrose was produced in a process which commenced with selective sulphonylation of sucrose with mesitylenesulphonyl chloride at -5 °C for 6 days. The major product was the 6,1',6'-trisulphonate and the desired 1',6'-disulphonate was separated out by silica gel column chromatography. Acetylation of the 1',6'-disulphonate followed by treatment with lithium chloride in dimethylformamide at 140 °C for 18 hours produced the 1',6'-dichloride which was de-acetylated to give 1',6'-dichlorosucrose shown in Figure 6. This was found to be approximately 80 times sweeter than sugar (1).

4,1'-Dichlorogalactosucrose was produced in a process which started by protecting the three primary hydroxyl groups with trityl groups and the secondary hydroxyls with acetate shown in Figure 7a and 7b. Detritylation of this tritryl derivative with boiling aqueous acetic acid removed the primary protecting groups and caused the acetate group on the 4 position to migrate to the 6 position producing the sucrose penta-acetate with the 4,1', and 6'-positions unprotected. Selective benzylation of this penta-acetate using benzoyl chloride in pyridine gave the 6'-benzoate which was chlorinated with sulphuryl chloride and lithium chloride and de-protected to give 4,1'-dichlorogalactosucrose. This product was found to be 120 times sweeter than sugar (1).

Other compounds in this series which were produced by similar protection sequences include 6,1',6'-trichlorosucrose which was 25 times sweeter than sugar (11), and 4,1',6'-trichlorogalactosucrose which was 650 times sweeter than sucrose, both shown in Figure 8.





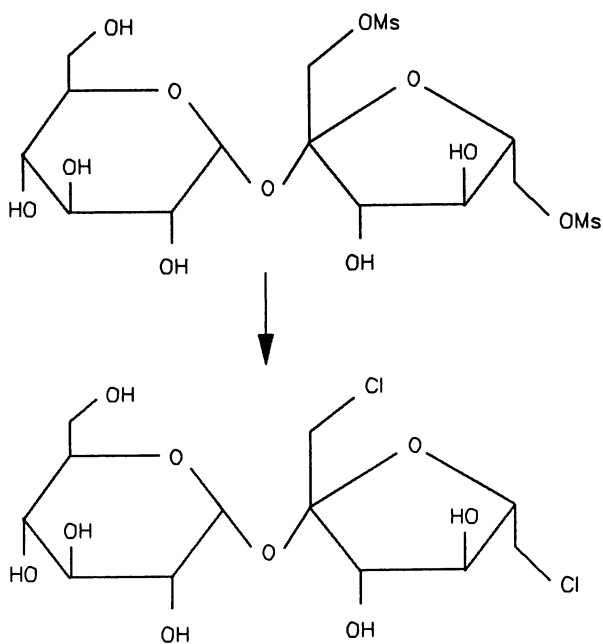


Figure 6. 1',6'-Dichlorosucrose

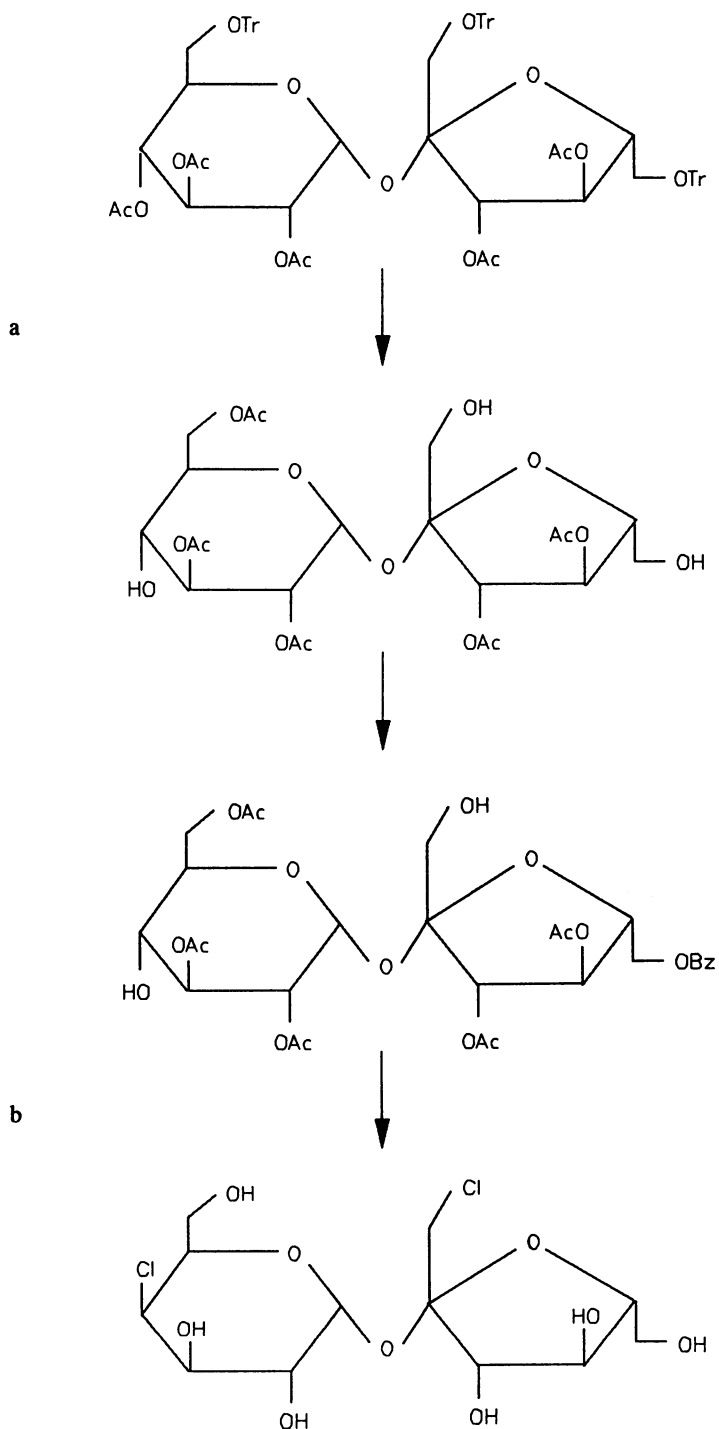


Figure 7. 4,1'-Dichlorogalactosucrose

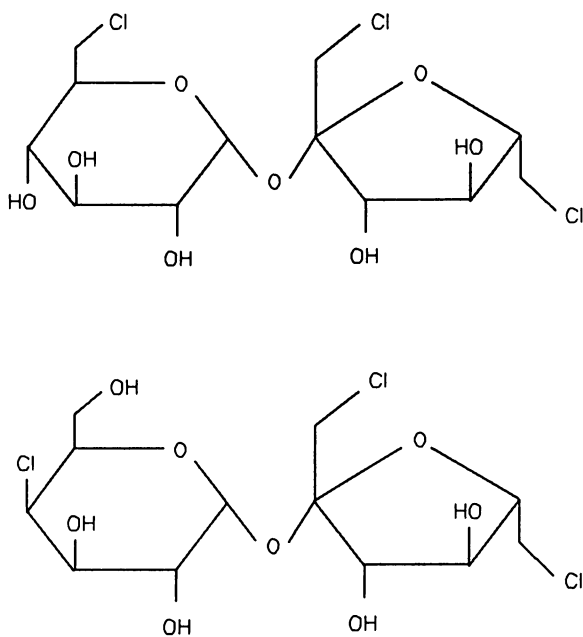


Figure 8. 6,1',6'-Trichlorosucrose (top); 4,1',6'-Trichloro-galacto-sucrose (bottom)

All of the compounds described so far are substituted at the 4,6,1' and 6'- positions. However, chlorination at the 4'- position also increases sweetness.

The 4,1',4',6'-tetrachloro-derivative was prepared by the sequence shown in Figure 9a and 9b. The 4,1',6'-trichloro-compound was blocked at the 6- position by treatment with tertiary butyl diphenyl silyl chloride in pyridine. Then the 3',4'-epoxide was formed by treatment with diethylazodicarboxylate and triphenylphosphine (12). The epoxide was cleaved using lithium chloride in dimethylformamide at 90 °C for 5 hours to give a 60% yield of the 4'-chlorinated derivative. Conventional de-esterification produced 4,1',4',6'-tetrachlorogalactosucrose. This was found to be 2200 times sweeter than sugar (13).

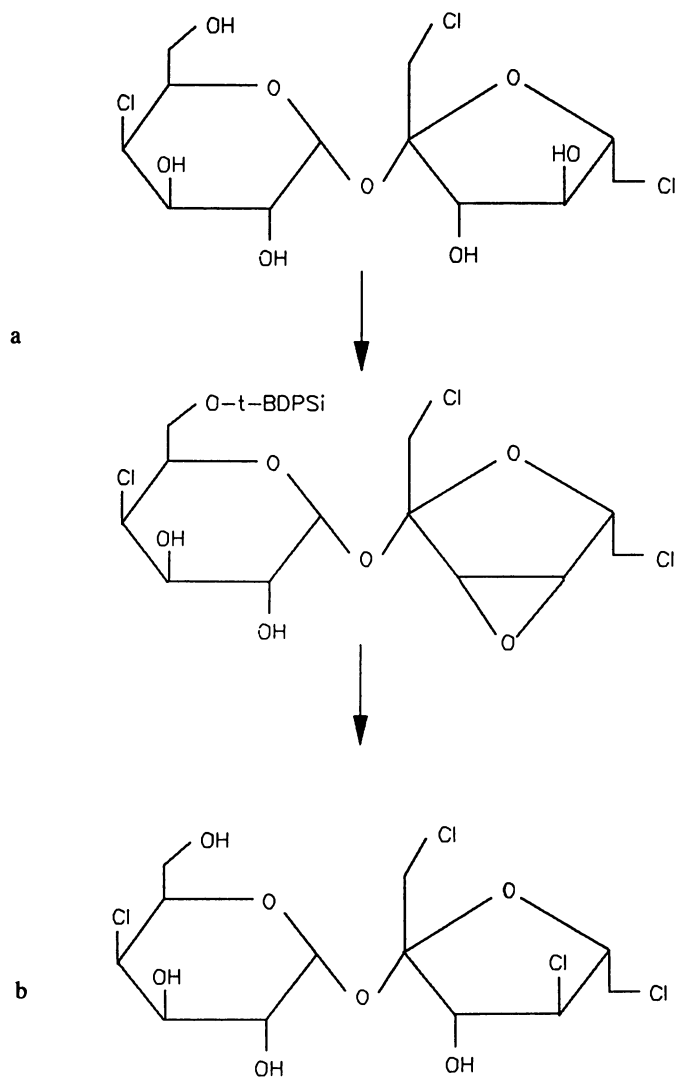
To summarise this group of compounds, it was clear that chlorination at any or all of the sites 4,1',4' and 6'- resulted in enhancement of sweetness while chlorination at the 6- position had a negative effect on sweetness.

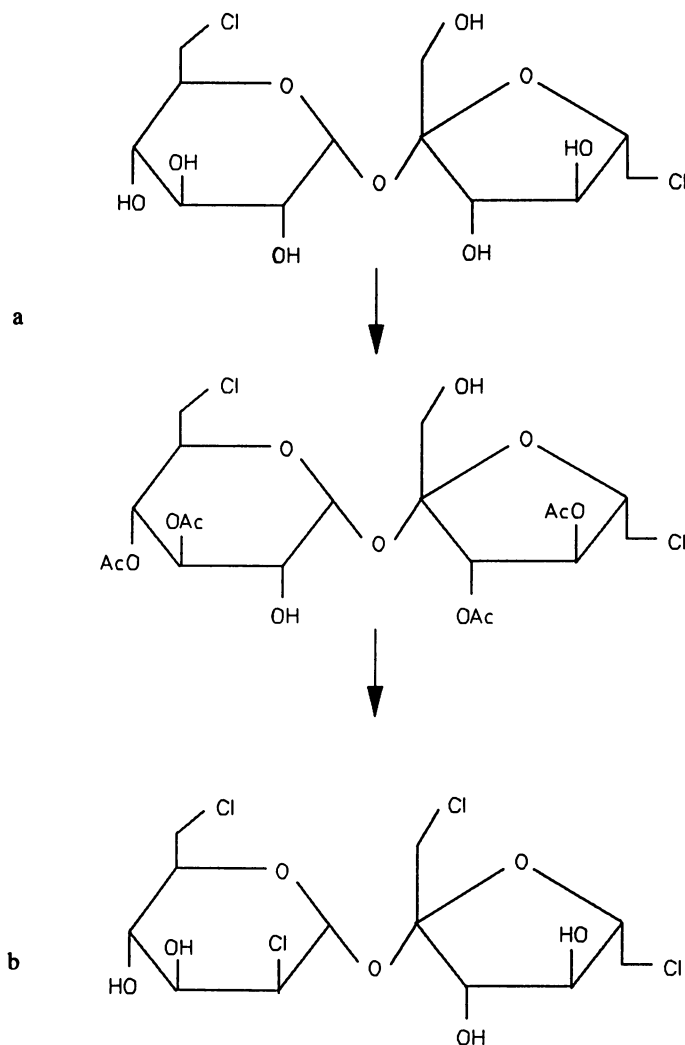
Effect of Substitution at the 6- Position

Based on another series of compounds, it was found that the influence of the substituent at the 6- position was more dependent on its size than on the presence or absence of an oxygen capable of participating in hydrogen bonding. Thus 6-deoxy-4,1',6'-trichlorogalactosucrose was found to be 400 times sweeter than sucrose, the 6-O-methyl derivative was 500 times sweeter, but the 6-O-isopropyl derivative was not sweet. Presumably in this last case, the size of the isopropyl function was such as to invade an essential structural boundary, thus preventing the molecule from binding with the sweetness receptor on the taste bud.

Effect of Substitution at the 2- Position

Chlorination at the 2- position also had a very profound effect on sweetness, as demonstrated by the 2,6,1',6'-derivative whose production is shown in Figure 10a and 10b. Sucrose was selectively chlorinated at the 6- and 6'- positions with a yield of about 50% using methane sulphonyl chloride and dimethylformamide, initially at -20 °C for 2 hours, then at 70 °C for 10 hours. Selective acetalation with 2,2-dimethoxypropane and paratoluenesulphonic acid in dimethylformamide at 20 °C for 4 hours followed by acetylation with acetic anhydride in pyridine and removal of the cyclic acetal gave a sucrose derivative with the 1'- and 2- positions unprotected. These were chlorinated using sulphuryl chloride and lithium chloride, and the ester groups removed to yield 2,6,1',6'-tetrachloromannosucrose. This was found to be exceedingly bitter (14) with a potency approximately equivalent to quinine, demonstrating that the presence of a hydroxyl group at the 2- position was essential for sweetness.





Effect of Other Halogens

In an attempt to determine the effect of size and electronegativity of substituents, other halogens were examined as follows. The 4,1',6'-tribromo- and the 4,1',4',6'-tetrabromo- derivatives (Figure 11) were produced using similar chemistry to that employed for the chlorides. The former was 800 times sweeter than sugar (15) while the tetrabromo compound was 7500 times sweeter than sugar. Clearly the size of the bromide substituent was such as to cause a better fit of the molecule onto the taste receptor. Both the more electronegative fluoride and the larger iodide did not result in such a great enhancement of sweetness. Thus the 4,1',6'-trifluoro-derivative was about 40 times sweeter than sucrose, whereas the 4,1',6'-triiodo-derivative was about 120 times sweeter than sugar (Figure 12). This is in contrast to the corresponding chloride which was 600 times sweeter and the corresponding bromide which was 800 times sweeter than sugar. It would appear, therefore, that bromine and chlorine have approximately the optimum molecular size and electronegativity.

To further elucidate the size and electronegativity effects of substituents, some compounds containing mixed halogens were produced. For example Figure 13 shows the 4-fluoro-1',4',6'-trichloro-compound which was 1000 times sweeter than sugar in contrast to the corresponding 4,1',4',6'-tetrachloro-derivative, which was 2200 times sweeter. This indicates that the size of the 4- substituent is particularly important: the small decrease in size from chloride to fluoride at this site caused a 50% reduction in sweetness.

Effect of Size of Substituent at the 4'- Position

From another series, it was found that increasing the size of the substituent at the 4'- position had a positive effect on sweetness. Thus, the 4'-iodo-4,1',6'-trichloro-derivative (Figure 14) was found to be 3500 times sweeter than sugar as against 2200 times for the 4,1',4',6'-tetrachloro- compound. A small increase in substituent size from chloro to iodo at the 4'- position resulted in an increase in sweetness of roughly 50%.

The chemistry involved in producing these mixed halogen derivatives incorporates a large number of separate processes, often employing fifteen or twenty steps. Such processes inevitably result in low yields and the majority of these compounds are clearly of academic interest only. However, following detailed evaluation including taste quality, stability and cost of manufacture, one compound was found worthy of further development.

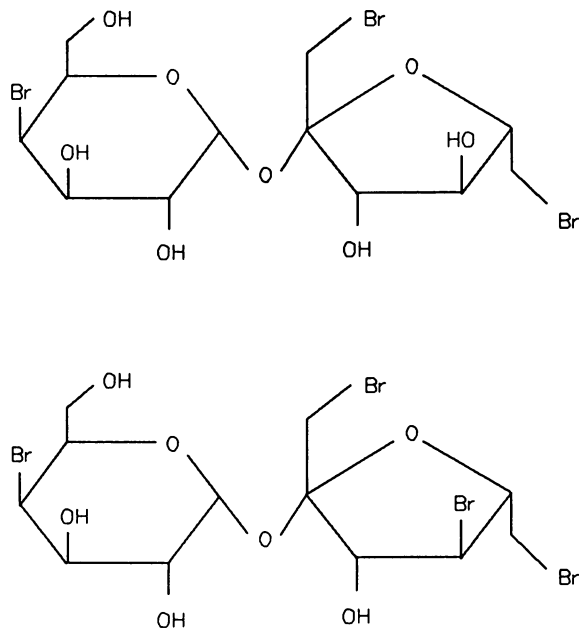


Figure 11. 4,1',6'-Tribromogalactosucrose (top); 4,1',4',6'-Tetrabromogalactosucrose (bottom)

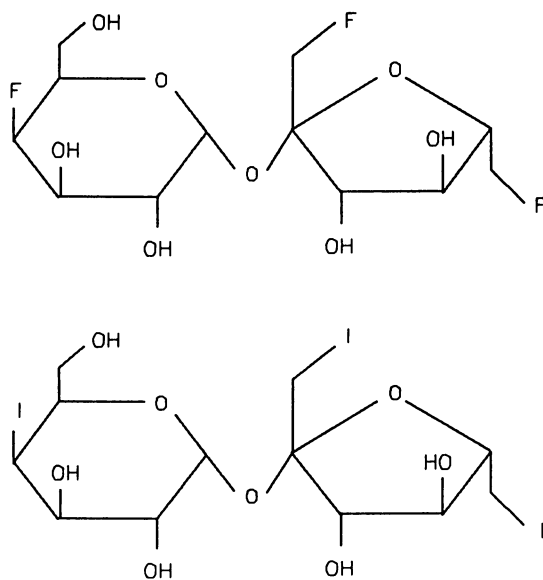


Figure 12. 4,1',6'-Trifluorogalactosucrose (top); 4,1',6'-Triiodogalactosucrose (bottom)

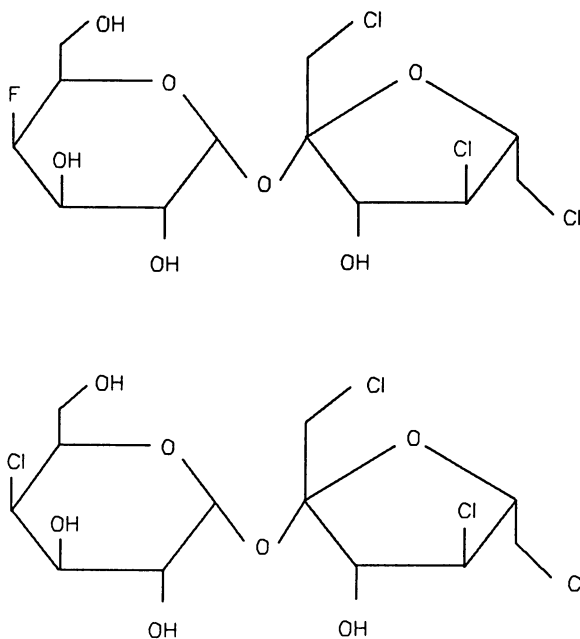


Figure 13. 4-Fluoro-1',4',6'-Trichlorogalactosucrose (top); 4,1',4',6'-Tetrachlorogalactosucrose (bottom)

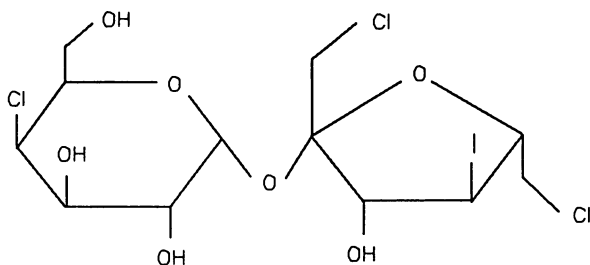


Figure 14. 4'-Iodo-4,1',6'-trichlorogalactosucrose

Identification of Sucralose

The 4,1',6'-trichloro-derivative of sucrose, shown in Figure 8, was called sucralose. Sucralose is roughly 650 times sweeter than sugar, is exceptionally stable in aqueous acidic conditions and has an excellent sweetness profile (16,17). These two attributes, stability and taste quality are of critical importance to the manufacturers of formulated foods containing high intensity sweeteners, and because of these characteristics sucralose is destined to become an outstanding commercial success.

Explanation of the Sweetness of Sucralose

In an attempt to explain the sweetness of sucralose and its relatives, Hough and Khan (18) have proposed the existence of two AH-B-X units which closely approximate the dimensions of the Kier (19) triangle. The proposed location of two of these AH-B-X triangles for sucralose is shown in Figure 15. In both cases, the equatorial 2-hydroxyl group on the galactopyranosyl unit must act as AH, the hydrogen bond donor to the receptor site. The top structure shows the involvement of the chloro group at the 1'-position as the proposed hydrogen bonding acceptor, B, with the axial hydrophobic group at the 4-position in the galactopyranosyl unit acting as the third binding site corresponding to the locking group X of the Kier triangle. The lower structure shows an alternative, or probably additional AH-B-X triangle as a consequence of the relatively free rotation around the 1'-position and the overall flexibility of the molecule. In this case the 2-hydroxyl group again acts as the hydrogen bond donor, A, with the 1'-chloro group

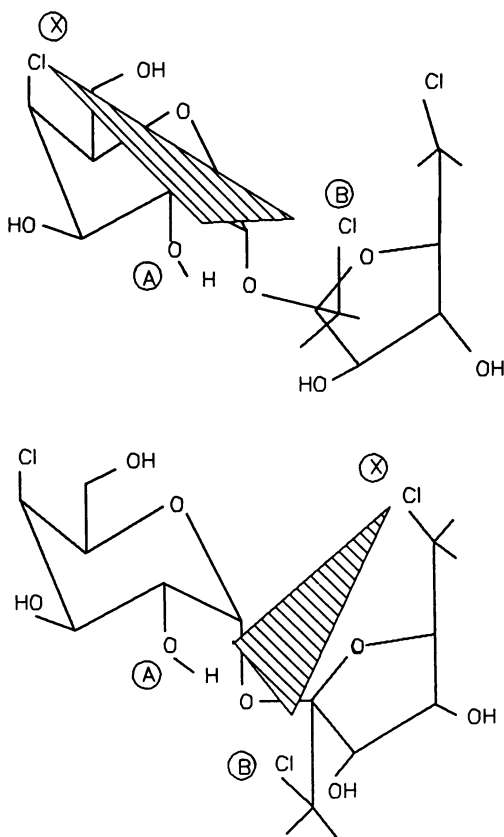


Figure 15. The 1',2,4-glycosidic (top); the 1',2,6-glycosidic (bottom) (Adapted from ref. 18).

participating as the electron accepting position, B. However, the conformation is now such as to allow the 6'-chloro group to act as the third binding site, X. It seems likely that the intense sweetness of some of these sugar derivatives is a direct consequence of the ability of the molecules to possess multiple AH-B-X binding sites. This seems to be confirmed by the intense sweetness of some of the 4'-halogenated compounds implying that a third AH-B-X triangle involving the 4'- position can participate.

In conclusion, a formidable amount of research chemistry has been devoted to sucrose and the enhancement of its sweetness, particularly by the groups at Queen Elizabeth College and Tate & Lyle. The main structural features which contribute to the sweetness of this series of molecules have been elucidated, the theory being consistent with the explanation of the sweetness of many otherwise unrelated molecules.

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Chapter 7

Design of Sweeteners A Rational Approach

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The successive discoveries in our laboratory of several series of hyperpotent sweeteners (higher than 40,000 times that of sucrose) are the result of a rational approach in their design. First we proved that CO_2^- and NO_2/CN groups, previously considered in sweeteners as identical interaction sites (B site in Shallenberger/ Acree's theory) in fact form two separate specific sites B and D (1980). We identified this D site in sweet β -alanine derivatives (1982), and designed a new predictive model which led to the first hybrids between sweetener series (sweet dipeptides and β -alanine derivatives). One of them, a thioureido derivative of aspartame was 50,000 times sweeter than sucrose (1983). Speculating on bioisosteric analogies, we synthesized the first guanidine sweeteners with potencies up to 50,000 (1985). Improving the hydrophobic site (G site), we reached a potency of 200,000 with sucrononic acid, the sweetest compound known (1987).

From saccharin to aspartame, the discovery of new synthetic sweeteners has resulted from the fortuitous tasting of compounds, an event rarely repeated in the same laboratory. During the last 10 years, we successively found several new synthetic sweeteners having sweet potencies from 3,000 to 200,000 times that of sucrose. We wish to describe the chronology of their discovery in relation with our progress in the understanding of the molecular features responsible for sweet taste.

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A Binding Site D Specific for NO₂ and CN Groups (1980)

The first theory which identified a common feature contained in sweeteners is that of Shallenberger and Acree (1967) (1). They proposed a bifunctional entity AH,B acting through intermolecular hydrogen bonds with a reciprocal unit of the sweet taste receptor which was responsible for sweetness. The exact distance between the AH hydrogen atom and the B atom was estimated to be 0.3 nm. According to this theory, nitro (NO₂) and carboxylic (CO₂H) groups were both considered the B entity.

One of the consequences was that structurally different sweeteners, amino acids, sweet dipeptides (aspartame) or nitroaniline derivatives (P 4000) would have a common binding at the sweet taste receptor (Figure 1).

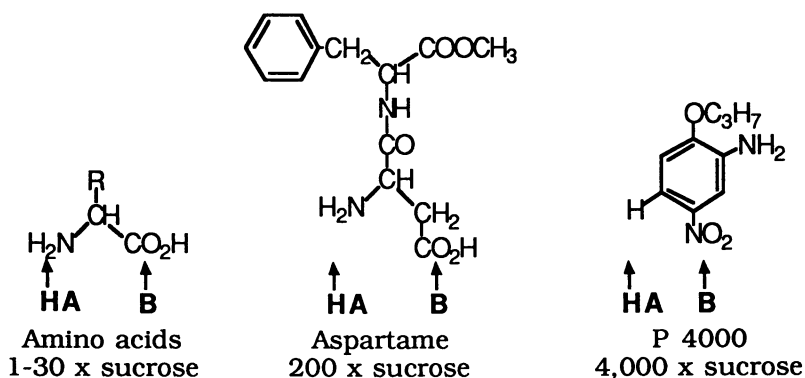


Figure 1. Typical interaction mode of amino acids, aspartame and P 4000 with the sweet taste receptor according to the AH,B concept of Shallenberger and Acree (1).

Despite the apparent general occurrence of this AH,B system in a large number of structurally different sweeteners, our attention was rapidly called to a few exceptions which suggested crucial differences in the role of the CO₂H and NO₂ groups.

Performing the same structural replacements as shown in Figure 2 in several other sweeteners, we demonstrated the generality of that initial observation i.e. the NO₂ and the CN groups are similar in their aptitude to induce a sweet taste activity but are different from the CO₂H group (Table I) (2).

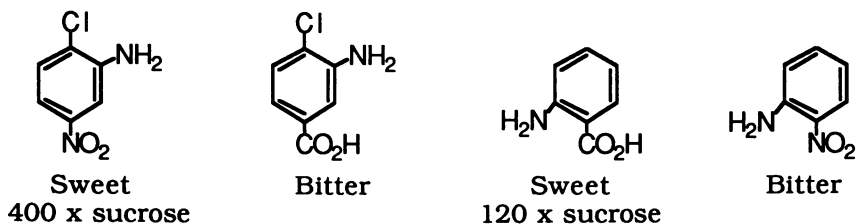


Figure 2. Some initial evidence in favor of non-equivalence between CO_2H and NO_2 groups for the sweet activity.

Table I. Effect of NO_2 , CN and CO_2H group permutation on the sweetening properties of N-carbamoyl β-alanine derivatives.

X	X'	Taste	
NO_2	CO_2H	700 (Suosan)	
CN	CO_2H	450 (Cyanosuosan)	
CO_2H	CO_2H	Not sweet	
NO_2	CN	Not sweet	

Our conclusion was that separate specific sites are involved in receptor binding, one site still named B, specific for the anionic CO_2^- group, and a second site named D, specific for the NO_2/CN groups.

As a consequence of this separation, sweeteners containing only a CO_2^- group (amino acids or aspartame) and sweeteners containing only a NO_2 group (P 4000) would bind separately with their corresponding specific sites of the receptor. On the other hand, a sweetener containing both the CO_2^- and NO_2/CN groups will bind simultaneously with these two specific sites. From this point of view, the suosan sweetener discovered by Petersen and Müller (1948) (700 times sweeter than sucrose) (3) was of great interest: this compound, an N-carbamoyl derivative of β-alanine, has both the B and D binding sites of aspartame and P 4000 respectively (Figure 3). Distances between AH, B and D groups contained in sweeteners were evaluated on the basis of this model (4).

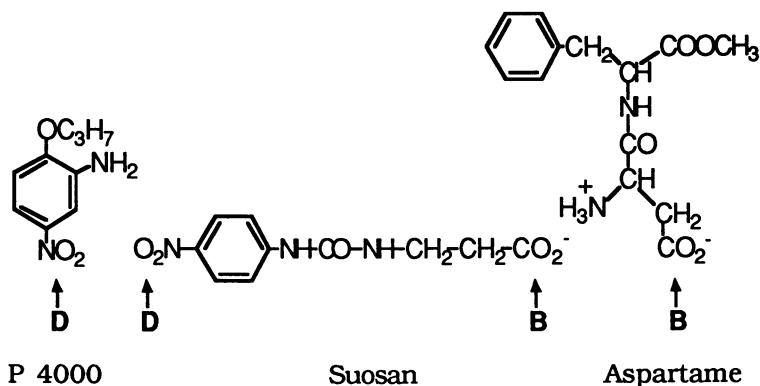


Figure 3. With a receptor containing specific sites for CO_2^- and NO_2/CN groups, suosan should have a common binding site with P 4000 and aspartame.

A New Model for Sweet Agonists (1982)

The sweet taste of dipeptides was attributed to the particular ability of their aspartic acid moiety to function as an efficient AH,B system with the $\beta\text{-CO}_2^-$ group optimally separated from the AH ($\alpha\text{-NH}_3^+$) part (Figure 1). Glutamic acid was not expected to induce a sweet taste, the $\gamma\text{-CO}_2^-$ group being too remote to constitute an effective AH,B system (5).

In two sweeteners, suosan and N-trifluoroacetyl aspartic acid derivatives, the B and D sites (CO_2^- and NO_2/CN) were identified, but an apparent discrepancy was observed in their respective B to D distances. Despite the negative influence suspected in the case of glutamic acid dipeptides, we synthesized the N-trifluoroacetyl glutamic acid derivative to mimic the B-D distance with suosan. The compound synthesized was very sweet with a potency 3,000 times that of sucrose (Figure 4) (6).

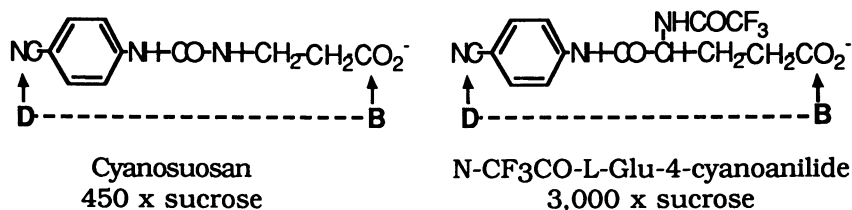


Figure 4. The unexpected sweet activity of the glutamic acid derivative is explained by an effective B to D distance, similar to that of the cyanosuosan model.

We then elaborated a preliminary model for sweet agonists on the basic concept summarized in the following points :

(i) A recognition site named D, specific for NO₂/CN groups, exists in sweeteners. The previous B site is still retained but is restricted to CO₂⁻ groups, the B to D distance being critical for the sweet activity. The binding of B and D sites with the receptor involves a hydrogen bond for the D site and an ionic bond for the B site.

(ii) The identified binding sites in sweeteners are optional and cooperative: their simultaneous binding is not required to induce a sweetness but their cumulative presence induces high potencies. As a result, if a minimum of two sites are stimulated, the simultaneous binding of the AH and B components according to the Shallenberger and Acree's theory is not a prerequisite to the sweet activity.

N-Carbamoyl Dipeptides: A Seminal Discovery (1982)

In addition to the AH,B system proposed by Shallenberger and Acree, a third complementary binding site involving dispersion forces was proposed by Kier (1972) (7). This hydrophobic component (here designated as site G) was postulated to induce higher sweet potencies in cooperation with the AH,B system. In the sweet dipeptide aspartame, Kier's hydrophobic component was well identified (the benzyl group) and its position relative to site B (the CO₂H group) was evaluated (8). Evidently, the suosan sweetener does not possess Kier's hydrophobic component, but contains the D site (the NO₂/CN group) which aspartame lacks. We then attempted to combine the identified AH, B, G and D sites within a single structure.

Upon comparing aspartame and cyanosuosan, we identified a common structural feature (the 3-aminopropanoate moiety). By preparing a hybrid between these sweeteners, the association of all the four sites was effectively made possible (9). With a potency of 14,000 times that of sucrose, this hybrid appeared much more potent than the parent compounds i.e. 30 times more than cyanosuosan and 70 times more than aspartame (Figure 5).

As previously observed in the case of suosan, replacing of the ureido unit (NHCONH) with a thioureido unit (NHCSNH) increased the potency to 50,000 times that of sucrose (9). It is noteworthy to recall that, with the exception of two compounds (10,11), substitution on the free amino group of aspartame was thought to alter the AH,B system, thus suppressing the sweet activity (5). In the present case, this N-carbamoyl substitution on the α-amino group was effective with almost all sweet dipeptides. The resulting increase in potency (generally between 10 to 100 fold) was essentially dependent on the size of the hydrophobic component contained in the parent dipeptide (9).

The N-carbamoyl dipeptides were the starting point for the subsequent discoveries of hyperpotent sweeteners. In fact, with

the four sites AH, B, D and the hydrophobic component G now connected within a single structure, an accurate evaluation of their relative distances was possible.

By comparing and superimposing this typical sweetener with a large variety of other sweetening agents, we completed the description of an ideal sweet agonist by adding four other binding sites to the model to reach an overall number of eight sites (Research Report at the G. D. Searle Company, USA, 1983). Superspartame was in this sense a very representative model since seven sites were tentatively identified in its structure as shown in Figure 5 (12).

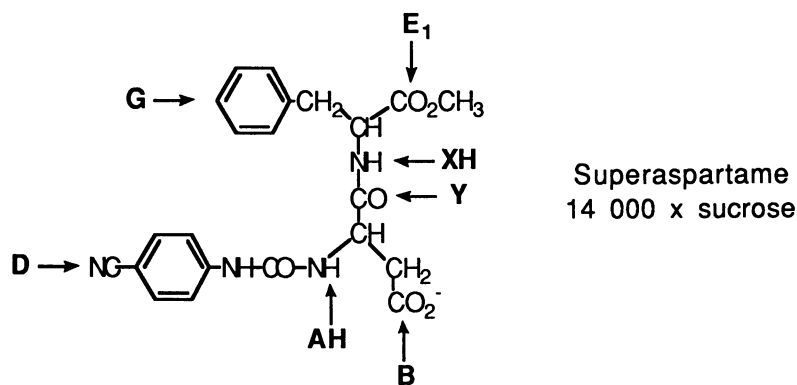


Figure 5. Superspartame, with its numerous binding sites, is considered as a reliable negative print of the sweet taste receptor.

Hyperpotent Guanidine Sweeteners (1985)

According to the proposed model, the major role attributed to the thioureido (NHCSNH) group contained in N-thiocarbamoyl dipeptides is to allow the binding of one of the NH group (see Figure 5) and to locate correctly the connected aspartame and 4-cyanophenyl components. Its subsequent replacement by other bioisosteric groups validated this assumption. From structure-activity relationships in histamine H₁ and H₂ receptor antagonists, it was known that an N-cyanoguanidine unit was an efficient bioisosteric substitute for the thioureido group (13). This replacement, attempted in an N-thiocarbamoyl dipeptide, led to a new sweetener with practically no change in sweet potency (Figure 6).

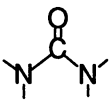
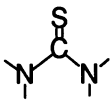
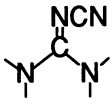
			
Radical name :	Ureido	Thioureido	N-CN guanidine
Potency in super-aspartame analogs	14,000	50,000	40,000
Potency in cyanosuosan analogs	450	2,100	900

Figure 6. Effect of replacing the ureido radical contained in superaspartame (Figure 5) and cyanosuosan (Figure 4).

Binding Site Y : Experimental Evidence (1985)

The same replacement in N-carbamoyl β -alanine derivatives also led to N-cyanoguanidine compounds having a similar potency (Figure 6) (14). It was known from Petersen and Müller's works on suosan that the replacement of the β -alanine residue with glycine resulted in a dramatic decrease of the sweet potency from 700 for suosan to 10 for the glycine analog (3,4). But in the N-cyanoguanidine derivatives, replacement of β -alanine with glycine resulted in a significant increase of the potency from 900 to 7,000 times that of sucrose (Figure 7) (14).

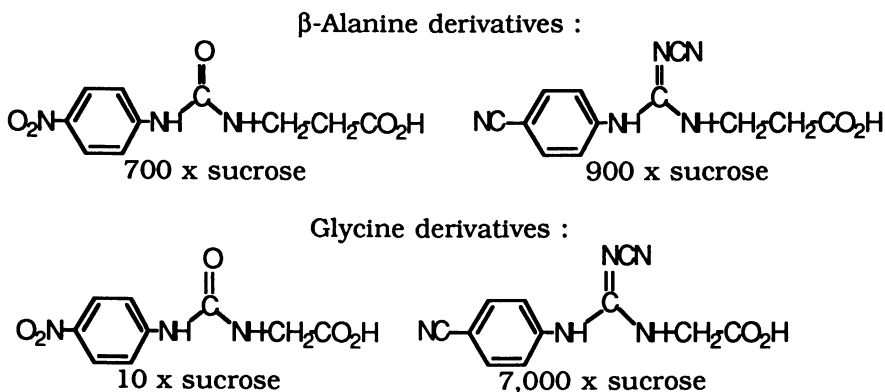


Figure 7. Correctly positioned only in the glycine derivative, the N-CN group allows an extra link with the sweet taste receptor (as site Y), thus increasing the potency from 10 for the ureido derivative to 7,000 for the N-cyano guanidine derivative.

We assumed that an increase in potency of this level (about eight fold) was the result of the extra link with the receptor allowed by the N-CN group (as a new site named Y), correctly positioned in the glycine derivative; this assumption was subsequently validated with the discovery of hyperpotent N-benzenesulfonyl guanidine derivative (Figure 8).

N-Alkylguanidine: The Most Potent Sweeteners (1986)

As already stated, no hydrophobic component could be identified in suosan and N-cyanoguanidine sweeteners. We succeeded in the addition of this missing component, with the synthesis of an N-benzenesulfonyl guanidine derivative, expected to combine the previous site Y (now the SO₂ group) with an additional hydrophobic component (the benzene ring) (15). The sweet potency increased from 7,000 for the N-cyanoguanidine compound to 45,000 for the N-benzenesulfonyl guanidine derivative, consistent with the existence of site Y, and its proximity with the hydrophobic component G (Figure 8).

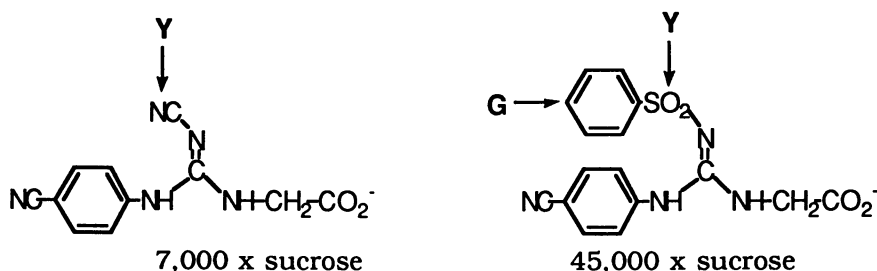


Figure 8. Addition of the missing hydrophobic component (site G) enables additional binding, increasing the potency from 7,000 to 45,000 times that of sucrose.

Studies on N-carbamoyl dipeptides taught us that binding sites in sweeteners have an optional characteristic : suppressing one of them in highly potent compounds only alters the potency of the resulting compound (9). As expected, the replacement of the SO₂ group (site Y) contained in the N-benzenesulfonyl guanidine derivative with a methylene group led to an N-benzyl guanidine which still had the high potency equal to 30,000 times that of sucrose (Figure 9) (16). This result suggested that the addition of the hydrophobic component (the phenyl ring) is the most important factor in the increase in potency of these compounds, site Y being of less importance.

The influence of the hydrophobic component on the potency of sweeteners has been widely investigated (8,17). The most representative compounds were the aspartyl-aminomalonyl diester sweeteners in which the sweet potency increases from 15 times

that of sucrose to 50,000, when the hydrophobic component change from the methyl ester group (COOCH_3) to the highly hydrophobic fenchyl ester group ($\text{COOC}_{10}\text{H}_{17}$) (18,19). Through similar extensive research, the hydrophobic component R (Figure 9) contained in sweet guanidines derivatives was optimized to reach, with a cyclononyl group, a potency of 200,000 times that of sucrose (20).

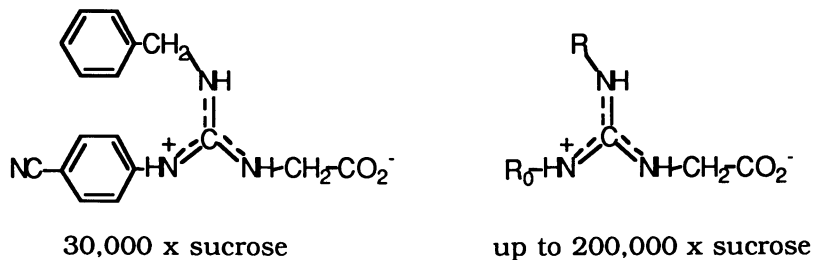


Figure 9. Replacement of the sulfonyl group (Figure 8) by a methylene group (N-benzyl derivative on the left side), led to the discovery of the N-cycloalkyl guanidine derivatives, the most powerful sweetening agents (on the right side a representative general formula).

This compound, now called sucrononic acid (Figure 10), an N-cyclononyl guanidine derivative, represents the most potent synthetic sweetener (a 0.01 mg/100 mL solution of this compound is sufficient to match the sweet potency of a 2 % sucrose solution).

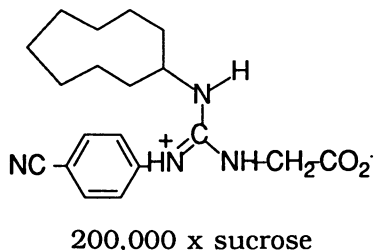


Figure 10. Sucrononic acid, the most potent sweetener with a potency of 200,000 times that of sucrose (zwitterionic form).

This exceptionally high potency results from two additional effects: the extreme efficiency of the different groups involved in the binding (nature, relative distances, size and shape) and the apparent rigid structure of the whole molecule induced by the rigid 4-cyanophenyl and the bulky cyclononyl substituent linked to a planar central guanidine group.

Substitutes for the 4-Cyanophenyl Group (1987)

To design a replacement for the 4-cyanophenyl component it was important to preserve the potential hydrogen bonding of the D site with the complementary feature of the receptor. An indazol-6-yl group, in which the nitrogen atom in the 2-position would just have the capacity to allow a hydrogen bond, was incorporated into the N-cycloalkyl guanidines (21). The resulting compound was found to be extremely sweet, with a potency of 130,000 times that of sucrose (Figure 11).

Potency in sweet N-cycloalkyl guanidine derivatives
(x sucrose) :

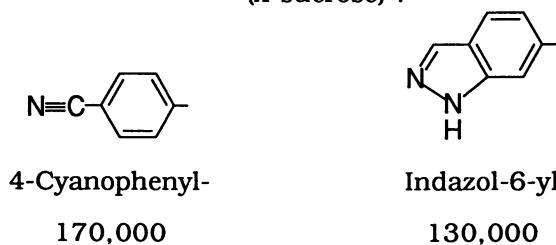
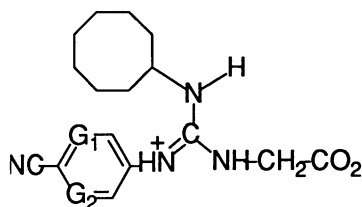


Figure 11. Structural analogs (R_0 in the general formula) of the 4-cyanophenyl group in guanidine sweeteners (formula of Figure 9 with $R = c-C_8H_{15}$).

The complexity of the aromatic substituent was also seen when the phenyl group was substituted by various groups or atoms in 3- and 5- positions (chlorine atom for example) (22) or replaced by a pyridyl or a pyrimidinyl ring (Figure 12) (21, 23). These results suggest this site is a more complex site than was suspected in the light of previous results.



$G_1 = G_2 = CH$	170,000 x sucrose
$G_1 = N, G_2 = CH$ (pyridyl ring)	100,000 x sucrose
$G_1 = G_2 = N$ (pyrimidinyl ring)	5,000 x sucrose

Figure 12. Potency of N-cycloalkyl guanidine derivatives containing or not nitrogen atoms in the aromatic substituent.

Conclusion

Starting from the notion that two recognition sites specific for the CO_2^- group and the NO_2/CN groups are present in the sweet taste receptor, we combined both these groups within a single molecule by preparing a hybrid between two sweet compounds, aspartame and suosan. This and related compounds were hyperpotent, with potencies up to 50,000 times that of sucrose. A model of an ideal sweet agonist containing up to eight optional and cooperative binding sites was then developed. With this predictive support, several series of hyperpotent sweeteners were found by the addition, in known sweet molecules, of the binding sites which were thought to be missing. Specifically, extremely potent guanidine derivatives were obtained, with potencies in excess of 100,000 times that of sucrose. By an accurate determination of each chemical group involved in the sweet activity, a potency of 200,000 times that of sucrose was finally reached with sucrononic acid, an N-cycloalkyl guanidine derivative. As a result of this strategy, many correlations between several different families of sweet molecules were discovered, for instance between amino acids, dipeptides, N-carbamoyl β -amino acids, nitroaniline derivatives (P 4000), N-carbamoyl dipeptides, aspartic or glutamic N-trifluoroacetyl derivatives and trisubstituted guanidines.

Acknowledgments

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Chapter 8

Tetrazoles as Carboxylic Acid Surrogates High-Potency Sweeteners

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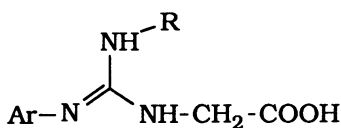
A recent patent by C. Nofre and J.-M. Tinti of the Université Claude Bernard disclosed disubstituted guanidine acetic acids as potently sweet compounds. Our work examined a number of analogs in this guanidine class of sweeteners to determine the effect of various structural modifications on sweet taste response. We examined various acid surrogates and found that tetrazolyl substitution for carboxyl gave sweet compounds. Requirements for size, degree of flexibility, and polarization effects in the other two regions of the molecule were examined through the synthesis and sensory evaluation of compounds. Maximum potencies of 30,000 times a 2% sucrose solution were discovered. Interesting conclusions to aid our understanding of sweet taste response can be drawn by comparing closely related analogs having dramatically different sensory properties.

Elicitation of a sweet response is a property shared by an astonishingly wide variety of molecular structures. Chemosensory detection of sweetness is present at birth and remains throughout a normal human lifespan (1). The universality of the response, the relatively poorly understood relationship of structure and activity (2,3), and the enormous commercial opportunity provided by the hedonic desire for sweet tasting products, have stimulated

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research and development directed toward identification of new sweeteners. A successful new entity will emulate sucrose with very similar maximum intensity, fidelity of taste quality reproduction, and time course of onset and extinction of response. It will elicit the response at very low concentrations, be stable in aqueous solutions from pH 2.5 to pH 8 and to temperatures above 100 °C. It must be water soluble and safe to consume as a food ingredient.

Principles of medicinal chemistry have been applied to the design of new sweeteners through systematic modification of structures in order to maximize taste quality, potency and the requisite physical properties. Recently the discovery of a new class of ultrapotent sweet compounds was disclosed by Nofre, Tinti and Ouar-Chatzopoulos (4). These compounds are disubstituted guanidine acetic acids represented by the general formula **1**. Potencies of up to 200,000 times sucrose (2% sucrose reference) were found for the most active examples disclosed.



1

R = alkyl group

Ar = aryl group

Acid Surrogates in the Guanidine Acetic Acid Series

The high potencies reported for compounds in this novel guanidine acetic acid structural class of sweeteners coupled with the relatively straightforward structures lacking asymmetric centers was of interest. Previous success in the use of tetrazoles as carboxylic acid surrogates (5) suggested examination of the effect of replacing the carboxylic acid moiety in the guanidine acetic acid sweetener class with acid surrogates of varying size and acidities. Results of replacing the carboxylic acid are shown in Table I along with data reported for the parent compounds. Of the various acid surrogates investigated we found substitution of carboxyl with sulfonic or phosphonic acid, which has a lower pK_a than carboxyl gave a bitter and no response, respectively. A similar result was obtained when succinimide, which has a higher pK_a was used. When tetrazole was substituted for carboxyl, a potentially sweet compound, albeit having reduced potency when compared to the carboxyl compound, was obtained.

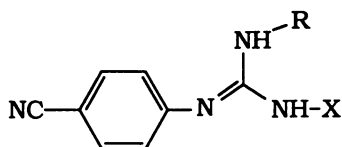
Previous sweetener research directed toward tetrazole analogs of D-tryptophan (6) showed that sweetener activity present with the parent amino acid compounds was retained. Examples exist in other physiologic areas where tetrazole may or may not be successfully substituted for carboxyl. Butler (7) and Singh (8) have

reviewed the literature describing the successful substitution of tetrazole for carboxylic acid in compounds having activity in a number of physiological areas including anti-inflammatory, analgesic, anti-bacterial, anti-allergic and hypolipidemic. However, other workers have found that tetrazole analogs of carboxylic acids have substantially lower activity or are totally inactive. For example, Crenshaw, et. al. (9) demonstrated the non-equivalence of tetrazole and carboxylic acid in the antifertility area and Almquist, et. al. (10), showed that the tetrazole analog of a potent angiotensin converting enzyme (ACE) inhibitor was much less active than the corresponding carboxylic acid. In addition, the tetrazole analog of γ -aminobutyric acid (GABA) was found to be inactive as a substrate for GABA transaminase. In fact, the tetrazole analog was a specific inhibitor of the enzyme (11). Therefore, this work was begun to further develop the structure-activity relationships (SAR) of the tetrazolyl guanidine class of sweeteners.

Synthesis of Tetrazolyl Guanidines. Of the numerous procedures available for the synthesis of guanidines (12-16), the three step isothiurea route employed by Nofre and coworkers (4) for the preparation of guanidine acetic acids was chosen. This route for the case of tetrazolyl methyl guanidines is outlined in Scheme I. Addition of an amine to the isothiocyanate gave a thiourea which was activated toward displacement by transformation to an S-methyl isothiurea. Displacement of methyl mercaptan with the final amine gives the desired guanidine. Preparation of tetrazole analogs of guanidine acetic acid sweeteners required the tetrazole analog of glycine. Synthesis of tetrazole analogs of amino acids has been described (17). Ammonium azide was added to a protected amino nitrile to prepare the tetrazole analog of glycine. This compound was then used as the final reactant in our synthesis.

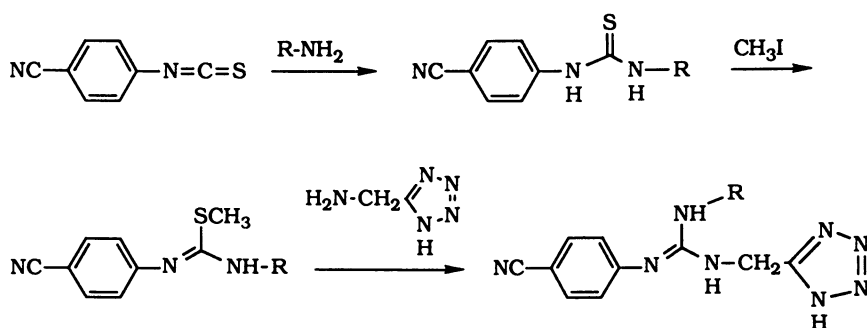
Comparison of Tetrazole Analogs With Guanidine Acetic Acids. Analogs in the series which spanned the range of potencies reported by Nofre, Tinti and Ouar-Chatzopoulos (4) were prepared in order to develop parallel structure-activity relationships. Nofre and coworkers had demonstrated the most effective aryl substituents were 4-cyanophenyl and 3,5-dichlorophenyl. Therefore, efforts were concentrated on the synthesis of analogs containing one or the other of these aryl moieties, the tetrazole analog of glycine and substituents spanning the range reported by Nofre and Tinti on the remaining guanidine nitrogen atom. All except one of the analogs were prepared by the isothiurea route indicated above and all of the tetrazole analogs were sweet. The potencies of the compounds prepared are given in Table II. It was somewhat difficult to compare potencies between the carboxyl and tetrazolyl series since the reference sweetener solutions were reported differently and potency is dependent on the reference sweetener concentration used (18). It is, however, readily apparent that while

Table I. Substitution For Carboxylic Acid



Compound Number	X	R	pKa	Potency
2	CH ₂ COOH	c-C ₆ H ₁₁	3-5	10,500(5) ^a
3	CH ₂ COOH	c-C ₈ H ₁₅	3-5	130,000(5) ^a
4	CH ₂ SO ₃ H	c-C ₈ H ₁₅	<1	bitter
5	CH ₂ PO ₃ H ₂	c-C ₆ H ₁₁	1-2	tasteless
6	CH ₂ CN ₄ H	c-C ₈ H ₁₅	~5	5,000(5)
7	Succinimide	c-C ₈ H ₁₅	9-10	bitter

^a Data from ref. 4.



Scheme I. Synthesis of Guanidines From Isothioureas

Table II. Rangefinding SAR Studies

<u>Compound Number</u>	<u>R</u>	<u>X=Tetrazole</u> <u>Potency</u> (% sucrose)	<u>X=COOH⁴</u> <u>Potency</u> (% sucrose)
8	H	50 (5)	2,700 (2)
9	Methyl	50 (5)	0
10	Benzyl	700 (7)	30,000 (2)
11	(S)-Phenethyl	500 (5)	28,000 (2)
12	Cyclohexyl	400 (4)	12,000 (2)
6	Cyclooctyl	5,000 (5)	170,000 (2)
13	Cycloheptylmethyl	800 (8)	---
14	1-Naphthyl	500 (5)	60,000 (2)
15	Benzenesulfonyl	2,000 (2)	45,000 (2)

<u>Compound Number</u>	<u>R</u>	<u>X=Tetrazole</u> <u>Potency</u> (% sucrose)	<u>X=COOH⁴</u> <u>Potency</u> (% sucrose)
16	Methyl	40 (4)	---
17	Benzyl	400 (4)	80,000 (2)
18	(S)-Phenethyl	3,000 (3)	120,000 (2)
19	Cyclohexyl	50 (5)	---
20	Cyclooctyl	400 (4)	60,000 (2)
21	1-Naphthyl	70 (7)	30,000 (2)
22	Benzenesulfonyl	600 (6)	---

tetrazoles retain sweetening activity, they are less potent than the corresponding carboxyl analogs by a factor of about twenty. A similar reduction in potency was observed in our previous work on tetrazole analogs in the suosan series (5).

Variations in the Tetrazole Chain. To maximize the sweet taste response in the tetrazole series, the SAR of the tetrazolyl guanidines was expanded by synthesizing analogs containing (S)- α -phenethyl or cyclooctyl as the representative functionality in the alkyl region, while varying the length of the tetrazole side chain as well as the aryl substituent.

The initial emphasis for examining the tetrazole chain length was based on the larger size of tetrazole compared with carboxylic acid. This steric difference could result in less efficient binding to a sweet taste receptor for the tetrazole analog, thus explaining the reduction in potency observed with tetrazole analogs. Molecular modeling showed that eliminating the methylene group in the tetrazole chain gave a molecule having an overall size more comparable to the carboxylic acid analog. However, electronically differences remained. In addition to this steric rationale, the 5-aminotetrazolyl analog was prepared to extend the SAR of this series, as the corresponding chain shortened carboxylic acid compound is unstable and cannot be prepared.

Attempts to prepare 5-aminotetrazolyl guanidines using the isothiurea route were unsuccessful, probably due to delocalization of electron density from the exocyclic nitrogen atom, resulting in decreased reactivity. It therefore became necessary to use the more reactive carbodiimide intermediate (13) in place of the isothiurea. The carbodiimide was conveniently prepared from the thiourea (19, 20) and it reacted smoothly with 5-aminotetrazole to give the desired guanidine in 38% yield (Scheme II).

In addition to this chain shortened analog, the β -alanine tetrazolyl analog was of interest due to the similarity in structure between this series and the suosan urea series (21, 22). Aminoethyl tetrazole, prepared from 3-aminopropionitrile and ammonium azide (17), was added to the isothiurea using the same conditions described in Scheme I. The compounds prepared in this series are shown in Table III along with the corresponding taste data. It was clear from the taste data that, of the analogs prepared, a one carbon spacer between the guanidine and tetrazole gives the optimum response.

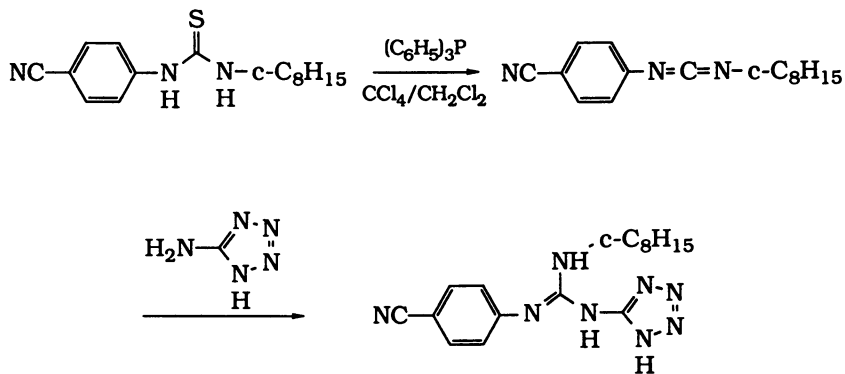
The chain shortened analog was found to be sweet, however, the potency was reduced by a factor of about ten from the initially prepared one carbon analog. Initial modeling considerations showed better steric overlap between the chain shortened tetrazole analog and the parent glycine compound. However, based on the taste data obtained, other considerations must be more important than a similarity in size. The electron density on the tetrazole ring is concentrated on the proximal nitrogen atoms, which are closer

to the guanidine nucleus than the corresponding electron density in the glycine case. This observation identifies portions of the molecule that are important for initiation of a sweet taste response and shows the importance of spacing between these interacting portions of the molecule with a taste receptor recognition unit.

In addition to the above possibility to explain the potency reduction found with tetrazolyl analogs, the tetrazole ring in the chain shortened case can derive added stability through conjugation with the guanidine nitrogen atom, by being in the same plane as the guanidine functionality. Increasing the preference for this planar conformation could disrupt any effective interactions between the anionic group and the receptor. If this were the case, it would indicate the active conformation of the carboxyl group of glycine may have a perpendicular orientation relative to the guanidine for efficient binding.

Variations in the Aryl Region

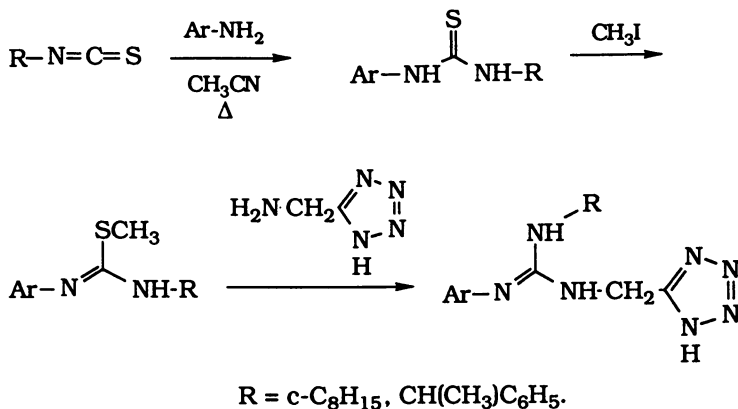
Having established the optimum chain length in the tetrazole series, the effect of the aryl substituent on the sweetness potency was examined. The synthesis of analogs having varied aryl groups was accomplished by the isothiurea route. Some of the desired aryl substituents were available as isothiocyanates (Trans World Chemicals is a supplier of a large number of isothiocyanates). In these cases, the synthesis of the guanidines followed the route outlined above in Scheme I. Addition of an aliphatic primary amine to the isothiocyanate proceeds readily to give a thiourea possessing two of the three substituents needed in the target guanidine. The final amine is added by first activating the thiourea through the formation of the S-methyl isothiurea followed by displacement of methyl mercaptan by the final amine. If the aryl isothiocyanate is not readily available, a modified route has to be employed as shown in Scheme III. Cyclooctyl and (S)- α -phenethyl isothiocyanates are commercially available and can be used to prepare the thioureas needed as guanidine intermediates. Because aromatic amines are less reactive than aliphatic primary amines, it is necessary to reflux the reaction to ensure complete conversion to product in a reasonable time period. Once the thiourea is obtained the remainder of the synthesis follows the isothiurea route described above. The compounds prepared and potency data for each are shown in Table IV. The results for variations in the aryl portion of the molecule show that cyanophenyl is the substituent giving the most potent compound, in addition to the cleanest tasting. Subsequent work, therefore, focused on analogs containing 4-cyanophenyl and aminomethyl tetrazole on the guanidine nucleus.



Scheme II. Synthesis of Guanidines From Carbodiimides

Table III. Effect of Anionic Substituent Chain Length

Compound Number	n	Potency (% sucrose)
23	0	400 (4)
6	1	5,000 (5)
24	2	bitter



Scheme III. Preparation of Aromatic Analogs

Table IV. Effect of Aromatic Substituents

<u>Compound Number</u>	<u>Ar</u>	<u>X=Tetrazole</u> <u>Potency</u> (% sucrose)	<u>X = COOH⁴</u> <u>Potency</u> (% sucrose)
18	3,5-Dichlorophenyl	3,000 (3)	120,000 (2)
25	3,5-Dimethylphenyl	600 (6)	30,000 (2)
11	4-Cyanophenyl	500 (5)	28,000 (2)
26	Phenyl	40 (4)	5,000 (2)
27	4-Carbomethoxyphenyl	30 (3)	700 (2)
28	2,6-Dimethyl-4-Pyridinyl	bitter	--

<u>Compound Number</u>	<u>Ar</u>	<u>X=Tetrazole</u> <u>Potency</u> (% sucrose)	<u>X = COOH⁴</u> <u>Potency</u> (% sucrose)
29	H	bitter	--
20	3,5-Dichlorophenyl	400 (4)	60,000 (2)
6	4-Cyanophenyl	5,000 (5)	170,000 (2)
30	Phenylsulfonyl	bitter	--
31	6-Aminoindazolyl	2,000 (2)	130,000 (2)
32	6-Aminoquinolinyl	bitter	10,000 (2)
33	2,6-Dimethyl-4-Pyridinyl	bitter	--
34	4-Carboxamidophenyl	300 (3)	--

Variations in the Alkyl Region

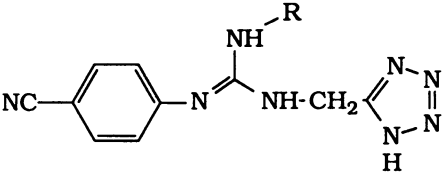
The final area to explore substituent effects on potency involved the alkyl region. Of the analogs described, the cyclooctyl compound, **6**, was found to be the most potent. Variations in the alkyl region were examined, attempting to improve the potency of these compounds. Using the cyclooctyl compound as a starting point, analogs with similar carbon numbers were prepared to determine what effect flexibility and entropic effects might have on potency. A series of analogs containing more flexible side chains were synthesized using the procedures described above. Taste results for these compounds are shown in Table V.

These results showed that more flexible analogs give a lower potency taste response, suggesting that interaction with a sweet recognition unit is less efficient. Since the monocyclic compounds initially evaluated, i.e. **6**, were more potent than the flexible analogs just described, further constraint of the structure to improve recognition in a dispersion binding region was investigated. This was accomplished by bridging the rings to form bicyclic structures. If a rigid analog is prepared and its shape closely resembles the receptor shape, enhanced potency would be expected. The bicyclic analogs shown in Table VI were prepared using the synthetic schemes shown previously. If the amines were unavailable commercially, they were conveniently synthesized by hydrogenation of the aromatic compound (compounds **40** and **41**) or from the ketones via reduction of the oximes (compound **42**, Scheme IV). The aromatic analogs can be viewed as bridged α -phenethylamine analogs, while the aliphatic bridged compounds are analogs of the cycloalkyl compounds **6** and **12**.

In all of the examples in Table VI, an increase in potency is observed when compared with the less rigid analog. The saturated ring system was identified as giving more potent sweet taste responses. It is noteworthy that the *cis*-endo-bicyclic ring systems gave rise to the most potent compounds reported within this series. Thus, conformational degrees of freedom, carbon numbers, and stereochemistry all affect the response in a more or less predictable pattern.

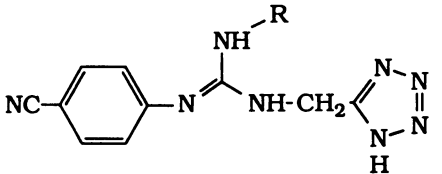
In summary, the structure activity relationships of a new class of ultra high potency sweeteners were expanded to provide additional novel sweetening agents. The viability of these materials as candidates for commercial products is being investigated. The nature of the comparative activity between the carboxyl and 5-tetrazolyl congeners developed so far, suggests similar agonist binding interactions with reduced receptor affinity or activation for tetrazolyl derivatives.

Table V. Effect of Flexible Lipophilic Substituents

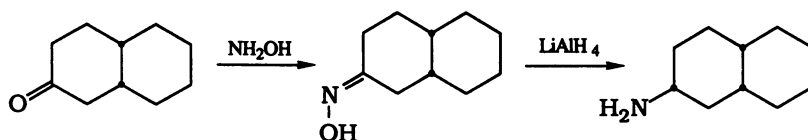


<u>Compound Number</u>	<u>R</u>	<u>Potency</u> (% sucrose)
6	Cyclooctyl	5,000 (5)
35	1-Dodecyl	20 (2)
36	1-Nonyl	200 (2)
37	5-Nonyl	bitter
38	Cyclohexyl-n-butyl	15 (2)

Table VI. Effect of Rigid Lipophilic Substituents



<u>Compound Number</u>	<u>R</u>	<u>Potency</u> (% sucrose)
39	1-Indanyl	2,000 (2)
40	2-Indanyl	2,000 (2)
41	1-Tetralyl	300 (3)
42	5-Tetralyl	bitter
43	<u>exo</u> -Norbornyl	200(2)
44	<u>endo</u> -Norbornyl	2,000(2)
45	cis- <u>endo</u> -2-Hydrindanyl	25,000 (2)
46	cis- <u>endo</u> -5-Hydrindanyl	30,000 (3)
47	cis- <u>endo</u> -2-Decalyl	20,000 (2)



Scheme IV. Synthesis of Bicyclic Amines

Acknowledgments

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Chapter 9

High-Potency Sweeteners Derived from β -Amino Acids

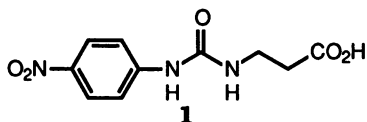
George W. Muller, Darold L. Madigan, J. Chris Culberson,
D. Eric Walters, Jeffery S. Carter, Carrie A. Klade, Grant E. DuBois,
and Michael S. Kellogg

The NutraSweet Company, Mt. Prospect, IL 60056

Over the last 20-25 years, intense effort has been focused on the discovery of novel sweeteners. Suosan, N-(4-nitrophenyl)-N'-(2-carboxyethyl)urea is a known synthetic sweetener which contains an aryl substituent and an acidic moiety. Aspartyl amide sweeteners contain a hydrophobic component and an acidic moiety. We designed analogues in which a hydrophobic unit was incorporated in suosan type sweeteners providing increased potency.

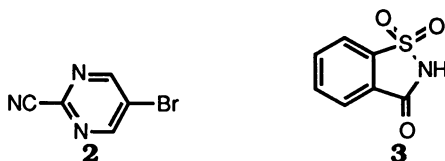
The commercialization of aspartame has revealed the large market for a safe, low calorie synthetic sweetener with a taste profile accurately reproducing that of sucrose. For a high potency sweetener to be commercially successful, it must meet the following criteria. It must: 1) be safe for human consumption under the conditions of approved use, 2) have a taste profile very close to that of sucrose, 3) have the required solubilities for commercial applications, 4) be stable to the application conditions, and 5) be economically competitive with sucrose and the high fructose corn syrups.

Herein, we wish to report a new series of high potency sweeteners related to suosan, **1** (1). Examination of the literature on sweeteners reveals several other high potency sweeteners containing electron deficient aryl moieties which may function as π -stacking recognition units(2-4) in a sweet taste perception mechanism.

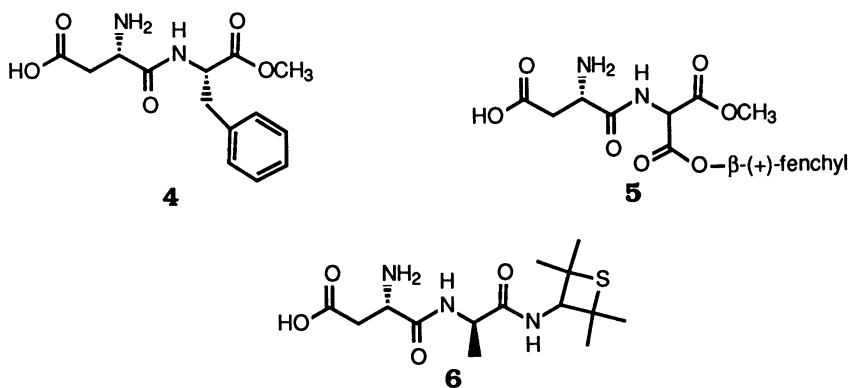


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Suosan, *N*-(4-nitrophenyl)-*N'*-carboxyethylurea (**1**), contains an aryl moiety and a short chain carboxylic acid. In 1972, Budesinsky and Vavrina reported 5-bromo-4-cyanopyrimidine (**2**) to be sweet (5). Compound **2** has a sweetness potency (5) of 700 times sucrose (on a weight basis) relative to a 7% sucrose solution [abbreviation: $P_w(7) = 700$]. The cyanopyrimidine **2** is an electron deficient aryl group. Saccharin (**3**) is a well known commercial sweetener exhibiting a $P_w(10) = 300$ which contains both aryl and sulfonamide moieties (6).

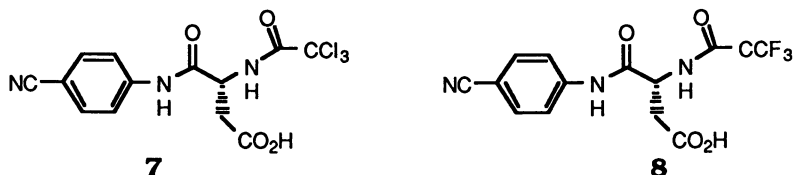


Examination of the aspartyl amide sweetener class reveals a great diversity of structure with sweetness potencies exceeding 30,000 times sucrose (7). The dipeptide ester known generically as aspartame (**4**) is the only aspartyl amide sweetener approved by regulatory agencies to date (8, 9). It has a sweetness potency ranging from 400 times sucrose versus a 0.34% sucrose solution to 100 times sucrose versus a 15% sucrose solution (8). The amino malonic acid derived sweetener **5** prepared by Fujino and coworkers has a reported sweetness potency of 22,000-33,000 times sucrose (7). Alitame (**6**), derived from *L*-aspartic acid, *D*-alanine, and a tetramethyl thietanylamine, exhibits a $P_w(10) = 2,000$ (10). These three aspartyl amide sweeteners have in common a free carboxylic acid and a large hydrophobic group.



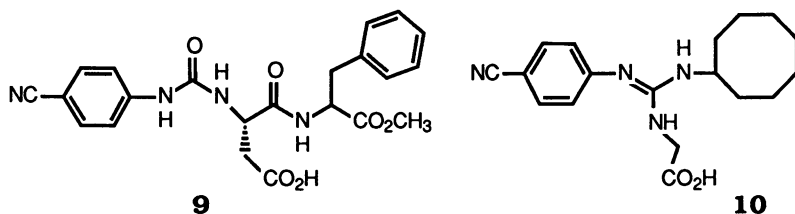
Combination of Aryl and Aspartyl Amide Sweeteners

Several examples of high potency sweeteners may be viewed as combining the attributes of both the aryl and aspartyl amide sweeteners. In 1973, Lapidus and Sweeney (11) reported that α -L-aspartyl anilide derivatives were sweet. *N*-Trifluoroacetyl- and *N*-trichloroacetyl- α -L-aspartyl-*p*-cyanoanilide (**7** and **8**, respectively) were reported to have sweetness potencies of 3,000 times sucrose (11). Inspection of structures **7** and **8** shows an electron deficient aryl moiety, a carboxylic acid and a hydrophobic substituent (trihaloacetyl).



In 1987, Nofre and Tinti at the Université Claude Bernard reported aryl ureido and thioureido derivatives of aspartame with $P_w(2)$ as high as 40,000 (12; potencies were recalculated from molar to weight ratios.). Ureido derivative **9** was reported to have a $P_w(2) = 7,800$. These derivatives contain an electron deficient aryl moiety, a carboxylic acid and a hydrophobic substituent.

More recently, Nofre and Tinti reported that *N*-aryl-*N'*-alkyl-*N''*-carboxymethylguanidines are high potency sweeteners with potencies up to 170,000 times that of sucrose (13). Compound **10** was reported to have a $P_w(2) = 170,000$ (13). Again, the same three recognition units are present.

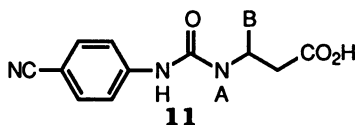


Thus, combination of the recognition units present in the prototype compounds yielded sweeteners with increased potency relative to members of either class.

Suosan as a Template

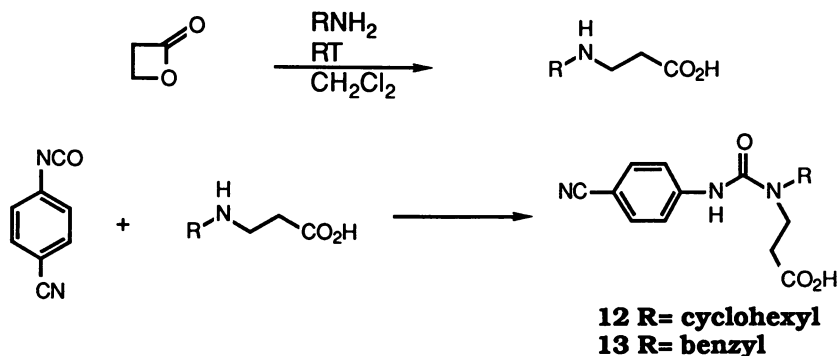
Nofre and co-workers had previously reported that a cyano moiety was a functional replacement group for the nitro group in suosan with only a small drop in sweetness potency (14; potencies were

recalculated from molar to weight ratios.). These workers reported a $P_w(2) = 870$ for suosan and a $P_w(2) = 600$ for the cyanophenyl substituted analogue. A report by Goodman and Rodriguez, along with the initial report by Lapidus and Sweeney, demonstrated that 4-nitrophenyl and 4-cyanophenyl were optimal groups for sweetness potency in the aspartyl anilide sweetener series (11, 15). We chose to use N-(4-cyanophenyl)-N'-carboxyethylurea (**11**, A = B = H) as our template instead of suosan.



Using examples from the above mentioned classes of sweeteners, a composite model of sweet taste agonists was developed at The NutraSweet Company (16). Suosan contains the requisite carboxylic acid moiety and an electron deficient aryl ring but lacks the large hydrophobic recognition unit common to a large number of high potency sweeteners. Examination of suosan in The NutraSweet Company sweet taste agonist model revealed the possibility of increasing the potency by incorporation of this hydrophobic recognition unit. Our examination of suosan suggested substitution at either of two positions, A or B of analogue **11**, might lead to higher potency sweeteners.

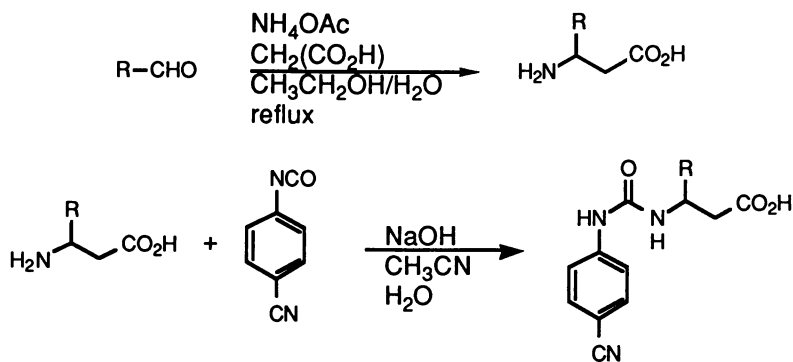
N-Substitution. The N-substituted compounds were prepared as shown in Scheme I. Treatment of β -propiolactone with amines yielded the desired N-substituted β -alanines (17). Treatment of 4-cyanophenyl isocyanate with the N-substituted β -alanines yielded the the desired analogues.



Scheme I

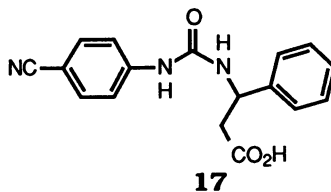
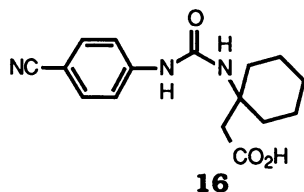
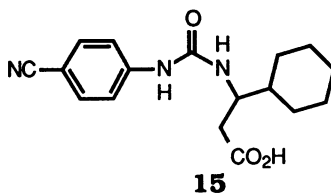
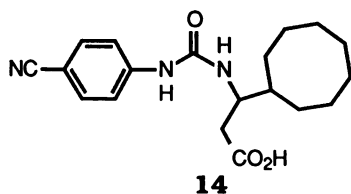
The analogues in which cyclohexyl and benzyl were substituted on the nitrogen were not sweet at concentrations up to 1 mg/mL in water. Both the cyclohexyl analogue **12** and benzyl analogue **13** were found to be bitter. These results confirmed that the β -NH moiety played a role in the recognition of an agonist for sweet taste perception.

β -Substitution. β -Substitution on the β -alanine portion of compound **11** incorporates a hydrophobic group while retaining the β -NH moiety. We first prepared analogues that were substituted with cycloalkyl groups via the route illustrated in Scheme II.



Scheme II

Among the analogues prepared were cyclohexyl, cyclooctyl, and spirocyclohexyl analogues **14**, **15**, and **16** respectively. Cycloalkyl substituted β -alanines were prepared by treatment of an aldehyde or ketone with malonic acid and ammonium acetate in refluxing 95% ethanol (18, 19). Treatment of 4-cyanophenyl isocyanate with the β -substituted β -alanine yielded the desired analogues (This reaction was run with either the neutral or sodium salt of the β -amino acid.). Analogues **14-16** were found to be very bitter, yet, interestingly all of these compounds exhibited a low level of sweetness in their taste profiles.



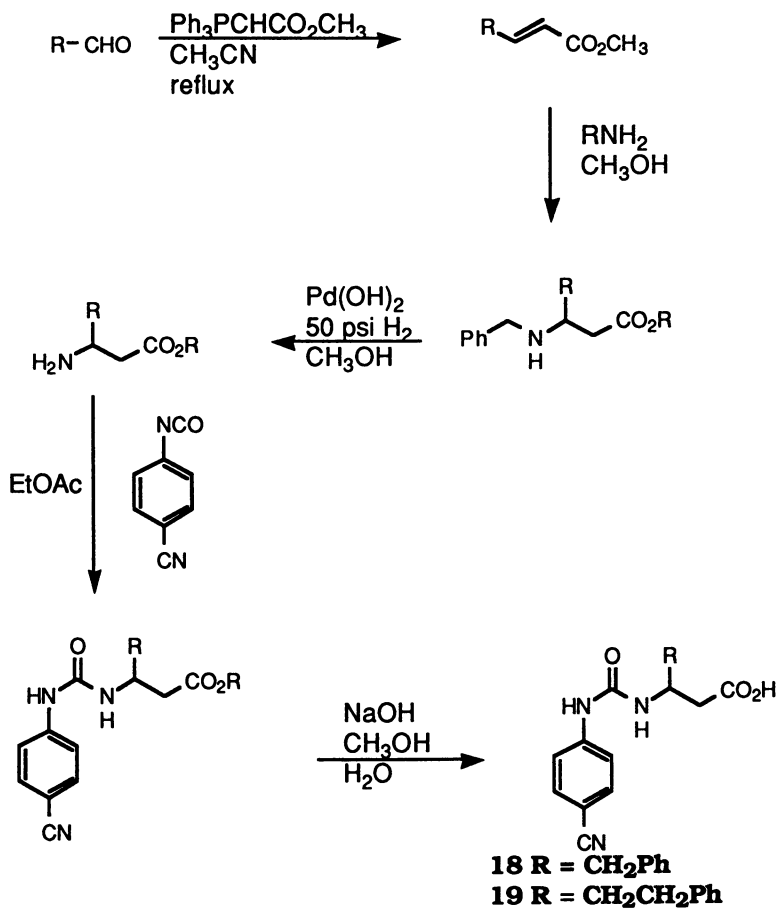
By an analogous procedure, the β -phenyl analogue **17** was prepared (3-amino-3-phenylpropionic acid is commercially available from Aldrich Chemical Company). Compound **17** was sweet with a $P_w(5) = 5,000$. The observed potency was over an order of magnitude greater than the parent compound **11** (**14**). The flavor profile of compound **17** was found to be very similar to sucrose with a clean sweet taste. The large increase in potency of **17** over **11** spurred exploration of the structure activity relationships (SAR) of this series.

To expand the SAR, we prepared homologues in which the phenyl ring was extended by methylene units. Thus, the benzyl and phenethyl analogues **18** and **19** were prepared. The synthetic route to **18** and **19** is illustrated in Scheme III.

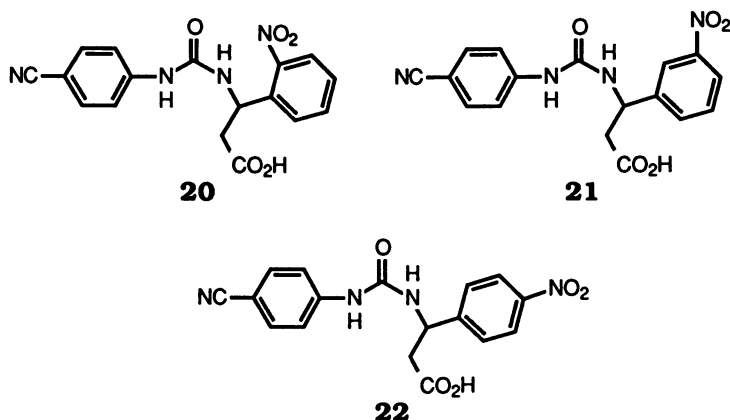
The desired β -substituted acrylates were prepared by treatment of the requisite aldehyde with a stabilized Wittig reagent (**20**). Amination was accomplished by Michael addition of benzyl amine (**21-23**) followed by catalytic hydrogenolysis (**24**). The resultant amino esters were then condensed with 4-cyanophenyl isocyanate followed by cleavage of the methyl ester with sodium hydroxide to yield the desired analogues.

Benzyl analogue **18** was sweet but less potent than the parent compound **17**, having a $P_w(3) = 3,000$. The phenethyl analogue **19** was empirically equivalent to compound **18** with a $P_w(2.5) = 2,500$. Extension of the aromatic group from the β position provided no advantage.

We also examined the effects on taste activity of various substitutions on the β aryl substituent of **17**. Electron withdrawing

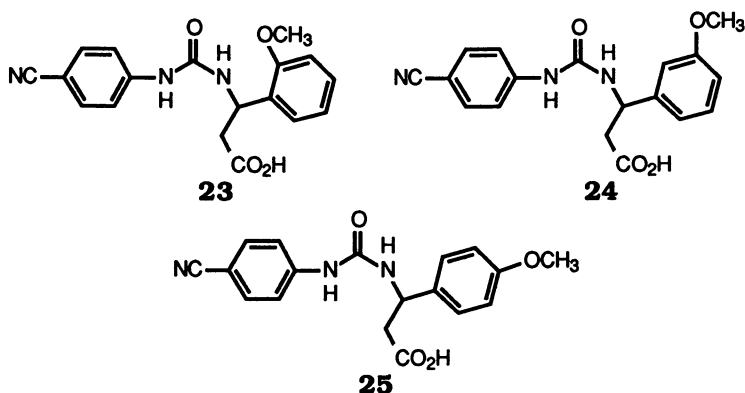
**Scheme III**

substituent effects were explored by preparing ortho-, meta-, and para-nitrophenyl analogues **20**, **21**, and **22**.



The meta analogue **21** had a sweetness potency [$P_w(5.5) = 5,500$] approximately equivalent to the parent compound **17** with para analogue **22** being slightly less potent [$P_w(4) = 4,000$]. However, the ortho analogue **20** retained only minimal activity with a $P_w(2.5) = 25$.

To examine the effects on taste activity of electron donating groups on the aryl ring, the ortho-, meta-, and para-methoxyphenyl substituted analogues **23**, **24**, and **25** were prepared.

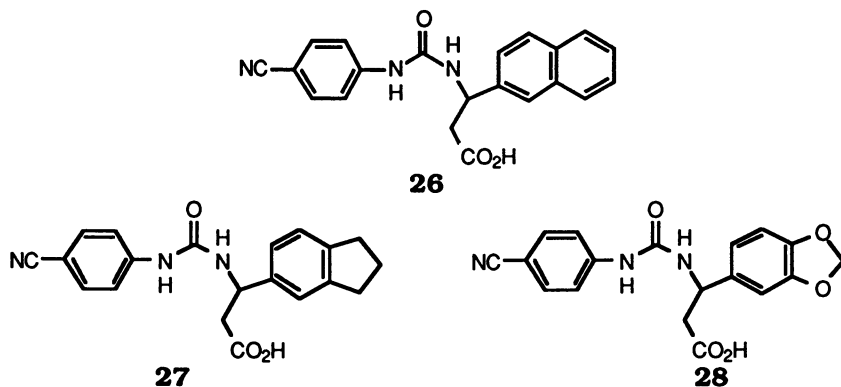


The meta analogue **24** had a $P_w(4) = 4,000$ and the para analogue **25** had a $P_w(5.5) = 5,500$. Again, as was observed for the nitro analogues **21** and **22**, there were only moderate effects on activity by meta and para substitution relative to **17**. Ortho-methoxy substitution resulted in complete elimination of sweet taste of the

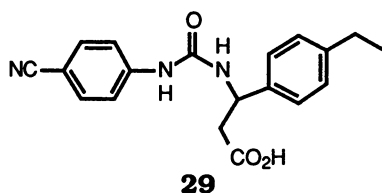
compound such that no sweetness was perceptible at 1.0 mg/mL in water.

Meta- and para-substitution with electron donating and electron withdrawing substituents on the aryl ring of **17** has minor effects on sweetness potency. However, ortho substitution obliterates the sweetness activity of these compounds.

The effects of substitution of fused ring systems for the β -phenyl moiety of **17** were probed by evaluation of analogues **26-28**.

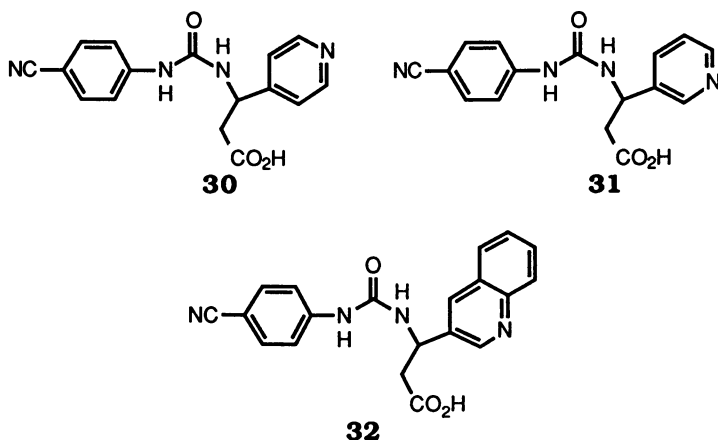


The 2-naphthalene analogue **26** exhibited a potency approximately equivalent to the parent compound **17**. The 5-indanyl analogue **27** was found to be less potent with a $P_w(2) = 4,000$. However, the piperonyl analogue **28** was found to be more potent than the indanyl analogue with a $P_w(2.5) = 25,000$. This finding is consistent with the enhancing effect of oxygen (and sulfur) which has been observed on sweetness potency in several other sweetener classes (25). To explore this effect further, the 4-ethylphenyl isosteric analogue **29** of the 4-methoxyphenyl analogue **25** was prepared. Consistent with the heteroatom potency enhancement theory, **29** was found to exhibit a $P_w(3) = 1,200$ while **25** exhibits a $P_w(5.5) = 5,500$.



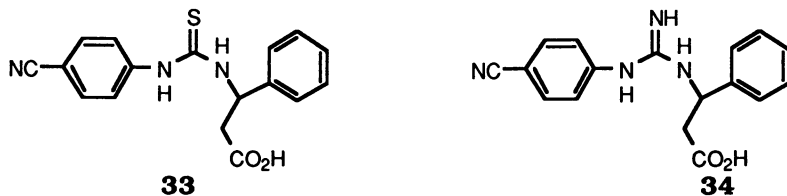
Also explored were the effects on taste activity of heteroaromatic substitution for the phenyl moiety of **17**. Specifically, the 4-pyridyl, 3-pyridyl, and 3-quinolyl analogues **30**, **31**, and **32** were prepared and evaluated. The 4-pyridyl analogue

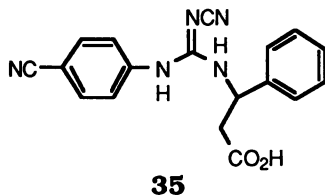
30 was found to exhibit a $P_w(2) = 2,000$. The 3-pyridyl analogue **31** and the 3-quinolyl **32** exhibited $P_w(2) = 20,000$. Heteroaromatic systems show potent activity in this series.



Isosteres

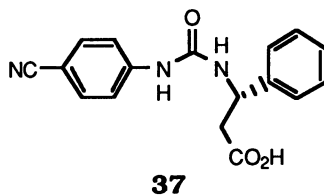
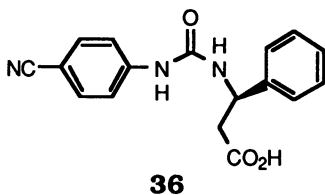
The concept of isosteric replacement of functional groups is widely used in the design of pharmaceutical products (26). Thiourea, guanidine, and cyanoguanidine may function as isosteric equivalents of the urea functionality. In the initial report on the suosan series, the isosteric replacement of the urea functionality by a thiourea moiety was reported to yield an increase in potency. Nofre and Tinti in 1985 reported that cyanoguanidine and guanidine isosteric analogues of aryl urea sweeteners were active (27). With these precedents, we prepared the thiourea, guanidine, and cyanoguanidine analogues of compound **17**. The thiourea **33** was sweet but empirically equivalent to **17**, with a $P_w(4) = 4,000$. The guanidine analogue **34** [$P_w(1) = 100$] and the cyanoguanidine analogue **35** [$P_w(3.5) = 350$] were weakly sweet. Disappointingly, these isosteric replacements did not lead to increases in activity in the parent compound **17**.





Chirality

All of the ureas previously discussed were prepared as racemates. In the aspartyl amide sweeteners, such as aspartame, only the enantiomer derived from L-aspartic acid is sweet (9). In the aspartyl anilides, initially reported by Sweeney and Lapidus, only the enantiomer derived from L-aspartic acid was found to have any sweetness activity (11). The method of Fischer was employed for the resolution of 3-amino-3-phenylpropionic acid (28, 29). Both R and S enantiomers **36** and **37** of urea **17** were prepared. Optical purity was determined by HPLC analysis on a chiral stationary phase column. Analysis of the R enantiomer by HPLC showed a small amount (<1%) of the S enantiomer. The R enantiomer **36** was detectably sweet only at high concentrations, whereas the S enantiomer **37** was found to be potently sweet [$P_w(7) = 7,000$]. The NutraSweet Company agonist model correctly predicted that the S-enantiomer would be the active compound. The low sweetness activity found for the R enantiomer is likely due to the trace amount of the S enantiomer present. It is of interest to note that the S configuration in this series correlates with asymmetric carbon configuration in the L-aspartic acid portion of the aspartyl amide and aspartyl anilide sweeteners.



Summary

Aspartyl amide (8, 9), aspartyl anilide (11, 15), aryl ureido β -amino acid (12), and guanidine (13) sweeteners were analyzed for chemical recognition components and compared in a computational agonist model system. This analysis led us to conclude that specific combinations of functionality could result in new sweeteners of enhanced potency. Using modern principles of medicinal chemistry, a new series of high potency sweeteners was discovered as a result of our analyses and subsequent studies of structure activity relationships. It remains to validate, at the

molecular level, the concepts that served as the basis for the construction of these promising novel sweeteners.

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Chapter 10

Molecular Basis of Taste

A Stereoisomeric Approach

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From the analysis of a number of aspartyl dipeptides by NMR and computer simulation we have arrived at a model which describes the conformational preferences of the sweet receptor. This review article attempts to document the synthetic and analytical approach utilized in arriving at this predictive structure for the basis of sweet taste.

The relationship between the structure and taste of sweet molecules has been studied by many groups. Shallenberger and Acree (1) proposed a model based on results obtained from a systematic study of unrelated sweet molecules such as saccharin, sugars, chloroform and unsaturated alcohols. Their model contained two structural features of the glucophore, namely, an electronegative atom B and a polarized system A-H. In the series of sweet molecules studied the distance between these two groups ranged between 2.5-4.0 Å. Following a study of substituted nitroanilines, Kier (2) proposed a third structural feature of the ideal glucophore, namely a hydrophobic moiety, X. He proposed that this hydrophobic group is involved in a dispersion bond at the taste receptor. By examining the taste profiles of eighteen amino acids in the L and D configuration he determined that the aromatic systems of phenylalanine, tyrosine and tryptophan in the D configuration were ideal candidates for the hydrophobic X group and that sidechains smaller than the isobutyl group of leucine did not fulfill the spatial requirement for the X moiety of the glucophore. The other structural features of the glucophore, namely the A-H-B system were adequately represented by the amino group and carboxylic acid. The Kier model was further refined by Ariyoshi (3) who added a configurational requirement for the X function.

The discovery of aspartame (L-aspartyl-L-phenylalanine methyl ester) at G.D. Searle (4) during the synthesis of the C-terminal region of gastrin, heralded the onset of the current interest in molecular structure as related to taste properties. The selection of aspartame as a potential marketable compound by Searle came after evaluating a variety of dipeptide analogs. Substitution of the aspartyl moiety invariably led to bitter or tasteless analogs (5) and in addition it was found that the α -amino and β -carboxylic acid functionalities must remain unsubstituted for activity. The phenylalanine methyl ester could be replaced by a variety of other amino acid methyl esters, the only requirement being that the sidechain replacement must be spatially comparable to the phenyl ring. By applying the Shallen-

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berger, Acree, Kier model to the known structural features of Aspartame it became apparent that the three glucophoric features are represented in the dipeptide. The A-H and B elements of the Shallenberger, Acree glucophore are present in the zwitterionic aspartyl residue as the NH_3^+ and β -carboxyl groups. The distance between these groups in the zwitterionic ring system as determined by molecular modelling is in close accordance with the predicted values (2.5-4.0 Å) from the Shallenberger-Acree model (1). The hydrophobic group proposed by Kier is accommodated by the phenyl sidechain of phenylalanine. However, the application of these models to the study of dipeptide sweeteners is restricted due to the two dimensional nature of the models as compared to the three dimensional nature of the peptides.

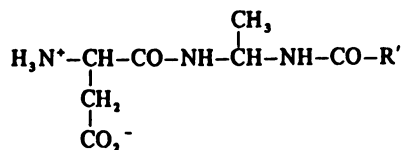
By analyzing the preferred conformation of aspartame using a combination of NMR and potential energy calculations Temussi *et al* (6) proposed an extended structure for the sweetener with the zwitterionic ring of the Asp residue and the sidechain of the Phe residue 180° apart in a flat parallel array. Van der Heijden *et al* (7-8) further refined the structural model for aspartame by observing that the length of side chains with respect to the AH/B moiety are extremely important for representatives of the aspartyl dipeptide ester series to taste sweet. Utilizing the conformations of aspartame reported by Lelj *et al* (9), van der Heijden (8) developed a model for the sweet taste that was different from that of Temussi *et al* but that allowed for differentiation of molecules by the size of the side chain and the end group.






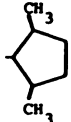
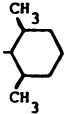
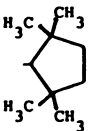
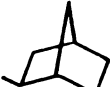


The Searle group reported the first departure from the dipeptide structures by preparing a series of substituted aspartyl amides (10), where the phenyl ring and methyl ester were represented by groups in the amide structure. However, the maximum potency achieved in this group of analogs was only 50 times that of a sucrose solution.

The potency of the Searle dipeptides was considerably enhanced when the phenylalanine was replaced by a substituted serine as the second residue. Ariyoshi (3) synthesized a series of substituted serine esters, where the hydrophobic X group was represented by the ester moiety and the carboxylic acid of the serine residue was masked by a methyl ester. The reported taste intensities were similar to aspartame. By replacing the ester by a hydrophobic ether Barnett *et al* (11) obtained dipeptides with reported potencies of eight hundred times (2,2,5,5-tetramethylcyclopentyl) and one thousand times (fenchyl) that of sucrose. Replacement of the serine derivative by an aminomalonic diester (12-13) resulted in molecules with impressive potencies as compared to sucrose. This structure-taste study culminated in the synthesis of a series of monosubstituted aspartyl-D-alanyl amides by the Pfizer group (14), with reported potencies between two hundred and two thousand times that of sucrose.

In this report we concentrate on the topochemical and stereoisomeric approach carried out primarily in our laboratories. Tsang *et al* (15) carried out a topological study of the taste receptor focused on substitution of the phenylalanine methyl ester by a series of homologous amino cycloalkane carboxylic acid methyl esters. The cycloalkane residues with increasing ring size were prepared *de novo* by a Bucherer-Lieb synthesis involving alkaline hydrolysis of the corresponding spirohydantoin. The methyl ester derivatives of these cycloalkyl amino acids were coupled to a suitably protected aspartic acid derivative and deprotected to yield the respective dipeptide. The results of this study are summarized in Table I. The cyclopropane and cyclobutane analogs are sweet. However, when the ring size is increased to a five membered system a critical spatial volume is achieved, resulting in a molecule which is sweet with a bitter aftertaste; cyclic structures larger than this exhibit bitter or tasteless profiles. It is postulated that the small cycloalkane rings can accommodate the hydrophobic requirement of the taste receptor whereas the larger six and seven membered rings have a greater effective interaction with

Table II. Properties of 1,1-diaminoalkane-derived sweeteners



R'	sweetness ^a	R'	sweetness ^a
-C(CH ₃) ₃	75-100		50-75
	500-700		35-50
	75-100		150-250 ^c
	300-400 ^b		150-200 ^c
	800-1000		75-100
"	600-800 ^c		5-15
	5-20		

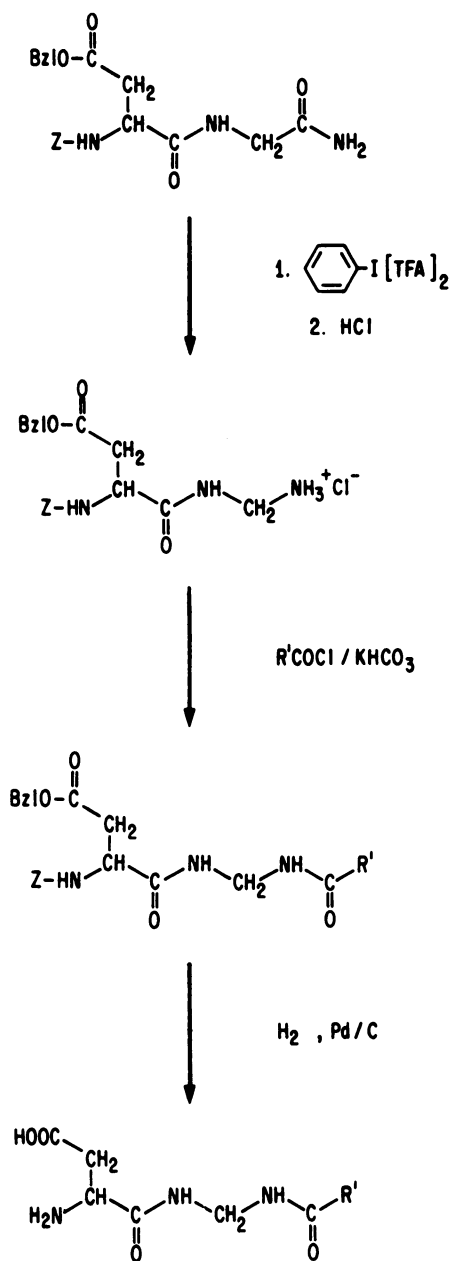


Figure 1. Synthesis of 1,1-diaminoalkane-derived sweeteners.

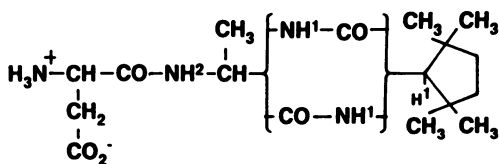
of these molecules are similar in quality to sucrose and depend on the nature of the R' group of the carboxylic acid used to acylate the gem-diamine.

The four stereoisomeric tetramethylcyclopentane compounds, comprising the L or D alanyl and the R or S diaminoethane derivatives, present a unique opportunity to study structure-taste relationships. Small changes in the overall topology affect the taste of these analogs (the L,L amide is bitter while the L,D amide and the retro-inverso analogs are intensely sweet). In addition, the bulky tetramethylcyclopentane group greatly decreases the conformational mobility of the peptide, allowing for a more complete analysis by NMR. With the assumption of a *trans* peptide bond and a nearly planar zwitterionic ring for the aspartyl moiety, the structure of the compounds can be determined from an extensive conformational analysis by NMR. The coupling constants, NOE values, and temperature coefficients used in defining the conformations of the four molecules are presented in Table III. The preferred minimum energy conformations are shown in Figure 2. Based on the results of this conformational study we have proposed a model (20-21) for sweet tasting analogs which contains elements of the models proposed by Kier (2), Temussi (6), van der Heijden (8) and Shallenberger and Acree (1). The conformation of a sweet molecule can be described as possessing an "L shape", with the A-H and B zwitterionic ring of the aspartyl moiety forming the stem, and the hydrophobic X group forming the base of the L (Figure 3). Planarity of the molecule in the x and y dimensions is critical for sweet taste, substantial deviation from this plane into the z dimension is correlated with tasteless (+z) or bitter (-z) molecules. The existence of the aspartyl zwitterionic ring cannot be proven conclusively but can be assumed *a priori* on the basis of evidence obtained from NMR experiments. The α -C β bond of the aspartyl residue possesses a staggered conformation with the carboxyl moiety and the amino group in the *gauche* position and the sp² plane of the terminal aspartyl carboxylate carbon atom and the α -C β bond coplanar. These conditions are conformationally favorable for the formation of the zwitterionic aspartyl ring.

The X-ray structure of aspartame has been solved by Kim *et al* (22). Crystallization was achieved in the tetragonal space group P4₁ with four aspartame molecules and one water molecule per unit cell. The molecule shows an extended conformation with *trans* peptide bonds. However, the phenyl ring is perpendicular to the peptide backbone and not coplanar with the zwitterionic ring of aspartic acid as would be predicted for a sweet dipeptide. This twisting of the phenyl ring is due to packing forces within the crystal structure which result in stacking of adjacent aspartame molecules into stable columnar structures. The isolated molecule from the crystal structure can be rotated 40° about the $\phi_{(\text{Phe})}$ bond, to achieve an isoenergetic conformation in which the rings are coplanar. This conformation correlates closely to our proposed model for the structure of sweet dipeptides in solution (Figure 3). Of course, in solution the aspartame molecule is solvated and devoid of packing forces. Thus the flexibility will easily allow for the "L-shape" conformation required by the model.

The X-ray structure of N-(L-aspartyl)-N'-[2,2,5,5-tetramethylcyclopentanyloxy]-(R/S)-1,1-diaminoethane has recently been determined (Benedetti, E. *J. Am. Chem. Soc.*, in press). The diastereoisomers were co-crystallized from a water/isopropanol mixture as colorless, solvent containing crystals. A suitable crystal fragment was sealed in a capillary together with some mother liquid. The monoclinic unit cell of the crystal was found to be of space group P2₁, containing a molecule of each diastereomer and two water molecules. Each isomer adopts an "L-shaped" conformation in the unit cell with the L,R isomer forming the regular "L" and the L,S isomer forming a "reversed L" as a result of crystal packing forces. This "reversed L" can be converted to the "L-shape" simply by a 180° rotation

Table III. NMR parameters of stereoisomeric retro-inverso and dipeptide amides



	Dipeptide Amides		Retro-inverso Amides	
	L,D-analog	L,L-analog	L,R-analog	L,S-analog
$J_{\text{NH}^1\text{-H}^1}$	10.3 Hz	10.2 Hz	—	—
$J_{\text{NH}^1\text{-AlaH}^\alpha}$	—	—	7.6 Hz	7.2 Hz
$J_{\text{NH}^2\text{-AlaH}^\alpha}$	< 1 Hz	< 1 Hz	< 1 Hz	< 1 Hz
NOE $\text{NH}^1\text{-H}^1$	—	—	9.0%	7.5%
NOE $\text{NH}^2\text{-AspH}^\alpha$	8.4%	absent	8.2%	1.2%
$\Delta \delta/\Delta T \text{ NH}^1$	5.5 ppb	4.9 ppb	5.9 ppb	7.5 ppb
$\Delta \delta/\Delta T \text{ NH}^2$	8.9 ppb	7.3 ppb	7.0 ppb	10.6 ppb

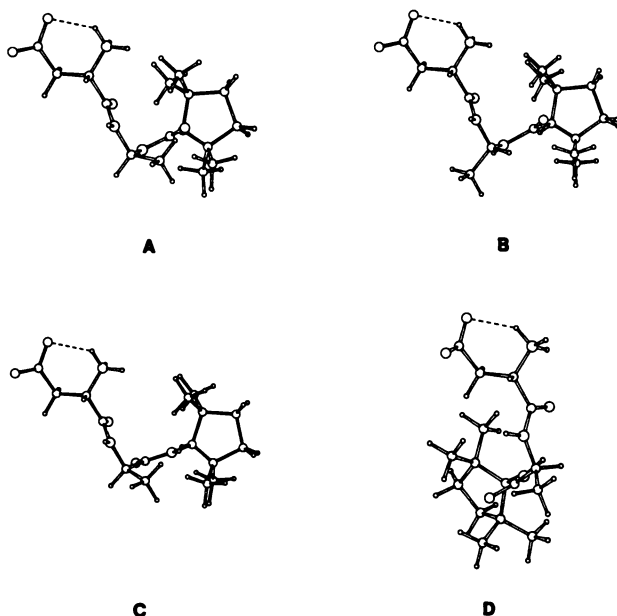


Figure 2. Preferred minimum energy conformations of (A) N-(L-aspartyl)-N'-[tetramethylcyclopentanoyl]-(R)-1,1-diaminoethane (L,R retro-inverso analog), (B) N-(aspartyl)-N'-[tetramethylcyclopentanoyl] (S)-1,1-diaminoethane (L,S retro-inverso analog), (C) L-aspartyl-D-alanyl-tetramethylcyclopentanylamide (L,D amide) and (D) L-aspartyl-L-alanyl-tetramethylcyclopentanylamide (L,L amide).

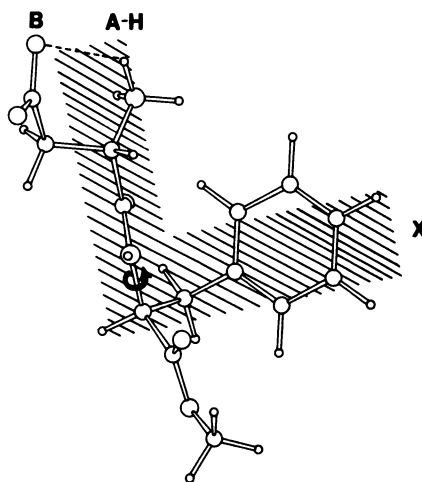
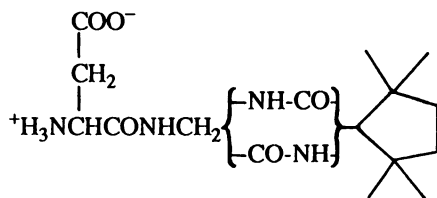


Figure 3. The model for the sweet taste with aspartame superimposed. The ϕ bond, shown by the arrow, has been rotated 40° from the X-ray diffraction structure. In addition, the hydrogen atoms have been added, with the standard bond lengths and angles. The AH-B and X groups of the molecule are illustrated according to the Schallenberger-Kier suggestions.

about ϕ_{gAla} . This rotation is energetically favorable and accounts for the preferred sweet structure of the L,R analog in solution (Figure 2).

We have also synthesized the analogous glycine and diaminomethane analogs of the aspartyl dipeptide amide where the X functionality is 2,2,5,5-tetramethylcyclopentane (Mierke, D.F., University of California, San Diego, unpublished data).



The synthesis of these molecules was accomplished in a similar manner to the alanine dipeptides. The Hofmann rearrangement was carried out by reacting [bis(trifluoroacetoxy)iodo]benzene with *Z*-Asp(OBzl)-Gly-CONH₂ to yield the diaminomethane residue. Both analogs have a pronounced sweet taste (potency 200 times 10% sucrose solution), although the glycyl analog has a moderately strong bitter taste concurrent with the sweet taste component. The comparison of the observed NOE's for the two analogs with the minimum energy conformations generated from computer simulations using DISCOVER (23-27) results in two different conformations for both of the analogs: one consistent with a sweet molecule and the other consistent with the bitter conformation determined from the NMR study of *L*-aspartyl-*L*-alanyl-tetramethylcyclopentyl amide. The energy difference between the two preferred conformations in the case of the retro-inverso analog is ~ 2.0 kcal/mol in favor of the "L shape" sweet conformer.

The conformations found for the glycyl amide analog are isoenergetic and although the overall topologies are different the observed NOE's do not allow exclusion of either conformer. The analog has a moderately strong bitter taste with a concurrent sweet taste component. The two isoenergetic structures are consistent with a sweet and a bitter conformation. Because of the increase in flexibility and enhanced conformational freedom of the glycyl residue, the analog can attain both the sweet and bitter conformations determined for the alanyl analogs. The observed taste properties combine the effects from both of these conformations.

We have also applied our proposed model to the study of a novel sweetener and its isomers developed by Pfizer (14). The sweet isomer, *L*-aspartyl-*D*-alanyl-(2,2,4,4)tetramethylthietanyl amide (alitime) is a modification of the tetramethylcyclopentane (TMCP) sweeteners where the TMCP moiety is replaced by the nearly planar tetramethylthietane (TAN) ring as the hydrophobic portion of the glucophore, providing a contrasting steric effect and degree of polarizability to the five membered ring system. The interest in this molecule stems from its high potency (2000x compared to sucrose) and enhanced stability towards decomposition. Computer analysis of the *L,D* (sweet) and *L,L* (bitter) isomers consisted of an extensive search for minimum energy conformations, including the global minimum conformation for both diastereomers. This was accomplished in a step-wise fashion, starting with smaller model compounds, such as Asp-NHCH₃ and Ac-TAN, and working toward the alitime structure. The minimum energy structures of the alitime isomers were selected on the basis of an energy difference of less than 5 kcal/mol from the lowest energy conformation. Conformations of the tasteless (*D,D*) species were obtained by inverting the conformations of the bitter (*L,L*) enantiomer.

The analysis of the sweet L,D isomer yielded seven unique minima whereas the L,L isomer yielded eight. By introducing the NOE constraints determined by NMR the range of computer-derived minima was reduced to four structures for the L,D molecule and seven for the L,L. All of the predicted structures for the sweet L,D isomer (Figure 4) are consistent with our proposed model for sweet taste. The zwitterionic ring of the aspartyl residue and the hydrophobic TAN group are in the same plane and perpendicular to each other, forming the stem and base of the L shape. The aspartic acid residue shows a *gauche*⁻ conformation about $\chi_1(\text{asp})$ which is consistent with the presence of the zwitterionic ring system. The conformational analysis of the L,L isomer indicated that the molecule shows significant distortion into the -z plane whereas the mirror image D,D tasteless isomer shows a +z distortion. Shallenberger *et al* (28) suggested that there is a 3 Å z barrier within which a potential sweet molecule must lie. Sufficient projection of the molecule over this barrier leads to bitter (-z) or tasteless (+z) responses. Our results obtained from the study of the alitame isomers confirm this observation.

Based on the rationale that the aspartyl dipeptides which exhibit sweet gustatory stimuli must, as a prerequisite, conform to an L-shaped molecule, we designed and synthesized a series of aspartyl dipeptides containing β -aminocyclopentanecarboxylic acid methyl ester as the second residue (Yamazaki, T., University of California, San Diego, unpublished data.). The structure, β -aminocyclopentanecarboxylic acid is unique as an amino acid isostere in that it contains two chiral centers associated with the peptide backbone. It also tends to form β -turn structures in an analogous fashion to proline when incorporated into a peptide chain. The *de novo* synthesis of this residue was undertaken employing two methodologies which specifically generated either *cis* or *trans* configurations and these were utilized in separate syntheses in order to obtain the all *cis* or all *trans* isomers. The *cis* isomers (1R,2S and 1S,2R) were obtained according to the method of Pleininger and Schneider (29-30) by Hofmann degradation of *cis*-2-carbamoylcyclopentanecarboxylic acid. The *trans* isomers (1R,2R and 1S,2S) were prepared by the Michael addition of ammonia to 1-cyclopentene-1-carboxylic acid (29-31). The methyl esters of the *cis* and *trans* isomers were prepared and coupled to Z-Asp(OBzl) using a HOBt ester coupling procedure. Deprotection by hydrogenolysis afforded the two racemic mixtures which were separated by reversed-phase hplc. The identity of each of the four configurational isomers was determined by ¹H-NMR and energy minimization calculations. The results obtained from these experiments and taste tests indicated that the conformations adopted by the various isomers in solution correlate closely with our predicted structural model (Table IV).

The synthesis of an analog of the retro-inverso alanine dipeptides has recently been reported. Zanno *et al* (32) claimed the synthesis of the analog N-L-aspartyl-N'-(2,2,5,5-tetramethylcyclopentanoyl)-2,2-diaminoacetic acid methyl ester. A preliminary minimized structure for the analog was obtained and suggested that the molecule should adopt a folded conformation in solution, consistent with the known structure of a sweet molecule. However, we were unable to prepare the analog using conventional retro-inverso methodology and instead decided to synthesize the molecule by means of a suitably protected diamino acetic acid.

The route to the formation of differentially protected diamino acetic acid has recently been reported by Bock *et al* (33). By modifying this published procedure we were able to prepare the peptide as a diastereomeric mixture about the central residue (Figure 5). The reactions are summarized as follows. Glyoxylic acid was condensed with tetramethylcyclopentyl carboxamide to yield the aminol derivative which was then allowed to react with isopropylmercaptan under acidic conditions. The sulphide adduct was then treated with benzylcarbamate in the presence of mercuric chloride to give the N-benzoyloxycarbonyl-N'-(tetramethylcyclopentanoyl)-

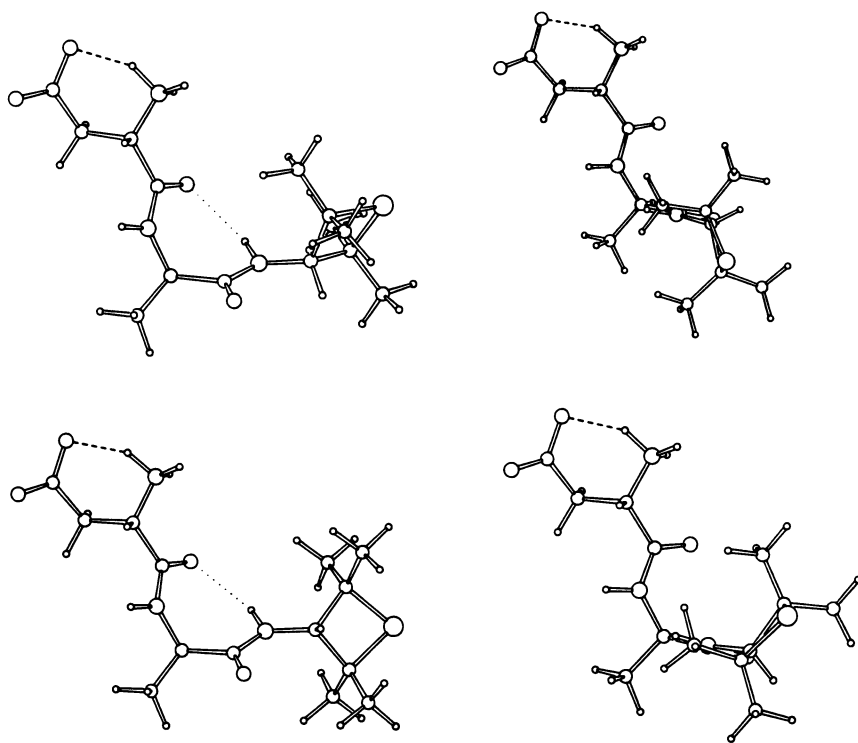


Figure 4. The four preferred conformations of L-Aspartyl-D-Alanyl-(2,2,4,4)tetramethylthietane. The structures differ only by an ca. 140° rotation about $\Psi_{(D-Ala)}$ and/or an ca. 60° rotation about the ϕ torsion of the thietane ring. All maintain a clear "L" shaped topology.

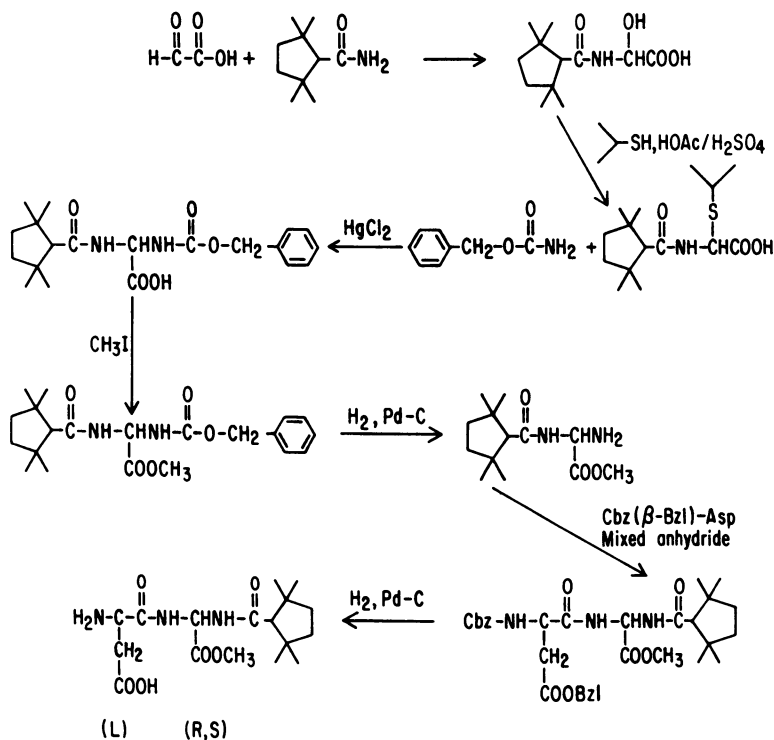
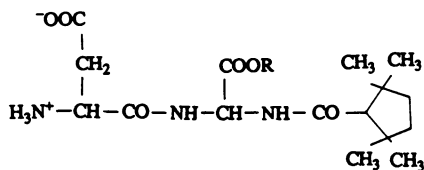


Figure 5. Synthesis of N-L-Aspartyl-N'-(tetramethylcyclopentanoyl)- 2,2-diaminoacetic acid methyl ester.

Table V. Structure-taste relationships of N-L-Aspartyl-N'-(tetramethylcyclopentanoyl)-2,2-diaminoacetic acid alkyl esters.



R	taste
CH_3-	sweet
$\triangle-\text{CH}_2-$	sweet
$(\text{CH}_3)_2-\text{CH}-$	sweet
$(\text{CH}_3)_2-\text{CH}-\text{CH}_2-$	bitter
$(\text{CH}_3)_3-\text{C}-\text{CH}_2-$	bitter

information concerning the exact orientation of the structural features required by the taste receptor.

Acknowledgments

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Chapter 11

Structure–Activity Relationship of Sweet Molecules

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The shape of the receptor active site of the receptor for sweet molecules, previously defined on the basis of a combination of rigid and flexible molds, has been refined using the shape of naphthimidazolesulfonic acids, a class of very large and rigid tastants. The new shape is still consistent with all previously examined sweet molecules. The model is illustrated by means of the fit of numerous rigid and flexible sweet and bitter tastants.

Sweetness is a stimulus imparted by a very large number of molecules of widely different chemical nature (1). The availability of many agonists and the practical relevance of sweeteners has stimulated numerous SAR studies and the development of general models of the receptor active site (2-12).

The oldest attempts to find common features among sweet molecules might be called one-dimensional structural approaches, meaning that all emphasis was placed on the chemical constitution without any reference to their actual three dimensional shape. They tried to attribute specific sweetening power to groups of atoms like CH₃, CHO, NO₂, COOH etc. (termed glucophores) that ought to modify basic taste properties of compounds much the way analogous substitutions modify optical spectra of the same compounds. All classifications (1,2) based on glucophores proved inadequate since, in general, it is not possible to attribute specific properties to single groups. Besides, there are several outstanding exceptions to this generalization; in particular, there are always numerous tasteless compounds containing one or more typical glucophores, e.g. N-methyl saccharin, sodium aniline sulfate, 3-nitro-4-methylaniline, etc.

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In Sweeteners; Walters, D., et al.;

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The first structural (and electronic) feature identified in most sweet molecules is the so-called AH-B entity (3). Shallenberger and Acree pointed out that nearly all sweet molecules (even those lacking any other constitutional similarity) have a hydrogen bond donor (AH) and a hydrogen bond acceptor (B) separated by ca. 0.3 nm. Thus the sweet taste of sugars, amino acids, saccharin, chloroform, olefinic alcohols and meta-nitroanilines was attributed to their ability to form two hydrogen bonds with a complementary B-AH entity of the receptor.

It must be noted that it is possible to find this AH-B entity in many other sweet compounds not quoted by Shallenberger and Acree (3), but also in many bitter and tasteless compounds. The main weakness of this theory however, is that the importance of both three-dimensional shape and volume is still overlooked. A trivial example is that molecules whose volume is larger than that of the receptor active site can never fit it even if they possess the correct electronic features (the AH-B entity and/or others).

Most models of the sweet receptor active site subsequently proposed (5, 9-12) include the AH-B feature and a third (dispersion forces) site at the apex of a triangle (5): they offer satisfactory explanations of many sweet compounds but fail to explain the relationship among sweet, bitter and tasteless compounds, even when they belong to the same chemical class.

A Receptor Active Site Model

We have tried to identify the main features of the receptor active site for sweet molecules by combining several empirical observations (taken from the literature) with rigorous geometrical criteria. The AH-B feature of Shallenberger was taken as a sufficient unifying criterion to assume that all sweet molecules (possessing it) interact with the same receptor. Another interesting observation of Shallenberger and Acree (4) was that simple amino acids can taste sweet only if their asymmetric α -carbon has a D configuration. Shallenberger and Acree attributed this behavior to the existence, in the receptor active site, of a steric barrier placed 0.3 nm below the plane of the AH-B feature, that prevents accommodation of L-amino acids with side chains bulkier than that of alanine. These hypotheses give the clue for a possible mapping of the receptor active site.

The key to a quantitative mapping was found through the peculiar relationship between sweet and bitter molecules. Simple amino acids and peptides apparently change their taste quantitatively from sweet to bitter when the chirality of the α -carbon adjacent to the AH-B entity (i.e. the amino and carboxyl groups respectively) is changed (4,5,13,14). This behavior, if taken at face value, would seem to point to the existence of two mirror-image receptor active sites, i.e., to two mirror-image receptor proteins. This is clearly a paradox if, as is necessary, the two

proteins are built of asymmetric residues of the same chirality, but can be overcome if the symmetry relationship between the two receptor proteins is of lower rank than the mirror-image relationship. In fact if one assumes that the two receptor active sites are essentially planar (or very flat) and very similar cavities, it is possible to account for the change in taste of amino acids with chirality simply by inverting the AH-B entities of the twin receptors for sweet and bitter tastes *via* a binary axis operation, as can be easily accomplished in nature by two single point mutations in the protein gene (6,7).

Figure 1 shows the relationship between the two enantiomers of a generic amino acid of formula $\text{HOOC-CH}(\text{NH}_2)\text{-CH}_2\text{-R}$ and the receptor active sites for sweet and bitter molecules, depicted here schematically as two identical boxes, whose right-hand side (shaded in the figure) represents the Shallenberger barrier. A C2 symmetry relationship relates the AH-B entities of the receptor active sites that are otherwise identical shallow cavities. Since the side of the receptor active site opposite to the Shallenberger barrier is completely open, the R moieties of the side chains of the two amino acids are almost completely outside the receptor active sites. The exact depth of the cavity representing the receptor active site can not be determined in a very detailed way, but it is possible to estimate it since it can accommodate the side chain of L-alanine, which is slightly sweet as well as the D-isomer (1,13,14). Accordingly all relevant agonist-receptor interactions are to be found only in the space comprised between the Shallenberger barrier and a parallel plane situated ca. 0.3 nm above it. On the other hand, it is not possible at the present time to estimate whether outside the open cavity the walls of the receptor are extending as a flat surface or, more likely, as the the surface of a wide funnel. This view of the receptor for sweet molecules is supported by the general observation that indeed a very large number of synthetic sweeteners are flat rigid molecules (1) and by the fact that there are both synthetic (15) and natural sweet macromolecules, i.e. the two sweet proteins monellin and thaumatin (16). In fact, with our model it is easy to envisage an interaction in which most of the sweet macromolecule is outside the receptor active site, whereas a protruding branch (a "sweet finger") is probing the receptor through the open side of the cavity.

A great asset of an active site model that can be treated as a pseudo-two-dimensional model is the possibility of using, quantitatively, the wealth of data from the literature on sweet rigid molecules to map the contours of the model. This task is also greatly facilitated by the hypothesis that all sweet molecules interacting with this receptor model have the AH-B entity (or at least half of it), since it will be possible to refer all molecules to the same orientation. We exploited the fact that many synthetic sweeteners show simple trends of taste intensity with substitution. For example, saccharins substituted in position 6 of the aromatic

ring remain sweet when the hydrogen is substituted with a methyl group, an amino group, a fluorine or a chlorine; retain a sweetish taste with bromine; but abruptly lose their taste when the hydrogen is substituted with an iodine or methoxy group. This behavior was interpreted as an indication of the existence of a wall in the receptor at approximately the distance of the van der Waals radius of iodine (6).

By analyzing several similar cases of substituted saccharins it was possible to identify the nearly complete shape of the receptor active site in the region of the plane surrounding the AH-B entity (6) and to delineate an upper hydrophobic region as a minimum shape of interaction by means of the structure of aspartame (17). Such a model could not be considered a complete geometrical model but rather a topological model, since only rigid molecules are fully reliable as molds and the flexibility of aspartame is very high (18). The final model was defined using a sweet molecule with the largest possible rigid area in the plane of AH-B, 3-anilino-2-styryl-3*H*-naphth(1,2-*d*)imidazolesulfonate (henceforth called SSN), whose shape was determined by conformational studies in our laboratory (18, 19).

A crucial point in comparing different sweet molecules, and hence in using an active site model, is the possibility of orienting their AH-B entities in the same way. This is not straightforward in the case of SSN since its AH-B entity is intrinsically different from those of most other sweet compounds. Strictly speaking, the anionic form of SSN has only the B part of the entity, i.e. the $-\text{SO}_3^-$ group. It can use two of the oxygens (like the oxygens of the SO_2 group of saccharin) as the B moiety of AH-B, but the shape of AH-B is slightly different from that of the same entity in saccharin. As a general rule, however, it is fair to say that an imperfect AH-B entity can be well tolerated by the receptor active site, provided the steric fit is otherwise very good. This last requirement is certainly met by SSN since its naphthimidazolesulfonic acid moiety has a shape exactly complementary to the lower part of the active site model derived from saccharins, and fills it completely. Actually, even the upper part of SSN (i.e. the styryl moiety) is very similar to that of the original model (6), only somewhat larger. It is important to emphasize that the styryl group is completely coplanar with the naphthimidazolesulfonic acid skeleton, thus assuring a very good hydrophobic interaction with the flat surface of the receptor active site corresponding to the Shallenberger barrier.

Figure 2a shows the quantitative two-dimensional contour of the new active site model, derived from a combination of the mapping with saccharins (6) and with SSN (18). The shape of the receptor active site, in the main plane of the flat cavity (xy), has been obtained optimizing the position of monoatomic apolar molecules, hereafter called 'S', interacting with the mold through a simple nonbond potential described by an $\exp-r^6$ function and a

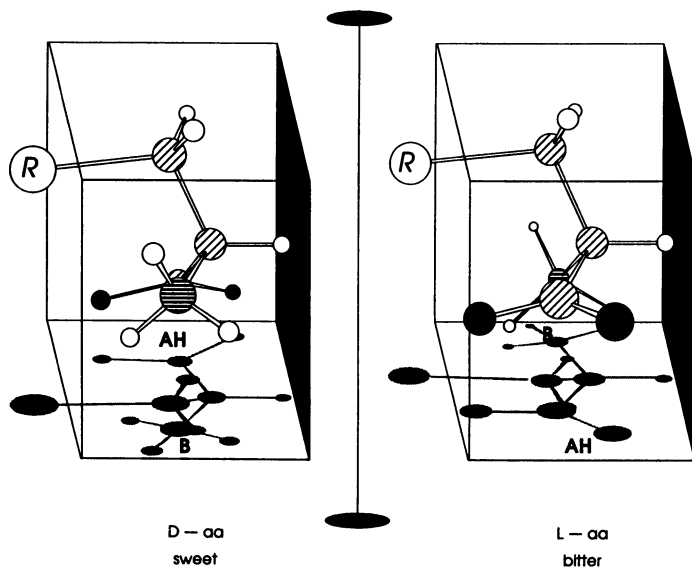
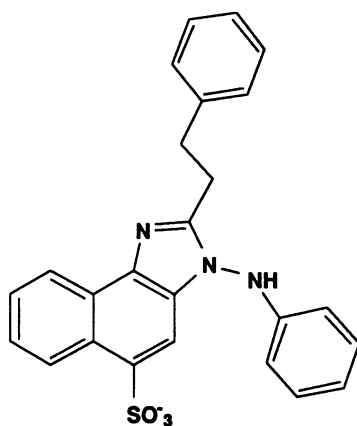


Figure 1. Schematic drawing of the active site models for sweet and bitter compounds, illustrated with reference to the enantiomers of a generic amino acid. The Shallenberger barrier is represented by the shaded wall of the box. The binary axis symmetry relationship is valid only for the AH and B groups of the receptors.



3-anilino-2-styryl-3H-naphth(1,2-d)imidazolesulfonate

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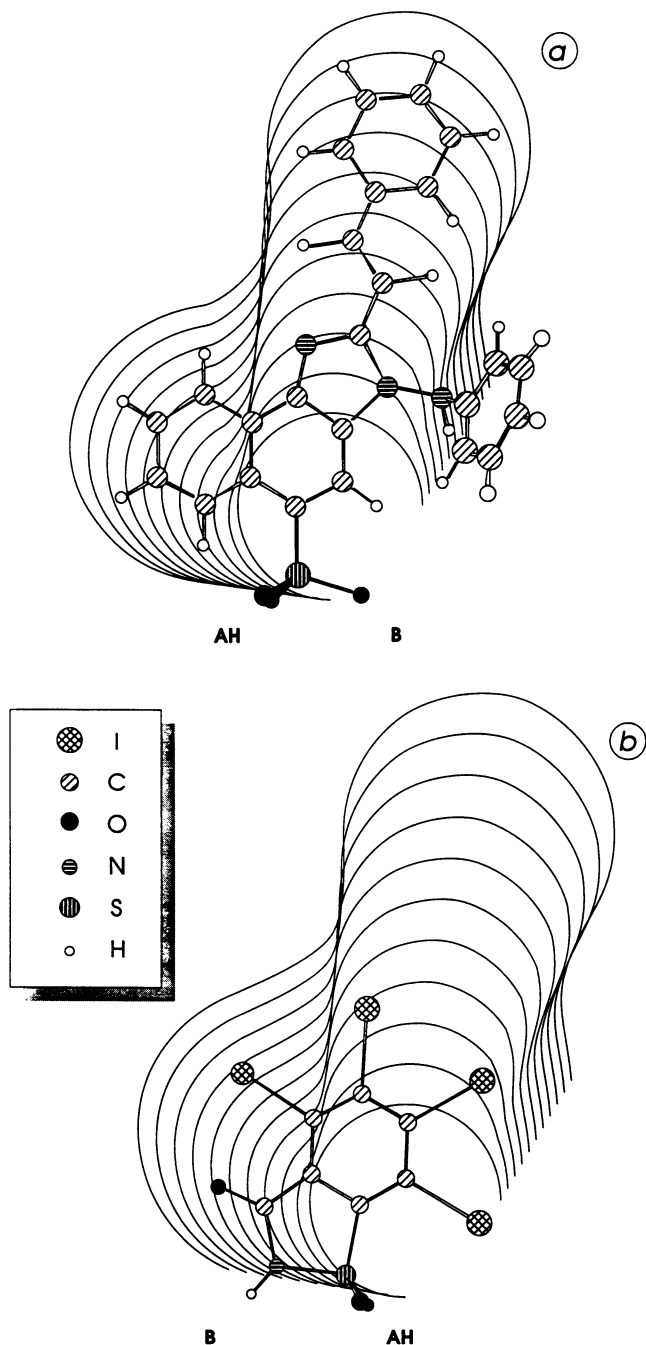
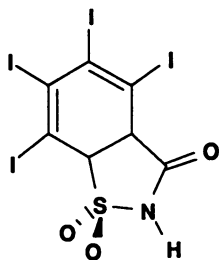


Figure 2. Models of the receptor active sites for sweet and bitter molecules, illustrated by the fit of 3-anilino-2-styryl-3H-naphth(1,2-d) imidazolesulfonate (a) and 4,5,6,7-tetraiodosaccharin (b), respectively.

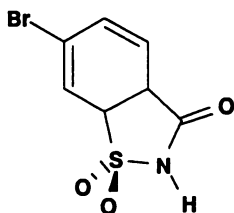
point charge coulomb interaction ($R^* = 2.111 \text{ \AA}$, $\epsilon = 0.202 \text{ kcal/mol}$, $q = 0. \text{ a.u.}$). For the apolar part of the sweet receptor active site, a total of 13 and 25 S units as a first and second shell respectively have been initially positioned around the mentioned mold molecules. For the polar part of the receptor active site, i.e. the lower part around the SO_3^- moiety, we have optimized (still in the xy plane) the position of a negative oxygen at ($R^* = 2.20 \text{ \AA}$, $\epsilon = 0.050 \text{ kcal/mol}$, $q = -1.0 \text{ a.u.}$) and of a positive nitrogen ($R^* = 1.82 \text{ \AA}$, $\epsilon = 0.055 \text{ kcal/mol}$, $q = +1.0 \text{ a.u.}$). In this second step the positions of the previously optimized positions of the 'S' units were kept fixed and the saccharin molecule position optimized with respect to the "receptor active site" derived by the SSN molecule. The upper hydrophobic part, corresponding to the area previously delimited by aspartame, although more rigorous than before (6), can only represent the minimum available area, since larger compounds were not available in this region. The main features of our receptor model can be summarized as follows: (i) the receptor active site of the receptor is a shallow, flat cavity with the outer side accessible even during interaction with the agonist (ii) the lower part of the cavity contains the main "electronic features", the most important of these being the AH-B entity; this part is always essential for binding (iii) the upper part of the cavity is hydrophobic and plays an important role in the modulation of sweetness intensity. A word of caution must be said about two points before attempting to use this model for drug design. The only electronic feature we used is the AH-B feature; it is very likely that other electronic features do contribute, in particular in a dynamic way, that is by inducing slight modifications in the region around the AH-B entity. Secondly, as we mentioned previously, the model gives no clues about the shape of the walls *above* the flat cavity: it is likely that they are funnel-shaped and, accordingly, it may be possible that compounds with substituents extending outside the flat cavity increase the binding constant; this can in fact be one of the reasons for the extraordinary activity of some supersweet compounds derived from aspartame (*vide infra*).

Considering that a fundamental point in the formulation of this model resides in the symmetry relationship between sweet and bitter receptors, it is easy to complete the picture with a twin model for bitter molecules (Figure 2b) that has the AH-B entity inverted by a C2 symmetry operation and a two-dimensional contour almost identical to that for sweet molecules, with the only difference that it is slightly larger, as indicated by the fact that 4,5,6,7-tetraiodosaccharin retains a slightly bitter taste (7).

Figure 3 shows the comparison of the fit of 5,6-benzo-saccharin (a bitter saccharin) in the two active site models. It is clear that, although possessing a perfect AH-B entity, it can only fit the bitter active site model (Figure 3b). Figure 4 illustrates another puzzling experimental observation, hitherto unexplained by any other receptor model, i.e. the fact that some sweet molecules have



4,5,6,7-tetraiodosaccharin



6-Br-saccharin

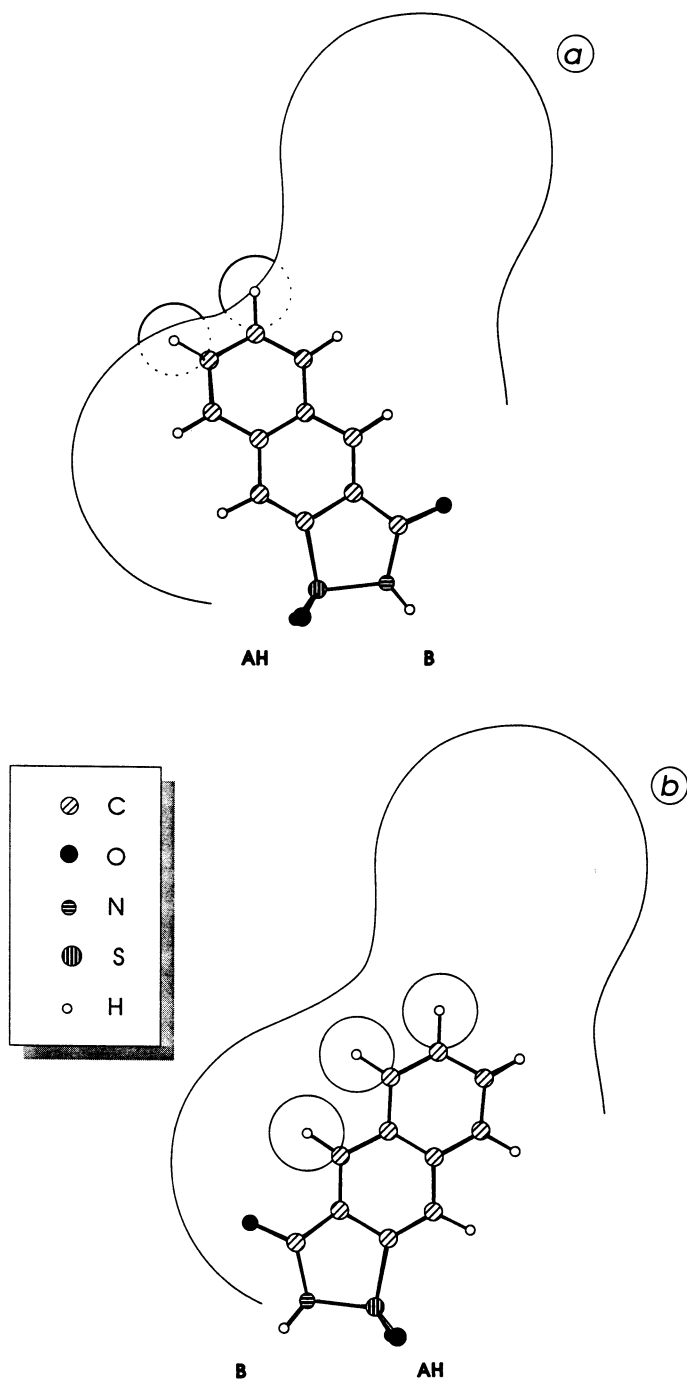


Figure 3. Comparison of the fit of 5,6-benzo-saccharin in the sweet (a) and bitter (b) receptor models.

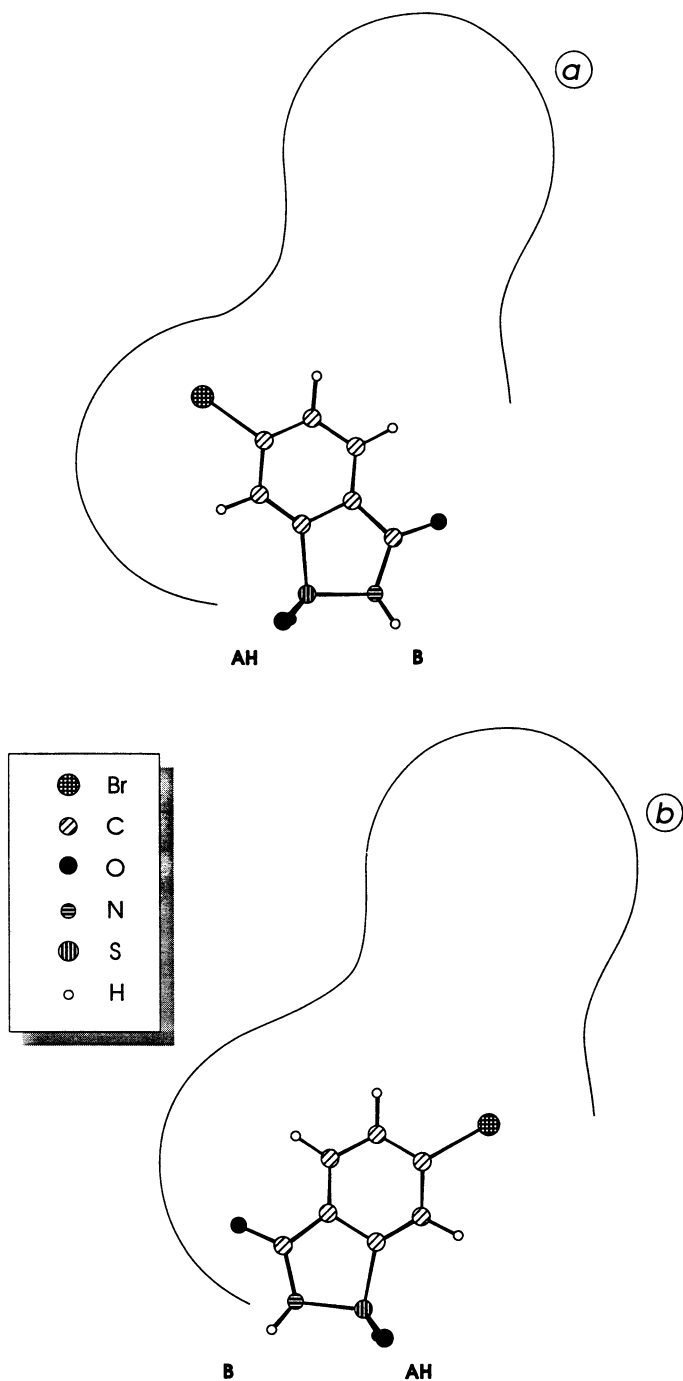


Figure 4. Comparison of the fit of 6-bromo-saccharin in the sweet (a) and bitter (b) receptor models.

a strong bitter aftertaste. The fact that 6-bromo-saccharin can fit both receptor active sites represents a simple and satisfactory explanation of its bitter aftertaste.

Most known sweet molecules, unrelated to the ones used for the mapping, do fit our model. Owing to space limitations we can only review some of them, divided into two sets: conformationally rigid and conformationally flexible tastants.

Conformationally rigid agonists

One of the most interesting cases of dependence of sweetness on geometrical isomerism is that illustrated by Verkade (20) for the isomers of ethoxy-nitroanilines. Out of the ten possible isomers only one (2-ethoxy-5-nitroaniline) is sweet, while all others are tasteless. A good model for the sweet receptor active site ought to be able to discriminate among the ten isomers.

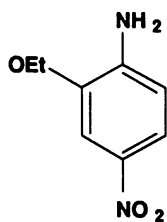
It is certainly reassuring that only this isomer can fit our active site model while all others invade the wall outlined by the two-dimensional contour or interfere with the AH-B entity. Figure 5 shows the fit of the sweet isomer (5d) in our active site model, together with three representative examples from the other isomers that do not fit. Equally significant is the fact that 2-halogeno-5-nitroanilines are all sweet, with an increasing sweetness from F to I that parallels the fit of the halogen atom in the upper part of the model, and other 2-alkoxy-5-nitroanilines are sweet, as long as they can fit the upper part of the active site, whereas they lose all sweetness when the upper wall is invaded (20).

Other interesting cases of rigid sweet molecules (1) are those of salicylic and anthranilic acids. These cases are readily explained by the simple application of the AH-B theory of Shallenberger and Acree (3), but it is impossible, on this basis, to explain why guaiacol carboxylic acid, whose chemical constitution is very similar, is bitter. Our model receptor active sites for the sweet and bitter receptors show that these are in fact trivial cases, since the first two compounds fit the sweet receptor whereas the third one can only fit the bitter receptor.

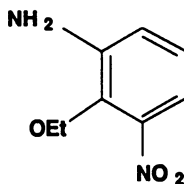
Other clear-cut cases of rigid sweet molecules were already illustrated when the receptor model was only a partial geometrical model (6).

Conformationally flexible agonists

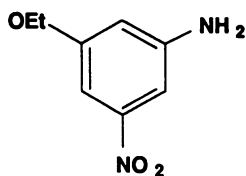
We have previously shown that our model active site model can be used to discriminate even among geometrical isomers of partially flexible tastants such as the three tolylureas (21). It is also easy to



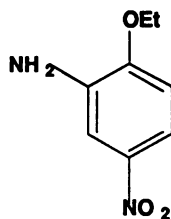
2-ethoxy-4-nitroaniline



2-ethoxy-3-nitroaniline



3-ethoxy-5-nitroaniline



2-ethoxy-5-nitroaniline

show that the taste of other ureas, either partially flexible (such as *p*-tolyl-*N*-methylurea or very flexible (such as suosan) can be interpreted on the basis of our model.

The most interesting conformationally flexible sweet compounds however, are probably aspartame and its analogs. We have recently shown that all low-energy conformers of aspartame are characterized by similar extended backbone conformations that are compatible with only one (*gauche*) Asp side chain conformation (D_{II} in the notation of ref. 18) but with all three (quasi-isoenergetic) side chain conformations of the phenylalanyl moiety found in vacuo (18), in solution (18) and in the solid state (22), i.e. F_I , F_{II} and F_{III} , respectively. Figure 6 shows that only the first conformation ($F_I D_{II}$, Figure 6a) is consistent with our active site model, as originally proposed by the semi-quantitative receptor model (6, 17, 18).

Moreover, the same conformation is the minimum energy conformation for the aspartame moiety of the *N*-(4-nitrophenylcarbamoyl)-*L*-aspartyl-*L*-phenylalanine methyl ester, one of the sweetest known compounds (23). This conformation was obtained from an energy minimization with respect to all internal torsional parameters for a total of 5832 conformations, using the AMBER force field (24). As starting conformations the set of most stable conformations obtained in the case of aspartame (17) and those obtained in the case of *N*-*para*-tolyl urea (21) were combined with the 12 most likely conformations deriving from the torsion around the C-N bond between the α carbon of Asp and the nitrogen of the urea moiety. As shown in Figure 7a, the aspartame moiety of this molecule fits perfectly into the flat cavity of the active site model, whereas the *p*-nitrophenylurea moiety contributes to the stabilization of the $F_I D_{II}$ aspartame conformation with the formation of a C_7 ring, but remains completely outside the active site model, protruding through the open side of the receptor. Even more remarkable is the fact that the *p*-nitrophenylurea moiety can fit into the flat cavity as well. However, in the latter case, the aspartame moiety extends outside the active site model (Figure 7b). This kind of dual mode of interaction with of the active site (i.e. the entropic factor it implies) together with possible additional interactions with the walls of a funnel-shaped receptor active site (above the flat bottom cavity), may well be the reason for the exceptional sweetness potency of this molecule.

It seems fair to conclude that the model receptor active sites we proposed for sweet and bitter agonists are, probably, the most general ones available at present and, in particular, the only models that can discriminate among sweet, bitter and tasteless isomers of several classes of tastants of widely different chemical constitution.

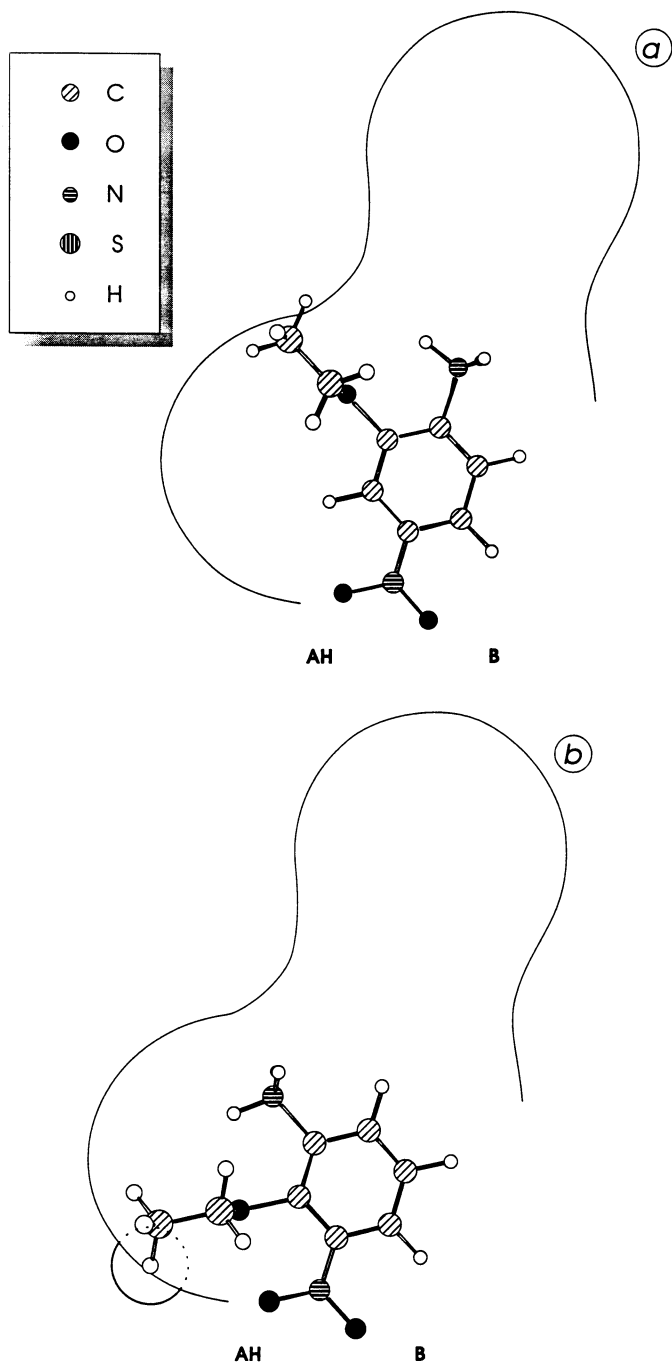


Figure 5. Comparison of the fit of four of the ten isomers of nitro-ethoxy-aniline in the sweet receptor. The only sweet isomer is 2-ethoxy-5-nitroaniline (d).

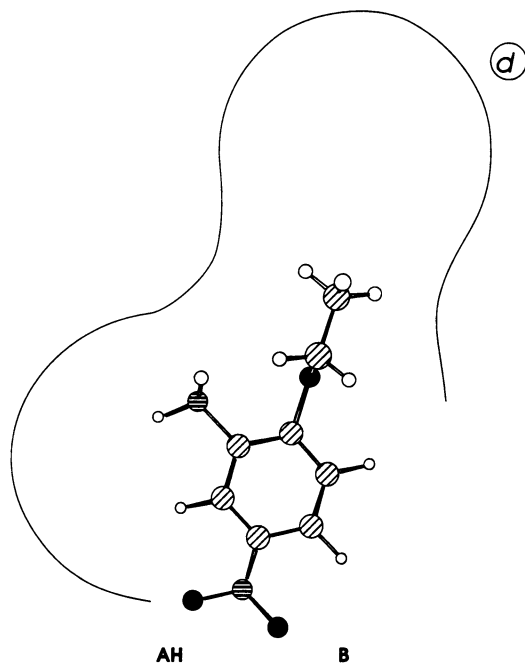
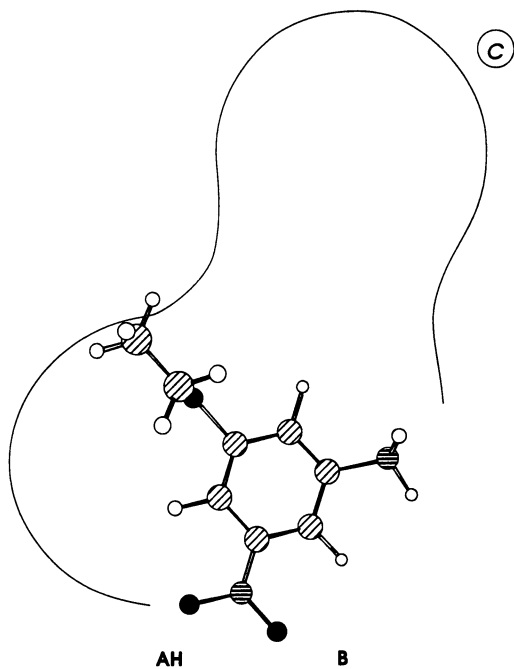


Figure 5. Continued.

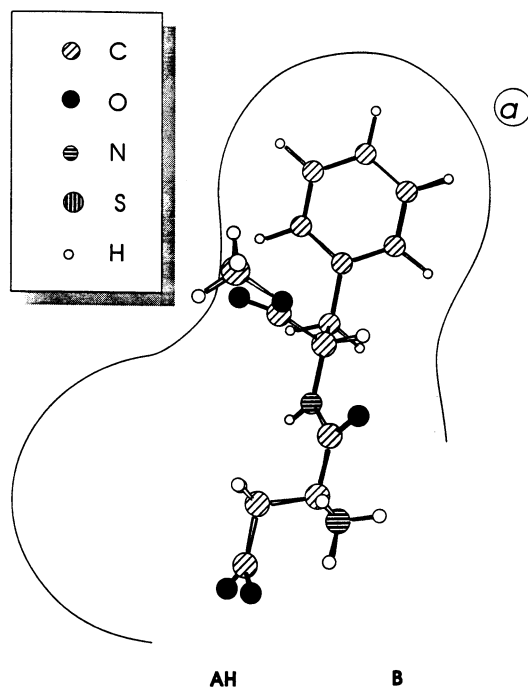


Figure 6. Comparison of the fit of the three low-energy conformers of aspartame, $F_I D_{II}$ (a), $F_{II} D_{II}$ (b) and $F_{III} D_{II}$ (c), in the sweet receptor.

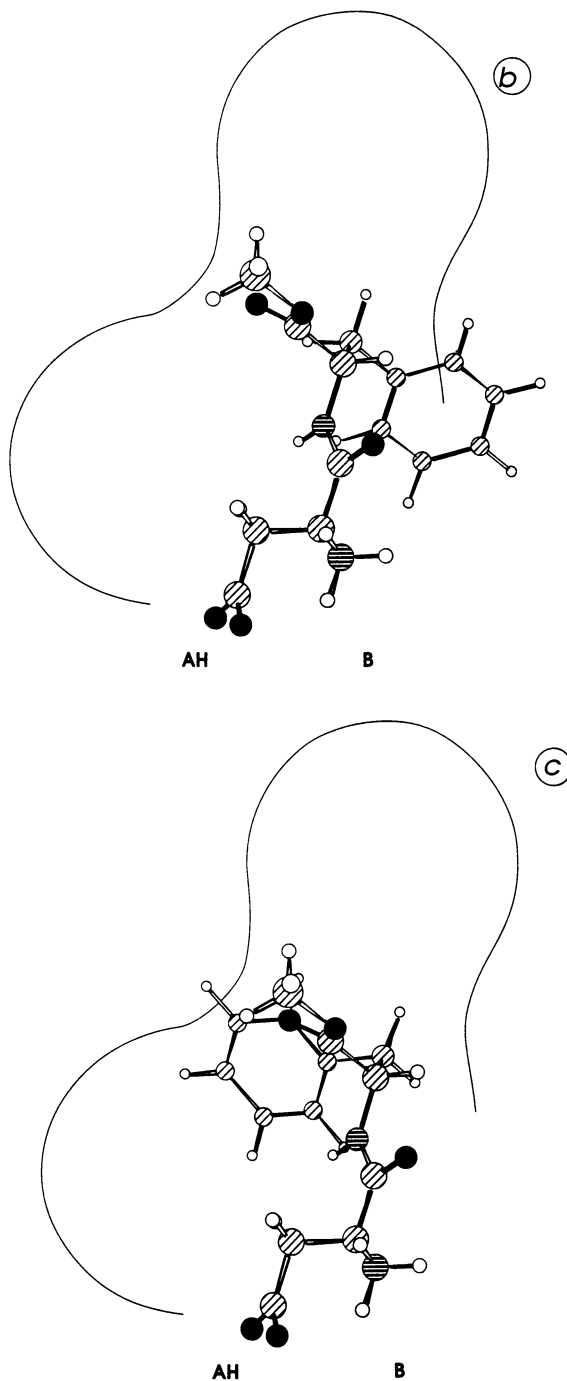


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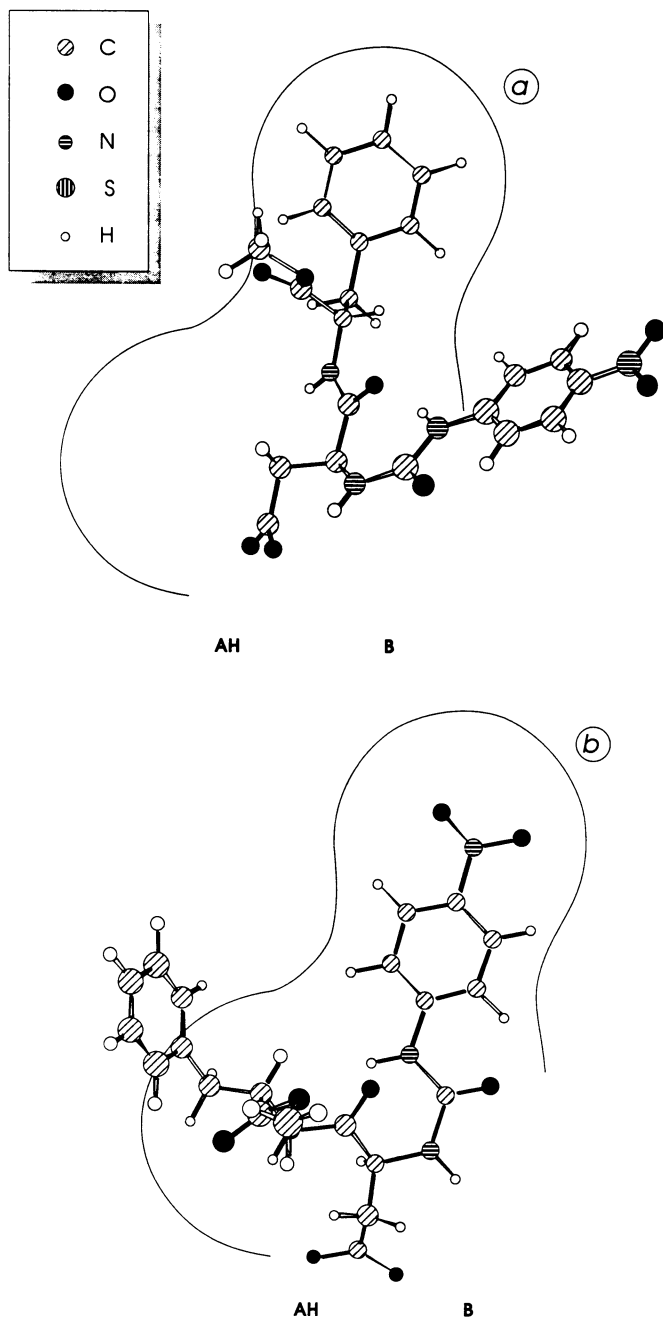


Figure 7. Comparison of the two possible interactions of the low-energy conformer of the N-(4-nitro-phenylcarbamoyl)-L-aspartyl-L-phenylalanine methyl ester with the sweet receptor.

Acknowledgments

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Chapter 12

Molecular Mechanics of Cyclopropane Peptide Analogues of Aspartame

Implications for Three-Dimensional Requirements of the Sweet Taste Receptor

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Molecular mechanics calculations on conformationally restricted cyclopropane peptide analogs of aspartame were used to develop a model for their binding to the sweet taste receptor, in which the ester alkyl group of the active analogs (aspartyl-1-aminocyclopropane carboxylic acid esters, Asp-Acc-OR) binds to the same site as the phenyl group of aspartame. The two possibilities for the active conformation correspond to the Temussi and Goodman models for the sweet taste receptor. The conformational restrictions imposed by incorporating isomers of 2,3-methanophenylalanine or 2,3-methanoproline into aspartame analogs are shown to be incompatible with either of these models, explaining their lack of sweet taste.

In recent years, significant progress has been made in our understanding of the stereochemical basis of sweet taste. In particular, the hypothesis of Shallenberger and Acree (1) regarding the importance of adjacent hydrogen bond donor and acceptor groups (the "AH + B" moiety) has gained wide acceptance and has been applied by various authors (e.g. 2-8). However, there is a lack of consensus (6-8) regarding the active conformation of the peptide sweetener aspartame (L-Asp-L-Phe-OMe) and, by implication, the three-dimensional shape of the sweet taste receptor recognition site.

The root of this problem is the conformational flexibility of peptide tastants like aspartame and many of its analogs, which precludes a unique and obvious hypothesis for the active conformation. Thus, in order to develop a three-dimensional receptor model, the conformational properties of a number of different analogs must be studied. Most useful in this regard are conformationally restricted or rigid analogs, which by their activity or inactivity can support or invalidate a hypothetical receptor model. This approach has been used by Temussi and collaborators (5-6), who have recently chosen relatively rigid non-peptide tastants to support their model of the active conformation of aspartame, and by Goodman and collaborators

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(7), who have used sterically restricted peptide and retropeptide analogs to develop a somewhat different receptor model.

One approach to conformational control in peptide analogs is the use of highly constrained amino acids, in which substitutions restrict the ϕ - ψ (ϕ - ψ) space to small, well defined regions of the Ramachandran plot. In particular, α , α -disubstituted amino acids (9), such as the well studied α -aminoisobutyric acid (Aib), are restricted to about 1% of the Ramachandran map, compared to over 15% for most amino acids, and 50% for glycine (10). Additional constraint is introduced when the α , α -disubstituents are bridged to form a cycloalkane system, the simplest examples being the 2,3-methano amino acids (2,3-MeAA), perhaps better known as the "cyclopropane amino acids", of which 1-aminocyclopropanecarboxylic acid (Acc) is the parent compound (11).

In this paper, we report on the conformational analysis of various aspartyl cyclopropane amino acid esters, the taste properties of which have been reported previously (12-14). Of these, only the parent compounds (Asp-Acc-OR) are sweet (12), whereas all four isomers of Asp-2,3-MePhe-OMe are tasteless (13), and Asp-2,3-MePro-OR analogs are bitter (14).

Based on molecular mechanics calculations, validated by comparisons with X-ray crystal structures where possible, we will propose probable modes of binding for the active compounds to the sweet taste receptor. These results will be interpreted in the light of published receptor models, in particular, the Temussi and Goodman models (5-7). Several other published models of the active conformations of aspartame were not considered as they have recently been shown to have poor predictive value (8).

Methods for the molecular mechanics studies

Computer-modelled structures of the Acc derivatives and aspartame were built using the standard molecular modeling options of the SYBYL program (Tripos Assoc., St. Louis), and the conformational energetics were evaluated using its SEARCH option.

Low energy conformers were determined by molecular mechanics calculations, using the Tripos force field (15), supplemented with some additional parameters for the cyclopropane system derived from experimental data. Following Allinger's approach in the MM2 force field (17), a special atom type was defined for the cyclopropane carbons. All parameters and force constants involving the cyclopropane carbon atom type were taken to be the same as those involving sp^3 carbon (the SYBYL C.3 atom type), except for one torsional parameter (described below) and the following altered equilibrium bond lengths and angles: cyclopropane C-C bond lengths of 1.50 Å and internal C-C-C angles of 60°. In optimized structures, these parameters resulted in values of about 118.5° for the exocyclic H-C-H angles and the $N-C_\alpha-C_1$ angle of Acc (sometimes called the τ angle). All of these are reasonably close to experimental values as reviewed by Barone *et al.* (16). In addition to allowing a more exact replication of experimental bond lengths and angles of the cyclopropane ring, use of the special cyclopropane carbon atom type permits the incorporation of a unique V_2 torsional term for the $O=C-C_\alpha-N$ torsional angle (ψ) of Acc (periodicity -2, barrier 4.4 kcal/mole). This addition to the force field, first used by Barone *et al.* in a

modified MM2 calculation (16), reproduces the stabilization of conformations in which a vector through the carbonyl C=O bond bisects the cyclopropane ring (i.e. $\psi = 0^\circ$ or 180°) which is observed experimentally in microwave spectra (18) and crystal structures (12,19) and predicted by *ab initio* quantum mechanics calculations (16). The recent comparative study by Barone et al. (16) demonstrates that standard force fields lacking such a torsional term fail to reproduce this conformational effect, which is electronic in origin and is due to the π character of the cyclopropane ring system. The parameter we have used for this V_2 torsional barrier (4.4 kcal/mole) is from a microwave study of cyclopropane carboxaldehyde (18).

For the coulombic potential energy term, the distant-dependent dielectric model was used (the default option in SYBYL), and charges for the neutral protected amino acid analogs were calculated by the Gasteiger-Marsili method (20), which, unlike semi-empirical quantum mechanics methods such as MNDO, is independent of conformation, and thus does not bias the conformational search in favor of the conformation upon which the initial charge calculation was performed. However, the preferred conformations and relative energies of various conformations of these compounds were found to be fairly independent of electrostatics, i.e. approximately the same results were obtained if the coulombic term was omitted from the calculations.

Grid search (which involves a rigid rotor approach) was used to calculate the total steric energy of the Acc derivatives over the entire ϕ - ψ space, using 5 or 10 degree angle increments. The resulting data files were contoured with respect to energy and plotted as ϕ - ψ plots. However, it should be pointed out that in our graphs the angles are plotted from 0° to 360° , rather than from -180° to 180° , the convention for traditional Ramachandran plots.

Validation of the force field model for Acc by comparison with published experimental data

The energy-contoured modified Ramachandran plot for the model peptide of the parent compound, i.e. N-acetyl-Acc-N-methylamide, is shown in Figure 1, and the minimum energy conformation of the protected Acc, indicated on Figure 1 by X at $\phi = 280^\circ$ and $\psi = 0^\circ$, is shown in Figure 2A. The minimum at ($80^\circ, 0^\circ$) is essentially isoenergetic to the global minimum indicated by X. Overall, our ϕ - ψ map for this compound is rather similar to that obtained by Barone et al. using AMBER (16), except that our calculations reproduce the ψ minima at 0° and 180° observed experimentally, due to our incorporation of the V_2 torsional term discussed previously. A similar map was calculated by Varughese et al. (21), but again incorrect minima for ψ were predicted due to lack of this torsional term. The force field model is further validated by the crystal structure of Asp-Acc-OPr (12) (Figure 2B). The conformation of the Acc portion of this molecule in the solid state is virtually identical to the global minimum energy conformation of the protected Acc model peptide (Figure 2A). The two molecules are shown superimposed in Figure 2C. Furthermore, a recent survey of crystal structures containing the Acc moiety (19) shows that, compared to calculations done with standard force fields (e.g. 16,21) the minima predicted by our force field model correlate exceptionally well with the observed structures.

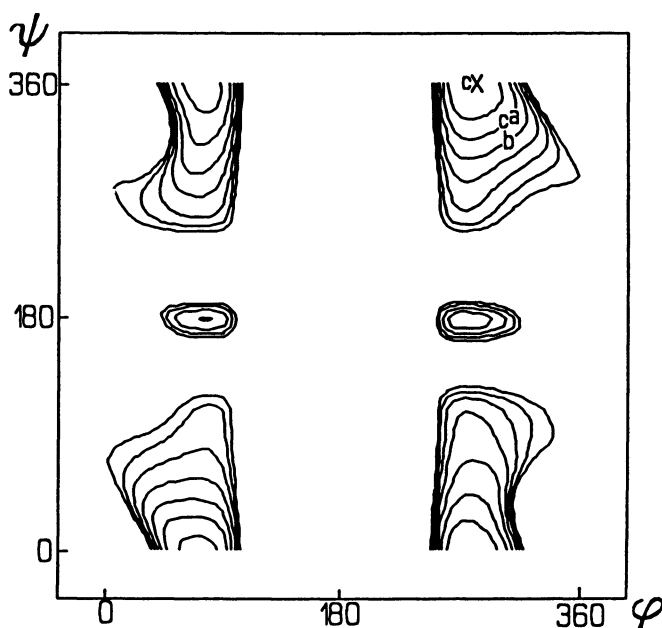


Figure 1. *Phi-psi* plot for the protected Acc model peptide (structure shown in Figure 2A). Energy above the global minimum (indicated by X) is contoured in 1 kcal/mole increments, up to 7 kcal/mole. The local minima for $\psi = 180^\circ$ are about 3.5 kcal/mole above the global minimum. Several common types of peptide structure are indicated as follows: a: 3_10 Helix; b: α Helix; c: Type I β turn.

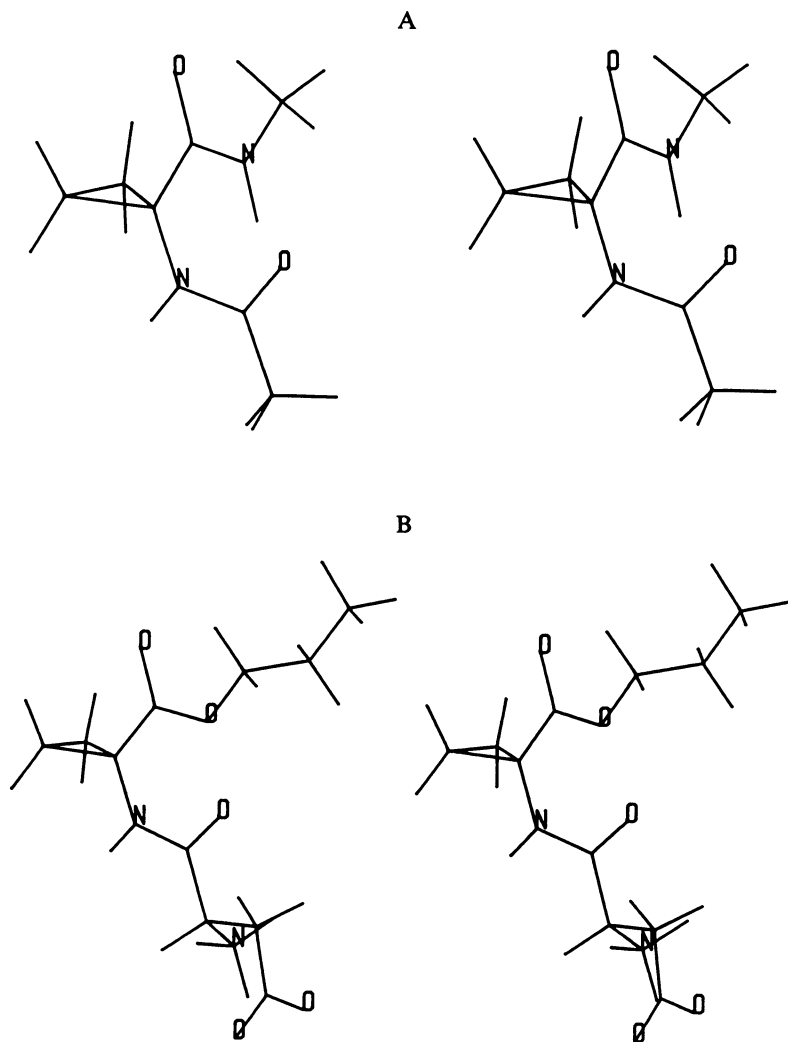


Figure 2A-2B. Stereoviews of structures of: **A:** The global minimum energy conformation of the Acc model peptide; **B:** The X-ray structure (12) of the sweet peptide analog aspartyl Acc propyl ester.

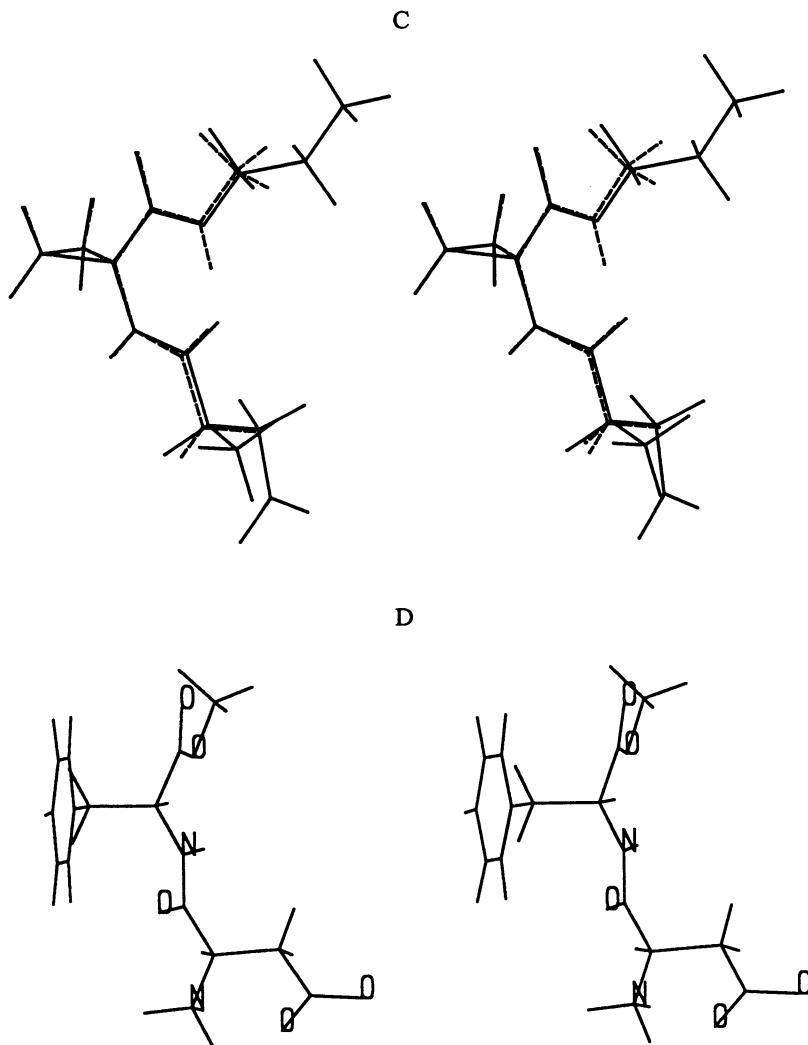


Figure 2C-2D. Stereoviews of structure of: **C:** Overlap of **2A** (dashed lines) and **2B** (solid lines), showing that the Acc conformations are virtually identical; **D:** The calculated global minimum energy conformation of the peptide sweetener aspartame.

Based on these calculations, it appears that the most optimal helical conformation for Acc in peptides is the 3_{10} helix (a, Figure 1), although α helices (b, Figure 1) and left-handed helices are also within 3 or 4 kcal/mole of the minimum. Most intriguing is the observation that Acc residues would be very effective in stabilizing a type I β turn (c, Figure 1), which to our knowledge has not yet been exploited in peptide drug design. However, it has recently been shown experimentally that tri- and tetra-Acc peptides fold into distorted type I β turns and 3_{10} helices (11), further validating the computational model, since these are the two classical secondary structural types that are closest to the calculated global minimum.

Conformational preferences of the Acc residue in Asp-Acc esters

Although the foregoing demonstrates the correspondence between this force field model for Acc derivatives and various experimental observations, the Acc peptide used to derive Figure 1 is not the best model for the compounds of interest, which are esters of Asp-Acc.

The energy-contoured modified Ramachandran plot produced by rotations about ϕ and ψ in the Acc residue of Asp-Acc-OPr is shown in Figure 3. It is apparent that the local minima for $\psi = 180^\circ$ are substantially lower for esters than they are for amides (Figure 1), being in fact isoenergetic to the conformations with $\psi = 0^\circ$. This is due to the fact that, in the amides, there are substantial steric interactions between the cyclopropane ring protons and the C-terminal amide proton when the amide bond is over the ring ($\psi = 180^\circ$). In the esters, there is no substituent equivalent to the amide proton on the ester oxygen, so the steric interaction is reduced relative to the amides when $\psi = 180^\circ$. The interactions of the ester oxygen lone pairs were taken into account in the calculation of Figure 3 by defining a special atom type with MM2 type lone pair parameters (17); this was necessary since SYBYL force field calculations ignore interactions of the default lone pair atom type.

Figure 3 indicates that for all practical purposes there are only four possible conformations of the Acc residue in the Asp-Acc-OR analogs. The two minima for ϕ are ca. $\pm 80^\circ$, and for ψ either 0° or 180° . The 4 possibilities are indicated by a-d in Figure 3. Considerations of which of these four conformations best overlap with the possible active conformations of aspartame should help suggest which one might be the actual active conformation.

Conformational preferences of the Aspartyl ψ angle

Like most investigators who have used semiempirical methods to study the conformations of aspartyl peptides, we have treated the Asp residue as a relatively rigid zwitterion. However, it is worthwhile to examine the flexibility around the Asp C_1-C_α bond (the ψ angle of the Asp residue). Figure 4 shows a plot of energy vs. ψ computed for Asp-Acc-OPr. The aspartyl group ψ angle is relatively restricted to a range of values from about $80-180^\circ$, energetically biased toward the higher values, which is consistent with the fact that in the crystal structures of both aspartame and Asp-Acc-OPr the observed value is about 150° . The minimum at -80° is not only about 1.3 kcal/mole higher, but is also in a quite narrow potential well and isolated by very high energy barriers. Thus this conformer would

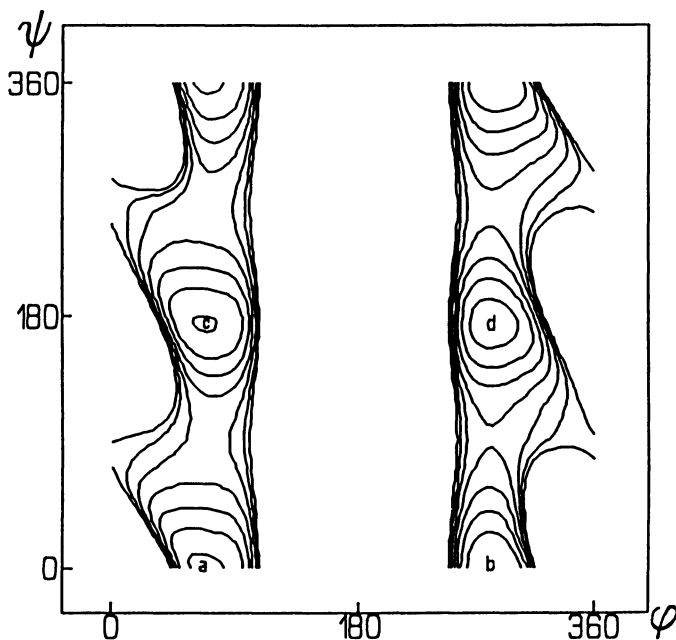


Figure 3. *Phi-psi* plot for the Acc residue in Asp-Acc-OPr (crystal structure shown in Figure 2B). Energy above the global minimum is contoured in 1 kcal/mole increments, up to 7 kcal/mole at the outer contour. The 4 preferred conformations are indicated by a-d. The crystal structure of Asp-Acc-OPr corresponds to conformation b.

make only a minor contribution to the overall conformer population at room temperature. Based on this analysis, the incorporation by previous authors (5-7) of the X-ray-like conformation of the aspartame Asp residue in their active site models appears to be reasonable. This restriction of the Asp *psi* angle is significant, since the aspartyl group functions as the essential AH + B moiety in sweet peptide analogs, and thus its orientation is critical in determining the receptor map. Since the same Asp *psi* angle is preferred in both aspartame and Asp-Acc-OPr, overlapping the aspartyl residue of preferred conformations of these two molecules should be the key to determining similarities in their mode of binding to the sweet taste receptor.

The global minimum energy state of aspartame

This was calculated by grid search, using the standard Tripos force field. The aspartate was treated as rigid, except for *psi*, and the other degrees of freedom were *phi*, *psi*, χ_1 and χ_2 of Phe. This gave the conformation shown in Figure 2D as the global minimum. This conformation is very close to the X-ray crystal structure, except

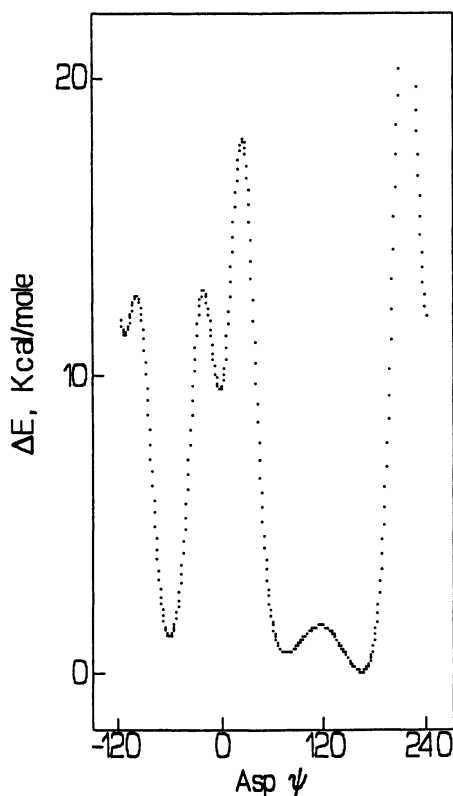


Figure 4. Potential energy of Asp-Acc-OPr as the aspartyl residue is rotated around the C1-C α bond (ψ). The global minimum at 160° corresponds approximately to the conformation seen in the crystal structures of aspartame and Asp-Acc-OPr.

that the ester group is rotated about 180°, and shows the phenylalanine side chain in the *least* populated conformation (F_{III}) as determined by the NMR studies of Temussi and collaborators (6). Nonetheless, our results seem to be consistent with the preference for this conformation in the solid state. It should be noted that the global energy minimum does not necessarily correspond to the most populated conformational type, since this will depend on the Boltzmann distribution of conformers in the regions of the various energy minima.

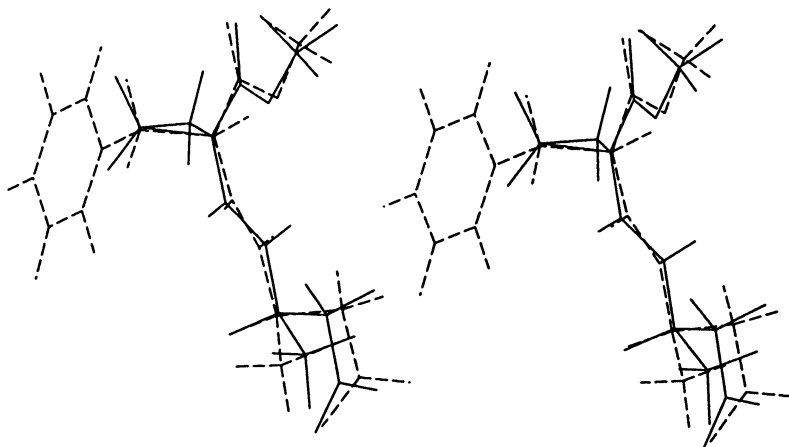


Figure 5. Stereoview of a common conformation for the sweet peptides aspartame (dashed lines) and aspartyl Acc methyl ester (solid lines), if the α -carbons and aspartyl residues are aligned (produced using the MULTIFIT option of SYBYL). These conformers are both within 2 kcal/mole of the global energy minima for each molecule. The Acc conformation is not far from conformation **b** of Figure 3. See text for discussion.

The mode of binding of Aspartyl-Acc esters

In the search for a possible common mode of binding for aspartame and Asp-Acc-OPr, which are approximately equivalent in sweetness, the most obvious approach initially is to attempt to align the aspartate residues, the Phe Ca with the Acc Ca, and perhaps the Phe benzylic carbon with the corresponding cyclopropane carbon atom. This leads to a model such as that shown in Figure 5. This was produced by starting from our calculated ground states for aspartame and Asp-Acc-OMe, defining common points to be overlapped (which included points on the peptide bond and the ester oxygens), and minimizing under zero distance constraints for those atom pairs (the MULTIFIT option of SYBYL). The conformers shown in Figure 5 are both within less than 2 kcal/mole of the global minima. However, this does not give a very convincing common model for their binding to the sweet taste receptor for several reasons. First of all, in this orientation, there is no part of Asp-Acc-OR occupying the space of the phenyl ring of aspartame. This, or some similar bulky hydrophobic group, is known to be critical for good activity. In addition, the structure-activity relationships of Asp-Phe-OR and Asp-Acc-OR esters are reversed with regard to the size of the R group; i.e., in the Acc analogs, activity increases from Me to Pr, whereas increasing the bulk of the aspartame alkyl ester group leads to a decrease in activity. This suggests that the ester alkyl group of Asp-Acc-OR actually serves as the bulky hydrophobic group, corresponding to the phenyl ring of aspartame. Taking these facts into account leads to a more satisfactory model

for the binding of the sweet cyclopropane analogs, obtained as follows: if the Acc ϕ angle is rotated from -80° , as seen in the crystal structure of Asp-Acc-OPr, to the other allowable value of $+80^\circ$ (conformations a or c on the isoenergy contour map of Figure 3, depending on whether ψ is 0° or 180°), convincing fits to the Goodman and Temussi models for the active conformation of aspartame are obtained. It should be emphasized that conformations b and d have been eliminated due to the impossibility of finding a fit to the possible active conformations of aspartame in which both the aspartyl and hydrophobic groups (phenyl and propyl) are aligned.

Best fit to the Goodman model: Figure 6 shows Asp-Acc-OPr in conformation a (Figure 3, $\phi = 80^\circ$, $\psi = 0^\circ$) fit to Goodman's model (7) of the active conformation of aspartame. The propyl group has been placed in an accessible staggered conformation emphasizing possible overlap with the phenyl ring of aspartame. This fit gives an excellent overlap of the aspartyl groups, and the propyl ester with the phenyl ring. This aspartame conformation is derived from the crystal structure by a 40° rotation of the Phe ϕ angle, after Goodman et al. (7). If the unmodified aspartame crystal structure is used for this fit to conformation a of Asp-Acc-OPr, an even better fit is obtained, with some overlap between the aspartame methyl ester and part of the cyclopropane ring.

Best fit to the Temussi model: Figure 7 shows Asp-Acc-OPr in conformation c (Figure 3, $\phi = 80^\circ$, $\psi = 180^\circ$) fit to the Temussi model (5-6) of the active conformation of aspartame; here, the propyl group has again been placed in an accessible staggered conformation emphasizing possible overlap with the phenyl ring of aspartame. This gives an excellent fit of the two molecules, with the cyclopropane ring mimicking the aspartame methyl ester.

It is not possible on the basis of these data alone to choose between the Goodman and Temussi models for the active conformation of aspartame, since conformations a and c (Figure 3) of Asp-Acc-OPr are essentially isoenergetic and at the global energy minimum. However, we can conclude that one of these two conformations is the probable active conformation of Asp-Acc-OPr, and may search for other evidence as to which of the two is the more likely candidate. However, it may be possible to discriminate between these two models on the basis of the little known fact that the amide analog Asp-Acc-NHPr is tasteless (Dr. James Sweeney, personal communication). The conformation shown as the fit to the Temussi model (Figure 7) would be much less accessible to the amide, i.e., the isoenergy map of Figure 1 would apply and an Acc ψ value of 180° would be 3.5 kcal/mole above the energy minimum, thus being much less likely to occur. However, in the fit to the Goodman model (Figure 6), replacement of the Asp-Acc-OPr ester by a peptide bond would be permissible, although it might restrict the flexibility of the propyl group as compared to the ester. Thus, it would be difficult to explain the lack of sweet taste of the amide analog with the Goodman model. According to our interpretations above, this may be evidence in favor of the Temussi model.

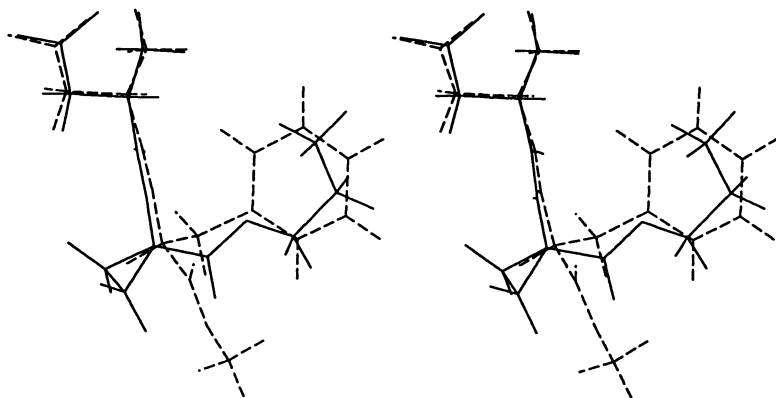


Figure 6. Stereoview of Asp-Acc-OPr (solid lines) in conformation a (Figure 3, $\phi = 80^\circ$, $\psi = 0^\circ$) fit to Goodman's model (dashed lines) of the active conformation of aspartame (oriented as in Figure 2 of reference 7). The propyl group has been placed in an accessible staggered conformation emphasizing possible optimal overlap with the phenyl ring of aspartame.

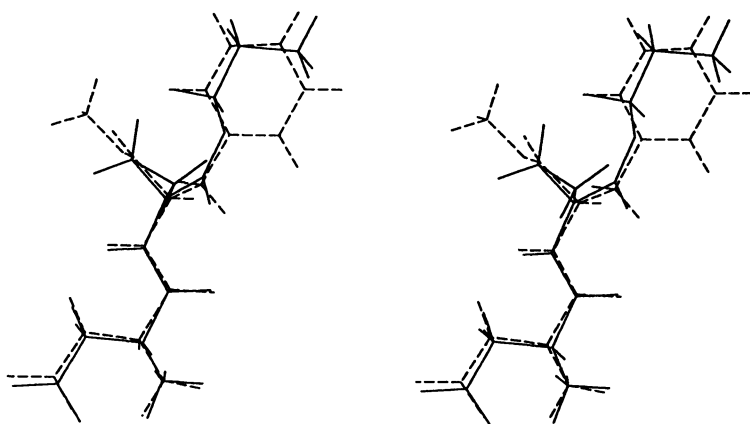


Figure 7. Stereoview of Asp-Acc-OPr (solid lines) in conformation c (Figure 3, $\phi = 80^\circ$, $\psi = 180^\circ$) fit to the Temussi model (dashed lines) of the active conformation of aspartame (oriented as in Figure 1 in references 5 and 6). The propyl group has been placed in an accessible staggered conformation emphasizing possible optimal overlap with the phenyl ring of aspartame.

The conformational basis for the lack of sweet taste of the four isomers of Asp-2,3-MePhe-OMe

The preferred conformational states of the four isomers of the 2,3-MePhe analogs of aspartame were calculated, and possible fits to the various receptor models were examined. These compounds will not overlap with aspartame in any of its populated conformers, for the following reason. In all four isomers of the cyclopropane analog of phenylalanine (2,3-MePhe) the structure of the cyclopropane ring forces the χ_2 bond (phenyl-cyclopropane carbon) to *eclipse* either the C1-C α or the N-C α bond, whereas in aspartame these are *staggered* in the three allowable conformations F_I, F_{II}, or F_{III} (6). This can be visualized for the cyclopropane hydrogen substituents in Figure 5. Thus, it is impossible for these analogs to precisely mimic any preferred conformation of aspartame. This is similar to the conclusion reached by Mapelli *et al.* (13) regarding the fit of these analogs to the Goodman model. Our current calculations give different ground states for the four isomers than those reported previously (13), due to our inclusion of the special cyclopropane V₂ parameter.

The conformational basis for the lack of sweet taste of the (\pm) isomers of Asp-2,3-MePro-OPr

The 2,3-MePro bicyclic ring system is relatively rigid, and there are two isomers, (2R,3S) or (2S,3R). Both of these were recently synthesized and incorporated into analogs of Asp-Acc-OPr; the taste (bitter) and conformational properties were also studied in detail, by theoretical and physical methods (14). Based on our current model of the binding of Asp-Acc esters to the sweet taste receptor, the lack of sweet taste of these compounds can be explained. The rigidity of 2,3-MePro restricts its *phi* angle to about -76° for the (2S,3R) analog, and $+76^\circ$ for the (2R,3S) analog. Thus, only the (2R,3S) analog of Asp-2,3-MePro-OPr can assume the putative active conformations, either a or c in Figure 3. However, the potential fit to either the Goodman or Temussi models is lost because the 2,3-MePro analogs favor a *cis* peptide bond (about 2 kcal/mole over *trans*), which is opposite to the preference shown by L-Asp-Acc-OPr. The preference of 2,3-MePro for a *cis* peptide bond is confirmed by both crystallographic and NMR studies (14). This places the aspartyl group (the important "AH + B" moiety) in a totally different position than that preferred by the sweet Acc analogs, thus providing a possible basis for the bitter rather than sweet taste.

Conclusions

The conformational restrictions imposed by the cyclopropane moiety, both steric and electronic in origin, make cyclopropane amino acid analogs of aspartame useful tools for probing the sweet taste receptor, particularly since some such analogs are very sweet, while others are bitter or tasteless. The force field model presented here gives excellent correlations with available X-ray data, and allows us to confidently state that the active conformation of the sweet analog Asp-Acc-OPr must be either conformation a or c (Figure 3), which correlate with the Goodman and Temussi models, respectively. However, on the basis of these compounds alone we are unable to discriminate

between the two models. The lack of sweet taste of the compounds containing the cyclopropane analogs of Phe and Pro can also be explained on the basis of their conformational preferences. We are currently working on incorporating the data on these compounds, active and inactive, along with other peptide and non-peptide analogs, in a full three-dimensional model of the sweet taste receptor.

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Chapter 13

Shape of Sweet Receptors Studied by Computer Modeling

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General models for sweeteners have been developed using a molecule building program by superimposition of the e/n-systems of a large number of sweet and nonsweet compounds from diverse structural classes. The models are based on psychophysical data (sweet thresholds) and clearly show the possible dimensions of the molecules compatible with sweet taste. The sweet potency depends strongly on the location of hydrophobic groups within the given sweet space. The models include amino acids, oxathiazione dioxides, benzisothiazolone dioxides, carboxyalkylbenzamides, naphthoimidazoles and related compounds.

Sweet taste is exhibited by a large number of compounds from diverse structural classes. Common structural elements of sweet compounds have been proposed and include the AH/B-system of Shallenberger and Acree (1) and the hydrophobic group X of Kier (2). The bipolar system can be more generally described as an electrophilic/ nucleophilic (e/n-)system in conjunction with a more or less extensive contact of the hydrophobic moiety with the corresponding portion of the sweet receptor (3,4). The quality and intensity of a given molecule's taste depends strongly on the e/n-system, the size and shape of the hydrophobic moiety and its position relatively to the e/n-system.

By superimposition of the e/n-systems of sweet and closely related nonsweet compounds, it is possible to elucidate the dimensions of a molecule compatible with sweet taste, and, thus to develop a basic model for sweet compounds, and to make inferences about the shape of a schematic sweet receptor (5). Since such a model for sweet compounds is based on psychophysical data (taste thresholds), it is integral in nature and may reflect a superimposition of several different real sweet receptor binding sites. A real receptor binding site, on the other hand, may only be a partial realization of the schematic integral receptor, derived by superimposition of different sweet compounds.

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The capability of this superimposition method shall be demonstrated by several examples.

Methods

The programs CORINA and MARYLIN were used for generation and superimposition of molecules (6). The 3D-coordinates of molecules were calculated by CORINA from the constitution formulae and stereochemical descriptors. Molecular models were generated and represented on a colour graphics screen MARYLIN. The program allowed various manipulations of these models, such as different colouring of atoms, translation and rotation of individual models, conformational changes at specific bonds, superimposition of several models and plotting the results in form of stereoscopic views.

Conformations were calculated with the programs MM2 (7), MOPAC (8), OPTIPOW (9) or SYBYL (10), depending on the size of molecules and the availability of parameters. Conformers which represent local or absolute energy minima were used for superimposition.

Taste qualities and sweet taste recognition thresholds were according to a previously established protocol (11).

e/n-Systems

A series of different e/n-systems are known, e.g. OH/OH in sugars, $\text{NH}_3^+/\text{COO}^-$ in amino acids and peptides, NH/SO_2 in oxathiazinone dioxides and isothiazolone dioxides, OH/CO in β -hydroxyketones, $\text{CONH}_2/\text{COO}^-$ in carboxyalkyl benzamides, CONH_2/OR in alkoxyphenyl ureas and NH_2/CO in ureas. In Table I the distances between the electrophilic and the nucleophilic groups are summarized for various sweet compounds. The data suggest that the compounds can be classified into two groups; the distance being in the range of 250 pm for group 1 and in the range of 800 pm for group 2. In the following, two different basic models were developed for these two groups.

TABLE I. e/n-Systems of Different Sweet Compounds

No.	Compound	e/n-System	Distance ^a [pm]
1	N-Alkyl(aryl) ureas	$\text{H}_2\text{N}-\text{CO}$	240
2	Oxathiazinone dioxides	NH/SO_2	241
3	Sulfamic acids	$\text{NH}-\text{SO}_3^-$	249
4	Benzisothiazolone dioxides	NH/SO_2	256
5	3-Aminocarboxylic acids	$\text{NH}_3^+/\text{COO}^-$	269
6	2-Aminocarboxylic acids	$\text{NH}_3^+/\text{COO}^-$	300
7	2-Aminobenzoic acid	$\text{NH}_3^+/\text{COO}^-$	304
8	3-Aminobenzoic acid	$\text{NH}_3^+/\text{COO}^-$	513
9	3-Carbamoyl-2,4,6-tribromophenylpropionic acid	$\text{H}_2\text{NCO}/\text{COO}^-$	761
10	4-Ethoxyphenyl urea	$\text{H}_2\text{NCO}/\text{OEt}$	770
11	3-Carbamoyl-2,4,6-tribromo cinnamic acid	$\text{H}_2\text{NCO}/\text{COO}^-$	773
12	Napththoimidazoles	NH/SO_3^-	839

^a Torsion angle of amino and carboxylic groups $\pm 60^\circ$.

Basic Model 1 (e/n-distance ~ 250 pm)

In Tables II and III the taste qualities and recognition thresholds of several sweet and nonsweet oxathiazinone dioxides and benzisothiazolone dioxides are summarized (11). The e/n-systems of these two groups of compounds were superimposed (Figure 1). The positions which are compatible and incompatible with sweet taste matched very well, and gave some information on the dimensions of the corresponding schematic sweet receptor. These dimensions are very clearly shown in Figure 2, which illustrates the incompatible, forbidden positions.

Data about the taste properties of several sweet and nonsweet amino acids are given in Table IV. The superimposition of several compounds is separately shown in Figure 3, while in Figure 4 it is included in the schematic receptor of Figure 2, derived from the oxathiazinone and benzisothiazolone dioxides. The amino acids give additional information about positions, which are forbidden for sweet taste. More than one apolar substituent in the β -position of sweet L- α -aminocarboxylic acids abolished the sweet taste (L-valine), while, on the other hand, polar groups were compatible with sweet taste in this position (L-threonine; the hydroxy group of L-4-hydroxyproline was located in the same area). In consideration of these facts, the sweet 1-aminocycloalkane-1-carboxylic acids fit into the model only with the amino group in equatorial position. From the literature (14) it is known that the zwitterion of the indicated cyclohexane crystallizes in this conformation. Calculations delivered very similar energies for both conformers.

TABLE II. Taste Qualities and Thresholds of Selected Oxathiazinone Dioxides (11) (cf. formula in Figure 17)

No	R ¹	Compound R ²	R ³	Quality ^a	c _{tsw} ^b [mmole/l]	f _{sac/g} ^c
1	H	H	Na	sw		10 ^d
2	H	CH ₃	K	sw/bi	0.08-0.12	130 ^{d,e}
3	H	C ₂ H ₅	H	sw/bi	0.06-0.09	150 ^d (Na)
4	H	C ₄ H ₉	Na	sw		30 ^d
5	H	C ₆ H ₅	H	bi	-(10) ^f	
6	CH ₃	CH ₃	Na	sw/bi	0.09-0.11	130 ^d
7	CH ₃	C ₆ H ₅	Na	bi	-(10) ^f	
8	C ₂ H ₅	C ₂ H ₅	K	sw/bi	0.1 -0.2	
9	C ₂ H ₅	C ₃ H ₇	K	sw/bi	0.07-0.11	70 ^d (Na)
10	C ₃ H ₇	CH ₃	H	sw/bi	0.3 -0.5	30 ^d (Na)
11	C ₃ H ₇	C ₄ H ₉	Na	bi	-(10) ^f	
12	C ₆ H ₅	CH ₃	Na	bi	-(10) ^f	
13		$\underbrace{\text{C}_6\text{H}_5\text{R}^g}_3$	H	sw/bi	0.08-0.15	

^a sw: sweet, bi: bitter ^b Sweet recognition threshold ^c f_{sac,g} = c(saccharose)/c(sweetener); the factor corresponds to a sweetener solution, which is isosweet to 4 % saccharose solution

^d According to ref. 19 ^e Relative sweetness potencies up to -180 are reported (20) ^f No sweet taste up to 10 mmol/l ^g R: -CH=CH-CH=CH-

TABLE III. Taste Qualities of Selected Benzisothiazolone Dioxides (cf. formula in Figure 17).

No.	Substitution Pattern					Quality		Ref.
	2	4	5	6	7	Sweet	Not Sweet	
1	X ^a						+	21
2		NO ₂				+		21-23
3		NHCOCH ₃					+	21,22
4		Cl				+		24
5			Cl			+		23,24
6			Br			+		23
7			NH ₂				+	21,23
8				F		+		16,22,24
9				Cl		+		16,22-24
10				Br		+		16,22-24
11				I			+	16,22,24
12					NO ₂		+	21,23
13					OH		+	23
14		┌─R ^b ─┐					+	24,25
15			┌─R ^b ─┐				+	24,25
16				┌─R ^b ─┐			+	24,26

^a All substituents without functional groups abolish the sweet taste ^b R: -CH=CH-CH=CH-

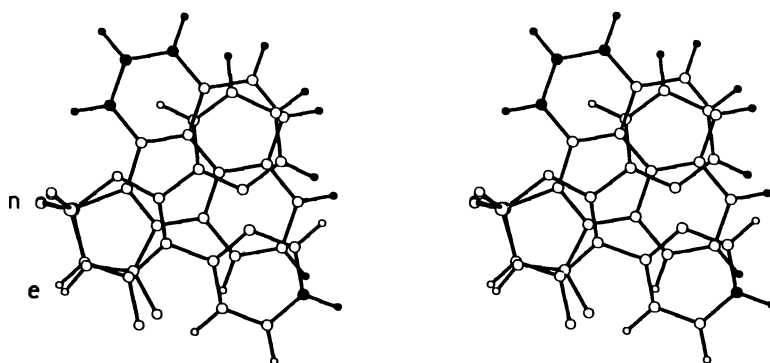


Figure 1. Superposition of Selected Sweet and Nonsweet Oxathiazinone Dioxides (Table II, No. 5,12) and Benzisothiazolone Dioxides (Table II, No.14-16). (o/o Positions allowed/forbidden for sweet taste, e,n: e/n-system, stereoscopic drawing)

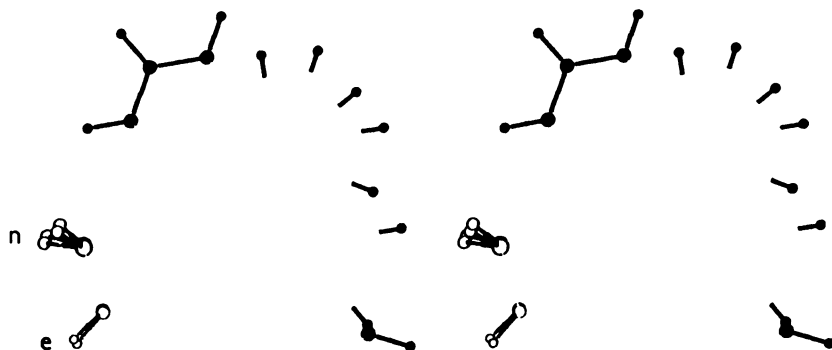


Figure 2. Superposition of Selected Sweet and Nonsweet Oxathiazinone Dioxides and Benzisothiazolone Dioxides. (Same compounds as in Figure 1; only the forbidden positions are shown)

The superimposition of the e/n-system of 2-aminobenzoic acids (Table IV, No.33-36) allows identification of some further positions incompatible with sweet taste (Figure 6).

From Figure 5 it can be seen that the sweet 1-aminocyclooctane-1-carboxylic acid is compatible with the schematic receptor, but the adamantane derivative is not, because it occupies a forbidden position for an apolar substituent, as previously discussed.

Several benzoates have been found to be sweet, and the taste quality and thresholds are described in Table V. In the case of these compounds, the cations and the carboxylate anions are assumed to act as e/n-systems. Other examples of sweet tasting substances, in which the e/n-system is provided by two separate components, are known from the literature (13). The benzoates matched very well with the schematic sweet receptor, with the exception of the nonsweet 3,5-dichloro derivative. Figure 7 shows that one of its chlorine atoms projects into the area forbidden for sweet taste.

A probable conformation of 6*S*,1'*S*-Hernandulcin, a sweet sesquiterpene from *Lippia dulcis* Trev. (14), is shown in Figure 8. The sweet isomer fitted very well into the schematic sweet receptor, with the isobutenyl group parallel to the receptor's backside (Figure 9). The terpene's area of hydrophobic contact with the schematic receptor corresponds to that of saccharin.

Since Hernandulcin is sweet, D-tert-butylglycine, formerly reported as tasteless (4), should be sweet too, or Hernandulcin would occupy a forbidden position within the schematic receptor, as is demonstrated in Figure 9. Reinvestigation of butylglycine at higher concentrations indeed revealed the sweet taste of this compound with a threshold of 60-80 mmol/l.

In Figure 10 it is shown that additional D-amino acids can be included in the schematic sweet receptor without overlapping any of the previously identified forbidden positions. The same is true for

TABLE IV. Taste Quality and Threshold of Selected Amino Acids

No.	Compound	Quality ^a	c _{tsw} ^b		Ref.
			[mmol/l]		
1	L-Alanine	sw	12	-18	27
2	L-2-Aminobutyric acid	sw/bi	12	-16	27
3	L-2-Aminovaleric acid	bi			27
4	L-Valine	bi			27
5	L-Isoleucine	bi			27
6	D-t-Butylglycine	sw	60	-80	
7	L-Proline	sw/bi	25	-40	27
8	D-Proline	ne			27
9	L-4-Hydroxyproline	sw	5	-7	27
10	L-allo-4-Hydroxyproline	ne			27
11	L-Serine	sw	25	-35	27
12	L-Threonine	sw	35	-45	27
13	D-Phenylalanine	sw	1	-3	27
14	L-Phenylalanine	bi			27
15	1-Aminocyclohexane-1-carboxylic acid	sw/bi	1	-3	28
16	1-Aminocycloheptane-1-carboxylic acid	sw/bi	2	-4	28
17	1-Aminocyclooctane-1-carboxylic acid	sw/bi	2	-4	28
18	1-Aminocyclononane-1-carboxylic acid	bi			28
19	D,L-3,5-Dibromophenylalanine	sw			16
20	D-Tryptophan	sw	0.2	-0.4	28
21	1-Methyl-D-tryptophan	sw	0.2	-0.4	18
22	6-Methyl-D-tryptophan		0.02	-0.03	18
23	5-Methoxy-D-tryptophan		0.2	-0.4	18
24	4-Methyl-D-tryptophan		0.04	-0.06	18
25	D,L-2-Amino-3-(1-naphthyl)propionic acid	sw	0.1	-0.2	
26	D,L-2-Amino-3-(2-naphthyl)propionic acid	sw	0.6	-1.0	
27	D,L-2-Amino-3-ethylpentane carboxylic acid	sw	10	-20	
28	1-Amino-4-methylcyclohexane-1-carboxylic acid	sw/bi	8	-10	28
29	1-Amino-4-ethylcyclohexane-1-carboxylic acid	bi			28
30	1-Amino-4-t-butylcyclohexane-1-carboxylic acid	ne			28
31	D,L-9-Aminobicyclo(3.3.1)nonane-9-carboxylic acid	bi			28
32	D,L-2-Aminoadamantane-2-carboxylic acid	ne			28
33	2-Amino-3-methylbenzoic acid	ne		-(50)	29
34	2-Amino-4-methylbenzoic acid	sw	5	-7	29
35	2-Amino-5-methylbenzoic acid	sw	1	-3	29
36	2-Amino-6-methylbenzoic acid	ne		-(50)	29

^a sw: sweet, bi: bitter, ne: neutral ^b Sweet recognition threshold concentration

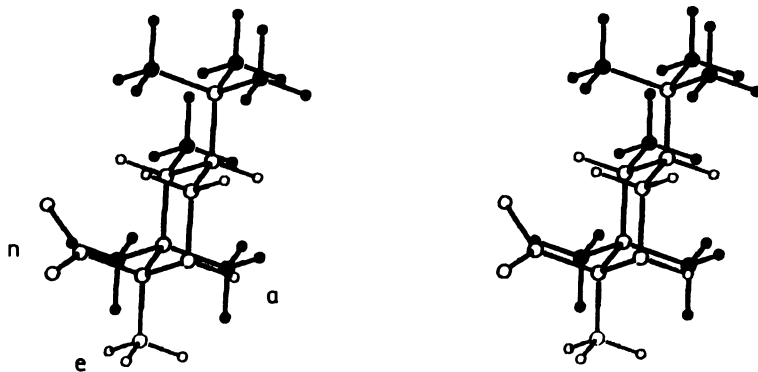


Figure 3. Superposition of Selected Sweet and Nonsweet Amino Acids (Table IV, No.2-4,7,9,10,12,28-30, a: allowed for hydrophilic, forbidden for hydrophobic groups).

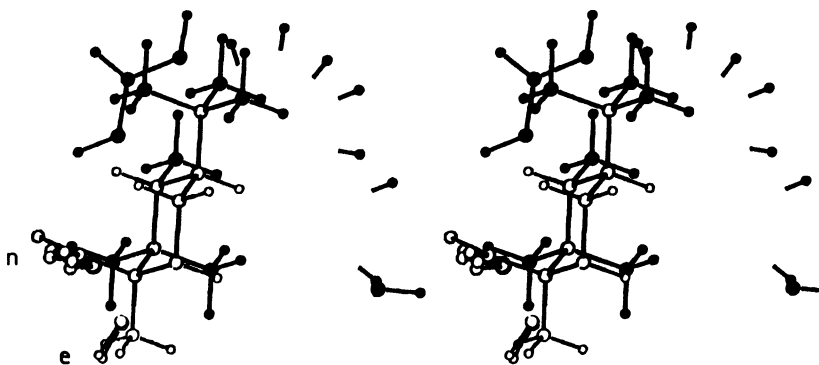


Figure 4. Superposition of the Sweet Receptor Model 1 (Figure 2) with Selected Sweet and Nonsweet Amino Acids

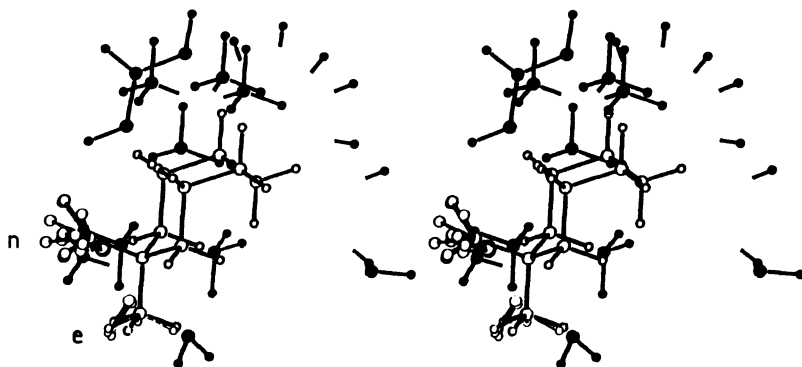


Figure 5. Superposition of the Sweet Receptor Model 1 (Figure 4, including an additional forbidden position, derived from D-proline (Table IV, No.8)) with 1-Amino-cyclooctane-1-carboxylic acid (Table IV, No.17)

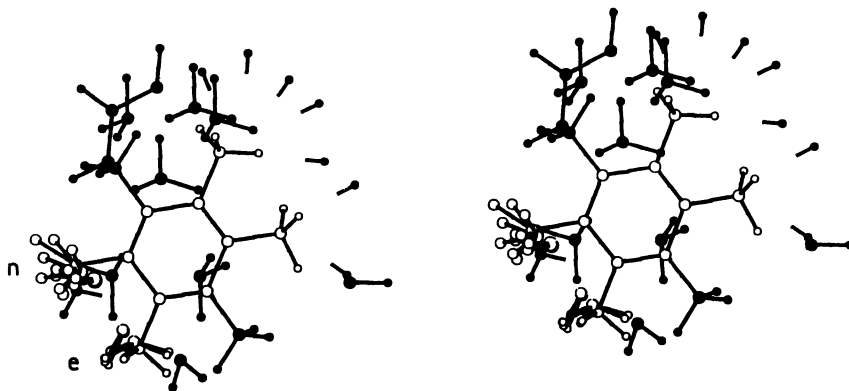


Figure 6. Superposition of the Sweet Receptor Model 1 (Figure 5) with 2-Aminobenzoic Acids (Table IV, No.33-36)

TABLE V. Taste Qualities and Thresholds of Selected Benzoates

No.	Anion	Cation ^a	Quality	c _{tsw} ^b [mmol/l]	Ref.
1	Benzoate	Li ⁺ (68)	sweet	10 - 15	29
2	Benzoate	Na ⁺ (97)	sweet	4 - 15	29
3	Benzoate	K ⁺ (133)	sweet	10 - 15	29
4	Benzoate	NH ₄ ⁺ (143)	neutral	- (100) ^c	29
5	Benzoate	Mg ²⁺ (66)	sweet	10 - 15	29
6	Benzoate	Ca ²⁺ (99)	sweet	10 - 15	29
7	Benzoate	Ba ²⁺ (134)	neutral	- (100) ^c	29
8	2-Methylbenzoate	Na ⁺ (97)	neutral	- (100) ^c	
9	3-Methylbenzoate	Na ⁺ (97)	sweet	5 - 8	
10	4-Methylbenzoate	Na ⁺ (97)	sweet	5 - 8	
11	3-Chlorobenzoate	Na ⁺ (97)	sweet	5 - 10	29
12	4-Chlorobenzoate	Na ⁺ (97)	sweet	2.5- 5	29
13	3,5-Dichlorobenzoate	Na ⁺ (97)	neutral	-(50) ^c	29

^a Cationic radius [pm] in parenthesis ^b Sweet recognition threshold concentration ^c Nonsweet up to the concentration in parenthesis

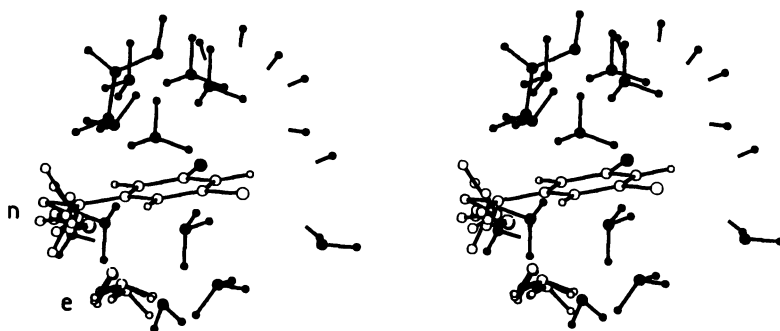


Figure 7. Superposition of the Sweet Receptor Model 1 (Figure 6) with 3,5-Dichlorobenzoate (Table V, No.13)

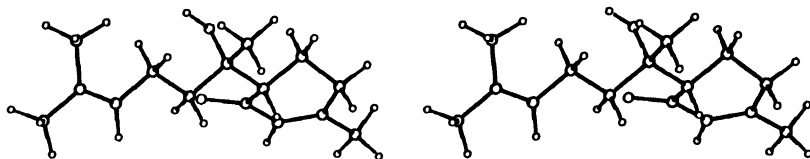


Figure 8. 6S,1'S-Hernandulcin

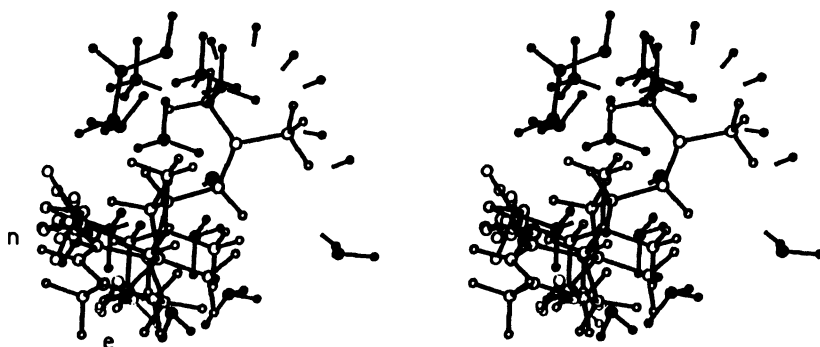


Figure 9. Superposition of the Sweet Receptor Model 1 (Figure 7) with 6S,1'S-Hernandulcin (Figure 8) and D-tert-Butylglycine (Table IV, No.6)

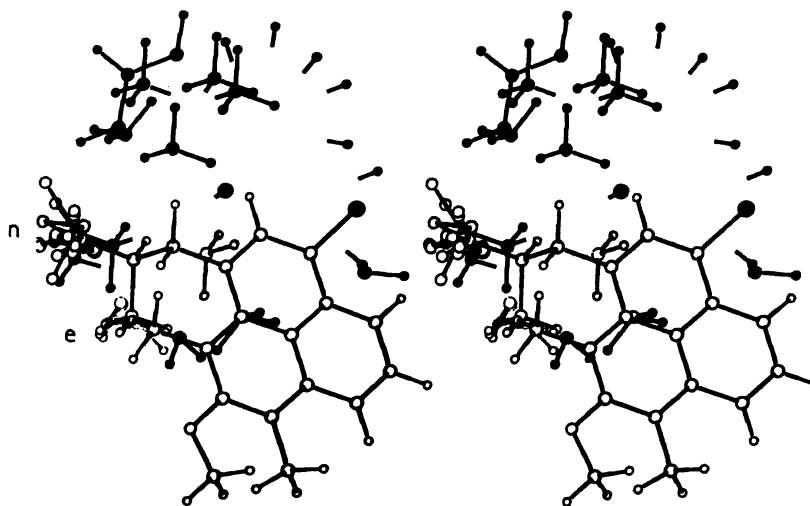


Figure 10. Superposition of the Sweet Receptor Model 1 (Figure 7) with Additional Amino Acids (Table IV, No.19-26)

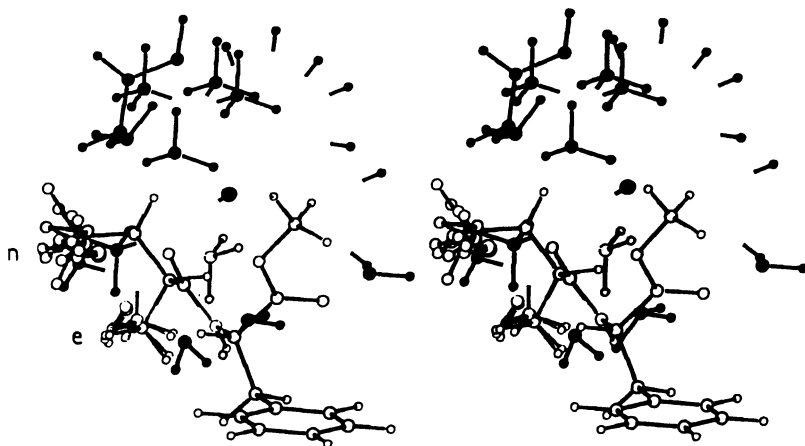


Figure 11. Superposition of the Sweet Receptor Model 1 (Figure 7) with Aspartame

aspartame and sucrose. One of the several possible conformers fitting into the schematic sweet receptor, is shown in Figures 11 and 12 for each of these compounds, respectively. On the other hand, Figures 10-12 illustrate clearly that the portion of the receptor occupied by the D-amino acids, aspartame and sucrose, is not particularly well defined. Additional investigation, with the inclusion of sweet and nonsweet compounds, is needed to further collaborate the location of the forbidden positions.

Basic Model 2 (e/n-distance ~ 800 pm)

Trihalogenated carboxyalkylbenzamides are potent sweeteners (15), with carboxamide and carboxylate groups acting as e/n-system (10). The sweet taste thresholds of some selected compounds of this class are summarized in Table VI. The distances between the central atoms of the e/n-system are in the range of 800 pm, as shown in Figure 13 for 3-carbamoyl-2,4,6-tribromocinnamic acid. Benzamides with carboxyalkyl side chains of different length can exist in conformations, in which the e/n-systems are approximately equidistant, while the positions of the benzene rings vary somewhat (Figure 14). An incorporation of the sweet benzamides into the basic model 1 (e/n-distance ~ 250 pm) was not possible.

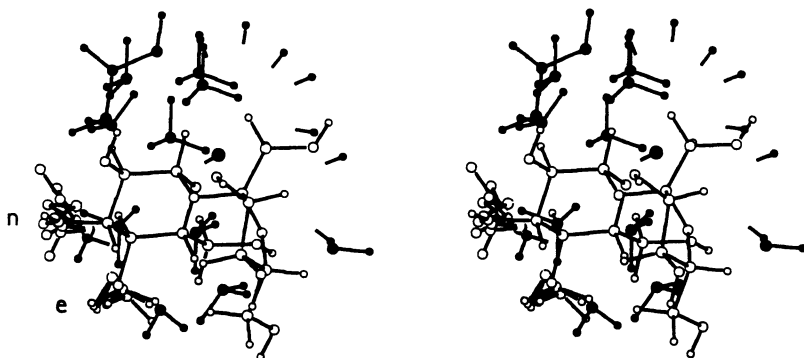


Figure 12. Superposition of the Sweet Receptor Model 1 (Figure 7) with Sucrose

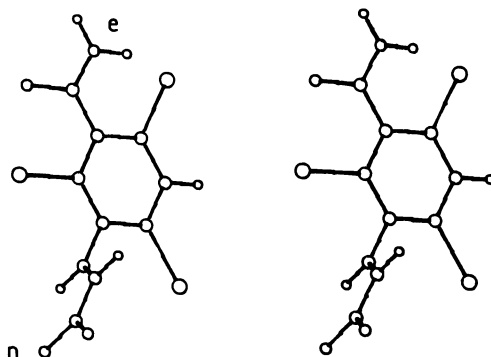


Figure 13. 3-Carbamoyl-2,4,6-tribromocinnamic Acid

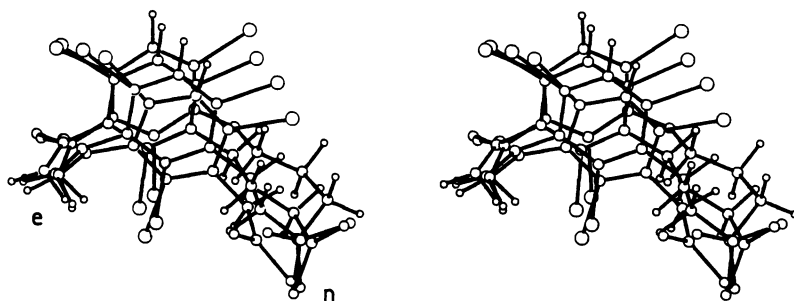


Figure 14. Superposition of Several Sweet 3-Carbamoyl-2,4,6-tribromophenylalkanoic Acids (Table VI, No.5-7,10)

TABLE VI. Taste Qualities and Thresholds of Selected 3-Carbamoyl-2,4,6-trihalogenophenylalkanoic Acids (10) (cf. formula in Figure 17)

No.	R ¹	R ²	R ³	R ⁴	R ⁵	R ⁶	ctsw ^a [μmol/l]
1	CONH ₂	H	(CH ₂) ₂ -COOH	H	H	H	-(20000) ^b
2	CONH ₂	Cl	O-CH ₂ -COOH	Cl	H	H	-(1000)
3	CONH ₂	Cl	O-CH ₂ -COOH	Cl	H	Cl	20 - 50
4	CONH ₂	H	O-CH ₂ -COOH	Br	H	Br	-(1000)
5	CONH ₂	Br	CH ₂ -COOH	Br	H	Br	40 - 55
6	CONH ₂	Br	(CH ₂) ₂ -COOH ^c	Br	H	Br	0.6- 1.4
7	CONH ₂	Br	(CH ₂) ₃ -COOH	Br	H	Br	5 - 15
8	CONH ₂	Br	(E)-CH=CH-COOH	Br	H	Br	0.5- 2
9	CONH ₂	Br	O-CH ₂ -COOH	Br	H	Br	5 - 20
10	CONH ₂	Br	O-(CH ₂) ₃ -COOH	Br	H	Br	30 - 60
11	CN	Br	(CH ₂) ₂ -COOH	Br	H	Br	^d
12	COOH	Br	(CH ₂) ₂ -COOH	Br	H	Br	-(1000)

^a Sweet recognition threshold ^b Nonsweet up to the concentration in parenthesis ^c The compound was tested as sodium salt; the threshold was calculated for the salt ^d Nonsweet.

From the literature several other sweet compounds are known, for which a longer-range e/n-system has to be assumed. Examples are several naphthimidazole derivatives, described by Cohn (16) and by Neri (17), dulcin and the 2-(3,6-dihydroxy-9H-xanthen-9-yl) benzoic acid (16), obtained by the reduction of fluorescein.

For the naphthimidazoles two different e/n-systems can be envisioned, either the NH/SO₃⁻ system of the sulfanyl residue, or the unsubstituted nitrogen of the imidazole ring and the SO₃⁻ group of the sulfanyl residue. The distance of the latter e/n system is in accordance with the e/n-system of the benzamides. The SO₃⁻ group of the naphthyl residue is not essential for sweet taste (16, 17).

In Figure 15, the e/n-systems of the sweet 2-phenyl-3-sulfanylnaphtho[1,2-d]imidazole-5-sulfonic acid (cf. Figure 18) and the 3-carbamoyl-2,4,6-tribromocinnamic acid (cf. Figure 18) are superimposed. The e/n-systems match very well and the benzene rings of the two compounds are located in adjacent areas. The dihydroxyxanthenyl benzoic acid (cf. Figure 18) also fit very well into basic model 2 (Figure 16).

Several ureas from the type R-NH-CO-NH₂ are sweet and bitter (Table VII), but only the 4-alkoxyphenyl derivatives (cf. Figure 18) exhibit a pure sweet taste (18). Because of these taste properties, it is obvious to assume the carbamoyl and alkoxy groups as the e/n-system. Figure 16 shows that the e/n-system of the alkoxyphenyl ureas matches very well with the e/n-systems of naphthimidazoles, carboxyalkyl benzamides and dihydroxyxanthenyl benzoic acid. On the other hand, the figure also demonstrates very clearly that a common hydrophobic area is built up by these sweet compounds with longer range e/n-systems.

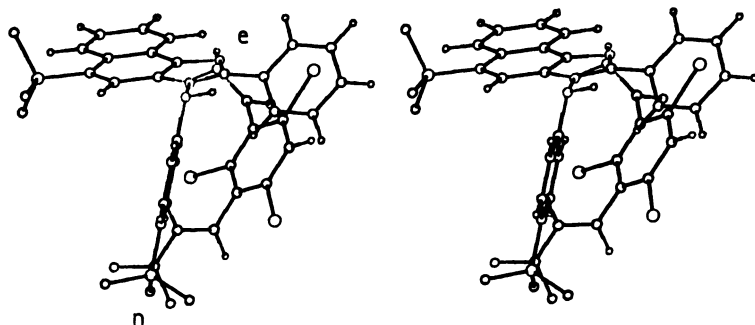


Figure 15. Superposition of 2-Phenyl-3-sulfanylnaphtho[1,2-d]imidazole-5-sulfonic Acid and 3-Carbamoyl-2,4,6-tribromocinnamic Acid

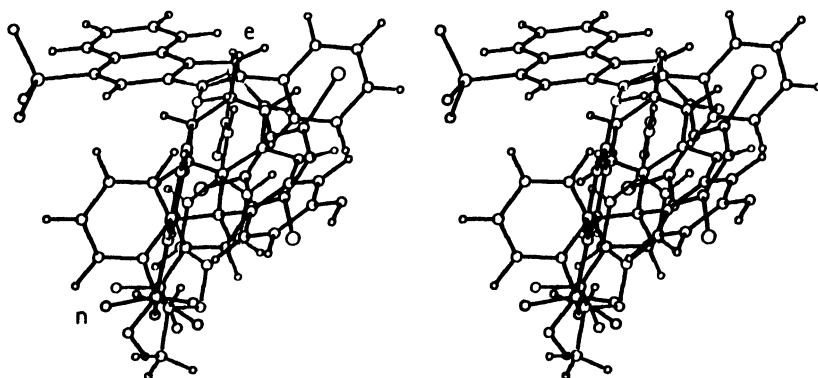


Figure 16. Superposition of the Sweet Basic Model 2 (Figure 14) with Dihydroxyxanthenyl Benzoic Acid and Dulcin (Table VII, No.7)

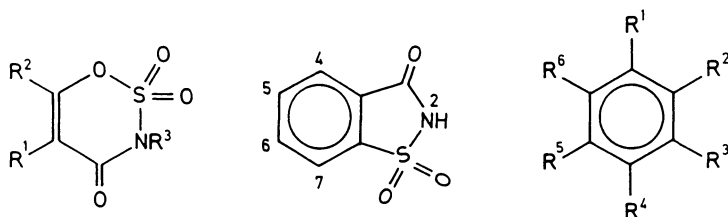


Figure 17. From left to right: Oxathiazinone Dioxides (cf. Table II), Benzisothiazolone Dioxides (cf. Table III), 3-Carbamoyl-2,4,6-trihalogenophenylalkanoic Acids (cf. Table VI)

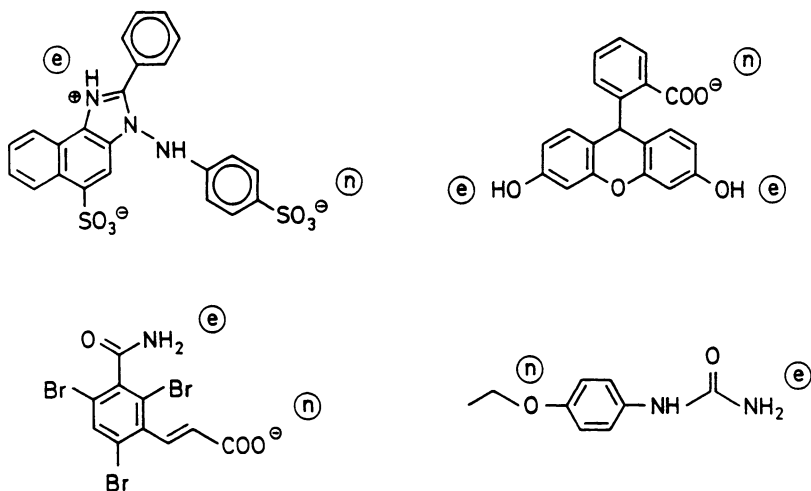


Figure 18. From left to right: 2-Phenyl-3-sulfanylnaphtho[1,2-d]imidazole-5-sulfonic Acid, 2-(3,6-Dihydroxy-9H-xanthen-9-yl)benzoic Acid, 3-Carbamoyl-2,4,6-tribromocinnamic Acid, (4-Ethoxyphenyl)urea

TABLE VII. Taste Qualities and Thresholds of Selected Ureas of the Type $R^1-C_6H_4-NR^2-CO-NHR^3$

No.	R ¹	R ²	R ³	ctsw ^a [mmol/l]	Ref.
1	H	H	H	- (40) ^b	18
2	Me	H	H	0.6 - 1.0	18
3	Et	H	H	- (10)	18
4	Pr	H	H	- (2)	18
5	OH	H	H	+ ^c	30
6	OMe	H	H	0.5 - 1.0	18
7	OEt	H	H	0.015-0.030	18
8	OEtBr	H	H	+	30
9	OEt	Me	H	+	30
10	OEt	H	Me	- ^d	30

^a Sweet recognition threshold ^b Nonsweet up to the concentration in parenthesis ^c Sweet ^d Nonsweet

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Chapter 14

Electrostatic Recognition Patterns of Sweet-Tasting Compounds

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Molecular electrostatic potential (MEP) patterns are determined for a select set of perillartine analogues. The optimal recognition pattern for these compounds is deduced from the electrostatic potential generated by the most potent 1,4-analogues. Similar patterns are found for the chemically related class of compounds, the 2-substituted 5-nitroanilines. The implications for tastant-receptor binding are discussed.

Electronic interactions play an important role in the interaction between sweet tastants and their receptors (1). Some electronic features postulated to be involved in the actual binding of sweet tastants to the receptor have been derived from a simple model suggested (2,3) by a consideration of a number of structurally diverse tastants such as fructose, saccharin, and chloroform. This model suggests that the binding takes place between an electronegative atom (B) on the tastant and an electropositive hydrogen on the receptor, as well as between a polarized system (A-H) on the tastant and an electronegative atom on the receptor. A distance of 2.0-3.0Å between A and B has been postulated as a requirement to initiate the taste response (2). An additional electronic feature of the tastant, identified by a hydrophobic site located on the tastant at a distance of 5.0-6.0Å from B, was assumed in the model in order to rationalize the taste potency (3). It has been pointed out (4) that many organic compounds possess such features and are clearly not very sweet. In addition, for tastants such as the perillartine analogues and the 2-substituted 5-nitroanilines, it is

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not possible to determine a polarized A-H moiety that would satisfy the above requirement and also form strong hydrogen bonds with the receptor (4). Furthermore, for the strong acids saccharin and acesulfame, it has been shown that it is the anionic form which is the biologically active species (5).

To investigate the electronic requirements for sweet-taste activation, we have initially focussed on the first stage of molecular recognition, i.e., the long range interaction of the tastant with the receptor which orients the tastant in the receptor's active site. This preliminary interaction between the receptor and tastant is dominated by long-range electrostatic interactions, which can be measured by the electrostatic potential pattern of the tastant. This pattern yields the electrostatic potential energy of interaction between a unit positive charge located at any point in space and the static charge distribution of the molecule (6). Since the molecular electrostatic potential (MEP) is a property of the molecule as a whole, it provides the basis for the development of a global model of electrostatic recognition. For a given class of tastants acting at the same receptor site, we assume that the MEP pattern of the most potent tastants defines the optimal pattern for electrostatic recognition by the receptor. The optimal molecular electrostatic potential pattern provides information about (1) the orientation of the tastant in the receptor cavity and (2) the electrostatic pattern associated with the receptor. This information is especially crucial in the case of sweet-taste recognition where the sweet-taste receptor has not been physically isolated.

Though the electrostatic energy is often the major contribution to the binding energy, other terms such as polarization, charge transfer, dispersion etc. may also be important to the binding energy (7). However, since the electrostatic potential determines the orientation of the tastant with respect to the receptor site, the molecular electrostatic potential patterns can be utilized to form a preliminary hypothesis concerning the molecular characteristics of tastant-receptor binding. Such a program has been used by Weinstein et al. (8) to determine the mechanism for the binding of 5-HT and its congeners and related compounds to the serotonin receptor.

Our initial work has dealt with the semi-rigid perillartine analogues for which structure-activity data are available (9). These analogues are characterized by a CC double bond in conjugation with the CN double bond of the oxime moiety. We focussed on a select set of compounds, characterized with one exception by their predominantly sweet taste. The compounds of interest are listed in Figure 1. Our first

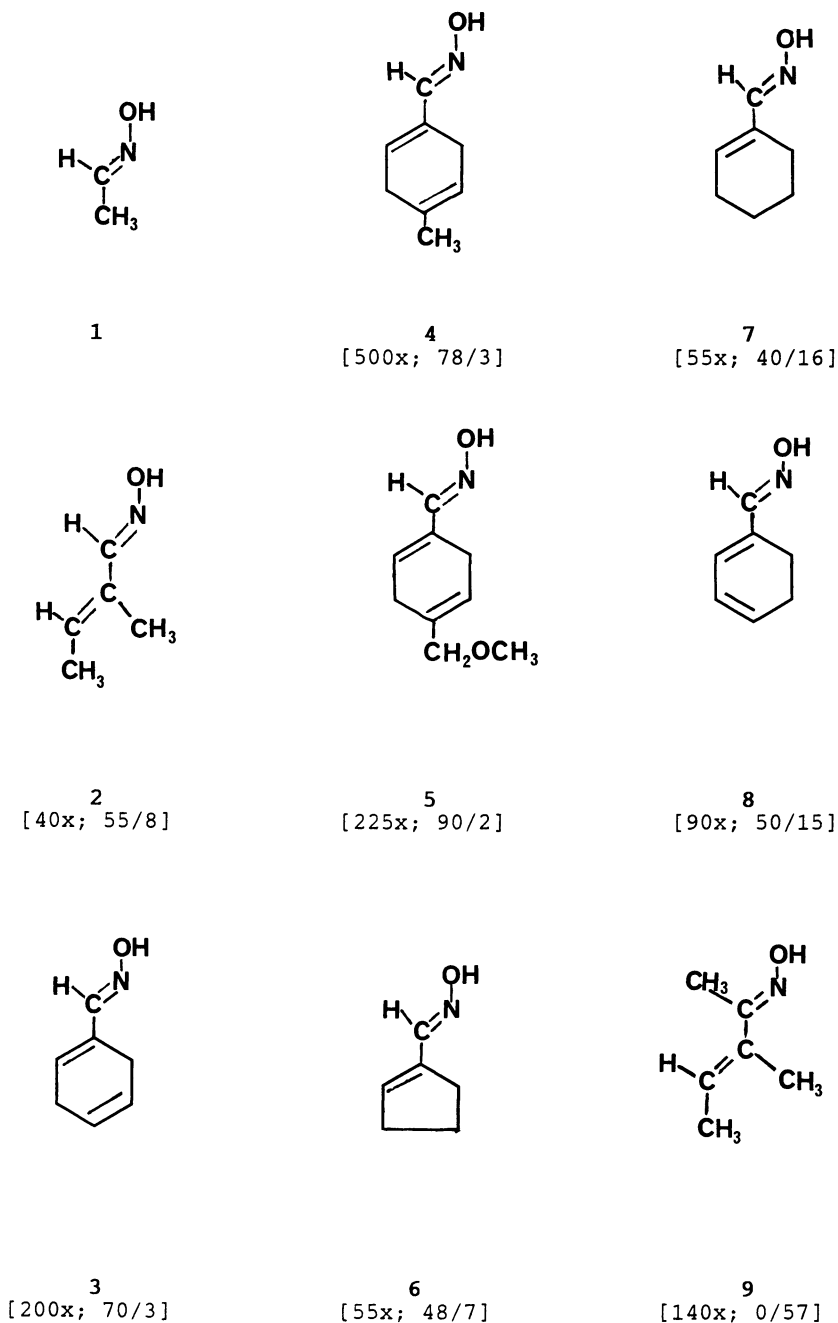


Figure 1. Chart of compounds. The taste potencies of the perillartine analogues are given by X (times sucrose). Also given are the ratios of sweetness to bitterness.

task was to identify the accessible conformations for these compounds. In order to do this, we performed a conformational analysis on the simplest compound, (E,E)-tiglaldoxime, 2, to determine the low-energy conformers (10). The torsional angles of interest were the CNOH and CCCN dihedral angles. The conformational analysis was done with the ab initio 3-21G basis set. A select set of calculations on tiglaldoxime with the 4-31G and 6-31G* basis sets yielded nearly identical results to those employing the 3-21G basis set. After identifying the low-energy conformers, the molecular electrostatic potential (MEP) patterns were calculated for the most potent compounds in Figure 1 in order to define the optimal recognition pattern (11). In addition, the MEP patterns were also calculated for a select set of the less potent compounds in Figure 1 (11). In the present work, we have also extended the MEP analysis to another set of sweet tastants, the 2-substituted 5-nitroanilines, for which structure-activity data are available (12). We selected this set of compounds not only for their topological similarity to the perillartines, but also because there is no experimental evidence to indicate that in lower animals they operate at different receptors (13). The electrostatic potential patterns of a small subset of these compounds were determined (Venanzi, T.J.; Venanzi, C.A., unpublished data) and are compared here to the MEP patterns generated by the perillartine analogues. All the MEP maps were calculated using the 3-21G basis set.

Conformational Analysis of Tastants

Electrostatic potential patterns are generally more dependent on torsional angle changes than on changes in bond angles and bond lengths. In the perillartine analogues two torsional angles are important, namely, the CNOH and CCCN angles. Since interaction with the receptor or solvent can provide energy of stabilization, the biologically active (accessible) conformers may, in fact, be at least 3-4 kcal/mol higher in energy than the most stable conformer in vacuo (14). We used the ab initio 3-21G basis set to study the relationship between the CNOH and CCCN angles and the energy of (E,E)-tiglaldoxime. The GAUSSIAN-82 package (15) was used for the conformational analysis of (E,E)-tiglaldoxime as well as for the calculation of the electrostatic potential of the compounds of interest. The geometry of (E,E)-tiglaldoxime was optimized for every angle in the study. For the optimization of the CNOH angle, the CCCN angle was set to 180 degrees. For the optimization of the CCCN angle the CNOH angle was set to 180 degrees. Our results are given in Table I.

Table I. Energy of (E,E)-tiglaldoxime as a function of <CNOH and <CCCN (3-21G basis set).

	<u>CNOH torsional angle (in degrees)</u>			
	180	120	60	0
Energy	0.0	3.5	8.9	8.4

	<u>CCCN torsional angle (in degrees)</u>				
	180	135	90	45	0
Energy	0.0	4.5	7.7	4.1	3.0

Energy, in kcal/mol, is given relative to the global minimum.

As can be seen from Table I the energy minimum for CNOH rotation is at 180 degrees, but this angle can vary by about 60 degrees and still yield an energy within 3-4 kcal of this minimum. The high energy value at 0 degrees is probably due to H--H as well as lone pair repulsions in the H-CNOH moiety. The results for the relationship of the energy of (E,E)-tiglaldoxime to changes in the CNOH angle were identical to those obtained for (E)-acetaldoxime (1 in Figure 1) (10). Thus, we assumed that the results obtained for the larger analogues would be similar to those obtained for (E,E)-tiglaldoxime. In addition, calculation of the CNOH rotational barrier in (E)-acetaldoxime using the 6-31G* basis set yielded results which were similar to those obtained from the 3-21G basis set (10). The only major difference was the value of the relative energy at 0 degrees; in the 6-31G* basis set this value was less, namely, 6.0 kcal/mol. The presence of the relatively low energy barrier in the region of the CNOH torsional angle around 180 degrees indicates that, in binding, the oxime hydrogen may leave the plane without a great cost of energy.

Table I also shows that, in the variation of the conformational energy of (E,E)-tiglaldoxime with respect to changes in the CCCN angle, the CCCN angle can vary on the order of 45 degrees around the minimum of 180 degrees and still yield a conformer which lies within 4 kcal of the minimum trans planar form (<CCCN and <CNOH =180 degrees). This variation in the CCCN angle will allow the NOH moiety to leave the plane, if necessary, when binding to the receptor occurs. In addition, Table I shows that the cis planar conformer (<CCCN=0 degrees and <CNOH=180 degrees) is within 3 kcal of the energy minimum. On the other hand, the barrier to trans-cis interconversion is on the order of 8 kcal/mol. Finally, if a change is made in the CCCN torsional angle the optimum CNOH angle is always 180 degrees with the global minimum at the CCCN and CNOH

angles equal to 180 degrees (Venanzi, T.J.; Venanzi, C.A. unpublished data).

In our previous work (11) we calculated the MEP patterns of the trans planar conformers of the perillartine analogues. From an analysis of the patterns of the most potent analogues we determined the important features of electrostatic recognition.

Electrostatic Potential Patterns of Perillartine Analogues

The most potent perillartine analogues are the 1,4 analogues (3, 4, and 5 in Figure 1), which have a relatively pure sweet taste. We assume that the MEP patterns of the trans planar form of these compounds define the optimal electrostatic recognition pattern for the perillartine analogues. In Figure 2 the molecular electrostatic potential is shown for two of these analogues. The results of the potential calculations for all the compounds are displayed as contour maps using the "SET MAP" facility of Chem-X (16). In Figures 2a and 2b the potential map for (E)-1,4-cyclohexadiene-1-carboxaldehyde oxime, 3, is shown at 1.6Å and 2.2Å away from the molecular plane. For the 1,4- and 1,3-analogs, the geometry of the oxime group is the fully-optimized 3-21G geometry of tiglaldoxime (see above) while the geometry of the ring system was taken from experimental results (11). The shorter distance represents the distance of maximum pi-electron density while the longer distance might be a more typical distance for an interaction between the ring and an amino acid residue (17). Figure 2 shows that the placement, extent, and orientation of the negative electrostatic potential regions are identical for both maps. There are two negative electrostatic potential regions: one near the oxime moiety and one over the hydrocarbon domain (the cyclohexadiene ring). The orientation angle between the two regions can be determined by drawing two straight lines which pass through the most negative contours in each of the two regions. Then, the orientation angle is defined as the angle formed by the intersection of these two lines. Figure 2 shows that the two negative electrostatic potential regions are nearly parallel, i.e. an orientation angle of 0 degrees. In addition, the two negative regions are separated by a slightly more positive region. The only difference between the MEP patterns at the two distances is the depth of the two regions, with more negative regions occurring at 1.6Å.

The same general pattern occurs for the substituted methyl derivative, 4, (11) and aldoxime, 5. For the choice of geometry of the methoxy group in aldoxime see (18). Figure 2c shows the electrostatic potential at 2.2Å of aldoxime. The only difference

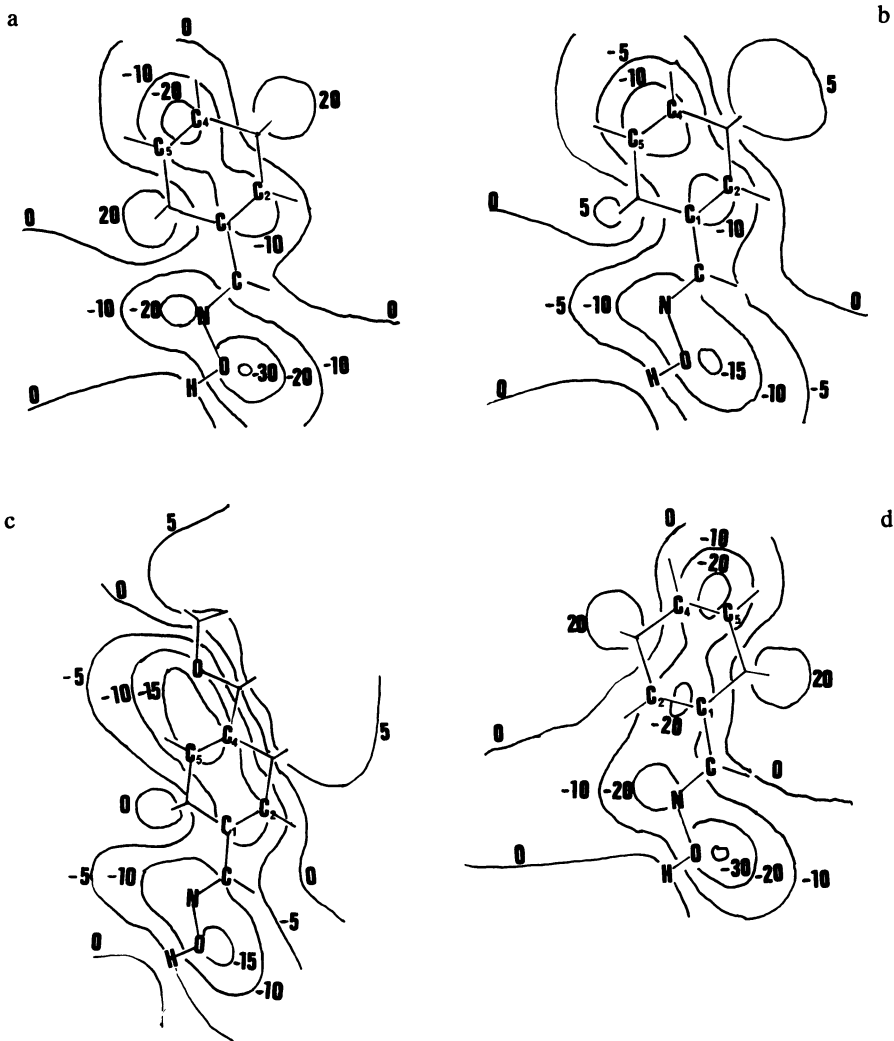


Figure 2a. Electrostatic potential map of 3 in a plane 1.6 Å away from the CNOH plane. Contour levels are in kcal/mol.

Figure 2b. Same as Figure 2a, except that the potential is calculated in a plane 2.2 Å away from the CNOH plane.

Figure 2c. Electrostatic potential map of 5 in a plane 2.2 Å away from the CNOH plane. Contour level units are the same as in Figure 2a.

Figure 2d. Same as Figure 2a, except that the potential is calculated for the *cis* conformer of 3.

However, the dramatic change in the orientation angle between the two regions in the cis conformer would suggest that this conformer does not have the optimal recognition pattern.

From the comparison between the MEP patterns of the cis and trans forms of the 1,4-analogue, one can deduce the MEP patterns of the other accessible conformers of the 1,4-analogue, i.e. conformers with CCCN dihedral angles only varying within 45 degrees of the trans planar form. Their MEP patterns would be nearly identical to the patterns generated by the trans conformer, with the only difference being a small change in the orientation of the two negative potential regions to each other. This result might suggest that there is a "spectrum" of conformations, with nearly identical MEP patterns and centered around the trans planar form, which are initially recognized by the receptor. On the other hand, in the process of binding, one of the accessible conformations would be eventually selected in the interaction with the receptor.

Finally, to determine whether the features of the electrostatic recognition pattern of the perillartines are shared by the MEP patterns of other tastants, we calculated the electrostatic potential patterns of a small select set of the 2-substituted 5-nitroanilines.

Electrostatic Potential Patterns of 2-Substituted 5-Nitroanilines

The substituted nitroanilines are a group of compounds with a molecular shape and volume similar to the perillartines. The potency data for these compounds (12) are not as extensive as that for the perillartines with the most potent member of this series being 5-nitro-2-propoxyaniline. In humans this substituted nitroaniline has a potency equal to 4000X (times sucrose) (13). Since the methoxy derivative should have a similar potential pattern to the propoxy derivative we used this compound as our model, since its smaller size minimized computational expense. The methoxy derivative has a similar potency to that of the 1,4-analogues. In this preliminary work, we took standard 3-21G values (19) for the geometry. We evaluated the pattern for the low-energy planar conformer. It is known from ab initio STO-3G studies that the torsional barrier around the CCNO dihedral angle is of the order of 5 kcal/mol (20). Therefore, like the perillartines, conformers other than the planar form may be accessible. For the methoxy group, we used a planar conformation with the oxygen atom near the amino group. Figures 3a and b give the 3-21G electrostatic potential of 2-methoxy-5-nitroaniline at 1.6Å and 2.2Å. Here, as in Figures 2a and b, the potential over the amino group and off the nitro group

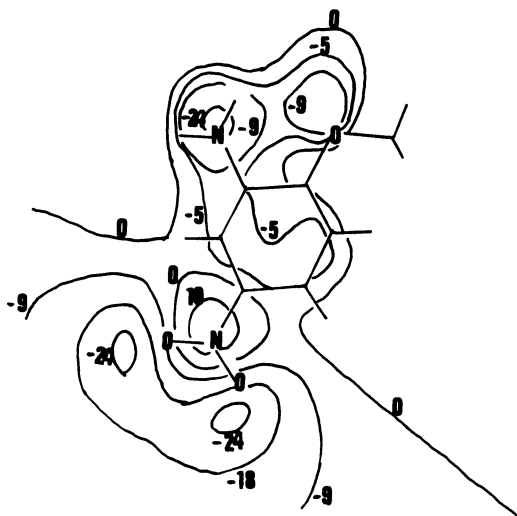


Figure 3a. Electrostatic potential map of 2-methoxy-5-nitroaniline in a plane 1.6Å away from the plane of the benzene ring. Contour level units are the same as in Figure 2a.

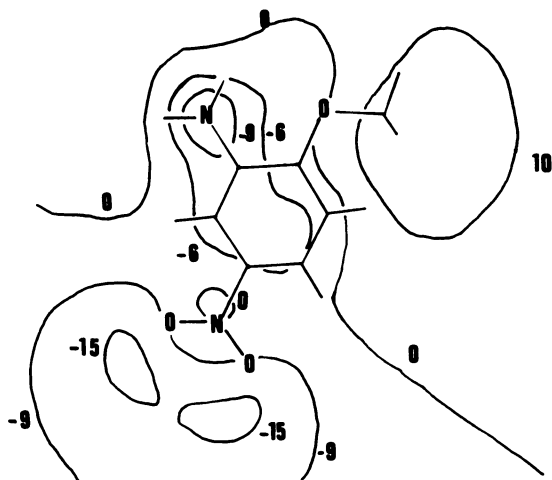


Figure 3b. Same as Figure 3a, except that the potential is calculated in a plane 2.2Å away from the benzene ring.

between the pattern of aldoxime and the unsubstituted 1,4-analogue is the slightly greater extension of the negative potential region over the hydrocarbon domain of the former. Otherwise, in terms of the placement of the negative regions and their orientation to each other, the MEP pattern of aldoxime is identical to that of the unsubstituted 1,4-analogue.

The molecular electrostatic potential pattern of the 1,4-analogues is assumed to be the optimal recognition pattern for the perillartines. The most significant features of this optimal recognition pattern are the extent, placement, and orientation of the potential region over the hydrocarbon domain. In contrast, the calculated MEP pattern of (E,E)-tiglaldoxime, 2, shows a smaller negative potential region in the hydrocarbon domain (11). One would predict that analogues 6 and 7 would have similar MEP patterns to 2. Interestingly enough, the potencies of 2, 6, and 7 are not only similar to each other but also considerably less than that of the 1,4-analogues. Likewise, the 1,3-analogue, 8, with two double bonds in the hydrocarbon domain positioned differently than those in the 1,4-analogues, produces a MEP pattern in that domain which is not oriented parallel to the region near the oxime moiety (11). This analogue has a potency which is also less than that of the 1,4-analogues. Finally, the bitter-tasting ketoxime analogue of tiglaldoxime, 9, has a pattern nearly identical to that of tiglaldoxime, 2. This suggests the possibility of similar complementary electrostatic recognition patterns for the perillartine sweet- and bitter-taste receptors. Also, this indicates quite clearly that the MEP cannot be used as a unique determinant of sweet-taste potency.

Although moderate changes in the CNOH torsional angle do not affect the basic MEP of the analogues, changes in the CCCN angle certainly would affect the placement and orientation of the potential region over the hydrocarbon domain. Recently, the MEP patterns of the *cis* planar forms of the analogues were determined (Venanzi, T.J.; Venanzi, C.A., unpublished data). In Figure 2d the MEP pattern of the *cis* form of the 1,4-cyclohexadiene analogue, 3, is given. As can be seen from the figure, two negative potential regions are present, one around the oxime moiety and one over the hydrocarbon domain. The depth, shape and extent of these regions are nearly identical to the depth, shape, and extent of the regions in the MEP pattern of the *trans* conformer (see Figure 2a). The only difference in the MEP patterns of the *trans* and *cis* forms is in the orientation angle between the two regions: the region over the hydrocarbon domain in the *cis* form is perpendicular to the region around the oxime moiety, rather than parallel as is found in the *trans* form.

decreases in magnitude going from 1.6Å to 2.2Å. On the other hand, in both cases, there are two negative electrostatic potential regions, one off the nitro group and a second over the benzene ring, with a positive region between them. In addition, the MEP pattern of the benzene ring is oriented fairly parallel to the MEP pattern off the nitro group. As can be seen from these maps, the electrostatic potential of the methoxy compound is more similar to that exhibited by the trans rather than the cis conformer of the 1,4-analogue of perillartine, 3. This same basic electrostatic potential pattern is found for other substituted nitroanilines (Venanzi, T.J.; Venanzi, C.A. unpublished data). We are currently investigating the low-energy conformers of these systems as well as the effect of conformational changes on the MEP of these systems.

In summary, our preliminary work on the nitroanilines indicates a similar MEP pattern to the 1,4-analogues of perillartine. This result is significant since from the viewpoint of substructure (functional groups and ring systems) these two sets of tastants are not chemically very similar.

Conclusions

The molecular electrostatic potential patterns of the perillartine analogues and the nitroanilines suggest the possibility of a common electrostatic recognition pattern for these compounds and, by implication, a common electrostatic potential pattern for their receptor(s). Two negative electrostatic potential regions are found in the MEP patterns of these two classes of tastants: (1) a deep negative well around the CNOH moiety in the perillartines and a comparable well off the nitro group of the nitroanilines and (2) a well of lesser depth associated with the ring system. Analysis of the electrostatic potential pattern provides data to begin formulation of a model of tastant-receptor binding. This two-step model may include features such as (1) an electrostatic interaction off the oxime or nitro moieties, followed by (2) the formation of a stacking complex between the ring system or hydrocarbon domain and a receptor residue. In the case of a stacking complex, the electrostatic component may not necessarily be the major term in the binding energy. In addition, dispersion forces and steric factors (21) are probably important for understanding not only tastant-receptor binding but also taste potency (such as the difference in taste potency between 5-nitro-2-propoxyaniline and 2-methoxy-5-nitroaniline). Yet the electrostatic component still determines the mutual orientation of the interacting partners. Thus, even though

non-electrostatic interactions are important in binding and activation, the electrostatic potential analysis is a necessary and informative first step to obtain information about (1) the complementary electrostatic features of the receptor, (2) the orientation of the tastant in the receptor site, and (3) the possible binding mechanism.

Our future work will involve the development of a binding model based on our electrostatic potential studies of these and other tastants. First, we will investigate rigid sweet-tasting systems such as saccharin and acesulfame (biologically active in the anionic form) to determine their electrostatic recognition patterns. In our earlier work on the electrostatic potential patterns of the acesulfame anion (22), we found two deep negative electrostatic potential regions with a shape and extent similar to the patterns of the perillartine analogues. On the other hand, the orientation between the two regions in acesulfame was different than the orientation in the perillartines. Although the perillartines can access a conformation which might yield a potential pattern similar to that of the acesulfame anion, it is not clear whether the "bare" anion is the correct structure for the MEP analysis. We have now begun a study of the hydrated anion. If the MEP patterns of the perillartines and the nitroanilines in certain accessible conformations are the same as the MEP patterns of these rigid tastants, that would imply common complementary receptor features for these diverse tastants. In addition, the possible conformations of the perillartines and nitroanilines at binding could be determined. Then, a quantum mechanical "supermolecule" study which would include a model of the receptor site could be undertaken to determine the electronic features of the interaction including electrostatic, polarization, and charge transfer effects. From this study the relative importance of dispersion forces in tastant-receptor binding may emerge.

Acknowledgments

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Chapter 15

Why Does a Sweetener Taste Sweet? A New Model

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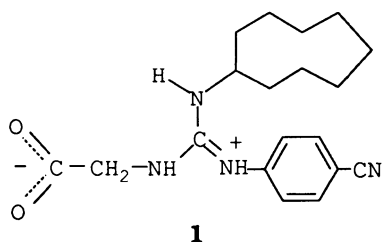
A model illustrating the molecular features which allow a molecule to initiate a sweet sensation in man is described. This model is mainly deduced from structure-activity relationships between new series of intensely sweet compounds recently discovered in our Laboratory. The model assumes the existence in the sweet receptors of eight recognition sites capable of identifying eight optional interaction sites in sweeteners, designated as AH, B, G, D, Y, XH, E₁ and E₂. The simultaneous binding of two of these sites can generate a sweet response, but four interactions (via sites AH, B, G and D) are necessary to induce extremely potent sweet activities.

Given that various attempts to find common stereochemical features between sweet substances have already been made (1-4), the proposed models have been too simple to explain the activity of complex molecules. They are either not specific enough (including too large a number of compounds, even non sweet molecules) or too specific (relevant to only a few active molecules) to provide a reliable predictive value. The most widely accepted and relevant molecular theory in the field of sweet taste is that put forward in 1963 by Shallenberger and Acree (1,2) who proposed the existence of an AH,B system in sweeteners, H being an acidic hydrogen linked to A, with A and B being two electronegative atoms separated by 0.28-0.30 nm. In this theory, AH was OH or NH, B an oxygen atom in groups such as CO₂H, SO₃H, SO₂, CO or NO₂, or the nitrogen atom of CN, or even a halogen atom.

Shallenberger and Acree suggested that this AH,B system can interact with a complementary AH,B system in the sweet receptors through two simultaneous hydrogen bonds to form the active

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complex. In 1972, Kier (3) proposed the existence of a third binding site involving dispersion forces. This hydrophobic site (now designated as site G) could cooperate with the AH,B unit to induce higher sweetening activities; the distances between this site and A or B atoms were estimated to be approximately of 5.5 nm for B-G and 3.5 nm for AH-G (3,4). In 1980, we demonstrated that CO₂H and NO₂ (or CN) groups, until then considered as the same B groups in the AH,B system, must in fact participate by specific and distinct interactions with the receptor (5,6). We concluded that, in addition to the above three interaction points, a fourth new binding site, named D, had to be retained (7). The significance of this finding was demonstrated when, in 1982, we prepared a new very potent sweetener which was 14,000 times as sweet as sucrose (compound **7**, Table I). This compound was a hybrid between two other sweeteners, the 4-cyanophenylcarbamoyl-β-alanine and aspartame, respectively 450 and 200 times as sweet as sucrose (potencies given on a weight basis) (compounds **12** and **14** in Table I) (8). For the first time, this high sweetening activity was obtained through the combination of the four above interaction sites in one structure. In 1987, the improvement of the various criteria required for these four binding sites led us to the discovery of the most potent sweetening agent known to this day. This compound, N-[N-cyclononylamino(4-cyanophenylimino)methyl]glycine, which we now call sucrononic acid (**1**), has a potency of 200,000 times that of sucrose (9,10).



On the basis of these findings, structurally different sweetening agents were reviewed (see Table I for some typical examples), in the search for common patterns of similar atoms or groups, and a new theory for the molecular mechanisms involved in the sweetening activity (Table I) is presented.

The existence of a receptor containing at least eight specific recognition sites is proposed. These sites correspond sterically and chemically to eight optional interaction sites in sweeteners and are designated as sites AH, B, G, D, Y, XH, E₁ and E₂. The spatial arrangement proposed for these sites is given in Figure 1, and their average relative distances and their Cartesian coordinates are given in Tables II and III.

The simultaneous interaction of a sweetening agent with all of these receptor recognition sites is not required in order to

TABLE I. Postulated interaction sites of some sweeteners. To elicit sweetness, the simultaneous binding of the eight sites assumed to be involved in the sweetener-receptor interaction is not a prerequisite.

Cpd No.	Abbr. Name or Formula*	Approx. potency (sucrose=1)	Interaction sites									
			AH	B	G	D	Y	XH	E ₁	E ₂		
1	CN.PhNH ⁺ =C(NHC-Nonyl)Gly	200 000	NH (Ph)	CO ₂ ⁻	c-Nonyl	CN						
2	diCl.PhNH ⁺ =C(NHCHMePh)Gly	120 000	NH (Ph)	CO ₂ ⁻	CHMePh	Cl						
3	(NO ₂ .PhNHCS)Aspartame	55 000	NH (α)	CO ₂ ⁻	Bz	CN	CO ⁺	CO ⁺	NH ⁺	CO (OMe)		
4	(CN.PyRNHCS)Aspartame	50 000	NH (α)	CO ₂ ⁻	Bz	CN	CO ⁺	CO ⁺	NH ⁺	CO (OMe)		
5	L-Asp-NHCH (CO ₂ Me)CO ₂ Fn	50 000	NH ₃ ⁺	CO ₂ ⁻	Fenethyl		CO ⁺	CO ⁺	NH ⁺	CO (OMe)	CO (OFn)	
6	CN.PhNH ⁺ =C(NHSO ₂ Ph)Gly	45 000	NH (Ph)	CO ₂ ⁻	Ph (SO ₂)	CN	SO					
7	(CN.PhNHCO)Aspartame	14 000	NH (α)	CO ₂ ⁻	Bz	CN	CO ⁺	CO ⁺	NH	CO (OMe)		
8	CN.PhNH (=-NCN)Gly	7 000	NH (Ph)	CO ₂ ⁻		CN	CN (NH)					
9	CN.PhNH ⁺ =C(NH)Gly	2 700	NH (Ph)	CO ₂ ⁻		CN						
10	NO ₂ .PhNHCSNH (CH ₂) ₂ CO ₂ ⁻	2 400	NH (CH ₂)	CO ₂ ⁻		NO ₂						
11	Suosan	700	NH (CH ₂)	CO ₂ ⁻		NO ₂						
12	CN.PhNHCONH (CH ₂) ₂ CO ₂ ⁻	450	NH (CH ₂)	CO ₂ ⁻		CN						
13	Saccharin	300										
14	Aspartame	200	NH ₃ ⁺	CO ₂ ⁻	Benzo		CO	CO	NH	SO	SO	
15	(6.Cl.Tryptaminy)5.tetrazole	100	NH ₃ ⁺	CN ₄ ⁻	Ph		CO ⁺	CO ⁺	NH ⁺	CO (OMe)	Cl	
16	Cyclamic acid	50	NH	SO ₃ ⁻	c-Hexyl							
17	D-Tryptophan	35	NH ₃ ⁺	CO ₂ ⁻	Indolyl							
18	Ethylene glycol	0.6	OH		Indolyl							OH
19	cis-1,4-c-Hexanediol	0.3	OH									OH

* Abbreviations : Ph = phenyl substituted; CN, Ph = 4-cyanophenyl; Gly = glycine (-NHCH₂CO₂⁻); diCl, Ph = 3,5-dichlorophenyl; NO₂, Ph = 4-nitrophenyl; Aspartame = L-aspartyl-L-phenylalanine methyl ester; Asp = L-aspartyl; Bz = Benzyl group; CN, Pyr = 2-cyanopyrimidin-5-yl; Fn = fenchyl; † Peptide bond. Cpd's 1 and 2 are described in (9); 3 and 7 in (8); 4 in (16); 5 in (17); 6, 8 and 9 in (18); 10 and 12 in (7); 11 in (19); 13, 14, 16-18 in (20); 15 in (21); 19 in (22).

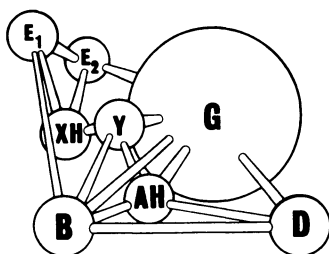


Figure 1. Spatial arrangement of the eight potential interaction sites of sweetening agents, with (i) four high-affinity sites designated as sites B for an anionic group (CO_2^- , SO_3^- or CN_4^-), AH for a hydrogen-bond donor group (NH, OH), G for a hydrophobic group (hydrocarbon group) and D for a hydrogen-bond acceptor group (CN, NO_2 , Cl) and (ii) with four secondary sites designated as Y, XH, E_1 , E_2 sites, Y, E_1 , E_2 being hydrogen-bond acceptor ligands (CO, halogen atoms) and XH a hydrogen-bond donor group (NH, OH).

generate a sweet response, a lower number of sites often being sufficient to initiate a sweet taste. The sweetness potency of a compound will depend both on the number of active sites involved in the molecule-receptor interaction and on the effectiveness of each individual interaction. The interaction efficiency, which is in fact the main factor, is strictly dependent on the space-filling properties of the molecule (size and shape) and on the nature, availability and orientation of the active groups involved in the interactions.

Table II. Average distances* (in nm) between the eight interaction sites identified in sweeteners.

Sites	AH	B	G	D	Y	XH	E ₁
E₂	0.70	0.70	0.45	1.15	0.45	0.30	0.30
E₁	0.80	0.75	0.70	1.25	0.50	0.35	
XH	0.45	0.45	0.55	1.10	0.32		
Y	0.35	0.50	0.45	0.80			
D	0.70	0.90	0.90				
G	0.60	0.80					
B	0.28						

* These distances are the intervals separating the points characterized, for B by the average point between the nuclei of the two oxygen atoms of a CO₂⁻ group, for AH and XH by the nucleus of the hydrogen atom in an NH or OH group, for Y, E₁, E₂ by the nucleus of the oxygen atom in a CO group, for D by the nucleus of the nitrogen atom in a CN group, for G by the center of a cyclononyl-type hydrocarbon group represented by an approximately spherical volume with a radius of about 0.4 nm.

Table II shows that several couples of hydrogen donor and hydrogen acceptor groups separated by approximately 0.30 nm are present in this model (AH-Y, AH-B, XH-Y, XH-E₁, XH-E₂). Such a feature was identified in sweeteners by Shallenberger and Acree and was the basis of their theory with the postulate of a unique AH,B system (1). Table II shows also that sites B (CO₂⁻) and D (NO₂/CN) are approximately equidistant (0.9 nm) from the hydrophobic component G. This particularity may explain the structural analogy initially attributed to these two groups (1-3), before we demonstrated in 1981 their difference for sweet activity (5-7).

Table III. Cartesian coordinates of the eight interaction sites identified in sweeteners.

AH	2.11	0.39	-1.79
B	0.00	0.00	0.00
G	3.55	5.94	-4.01
D	8.86	0.00	-0.00
Y	2.00	4.11	-0.49
XH	-1.01	3.86	-1.83
E₁	-1.78	7.04	-0.22
E₂	-0.83	6.20	-3.03

Table I shows the chemical homogeneity of the ligands retained as AH, B, G, D... sites in sweeteners and allows us to consider the probable nature of the physical interactions involved in the receptor recognition. Site AH is a hydrogen-bond donor group, and is represented by NH or OH groups. Site B is an anionic group such as CO₂⁻, SO₃⁻ or CN₄⁻ (tetrazolyl) groups, forming an ionic bond with the receptor. Site G is a hydrophobic group, such as the alkyl, cycloalkyl or aryl groups, involved in London-van der Waals forces and hydrophobic interactions. Site D is a hydrogen-bond acceptor moiety principally represented in Table I by CN, NO₂ or Cl. Site Y is a hydrogen-bond acceptor ligand such as CO, SO₂, NO₂, CN or halogen. Site XH, structurally similar to site AH, is a hydrogen-bond donor group such as an NH or OH group. Finally, sites E₁ and E₂, often working simultaneously, are hydrogen-bond acceptor groups such as CO, SO₂, intramolecularly hydrogen-bonded hydroxyl groups, or halogen atoms.

Sites AH, B, G and D are defined as high-affinity interaction sites since they are frequently present in sweet-tasting compounds, and their simultaneous presence leads to the most potent compounds (compounds 1-4 in Table I). The importance of the D group, which had been ignored until our initial observations (5-7), is now well established. This group is present in almost all compounds having sweet potencies greater than 2,000 times that of sucrose (compounds 1-11; Figure 2).

The other sites, Y, XH, E₁ and E₂, are generally associated with one or several of the other AH, B, G or D sites and are not as crucially important to elicit potent sweetening activities. The binding of a molecule containing two or three of these low-affinity interaction sites, sensitive to the other molecular characteristics (size, shape, electronic and space-filling properties) is expected to induce only low sweetness.

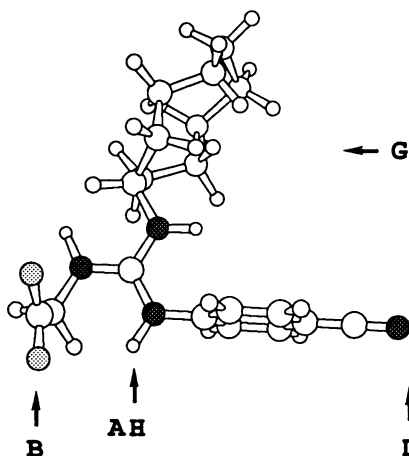


Figure 2. Sucronic acid according to the model of Figure 1 (**B** is CO₂⁻, **AH** is NH, **D** is CN and **G** the cyclononyl group; ● represents nitrogen atoms and ● oxygen atoms).

Conversely, binding of several high-affinity interaction sites (AH, B, G or D) will induce significantly high sweetness. In this case, the overall characteristics attached to each binding site (precise chemical nature and relative distances) as well as the whole environmental hindrance of the complementary receptor features are sufficiently specific to explain the effect of chirality on sweet activity and to minimize the existence of inactive compounds which fits the model.

Is the sweet taste activity mediated through a single receptor or several distinct receptors? The answer is still pending. Some authors have suggested that only multiple receptors can explain the differences existing in chemical structures, threshold sensitivities or other physiological data for various sweeteners (11-13). Some other physiological data are standing in favor of a single type receptor, for instance the same inhibition by gymnemic acid for different classes of sweeteners (14,15). This model suggests the existence in humans of a single type of receptor with at least eight recognition sites and able to interact with sweeteners of different structural classes. This possibility does not, however, exclude the existence in man or animals of other sweet receptors containing a lower number of these recognition sites, and interacting with a more limited number of sweeteners.

Conclusion

The present model describes the molecular features which allow a molecule to initiate a sweet sensation in man. It includes and clarifies the three previously postulated sites (Shallenberger and

Acree's AH,B sites and Kier's lipophilic site). It assumes the existence in the sweet receptors of at least eight recognition sites with the capacity to identify at least eight complementary and optional interaction sites in sweeteners. The spatial arrangement and relative distances between these sites are also given.

Acknowledgments

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Chapter 16

Three-Dimensional Model for the Sweet Taste Receptor

Development and Use

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A model has been developed to aid in understanding the structure-taste relationships of dipeptides, guanidines, urea-dipeptides and related compounds. This model was built using conformational analysis and electrostatic potential calculations on a few highly potent representative compounds. It is consistent with known structure activity relationships in these series of compounds, and has been used to (1) design new potent analogues of known sweeteners; (2) identify structural and electronic features leading to bitterness; (3) design inhibitors of sweetness which may be useful in classifying sweeteners on the basis of receptor type. It is currently being used to design novel sweeteners.

The transduction of sweet taste is believed to be initiated by receptor protein(s) located on the surface of the taste cell. To date no receptor has been isolated, although many attempts have been made (1-3). Given the lack of an isolated receptor protein, models of the sweet taste receptor must be inferred from the available structure activity relationships (SAR). Models of the sweet taste receptor have grown in their complexity over time as new sweeteners have been discovered.

Shallenberger and Acree were the first to try to explain sweetness in terms of a model. Their model contains two recognition units, a hydrogen bond donor (AH) and a hydrogen bond acceptor (B) located from 2.5 to 4.0 angstroms apart (4). This model allows one to rationalize the sweet taste of most sweet compounds, but a large number of non-sweet compounds would be predicted to be sweet by this model. Shallenberger and coworkers later added a "steric barrier" to their model (5) to account for the sweetness of D but not L amino acids. Even with this additional constraint, the model can not be used to reliably predict new sweet

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compounds. The Shallenberger model has been used as a starting point for several other models. Kier (6) added a "dispersion binding" or hydrophobic site, 3.5 angstroms from the AH site and 5.5 angstroms from the B site. As the number of sweetener classes has increased, so has the optimal length of the hydrophobic region. Van der Heijden and coworkers (7) claim the optimal AH to hydrophobic site length to be 10.8 angstroms.

Mazur et al. (8) noticed the sweetness of aspartic acid based sweeteners depends on the stereochemistry and size of the substituents on the carbon atom attached to the amide nitrogen. Sweetness occurs when the groups are arrayed as shown in Figure 1. Potencies are maximized when the largest substituent is hydrophobic and polarizable. These observations led several groups to further investigate the structural requirements of the large hydrophobic group (9-11).

Temussi and coworkers selected a conformation of aspartame which they believe to be the active (sweet) one, using NMR and conformational energy calculations (12-14). Based on this conformation, they have developed a receptor site model. This model is consistent with the majority of peptide based SAR but was found to be inconsistent with NutraSweet internal SAR (D.E. Walters and D.A. Jones, unpublished observations). The Temussi NMR data was re-interpreted by van der Heijden et al. (15) to produce a model close to the one described by Kier (6) with a hydrophobic group consistent with the work of Brussel (9). Unfortunately, van der Heijden's work yields a model that is not consistent with the observed sweetness of aspartyl anilides (16), trifluoroacetyl-aspartame (17), and the arylurea-dipeptides (18).

Many models for the sweet taste receptor have been proposed, but all of the models are limited in their value as tools to design new sweeteners. Many of the models are two dimensional in nature, and most do not account for the non-local charge distribution. In the work summarized in this paper, we have constructed a model which is consistent with SAR of the dipeptide and related sweeteners. The model is inherently three-dimensional and does account for non-local electronic effects. In addition to rationalizing known SAR, we will show the use of this model in a predictive and sweetener design mode.

Development of the Model

The construction of our model can be divided into four phases. The first phase was to select appropriate compounds upon which to base the model. Second, conformational analysis was done to identify low-energy conformers of these compounds. The next phase involved superimposing low energy conformers of the chosen compounds in such a way as to give a common pattern of functional groups. The final step was to partition the model into molecular recognition sites.

Compound selection. It is not clear whether there is a single sweet taste receptor or multiple receptors (19). We chose compounds which we believe may act at a common site since they have similar molecular recognition units (carboxylate group, several amine/amide groups, a large hydrophobic group). In addition, we chose compounds with high potencies, since we expect such compounds to have better fit to the receptor site. Compounds **1-5** shown in Figure 2 were selected as starting points for our model. Compounds **1** and **2** are guanidine-acetic acid derivatives discovered by Nofre and Tinti (20). Compound **3** is an arylurea derivative of aspartame, also discovered by Nofre and Tinti (18). The aspartic fenchyl ester derivative **4** is the most potent of the aspartic-aminomalonyl diesters described by Fujino (10). Finally, we included aspartame, **5** (8), as the prototype compound in this series.

Conformational analysis. In our initial work, we used the MMFF (molecular mechanics force field) program of Chemlab (21). This is a modified version of Allinger's MM2 program (22). We generated force field parameters where necessary (urea and guanidinium groups) using the method of Hopfinger and Pearlstein (23). Later calculations were done using Still's MacroModel program (24), again using parameters we generated for urea and guanidinium groups. A working assumption was made that the "active" conformation should be near a local energy minimum (not necessarily the global minimum). Another assumption was that carboxylic acid, amine, and guanidine groups exist in the ionic form in aqueous solution.

Superimposition of conformers. The high-potency guanidine sweeteners of Nofre and Tinti (20) were particularly useful in identifying the best way to superimpose the compounds in our series. Whereas dipeptides such as aspartame have hundreds of accessible low energy conformers, the planar guanidinium group limits the number of conformers available. We calculated the electrostatic potential (EP) for each of these molecules to further aid in the conformation selection process. The EP provides a means of unambiguously overlaying structures that do not have identical functional groups. The EP was calculated using a point charge approximation. The charges were obtained using the INDO/S method (25). Low energy conformers were superimposed visually on a computer graphics screen to provide optimum steric overlap and electrostatic matching. Finally, a van der Waals surface over the superimposed compounds was generated, with a composite EP mapped onto the surface.

Molecular recognition sites. The use of EP calculations allows for the logical division of the model into several regions. The first

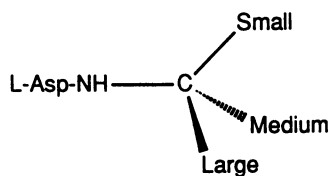


Figure 1. Stereochemistry of L-aspartyl-derived sweeteners

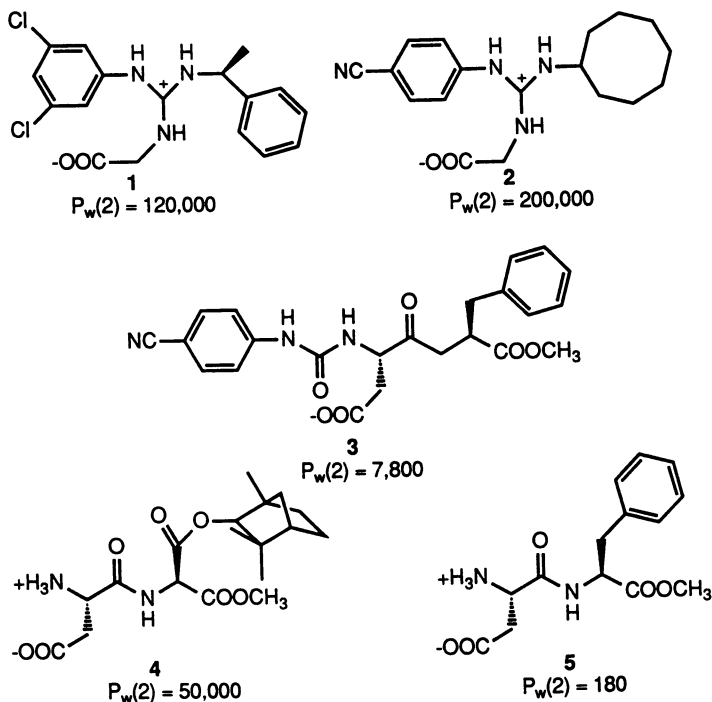
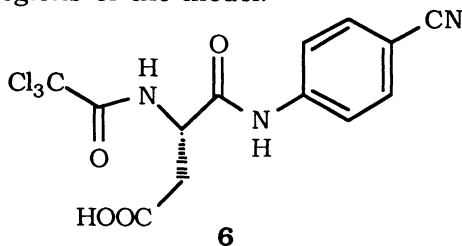


Figure 2. Compounds used as templates for the model.

region is dominated by an acidic group defined by the carboxyl groups of the model compounds. Region two, the major NH site, is defined by the free amine of aspartame and the aryl-NH of the guanidine sweeteners. A second NH site is defined by the dipeptide sweetener's amide NH and the hydrophobic NH of the guanidine sweeteners. A large hydrophobic region dominates one side of the model and its shape is quite well defined. The methyl esters of the dipeptide sweeteners define the ester region of the model. The final region of the model is characterized by an electron deficient aryl ring in the guanidine and arylurea-dipeptide sweeteners. We designate this region the pi-stacking region. Figure 3 show a schematic representation of the model with the parts described above labeled.

Applications of the Model

We proceeded to test the model to see whether it is consistent with known structure activity relationships in the aspartic dipeptides, aspartic amides, aspartic anilides, arylurea-dipeptides, and guanidine classes. The model is consistent with all of these classes. Compounds which have the correct pattern of EP have a sweet taste. Compounds which effectively fill the hydrophobic region tend to have the highest potencies; compounds which only partially occupy the hydrophobic region and compounds which extend beyond the boundaries of this region tend to have low potencies or non-sweet tastes. Compounds which have an electron-deficient aromatic ring in the pi-stacking region tend to have enhanced potencies. Figure 4 illustrates the case of an aspartyl anilide, trichloroacetyl-L-aspartyl-*p*-cyanophenyl anilide **6**. The steric fit into the model is quite good. The electronic fit is also quite good since the molecular recognition sites are close to corresponding regions of the model.



To test the predictive value of the model, we made predictions of the sweetness of compounds **7** and **8**. Based on its good steric and electronic fit, we expected compound **7** to have a relatively high sweetness potency. On the other hand, the isopropenyl group of compound **8** extends well beyond the steric boundary of the model and was therefore not expected to be very potently sweet. Compound **7** was subsequently found to have a potency of >20,000 times sucrose at a 2% sucrose sweetness level; compound **8** was

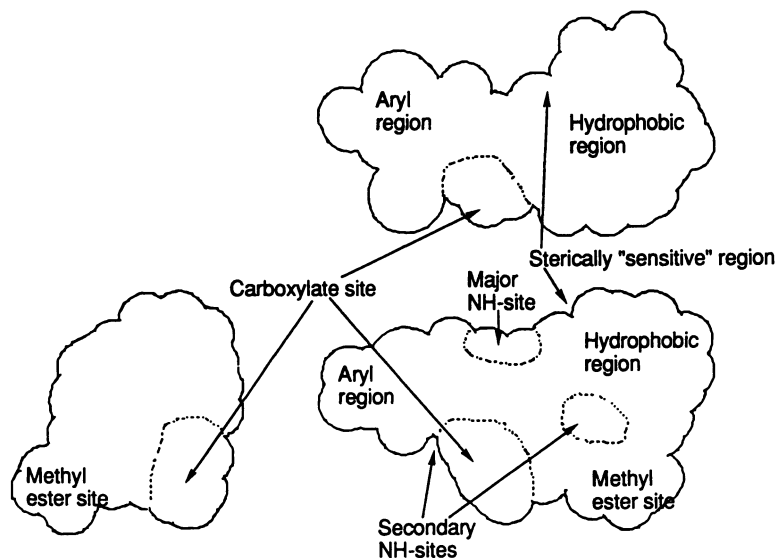
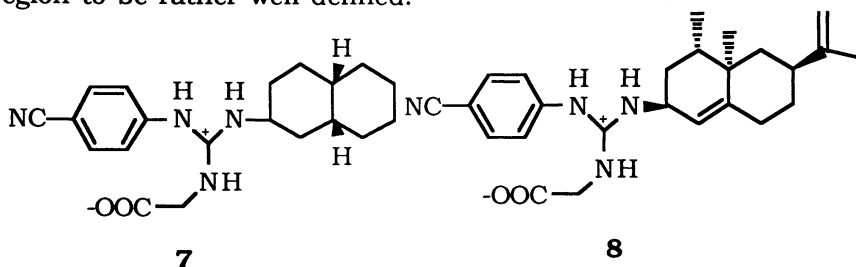


Figure 3. Three orthogonal views of the van der Waals surface of the receptor site model, with recognition sites labeled.

found to have a potency of <100 times sucrose. We have consistently found the spatial requirements of the hydrophobic region to be rather well defined.



The importance of the EP fit is illustrated by compounds **9** and **10**. These compounds both fit the model well sterically but have an aromatic nitrogen atom near the major NH site of the receptor model. The lone pair electrons on the ring nitrogen atom dramatically influence the EP of the adjacent aryl-NH group. Compounds **9** and **10** are quite bitter, in contrast to their carbon-analogs which are intensely sweet. In general, changes in the EP around the major NH site result in bitter-tasting compounds.

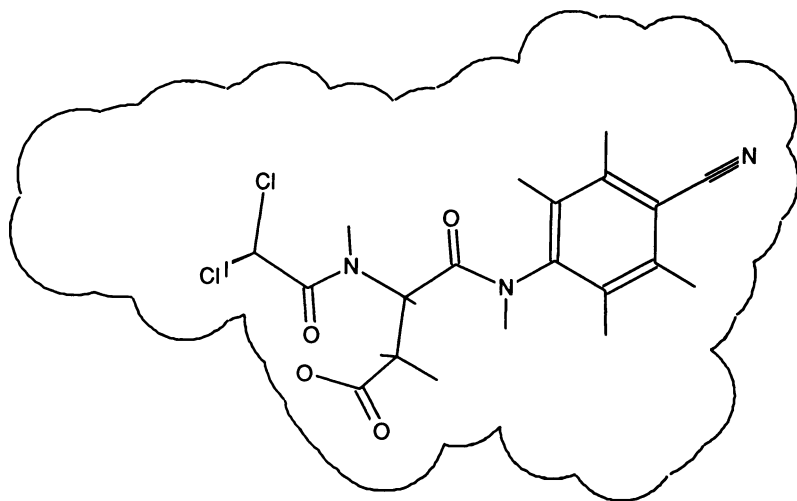
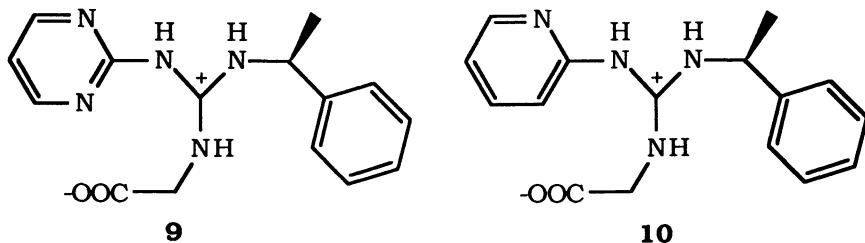
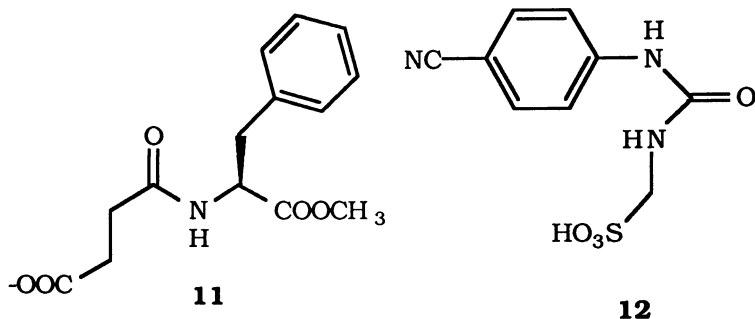


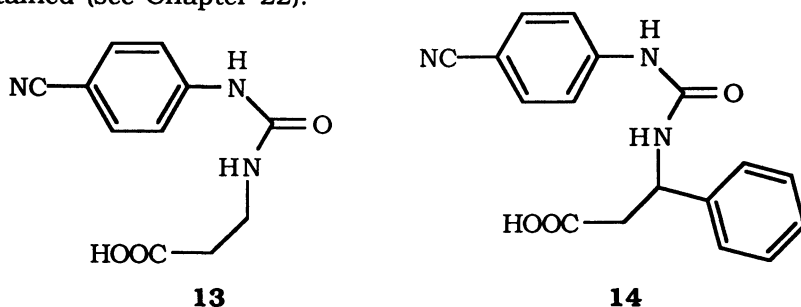
Figure 4. Trichloroacetyl-L-aspartyl-*p*-cyanophenyl anilide in the receptor model.



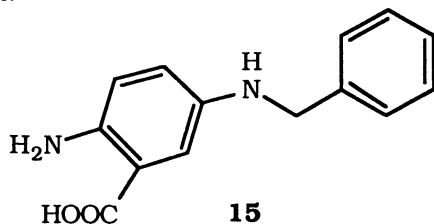
We expected that the removal or modification of certain recognition elements from a sweet compound would lead to sweet taste inhibitors, compounds which could bind to the receptor site but not trigger the sweet response. This prediction was born out in compounds **11** and **12**. Removal of the amino group of aspartame (**5**) led to compound **11**, succinyl-L-phenylalanine methyl ester, which blocks the sweet taste of aspartame. Changing the nature of the acidic group also produces a compound that effectively blocks sweet taste. Compound **12** (**26**) is an analog of the sweet compound **13** (**27**), in which one methylene group has been removed and the carboxylate group is replaced by a sulfonic acid.



In another instance, the model was used to improve the potency of a known sweetener. Compound **13**, an analog of suosan synthesized by Nofre et al. (18), lacks any functional groups to fill the hydrophobic group of the model. The addition of a phenyl group to compound **13** at the carbon beta to the carboxylate group yields compound **14** (28). By occupying the hydrophobic region of the model, an increase in potency of one order of magnitude was obtained (see Chapter 22).



The model has also been used to design new lead compounds, which are not simple modifications of known sweet compounds. Compound **15** was designed using the model and found to be moderately sweet.



Summary

We have constructed a model of the sweet taste receptor using molecular design techniques. This allows us to define both steric and electronic criteria necessary for sweetness within the dipeptide and guanidine series. The model has been applied to the rationalization of known SAR, the design of higher potency analogs

of known sweeteners, and the design of novel sweeteners. The method of model construction outlined above can be applied to other classes of sweeteners as well, in order to assist in the design of new sweeteners.

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Chapter 17

Sweet Taste Transduction

A Molecular-Biological Analysis

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While the chemistry of sweet tasting compounds has been extensively studied (1-5), precious little has been known until recently on the cellular mechanisms of sweet taste transduction. Work in the authors' laboratory, as well as in several others, has begun to shed light on this problem. Specifically, evidence has accumulated in the last three years, suggesting that sweet taste receptor proteins (as yet unidentified) activate a membrane transduction cascade. This molecular chain of events appears to be very similar to that which is associated with receptors for hormones and neurotransmitters, as well as visual photoreceptors and olfactory receptors (6-8). The proposed transduction cascade includes (see Figure 1):

- (1) A transmembrane protein receptor that binds sweet compounds stereospecifically and subsequently undergoes a conformational transition.
- (2) A membrane amplifier GTP-binding protein (G-protein) of the stimulatory type (G_s).
- (3) The membrane enzyme adenylyl cyclase, that produces an intracellular second messenger cyclic AMP (cAMP).
- (4) The enzyme cAMP-dependent protein kinase (PK-A), which is activated by cAMP and catalyses protein phosphorylation.
- (5) A phosphorylation-gated potassium channel, closed when acted upon by PK-A, thereby leading to sensory cell depolarization and neurotransmitter release.

The initial elucidation of such a mechanism represents a major breakthrough in our understanding of sweet taste transduction.

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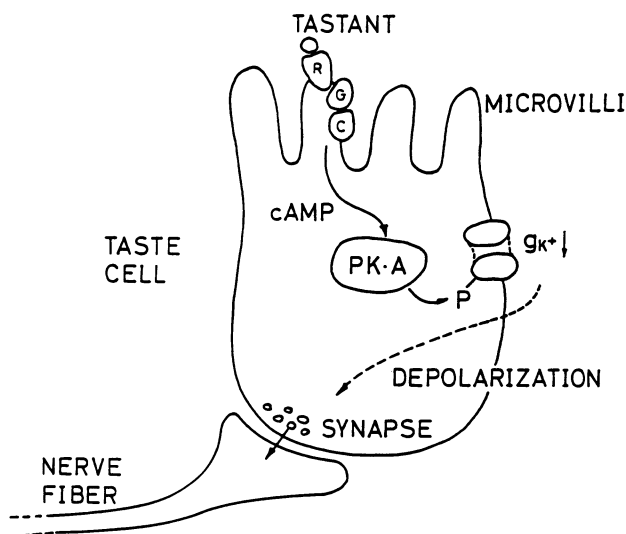


Figure 1. A scheme showing the main molecular components of the mechanism proposed for sweet taste transduction. The tastant (sweet agonist) binds to a receptor (**R**) and activates it. In its active conformation, the receptor can interact with the G-protein (**G**) and catalyze its conversion to the active, GTP-bound form. The complex of active G-protein and adenylyl cyclase (**C**) is catalytically active, generating the second messenger cAMP from ATP. cAMP binds to cAMP-dependent protein kinase (**PK-A**) and stimulates its activity by inducing subunit dissociation. The active catalytic subunit of PK-A catalyzes the covalent attachment of phosphate groups ($\sim\text{P}$) to proteins, specifically to a potassium channel. In this case, channel phosphorylation results in its closure and a decrease in potassium conductance (g_{K^+}), leading to cell membrane depolarization. The latter may cause calcium influx and neurotransmitter release at the synapse between the taste cell and the outgoing afferent nerve fiber leading to the brain. **R**, **G** and **C** are attached to the apical, microvillar membrane of taste cells. PK-A is a cytoplasmic, soluble protein. The potassium channel and the synapse are in the basolateral membrane of the taste cell.

The Enigma of Sweet Reception

Sweet taste reception is unique among biological receptor mechanisms. On one hand, there is obvious stereospecificity, as attested by the fact that only relatively few compounds elicit sweet sensation. The "pharmacology" of sweet taste is highly analogous to that of drugs, as well as neurotransmitter and hormone analogs. Slight chemical variations lead to pronounced changes in sweet "agonist" potency, and often transform a sweet compound into a bitter one. Such transformation is reminiscent of that from an agonist to an antagonist in classical pharmacology.

On the other hand, sweet reception probably represents the "weakest" known receptor mechanism. The affinity toward the natural agonists, mono- and di-saccharides, is represented by a K_d of hundreds of millimolar. This is apparent in the fact that the half maximal electrophysiological, behavioral and biochemical effects of such sugars is attained at concentrations between 0.1 and 1.0 molar (9-13). No other receptor mechanism is known to have such an attribute. Presented with such a high apparent K_d , any pharmacologist would invoke terms such as "non-specific" and "artifactual." How can the contradiction between extreme specificity and very low affinity be reconciled?

It is useful to consider the evolution of sweet taste. The most likely explanation for the low apparent affinity associated with sweet taste function is that the natural ligands need be recognized at very high concentration. An organism satisfied with very dilute sugar solutions cannot fulfill its nutritional needs, and therefore will have a selective disadvantage. On the other hand, chemical stereospecificity is just as important here as it is in any other receptor system: consuming the wrong chemical will surely be detrimental. Thus, it is possible that the special attributes of sweet taste reception evolved under a unique set of evolutionary constraints, which require high specificity concomitant with very low affinity.

What could be the unique molecular properties of sweet taste receptors? All receptors function by way of multiple "Elementary Interactions" (EI's). The pharmacology, x-ray crystallography and molecular modeling of receptor-ligand interactions strongly suggest that receptor specificity and affinity are generated through simultaneous noncovalent interactions, each bearing a small amount of binding energy. EI's could be any of the following: hydrogen bonds, dipole-dipole interactions, ionic pair interactions, hydrophobic interactions and other weak interactions, e.g., van der Waals forces. Each of these EI's typically contributes 1-2 kcal/mole to the free energy change associated with ligand-receptor binding (14). To generate a typical affinity characterized by a dissociation constant K_d in the range of 1 nM to 1 μ M, it is necessary to have 6-9 EI's. This is based on the assumption of a free energy of 1.4 kcal per EI, using the formula for ΔG , the free energy of interaction:

$\Delta G = -2.3 \cdot R \cdot T \cdot \log K_d$ (R is the gas constant). It is the fact that such multiple interactions are sterically ordered that confers specificity upon ligand-receptor binding.

Now, for sweet receptors, a K_d of 100 mM amounts to only one "standard" EI! This, of course, cannot be reconciled with the documented stereospecificity. Furthermore, sweet agonists (similar to other agonists) have to induce the correct conformational transition in their receptor, converting it to the active state, capable of G-protein stimulation. In other words, it is necessary to provide the specificity and allosteric capacity in the ligand-receptor interactions, most likely by multiple EI's, *without* producing excessive free energy of binding. One way by which this contradiction could be reconciled is through the following model (Figure 2): *Low affinity sweet taste agonists interact with their receptors via multiple elementary interactions (EI's), each having a very low free energy of binding..* Such a situation will obtain if the interactions are constrained so that no EI is optimal. Strained non-covalent bond angles and distances as well as steric hindrance effects could jointly produce the desired effect. Some EI's may actually contribute *negatively* to the binding energy (that is, have a repulsive nature). Another possible contributing factor could be conformational constraints, whereby the free energy of EI's is partially expended to induce conformational transitions in the receptor. At present it is impossible to predict with certainty the actual number of EI's in a typical sweet receptor binding site, but it is not impossible that it has even more EI's than a typical drug receptor. The foregoing model has some interesting consequences and predictions:

- (1) Sweet agonists will have a multiplicity of functional groups. Sugars, which are polyhydroxyl compounds, are a case in point.
- (2) The sweet receptor binding site will be large and relatively complex. This point is borne out by recent developments in the field of artificial sweeteners.
- (3) The stereospecificity requirements for sweet compounds will be harder to define, and models for the sweet receptor binding site more difficult to develop. This indeed appears to be the case.

G-Proteins, Adenylyl Cyclase and Sweet Taste Reception

The first evidence that G-proteins are involved in any receptor mechanism came through the realization that some hormones will activate intracellular second messenger production only in the presence of the nucleotide guanosine triphosphate (GTP) (6,7,15). The most well known example of a second messenger-producing enzyme is adenylyl cyclase, which catalyses the formation of cyclic AMP (cAMP). This led to the hypothesis that the receptor contained a GTP binding site, as well as an enzymatic site catalyzing second messenger production. Later, it was found that the

receptor, GTP binding entity and the enzyme constitute separate proteins. Subsequently, the receptor-coupled GTP binding proteins (G-proteins) were identified, purified, and later subjected to molecular cloning.

G-proteins are now known to constitute a very broad family (Figure 3), and their occurrence spans receptor mechanisms from visual photoreceptors through brain neurotransmitters, neuropeptides, hormones, growth factors, and chemotaxis receptors. Functionally, such G-proteins are classified according to the distal mechanism they modulate, and by whether they activate or inhibit such mechanism. Notable among these distal mechanisms are the enzymes adenylyl cyclase, cyclic GMP phosphodiesterase (in vision), phospholipase C (the enzyme that catalyzes phosphatidyl inositol turnover) and several types of ion channels. Major types of G-proteins are: G_s , the stimulatory G-protein that activates adenylyl cyclase; G_i , the inhibitory G-protein that inhibits the same enzyme; G_o , a brain-specific G-protein akin to G_i ; transducin (G_T), the visual G-protein. The G-proteins that activate phospholipase C and ion channels generally belong to the G_i family.

The molecular understanding of chemosensory transduction lagged behind that of other processes. About five years ago evidence began to emerge that a mechanism, including a stimulatory type G-protein and adenylyl cyclase, is central in olfactory transduction. The current evidence for this mechanism is rather extensive, and includes biochemical and molecular cloning reports (16-20), as well as electrophysiological experiments (21).

More recently, attention has begun to be directed toward the molecular basis of taste reception. Considerable indirect evidence has accumulated in the last two decades, suggesting the involvement of cyclic nucleotides in sweet and bitter taste. This includes biochemical, histochemical and electrophysiological reports (22-29). In the last four years experiments have been conducted that more directly address the question of whether sweetener receptors could exert their action via a G-protein/adenylyl cyclase mechanism, as detailed below.

Biochemical Evidence. The first biochemical experiments suggesting the involvement of a G_s -protein in sweet taste reception (13,30) utilized a membrane preparation derived from rat tongue tips, rich in fungiform papillae. Nevertheless, this preparation is not highly enriched in taste cell membranes, since a large fraction of the tongue surface is covered with non-sensory epithelium, and the preparation contains muscle membranes as well. Furthermore, such a preparation is not specifically enriched in that part of the taste cell membranes (the apical microvilli) thought to be related to signal transduction. This is an obvious drawback of the presently available membrane preparations, when compared to the much purer isolated cilia preparations routinely used in olfactory

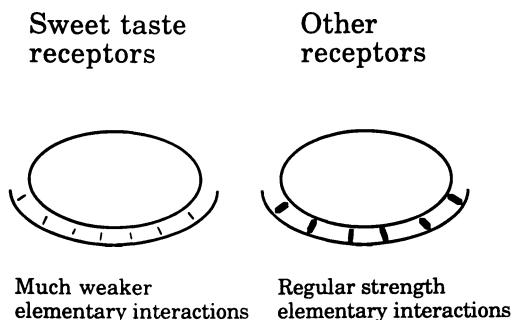


Figure 2. A hypothetical model for the way by which sweet receptors maintain stereospecificity despite their very low affinity (see text).

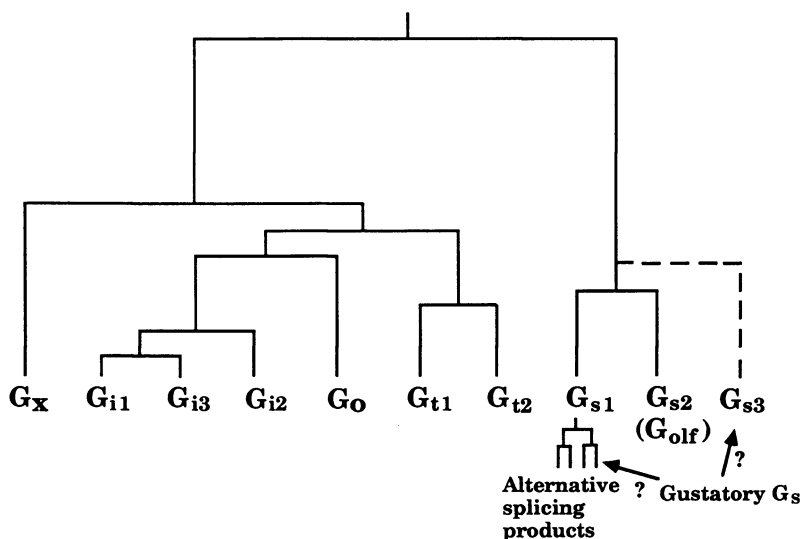


Figure 3. A tree showing the homology relations for different G-proteins heavy (α) subunits. The stimulatory GTP-binding proteins are on a separate "branch," distinct from all others which generally belong to the inhibitory GTP-binding protein type. At present, two genes are known for stimulatory G-proteins: G_{s1} which gives rise to at least four different polypeptide products expressed in different tissues, and G_{s2} (G_{olf}) which is uniquely expressed in olfactory neurons. Two possible scenarios are depicted for the G_s protein involved in sweet taste transduction: (1) it is a new G-protein [G_{s3} "]; (2) it is one of the known G_{s1} products.

research. Future developments will have to resolve this problem. An important step forward is the development of an isolated rat taste papillae preparation, used in conjunction with a cellular cAMP accumulation assay (31).

The rat tongue tip preparation has an adequate specific activity of adenylyl cyclase, comparable to that of many other preparations, e.g., liver and erythrocyte membranes. This specific activity is however considerably (100-1000 fold) lower than that seen in olfactory cilia.

When guanine nucleotides, such as GTP or its non-hydrolyzable analog GTP γ S, are added, the generation of cAMP is enhanced, as expected for a system that includes a G-protein. This in itself is rather common, and cannot be taken as evidence for receptor coupling to a G-protein. The crucial experiment must involve addition of a functional ligand. Here a problem arises: the most obvious sweet-tasting ligands are sugars, and these must be used at their physiological concentrations, namely 0.1-1.0 M. Planning such experiments is thus rather tricky. The exceedingly high ligand concentrations could have various nonspecific effects on the membrane enzymes, effects related to high osmotic pressure, viscosity and simply non-specific binding to proteins. This problem is reminiscent of that encountered in the first biochemical experiments with odorants, where it was found that the necessary concentrations approach 1 mM, which could exert nonspecific effects, e.g., on membrane fluidity.

When the experiments with sugars were performed, much care was taken to carry out the correct controls. Several mono- and disaccharides were found to activate the tongue membrane adenylyl cyclase. That such activation was physiologically significant was supported by the following (13):

- (1) Activation was observed only in the presence of guanine nucleotides. Interestingly, both GTP and GTP γ S were effective synergizers, unlike most other systems, and similar only to turkey erythrocyte membranes.
- (2) Since both guanine nucleotides tested synergized with sucrose and other saccharides, it became important to identify a G-protein activator that could fully activate the tongue adenylyl cyclase, irrespective of the presence of receptor-activating ligands. This indeed was found to be the case for aluminum fluoride (AlF $_4^-$), another known activator of G-proteins. This confirmed that the synergy with guanine nucleotides was not artifactual.
- (3) Sugar activation was tissue specific. It was observed to the full extent only in tongue tip membranes and not in several other membrane preparations: tongue muscle, tongue non-sensory epithelium, skeletal muscle and olfactory cilia.
- (4) Different sugars activated to largely different degrees in the range of 20-150%. This is inconsistent with any explanation in terms of nonspecific, physical effects.

- (5) The order of potency of three different sugars (maltose < glucose < sucrose) was similar to that previously obtained by electrophysiological recordings from the rat chorda tympani nerve (10-12).
- (6) Certain bivalent cations (Cu^{+2} and Zn^{+2}) inhibit the activation of adenylyl cyclase by sucrose, in parallel to their inhibitory effect in electrophysiological recordings (32,33).
- (7) The sugar analog methyl-4,6-dichloro-4,6-dideoxygalactopyranoside inhibits the activation of adenylyl cyclase by sucrose, again in parallel to its inhibition of electrophysiologically recorded responses (34).

A strong corroboration for the significance of the cAMP mechanism in sweet taste comes from the more recent results obtained in isolated rat papillae, monitoring cAMP generation in intact cells. Highly significant enhancement of second messenger generation was observed in response to extracellular sucrose (31).

The Possible Nature of the Gustatory G-Protein and Adenylyl Cyclase. The fact that sugars activate adenylyl cyclase rather than inhibit it suggests the participation of a G-protein belonging to the stimulatory (G_s) class. It is presently unknown which subtype of G_s is coupled to sweet taste receptors. Olfactory reception utilizes a novel, specific G-protein, known as G_{s2} or G_{olf} . This protein is coded for by a separate gene, highly (~90%) homologous to, but different from, the G_{s1} gene. G_{s1} codes for several polypeptide products generated by a process known as alternative mRNA splicing, and expressed in many different tissues. The biochemical properties of the putative gustatory G_s are different from those of G_{s2} (G_{olf}): the latter is strongly activated by both $\text{GTP}\gamma\text{S}$ and AlF_4^- (35,36), while the former shows partial activation with agonist synergy for $\text{GTP}\gamma\text{S}$ and full activation only with AlF_4^- (13). It is possible that gustatory G_s is coded for by a new gene, but more likely it is derived from the G_{s1} gene (Figure 3). If the latter possibility holds, gustatory G_s may be identical to one of the gene products expressed in other tissues, or a new protein variant. Molecular cloning will allow resolution of this problem.

An intriguing correlation with the notion that a transduction mechanism akin to that for hormone receptors is involved in sweet taste responses is afforded by a report showing that an inability to taste sweet (aglycogeusia) is associated with a metabolic genetic disease, pseudohypoparathyroidism (37). Future investigations should determine the exact molecular basis for this rare deficiency. Incidentally, the report that pseudohypoparathyroidism type 1a, arising from a G_{s1} deficiency (38), is associated with olfactory deficits, cannot simply be explained by a break in the peripheral chemosensory transduction cascade. This is in view of the evidence that olfactory G-protein is coded by a different gene, G_{s2} .

Thus, an explanation in terms of the central nervous system should be favored.

No information is available on the nature of gustatory adenyl cyclase. The enzyme has recently been identified in several tissues by a combination of antibody binding, purification and molecular cloning. Brain has two major types: a 110 kDalton calmodulin-sensitive form and a 150 kDalton calmodulin-insensitive form. Olfactory cilia have their unique form, AC_{olf}, which is 180 kDalton in size (39). The taste system may have one of the known forms or a novel one.

Gustatory Ion Channel Modulation by cAMP

Assuming that cAMP is a second messenger for sweet taste responses, one may wonder how changes in this cytoplasmic compound lead to the known taste cell responses, i.e., depolarization and release of neurotransmitter. At present, answers to this question rely solely on recent single cell electrophysiological recordings (40-42). cAMP was found to induce transmembrane depolarization similar to that caused by sweet tastants such as sucrose. This depolarization was found to be induced by the closure of 44 picoSiemen potassium channels in the apical membranes of taste cells. The molecular basis for such cAMP channel conductance modulation has also been established by patch clamp recordings. This effect is blocked by the Walsh-Krebs protein kinase inhibitor, known to inactivate cAMP-dependent protein kinase. Furthermore, the active catalytic subunit of such kinase can mimic the effect of cAMP or tastant. This lends strong support to the last steps in the transduction mechanism shown in Figure 1. The mechanism is clearly different from that found in olfactory sensory neurons, where cAMP directly binds to cation channels and induces their opening (43).

Summary and Prospects

Our understanding of the molecular basis of sweet taste reception has proceeded tremendously in the last few years. Added to a burgeoning discipline related to the fine structure of sweeteners and their binding site(s) is a view of the enzymatic cascade activated by gustatory receptor proteins in sweet-sensitive taste cells. These two bodies of knowledge should complement each other, and lead to an emerging field of "taste pharmacology," akin to that which has long existed for the action of drugs and hormones.

A most awaited development is the future identification, isolation and characterization of the protein receptors themselves. It is most likely that the receptors for sweet compounds will turn out to be homologous to other receptors known to be coupled to G-proteins (cf. ref. 18). Examples are rhodopsin, the visual

photoreceptor, α - and β -adrenergic receptors, muscarinic acetylcholine receptors, dopamine receptors and many other neurotransmitter and neuropeptide receptors. All of these share a structure that includes seven transmembrane helical segments, extracellular glycosylation and numerous intracellular phosphorylation sites (44). Sweet taste receptors may belong to the same receptor superfamily. Some of the molecular-biological strategies that are currently used for attempting the isolation of genes coding for sweet taste system receptors are based on this notion. When isolated, such genes could reveal the fine structure of the sweet compound binding site, as well as the possible multiplicity of sweet taste receptors, and aid in future development of new non-nutritive sweeteners.

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Chapter 18

Mechanisms of Sweet Taste Transduction

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Models of saccharide-stimulated responses in taste cells are presented based on electrophysiological, pharmacological and morphological investigations. Two models are most consistent with the available data. In both these models, saccharides bind to receptors on the apical membrane of taste cells causing them to depolarize. This depolarization results in the generation of action potentials in nerves synapsed to them. In the first model, the binding of saccharides to receptors opens an amiloride-inhibitable channel permitting small cations to enter and depolarize taste cells. Such a model is consistent with salt-modulating saccharide responses. In the second model the binding of saccharides to receptors activates a second messenger cascade that either closes potassium channels and/or opens chloride channels. In the second model, taste transduction can occur in the absence of salts.

There are several classes of sweet tasting compounds, with different structures, potencies and taste qualities, that may interact with lingual epithelia using quite different mechanisms. Understanding the physiological mechanisms by which these molecules interact with taste cells represents a fascinating challenge to researchers. The scope of this report will be limited to describing the responses elicited by two of the most commonly used saccharides, glucose and sucrose, in the presence and absence of salts.

ARE THERE RECEPTORS FOR SACCHARIDES?

To understand saccharide-stimulated transduction mechanisms, it is first necessary to determine whether saccharides bind to specific receptor(s) on taste cells or whether they interact via less specific mechanisms. Since receptors for naturally occurring saccharides have not yet been isolated from lingual epithelia, there is no direct evidence for their existence. One reason for questioning the existence of receptors is that D- and L-glucose are equally sweet. Another is that

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relatively large concentrations of saccharides are required to elicit taste responses. This latter point can be made by comparing the apparent dissociation constants, K_d , for glucose interacting with various glucose binding proteins with the K_d for glucose eliciting taste responses. The K_d 's (in parentheses; in mM) are: D-galactose binding protein, (0.0002) (1); hexokinase (yeast), (0.1) (2); Na-glucose transporter, (0.68 and 69) (3). The apparent K_d 's for glucose interacting with taste systems are: sugar receptors (fly), {(360) binding study (4), (~300) nerve recording (5)}; whole chorda tympani (rat), (~200) (6); lingual epithelia (dog), (280) (7); whole chorda tympani (dog), (~300) (Kumazawa, T. and Kurihara, K.; *J. Gen. Physiol.* 1990, in press); human, (200-300) (8). The high K_d 's of saccharides required to elicit taste responses implies that the free energy difference between a saccharide in aqueous solution and a saccharide at its "site of action" in the taste cell membrane is small, about -1 Kcal/mol. However, as described below, these low affinities or free energies of transfer do not exclude the possibility of saccharides binding to receptors.

The free energy, ΔG , for transferring a saccharide in water to a putative taste receptor reflects primarily the difference in the free energy to completely or partially dehydrate the saccharide and the free energy of association with the ligands of the receptor. Also included in ΔG are any conformational changes resulting from binding of the saccharide. The interaction of glucose to the D-galactose binding protein from *E. Coli* (1) results in the almost complete dehydration of D-glucose. For this interaction, $K_d = 0.2 \mu\text{M}$, corresponding to a free energy of transfer, $\Delta G = RT \ln K_d = -9.2 \text{ Kcal/mol}$. Consequently, as illustrated below, relatively small changes in ΔG are sufficient to decrease significantly the affinity (K_d) of a receptor. That is, if a single hydrogen bond between the saccharide and the receptor either did not form or was strained then ΔG would be reduced to about -5 Kcal/mol and K_d would increase about a thousandfold, to about 0.2 mM, a value similar to the weaker binding of glucose to hexokinase. It follows directly that a small change in amino acid composition from that of hexokinase, for example, could form a saccharide receptor with K_d 's of 200 mM like those found in lingual epithelia. Therefore low affinity sites cannot be used as arguments against receptors for saccharides. Consequently, in this report we will consider models for saccharide stimulated taste transduction with and without receptors.

Additional information about saccharide binding sites has been obtained from electrophysiological studies where it was demonstrated that the interaction of saccharides with taste cells depolarizes them (9). The depolarization of taste cells implies that ions must flow across their cell membranes. Hence ions (salts) are involved in the response to saccharides. The remainder of this manuscript will deal with the ion currents that are induced by the interaction of saccharides with taste cells.

I will restrict this manuscript to studies involving saccharide-stimulated responses in mammals. Among mammals there are large variations in responses to saccharides that possibly involve a variety of transduction mechanisms (5,10). Moreover, the response may depend on the salt type and concentration. I will emphasize the responses of dogs since, as described in this section, dogs and humans exhibit similar responses to saccharides. Chorda tympani responses in dogs and

humans exhibit a similar order of responsiveness to sucrose, glucose, fructose, galactose, maltose, and lactose (10). Chorda tympani responses in dogs elicited by saccharides in the presence of 30 mM NaCl are partially (~60%) inhibited by the epithelial cation (Na and Ca) channel inhibitor, amiloride (7), (Figure 1A). Psychophysical experiments yield conflicting results regarding amiloride's ability to decrease the intensity of sweet tastants. One study showed that amiloride inhibited saccharide responses about 50% (11), whereas another study failed to observe any inhibition by amiloride (12). These necessary psychophysical experiments should be repeated under conditions where the same area of the tongue is stimulated and where the pH, temperature, and salivary concentrations of NaCl and CaCl₂ are carefully controlled. Humans and dogs share another similarity in that gymnemic acid inhibits their chorda tympani responses to saccharides (10). Relative potencies of saccharides, and sensitivity to inhibitors such as amiloride and gymnemic acid provide three important criteria to assess whether dogs provide a suitable model for studies of human saccharide taste. Few animals fulfill all three conditions. For example, saccharide-stimulated responses in rodents are amiloride-insensitive (13) but are inhibited by gymnemic acid whereas in rabbits the opposite occurs. Finally, behavioral studies show that dogs, like humans, exhibit a preference for cyclamate over saccharin (7).

DO SACCHARIDES BIND TO THE APICAL OR BASOLATERAL MEMBRANE? A popular hypothesis for the mechanism of saccharide-stimulated taste transduction is the suggestion that saccharides bind to receptors of microvilli on taste cells; which, in some manner, depolarizes them thus generating action potentials in nerves synapsed to taste cells. However, an alternative possibility, that has not been seriously considered, is that small saccharides may diffuse across tight junctions between taste cells and interact with putative receptors on the basolateral membrane. Evidence for this mechanism comes from three sources: morphological studies of canine fungiform papillae using lanthanum as an electron dense extracellular space marker, electrophysiological studies on the anterior region of isolated canine lingual epithelia, and isotopic flux studies (14,7). In the morphological studies, precipitates of lanthanum were visualized in the taste pore after lanthanum was added from the serosal or basolateral side (Holland, V. F., Zampighi, G.A. and Simon, S.A. submitted for publication 1990), indicating that lanthanum diffused across the tight junctions between taste cells and accumulated in the taste pore. These data indicate that some tight junctions between taste cells exhibit a focal, rather than zonular, arrangement. The opposite is true for the tight junctions in the epithelium. Electrophysiological studies on isolated lingual epithelia showed that ouabain, a specific inhibitor of Na-K-ATPase, inhibited D-glucose-stimulated transport, as expected, when added to the serosal solution (Figure 1B) but also when added to the solution bathing the apical membranes (15). Since the Na-K-ATPase is normally found in the basolateral membrane of epithelial cells having tight junctions, and not in their apical membranes, these experiments suggest that ouabain must have diffused across the tight junctions of canine lingual epithelia into the extracellular space. Similarly, 0.1 mM amiloride-inhibited D-glucose stimulated transport when added either to the apical (Figure 1C) or basolateral membranes

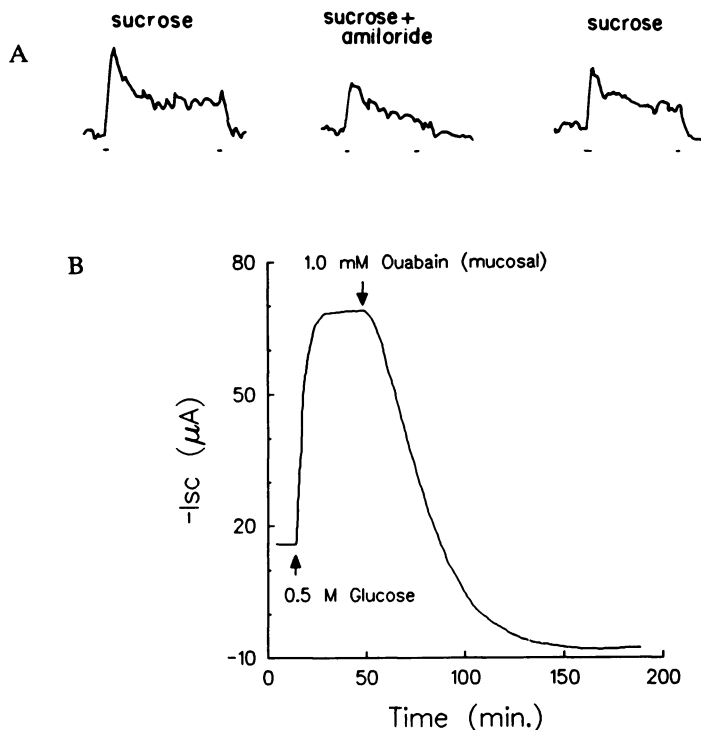


Figure 1. Responses of canine lingual epithelia. A. Integrated responses of chorda tympani to 1.0 M sucrose in 30 mM NaCl (left panel). The response was inhibited about 50% by 0.8 mM amiloride (middle panel). This inhibition by amiloride was reversible (right hand panel). (Reproduced with permission from Ref. 7, p. 101. Copyright 1988 Rockefeller University Press.) B. Trace of ouabain inhibition of D-glucose-stimulated short circuit current, I_{sc} (Reproduced with permission from Ref. 15, p. 15. Copyright 1990 Oxford University Press.) C. D-glucose-stimulated I_{sc} inhibited by $BaCl_2$ added to the serosal solution and by 0.1 mM amiloride added to the mucosal solution. The combination of these two inhibitors completely inhibited I_{sc} . (Modified from Ref. 15.) D. Inhibition of the open circuit potential, V_{oc} . I_{sc} decreased in parallel with V_{oc} by 8-bromo-cAMP added to the serosal solution. (Reproduced with permission from Ref. 14, p. R399. Copyright 1989 The American Physiological Society.) In B, C and D chamber area = 3.1 cm², temperature = 36±1°C and the mucosal solution contained 0.05 NaCl, 2 mM HEPES at pH 7.4 in the presence and absence of stimuli.

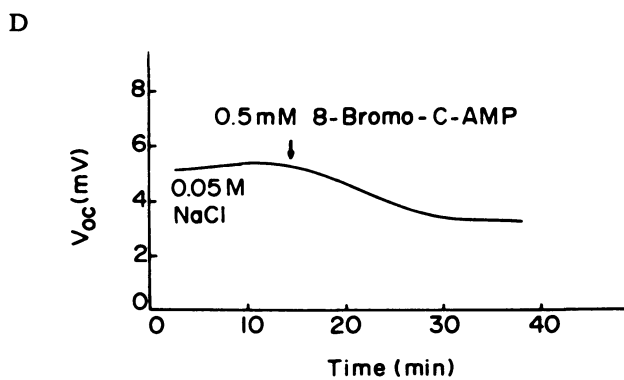
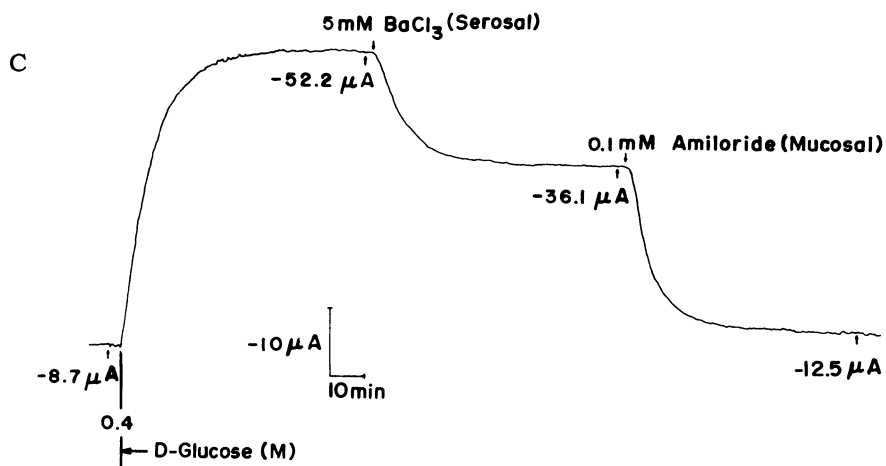


Figure 1. Continued.

(15). Further evidence that small saccharides can diffuse across tight junctions in isolated canine lingual epithelia consists of the measurement of a ouabain-insensitive influx of 3-O-methylglucose (7). These data suggest that molecules at least the size of ouabain (MW 586) can diffuse from the oral cavity into the extracellular space where they may interact with taste cells or nerves. Thus, since tight junctions between taste cells are permeable to small non-electrolytes, one must consider the possibility that saccharides interact with (amiloride-inhibitable) receptors on the basolateral membranes of taste cells.

ELECTROPHYSIOLOGICAL MEASUREMENTS OF SACCHARIDE STIMULATED TRANSPORT

The two electrophysiological methods used are measurements from the whole chorda tympani nerve (CT) and measurements of the short circuit current (Isc) and open circuit potential (Voc) across an isolated anterior region of the lingual epithelium. Measurements from the CT are obtained by placing the entire nerve over a metal electrode connected to an amplifier and applying stimuli to the tongue. The Isc is measured by placing an isolated lingual epithelium in an Ussing chamber, where the apical and basolateral solutions are separated, and "clamping" the transepithelial potential to 0 mV. Voc is the transepithelial potential when Isc = 0. With Krebs-Henseleit buffer bathing both surfaces, 1 mM ouabain completely inhibits the Isc. Heck et al. (16) measured the Isc and CT responses simultaneously and showed they are correlated in both their magnitude and temporal responses. The addition of saccharides to the dorsal surface of dog tongue increases Isc. Measurements of Na⁺ and Cl⁻ influx and efflux across isolated dog tongue (in hyposmotic NaCl solutions) after the addition of saccharides show that the majority of the increase in Isc is due to an amiloride-inhibitable increase in Na influx (7,14). Cl⁻ flux also changes upon saccharide stimulation although the manner in which taste cells regulate Cl⁻ transport is completely unknown. In summary, addition of saccharides such as D- or L-glucose, sucrose, fructose, 3-O-methylglucose, 2-deoxyglucose, and maltose results in an amiloride-inhibitable influx of cations into canine taste cells (7). The increase in Voc is proportional to the change in receptor potential, Vr, measured with microelectrodes impaled in taste cells since Voc = Vmc + Vcs, where Vmc is the difference in potential between the mucosal solution and the taste cell cytoplasm and Vcs is the potential difference between the taste cell cytoplasm and the serosal solution and Vcs \propto Vr.

TRANSPORT PROTEINS IN TASTE CELLS

Models depicting the interaction of small saccharides with taste cells and their associated tight junctions (TJ) are shown in Figure 2. The models need not be mutually exclusive and, more than likely, all contribute, to various extents, to saccharide-stimulated responses. Five model taste cells have the following common features: amiloride-binding proteins, Na-K-ATPase in their basolateral membrane, Ba⁺⁺-inhibitable K⁺-channels in the basolateral membrane, and synaptic vesicles. Moreover, in these models the tongue is bathed in hyposmotic concentrations of NaCl much like those found in saliva. In the

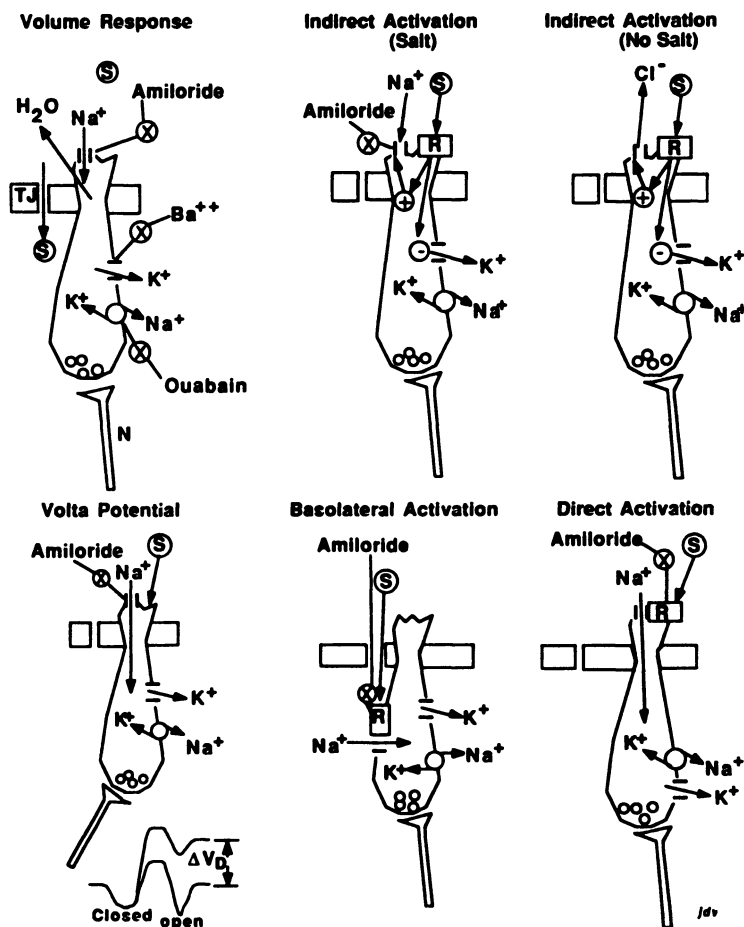


Figure 2. Six models of saccharide-stimulated transport in taste cells. Components of taste cells include: nerves (N), synaptic vesicles (open circles), saccharides (S) and saccharide receptors (R) and tight junctions (TJ). The symbols, ⊕ and ⊖ indicate activation and inhibition, respectively.

remaining model (Indirect Activation model (no salt)) salts are absent from the dorsal surface of the tongue and the saccharide-stimulated response is insensitive to amiloride. Evidence for the amiloride- and ouabain-inhibitable pathways is given in Figure 1. Their presence is also inferred from immunocytochemical studies (17) and (in rats) from patch clamp studies (18). In addition, histochemical studies also show the presence of a Ca^{++} -ATPase responsible for maintaining low intracellular calcium concentrations (17). Evidence for a Ba^{++} -inhibitable K-pathway on the serosal side is given in Figure 1C and in Simon et al. (19,15). There is no direct evidence for synaptic vesicles in taste cells in canine fungiform papillae. However, since synaptic vesicles have been found in every other mammalian taste cell examined (20,21) we tacitly assume that they are also present in canine taste cells.

Taste cells also contain transport pathways not mentioned above. These include Ca^{++} -channels involved in synaptic vesicle release, K^{+} -channels as receptors for bitter tastants (22), and pathways involved in the regulation of intracellular H^{+} , Cl^{-} and Ca^{++} . These pathways are not shown in Figure 2 as their presence, location on the taste cell, and putative role in saccharide-stimulated transport are not known. In addition, the resting potential of dog taste cells has not been measured although it is reasonable to assume that dog taste cells are similar to other taste cells in that their resting potential is about -60 mV (23).

MODELS FOR SACCHARIDE-STIMULATED ION TRANSPORT.

Let us now look in detail at the experimental evidence for or against each of the models for saccharide-stimulated ion transport shown in Figure 2.

THE "VOLUME RESPONSE" MODEL. This model, having no specific receptors for saccharides, predicts that application of saccharides to taste cells will either increase or decrease their volume depending on their membrane permeability and osmolality of the saccharide solution bathing them. Taste cells would respond to osmotic pressure changes of the saccharide solution by activating an amiloride-inhibitable, cation-selective pathway. If saccharides are relatively impermeant to taste cell membranes, as expected for sucrose or L-glucose, they should, at hyperosmotic concentrations, decrease the volume of the taste cells. (More complicated models involving streaming potentials can also be constructed). In contrast, if saccharides are relatively membrane-permeable, as would be expected for D-glucose or fructose, then the volume of taste cells would be expected to increase (after it initially decreased if the concentrations were hyperosmotic). A subset of the Volume Response model is a model in which taste cells have a channel that is activated by a change in membrane tension that arises as a consequence of volume changes. Channels activated by changes in membrane tension induced by cell volume changes have been described (24). To test the Volume Response model, the apical side of an isolated dog tongue was bathed in a buffered 50 mM NaCl solution (to simulate saliva) and the serosal side was bathed in Krebs-Henseleit buffer. We (15) tested whether increasing the osmolality of the serosal solution, by adding saccharides to the Krebs-Henseleit buffer or decreasing the osmolality of the serosal solution by removing about half the NaCl,

would simulate the increase in *I*_{sc} measured after addition of saccharides to the apical solution. Decreasing the osmolality of the basolateral solution caused a transient decrease in *I*_{sc} whereas increasing the osmolality of the serosal solution did not produce a significant change in *I*_{sc} (15). Thus, the Volume Response model is not consistent with these data. This does not imply, however, that taste cells do not undergo volume changes when exposed to stimuli of widely ranging osmolalities or that increasing the osmolality of the basolateral solution could not in some instances change the receptor potential (25).

THE "INDIRECT ACTIVATION" (SALT) MODEL. The Indirect Activation model proposes that saccharides initially bind to a receptor in the apical membrane of a taste cell. This binding would activate one or more second messengers in the cell's cytoplasm. The second messenger(s) would then activate (open) an amiloride-inhibitable pathway (on either the apical or basolateral membrane), permitting Na⁺ to enter and causing a depolarization of the taste cell. Here, the pathway is distinct from the saccharide receptor. The same or a different second messenger(s) could also lead to the closing of a K⁺-channel in the basolateral membrane which would also result in a membrane depolarization. It is important to note that in the complete absence of salts, a saccharide induced mechanism involving Na⁺ (or cation) influx is not possible and that taste cells could, in principle, become depolarized either by a Cl⁻ efflux or via the influence of a second messenger closing a K⁺-pathway as described above. We tested the Indirect Activation model in dogs by adding membrane-permeable second messengers to the Krebs-Henseleit buffer of the serosal side while maintaining the apical side in a buffered 50 mM NaCl solution (14). We reasoned that, if second messengers were activating or inhibiting channels, we would observe the same stimulation of *I*_{sc} as when saccharides were added to the apical bathing solution. The addition of the membrane-permeable form of c-AMP produced a transient decrease in *I*_{sc} and *V*_{oc} (Figure 1D, 14); whereas increasing the intracellular concentrations of either Ca⁺⁺, c-GMP, or an analogue of diacylglycerol did not produce significant changes in *I*_{sc} (14). From these data it is evident that increasing the concentration of c-AMP and, by implication, the other second messengers into dog taste cells (in the presence of NaCl) does not affect saccharide-stimulated currents. As nothing is known about the basal levels of second messengers in taste cells it is not known what effect additional increases or decreases in their concentration will have.

THE "INDIRECT ACTIVATION" (NO SALT) MODEL. Many experiments testing the effects of saccharides are performed after adapting the tongue to deionized water and then adding saccharides (in deionized water) to the apical surface of the tongue. Under these conditions, taste cells can depolarize as a consequence either of the closing of basolateral K⁺-channels and/or of the opening of Cl⁻-channels. Since Na⁺ entry is not involved in this process it is expected that the saccharide responses be amiloride-insensitive. The Indirect Activation Model may reflect the following situations: the amiloride-insensitive CT component seen in Figure 1A, saccharide responses elicited in the presence of large

cationic salts, and saccharide responses elicited in the presence of low or no salt.

The CT responses of saccharides elicited by rodents in the absence of salts are amiloride-insensitive (13) and hence reflect an intrinsically different mechanism than those heretofore described. Hints about such a mechanism come from intracellular recordings from rodent taste cells. In these studies the interaction of saccharides with taste cells activates second messengers which in turn may depolarize them by inhibiting K^+ -channels on their basolateral side. The closing of channels would increase the taste cell resistance (26,9). Alternatively, the second messengers could also activate Cl^- -channels which would depolarize taste cells as the electrochemical potential gradient favors Cl^- efflux. Presently, no evidence has been presented to support the Cl^- hypothesis. An elegant patch clamp study with frog taste cells supports the Indirect Activation Model (27). In these studies the interaction of saccharides stimulated a second messenger cascade resulting in the closing, by phosphorylation, of a 44 pS K^+ -channel. Biochemical studies on rat lingual epithelia also support the second messenger hypothesis since adenylate cyclase was stimulated by the addition of hyperosmotic concentration of saccharides (28). However, this stimulation could be a consequence of volume regulation by taste or epithelial cells.

THE "VOLTA POTENTIAL" MODEL. This model has been suggested to explain transduction events in taste and olfaction by Kurihara and colleagues (29) and also to explain changes in the conductance-voltage curves and kinetic properties of voltage-dependent K^+ channels induced by phloretin (30). The Volta potential is the potential arising from fixed or adsorbed charges and dipoles associated with any solvated surface (31). It is equal to the sum of the double layer potential $\psi(0)$, that arises from fixed or adsorbed charges on the surface, and the dipole potential, ΔV_d , that arises from the perpendicular components of the dipoles (and multipoles) in the membrane and the membrane-associated water molecules (31). In the Volta Potential model, the transduction mechanism for saccharides differs in a subtle, but testable, manner from transduction mechanisms in other models outlined in Figure 2. In other models, saccharides bind (albeit weakly) either to a receptor (Indirect Activation Model) or to a receptor-channel complex and as a consequence of either a conformational change or the activation of a second messenger, channels open (or close) and ions diffuse down their electrochemical gradients. *In the Volta Potential model there are no receptors for saccharides.* In this model, saccharides "gate" or open an amiloride-inhibitable, cation-selective pathway (or another ion-selective pathway, depending on species) by lowering the energy of the open state relative to the closed state thus changing the probability that the channel will be in an open state (see inset in Figure 2). For voltage-dependent channels, changing the membrane potential, V , across a membrane of thickness, d , changes the electric field ($E=V/d$) across the membrane. Typical electric fields are 10^5 V/cm. The dipolar fields, produced by the solvent and membrane multipoles, are of sufficient magnitude to couple with the "gating" charges or gating dipoles in channel proteins and provide the conformational energy to alter the probability that a channel will be either closed or open. The potential

produced by this field is commonly called the dipole potential, ΔV_d . It can have a magnitude of several hundreds of millivolts (31). Changing ΔV_d could alter the probability that a channel will be in a given state (30). Thus, the addition of saccharides, could in principle, change ΔV_d and thus affect the probability that the saccharide-stimulated channel is converted from its closed to its open state. An interesting feature of this model is that the magnitude of change in Volta potential may depend on the stereochemistry of the molecule added to the solvent phase. In this regard, Reyes et al. (32) used a series of phloretin analogues to show that small substitutions in the phloretin molecule produce large changes in the Volta potential. Sweet tasting molecules such as glycerol have been shown to decrease the Volta potential (33).

The Volta Potential model was tested by first measuring ΔV_d in lipid monolayers of egg phosphatidylcholine (EPC) at 65\AA^2 spread over a buffered subphase containing 0.05 M NaCl. Under these conditions $\Delta V_d = 415\text{ mV}$ (31). We then measured the change in ΔV_d produced by the addition of 0.5 M D-glucose or 0.5 mM phloretin or phloridzin to the subphase. 0.5 M D-glucose decreased ΔV_d by 20 mV and 0.5 mM, phloretin or phloridzin decreased ΔV_d by 195 and 145 mV, respectively. (For uncharged or zwitterionic lipids, such as EPC, the Volta potential is equal to the dipole potential since $\psi(0) = 0$). Thus if the Volta Potential model were correct, 0.5 mM phloretin (or phloridzin) should produce the same (or a higher) increase in I_{sc} induced by D-glucose. The addition of 0.5 M D-glucose (in 50 mM NaCl) increased I_{sc} about $11\ \mu\text{A}/\text{cm}^2$ whereas the addition of 0.1 mM phloretin or phloridzin produced $<1\ \mu\text{A}/\text{cm}^2$ increase in I_{sc} . Thus, the Volta Potential model is inconsistent with the expectations of this experiment.

THE BASOLATERAL AND DIRECT ACTIVATION MODELS. These models differ only in the location of the amiloride-inhibitable receptor-channel complex. In the Direct Activation model, the amiloride-sensitive channel is confined to the microvilli of taste cells whereas in the Basolateral Activation model the amiloride-sensitive channel is confined to the basolateral membrane. In both cases, saccharides first bind to the receptor-channel complex causing it to open and permitting cations to flow down their electrochemical gradient. It is not known how many saccharide molecules are required to bind to the receptor for the channel to open. It is important to note that, if the amiloride-inhibitable channel is localized to the basolateral surface, then it would have to be in its closed state in the absence of significant concentration of saccharides, since otherwise taste cells would be permanently depolarized as a result of the high Na concentration ($\sim 120\text{ mM}$) in the extracellular space.

Evidence in support of the Basolateral Activation model is that:

1. Small molecules, such as glucose and amiloride, can diffuse across tight junctions between taste cells and enter the extracellular space where they can subsequently interact with putative saccharide receptors.
2. Immunocytochemical studies on canine lingual epithelia showed that antibodies formed against amiloride-inhibitable channels from kidney cells bind to the basolateral membranes of taste cells (17).
3. Patch clamp studies of rat taste cells in intact fungiform papillae show that amiloride-inhibitable currents can be recorded from their basolateral membrane (18).

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The only convincing argument against this model is that saccharides added to the Krebs-Henseleit buffer bathing the serosal solution did not elicit a significant change in *I*_{sc} (15) as previously described in the Volume Response model. Consequently, in the presence of salts, the Direct Activation Model is more consistent with the data for dogs (and presumably humans) than the Indirect Activation Model.

The Direct Activation Model also predicts that the response to saccharides should depend on the type and concentration of salt present. In isolated dog tongue the increase in *I*_{sc} induced by 0.5 M D-glucose was reduced from about 11 $\mu\text{A}/\text{cm}^2$ in 50 mM NaCl to about 7.4 $\mu\text{A}/\text{cm}^2$ in 1 mM NaCl. In 1 mM NaCl, the D-glucose-stimulated *I*_{sc} was inhibited about 28% by 0.1 mM amiloride as compared with 60-70% inhibition by 0.1 mM amiloride in 50 mM NaCl. These data, together with the saccharide-stimulated CT responses which exhibit amiloride-sensitive and -insensitive components (Figure 1A) suggest there are two saccharide-stimulated pathways that can be distinguished by their amiloride sensitivity. Thus it appears that the amiloride-sensitive component is salt dependent and the amiloride-insensitive component is salt (mucosal) independent. Therefore, one would predict that in the presence of large impermeant cations, only the amiloride-insensitive component would remain, as the large cations would not be able to be transported into taste cells via the cation-selective amiloride-sensitive pathway and as a result the *I*_{sc} would be reduced. This indeed occurs as the addition of 0.5 M glucose in the presence of 0.05 M Tris Cl (Simon, unpublished observation) or 0.15 M N-methyl-D-glucammonium Cl (GACl) (7) to these same salts increased *I*_{sc} only about 3 and 2 $\mu\text{A}/\text{cm}^2$, respectively, compared with 11 $\mu\text{A}/\text{cm}^2$ when glucose was added to 0.05 M NaCl. Also consistent with the hypotheses that there are (at least) two saccharide-stimulated pathways that can be distinguished by their responses to salts are the studies by Anderson et al. (34). These researchers measured the responses of single canine CT fibers in the presence of salts and saccharides. They identified three classes of individual fibers: a class which responded only to saccharides, a class which responded only to salts (NaCl) and a class which responded to both salts and saccharides.

In humans as well as in dogs there is a dependence of salt on the saccharide response (35,34). That is, beginning at low NaCl concentrations, one would predict that increasing the salt concentration should increase the sweetness of saccharides, since in the absence of salts one saccharide-stimulated pathway will be activated, whereas in the presence of salts the other (if present) will be activated. Thus adding salt should increase the response to saccharides. In isolated dog tongue, the dependence of the D-glucose-stimulated *I*_{sc} showed a maximum at 0.1 M NaCl as does the tonic component of chorda tympani responses from dogs elicited by solutions of 0.5 M sucrose with different NaCl concentrations (14), (Kumazawa, T. and Kurahara, K. J. Gen. Physiol. 1990, in press).

SUMMARY

Saccharide-stimulated responses show two saccharide-stimulated pathways that can be distinguished on the basis of their amiloride sensitivity and salt dependence. The saccharide-stimulated, amiloride-

sensitive pathway is operational in the presence of NaCl whereas the saccharide-stimulated, amiloride-insensitive pathway is independent of salt but is modulated by second messengers.

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Chapter 19

Phenoxyalkanoic Acid Sweetness Inhibitors

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Substituted phenoxyalkanoic acids are potent inhibitors of the sweetness response. The degree of inhibition of sweetness is dependent on the concentration of substituted phenoxyalkanoic acid in a sweetener-inhibitor mixture. In contrast to some other sweetness inhibitors, pre-treatment of the tongue is not necessary and the inhibitory effect is immediate and rapidly reversible. The acidic group, or its salt, is necessary, and the nature, position and degree of substitution on the phenyl moiety influences potency. The results of sensory studies designed to determine the possible mechanism of action are described, and the implications for our understanding of the mechanism whereby sweeteners induce their effects are discussed.

The existence of compounds capable of inhibiting the perception of sweetness have been known for many years, but they have not been the subject of intense study. Nonetheless, the mechanisms whereby known inhibitors exert their effects have, to some degree, been examined and a variety of mechanisms shown to be operating. For example, *N*-ethylmaleimide, iodoacetic acid, and *p*-chloromercuribenzoate (1) probably inhibit sweetness perception by covalent interaction at the taste cell membrane (2). Gymnemic acid and the more recently discovered ziziphins are believed to inhibit sweetness through their surface active, detergent like properties (3). Similarly, sodium dodecyl sulphate is capable of penetrating and disrupting the phospholipid membranes that are believed to be components of sweetness receptors (4). Methyl-4,6-dichloro-4,6-dideoxy- α -D-galactopyranoside inhibits sweet taste

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responses in gerbils, and there is some evidence to suggest that the mechanism of inhibition may be competitive (5). Finally, the diuretic amiloride is reportedly an inhibitor of sweetness, although there are conflicting results using conventional sensory methodology. Its mechanism of action has been suggested as that of an inhibitor of ion transport mechanisms (6).

Recently, a number of other classes of chemicals have been discovered to have sweetness inhibiting properties (7-9). In common with other sweetness inhibitors, these substituted phenylalkanoic acids, phenoxyalkanoic acids, and benzoylalkanoic acids inhibit the perception of sweetness of differing chemical structures. However, they exhibit no evidence of disrupting taste cell membranes, and their effects are immediately reversible. Therefore, they are potentially valuable tools to increase our understanding of the chemical structures of sweetness perception.

Structure-Activity Relationships

The initial observation that these phenoxyalkanoic acids can inhibit the perception of sweetness was made on tasting a solution of sucrose at 5% (w/v) concentration which contained 0.05% (w/v) 4-hydroxy-3-methoxy-phenylpropanoic acid. The mixture was perceived as being equi-sweet to a 3% (w/v) sucrose solution, thus, the degree of inhibition was approximately 40%. This initial observation led to what was merely a standard structure-activity relationship program. Derivatives were prepared synthetically and their inhibitory action assessed using either the methodology described or one in which sucrose was at 10% (w/v) and the inhibitor under test at 0.005% (w/v).

Synthetic procedures followed general published guidelines (10-12). In outline, phenylpropanoic acid derivatives were prepared by reduction of the corresponding cinnamic acid derivative, itself obtained by a Perkin condensation of the appropriate benzaldehyde with acetic anhydride in the presence of sodium acetate. Phenoxyalkanoic acid derivatives were prepared by reduction of the appropriate phenol with a haloalkanoic acid or an ester of a haloalkanoic acid.

It was unfortunate that the scope of this programme was limited by its objective. It was not designed either to unravel the comprehensive relationships presumed to exist between structure and effect, or to develop structural correlations between sweetness inhibitors and known sweeteners. The objectives were to identify the optimum compound to develop commercially. Nonetheless, there were many interesting observations arising from the limited structure-activity program, some of which are seemingly conflicting and have not yet been rationalised.

For example, within the phenylpropanoic acid series, the position of a methoxy-substituent has no influence on the effectiveness of the resulting compound as an inhibitor of

sweetness (Table I). However, within the 2-phenoxypropanoic acid series, positioning of a methoxy-substituent has a major impact on inhibitory power (Table II) and it is not immediately obvious why this difference exists.

Table I. Effect of Substituent Position on Inhibition by Phenylpropanoic acids.

Position/Substituent	Inhibition (%) (0.05% Inhibitor in 5% Sucrose)
2-Methoxy	40
3-Methoxy	40
4-Methoxy	40
3-Ethoxy	60
4-Ethoxy	60

Table II. Effect of Substituent Position on Inhibition by Phenoxypropanoic Acids

Position/Substituent	Inhibition (%) (0.05% Inhibitor in 10% Sucrose)
2-Methoxy	15
3-Methoxy	30
4-Methoxy	50

Similarly, α -naphthyl phosphate does not inhibit sweetness, whereas the corresponding sulphate (α -naphthyl sulphate) does. In contrast, α -hydroxy benzylphosphinic acid does inhibit sweetness, but α -hydroxy- α -toluene sulphonic acid does not (Figure 1).

Other structure-activity relationships developed follow a more consistent pattern. Increasing the lipophilicity of substituents on the phenyl or phenoxy moiety increases the effectiveness up to a maximum, when, with further increases in lipophilicity, there is a drop in inhibitory efficiency. As long ago as 1966, similar observations had been made on the relative sweetness of derivatives of 2-amino-4-nitrobenzenes. Deutsch and Hansch had observed that relative sweetness was highly dependent on the hydrophobic binding constant (13). Here, the inhibitory effectiveness of compounds was found to correlate with their calculated log P values in a similar way to that observed by Deutsch and Hansch for sweeteners. As the lipophilicity of a substituent increases, so does the compound's measured "biological response", up to a maximum, following which there is a steady fall in effectiveness (Tables III and IV). Interestingly, even this relationship only holds within the alkyl

substituent or alkoxy substituent series, not across those structural boundaries.

Table III. Correlation of the Nature of the Substituent with the Biological Response of Phenoxypropanoic Acids.

R	Log P_{calc}	Inhibition (%) (0.005% Inhibitor in 10% Sucrose)
H	1.60	11
CH ₃	2.16	41
C ₂ H ₅	2.62	63
iC ₃ H ₇	3.13	45
tC ₄ H ₉	3.58	33

Table IV. Correlation of the Nature of the Substituent with the Biological Response of Phenoxypropanoic Acids.

R	Log P_{calc}	Inhibition (%) (0.005% Inhibitor in 10% Sucrose)
OH	0.93	0
OCH ₃	1.58	48
OC ₂ H ₅	1.98	34
OC ₃ H ₇	2.65	13
OC ₄ H ₉	3.14	<10

The key feature of these observations is that they provide a link between the structural requirements for sweetness induction and sweetness inhibition. An additional, perhaps key, observation that links sweetness induction with inhibition structurally is that for optically active sweetness inhibitors, one enantiomer is an inhibitor of sweetness while the other enantiomer is inert. Identical observations have been reported with, for example, amino acids of certain dimensions (14).

While these structure-activity relationships are intrinsically interesting, they have done little to help our understanding of what type of receptor event, if indeed it is a receptor event, is involved in sweetness inhibition by these compounds. However, from within the compounds evaluated as part of this structure-activity relationship program, and drawing on the literature, it is possible to draw some tenuous structural analogies between selected sweeteners and inhibitors (Figure 2).

Although description of these structural similarities as "analogues" is rather simplistic, if the objective had been to identify closer analogies, there is every likelihood that they would have been found. However, that was not the purpose of these structure-

NON-INHIBITORS

SWEETNESS INHIBITORS

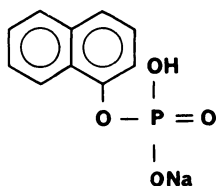
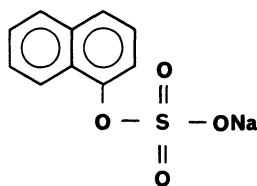
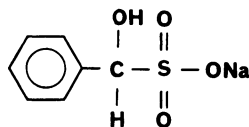
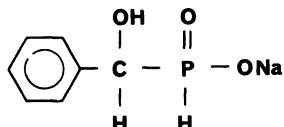
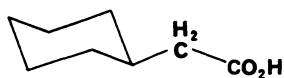
 α -Naphthyl phosphate α -Naphthyl sulfate α -Hydroxy toluene sulfonic acid α -Hydroxy benzyl phosphinic acid

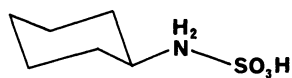
Figure 1. Examples of compounds which inhibit and do not inhibit sweetness, illustrating the anomalous nature of some structure-activity relationships.

SWEETNESS INHIBITORS

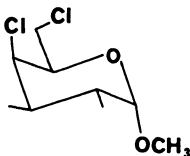
SWEETENERS



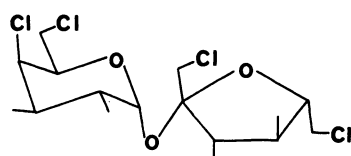
Cyclohexyl acetic acid



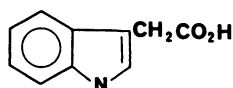
Cyclohexyl sulfamic acid



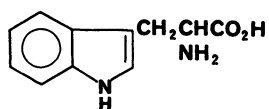
Methyl-4,6-dichloro galactoside



461 6' Tetrachloro galacto-sucrose



Indolyl acetic acid



D-Tryptophan

Figure 2. Structural analogies between some sweeteners and sweetness inhibitors.

activity relationship studies. One conclusion from these observations is that a similar mechanism may be operating to that whereby sweeteners are thought to induce their effects; that is, a ligand-receptor interaction. Examining the structures illustrated in Figure 2 it can be seen that these inhibitors can all be described as being "structurally deficient". Early attempts to rationalise the relationships between structure and sweetness centered on the AH,B concept of Shallenberger and Acree (15). All of the inhibitors illustrated can be considered to lack a putative AH moiety. This may be no more than a coincidence, but is worthy of more detailed study.

Sensory Studies

From the structure-activity relationships that have been described, 2-(4-methoxyphenoxy)propanoic acid [PMP] was selected for commercial development. The sodium salt (Na-PMP) was subsequently accorded FEMA GRAS status for use in confectionary/frostings, soft candy and snack products. Its function is to enhance flavor through modulation of frequently excessive sweetness in formulated products within these market sectors. Na-PMP has been used in sensory experiments designed to identify the mechanism whereby this type of inhibitor induces its effect.

Dose-response experiments following standard sweetness matching techniques illustrate graphically the potency of this inhibitor (Figure 3). Increasing concentrations of Na-PMP were added to fixed concentrations of sucrose (12%, w/v and 8%, w/v) and the resulting mixtures matched for sweetness intensity with standard sucrose solutions. As can be seen, the perceived intensity of a 12% sucrose solution is reduced to that of a 4% sucrose solution by as little as 100 ppm Na-PMP. Although 100 ppm Na-PMP has a slightly salty taste, in sweet solutions, there is no detectable salty taste. Therefore, mixture suppression cannot account for these observations. With the same amount of inhibitor, an 8% sucrose solution is equi-sweet to a 2% sucrose solution. Interestingly, the dose-response curves are essentially parallel.

In addition, Na-PMP is also a potent inhibitor of the sweetness elicited by non-sucrose sweeteners. A 0.07% solution of aspartame, which is approximately equi-sweet to a 12% sucrose solution, is inhibited to the same degree as the concentration of Na-PMP increases. Although not illustrated in Figure 3, other non-sucrose sweeteners follow precisely the same pattern. Carbohydrate sweeteners, e.g., fructose; sugar alcohols, e.g., sorbitol; and intense sweeteners, e.g., saccharin, neohesperidin dihydrochalcone, and thaumatin that have been matched for perceived sweetness intensity are all inhibited to the same degree by the same concentration of inhibitor.

These results, taken in isolation, can be rationalised in a variety of ways. Either Na-PMP has a greater affinity for the sweet

receptor(s) than sweeteners, or it may interact at a complementary (allosteric) site, or it may interfere with secondary transduction mechanisms. In addition, it might interact with the sweeteners themselves, thus making sweetener-receptor interactions impossible.

The mechanism of action was investigated in appropriate sensory experiments. In one such experiment, an attempt was made to determine if the inhibition mechanism involved with this type of compound is competitive, non-competitive or uncompetitive. A suitable range of fructose solutions were matched for perceived sweetness against sucrose standards. To these fructose solutions were added increasing amounts of Na-PMP and the resulting mixtures matched to sucrose standards. The data, equivalent to enzyme-kinetics experiments, were amenable to Lineweaver-Burk manipulation. The results are summarised in Figures 4 and 5.

The data suggest a competitive mechanism of inhibition. Although these data do not prove a competitive mechanism, taken with the following additional evidence, they are consistent with that explanation.

1. The inhibitory effect and its degree are independent of pH. The identical Na-PMP/sucrose mixture buffered at pH values from 3-8 is equi-sweet with the identical sucrose standard.
2. There is no general taste quality inhibition with Na-PMP. It is a specific inhibitor of sweetness and has no impact on salty, sour, or bitter tastes.
3. There is no evidence that Na-PMP interacts with sweeteners, so reducing their affinity for the sweetness receptor(s). For example, the optical activity of a sucrose solution is unaffected by addition of Na-PMP.
4. The occurrence of inhibition, and its degree, are highly dependent on the structure of the inhibitor. This is best exemplified by the activity of S(-)PMP and the complete lack of effect with R(+)-PMP.
5. Inhibitor and sweetener must be mixed in solution for the effect to be observed. Pretreatment of the tongue, even extensive rinsing in a solution of Na-PMP, has no impact on the response to a given concentration of sweetener tasted subsequently.
6. The inhibitory effect is immediate, and is immediately and completely reversible on rinsing. There appears to be no carryover effect and therefore no evidence for any membrane disruption events being involved.
7. Each successive concentration of inhibitor depresses the sweetness response of any sweetener to a like degree and in a parallel fashion (see Figure 3).

Therefore, in view of these observations, taken together with the noted structural "similarities" between agonists and antagonists,

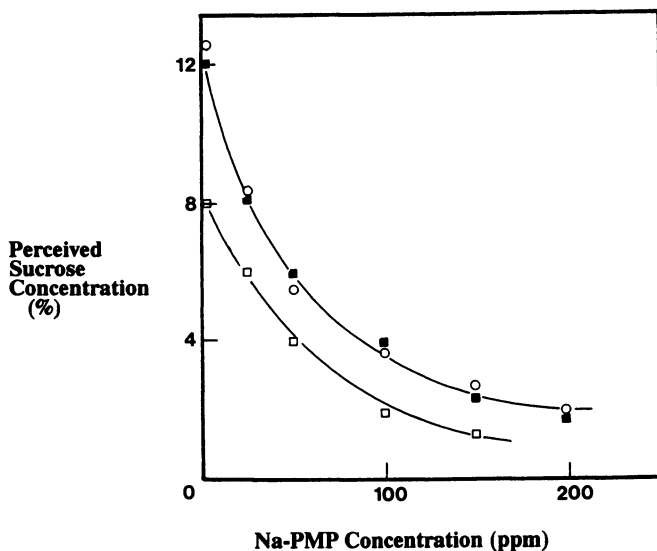


Figure 3. Dose-response characteristics of Na-PMP and its inhibitory action on sucrose and aspartame. (■) 12% sucrose; (□) 8% sucrose; (○) 0.07% aspartame.

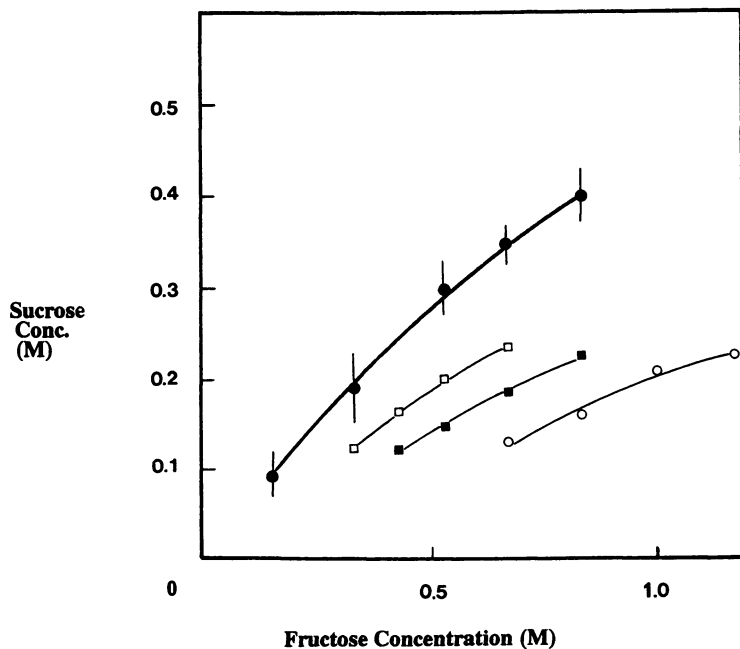


Figure 4. Inhibition of fructose by increasing concentrations of Na-PMP. (●) No inhibitor; (□) 0.15 mM Na-PMP; (■) 0.30 mM Na-PMP; (○) 0.60 mM Na-PMP.

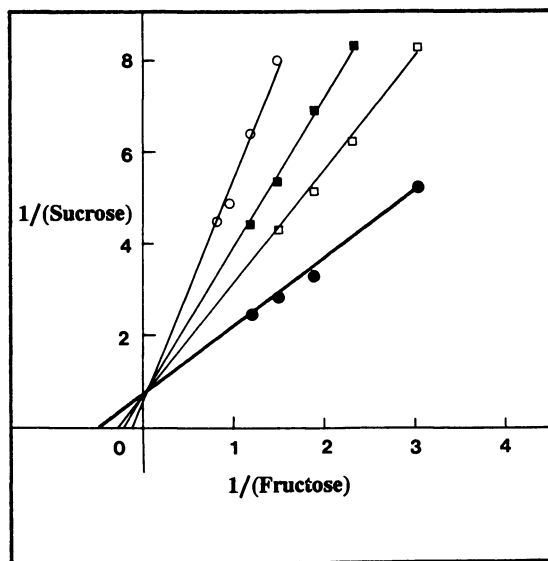


Figure 5. Lineweaver-Burk double reciprocal presentation of the data in Figure 4. (●) No inhibitor; (□) 0.15 mM Na-PMP; (■) 0.30 mM Na-PMP; (○) 0.60 mM Na-PMP.

competitive inhibition appears to be the most likely mechanism whereby Na-PMP and the other phenyl- and phenoxyalkanoic acids induce their sensory effects.

Discussion

It is interesting to speculate on the implications of these observations for our understanding of the peripheral mechanisms involved in sweetness reception. For example, do different structural classes of sweetener induce their effects through interaction at different receptor sites on the tongue? In other words, does each sweetener or structural class of sweetener have its own unique receptors? Or is there a single sweetness receptor type that responds to the multitude of widely differing chemical structures which elicit sweetness?

The data presented are consistent with, but do not prove, competitive inhibition. However, if competitive inhibition is assumed to be the mechanism of action, then it is a logical extension to conclude there is a single receptor structure that "codes" for sweetness. However, this would not preclude the existence of structural variants of this central receptor capable of coding for quality of sweetness which thus permit sweeteners to be readily distinguished one from another.

The issue remains open to debate. One difficulty of resolving it has been the lack of suitable ligands to help isolate and study receptors for sweeteners. If nothing else, 2-(4-methoxyphenoxy)-propanoic acid and other structurally similar sweetness inhibitors are additional tools which are now available to study the chemistry and biochemistry of sweetness perception and its inhibition.

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Chapter 20

Concentration–Response Relationships of Sweeteners

A Systematic Study

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Sweetness intensity ratings were made by a trained panel for a range of concentrations of nineteen sweeteners. Panelists were trained to make sweetness ratings relative to six sucrose standards (2%–16%). The shapes of the concentration–response plots were sweetener-dependent. Sugars and sugar alcohols yielded linear concentration–response relationships for intensities up to that of a 16% sucrose standard. High-potency sweeteners including aspartame, acesulfame-K and alitame yielded hyperbolic concentration–response plots.

The purpose of this study was to utilize a systematic approach for determining concentration–response relationships for a broad range of sweeteners relative to a sucrose reference. Trained panelists evaluated sugars (sucrose, glucose, fructose, fructo-oligosaccharide sweetener), sugar alcohols (maltitol, lactitol, isomalt), terpenoid glycosides (monoammonium glycyrrhizinate, stevioside, rebaudioside-A), dipeptide derivatives (aspartame, alitame), a sulfamate (sodium cyclamate), a protein (thaumatin), a chlorodeoxysugar (sucralose), two N-sulfonyl amides (sodium saccharin, acesulfame-K), a dihydrochalcone (neohesperidin dihydrochalcone), and an amino acid (glycine). Although concentration–response relationships have previously been obtained for some of these sweeteners (1–6), the present methodology expands upon prior work in that: 1) a wider range of structural types is evaluated; 2) the intensity ratings are referenced to standard sucrose concentrations by a trained taste panel; and 3) the mathematical forms of the concentration–response relationship are examined.

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Method

Subjects: Screening and training. The subjects were screened for normal taste and odor acuity and the ability to recognize and discriminate among various taste stimuli (7). The final panel participants were selected for their ability to: correctly identify the tastes sweet (2.0% sucrose), salty (0.2% NaCl), sour (0.07% citric acid), and bitter (0.07% caffeine); accurately rank order a series of four concentrations of each taste stimulus; successfully identify the odd sample in a series of triangle tests; and recognize and describe the aroma of six common odorants.

Five female and thirteen male students and employees of Duke University, Durham, N.C., (mean age 36.5 years \pm 13.3) were trained across seven weeks (17 one-hour sessions) in a modified Spectrum™ descriptive flavor profile method (8). The purpose of the training was to familiarize the panel with taste profiles of sweeteners. The panel was trained in techniques to recognize, describe and quantify the tastes and aromatic characteristics of sweeteners.

The training began with an overview of basic taste and olfactory physiology as well as the psychophysical principles of vocabulary development and scaling. Through the use of flavor attribute references (e.g., metallic = .0003 g Ferrous sulfate in 150 mL 500 ppm aspartame solution; licorice = 1 drop McCormick anise extract in 100 mL 500 ppm aspartame solution), the panel learned standard flavor vocabulary to describe tastes and odors of sweeteners. Six concentrations of sucrose (2%, 5%, 7.5%, 10%, 12%, and 16%) were used to standardize sweetness intensity ratings on a 15 cm line scale (Figure 1). These sucrose standards were assigned the intensity values 2, 5, 7.5, 10, 12, and 15, respectively. Two concentrations of caffeine were used to standardize bitterness intensity ratings: 0.05% (assigned a value of 2 bitter) and 0.08% (assigned a value of 5 bitter). The panel evaluated seven sweeteners at various concentrations during the training: acesulfame-K, aspartame, sodium cyclamate, sodium saccharin, sucrose, and thaumatin. Two mixtures were also used in training: aspartame plus caffeine, and sucrose plus caffeine.

Stimuli. The stimuli, classification, dilution range, and number of concentrations tested are given in Table I. Samples were dissolved in deionized water at room temperature within twelve hours of evaluation. Concentrations are reported on a weight/volume basis, correcting the weight for analyzed purity of the sample (e.g., Na-saccharin, Sigma, lot 76F0079, contained 14.3% water according to the supplier). Panelists received 20 mL aliquots of test stimuli, served at room temperature in 30 mL odor-free plastic cups coded with randomly selected three-digit numbers. The panel was conducted under natural lighting in a quiet, odor-free room.

FLAVOR PROFILE ANALYSIS

Sample # _____

AROMATICS

_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____

BASIC TASTES

Sweet	_____	_____	_____
Bitter	_____	_____	_____
Sour	_____	_____	_____
Salty	_____	_____	_____

FEELING FACTORS

Metallic	_____	_____	_____
Astringent	_____	_____	_____
Cooling	_____	_____	_____
Other ()	_____	_____	_____

TIME OF MAXIMUM INTENSITY (CIRCLE ONE):**EARLY MIDDLE LATE****COMMENTS:**

Figure 1. Panelist response sheet.

Table I. Sweeteners used in this study

Compound	Classification	Dilution Range (ppm)	No. of concs.
Acesulfame-K	N-Sulfonyl amide	100-1560	10
Alitame	Dipeptide	5-200	10
Aspartame	Dipeptide	60-3000	28
Fructo-oligosaccharide sweetener	Polyol*	13,000-100,000	6
Fructose	Polyol	6,400-35,000	11
Glucose	Polyol	9,600-50,000	10
Glycine	Amino acid	13,000-200,000	7
Isomalt	Polyol†	13,400-300,000	8
Lactitol	Polyol	20,000-500,000	10
Maltitol	Polyol	6,500-500,000	10
Monoammonium glycyrrhizinate	Terpenoid glycoside	200-3000	7
Neohesperidin dihydrochalcone	Polyketide	32-875	9
Rebaudioside-A	Terpenoid glycoside	70-1500	13
Sodium cyclamate	Sulfamate	500-9000	8
Sodium saccharin	N-Sulfonyl amide	20-1200	17
Stevioside	Terpenoid glycoside	70-1500	12
Sucralose	Chlorosugar	19-1200	11
Sucrose	Polyol	16,000-160,000	53
Thaumatococin	Protein	2.8-60	18

* Fructo-oligosaccharide sweetener is a mixture of kestose, nystose, and 1-O- β -D-fructofuranosyl-nystose.

† Isomalt is a 1:1 mixture of 6-O- α -D-glucopyranosyl-sorbitol and 6-O- α -D-glucopyranosyl-mannitol

Deionized water and unsalted crackers were available to clear the palate between stimuli.

Procedure. The concentration-response data were obtained by presenting four concentrations of a sweetener in one session. A fifth sample containing sucrose or aspartame was presented as a control. In each session, the panelists first tasted their sweet and bitter references: 2%, 5%, 7.5%, 10%, 12% and 16% sucrose, and 0.05% caffeine. They then tasted the first of five test samples, followed by a water rinse. The panelists were instructed to taste the samples, holding and swirling in the mouth for ten seconds, and to rate the maximum intensity each attribute reached before discarding the sample. Intensity scores were recorded on a response sheet individually coded for each sample. Panelists rinsed with water three times and waited approximately 60 seconds, or until all taste sensation subsided, before proceeding to the next sample. Evaluation of the four remaining samples was conducted in a similar fashion. The order of the five stimuli was randomized across subjects.

Statistical treatment. Each data point on the concentration-response plots represents the average of all panelist intensity responses within a session. While only sweetness and bitterness data are presented here, panelists provided a full sensory profile of each sweetener sample. This included quantification of any additional tastes that were detected, such as salty, sour, or metallic. Aromatic notes and "feeling factors" such as burning, viscous or smooth were also rated for intensity. In addition, panelists judged the time of maximum sweetness intensity as early, middle or late in onset.

The sweet intensity panel means for each sweetener were tested for their fit to three different mathematical models: (a) linear; (b) Beidler equation; (c) extended Beidler equation. Equation 1 is the Beidler equation (9), which is analogous to the Michaelis-Menten equation for enzyme-substrate interaction. This equation has often been used to fit concentration-response data for tastants. The third model we used (equation 2) is a modification of the Beidler equation which is equivalent to the Hill equation (10) for a receptor with multiple sites. The Hill-type equation, when the exponent $n = 1$, reduces to the Beidler equation.

$$R = \frac{R_m \cdot C}{1/K + C} \quad (1)$$

where R is the observed response; R_m is the maximal response; C is the sweetener concentration; and $1/K =$ concentration which yields half-maximal response, equivalent to the reciprocal of the receptor-sweetener association constant.

$$R = \frac{R_m \cdot C^n}{(1/K')^n + C^n} \quad (2)$$

where n is the apparent number of binding sites per receptor molecule, and $1/K'$ is still the concentration which yields half-maximal response, but it is no longer directly related to the binding efficiency as it is in equation 1.

If we assume that the observed response to sucrose increases with concentration according to a Beidler-type relationship, then the panelists are being trained to linearize a hyperbolic function. The result of this is that compounds with a maximal response comparable to that of sucrose will produce a linear concentration-response relationship (with slope greater than 1 if the potency is greater than that of sucrose, and less than 1 if the potency is less than that of sucrose). Compounds with maximal response less than that of sucrose will produce a hyperbolic curve (of the same form as the Beidler model, equation 1). A manuscript showing the mathematical derivation of these relationships is in preparation (K. Gibes).

Concentration-response data were analyzed by nonlinear regression modeling techniques using SAS Institute's PROC NLIN (11), which uses least squares as a fitting criterion. To test whether the Beidler model (equation 1) was sufficient (i.e. exponent=1 in equation 2), an F-test was performed by the "extra sum of squares" principle (12). In addition, for aspartame and sucrose, sufficient concentration replications were done to allow for an F-test for "lack of fit" of the model (13). Finally, for sucrose an additional t-test was performed to determine whether the slope of the linear fit was equal to 1 as would be expected since sweetness was measured on the sucrose equivalency scale.

Results

Sugars and sugar alcohols. Sucrose gave a linear response with concentration ($p = 0.1043$, lack of fit F-test). It can be seen in Figure 2 that the slope of this line is close to 1.0 ($p = 0.2833$, t-test for equality with 1.0). In other words, the mean panel responses closely matched the actual concentrations of the samples. Since panelists had been trained to make their intensity judgments based on a sucrose-referenced scale, this one-to-one correspondence of sucrose concentration and intensity rating confirms the reliability of the scaling methodology employed in this study.

The other sugars and sugar alcohols were best fit to the linear model over the concentration ranges studied. The graph for fructose has an initial slope that is greater than one, consistent with its increased potency relative to sucrose (Figure 3a). Glucose and fructo-oligosaccharide sweetener, however, exhibit slopes of less than one; this is indicative of sweetness potencies lower than

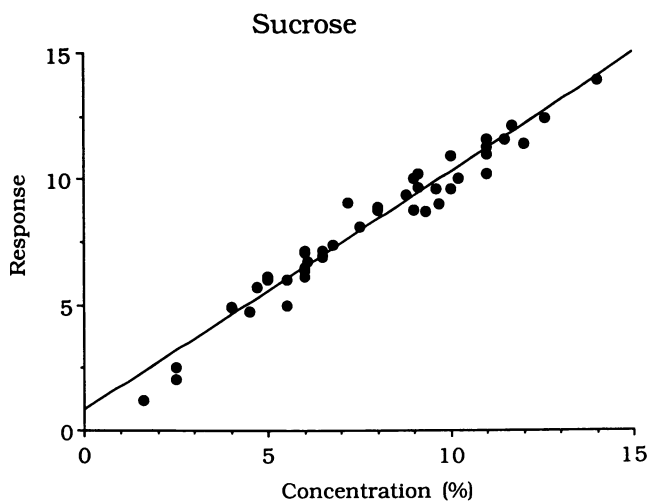


Figure 2. Concentration-response data for sucrose. The equation for least-squares fit of a straight line to the data is $R = 0.80 + 0.94(C)$; $R^2 = 0.96$.

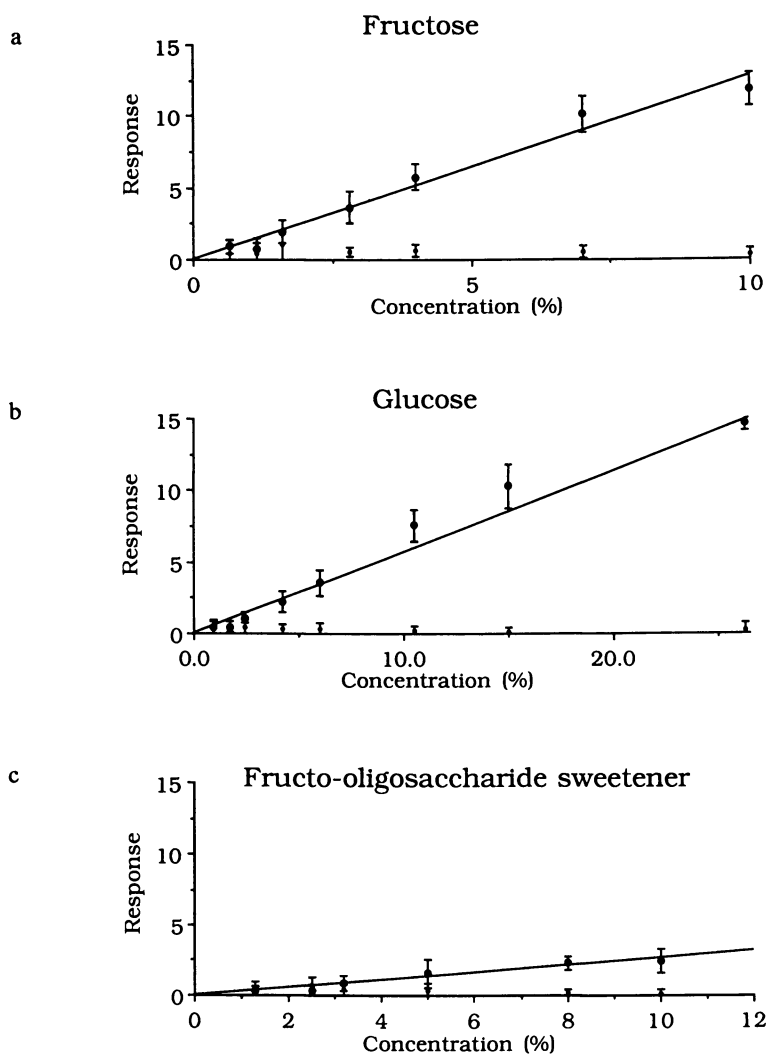


Figure 3. Concentration-response data for fructose, glucose, and fructo-oligosaccharide sweetener. For figures 3-8, large circles indicate sweetness response and small circles indicate bitterness response; error bars are Least Significant Difference, $[(2 \cdot \text{std.dev.}) / (\sqrt{2/n})]$, where n = no. of panelists. (a) Fructose, $R = 0.04 + 1.27 C$; $R^2 = 0.973$. (b) Glucose, $R = -0.02 + 0.60 C$; $R^2 = 0.974$. (c) Fructo-oligosaccharide sweetener, $R = -0.03 + 0.27 C$; $R^2 = 0.949$.

that of sucrose (Figures 3b and 3c). Sugar alcohols were also found to be less potent than sucrose. Isomalt, lactitol and maltitol all have slopes of substantially less than one (Figure 4).

High potency sweeteners. The shape of the concentration-response functions obtained for high potency sweeteners differs substantially from those obtained for the polyols discussed above. Aspartame exhibited a concentration-response function which was hyperbolic on the sucrose reference scale ($p = 0.7832$, lack of fit F-test).

The other high potency sweeteners evaluated in this study behaved similarly, asymptotically approaching maximal responses. The Beidler equation (equation 1) gave the best fit to the experimental data except in the cases of sodium cyclamate, sodium saccharin, and sucralose. For these three compounds, the Hill-type equation (equation 2; $n = 1.8, 1.4, 1.4$, respectively) gave a slightly better fit, but in each case a single data point was responsible for the better fit of the Hill-type equation. While we cannot rule out the possibility that a Hill-type equation may be required for some compounds, our data indicate that the standard Beidler equation is an adequate model of the sweetener-receptor interactions for the high-potency compounds. As shown in Figure 5, the highest maximal responses were observed for the dipeptide sweeteners aspartame and alitame (16.0 and 14.6, respectively) and for sucralose (13.0). These three compounds have the lowest incidence of non-sweet tastes among the high-potency sweeteners. The remaining high-potency sweeteners (Figure 6-8) had R_m values less than 12. These compounds all had significant concentration-dependent non-sweet tastes (acesulfame-K, sodium cyclamate, monoammonium glycyrrhizinate, neohesperidin dihydrochalcone, rebaudioside-A, sodium saccharin, stevioside) or limited solubility (glycine) which may have prevented attainment of higher sweetness intensities. Table II lists the values for maximal response (R_m) and apparent receptor-sweetener association constants ($1/K$) for all of the high-potency sweeteners obtained from fitting to the Beidler equation.

Discussion

The linearity of the sucrose response with concentration is a result of the panel training. The linearity observed for the other sugars and sugar alcohols indicates that they should exhibit a maximal sweetness similar to that of sucrose. In some cases (e.g., fructo-oligosaccharide sweetener and isomalt) the potency and/or solubility is too low to ever actually achieve the same sweetness intensity as that of a concentrated sucrose solution. The ability of the Beidler equation to fit concentration-response data for the high-potency sweeteners is consistent with a one-to-one sweetener-to-receptor interaction for these compounds. It is less clear how to interpret the apparently improved fit of the Hill-type

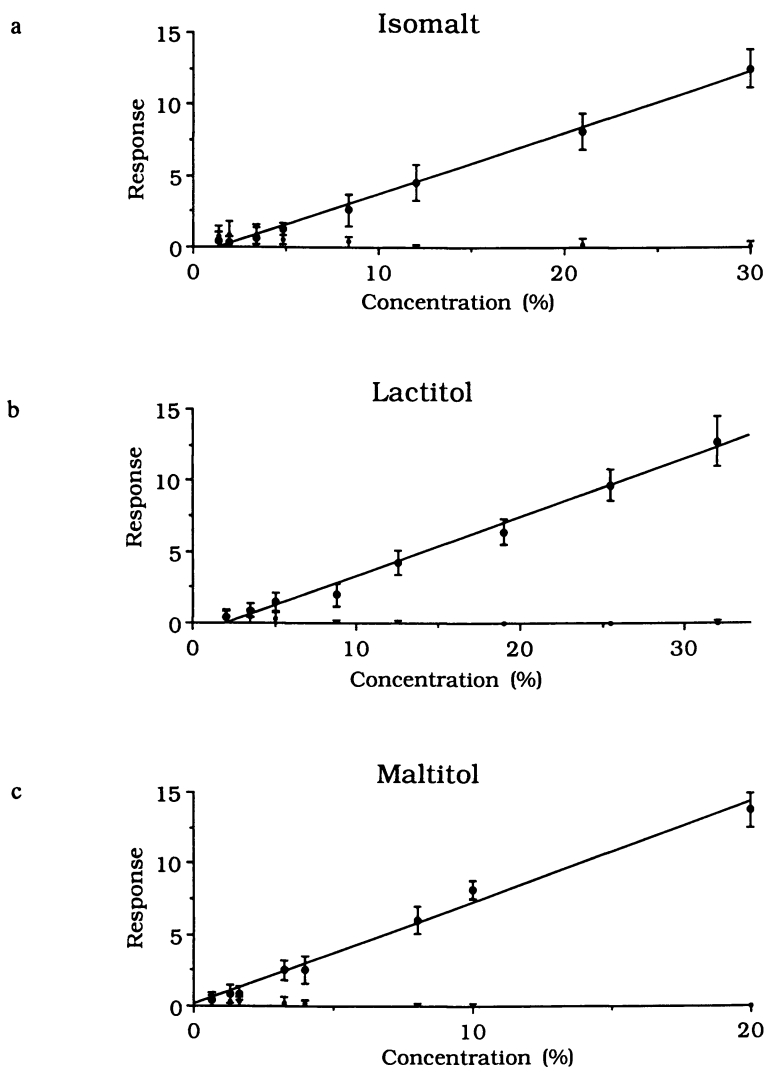


Figure 4. Concentration-response data for isomalt, lactitol, and maltitol. (a) Isomalt, $R = -0.63 + 0.43 C$; $R^2 = 0.996$. (b) Lactitol, $R = -0.82 + 0.41 C$; $R^2 = 0.990$. (c) Maltitol, $R = 0.05 + 0.71 C$; $R^2 = 0.990$.

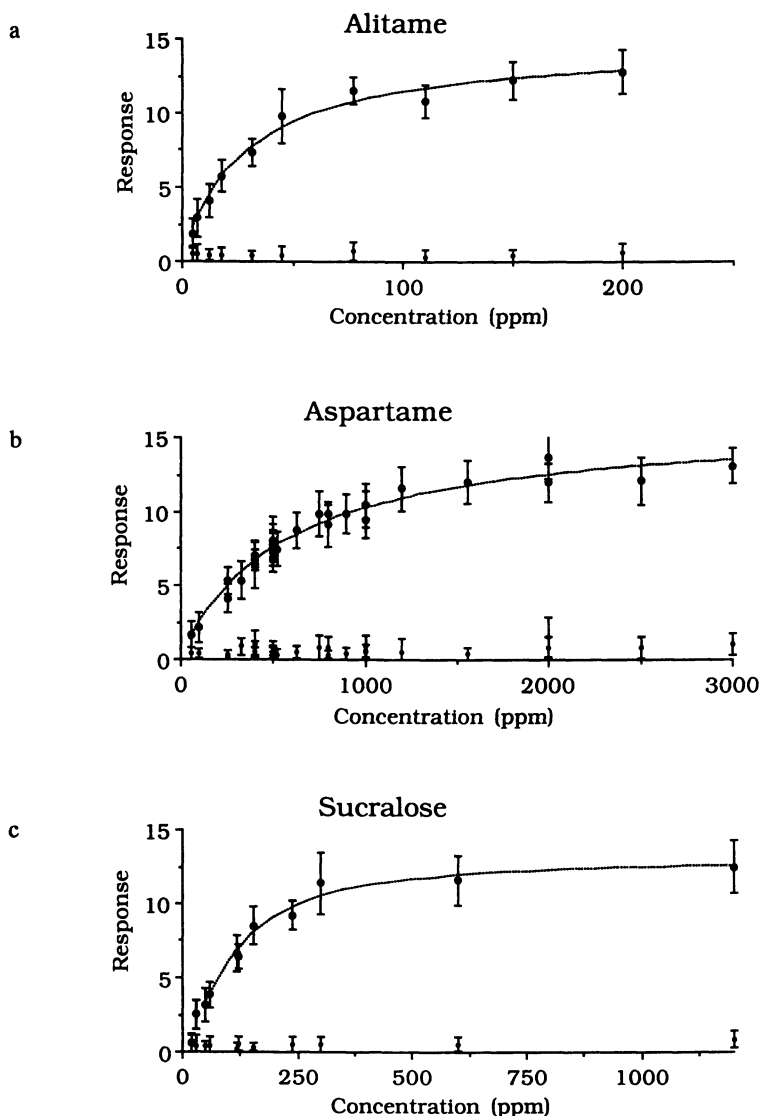


Figure 5. Concentration-response data for alitame, aspartame, and sucralose (Hill-type equation). For figures 5-8, the calculated Beidler (or Hill-type) curve are shown.

(a) alitame: $R = \frac{(14.6)(C)}{28 + C}$; (b) aspartame: $R = \frac{(16.0)(C)}{560 + C}$;

(c) sucralose: $R = \frac{(13.0)(C)^{1.4}}{110^{1.4} + C^{1.4}}$

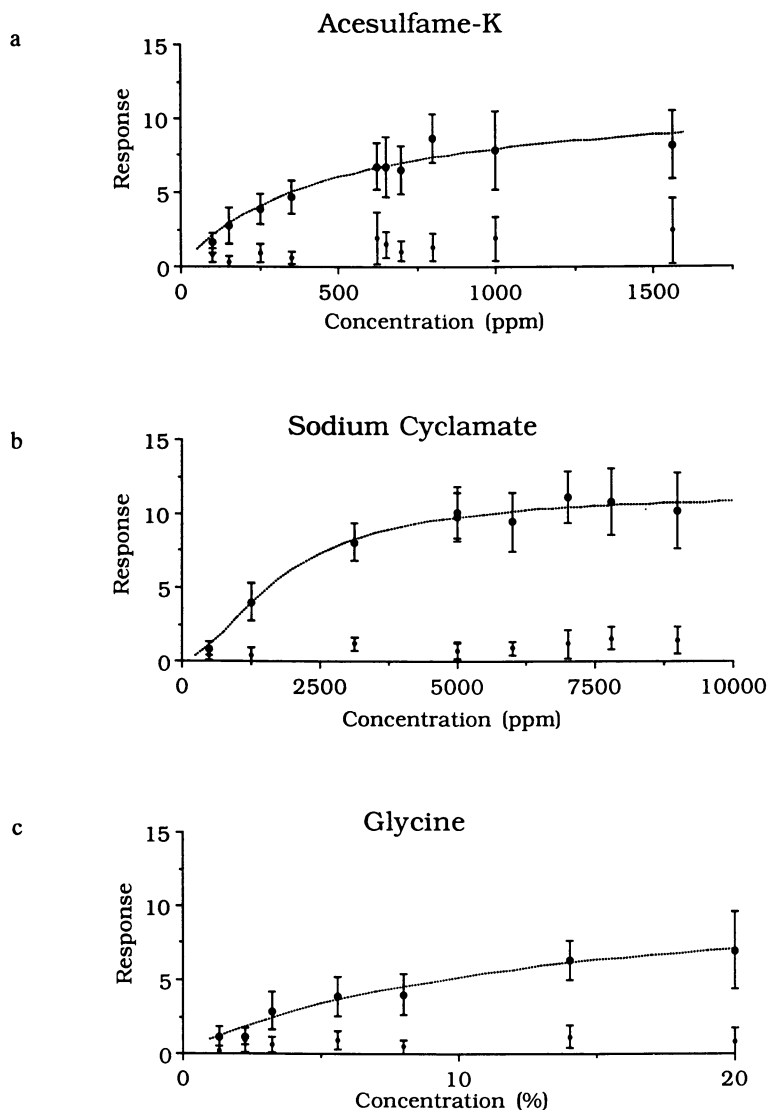


Figure 6. Concentration-response data for acesulfame-K, sodium cyclamate (Hill-type equation), and glycine. (a) acesulfame: $R = \frac{(11.6)(C)}{470 + C}$; (b) cyclamate: $R = \frac{(11.3)(C)^{1.8}}{1800^{1.8} + C^{1.8}}$; (c) glycine: $R = \frac{(11.3)(C)}{12.0 + C}$

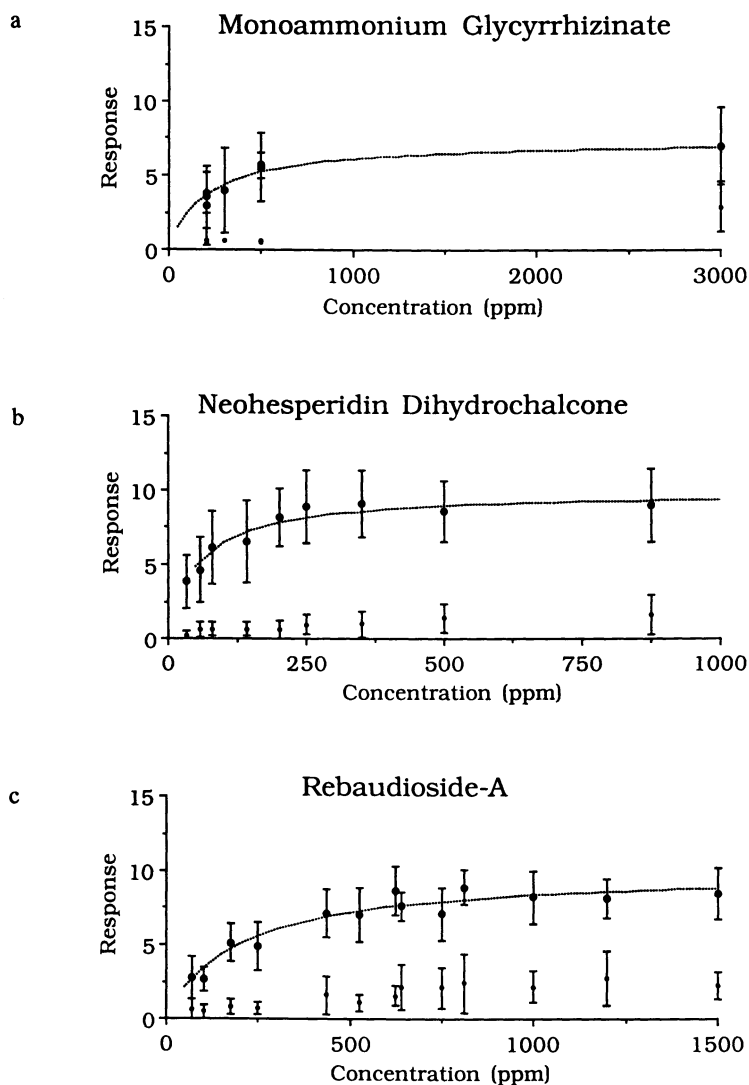


Figure 7. Concentration-response data for monoammonium glycyrrhizinate, neohesperidin dihydrochalcone, and rebaudioside-A. (a) monoammonium glycyrrhizinate:

$$R = \frac{(7.3)(C)}{210 + C} ; \text{ (b) neohesperidin dihydrochalcone: } R = \frac{(9.8)(C)}{53 + C} ;$$

$$\text{(c) rebaudioside-A: } R = \frac{(10.0)(C)}{200 + C}$$

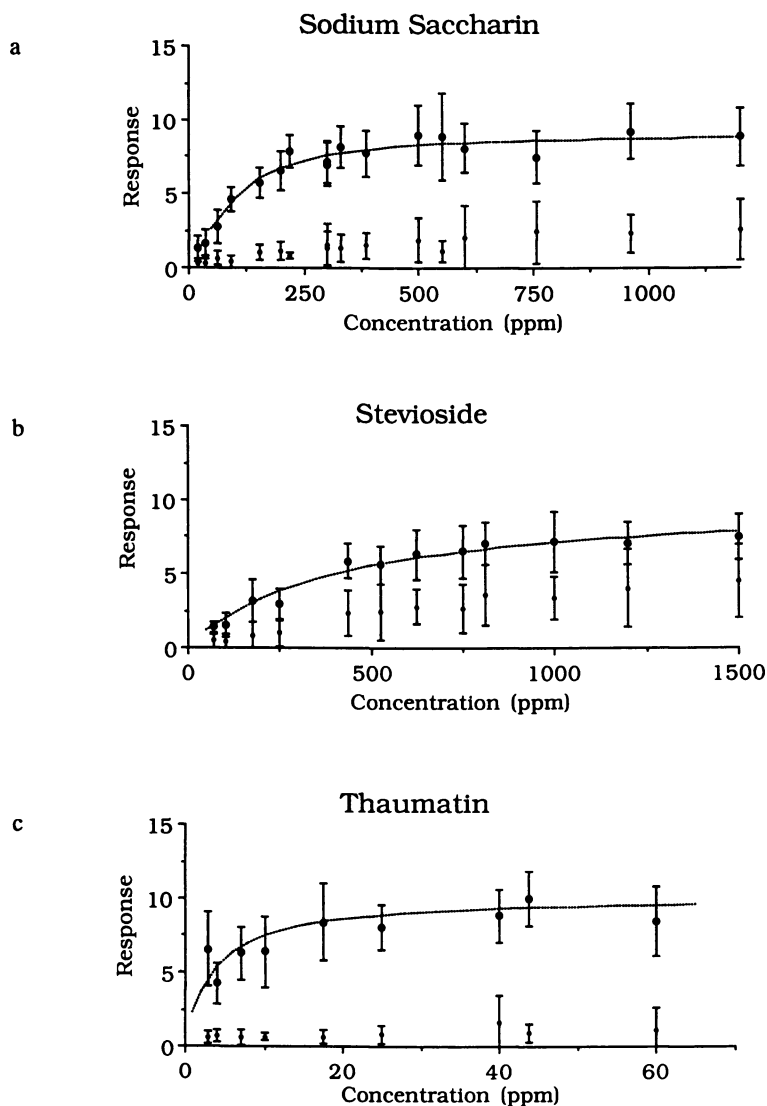


Figure 8. Concentration-response data for sodium saccharin (Hill-type equation), stevioside, and thaumatin.

(a) saccharin: $R = \frac{(9.0)(C)^{1.4}}{96^{1.4} + C^{1.4}}$; (b) stevioside: $R = \frac{(9.9)(C)}{410 + C}$

(c) thaumatin: $R = \frac{(10.1)(C)}{3.6 + C}$

Table II. Beidler equation (or Hill-type equation) parameters derived from the concentration-response data

Compound	1/K	R _{max}
Acesulfame-K	470 ppm	11.6
Alitame	28 ppm	14.6
Aspartame	560 ppm	16.0
Glycine	12%	11.3
Monoammonium glycyrrhizinate	210 ppm	7.3
Neohesperidin dihydrochalcone	53 ppm	9.8
Rebaudioside-A	200 ppm	10.0
Sodium cyclamate	1800 ppm ^a	11.3
Sodium saccharin	96 ppm ^a	9.0
Stevioside	410 ppm	9.9
Sucralose	110 ppm ^a	13.0
Thaumatococin	3.6 ppm	10.1

^a Value listed is for 1/K' (equation 2) rather than 1/K.

equation (with non-integral exponents) for three of the compounds. Ariëns points out that interactions which appear to have a higher order than one-to-one are often the result of a sequential series of interactions (14). Multiple receptor types and receptor cooperativity are hypotheses which could be considered.

All of the sugars and sugar alcohols have in common a large number of hydroxyl groups (hydrogen bond donors/acceptors) and a requirement for high concentrations in order to elicit sweet taste. It is conceivable that these compounds activate receptor cells in some non-specific way (e.g., by osmotic or conformational perturbation of cell membranes) rather than by direct interaction with receptor protein(s). High-potency sweeteners, on the other hand, might interact specifically with some subset of the receptor population. This would account for the lower maximal sweetness levels for these compounds. Alternatively, high potency sweeteners and polyol sweeteners may all activate the same receptor protein where the polyols are full agonists and the high potency sweeteners are partial agonists. Mediation of sweet taste by more than one receptor protein or more than one cellular activation system cannot be ascertained by analysis of the data presently in hand. It appears clear, however, that polyol sweeteners as a group are distinct in their behavior from high potency sweeteners. Thus it is tentatively concluded that at least two routes to receptor cell activation must exist.

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Chapter 21

Time–Intensity Profiles of Dipeptide Sweeteners

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We have prepared a number of potentially sweet L-aspartyl-D-phenyl and D-heteroaromatic glycine esters and amides. Many of these compounds display a slightly delayed onset of sweetness and a persistent sweet aftertaste. The time-intensity profiles (TIP) of these and other aspartic acid amides were determined using a cross-modal matching technique where sweetness intensity was correlated with sound intensity. The persistence of the sweet taste appears to be related to hydrophobicity.

Since aspartame was accidentally discovered at G. D. Searle in 1965 (1), over 1000 dipeptide derivatives have been prepared in an effort to determine the structural features required for sweetness (2). Until recently, most replacements or modifications of the aspartic acid portion of aspartame led to a loss of sweetness. In contrast, many modifications of the phenylalanine methyl ester portion of aspartame result in sweet compounds. For example, the methyl ester group can be replaced by a variety of small groups such as methyl. Likewise, the benzyl portion can be replaced by a wide variety of relatively large groups. Improved stability and increased potency have been the goals of much of this research. Fujino et al. (3) were the first to demonstrate that large differences in potency can be obtained by replacement of the benzyl portion of aspartame by ester groups. Esters derived from appropriately methylated alcohols were particularly potent. For example, the esters derived from (-)- α -fenchol and (+)- β -fenchol were 100 and 250 times more potent than aspartame, respectively.

However, the Fujino sweeteners, like aspartame, bear a methyl ester group which results in limited stability. At acidic pH, the ester hydrolyzes to the carboxylate while at neutral and basic pH, cyclization to a diketopiperazine takes place. In an effort to improve stability while retaining high potency, we examined analogs of the Fujino sweeteners where the labile methyl ester was replaced

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with a stable group. Isosteric replacements for the methyl ester (CONHMe, CH₂OMe) gave compounds of modest potency (R. B. Blum, C. R. Riley, unpublished results) at least partly due to the choice of (+)- α -fenchol instead of (-)- α -fenchol, a feature which was later shown to greatly affect potency. Other simple alternatives to the methyl ester group were examined, among them aromatic groups such as phenyl. Both ester and aromatic groups have an sp² carbon directly bonded to the chiral center at the C terminal side of the dipeptide derivative. The phenyl group is larger than precedented replacements for the methyl ester group, and its planarity is important for eliciting the potently sweet taste. We prepared a variety of L-aspartyl-D-phenyl and D-heterocyclic glycine esters (4,5) and amides. As observed by Fujino, the fenchyl esters were the most potently sweet.

The quality of the sweet taste, however, was compromised by a non-sucrose-like time-intensity profile. The sweetness showed a slightly delayed onset and a lingering sweet aftertaste. The aftertaste was described by some panelists as cloying, licorice-like or rock candy-like and was perceived primarily on the sides and back of the tongue. We then devoted considerable effort toward quantifying the time-intensity profiles of our sweeteners in an effort to define the structural and physical properties responsible for the temporal qualities.

Time-Intensity Profile (TIP) Methods

One of the earliest methods to continuously measure the TIP of sweeteners employed a strip-chart recorder. Sweetness intensity was recorded manually by guiding a pen along the paper cutter bar which was labeled "none" at one end and "extremely" at the other (6) or by moving a pointer connected to the recorder pen along a line scale (7). With these methods, the strip chart records a curve describing the time-intensity profile. This strip chart method was further developed by Swartz (8) with the addition of a foot pedal to start the chart. Birch et al. (9) used a calibrated potentiometer dial box in place of manual recording with a pen. This apparatus, which reduces the distractions from the pen and moving chart paper, was described as a SMURF (Sensory Measuring Unit for Recording Flux). Further improvements of these methods included digitized input of data from the time-intensity curve by Schmitt et al. (10), followed by replacement of the strip chart method by direct input into a personal computer. Guinard et al. (11) used a joystick for data input to measure the bitterness of iso- α -acids, while Lee (12) used a game paddle for data input in a study of the taste attributes of chocolate. Yoshida (13) described the application a similar computer technique to sweeteners. A mouse was used for input of sensory data. Intensities were assessed by magnitude estimation, with 2.5%, 5.0%, and 10% sucrose corresponding to defined intensities. Lee and Pangborn (14) have summarized the development and application of time-intensity techniques for sensory evaluation.

We also employed a personal computer-based time-intensity system developed by Moese and Bunger at Procter & Gamble (S. A. Moese, J. R. Bunger, unpublished results). This system is composed of a set of Apple II plus computers used for protocol planning and collection and analysis of sensory data. The system provides flexible protocol design for single or multiple product evaluations and complete test documentation. The feature which distinguishes this system from those mentioned above is the cross-modal matching technique in which panelists match perceived sweet taste intensity with sound intensity.

In practice, panelists seated in private booths are greeted by user-friendly software. They are instructed to enter identification data and then to put on the earphones provided. The software then guides panelists through a training session which teaches cross-modal matching and magnitude estimation by asking the panelist to match sound intensity with squares of various sizes, the extent of liking of common objects and activities, and the dynamic expansion of the lungs while breathing. Panelists control a 1000 Hz sound between 0 and 90 dB by the use of an uncalibrated thumbwheel which goes through several complete revolutions to span the entire range of sound intensity. Panelists typically only use a portion of the total range. The thumbwheel is mounted on a box with an on-off switch used to indicate the beginning and end of each evaluation. At the end of the session, the panelist is offered the opportunity to see the results displayed graphically. The data is stored on floppies and transferred to a mainframe for statistical analysis. A concise summary report containing the eight parameters shown in Figure 1 can be obtained. Three parameters relevant to this report (I_{\max} , AT, and $t_{1/2}$) are encircled on the Figure.

Panelist Selection and Panel Procedure

Before paneling, each sweetener was submitted for acute toxicity testing in the rat. A single oral dose of >100 times the intended human exposure, calculated on a mg/kg basis, was given. The animals were observed for two weeks for gross signs of toxicity. At the end of this period, the animals were sacrificed and necropsied. Sweeteners showing no evidence of acute toxicity were paneled. In addition, only male panelists were used to eliminate any imaginable risk to women of child-bearing age. The panelists for the TIP sessions were those used to establish the potency of our sweeteners by magnitude estimation versus coded sucrose standards. Panelists could discriminate among the primary tastes and could distinguish changes in taste intensity for differing concentrations of sucrose. None had prior TIP experience. Six TIP training panels were run with aqueous solutions of sucrose and aspartame. Samples (10 mL in 30 mL plastic cups) were prepared in commercial (Talawanda) spring water. Panelists sampled the entire 10 mL of solution and flipped the switch to begin the session. As the sweet taste grew in intensity, panelists rotated the thumbwheel to produce a similar increase in sound intensity. The sample was expectorated after 5 sec as

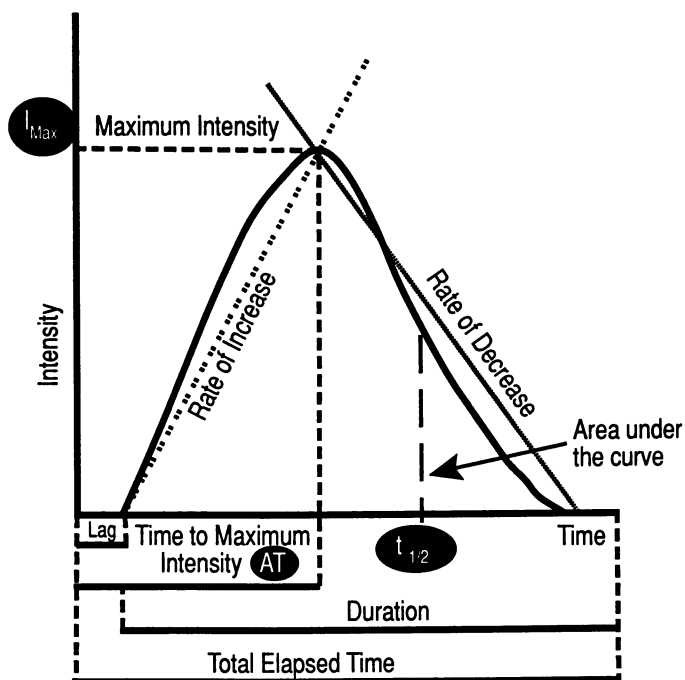






























Fig. 1. Time-intensity profile parameters.

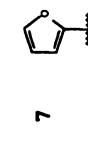
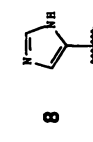
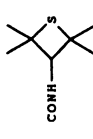
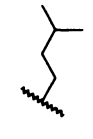
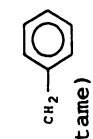
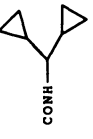
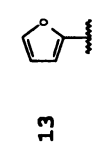
indicated by a countdown clock on the computer screen. Panelists continued to match the change in sweet taste intensity with sound intensity until the sweet taste was gone and then they flipped the switch to off. The range of sound intensity selected by each panelist during the session was completely subjective. No effort was made to standardize the range and there were large variations among the panelists. Panelists were selected on the basis of their ability to determine the duration of sweet taste ($t_{1/2}$) to within two standard deviations of the panel mean. In practice, only one of ten panelists failed to meet this criterion. Seven to nine panelists were used for each session. Three or four samples were evaluated in a balanced pattern unless it was suspected that a sample had a severe lingering aftertaste, in which case it was tasted last. Generally, a single evaluation was made for each sweetener, except for sucrose which was run as a control in each panel. The concentrations of the sweeteners were selected to match the sweetness intensity of 10% sucrose based on potencies (Table I) determined in earlier panels. Between samples, panelists were instructed to eat an unsalted soda cracker and to rinse their mouth thoroughly with water. The software imposed a minimum wait period of two minutes between samples.

Normalization Procedure

The mean maximum sound intensity, I_{\max} , determined by averaging the maximal sound intensity chosen by each panelist for each sweetener (corresponding to the mean maximum in sweetness intensity) is given in Table I where each unit corresponds to 0.022 dB. Since the sound intensity range chosen by each panelist varied greatly, the intensity data were normalized to avoid biasing the results by panelists who chose higher intensities (15). We assigned a value of 150 units to the highest sound intensity chosen by each panelist for each sweetener and scaled the remaining intensity data accordingly. The time course of each panel was determined by averaging the normalized sound intensities at 1 second intervals across all panelists. Because panelists often perceived maximal sweet taste intensity at different times, the mean normalized intensity was always less than 150. This normalized data was used to determine two important descriptors of the time intensity profile: the appearance time, AT, (time to maximum intensity) and the persistence, $t_{1/2}$, (the time for taste intensity to decline to half its maximal value). This particular measure of persistence was previously used by Lawless and Skinner (7). Values for the total duration of the sweet taste were more variable, as panelists were often uncertain about when the last traces of sweetness were gone. This was a particular problem with very persistent sweeteners. With a method for quantitating the time-intensity profile established, we turned our attention to the measurement of physical properties which might be responsible for the observed temporal properties.

Table I. Taste and Physical Properties of Aspartic Acid Amide Sweeteners

Cmpd	R ₁		R ₂		Potency ^a	I _{max} ^b	AI ^c	t _{1/2} ^d	R _m ^e	logP ^f
										
1					1200	700	12 ± 5	27 ± 11	1.12	4.94
2					3400	630	9 ± 5	22 ± 10	1.00	4.94
3					2000	1090	20 ± 8	41 ± 12	0.79	4.58
4					16000	830	18 ± 8	39 ± 12	0.79	4.11
5					400	700	10 ± 5	20 ± 6	0.55	4.27
6					500	1000	10 ± 4	26 ± 6	0.27	3.28

7		400	560	9 ± 3	15 ± 2	0.25	2.71
8		350	650	8 ± 2	13 ± 3	0.23	2.75
9	CH ₃ 	2000	540	9 ± 3	15 ± 3	0.02	2.38
10		80	510	7 ± 3	18 ± 6	-0.19	2.36
11	CO ₂ Me (aspartame) 	180	840	8 ± 2	16 ± 6	-0.27	1.86
12	CH ₃ 	1200	700	8 ± 3	17 ± 5	-0.35	0.95
13		600	540	8 ± 3	12 ± 2	-0.39	1.37
	sucrose (10%)	1	660-1260	8 ± 1	15 ± 2		

*10% Sucrose = 1. ^bMean maximum sound intensity. ^cAppearance time (sec) as mean ± std. dev. ^dTime (sec) for intensity to decline to half its maximal value as mean ± std. dev. ^eR_m = log (1/R_t - 1). See text. ^fPartition coefficient (MEDCHEM).

Hydrophobicity Estimates

During our earlier taste panels to determine the potency of our sweeteners, we noticed that the persistence of the sweet taste was greatest for sweeteners containing the most hydrophobic groups. To assess the significance of this observation, hydrophobicity was estimated in two ways.

First, reversed-phase thin layer chromatography was used to determine R_f values, the ratio of the distance traveled by the sweetener spots to the distance traveled by the solvent front (Whatman LKC18F plates 5 x 20 cm, 200 μ thickness). Five compounds were spotted per plate, with aspartame common to each plate. The plates were developed together by ascending elution in a filter paper-lined closed glass tank with 60% MeOH/H₂O as eluent. After drying the plates, the spots were visualized with ninhydrin and the R_f 's were measured. The observed R_f values were converted to R_m values given in Table I using the relationship $R_m = \log(1/R_f - 1)$ (16). R_m is defined as $\log k'$, the capacity factor in liquid chromatography which is related to the partition coefficient between an apolar stationary phase and the polar mobile phase. Similar partitioning behavior in thin layer chromatography allows R_m to be related to R_f as shown above. This converts the experimental partitioning data to the same logarithmic form as the $\log P$ values given below. In a crude way, the partitioning of sweeteners between the mobile phase and the hydrophobic layer on the TLC plate may be related to the partitioning of sweeteners between aqueous solution and the membrane-bound sweet taste receptor.

Partition coefficients, $\log P$, were also calculated for the variable part of the sweeteners, i. e., the AspNH fragment was excluded from the calculation. $\log P$ values were determined from Rekker's fragment constants (17) and from the MedChem program (18) based on the Pomona College database. The values from the latter method are given in Table I.

Results and Discussion

The time-intensity profiles for sucrose, aspartame, and L-aspartyl-D-furylglycine amide **13** are shown in Figure 2. These three sweeteners have similar temporal properties and show relatively minor differences in the appearance time, AT, and persistence, $t_{1/2}$. Figure 3 shows the TIP for a group of sweeteners with differing temporal profiles. The TIP of sucrose and aspartame are again similar but the phenyl and furylglycine esters **1** and **4** show a delay in the appearance and decay of the sweet taste as indicated by the increased values of AT and $t_{1/2}$ (Table I). Note that the normalization procedure used to derive Figures 2 and 3 serves only to adjust the intensity scale along the y-axis.

Data for the temporal and partitioning properties of thirteen sweeteners are summarized in Table I. Persistence is known to be related to intensity and the $t_{1/2}$ data should be corrected to reflect persistence at equal perceived intensities (I_{max}). This has been done in cases where panelists are trained to assign defined intensity

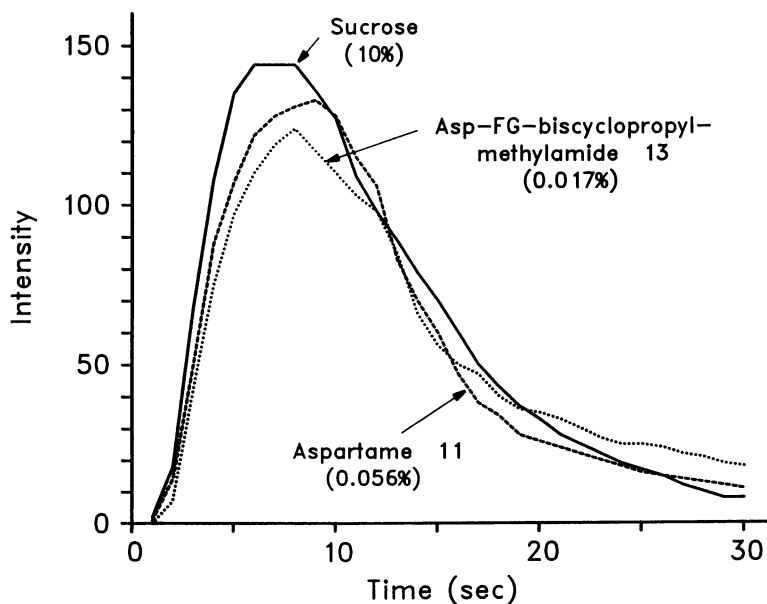


Fig. 2. Time-intensity profile (normalized). FG is furylglycine. Structures are given in Table I.

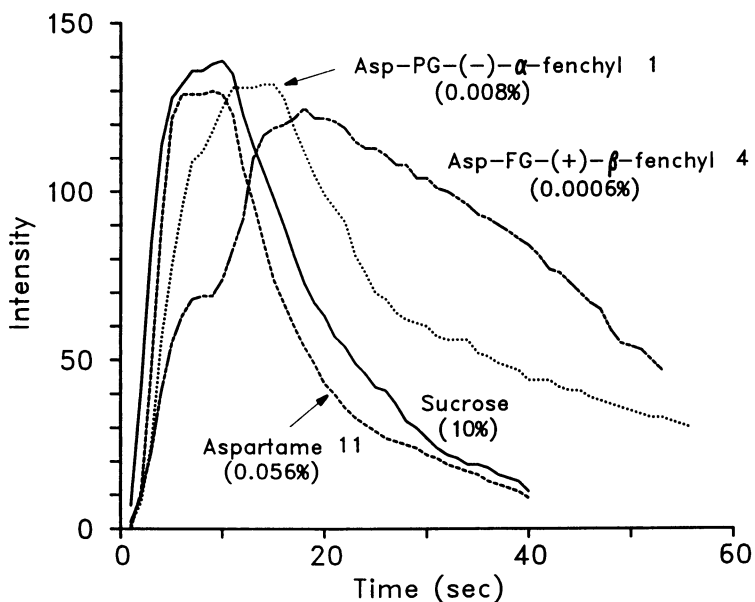


Fig. 3. Time-intensity profile (normalized). PG is phenylglycine and FG is furylglycine. Structures are given in Table I.

values to a given concentration of taste stimulant (19, 20). However, in our case, panelists were free to choose any sound intensity to match the perceived sweetness intensity and the resulting large variation in mean maximal intensity (standard deviations are of the same order of magnitude as I_{\max} itself) precludes a meaningful correction. It is somewhat reassuring to note that the range of I_{\max} values for sucrose (660-1260) and for the sweeteners in Table I (510-1090) are similar.

Figures 4 and 5 show the correlation of $t_{1/2}$ with hydrophobicity. The thienyl- and furyl-glycine sweeteners **3** and **4** were considered outliers and were excluded from the regression analysis. The fact that the lines can only account for about half of the variance in the data is not too surprising given the imprecise nature of the time-intensity data. The combination of a hydrophobic R_1 and R_2 groups as for sweeteners **1-5** generally results in an increase in appearance time and persistence. The reason for the extreme persistence for the thienylglycine ester **3** is not apparent. The furyl-glycine sweetener **4** also showed an anomalously large AT and $t_{1/2}$. This cannot result solely from the presence of the furyl group as the furyl-glycine amides **7** and **13** showed sucrose-like time-intensity profiles. The imidazolylglycine sweetener **8** demonstrates that fenchyl esters can possess sucrose-like time-intensity profiles when the R_1 group provides sufficient hydrophilicity. The imidazolyl group of **8** should be protonated at pH 7 which would increase its hydrophilicity further. The sweeteners **9-13** are all relatively hydrophilic and show sucrose-like time-intensity profiles.

Certain classes of sweeteners such as the dihydrochalcones have been associated with delayed (slow onset, lingering aftertaste) time-intensity profiles. DuBois and co-workers (19) prepared a large variety of dihydrochalcone analogs to assess the relevance of metabolism, conformation, chelation and hydrophobicity to their temporal properties. They concluded that none of the four proposed rationales was supported. Of particular note was a group of A-ring C-4 hydroxyl modifications which varied the hydrophilic character. Among four amino acid ethers prepared, the homoserine ether showed a significant decrease in persistence, although it was still more persistent than sucrose or saccharin (21). The hydrophobicity of these four compounds was estimated using the liquid chromatographic parameter k' , which reflects the partitioning of solute between the stationary and mobile phases. While potency appeared to be related to hydrophobicity, persistence was not. The improved time profile for the homoserine ether remained unexplained.

The aspartic amide class of sweeteners has not generally been considered a persistent class. The persistent phenyl-, furyl-, and thienylglycine esters demonstrate that, in fact, time-intensity profiles ranging from sucrose-like to dihydrochalcone-like can be found within the dipeptide class. The structural variations for the compounds listed in Table I involve variations in groups which are uncharged and can affect only the hydrophobic binding and, in some cases, hydrogen binding in and around the taste receptor. Limiting the source of the variation in hydrophobicity to groups which cannot participate in ionic binding distin-

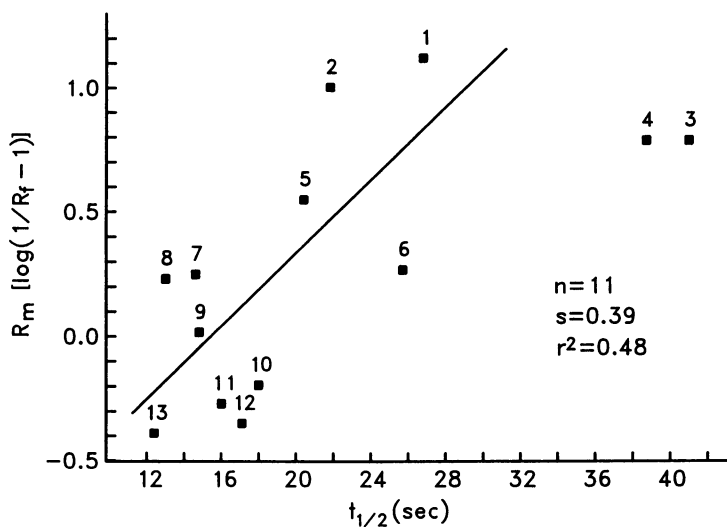


Fig. 4. Hydrophobicity (R_m) versus persistence ($t_{1/2}$) for compounds in Table I.

Compounds 3 and 4 are outliers and are not included in the analysis.

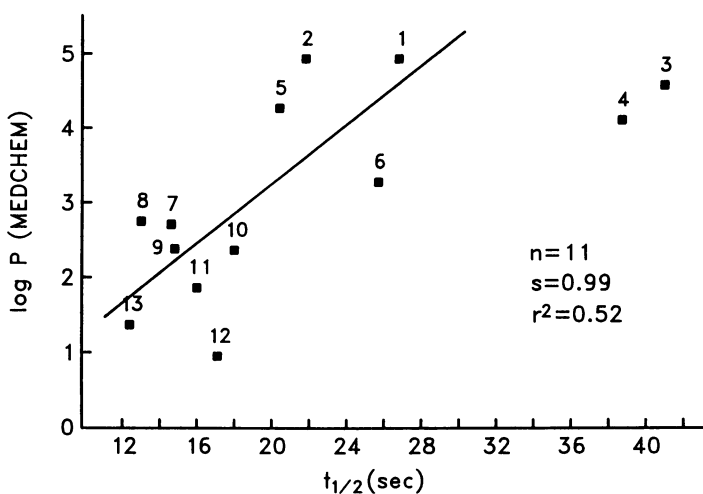


Fig. 5. Hydrophobicity ($\log P$) versus persistence ($t_{1/2}$) for compounds in

Table I. Compounds 3 and 4 are outliers and are not included in the analysis.

guishes these compounds from the dihydrochalcone derivatives mentioned above and may be responsible for the correlation of persistence with hydrophobicity seen in this study.

There are currently several hypotheses to explain sweetener persistence. Birch and co-workers (22, 23) have proposed the formation of an "orderly queue" of stimulus molecules at the receptor. Nonpersistent molecules pass through the queue quickly while persistent molecules pass through slowly. It is not apparent why the compounds listed in Table I should differ greatly in their ability to travel such a queue and the relevance of this explanation, at least in the present case, is doubtful. Jakinovich and Sugarman (24) has suggested that different binding rate constants at a single receptor may account for changes in persistence. Dubois and Lee (20) have proposed that sucrose-like sweeteners bind specifically and rapidly to the sweet taste receptor site while persistent sweeteners bind to nonreceptor binding sites during diffusion to and from the sweet taste receptor site. The result is a delayed onset of sweet taste and a persistent sweet aftertaste. Boudreau (25) has suggested that there exist two types of sweet taste receptors: sweet₁ which resides on the front of the tongue and interacts with nonpersistent sweeteners such as sucrose, and sweet₂ which resides on the back of the tongue and interacts with persistent sweeteners such as the dihydrochalcones. Sucrose-like aspartic acid amides are sensed on the front of the tongue, while the persistent amides are initially sensed on both parts of the tongue, with the lingering sensation primarily on the back of the tongue.

These ideas provide possible rationales for the temporal properties of the compounds in Table I. The variations in temporal properties might be explained by interaction with two different receptors. Alternatively, the receptor may be identical, with only the environment differing between the front and back of the tongue. If the receptor environment at the back of the tongue is more hydrophobic, nonspecific hydrophobic binding would delay the arrival of the tastant at the receptor giving rise to an increase in the appearance time. Hydrophobic sweeteners would only slowly partition out of the receptor environment resulting in a persistent aftertaste, in accordance with Dubois and Lee's proposal.

In summary, we have demonstrated that the computer-guided cross-modal matching technique, which correlates sound intensity with sweetness intensity, is useful in quantitating the time-intensity profiles of sweeteners. Using this technique, various aspartic acid amides were found to display temporal properties ranging from sucrose-like to dihydrochalcone-like. Correlations with measured and calculated hydrophobicities suggest for the first time that hydrophobicity may be one important factor in determining the time-intensity profiles of aspartic acid amide sweeteners.

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Chapter 22

Electrophysiological Evaluation of Sweeteners

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A method is described for the electrophysiological evaluation of the temporal profiles of sweeteners. Temporal profiles are difficult to quantitate in human taste panels because of the need for extensive panelist training and because time resolution is not adequate, particularly in the early portion of the profile. These problems can be circumvented by directly measuring nerve activity in the sensory nerves, particularly in the chorda tympani nerve. Results obtained from chorda tympani recordings in rhesus monkeys are in agreement with published human taste panel results.

In contrast to salty taste, which is elicited cleanly only by NaCl and LiCl, there are numerous structural classes of compounds which taste sweet. These include dipeptides (aspartame, alitame), amino acids (D-tryptophan), kynurenines, aryl ureas (suosan), aryl guanidines, carbohydrates, halogenated carbohydrates (sucralose), N-sulfonylamides (saccharin, acesulfame), and proteins (thaumatin, monellin, pentadin). There are few areas of new product research which receive more industrial effort than high-potency sweeteners (1). As a result, the number of new sweeteners has grown substantially over the last decade, and it is safe to say that an even larger number of sweeteners is currently under development.

Three characteristics need to be evaluated psychophysically before a new compound can find use as a sweetener: the flavor profile (taste quality), the potency profile (concentration/response function), and the temporal profile (time/intensity function). The standard way of quantitating these characteristics is to carry out psychophysical evaluation with a human taste panel. However, safety tests must be carried out before human panels may evaluate a new compound, and extensive training is required in order to get reproducible results (2). Among the properties listed above, the

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temporal profile is particularly difficult to assess. First we define the temporal profile, then we review approaches to temporal profile analysis in human subjects, and finally we describe an electrophysiological approach in which temporal characteristics are precisely quantitated by measurement of the chorda tympani nerve activity in anesthetized monkeys.

The Temporal Profile

The sweetness of a sweetener changes during the time it is on the tongue, sometimes dramatically. The way this happens can be very different for different sweeteners. Sucrose and thaumatin provide an illustrative contrast. A 10% solution of sucrose is as sweet as a 0.025% solution of thaumatin, but this is not evident during the first seconds after the thaumatin is tasted. Sucrose reaches its maximum sweetness within a few seconds. Thaumatin has no taste at all for the first seconds, then its sweetness intensity gradually builds to a maximum over a period of about 10 seconds. Disappearance of the sweetness is also very different; the sweetness of sucrose clears rapidly, while the sweetness of thaumatin persists for several minutes after swallowing (3). Other sweeteners have temporal profiles intermediate between these extremes (4). The temporal profile of a sweetener is an important parameter, since it has a substantial impact on acceptability in food systems.

Psychophysical Evaluation of Sweeteners

Several methods have been used to record temporal profiles of sweeteners. Early time-intensity work (5-8) was done with panelists rating sweetness intensity at time intervals determined with a clock or stopwatch. Strip chart recorders (9-12) and potentiometers (13) have also been used to permit continuous recording of time-intensity data. However, there are several limitations to temporal profile analysis with human panels. Even with extensive training, it is difficult to get reproducible quantitative data. Variables such as rate of mixing with saliva and the saliva flow rate are hard to control. In addition, time resolution is limited to several seconds, which may not be adequate for early portions of the time-intensity profile. There exists the complication of subjective interpretation by the panelist. In addition, panelist fatigue limits the rate at which data can be acquired. Therefore, we describe an alternative approach to direct measurement of temporal profiles of sweeteners: electrophysiological recording from the taste nerves. This method is based on several physiological and anatomical conditions. The first is that all sensations (including taste) are carried by nerve impulses from peripheral sense organs to the brain. The sense organs for taste are called taste buds. Taste information is carried from the taste buds to the brain primarily in the chorda tympani and glosso-

pharyngeal nerves. The chorda tympani nerve carries signals from the anterior part of the tongue. This nerve is relatively easy to locate surgically and to utilize for electrophysiological recording from anesthetized animals.

Electrophysiological Recordings of Taste

Figure 1 shows typical recordings of chorda tympani nerve activity from a rhesus monkey on stimulation with a series of sweeteners. These are not direct recordings of the individual nerve pulses, as individual nerve pulses are difficult to quantify. Instead, the impulse activity is electrically summated and the nerve activity is displayed as a voltage instead of as a frequency. In Figure 1, responses to xylitol, sucrose, aspartame, acesulfame-K, NaCl, and protein sweeteners are recorded. In the first two series, native monellin and thaumatin (both very sweet) were used. In the third series, the protein sweetener is thaumatin in which six of the lysine residues are methylated (14); this compound is still sweet, and it still gives a nerve response. In the fourth series (thaumatin with iodinated tyrosines, ref. 15) and the fifth series (thaumatin with acetylated lysines, ref. 14), loss of sweet taste correlates with loss of nerve response.

Several features of the nerve recordings should be noted. First, the responses are very reproducible for any given stimulus. Second, tasteless compounds give no nerve response. This result is supported by behavioral experiments in monkeys and taste tests in humans. Third, cross-adaptation can be seen between the sweet proteins and the other sweeteners. The magnitude of the sucrose response following the protein sweetener is substantially reduced because of the prolonged stimulation by the protein. Finally, the temporal profiles of the protein sweeteners are considerably different from those of the other sweeteners. The temporal profiles will be further examined in the following section.

The Temporal Profile of the Electrophysiological Taste Response

The temporal profile of a sweetener can readily be observed in these electrophysiological recordings, particularly if the recording is displayed with an expanded time scale. Several quantitative parameters can be obtained from such recordings, as illustrated in Figure 2. The *maximum amplitude* is the signal intensity attained, corresponding to the maximum intensity of sweetness. The *delay time* is the time from initial stimulation to signal 10% above baseline, corresponding to latency between application and first taste sensation. *Rise time* is the time between initial stimulation and maximal response amplitude, i.e., the time required to reach maximal intensity. *Slope (maximum amplitude/rise time)* is the change in magnitude with respect to time; it is an indicator of the rate of change of intensity. *Resume time* is the time which elapses

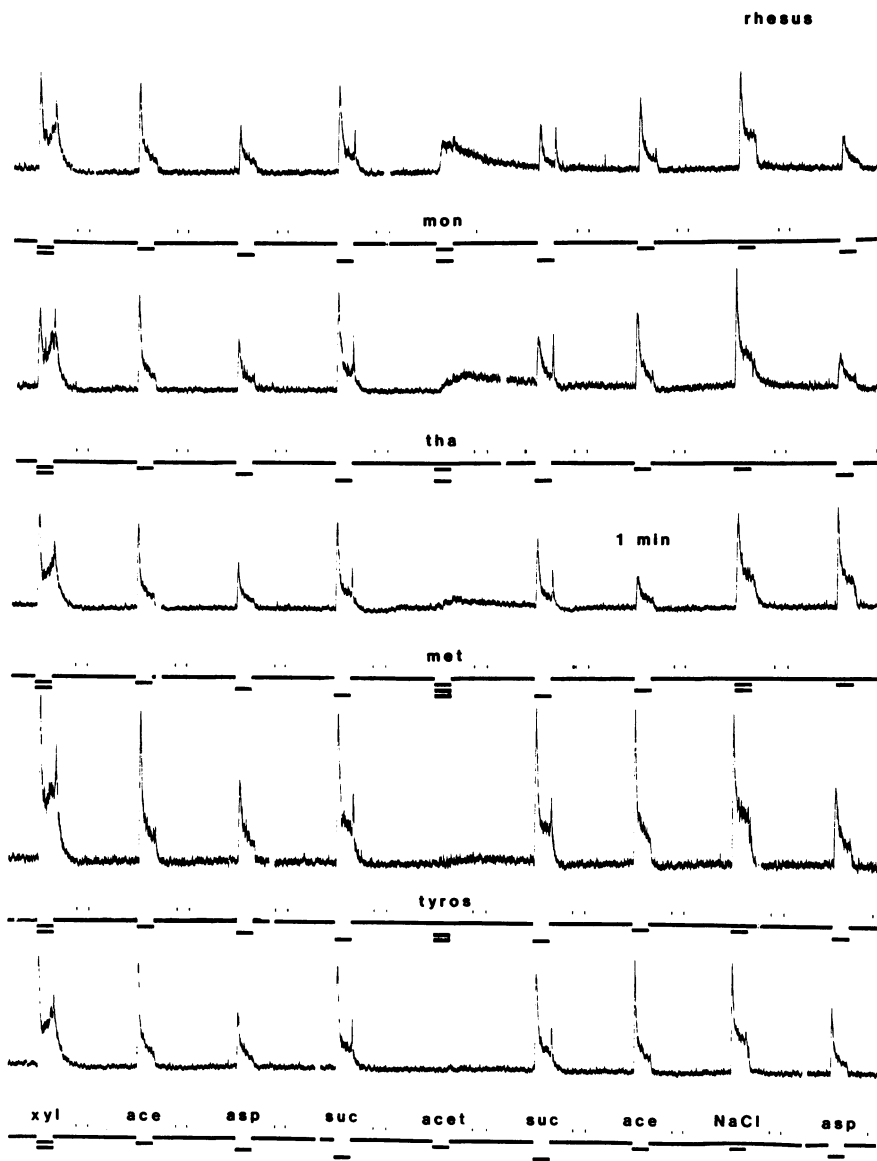


Figure 1. A series of summated chorda tympani nerve recordings during taste stimulation in a rhesus monkey. Except for changes in the protein sweetener, the same sequence of stimuli was used in all 5 recordings: xylitol, acesulfame-K, aspartame, sucrose, *protein sweetener (0.02%)*, sucrose, acesulfame-K, NaCl, aspartame. The protein sweeteners used were: (a) monellin; (b) thaumatin; (c) thaumatin in which lysine residues are methylated [still sweet]; (d) thaumatin in which tyrosines are iodinated [tasteless]; (e) thaumatin in which lysines are acetylated [tasteless].

from the conclusion of stimulation until signal returns to baseline. *Area* is the integrated area under the recording.

The Choice of an Animal Model--Phylogenetic Differences

A very important consideration when choosing an animal model is that the sense of taste in mammalian species differs. Many compounds which are sweet to the human tongue are not sweet to common experimental animals such as rats or dogs (16). There is a strong phylogenetic relationship in taste (16-21), so that the more closely related to humans the animal species which is used for the electrophysiological assay, the more similar is its sense of sweet taste to that of humans. Table I summarizes some of the results found in primates.

From Table I it can be concluded that the squirrel monkey can not be used for the evaluation of the sweetness of analogs of aspartame or thaumatin, since these compounds do not taste sweet to New World monkeys. On the other hand, results in Table I indicate that an Old World monkey (e.g., rhesus) is a reasonable choice as an animal model for human taste. Only one difference (effect of gymnemic acid) has been found between the sense of sweet taste in Old World monkeys and humans (22,23).

Comparison Between Temporal Profiles in Humans and Monkeys

In order to evaluate this method of temporal screening, we chose sweeteners for which temporal profiles have been studied in humans. Acesulfame-K (rapid onset) and monellin (very slow onset, lingering sweetness) represent the extremes in temporal profile. Sucrose, aspartame, and an example of the highly potent guanidines (compound 1) discovered by Nofre and Tinti (24) were included in order to assess the utility of this method in ranking sweeteners by temporal profile. In order to cover a range of concentrations, we selected for each sweetener a concentration equivalent in sweetness to 10% sucrose (in humans); we then used samples with twice this concentration and one-half of this concentration. The data from the electrophysiological measurements is summarized in Table II and Figure 3. The data for each sweetener concentration were obtained from at least three stimulations of the monkey's tongue, and the neural recordings were digitally summed to enhance the signal-to-noise ratio. Temporal parameters were measured from the summed recordings. The data shown in Table II is taken from a representative monkey; data from other monkeys are comparable.

Acesulfame-K showed the most rapid onset followed, in turn, by sucrose, compound 1, aspartame, and monellin. Figure 3 shows that the taste of monellin lingers after stimulation is stopped. In this respect, monellin is quite different from the other sweeteners, which return to baseline rapidly after stimulation.

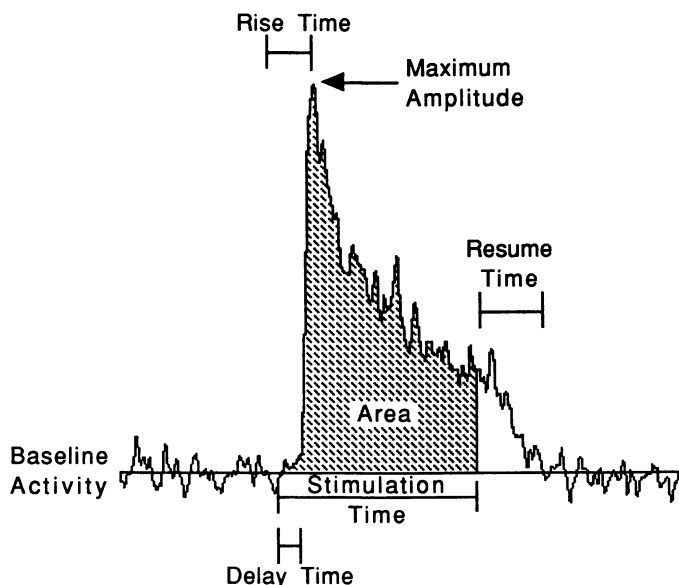


Figure 2. A representative summated recording from the chorda tympani nerve of a rhesus monkey during a 10 sec stimulation with a sweetener. The quantitative parameters *maximum amplitude*, *delay time*, *rise time* (dt), *resume time*, and *integrated area* are indicated.

Table I. Effects of some sweeteners and taste modifiers in primates

	Gymnemic acid effect on sweeteners	Thaumatococcus and aspartame	Gymnemic acid effect on miraculin	Miraculin effect on acid taste
Humans, apes (Superfamilia Hominoidea)	abolishes sweetness	taste sweet	abolishes sweetness	induces sweetness
Old World monkeys (Superfamilia Cercopithecoidea)	no effect	taste sweet	abolishes sweetness	induces sweetness
New World monkeys (Infraordo Platyrrhina)	no effect	no sweet taste	no effect	induces sweetness
Half-monkeys (Ordo Prosimiae)	no effect	no effect	no effect	no effect

Table II. Temporal parameters measured for acesulfame-K, aspartame, monellin, guanidine sweetener **1**, and sucrose

Sweetener	Max Amp ^a	Area ^a	Delay Time ^b	Slope ^a	Rise Time ^b	Resume Time ^b
Acesulfame						
0.35 g/L	122	1192	250	21.8	560	1520
0.70 g/L	172	1608	190	41.0	420	1920
1.40 g/L	206	1580	270	54.2	380	4980
Aspartame						
0.35 g/L	42	492	330	8.2	510	1580
0.70 g/L	51	717	340	9.4	540	1440
1.40 g/L	89	809	280	22.3	400	1440
Monellin						
13 mg/mL	24	446	410	0.5	5270	12500
26 mg/mL	33	1085	360	0.6	5300	19520
52 mg/mL	37	837	470	3.5	1070	19520
Guanidine 1						
7.1 mg/mL	29	447	350	3.8	770	920
14.2 mg/mL	69	773	320	14.7	470	1480
28.4 mg/mL	65	816	340	11.8	550	1740
Sucrose						
0.15 M	89	1481	300	16.8	530	2060
0.30 M	121	2237	300	25.2	480	5440
0.60 M	113	1837	300	27.6	410	8100

^a Units of measure are arbitrary.

^b Milliseconds.

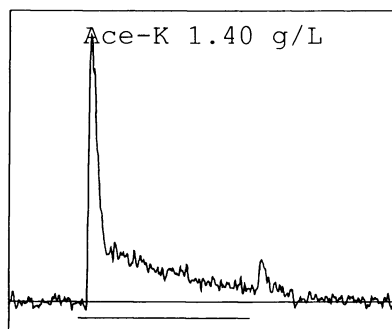


Figure 3. Summated chorda tympani nerve recordings for acesulfame-K (1.4 g/L), sucrose (0.60 M), aspartame (1.4 g/L), compound **1** (28.4 mg/L), and monellin (52 mg/L). The bar at the bottom of each recording indicates the 10 sec time during which sweetener solution flowed over the tongue. Spikes in neural activity occurring at the end of the stimulation with sucrose and acesulfame in the figures above do not occur in all recordings; they appear to be occasional artifacts of the magnetic valve switching.

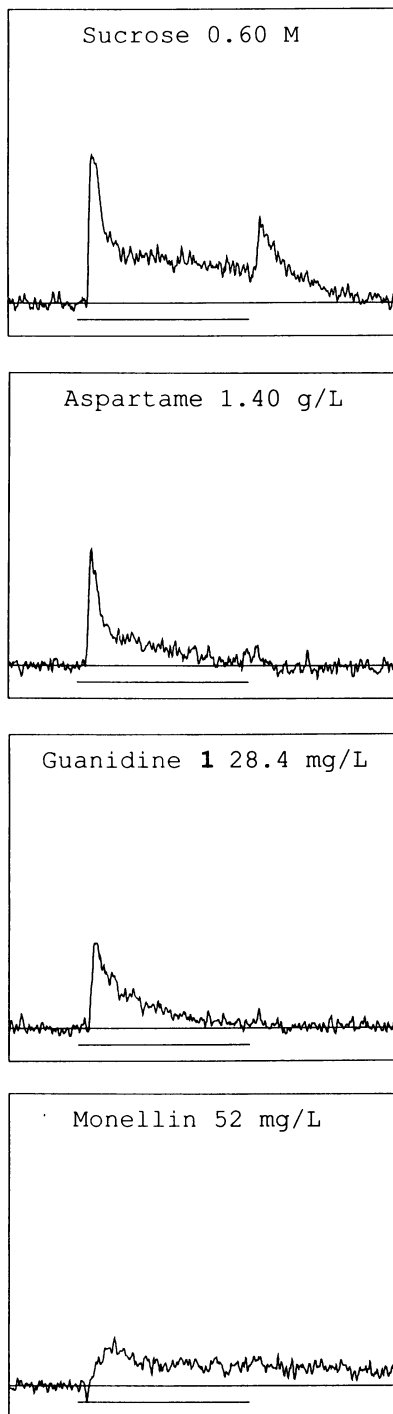
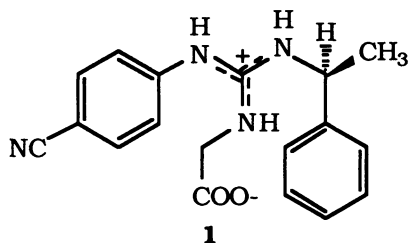


Figure 3. Continued.



In studies with human panelists, DuBois and Lee (11) characterized the temporal profile of sweeteners in terms of *appearance time* (time required to attain maximum sweetness intensity) and *extinction time* (time required for sweetness intensity to return to a defined low level). They found sucrose (10%) and aspartame (750 ppm) to have similar appearance times (4 and 5 sec, respectively), while aspartame showed a longer extinction time (13 and 19 sec, respectively).

Ott and coworkers (12) have measured time-intensity profiles of sucrose, aspartame, alitame, and acesulfame-K with a human panel. They derived a number of quantitative parameters from their recordings. They found a more rapid response to acesulfame-K than to sucrose or aspartame (*TMAX*, time to maximum intensity, = 7.9, 9.7, 9.6 sec, respectively; *RATEMAX*, maximum intensity divided by *TMAX*, = 8.4, 7.1, 7.8, respectively). They also found that the sweetness of aspartame persists longer than that of sucrose or acesulfame-K (*RT*, recording time, = 53.0, 36.3, 34.7 sec, respectively).

Quantitative temporal profiles have not been reported for monellin, but its sweetness is reported to persist for an unusual period of time (25).

Discussion

Overall, results with human panelists correlate well with our electrophysiological findings. It was of interest to determine if any of the measured temporal parameters might be independent of concentration, thus allowing a judgment of a compound's temporal acceptability (similarity to sucrose and aspartame) from data on a single concentration of material. The effect of sweetener concentration on temporal profile is illustrated in Table II. An increase in sweetener concentration leads to an increase in the maximum point reached, slope, and the resume time. In other words, a more concentrated solution results in a more intense response which is realized more quickly and which lasts longer. Our electrophysiological findings are in good agreement with the human taste panel results of Ott *et al.* (12) with respect to the rapid onset (short rise time, large slope) of acesulfame relative to sucrose and aspartame.

At this point in our development of this methodology, the best method for the judgment of temporal acceptability is visual inspection of the response profiles. Responses characterized by a rapid onset (large slope, small delay) and a rapid return to base line (short resume time) after stimulation are acceptable to consumers, since they are characteristic of sucrose. Figure 3 shows response profiles for the five sweeteners used for this study. All of the sweeteners except monellin show sucrose-like summated nerve responses. Since the temporal profile shows some concentration dependence, it is probably necessary to make measurements at several concentrations (or at a concentration which gives a response intensity in the desired range). Work is continuing in the quantitation of more subtle temporal differences.

Conclusion

The method outlined provides a technique for evaluating the temporal properties of sweeteners. This technique has the advantage that it provides quantitative data on the entire temporal profile (both onset and lingering) without the complication of subjective interpretation which occurs in human psychophysical experiments.

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Chapter 23

Novel Sweeteners

Regulatory Issues and Implications

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Novel sweeteners, in common with other novel substances intended for use in the food supply, must undergo rigorous review by government health agencies as a prerequisite to market approval. Novel sweeteners - that is to say sweetening materials not previously known or used in the food supply - would generally be classified as food additives and as such would normally be reviewed within existing regulations which deal with food additives. Thus, the process of review and evaluation of novel sweeteners does not, *a priori*, involve any new regulatory needs. The regulations under which other substances such as novel preservatives or emulsifiers can be examined also encompasses materials which function to impart a sweet taste to foods.

This overview of such regulatory requirements, although based on United States and Canadian regulations, will also serve to illustrate the situation in most developed countries. The focus will deal with food additive concerns and will also address some additional needs specific to novel sweeteners as a class.

Food Additive Preclearance Requirements

National governments have a responsibility to ensure the health and safety of their populations, and nowhere is this more important or visible than in relation to the food supply. Consumer concerns about food safety and the proliferation of food additives provide an ongoing backdrop against which government regulatory agencies must make informed decisions as to the safety and acceptability of additives, often in controversial circumstances. Sweeteners, as a class, have in the past engendered considerable controversy, particularly in the case of saccharin and cyclamates because of their association with carcinogenic effects and, as a consequence, regulatory agencies take a very conservative approach when dealing not only with these substances but with food additives generally.

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It is instructive to note a typical definition of a food additive. Under Section B.01.001 of the Canadian Food and Drug Regulations, a food additive is defined as "any substance, the use of which results or may reasonably be expected to result in it or its by-products becoming a part of or affecting the characteristics of a food." This definition, which also goes on to define some exclusions such as drugs or agricultural chemicals that are covered under other requirements, is quite broad and can be seen to include all substances which are not foods themselves or which are covered by other classifications. For example, in the United States GRAS [Generally Recognized as Safe] food ingredients are substances which may be added to foods but which, because of a history of use, differ from food additives in their legal requirements respecting safety evaluation.

In general, government agencies evaluating a novel food additive such as a novel sweetener will need to address five main areas as part of the premarket review process: 1. The identity and nature of the substance; 2. The types of intended use and potential intake levels; 3. Technical effects of the additive; 4. Analytical methodology in food; 5. Studies to determine safety.

Details of the statutory requirements for food additive petitions in the United States are outlined in Section 409(b)(2) of the United States Federal Food, Drug and Cosmetic Act, and the data requirements are described in greater detail in Section 171(c) of Title 21, Code of Federal Regulations. Similarly, Section B.16.002 of the Canadian Food and Drug Regulations describes the requirements which a petitioner must meet when requesting amendments to the Food Additives Tables of the Food and Drug Regulations.

Identity and Nature of the Substances

A prerequisite in the regulatory evaluation of any novel substance is the need for a complete dossier of information on its identity, stability, composition, method of manufacture and specifications. This requires the petitioner to compile and submit data including the common or usual name, IUPAC or chemical abstracts names, chemical formula, molecular weight, compositional analysis and purity, and properties of the substance, e.g. melting point, specific gravity and optical rotation.

It is of particular importance to provide food-grade specifications which will encompass the product intended for commercial distribution and which will also relate to the material that was employed in the toxicological testing program. Specifications in the format of the Food Chemicals Codex, 3rd edition (1), are acceptable in both the United States and Canada and should include a description of the substance, identification tests, an assay test and limits of impurities together with validated methods of analyses for the parameters in question. The results of

analysis of several typical production batches of the substance should also be provided to indicate conformity with the proposed specifications.

Also required is a description of the manufacturing process, including a list of reagents, solvents, catalysts, reaction conditions, purification procedures, etc. Regulatory agencies are particularly interested in assurances that the product will be routinely prepared under conditions which control its composition and properties and that specifications will consistently be met. It is to be expected that different methods of manufacture will give rise to differences in impurities and by-products. For example, in the case of saccharin, o-toluenesulfonamide (OTS) can arise as an impurity in certain manufacturing processes and not in others. At one time, OTS was considered a causative factor in bladder tumor induction in animals. As a result, the need to control the presence of OTS in saccharin was considered imperative and strict specification limits (not more than 0.0025%) were imposed on its presence in the product (OTS was later ruled out as the causative factor). A typical specification would also include limits for heavy metals and arsenic.

As more interest is shown in biotechnology as a route to the biosynthesis of novel chemical entities, cognizance should also be taken of the need to address the differences, if any, in specifications arising out of differences in method of manufacture. Of equal importance is the question of stability of the compound, i.e. does it decompose over time, is it sensitive to humidity, does it react to other compounds, etc. In the case of aspartame, for example, the presence of 5-benzyl-3,6-dioxo-2-piperazineacetic acid (a cyclization product of aspartame) was recognized as an impurity in the commercial product with the result that a specification limit was deemed necessary and set at "not more than 1.5%".

Although specifics may differ in different jurisdictions, the essence of what regulatory agencies will require relates to information that describes the substance, indicates that it is prepared in accordance with good manufacturing practices and that quality control will be exercised to ensure consistency of product in conformity with accepted specifications.

Uses and Technical Effects of the Substance

The proposed uses of the substance in question depends to a large extent on its intended effect and its properties. In the case of sweeteners, the objective is clear, that is, to impart a sweet taste to foods, and thus a potentially wide range of applications can be foreseen.

Regulatory agencies require information on the amounts of the substance to be added to foods, the types of foods, fate of the compound in the various food applications, the amount of the substance and its by-products that are likely to be consumed and data to support technological efficacy, i.e., will the compound

accomplish the intended effect of imparting sweetness to the food in question over a normal expected shelf life? Two aspects are particularly important. The first relates to the fate in food. While some substances may be stable at room temperature, they may be prone to rapid degradation by heat and/or low pH. Thus, they may be inherently unsuitable in soft drinks or in products that are baked. Evidence must be supplied to justify the level of use of the substance, and types of applications including data demonstrating extended stability of the compound under conditions of use. Areas which are technologically inappropriate or self-limiting should also be pointed out.

A second key area dealing with the proposed uses of a substance such as a novel sweetener relates to the need to provide estimates of intake of the substance and possibly of its by-products by consumers. This should include not only the average or typical consumer but also the so-called high consumer. From this information, regulatory agencies determine the Estimated Daily Intake (EDI) which is of critical importance in relation to safety considerations and in regard to determining the extent and range of uses that can be permitted. Table I indicates the uses of aspartame currently permitted in Canada.

Table I. Permitted Uses of Aspartame in Canada

Additive	Permitted in or upon	Purpose of Use	Maximum Level of Use
Aspartame	Table-top sweeteners	Sweetener and flavour enhancer	Good Manufacturing Practice
	Breakfast cereals	Sweetener and flavour enhancer	0.5%
	Beverages; Beverage concentrates; Beverage mixes	Sweetener and flavour enhancer	0.1% in beverages as consumed
	Desserts; Dessert mixes; Toppings; Topping mixes; Fillings; Filling mixes	Sweetener and flavour enhancer	0.3% in product as consumed
	Chewing gum; Breath-freshener products	Sweetener and flavour enhancer	1.0%

Source: Adapted from Food and Drug Regulations Section B.16.100, Table VIII, p. 67-22, 1989.

Analytical Methodology in Foods

In addition to the analytical methodology that is required with respect to the specification of the substance and its impurities, analytical methods to determine the quantity of the material or its by-products in foods must also be developed by the petitioner.

This is particularly important in cases where regulatory limitations have been established on the amount of the substance that can be permitted in particular foods. Thus, the methods have a role in the enforcement of tolerances as well as in the assurance of the accuracy of estimates of exposure or EDI considerations.

Methods must be easily reproducible under normal laboratory conditions without the need for sophisticated equipment and certain specially trained personnel. Methods must be specific, accurate, precise and reliable and have the required sensitivity. Most importantly in a regulatory context, the methods must be able to withstand scrutiny in court room settings.

Safety Studies

The area which is most critical for decision-making by regulatory agencies and which is also the most demanding and time consuming for the industry concerns the need to provide assurance of the safety-in-use of the proposed novel substance. The principal methodology employed in the generation of this type of support data involves the use of experimental animals as surrogates for man and the application of a wide range of toxicological tests.

These encompass both acute, short term and chronic studies usually designed to assess carcinogenic potential. Further tests include genotoxicity tests, to determine the potential for adverse effects on the genome in germ and somatic cells, mutagenicity studies, teratogenicity studies to examine potential for congenital malformations and reproduction studies to assess potential effects on the reproductive capacities of both the parental generation and their offspring. In addition, pharmacokinetic studies which address the absorption, distribution, metabolism and excretion of the substance are pivotal to the selection of the most appropriate animal species for toxicity testing and in the determination of the most appropriate dose levels to be employed. Depending on the nature of the compound and what is known about its chemical structure, properties, metabolism, potential exposure and relationship to other tested chemicals, all or only some of the battery of studies indicated may be required.

The overall objective of the toxicological testing of a novel chemical entity is to determine the maximum dosage level which produces no discernible injury when the substance is administered over the major portion of the life span of the test animals. This level is called the "No Observable Adverse Effect Level" (NOAEL) and is expressed as the amount ingested in mg/kg body weight/day

by the animals in question in a given test procedure. The value is then used to establish a critical parameter for the estimation of risk to the human population. The parameter, called the Acceptable Daily Intake for man or (ADI) is defined as the amount of a food additive (expressed in mg/kg body weight) that an individual can consume daily in the diet, over a lifetime, without risk. The ADI is usually determined by dividing the NOAEL for the most sensitive animal species and applying a safety factor or margin of safety. While the safety factor may vary depending on the nature of any effects noted, a value of 100-fold is usually employed to derive an ADI. Thus, in the case of aspartame, Canadian authorities considered the NOAEL from long-term animal studies to be 4 g/kg body weight/day. With the application of a 100-fold safety factor to allow for extrapolation from animals to man, the Canadian authorities established an ADI of 40 mg/kg body weight/day. Table II indicates internationally established ADIs for some common sweeteners.

Table II. Acceptable Daily Intake (ADI) Values for Some Sweeteners as Established by JECFA^a

Aspartame	0-40 mg/kg/day ^b
Saccharin and its Ca, K and Na Salts	Temporary Group ADI 0-2.5 mg/kg/day ^c
Thaumatococin	ADI Not Specified ^d
Mannitol	ADI Not Specified ^e

^a FAO/WHO Joint Expert Committee on Food Additives (JECFA)

^b 25th Report of JECFA, WHO Technical Report Series (TRS) No. 669, 1981.

^c 28th Report of JECFA, WHO TRS No. 710, 1984. Temporary ADI allocated--further safety data required.

^d 29th Report of JECFA, WHO TRS No. 733, 1986. ADI not specified--no upper limit of intake assigned in view of very low toxicity.

^e 30th Report of JECFA, WHO TRS No. 751, 1987.

The ADI is the toxicological endpoint which governs the allocation of permitted uses of a food additive assuming technological efficacy has been demonstrated. In the evaluation of requests for specific use allocations of a given food additive, the estimated daily intake (EDI) figures are compared with the ADI of the additive or, in the case of a major impurity, to the ADI of that impurity. As a matter of prudence, the estimated or probable daily intakes (EDI) should not exceed the ADI. In those instances where

the estimated intakes are low or where an ADI is high or not specified, the risk to human health in permitting the proposed uses of the substance would be low.

However, a health risk from a food additive is, *inter alia*, governed by the two factors of inherent toxicity of the additive and its exposure pattern. In those cases where potential exposure of the human population can be expected to be as high as would be the case with novel sweeteners, regulatory agencies will always take a cautious approach in the allocation of permitted uses. This would be particularly so in the case of additives with low ADIs.

Special Considerations for Sweeteners

The requirements for the approval of a food additive are onerous indeed and involve a considerable expenditure of scientific effort, time and money. However, the addition of a novel chemical entity to the food supply is a serious matter which could have a significant impact on the health and welfare of the consumer. It is, therefore, no surprise that government health agencies are quite conservative when dealing with these substances. In the case of novel sweeteners, some additional considerations are germane to the evaluation process for the following reasons: i) novel sweeteners without the caloric content of sugar or other traditional sweeteners have the potential for extremely wide scale exposure across all sectors of the population; ii) because of this exposure, the potential for a significant number of the population to exhibit effects not discernible by present day animal toxicological testing protocols - specifically allergic or hypersensitivity reactions - cannot be ignored; iii) novel non-carbohydrate sweeteners may be of significant interest to specific vulnerable population subgroups - e.g., diabetics - for which groups additional studies may be required; iv) specific labelling may be required to advise on potential adverse effects for specific population subgroups and to advise consumers of nutrient changes.

Exposure Considerations

In a recent publication (2), the United States Calorie Control Council noted the results of a nationally sponsored survey which indicated that more people are leading "low-cal" lifestyles and are controlling calories as part of the adoption of an overall healthy lifestyle. Some ninety-three million Americans now consume low-calorie foods and beverages. This figure represents an increase of some 15 million consumers since 1986, and is more than double the number of such consumers since 1978.

With an ever-increasing health and calorie-conscious population as an eager and receptive group for low-calorie food and beverage products, novel intense sweeteners, together with novel fat substitutes, have the potential for extensive and escalating

distribution and use in the food supply. This factor was recognized in Canada during the deliberations pertaining to the approval process for the use of aspartame in foods.

Thus, the concept of "post-market surveillance" in addition to the traditional "pre-market evaluation" was introduced in 1979 as part of Health and Welfare Canada's proposal on the regulatory status of aspartame. At the time there was a concern that the acceptable Daily Intake (ADI) would be exceeded in certain population subgroups. It should be stressed that exceeding an ADI by individuals on occasion is of no practical consequence in terms of overall risks to health. It is, however, a matter of concern for health agencies when habitual intakes exceed ADI values particularly in vulnerable subgroups such as children.

Canadian regulators subsequently decided that the use-pattern and intake of aspartame should be monitored by industry to assess its safety under practical conditions of use. Such post-market surveillance studies were to be designed to provide data regarding the distribution of aspartame intake, including mean and upper percentiles for high users. It was also not considered useful to initiate any such program until it could be shown that adequate information on consumption patterns could be obtained, i.e. after product sales and use had been established. Further, and most important both for the industry and the Health Department, it was indicated as a matter of policy that monitoring consumption data would be taken into consideration as part of the assessment of any requests for future extensions of aspartame use.

In compliance with this policy, the NutraSweet Company contracted to undertake a dietary survey to measure aspartame intake in Canada (3). This survey was undertaken in 1987 after use patterns were considered to have been sufficiently established and involved two time periods, utilizing nationally representative samples of 5544 and 4872 Canadians. The results indicated the average daily intake of the total sample to be 0.6 mg/kg per day. Extreme intakes at the 90th and 95th percentiles for the total population were 3.8 and 5.2 mg/kg body weight/day respectively. Subgroups such as diabetics were also studied. In all cases, the intake of aspartame was found to be far below the ADI of 40 mg/kg/day.

This study, which may well serve as a model, provides results which are of importance not only as a general review of consumption and intake patterns but which will be particularly relevant in regard to the evaluation of new requests for additional uses of aspartame. While all new sweetener petitions will be examined on a case-by-case basis, the need for post-market surveillance data particularly for low ADI substances will remain a general policy requirement in Canada.

Potential for Adverse Reactions

In the assessment of safety of a novel substance, animal species have traditionally served as surrogates for man and thereby as tools for the prediction of potential adverse consequences in the human. These toxicological approaches, supplemented by newer in vitro techniques such as the Ames test and other tests not involving large scale animal models have, in the main, been good predictors of the safety of a variety of chemical substances. However, tests are not yet available for the pre-market prediction of a range of consequences usually classified as "adverse or hypersensitivity reactions" that may be caused by or associated with the use of a particular additive in man. Examples include reported adverse reactions due to the food colour tartrazine, the seasoning monosodium glutamate and the sweetener aspartame.

As a consequence, the reporting and monitoring of adverse reactions is also seen as a necessary post-market requirement for large exposure additives. It is seen as an exercise which involves not only the public, medical practitioners and health agencies, but the industry as well. For example, an aspartame adverse reaction report is compiled quarterly by the United States Food and Drug Administration Center for Food Safety and Applied Nutrition. This monitors the nature, severity and numbers of complaints from a variety of sources. Results are analyzed as to trends and as determinants of future policy. Similar reports are also monitored by other governments for a range of substances.

From a policy perspective, the question arises as to the acceptability of a substance for use in food which has the potential to cause allergies or hypersensitivity reactions. This matter is one which is of considerable concern to governments. Some guidance has been provided by the FAO/WHO Joint Expert Committee on Food Additives (4, 5). In considering this problem the Committee was of the view that no approval should be given to substances causing serious or widespread hypersensitivity reactions. For substances capable of causing only a low incidence of, or minor hypersensitivity reactions, the Committee was of the view that ADIs should be established. However, the Committee stated that appropriate labelling should also be considered as a possible mechanism to enable sensitive individuals and their physicians to identify possible sources of allergic reactions and to minimize such hazards. While the specific allergy-related labelling would be case dependant and could range from a simple label declaration of the presence of the compound in a food to cautionary statements detailing the symptomatology, labelling would at least afford the consumer with some basic information and thereby with options to remedy his or her situation as a matter of individual choice.

These Committee views are compatible with most regulatory agency thinking and are in line with the type of precautionary measures that would appear reasonable assuming a low incidence of

mild reactions. It is recognized, however, that test methodologies to predict hypersensitivity and adverse reactions together with regulatory mechanisms to deal with them will continue to be the focus of considerable attention.

Vulnerable Subgroup Considerations

An additional consequence of extensive and potentially escalating use of novel sweeteners in the food supply relates to their consumption by population subgroups with particular health concerns. Diabetics, for example, who must exercise care respecting sugar intake are particularly interested in sugar substitutes. Because of particulars related to their condition, population subgroups that may constitute target groups for potential large scale consumption should be considered for specific studies such as metabolism, etc. Groups such as the very young and the obese may also be useful in assessing the nature of any potential untoward effects that may be associated with particular novel sweeteners. With regard to aspartame, such studies were carried out in normal, obese, diabetic and phenylketonuric adult humans. Persons with phenylketonuria, an inborn error in metabolism, must restrict their intake of L-phenylalanine from common foods as well as from aspartame in which it is a constituent amino acid. Canadian and U.S. regulators considered it necessary to advise this vulnerable subgroup of the presence of phenylalanine and, thus, the Canadian and U.S. label of any food containing aspartame must carry on its principal display panel the statement "contains phenylalanine".

Labelling Considerations Pertaining to Novel Sweeteners

It is readily understandable that the presence of a novel sweetener or any combination of novel sweeteners in any foodstuffs would be expected to be indicated on the list of ingredients of that product. In addition, consumers might find a statement of the sweetness of a serving expressed in terms of the amount of sugar producing an equivalent degree of sweetness to be quite useful.

The use of high-intensity novel sweeteners in foods may also result in considerable shifts in the macronutrient composition of protein, fat and carbohydrate in the final product compared with a similar product containing caloric sweeteners. Such products may be widely consumed by diabetics who require information on the macronutrient content as a basis for the calculation of exchange values. Furthermore, compositional information on new products sweetened with novel substances will not be readily available in food composition handbooks.

Because of such considerations, a label statement of the quantities of all macronutrients together with the energy value should be required on all foods containing novel sweeteners. In addition, such foods may be subject to other specific labelling

requirements depending on their categorization as, for example, "foods for special dietary use" or "table-top sweetener preparations." Full nutrition labelling, including the declaration of micronutrients, e.g. vitamins and minerals contained in the food product, is also an option to be explored in view of increasing consumer interest in nutrition. Manufacturers of foods containing novel sweeteners should be aware of the considerable body of specific regulatory requirements that pertain to both labelling and to the use of claims in both American and Canadian regulations.

Conclusion

The discovery of a novel sweetener is but the initial step in a long process leading toward potential commercialization. As has been discussed, all novel sweeteners must undergo a complex, extensive and time consuming battery of tests as part of the pre- and post-market review process and evaluation of the safety and acceptability of these substances.

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Chapter 24

The Future of Synthetic Sweeteners

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A major problem with this subject is aptly summarized in a quotation from Niels Bohr, "Prediction is very difficult, especially about the future." What are my credentials for attempting to guess the future of synthetic sweeteners?

I was in on the ground floor of aspartame because, in 1965, James Schlatter, a chemist who worked for me discovered the amazing taste of this rather simple molecule. Our employer, G.D. Searle & Co., decided to develop the material, and I have kept abreast of what has happened with synthetic sweeteners over the last 25 years.

Market

It doesn't require much insight or expertise to realize that synthetic sweeteners are booming. Sales figures for The NutraSweet Company since approval for wet uses in 1983 (1984: \$568,000,000; 1989: \$875,000,000) show this clearly. These impressive numbers certainly have benefited from the quality of aspartame's taste and from an inspired marketing strategy. However, people really want synthetic sweeteners and, when offered a product with reasonable properties, will rush to put down their money.

For diabetics, the rationale for existence of a good-tasting synthetic sweetener is obvious. It can change a patient's attitude toward his disease and toward his life in general. The NutraSweet Company has in its files hundreds of spontaneous letters from diabetics and their relatives describing how aspartame has transformed the lives of these unfortunate people. Many of these letters are highly emotional. So, here is a product that is a genuine boon to a certain segment of the population.

What about the normal population? Is this simply a fad market? How can one justify enormous research and development

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expenditures followed by the agony of registration for the person who consumes diet beverages in order to feel good about eating a hot fudge sundae?

People seem to want low calorie palatable products. Their motives may be confused and not always defensible or easy to analyze, but the demand is real and growing and it will not go away. Synthetic sweeteners are part of the answer to that demand. The demand is part of a new preoccupation with health and fitness. A cynic might say that in countries where people don't have to work very hard anymore, they have time to worry about marginal and rather unimportant aspects of their lives.

Regulatory agencies of the government respond to these trends by aiding and abetting them, rather than fighting back. Labeling proposals generally tend to make consumers even more aware of the ingredients and composition of their food. In the process of regulating health claims on food packages, the government may be willing to permit health claims for specific chemicals that have specific beneficial effects on specific diseases. Other proposals would change officially recommended percentages of the basic food types in the national diet. For example, convincing people to lower the percentage of fat in their diet might have simply gigantic economic consequences.

The conventional wisdom is that in the near future, diet soft drinks in the United States will rise from about 30% of the market to at least 50% of the market. When you consider that, to a large extent, a soft drink company that makes this transition does so by cannibalizing its own existing non-diet business, and may lower its profit margin in the process, there must be powerful social forces behind the change. In the 1960's, cyclamate-saccharin sweetened soft drinks commanded about 15% of the market. Of course, aspartame has a better taste profile than a cyclamate-saccharin combination, but the rapid increase in market share can in no way be accounted for only by improved quality of taste.

Most of the changes in the market place occurred in the United States. The factors that have brought on the present state of affairs in this country have nothing to do with race, religion, politics, or sexual preference. It is part of the general world-wide spread of consumerism, environmental concerns, and related items. The United States, with its high standard of living and leisure time for its citizens, seems to be in the forefront of the movement that relates food ingredients, as distinguished from general nutrition, to health.

Prediction: The world market for synthetic sweeteners is on its way up, up, and out of sight. Present projections are probably too conservative.

Discovery

How are we to discover new sweeteners to satisfy this possibly rapidly expanding market? There are three categories of sweeteners: natural substances, accidental discoveries, and analogs of known sweeteners.

Naturally occurring compounds known to be sweet. These may be available as pure chemicals or as mixtures of closely related structures. Their discovery is generally lost in antiquity. In some cases, the properties were known to native populations and the information passed on to explorers, colonists, and the like. Carbohydrates are the most famous of naturally occurring sweeteners with sucrose dominating that market.

All natural carbohydrate sweeteners known to date have low potency. This requires administering very high doses to put it in a medical perspective. Therefore, toxicological studies would be difficult because of required safety factors. One can speculate that if sucrose came along today as a new chemical entity, it might never gain FDA approval. Particularly, if some enterprising researcher stumbled across the fact that sucrose is associated with dental caries.

Some terpenoid glycosides are sweet. Stevioside is available in a few countries. So far, terpenoid glycosides have a marginally acceptable taste profile with a suspected toxicity. No one seems to want to spend the money to gain regulatory approval for stevioside in the United States, for example.

The discovery of sweet proteins upset a widely held belief that a high molecular weight protein couldn't have a taste. Examples are monellin and thaumatin which have been intensively investigated. Their taste profiles are unsuitable for use alone as sweeteners, but they may have some application as taste modifiers and enhancers. Thaumatin has been approved for this purpose.

To improve a protein sweetener taste profile requires a point mutation approach which can now be done nicely by genetic engineering techniques. However, there are no convincing clues as to where to start. In spite of some ingenious efforts, the active site for triggering sweet taste of either thaumatin or monellin is still unknown. The elegant research on monellin described by Dr. Sung-Hou Kim in this Symposium was aimed at improving stability of the molecule, not taste.

In summary, the prospects for exciting advances in the area of naturally occurring sweeteners are dim even though one can never rule out a new substance isolated from a sweet plant. Even if this were to happen, it is problematical whether the compound would be useful. In general, natural sweeteners are plagued by a combination of low potency, bad temporal properties, or distracting flavor. The funding for a massive search for naturally occurring sweet substances seems not to be forthcoming.

Synthetic organic compounds unrelated to previously known sweeteners. Unrelated simply means that the substances can't look chemically very much alike. Any structural resemblance is a coincidence because none resulted from a search for sweeteners or even from research in the general sensory area. Each compound has a story connected with its accidental discovery. Some of the stories have been recorded. For example:

- Saccharin. Fahlberg ate his lunch in the lab.
- Cyclamate. Sveda smoked in the lab.
- Aspartame. Schlatter licked his finger to pick up a piece of weighing paper.
- Acesulfame-K. Clauss licked his finger to pick up a piece of weighing paper.
- P-4000. Circumstances not known. A sweet orange solid was isolated from a red oil. This happened in Holland during World War II. Verkade, who was associated with the project, reported that getting a Dutch patent was easy because orange was associated with the House of Orange, the royal family of Holland, while red was associated with the German flag.
- Suosan. Circumstances not known.

Serendipitous or accidental discoveries are probably the most promising at the present time for finding novel sweeteners. However, research based on accidents has no value whatsoever and is a contradiction in terms. My recommendation is to return to a nineteenth century characterization practice: taste every compound you synthesize.

Structural modifications of known sweeteners or other tastants. This approach has been used by medicinal chemists. Modifications also attract the efforts of academic chemists interested in structure-activity relationships.

Analog synthesis is popular and there are a number of successful examples. At the moment, with the minor exception of neohesperidine dihydrochalcone, sweetener analogs have not achieved regulatory approval. Undoubtedly, analog research will continue. But, there are at least three special problems in addition to the obvious task of finding a compound that meets performance requirements. These are cost, safety, and novelty.

Cost. Since the starting material is already a sweetener, any structural change increases the cost unless the change dramatically enhances potency, hopefully by an order of magnitude or even two. The literature contains examples of this happening. But, every lab's notebooks are full of failures.

Safety. Any doubts or weak spots or vulnerable places in a prototype sweetener's safety profile may carry over to the analog. Worse, because it is a new compound, you have introduced new toxicity questions in addition to the old ones. The safety problem may actually be the single greatest deterrent to research on new sweeteners.

Novelty. Since the starting sweetener is a known compound, the improved versions may be obvious modifications in the eyes of the patent department. A good example is alitame. It is a dipeptide amide where the amine part is rather complex. Structurally similar, sweet, low potency, dipeptide amides from simple amines were already known. On applying for a U.S. patent, Pfizer received a first rejection and a second rejection. The alitame application was eventually allowed, an outcome that was by no means certain. Patentability considerations do encourage radical structural changes in known sweeteners which then ordinarily give the least chance to retain sweet taste.

Can our chances to find sweeteners on purpose be improved? What new knowledge is needed in order to bypass collecting plants from all over the world, tasting possibly unpleasant substances, or synthesizing complex molecules.

Three approaches discussed in this symposium are: computer generation of a receptor model, investigation of the mechanism of taste message transduction, and design of chemicals that may be used to label, isolate, and characterize a sweet taste receptor. As of now, although these are exciting areas, they have not pointed the way to the design and synthesis of really new, novel sweeteners completely unrelated to existing prototypes.

Prediction: My prediction for the discovery of new sweeteners is twofold. First, the low tech approach of everyone tasting every compound they prepare would uncover an endless stream of new leads for excellent synthetic sweeteners, but this simple activity risks megadollars liability lawsuits. Second, a primate sweet taste receptor will be isolated that will allow setting up a screening assay for compounds that bind to the receptor.

Other Food Additives

Synthetic sweeteners are one member of a large class of food additives. A synthetic sweetener, for example, is usually a single pure chemical that replaces the sweet taste of sucrose, another single, pure chemical. A synthetic flavor additive may be a unique entity that typically replaces a natural flavor, the latter often being a complex mixture of compounds. The latter uses smell as its mechanism of action as well. Artificial colors are defined

substances that can add desirable color to a food product or could substitute for a natural color.

There is also the situation where complex substances, often polymers, can provide functional properties other than taste, flavor, or color. An example is polydextrose, a bulking agent for use with high potency sweeteners. A cake baked with saccharin simply looks pitiful. If sucrose is replaced in many products, a substitute for sucrose's space filling or bulking properties is needed. Another example is a fat substitute where, ideally, the substitute should feel exactly like fat but without the negative health consequences of fat.

Thus, there are opportunities for food additives that depend for their success on specific effects on four of our five senses, namely taste, smell, sight, and touch. This situation strongly invites dedicated, targeted, scientific research. The traditional food industry is not carrying out this research. Virtually all the past and present synthetic sweeteners were discovered in the laboratories of pharmaceutical companies or university chemistry departments. Some of the discoveries may have been accidents but these accidents resulted from the nature of the research that was in progress.

The food industry could do the same type and quality of research as the pharmaceutical industry. They could synthesize new compounds to explore structure-activity relationships; they could isolate and characterize receptors and use them in sophisticated food additive screening assays; they could exploit the power of computational chemistry to postulate models for desired taste or functional properties. There is almost no activity at this level of basic research under way in food company laboratories.

The reason, in overly simple terms, is that the food industry doesn't believe research will pay off while the pharmaceutical industry knows it does. Some of the factors involved are need, safety, and profits.

Need. The need for medicine and the provision of medicine is found in the oldest records. A similar health-based need for food additives is only now becoming apparent.

Safety. The pharmaceutical industry is cautious about safety for new drug approvals. In spite of what you may hear, though, proving safety of a drug is relatively easy. The Physicians' Desk Reference (PDR) which describes properties of drugs has about 5,000 entries. Even with allowance for multiple dosage forms and, frequently, several manufacturers of one drug, there are unquestionably a lot of drugs on the market.

Getting approval for a new food additive chemical can be far more difficult than for a new drug. Drugs are often used acutely; food additives are not. Even chronic drug administration may be temporary; you will probably eat for the rest of your life. The

prescription system exerts control over drug distribution whereas no such mechanism exists for food additives. Also, because of fear of chemicals in the food supply, post-marketing criticism of the government can be intense.

Profits. In the pharmaceutical industry, the existence of third party payers and direct connection of drugs with alleviation of disease makes possible enormous mark-ups and rapid recovery of investment. People who are sick are more concerned with getting well than with the cost of drugs.

On the other hand, food is eaten overwhelmingly by people in normal health. Consumers are very much aware of the cost of food and are extremely unhappy about price increases. It follows that food and beverage producers strongly resist cost increases. However, the situation is changing. Food manufacturers, particularly cereal makers, have discovered health claims on their packages sell products. The government is not going to stop this kind of advertising. Instead, they are eventually going to adopt regulations that will control the claims. You can be sure, that having once experienced the sales increases associated with food health claims, marketing people will find ways to associate their products with medical benefits.

If food companies make claims for improved health, claims for improved taste and flavor, improved odor, improved appearance, improved feel will follow. The attributes need not at all be substitutes for expected well-known existing attributes. They could be promoted as novel tastes, flavors, odors, appearances, feels. Especially in a society that equates new with better, discovery and development of food additives with novel properties has considerable potential.

Prediction: With the almost certain advent of documentable health claims for foods, will come the stimulus for excellent research looking for new compounds that can improve foods in a number of areas. The traditional food industry is unlikely to undertake the kind of research needed to lead to these discoveries. Instead, present pharmaceutical companies will set up sections and divisions whose mission is to discover new chemical entities for use in foods.

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