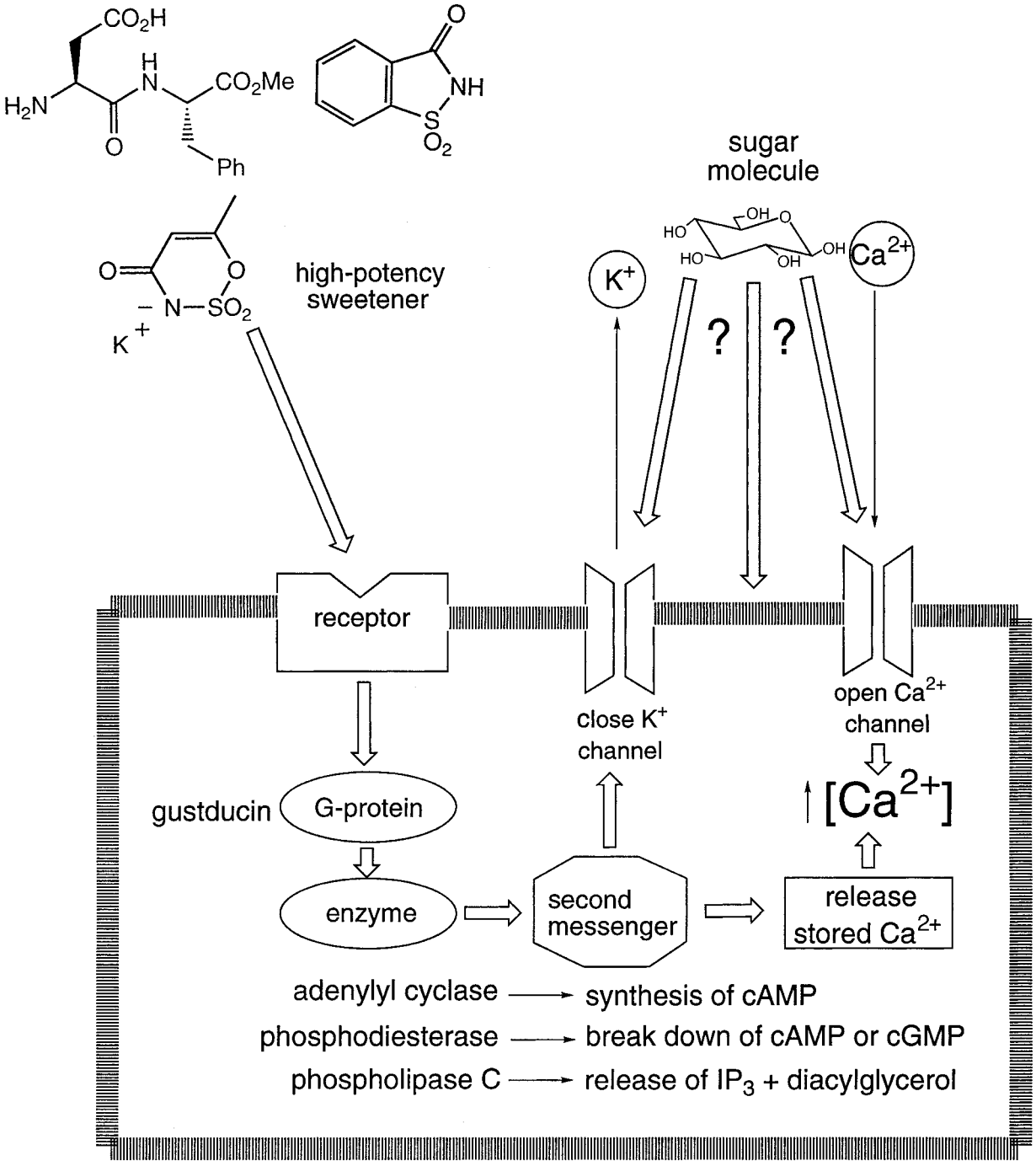
The "sweet" taste can be obtained not only from sugar (top right) but also from synthetic sweetners (top left).



The uses and the syntheses of such sweetners are discussed in the review by D. Ager et al. in the following pages.

Commercial, Synthetic Nonnutritive Sweeteners

David J. Ager,* David P. Pantaleone, Scott A. Henderson, Alan R. Katritzky, Indra Prakash, and D. Eric Walters

Nonnutritive sweeteners represent a large commercial market, and both in terms of volume and value of sales they fall between the majority of pharmaceutical compounds and bulk chemicals. The synthesis of these sweeteners can offer some unique challenges. The current large-scale methods used for the preparation of sweeteners that are currently allowed for usage in North America and Europe have been highlighted. The elegance of some of these syntheses is the simplicity of the methodology and is a reflection the amount of resources that have been expended to discover the approach and then reduce it to practical scale. Alternative approaches to some of the sweeteners, in particular biochemical methods, have been included as these could supercede total chemical synthesis.

Keywords: enzyme catalysis · sweeteners · synthetic methods

1. Introduction

Nonnutritive sweeteners have established a significant market. This large-scale usage has presented some unique problems with regard to their chemistry and biological properties.[1] We will only cover synthetic commercial sweeteners and all of them have been subjected to large amounts of biological testing.[2] Thus, their entry on to the market is somewhat reminiscent of an over-the-counter drug. Despite the large number of compounds that are known to be sweet, relatively little is known about their mode of action. Although we have defined these sweeteners as nonnutritive, many, such as aspartame, are caloric. However, the amount of the sweetening agent used in a specific food application is usually so low that it makes no significant caloric contribution.

In addition to being sweet, a compound has to have a number of physical attributes associated with it before it can be considered a good sweetening agent, fit for human consumption: Obviously, the sweet character should have no off-tastes; the compound must be safe and stable under use conditions; and as economical issues are also of importance, the compound should be sweeter than sugar in order to offset synthesis costs. We will concentrate on the commercial-scale syntheses of synthetic, nonnutritive sweeteners that have been approved for use in North America or Europe. The amounts of sweeteners produced, especially aspartame, means that these compounds are on the borderline of fine and bulk chemicals. The practice of large-scale synthesis in this arena has led to some elegant approaches and both chemical and biochemical methods are covered in this review.

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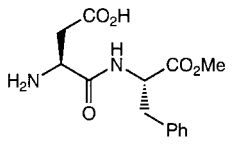
1.1. Mode of Action

Taste receptor proteins have not yet been isolated or unequivocally identified. There are two major reasons why this is so:

1. Traditional receptor isolation methods have usually involved the use of a high-affinity ligand (half-maximal binding at nanomolar concentrations or less) which can be used to monitor the progress of purification. Sugars trigger sweetness only at concentrations of ~0.1 molar; aspartame (**1**), saccharin (**2**), and acesulfame-K (**3**) elicit sweetness at concentrations of a few millimoles per liter. At these concentrations, it is nearly impossible to distinguish receptor-specific binding from nonspecific binding to other



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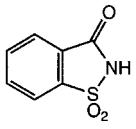

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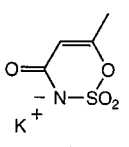

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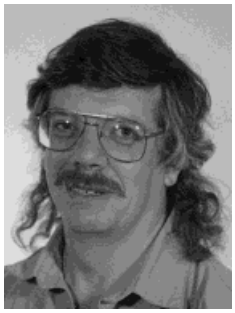
components of the tissue from which the receptors might be extracted.

2. The difficulty in isolating taste-active cells. These are specialized epithelial cells which are embedded in taste buds among a large number of nontaste-active cells. When these cells are separated from their nerve cells, they rapidly de-differentiate back into ordinary epithelial cells. Thus, it has not been possible to grow taste-active cells in cell cultures.

Despite these difficulties there is now a large body of evidence indicating that sweetness is mediated in many cases by G-protein-coupled receptors (GPCR). GPCR are respon-



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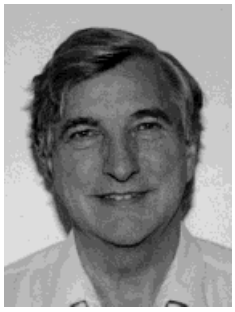

A. R. Katritzky

I. Prakash

D. E. Walters

David J. Ager was born in Northampton, England in 1953. He received a B. Sc. from Imperial College, London, and a Ph.D. from the University of Cambridge, working for Dr. Ian Fleming on organosilicon chemistry. In 1977 he was awarded a Science Research Council Postdoctoral Fellowship that allowed him to collaborate with Professor Richard Cookson at the University of Southampton. In 1979 he joined the faculty of the University of Liverpool as a Senior Demonstrator. This was followed by an assistant professor position at the University of Toledo in Ohio. In 1986 he joined the NutraSweet Company's Research and Development group, that has now become NSC Technologies, part of Monsanto Growth Enterprises. Dr Ager is now responsible for the chemical development of new products, for the fine chemical intermediates business.

David P. Pantaleone received his B.A. in Chemistry from Lewis University, Romeoville, IL in 1977 and his Ph.D. in Chemistry from The Ohio State University in 1983. He joined G. D. Searle in 1983 where he studied the biological preparation of aspartame. Currently he is Group Leader in Protein Biochemistry at NSC Technologies and studies biotransformation approaches to speciality chemicals.

Scott A. Henderson was born in 1968 in Melbourne, Australia, and received his Ph.D. in 1996 from Swinburne University of Technology, under the supervision of Ms. Jacqueline O'Connor and Dr. G. Paul Savage of CSIRO Division of Chemicals and Polymers. Currently he is senior postdoctoral researcher with Professor Alan Katritzky at the University of Florida.

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Indra Prakash was born in Muzaffaranager, India in 1956. Receiving an M.Sc. degree from the University of Roorkee, India, in 1977, he was awarded the Gold Medal for academic distinction. In 1982, he received a Ph.D. degree from Kurukshetra University, India, under the direction of Professor S. P. Singh. He also worked at Union Carbide at Bhopal, India. After coming to the USA, he worked for Professor Kagan, Professor Moriarty, and Professor Sosnovsky. In 1987 he joined Aldrich Chemical Company as a Senior Chemist and then became a Principal Investigator for a National Cancer Institute contract with Aldrich. Currently, Dr Prakash is a project leader developing processes for new sweetener candidates for The NutraSweet Kelco Company, a sector of Monsanto.

D. Eric Walters earned a B.Sc. in Pharmacy from the University of Wisconsin, followed by a Ph.D. in Medicinal Chemistry from the University of Kansas. He spent 12 years carrying out research in the pharmaceutical industry and food industry, particularly in the field of sweetener research while working for the NutraSweet Company. In 1991 he joined the Biological Chemistry faculty at the Chicago Medical School. He coedited a book on sweeteners.[11a] His current research includes the search for new anti-HIV and anti-parasitic drugs as well as development of new methods for computer-aided drug discovery.

sible for detection of many neurotransmitters, hormones, odorants, and even photons. The GPCR are proteins having seven α-helical segments embedded in the cell membrane. When extracellular ligands bind to the receptor (Figure 1),

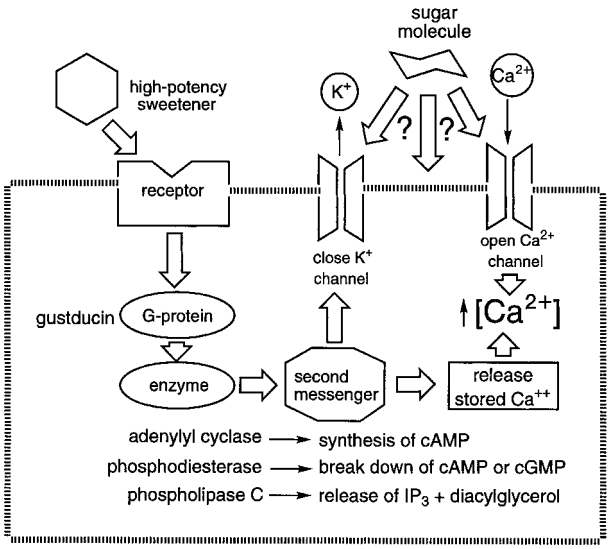


Figure 1. Schematic representation of a taste cell, indicating likely components of the taste transduction system. The initial stimulus may be either a high potency sweetner or a sugar; the final step is cell depolarization, which may involve loss of potassium ions and a sharp increase in the concentration of free intracellular calcium ions.

they induce a conformational change in the protein causing the intracellular portion to interact with one or more GTP-binding proteins (the G proteins). The G proteins may, in turn, modify the activity of an enzyme, which increases or decreases the amount of a "second messenger" molecule: Adenylyl cyclase may be induced to make higher levels of cyclic AMP (cAMP), or a phosphodiesterase may degrade cyclic nucleotides, or a phospholipase may convert phosphatidylinositol lipids to diacylglycerol and inositol trisphosphate (IP_3). These second messengers may alter calcium ion concentrations or modify ion channel activities leading to cell depolarization which, in turn, sends a nerve signal to the brain.

Margolskee et al. characterized a taste cell-specific G protein which they named gustducin because of its similarity to the retinal G protein, transducin.[3] Gustducin is able to activate a phosphodiesterase.[4] These workers found that mice in which the gustducin gene is inactivated have diminished responses to both sweet and bitter tastes.[5]

The second messenger cAMP increases in rat taste-cell membrane preparations upon treatment with sucrose or saccharin.[6] In these experiments aspartame had no effect upon cAMP levels, consistent with the observation that rats are not able to taste aspartame.

The transduction of sweetness may also involve the second messengers IP_3 and calcium. Very recently, both sucrose and high-potency sweeteners were shown to lead to high intracellular levels of calcium ions.[7] The high-potency sweeteners do so through the second messenger IP_3, which releases *intracellularly* stored calcium ions. Sucrose in contrast opens ion channels on the cell surface that let *extracellular* calcium enter the cell. Results from Kinnamon's research group indicate that sucrose, high-potency sweeteners, and cyclic nucleotides close a potassium channel, and cause cell depolarization.[8] Closing potassium leak channels should lead to an increase in the cell's membrane potential; it is not yet clear whether this causes cell depolarization directly, or if the increased positive potential triggers voltage-gated calcium channels.

Another issue which is not yet resolved concerns sugars. Do they act directly on a receptor protein or ion channel? There are reasons to suspect that sugars do not bind to a specific receptor site. First, sugars taste sweet only at high concentration (about 0.1M). This is in sharp contrast to the concentrations required for hormones, neurotransmitters, etc. to trigger their receptors (typically nanomolar or picomolar concentrations). Furthermore, it is well known that D- and L-sugars have comparable sweetness potencies; for example, the taste of L-glucose is comparable to that of D-glucose. These observations suggest that sugars might act *indirectly* on receptors or ion channels, through effects on the properties of the surrounding membrane lipids.

Are there multiple receptors for sweet taste? A review of the evidence indicates that there must be more than one receptor type.[9] First, the chemist is immediately struck by the diversity of chemical structures (including sugars, sulfamates, heterocycles, ureas, arylguanidines, peptides, proteins, oximes, and terpenes) which can evoke sweet taste. It is unlikely that a single receptor binding site could accommodate all of the known sweet-tasting compounds. Second, we know that it is easy for humans to distinguish different taste qualities for different high-potency sweeteners. Most consumers can readily tell the difference between sugar and high-potency sweeteners and trained panelists can detect taste differences among different high-potency sweeteners. Third, blends of structurally different sweeteners (aspartame plus acesulfame, for example) produce synergy, a higher-than-predicted sweetness intensity based on potencies of the two individual sweeteners.[10] On the other hand, structurally similar sweeteners, such as saccharin and acesulfame, do not show synergy; they are simply additive. Many more studies of this type would be required to get an estimate of the number of different types of sweetness receptor which might be involved.

In summary, the available evidence indicates that high-potency sweeteners utilize a different mechanism than sugars to trigger the response of taste cells. In addition, it appears that the receptors which mediate the sweetness of aspartame are different from those which respond to saccharin and acesulfame.

1.2. Measurement of Sweetness Potency

Quantitation of sweetness must be carried out by human panelists. Experimental animals cannot be used because it is impossible to evaluate the quality of taste which an animal detects. Also, there are substantial differences between species with respect to their response to high-potency sweeteners. Typically, taste panelists use a 15 cm line scale to rate

the sweetness of solutions of sweeteners.[11] Panelists have been trained to rate a 5% sucrose solution at the 5 cm mark, a 10% sucrose solution at the 10 cm mark, and so forth. With practice, such data can be quite reproducible (usually ± 1 unit). Panelists then evaluate test solutions to determine what concentration of a high-potency sweetener matches a given concentration of sucrose.

Sweetness potency is usually expressed relative to a standard concentration of sucrose, on a weight basis. For example, 0.025% aspartame is equivalent in sweetness to 5% sucrose, so the *potency* is $5/0.025 = 200$ times higher than sucrose. High-potency sweeteners do not show a linear response relative to sugars. At the sweetness level of a 2% solution of sucrose (about the taste recognition threshold), aspartame has a potency of 625 while at 10% sucrose (about the level used in carbonated soft drinks), its potency is 110. Acesulfame has potencies of 204, 140, and 34, relative to 2%, 5%, and 10% sucrose, respectively. Potencies reported in the literature are often measured near the threshold value. This is probably due to the tendency to want one's new sweetener to have the highest potency possible. The nonlinear response means that, at some point, increasing the concentration no longer produces increasing sweetness; the response asymptotically approaches a maximal value. Mathematical analysis of concentration–response curves indicates that aspartame, acesulfame, and saccharin have a maximal sweetness level of 16.0, 11.6%, and 9.0% sucrose, respectively.

2. Aspartame

Aspartame (L-aspartyl-L-phenylalanine methyl ester, APM; **1**) is currently the most widely used nutritive sweetener worldwide. Although this compound was known in the literature, its sweetness was discovered by James Schlatter, a chemist at G. D. Searle (now part of Monsanto), who in 1965 while working on an antiulcer compound licked his finger.[12] Several analogues, especially more stable esters,[13] were made and evaluated for sweetness potencies, taste profile, and toxicities. In the end, however, it was the first compound that was chosen for commercial development.

Aspartame is 160–180 times sweeter than sucrose. It has a good, clean sweet taste but its time–intensity profile differs from sucrose. It has a slower onset and a lingering sweet taste. Aspartame has also been reported to enhance the flavor of the foods. Therefore, when converting a sugar-based food to aspartame, the amount of aspartame needed is not necessarily 1/160–1/180 of the weight of sugar. Aspartame is caloric and yields about 4 kcal g^{-1}; however, because of its sweetness intensity, only a minute quantity is consumed resulting in a negligible calorie contribution. It is slightly soluble in water (about 3 g per 100 mL, pH 3, room temperature). The solubility increases with higher or lower pH as well as with increased temperatures, but degradation also occurs under these conditions; at high pH it cyclizes to the diketopiperazine. In aqueous solution, the relationship between pH and stability of aspartame is a bell-shaped curve with the maximum stability at pH 4.3.[14] At higher or lower pH, the half-life of aspartame in aqueous solution diminishes quickly. Currently, most soft drinks are formulated at pH 2–3. The ester group of the aspartame is very susceptible to hydrolysis and hence avoidance of excessive heat is desirable as degradation with concomitant loss of sweetness can be rapid. Aspartame was approved by the FDA (Food and Drug Administration) in 1981 for use in dry goods. Two years later it gained additional approval for use in carbonated beverages, and in 1993 was approved for use in baked goods, candies, and still beverages. It can be used in almost all food categories, but the major consumption is in beverages.

2.1. Chemical Synthesis

Aspartame (**1**) is a dipeptide composed of the two amino acids L-aspartic acid and L-phenylalanine. Many chemical syntheses have been reported; most involving the coupling of the two amino acid units having appropriate functional group protection with conventional peptide synthetic reagents.[15] Research directed towards the development of synthetic processes has been concerned largely with the problems of increasing the yield and purity of the desired product while suppressing the formation of the by-products, especially those arising from the β-coupling of the aspartyl unit. A significant amount of effort has been expended to maximize the desired α-coupling through changes in reaction conditions and protection on the aspartyl nitrogen atom.[16] It is fortuitous that the desired product crystallizes from the reaction mixture as the hydrochloride salt, leaving all of the undesired isomers in solution.[17]

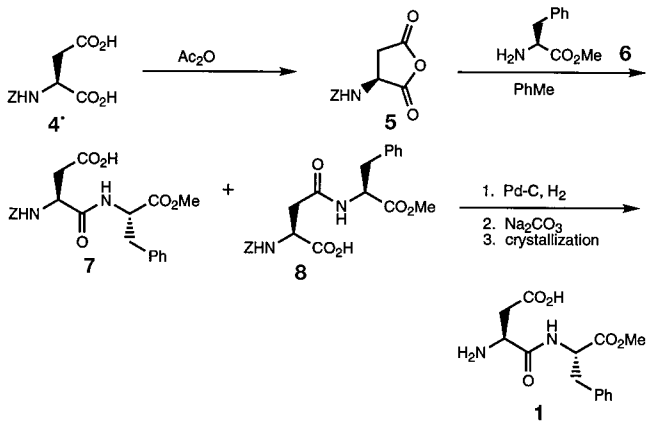
The chemical methods of industrial significance involve the dehydration of aspartic acid to form an acid anhydride, which is then coupled with the phenylalanine or its methyl ester to give the desired product. The two major processes are known as the Z- and F-processes, named after the protecting group used on the aspartyl moiety. Both of these processes produce some β-coupled products together with the desired α-aspartame (**1**), but the selective crystallization removes the undesired isomers. However, since the amino acid raw materials are expensive, they must be recovered from the by-products and waste streams for recycling.

2.1.1. The Z-Process

This process involves the dehydration of the benzyloxycarbonyl-L-aspartic acid (**4**) with acetic anhydride.[18] The anhydride **5** is then coupled with the methyl ester of L-phenylalanine **6** in toluene to give a mixture of benzyloxycarbonyl α- and β-aspartames **7** and **8** (Scheme 1). The protecting group is removed by hydrogenolysis with Pd on carbon as the catalyst. Addition of sodium carbonate to adjust the pH of the resultant mixture of aspartame isomers to about pH 5 gives APM **1** upon crystallization.

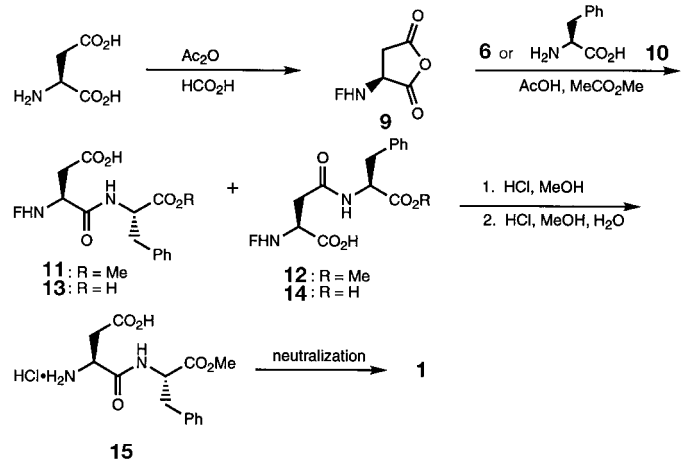
2.1.2. The F-Process

This process involves the protection of the amino group of aspartic acid with a formyl group (by treating aspartic acid



Scheme 1. Synthesis of aspartame (**1**) by the Z-process (Z = benzyloxycarbonyl).

with acetic anhydride in the presence of formic acid) and concomitant dehydration to form the anhydride **9** (Scheme 2). The anhydride is then coupled either with L-phenylalanine (**10**)[19] or its methyl ester **6**[20] and the formyl group removed by acid hydrolysis. This reaction is accompanied by a number



Scheme 2. Synthesis of aspartame (**1**) by the F-process (F = formyl).

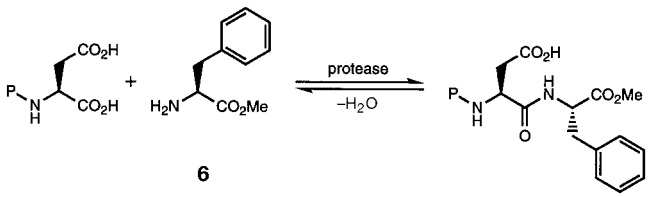
of other reactions that includes the hydrolysis of the esters **11** and **12**, and esterification of the acids **13** and **14**. The resultant mixture of α- and β-products is subjected to the esterfication conditions of aqueous methanol in the presence of hydrochloric acid. α-APM hydrochloride **15** preferentially crystallizes out from this mixture and is then neutralized to yield APM **1**. This coupling procedure can be performed as a "one-pot" reaction.[21] L-Aspartic anhydride, without amino group protection, has also been used in the synthesis of α-APM but the reaction produces a large amount of oligopeptides.[22]

2.2. Enzymatic Synthesis

The application of biotechnology and biocatalysis toward the synthesis of the synthetic sweetener aspartame (**1**) has been extensively explored. Because of the presence of two carboxylic acid groups in the aspartic acid unit the chemical process forms both α- and β-products, the use of enzymes that are both regio- and stereoselective is very desirable. As a result of its dipeptide structure, many variations of reverse proteolysis, that employ both kinetically and thermodynamically controlled approaches, have been investigated with different enzymes and under various reaction conditions. In addition, there have been many other approaches that utilize biocatalytic methods directed toward APM synthesis. These include enzymatic deprotection of an APM precursor, amination of methyl fumarylphenylalaninate, enzymatic esterification of α-L-aspartyl-L-phenylalanine, and the use of a synthetic polypeptide, poly(aspartate-phenylalanine). A number of these biocatalytic approaches to APM synthesis will be discussed below, but as this field is quite large, the reader is referred to the following review articles.[23–25]

2.2.1. Peptide Coupling

The use of proteases to catalyze the synthesis of a peptide bond has been known for many years and is the major biocatalytic route to prepare precursors of APM. This thermodynamic approach is illustrated in Scheme 3. An *N*-



Scheme 3. Protease-catalyzed synthesis of an aspartame precursor (P = protecting group).

protected L-aspartic acid is reacted with methyl L-phenylalaninate (**6**) in the presence of a protease, usually a metalloprotease, to form an *N*-protected-APM derivative. This product is very often insoluble and thus shifts the equilibrium to favor synthesis of the peptide. The protecting group can then be removed either chemically or biologically to yield APM **1**.

In another type of peptide coupling reaction serine or cysteine proteases, which form acyl–enzyme intermediates when they hydrolyze peptide bonds, are used. The serine protease from yeast, carboxypeptidase Y, has been investigated extensively for this purpose.[26] For APM, a blocked aspartic acid, usually the α-methyl ester, reacts to acylate the enzyme. This acyl–enzyme intermediate will form APM, in the presence of a competing nucleophile to water, such as methyl L-phenylalaninate (PM). Although conversions are generally low using this type of peptide coupling method, this approach has been investigated for APM synthesis using the endopeptidase from *Staphylococcus aureus* strain V8.[27]

Over the past 20 years, there have been many variations of this reaction described in the literature and numerous review articles on reverse proteolysis utilizing both kinetic and thermodynamic approaches.[28] The most common variations for the thermodynamic approach have been with the protecting group, P, the enzyme and the reaction conditions. Some of the more common protecting groups that have been used to enzymatically synthesize APM are shown in Table 1. Of these

Table 1. Protecting groups (P) used to synthesize APM-precursors by reverse proteolysis.

Protecting group	Abbreviation	Reference
acetoacetyl	AcAc	[25]
acetyl	Ac	[29]
benzoyl	bz	[30]
benzyloxycarbonyl	Z	[23, 24, 31, 32]
tert-butyloxycarbonyl	Boc	[33]
formyl	F	[34]
isovaleryl	Iv	[25]
(*p*-methoxybenzyl)oxycarbonyl	Moz	[33, 35]
phenylacetyl	PhAc	[36, 37]

protecting groups, the one that has been most commonly used is the Z group. When the stoichiometry of the reaction is such that two equivalents of methyl phenylalaninate are used with one equivalent of Z-asp, the Z-APM · PM product precipitates out and shifts the equilibrium to greater than 95% conversion.[35] This is the basis of the commercial TOSOH process with thermolysin operated by Holland Sweetener (see below).[24] One significant variation has been the use of racemic PM instead of only the L-isomer. Since the enzyme will only recognize the L-PM isomer to form the peptide bond, the unreacted D-PM isomer forms a salt and then, after acidification, the D-PM can be chemically racemized and recycled. A further improvement of this process employs a water-immiscible solvent, such as toluene or 4-methylpentan-2-one, which is added once the Z-APM · D-PM salt begins to precipitate. This results in lower enzyme inactivation and allows the process to be run in a continuous mode.[38]

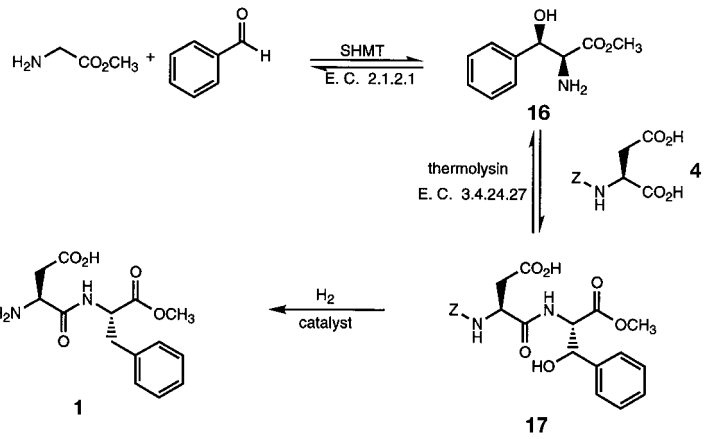
The most commonly used enzyme for this biosynthetic reaction is the metalloprotease thermolysin (EC 3.4.24.27) isolated from *Bacillus thermoproteolyticus*. The commercial source of the enzyme is called thermoase and is available from Daiwa Kasei Co. (Osaka, Japan).[23] As the name implies, the enzyme is tolerant of high temperatures for extended periods of time as well as withstanding a variety of organic solvents. These features make it an excellent choice for industrial applications. In addition, immobilized enzyme[32, 39–41] and membrane processes[42,43] have been developed using thermolysin. A stablized form of the enzyme called a CLEC (cross-linked enzyme crystal) has also been shown to have utility to couple Z-asp and PM.[44]

P-APM precursors have been synthesized by other enzymes such as papain[33], pepsin,[43, 45] *Pseudomonas aeruginosa* elastase,[46] and aminopeptidase A.[47] Most recently, site-directed mutagenesis of the gene encoding a neutral protease from *Bacillus proteolyticus* has been used to improve the coupling of Z-asp and PM relative to thermolysin.[48]

Many solvent systems, both miscible and immiscible with water, have been explored in order to shift the equilibrium of this reaction.[49] One method described by Homandberg and co-workers utilized organic cosolvents such as glycerol and other glycols to reduce the water content to favor synthesis.[50] Other solvents such as acetonitrile,[51] ethyl acetate,[32, 52] *tert*-amyl alcohol,[40] and triethyl phosphate[53] have been explored.[54] A mixed solvent system of ethyl acetate and *tert*-amyl alcohol has also been shown to be quite effective with yields of 99%.[41]

2.2.2. Non-phenylalanine Route[55]

Early in the development of APM processes, the high cost and lack of availability of L-phenylalanine was a key focus of chemists and biochemists alike. One approach was to prepare L-phenylalanine as a starting material and then couple it to form the sweetener **1**. An alternative approach was to build the L-phenylalanine side chain from inexpensive starting materials. Workers at W. R. Grace and Company explored a non-phenylalanine route to APM that utilized the enzyme serine hydroxymethyltransferase (SHMT) to prepare methyl L-*erythro*-phenylserinate (**16**) from methyl glycinate and benzaldehyde (Scheme 4). Although the enzyme is highly



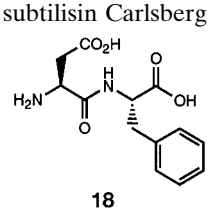
Scheme 4. Reaction sequence of a non-phenylalanine requiring route to aspartame (**1**) using two enzymatic steps followed by chemical hydrogenation.

stereospecific for the α-carbon, forming the *S*-isomer, the stereospecificity of the β-carbon is not as strict and as a result L-*threo*-phenylserine forms. However, under kinetically controlled conditions the formation of L-*erythro*-phenylserine is favored. Next, the methyl ester was coupled enzymatically with Z-aspartic acid (**4**) to form the adduct **17**. Catalytic hydrogenation was then used to remove the Z-protecting group as well as the hydroxy group to yield APM **1**.[56]

2.2.3. Other Biocatalytic Reactions

Enzymatic Esterification

The enzymatic esterification of the dipeptide aspartylphenylalanine (AP, **18**) and *N*-blocked derivatives was described by Davino who used the enzyme subtilisin Carlsberg in the presence of high concentrations (60%) of methanol for long periods of time.[57] Due to the low concentration of AP used, the throughput for this type of reaction would be very low and not likely to



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be practical. Another esterification procedure was described by Choi and co-workers.[58] A number of proteases were tested and the best results (~40% yields) were obtained with α-chymotrypsin in 25% methanol.

Enzymatic Deprotection

As described in Table 1, many different protecting groups have been utilized to synthesize APM enzymatically. Some of these same protection methods have also been employed in chemical approaches to **1**. The Z group is removed by catalytic hydrogenation, a process that is very efficient and does not cause degradation of the resultant dipeptide. Other protecting groups are not as easy to remove due to the harsh conditions required, and the stability of the product under the reaction conditions can be of major concern. The use of enzymes or microorganisms to effect deprotection can, therefore, offer some significant advantages for deprotection over chemical methodologies.

The use of the enzyme penicillin acylase (EC 3.5.1.11) to deprotect *N*-phenylacetyl-blocked peptides has been well documented.[59] The enzymatic removal of this protecting group from an APM precursor, PhAc-APM, has been described by Fuganti and Grasselli.[36] More recently, *p*-substituted phenylacetyl APM derivatives were deprotected with this acylase as described by Stoineva and co-workers.[37] Another protecting group, the phthalyl group, has been shown by Zmijewski and co-workers at Lilly to be removed from phthalimido-APM by an enzyme isolated from *Xanthobacter agilis*.[60]

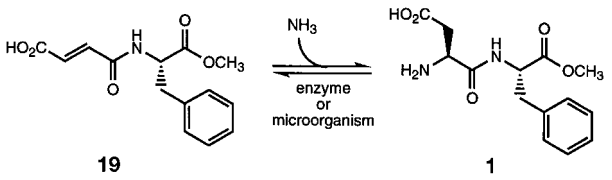
Poly(aspartyl-phenylalanine)

With the advent of recombinant DNA technology, several literature reports have cited the use of synthetic genes expressed in bacterial systems to form a polymer of aspartic acid and phenylalanine. This polymer can then be cleaved enzymatically to form the dipeptide α-L-aspartyl-L-phenylalanine (**18**) which can then be enzymatically esterified to form APM. The first report was by Searle Research in High Wycombe which described clones containing genes that coded for about 150 repeats of aspartylphenylalanine.[61]

Cleavage of a polymer composed of aspartylphenylalanyllysine was achieved with trypsin producing the tripeptide Asp-Phe-Lys. Subsequent treatment with carboxypeptidase B yields the dipeptide **18**.[58] APM was formed by enzymatic esterification as described above. A gene encoding 167 amino acids from the *N*-terminus of prochymosin followed by $(Asp\text{-}Phe)_{61}$ has been described.[62] It was inserted into a plasmid and expression in *E. coli* yielded inclusion bodies of the fused poly(Asp-Phe) which was calculated as 11.2% of the total cellular protein. Although this is a clever approach, the practicality is limited due to the low expression efficiency and the need for enzymatic cleavage and esterification steps.

Amination of Methyl Fumarylphenylalaninate

With the relative ease of chemically synthesizing methyl fumarylphenylalaninate (**19**) from maleic anhydride and phenylalanine, the notion of amination with an ammonia lyase-type enzyme to yield APM **1** has been contemplated for some time (Scheme 5). Because the substrate specificity for



19 **1**

Scheme 5. Amination of **19** to aspartame (**1**).

the known ammonia lyases is very strict, the enzyme activity was discovered through screening.[63] This process, however, has not been practiced commercially, as the yields are extremely low.

Direct Coupling

The ideal enzymatic coupling scheme would utilize L-aspartic acid and methyl phenylalaninate in the presence of an enzyme. This would alleviate the need for expensive protecting groups as well as a prior chemical step to synthesize the APM precursor and a latter step to remove them. However, this would mean that the enzyme preparation must be free of esterase activity, since one substrate contains a methyl ester. Paul and co-workers at BioEurope (Toulouse, France) identified an enzyme from *Micrococcus caseolyticus* that was capable of carrying out this coupling reaction.[64] Starting with 0.2 M L-aspartic acid and 0.5 M methyl phenylalaninate, the maximum APM yield was 35 mM. More recently, the BioEurope group with other colleagues have purified and characterized the aminopeptidase A (EC 3.4.11.7) from that strain, now called *Staphylococcus chromogenes*.[47] The initial rate of APM synthesis with purified enzyme was 0.23 mM h^{-1}. Before this enzyme could be used in a process to prepare APM in quantity, the rate will undoubtedly need to be substantially increased.

Fermentation

Two Japanese companies have reported on fermentation routes to produce APM directly by incubating microorganisms with L-asp and PM. In the case of the Ajinomoto Company, a solution of L-asp and PM was incubated with a selected bacterial strain and allowed to react, usually between 16 and 24 h. Yields of these reactions ranged from 73–576 mg dL^{-1}.[65] Toya Soda Manufacturing Company, Ltd. was issued a patent for a fermentation route that claimed a number of bacterial and yeast strains which were capable of synthesizing APM.[66] In a typical example, 1.0 M L-asp plus 1.0 M PM in phosphate buffer was incubated with mycelium from a 500 mL culture of a specific strain for 16 h at 37 °C with shaking. The yield of APM was reported to be 100 mg (1.4%).

The work described here on fermentation approaches for the synthesis of APM **1** is interesting and scientifically intriguing, but practically speaking the yields are just too low to be of commercial significance. Much additional work, therefore, would be needed to substantially increase the reaction yield before a commercial process could be realized.

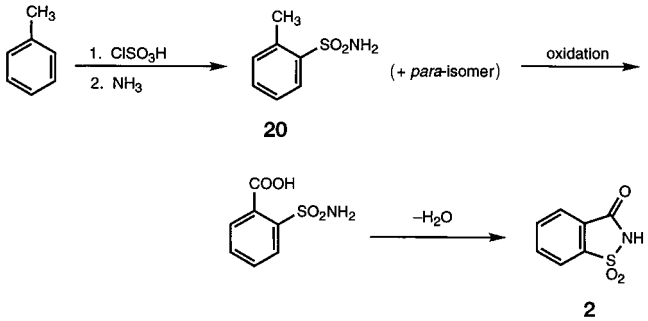
2.3. Summary

As evidenced here, a very large amount of research has been, and continues to be, directed toward the biochemical production of APM. The Holland Sweetener Company does use a process that utilizes this technology. As further advances in biotechnology are made, additional "green" processes to **1** are likely to be developed.

3. Saccharin

Saccharin (1,2-benzoisothiazol-3(2*H*)-on-1,1-dioxide, **2**) is currently the most widely used nonnutritive sweetener in the world.[67] This compound was accidently discovered by Remson and Fahlberg in 1878 at the Johns Hopkins University, Baltimore, and has been sold since 1885.[68] It is about 300 times sweeter than sucrose which, on a cost per sweetness basis, makes it cheaper than sugar. Thus, saccharin has a high usage in third world countries. It also had considerable usage during the World Wars when sugar was in short supply.[67] The chemical stability of this sweetener and low cost make it suitable for use in a wide variety of applications such as toothpaste, mouthwash, and pharmaceuticals, as well as in food and beverages. Saccharin, however, does not provide a "clean" sweet taste, and some users can detect a bitter or metallic off-taste. As the parent compound **2** is only sparingly soluble in water, the sweetener is usually used as the sodium or calcium salt. The safety of saccharin has been questioned due to the report of problems associated with bladder cancer in rats. However, the interpretation of these results is still being debated.[67] It is excreted unchanged from man with little absorption.[69]

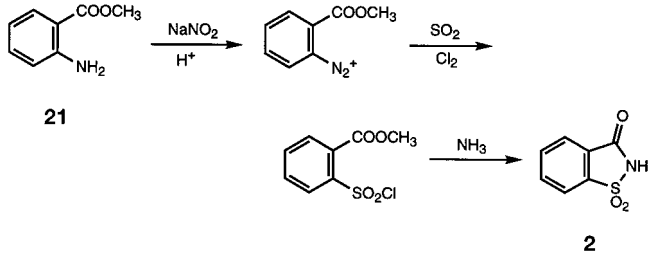
There are two major approaches to the chemical synthesis of saccharin (**2**), namely the oxidation of the *o*-toluenesulfonamide **20** or the replacement of the amino group of anthranilic acid by a sulfur moiety.[70] Treatment of toluene with chlorosulfonic acid gives *o*- and *p*-tolulenesulfonyl chlorides, which are then converted to the sulfonamides by reaction with ammonia; the *ortho*-isomer predominates (Scheme 6). Separation of the isomers can be accomplished



Scheme 6. Synthesis of saccharin (**2**) from toluene.

at this or a later stage.[71, 72] The *o*-toluenesulfonamide can be oxidized to the corresponding carboxylic acid with a variety of agents including potassium permanganate,[71] potassium chromate,[73] chromic acid,[74] electrochemically,[75] air,[76] or oxygen.[77] The *ortho* isomer is dehydrated to give the sweetener **2**.[73, 78]

The alternative sequence (Scheme 7), a variation on the Maumee process, starts from anthranilic acid. The process involves diazotization of methyl anthranilate (**21**) and then

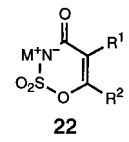


Scheme 7. Synthesis of saccharin (**2**) from methyl anthranilate **21**.

treatment of the diazonium salt with sulfur dioxide and chlorine gas to give the sulfonyl chloride which is then treated with ammonia to give **2**.[79] Variations have been advocated to improve yields and impurity profiles.[78] The original Maumee process involved reaction of the intermediate diazonium salt with sodium disulfide and subsequent reaction with chlorine.[67, 80]

4. Acesulfame-K

Acesulfame-K (Ace-K, **3**) has been developed as a sweetener by Hoechst[81] as a result of the discovery of oxathiazinone dioxides **22** by Clauss and Jensen in 1970.[82] Oxathiazinone dioxides **22** that have hydrogen or lower alkyl groups as the R^1 and R^2 substituents taste sweet. Although the derivative **23** ($R^1 = Et$, $R^2 = Me$) is the sweetest oxathiazinone dioxide yet discovered, Ace-K **3** was chosen for development as the most suitable sugar substitute because it has a more pure sweet taste than the other oxathiazinone dioxides. It is approximately 120 times sweeter than sucrose and has a high water solubility, whereas most synthetic sweeteners have unsatisfactory water solubility.[83] For practical purposes, when formulated in food products, it is stable to hydrolysis, even in highly acidic beverages, and is nontoxic.[84, 85] Metabolism involves ring cleavage to give acetoacetamide-*N*-sulfonic acid, which is further degraded to give physiologically benign substances.[86]

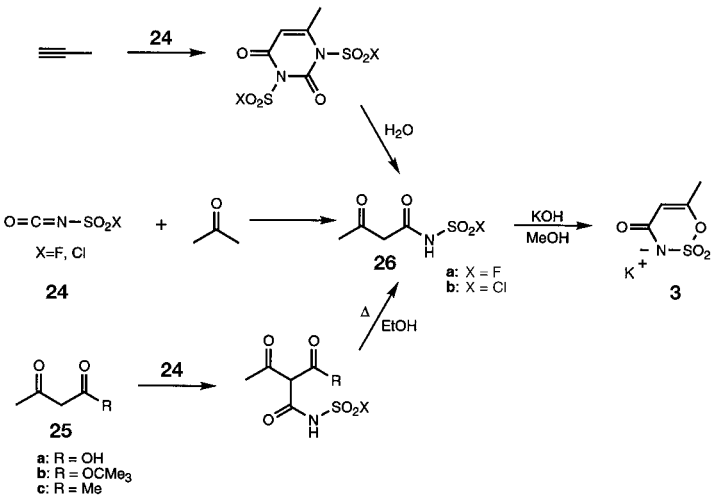


3 R^1=H, R^2=Me, M=K
23 R^1=Et, R^2=Me, M=K

4.1. Chemical Approaches

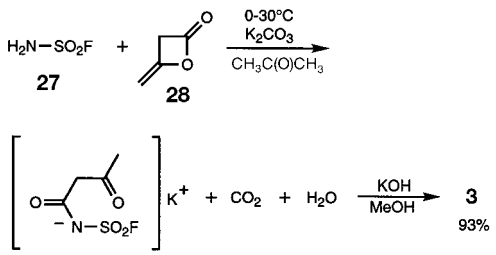
Early methods for Ace-K synthesis used chlorosulfonyl or fluorosulfonyl isocyanate (**24**) as the starting material. Although these methods allow the formation of a wide range of oxathiazinone dioxide derivatives, only Ace-K **3** formation will be discussed here. A detailed review of oxathiazinone

dioxides synthesis was published by Clauss and Jensen in 1973.[84] Reactions of chloro- or fluorosulfonyl isocyanate (**24**) with propyne,[82] acetone,[81, 87, 88] acetoacetic acid (**25a**),[89] *t*-butyl acetoacetate (**25b**),[90] or acetylacetone (**25c**)[87] give *N*-chloro or *N*-(fluorosulfonyl)acetoacetamide (**26**), which is then cyclized by methanolic potassium hydroxide to give Ace-K **3** (Scheme 8).



Scheme 8. Synthesis of Ace-K **3** from halosulfonyl isocyanates **24** through sulfonyl acetoacetamide **26**.

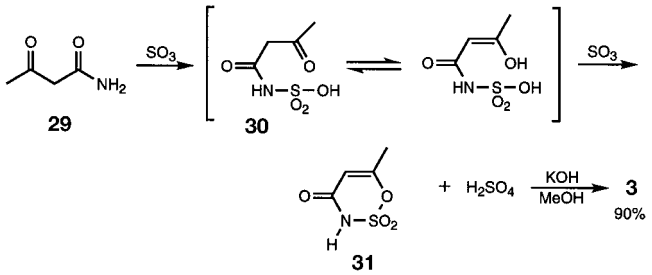
Salts of *N*-(fluorosulfonyl)acetoacetamide (**26**) can also be formed by treating diketene **28** with amidosulfonyl fluoride **27** (the product of partial hydrolysis of fluorosulfonyl isocyanate) in the presence of organic nitrogen bases[91] or inorganic bases (Scheme 9).[92] The use of potassium carbonate, under hetero-



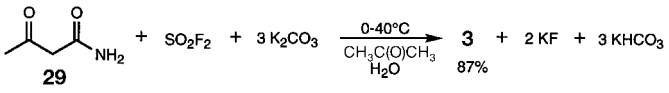
Scheme 9. Synthesis of Ace-K **3** from amidosulfonyl fluoride **27**.

geneous reaction conditions, is preferred because the *N*-(fluorosulfonyl)acetoacetamide salt formed is a crystalline solid.[92, 93] Although high yields of Ace-K **3** can be obtained by the above methods, the difficulty in the preparation and handling of chloro- or fluorosulfonyl isocyanate limits their industrial utility.

An alternative method involves the treatment of acetoacetamide (**29**) with at least two equivalents of sulfur trioxide (Scheme 10).[94] The initial addition of sulfur trioxide to acetoacetamide (**29**) presumably forms *N*-sulfoacetoacetamide (**30**), which is then dehydrated by sulfur trioxide to form oxathiazinone dioxide (**31**). Neutralization with potassium hydroxide gives Ace-K **3**.[96] Reaction of acetoacetamide (**29**) with sulfuryl fluoride and potassium carbonate gives Ace-K **3** in high yield (Scheme 11).[96] Some other fluorosulfonyl



Scheme 10. Synthesis of Ace-K **3** from acetoacetamide (**29**) and sulfur trioxide.



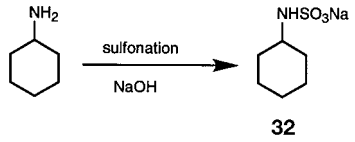
Scheme 11. Synthesis of Ace-K **3** from acetoacetamide (**29**) and sulfuryl fluoride.

compounds (i.e. chlorosulfonyl fluoride, pyrosulfuryl fluoride, and chloropyrosulfuryl fluoride) can also be used, but sulfuryl fluoride gives the highest yield of Ace-K. Yields are even higher when aqueous acetone (1 – 12 % w/w in water) is used as solvent.

5. Cyclamates

Cyclamates are salts of cyclohexylaminosulfonic acid (cyclamic acid). Sodium cyclamate (**32**, often also called cyclamate) is used as a nonnutritive sweetener and the analogous calcium salt is used especially in low sodium diets. This product is available only in some parts of the world, such as Canada. It was accidently discovered by a graduate student, Michael Sveda, in 1937 at the University of Illinois.[73, 97] The patent was issued to Du Pont in 1942, but later it became the property of Abbott Laboratories, Illinois when Abbott submitted an NDA (New Drug Application) in 1950. In 1958, the FDA classified sodium cyclamate as a GRAS (Generally Regarded As Safe) sweetener. Later in 1966, it was reported that sodium cyclamate could be metabolized in the intestine to cyclohexylamine, which had been shown to cause chromosomal damage in animals and, when administered with saccharin (10:1), tumors in rats.[98] In 1970, the FDA banned the use of cyclamate. Abbott Laboratories did additional safety studies and submitted a petition twice but it was denied by the FDA. Cyclamate use is not permitted in the USA.

Cyclamate is 30 times sweeter than sucrose. It has a bitter off-taste, but has good sweetness synergy with saccharin. It is soluble in water, and its solubility can be increased by preparing the sodium or calcium salt. It is stable over a wide range of pH and temperatures. Many chemical syntheses have been reported of this simple compound **32**; all of them use the sulfonation of cyclohexylamine approach (Scheme 12). Sulfonation can be achieved with sulfamic acid, a salt of sulfamic acid, or sulfur trioxide.[99] With the current regulatory ban in the USA, and the simple nature of the molecule, little work is being done on this sweetener.



32

Scheme 12. Synthesis of sodium cyclamate.

6. Sucralose

Sucralose (TGS) **33**, 4,1′,6′-trichloro-4,1′,6′-trideoxygalactosucrose, is a trichloro disaccharide nonnutritive sweetener that is currently used in Canada and Australia and recently approved in the USA. This compound was discovered through a systematic study where sucrose derivatives were prepared. It was found that substitution of certain hydroxy groups by a halogen atom increased the sweetness potency dramatically.[100] Sucralose was chosen as the development candidate by Tate and Lyle.[101, 102] It is licensed to McNeil Speciality (a Johnson & Johnson subsidary) in the USA.

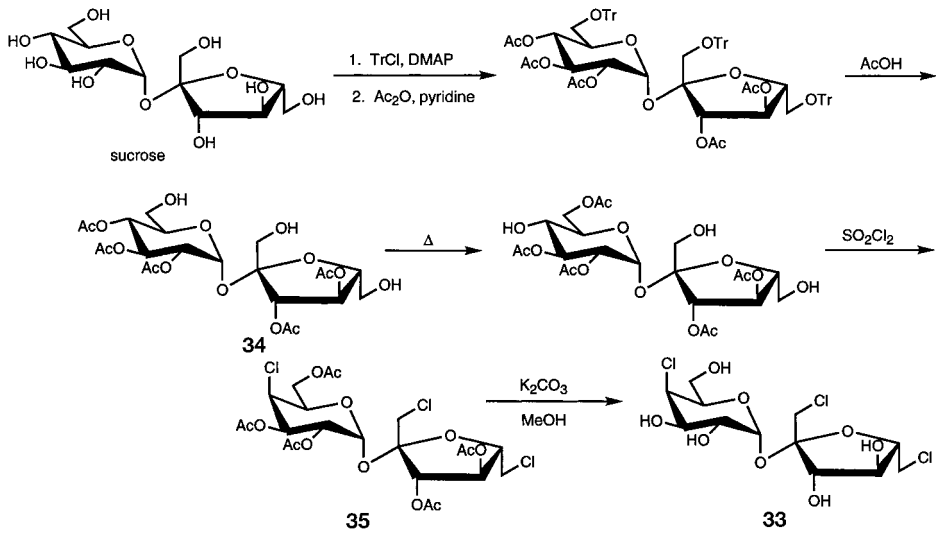
Sucralose is 450–650 times sweeter than sucrose. It has a pleasant sweet taste and its taste quality and time intensity profile is very close to that of sucrose. It has a moderate synergy with other nutritive[103] and nonnutritive sweeteners.[104] It is very soluble in water and is stable over a wide range of pH and temperatures. There is some discolorization and it does liberate HCl when stored at high temperatures.[105]

6.1. Chemical Approaches

The synthesis of TGS **33** involves a series of selective protection and deprotection steps so that the 4-hydroxy group can be converted to a chloro atom with inversion of configuration (Scheme 13).[102, 105] Differentiation between the primary hydroxy groups of the two sugar moieties is also required. The primary hydroxy groups are protected with the bulky trityl group, and then the secondary hydroxy groups are acetylated with acetic anhydride or acetyl chloride.[107] Deprotection of the primary hydroxy groups provides **34**. The acetyl group at the C-4 position of the glucose moiety migrates to the primary hydroxy group of the glucose moiety when **34** is heated under reflux at 125 °C in 4-methyl-pentan-2-one and acetic acid. This key migration allows the primary hydroxy group of the glucose unit to be protected in the subsequent chlorination step and also provides for the selective deprotection of the secondary hydroxy group at C-4. Treatment of the free hydroxy groups with sulfuryl chloride affords the corresponding trichloro disaccharide **35** which is then deprotected to give the sucralose.[108] Since there is a stereochemical inversion at the C-4 center of the glucose moiety, the product formed is a trichlorogalactosylfructose. Other chlorinating agents, such as phosgene and thionyl chloride in the presence of triphenylphosphane oxide or sulfide, have also been advocated for the chlorination reaction.[109] The usefulness of sucrose 6-esters in the preparation of sucralose has prompted a number of chemical and enzymatic approaches to these compounds.[110] The chemical methods are based on regioselective cleavage of a 4,6-orthoester.[111]

6.2. Enzymatic approaches

Due to the lack of synthetic methodology that offers stereochemical and regioselective control, chemical methods to prepare derivatives of carbohydrates are generally much more difficult than reactions with amino or nucleic acids.[112] The use of enzymes or microbial cultures to augment synthetic organic chemistry and carry out selected functionalization of complex molecules has been widely documented in the growing field of biocatalysis.[113] Such regio- and stereochemical control is needed in the synthesis of the synthetic sweetener sucralose **33**.

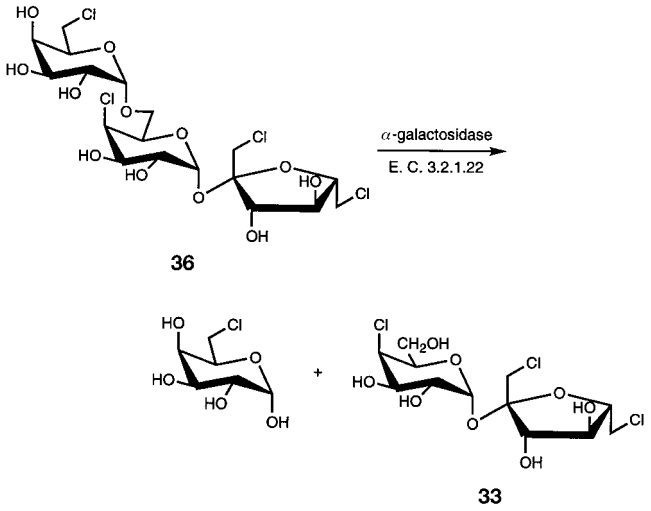


34 35 33

Scheme 13. Synthesis of TGS **33** from sucrose. The 4-hydroxy group is selectively deprotected by chemical transformations. DMAP = 4-dimethylaminopyridine, Tr = triphenylmethyl (trityl).

6.2.1. *Trisaccharide cleavage*

This process begins with the trisaccharide raffinose (6-*O*-α-D-galactopyranosyl-sucrose) followed by chemical chlorination with thionyl chloride in the presence of triphenylphosphane and triphenylphosphane oxide to yield tetrachlororaffinose **36** (TCR, *O*-α-D-6-chloro-6-deoxy-galactopyranosyl-(1→6)-α-D-4-chloro-4-deoxygalactopyranosyl-(1→2)-β-D-1,6-di-chloro-1,6-dideoxyfructofuranoside)(Scheme 14). The TCR



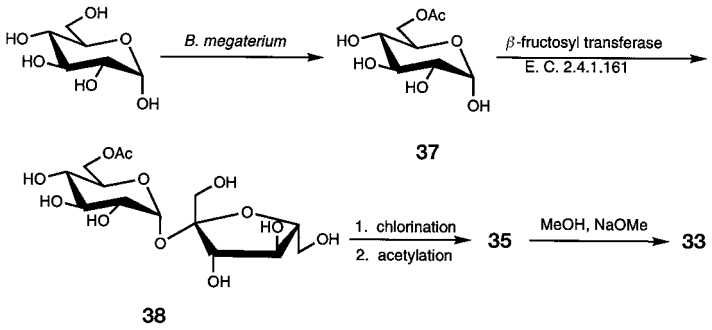
Scheme 14. Cleavage of 6-chloro-6-deoxygalactose by enzymatic hydrolysis with an α-galactoside.

is isolated as the heptaacetate ester, which after ester hydrolysis, is enzymatically treated with an α-galactosidase to remove the 6-chloro-6-deoxygalactosyl moiety from the 6-position to yield TGS (**33**).[114, 115]

The α-galactosidase (EC 3.2.1.22) employed here is the same one that is utilized in the sugar beet industry to hydrolyze raffinose in beet sugar molasses to aid in the production of sugar.[116] This commercial enzyme was derived from the fungal strain *Mortierella vinacea* var. *raffinoseutilizer* (ATCC 20034). In addition to this strain, other fungal strains possessing this activity have also been isolated which include *Circinella muscae*, *Absidia griseola* and *Aspergillus niger*.[114] Reported conversion of TCR **36** to TGS **33** is in the 60–70% range, depending on the reaction conditions used.

6.2.2. *Bioorganic synthesis*[117]

Another route to TGS utilizes both chemical and biocatalytic transformations.[118] Glucose was converted to glucose-6-acetate (**37**) by *Bacillus megaterium* with a maximum concentration of 15 g L^{-1} (Scheme 15). A specific β-fructosyl transferase (EC 2.4.1.161) isolated from *Bacillus subtilis*, using sucrose as the fructosyl donor with **37** as the acceptor, formed sucrose-6-acetate **38** with maximum concentrations of 120 g L^{-1}, representing a yield of 58%. Following chemical chlorination and acetylation, 4,1′,6′-trichloro-4,1′,6′-trideoxy-*galacto*-sucrose pentaacetate (**35**) formed with a 39% molar yield. Chemical deesterification yielded TGS **33** with a 90% molar yield. Although this route to TGS is comprised of two

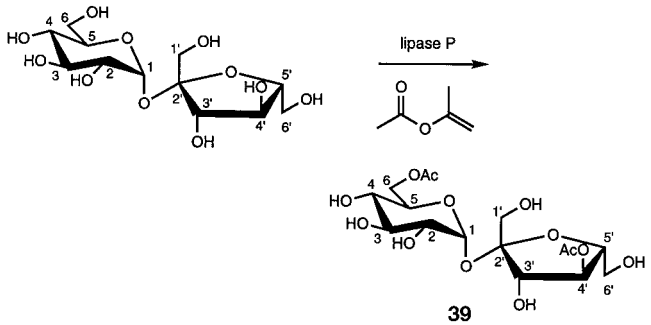


Scheme 15. Synthesis of TGS **33** from glucose through a combination of chemical and biochemical steps.

biocatalytic and two chemical steps, it does show the versatility of combining both approaches into one manufacturing process.[119]

6.2.3. *Regioselective Acylation*

As discussed by Klibanov, regioselective chemical reactions of carbohydrates are particularly difficult due to the large number of hydroxyl groups in these types of molecules.[120] The use of enzymes in organic solvents to catalyze these types of reactions has been well documented and offers the synthetic chemist an alternative approach to this complex type of reaction.[121] In the case of TGS, Dordick *et al* have described the enzymatic preparation of a useful intermediate **39** from sucrose, (Scheme 16).[122] In this reaction, lipase P



Scheme 16. Regioacylation of sucrose with isopropenyl acetate to form 4′,6-di-*O*-acetylsucrose.

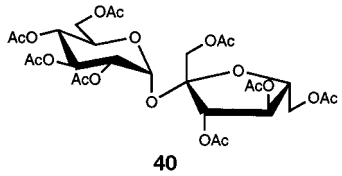
(*Pseudomonas sp.*) from Amano was used with isopropenyl acetate to acylate the 4′ and 6 position of sucrose. Chlorination of this intermediate yields TGS **33**.

6.2.4. *Regioselective Deacylation*

This approach has been used to synthesize intermediates of TGS, described by Palmer and Terradas and Bornemann *et al*.[122] As shown in Table 2 selected enzymes under specific reaction conditions, i.e. organic solvent and pH, will deacylate sucrose octaacetate **40** regioselectively to produce a number of potential intermediates in the preparation of TGS. These include 2,3,4,6,1′,3′,6′-heptaacetyl-sucrose, 2,3,4,3′,4′-pentaacetyl-sucrose and 2,3,6,3′,4′-pentaacetyl-sucrose.

Table 2. Regioselectivity of selscted lipases, proteases, and carbohydrates toward **40**.[123]

Enzyme	Company	Source	Position deacylated
lipases			
AY30	Amano	*Candida cylindracea*	4′, 1′
PPL	Sigma	porcine pancreas	4′
AP12	Amano	*Aspergillus niger*	4, 6
SP-435	Novo	*Candida antarctica*	6′, 1′
proteases			
alcalase	Novo	*Bacillus licheniformis*	1′, 6′
proleather	Amano	*Bacillus subtillis*	1′, 6′
carbohydrases			
α-galactosidase	Amino	*Aspergillus niger*	4



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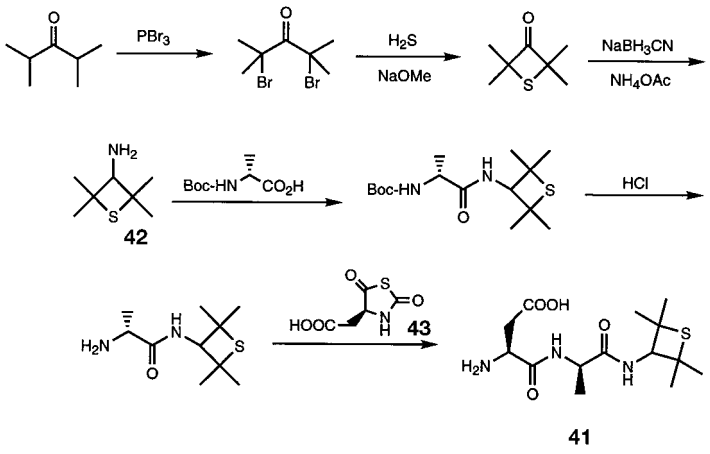
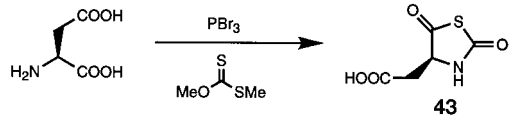
7. Alitame

Alitame **41** is a dipeptide-derived, nutritive sweetener found by Pfizer, through a discovery program, in 1983.[124-126] It is currently used in Australia, China, and Mexico with approval in the USA pending. Alitame is 2000 times sweeter than sucrose. It has a pleasant sweet taste with no aftertaste. Its time – intensity profile is similar to that of aspartame but is more stable. At pH 2 – 4, its half-life is twice that of aspartame. It has been reported to develop an occasional off-flavor in soft drinks that can be minimized, or even avoided, by the addition of EDTA.[127] It has sweetness synergy with saccharin.

Alitame contains the two amino acids L-aspartic acid and D-alanine and a tetramethylthietane moiety. The synthesis involves the coupling of the two amino acids and thietane moiety by conventional peptide synthesis techniques.[125] The order in which the three units are coupled is not important. The aminothietane moiety is prepared from diisopropyl ketone (Scheme 17). Diisopropyl ketone is brominated with phosphorus tribromide to give 2,4-dibromo-2,4-dimethylpentane-3-one. This dibromo compound is cyclized by treatment with hydrogen sulfide in presence of sodium methoxide to give 2,2,4,4-tetramethyl-3-oxothietane. The reductive amination of the ketone with ammonium acetate and sodium cyanoborohydride or by heating it in the presence of formamide and formic acid (Leuckart reduction) gives the aminotetramethylthietane moiety **42**. Scheme 18 shows the activation of the aspartyl unit as the thiocarboxylic anhydride (**43**).[124, 125, 128]



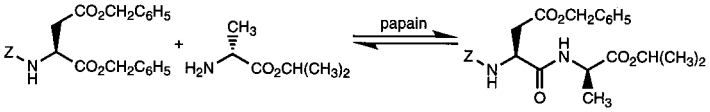
Scheme 17. Synthesis of alitame **41**.



Scheme 18. Synthesis of the thiocarboxylic anhydride **43** from L-aspartic acid.

7.1. Enzymatic Approaches

For the most part, the synthesis of alitame has generally been carried out by chemical means. However, as alitame is a dipeptide amide this sweetener is a candidate for peptide coupling. A synthetic pathway has been described by Barbas and Wong utilizing the enzyme papain for the synthesis of an alitame precursor (Scheme 19).[129] Upon hydrogenation, the

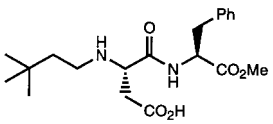


Scheme 19. Coupling of dibenzyl Z-L-aspartate with isopropyl D-alaninate in the synthesis of alitame.

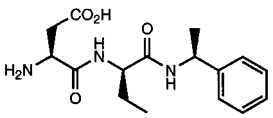
Z-and benzyl-protecting groups are removed and the remaining dipeptide ester, L-Asp-D-Ala-(O*i*Pr), can be converted into alitame by chemical coupling to 3-amino-2,2,4,4-tetramethylthietane (**42**, see Scheme 17).

8. Summary and Outlook

A number of companies and universities are still actively pursuing the search for, and development of, new nonnutritive synthetic sweeteners. Economic issues are driving these compounds to be extremely potent on a sweetness-to-weight basis when compared to sucrose. Many of these compounds have not been approved for use in commercial products and their commercial development still has to be seen. Indeed, these potential next-generation sweeteners contain a diverse array of functionality ranging from dipeptide derivatives such as **44** and **45**[130, 131] to guanidines[132] and ureas.[133] Several of the new dipetpide sweeteners have had X-ray structures reported as exemplified by **44** and **45**.[133] There is still much work to be done before any of these next-generation sweeteners are approved for use. This includes the development of viable processes, although with the significant increases in potencies the volumes at commercial scale are lower significantly relative to the sweeteners discussed above. Refine-



44



45

ments in smaller scale processes often have less commercial impact because of the smaller volumes involved.

However, one of these next-generation sweeteners, Neotame **44**, has been submitted to the USA FDA for approval as a tabletop sweetener by Monsanto. This compound is 8000 times sweeter than sucrose and has a clean sweet taste profile. It can be prepared by a reductive alkylation of aspartame (**1**).[131] This new sweetener also appears to have increased stability under a variety of conditions relative to **1**. These improved properties, as well as the cost advantages, make Neotame **44** a strong commercial, next-generation sweetener candidate.

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