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REVIEWS

Applying Combinatorial Chemistry and Biology to Food Research

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In the past decade combinatorial chemistry has become a major focus of research activity in the pharmaceutical industry for accelerating the development of novel therapeutic compounds. The same combinatorial strategies could be applied to a broad spectrum of areas in agricultural and food research, including food safety and nutrition, development of product ingredients, and processing and conversion of natural products. In contrast to "rational design", the combinatorial approach relies on molecular diversity and high-throughput screening. The capability of exploring the structural and functional limits of a vast population of diverse chemical and biochemical molecules makes it possible to expedite the creation and isolation of compounds of desirable and useful properties. Several studies in recent years have demonstrated the utility of combinatorial methods for food research. These include the discovery of synthetic antimicrobial, antioxidative, and aflatoxin-binding peptides, the identification and analysis of unique flavor compounds, the generation of new enzyme inhibitors, the development of therapeutic antibodies for botulinum neurotoxins, the synthesis of unnatural polyketides and carotenoids, and the modification of food enzymes with novel properties. The results of such activities could open a large area of applications with potential benefits to the food industry. This review describes the current techniques of combinatorial chemistry and their applications, with emphasis on examples in food science research.

Keywords: Combinatorial chemistry; food safety; nutrition; natural products

INTRODUCTION

Pharmaceutical research has traditionally relied on the screening of natural substances for "lead" compounds that possess desirable biological activities. The structure and activity of various derivatives of these compounds are further investigated by means of "rational design" to systematically synthesize a small number of candidate drug molecules and evaluate them one at a time. In recent years, this relatively slow discovery process to find new drugs has largely been supplemented by techniques derived from the concept of combinatorial chemistry. The central idea of combinatorial chemistry is to synthesize a vast library of all possible variants of the molecule of interest and screen the population for the few variants that exhibit the desirable and useful property.

The power of the concept arises from the immense number of candidate molecules that can be assessed. For example, a simple organic molecule with a core structure carrying five randomly arranged substituents theoretically produces a library of 3125 variants. The diversity of a tripeptide library of randomizing the 20 natural amino acids at each position will amount to 8000 distinct peptides. A library of hexapeptides will have a diverse population of 64 million sequence variants. In contrast to the traditional method, the combinatorial approach relies on molecular diversity and high-throughput screening, and obliterates the need for prior knowledge of the structure−function relationship of the molecules created.

Methods for creating diverse molecular libraries can be divided into two categories. The first or chemical approach is based on random or directed synthesis of chemical compounds, typically displayed on solid supports, such as activated beads, pins, or batch arrays (*1*−*4*). Libraries can also be synthesized in liquid-phase using a soluble linear homopolymer [poly(ethylene glycol) monomethyl ether] (*5*). The second or biological approach uses genetically encoded and expressed systems to generate diverse libraries of RNA, DNA, proteins, and peptides (*6*−*8*). In certain biological systems, the molecules selected in the first round of library screening are again subjected to mutation and amplification to create a new pool of diverse molecules for another round of screening. The iterative process of mutation, selection, and amplification to evolve the best-fit molecule is known as "directed evolution".

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In the past few years, the initial emphasis on random or diversity-driven libraries for high-throughput screening, in which a huge number of random variants are assayed for a "hit", has been shifted to more focused strategies. Libraries are now more frequently directed toward the biochemical function or chemical structure/activity of a specific target for knowledge-based or focused screening (*9*, *10*). Design methods are more focused on delivering maximum success rates using multiple, moderate scale libraries. Also emerged as a new and promising approach is the *in silico* design of focused libraries intended to hit a single biological target or families of related targets (*11*). The merging of molecular modeling information with combinatorial chemistry focuses the synthetic efforts to increase the number of hits in combinatorial libraries. The combined use of the traditional high-throughput approach and the screening of focused or targeted-directed libraries has become the current trend for drug discovery and development.

A key to success in the use of combinatorial chemistry is the development of high-throughput screening techniques capable of the rapid selection of molecules with a target property in libraries containing hundreds or thousands of compounds. The chemistry of detecting a biochemical function, such as ligand affinity against a receptor or selection of catalysis of an enzyme, varies with each experimental design, but sensitivity and efficiency of the detection method are the key features underlying all process development. A popular method employs arraying the compounds in a microtiter plate format for functional assay. The density of screening formats ranges from 96 to 9600 wells and higher, but in the past few years, the use of "lawn" or "well-less" screening formats has been reported. This assay format is characterized by a continuous biological test matrix on a gel lawn instead of divided into wells in traditional microtiter plates (*12*). A no-well format is the densest format possible. When automated with robotic systems for chemical synthesis, reagent handling, and arraying, the process can rapidly screen huge libraries of compounds at ultrahigh speed.

Another important aspect for screening libraries is to develop reliable high-throughput analytical techniques capable of assessing the occurrence, structure, and purity of the products. For the biological approach, characterization of the active product can be achieved by sequencing the DNA, peptide, or protein. The conventional Edman reaction used for peptide sequencing is laborious and costly and has been succeeded by the more sensitive, high-throughput, and high-specificity mass spectrometry (MS) methods (*13*−*15*). However, it is in the screening of libraries of small organic molecules that mass spectrometry has found wide applications. MS analysis (using TOF-SIMS or MALDI-FTMS, e.g.) is employed for the screening, identification, and structural characterization of discrete compounds in solution libraries, solid-support libraries, and one-pot synthesis (*16*, *17*). The emphasis is also placed on the characterization of standards, products, and byproducts in the development phase of the chemical library to ensure the quality of the final library (*18*). Emerging techniques, such as flow NMR spectroscopy in which the sample solution can be introduced into the NMR probe as a flowing stream to facilitate continuous structural analysis, may potentially become a powerful tool in combinatorial chemistry (*19*). Recent efforts have also been directed to the automation of X-ray crystallography and data collection as a viable utility for high-throughput structural determination (*20*).

We have previously proposed that agricultural and food research could benefit from the technology of combinatorial chemistry (*21*). We have also described the potential applications of combinatorial chemistry in four major areas of agriculture and food: (1) food safety and nutrition, (2) food ingredients and additives, (3) bioconversion of natural products, and (4) modification of proteins and enzymes (**Figure 1**) (*22*). These ideas were further elaborated in an ACS Symposium, "Combinatorial Methods for Agriculture and Food Processing Applications" (*23*). This paper reviews the techniques of combinatorial chemistry and recent examples of applications in food science.

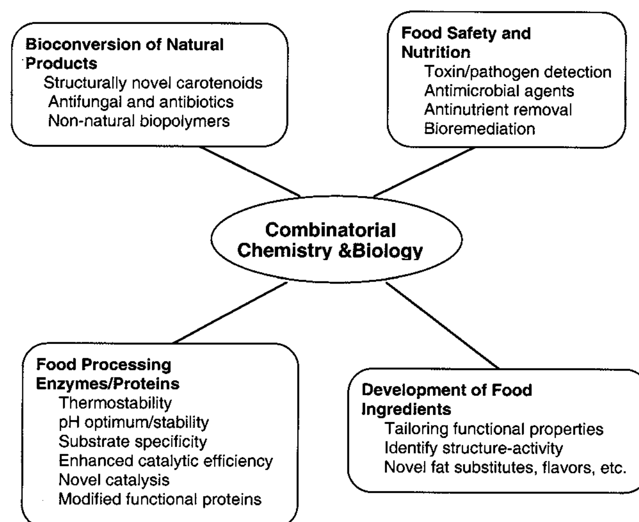

Figure 1. Potential applications of combinatorial chemistry and biology in food research (*22*).

CHEMICAL APPROACH

The chemical synthesis approach was initially used to create peptide libraries for the selection of peptide variants with unique target properties. The underlying chemistry is the solid-phase synthesis, pioneered by Merrifield (*24*, *25*) with subsequent refinements (*26*). The commonly used solid polymers include the bead-shaped conventional resins, such as polystyrene resins cross-linked with poly(ethylene glycol). In addition, paper disks (*27*), combinable cartridges (*28*) and columns (*29*), and double-walled permeable Teflon wafers (*30*) have also been used as solid supports.

Synthetic Peptide Libraries. Libraries are created by the synthesis of compounds in parallel or simultaneously in mixtures, in contrast to the traditional synthetic chemistry of preparing one compound at a time. For example, chemically coupling a series of homologues A with a series of homologues B would produce a library consisting of a range of A−B compounds of all combinations. The process of using parallel synthesis on solid supports has been the preferred procedure. The method of simultaneous synthesis in mixtures (also known as the "one-pot synthesis") is less desired in some cases. It requires deconvolution to identify the lead compound in the reaction mixture (see below).

The first example of using multiple parallel synthetic procedures for the rapid concurrent synthesis of hundreds of peptides was reported by Geysen et al. (*1*). The coupling reactions of amino acid mixtures were conducted on the surface of polyethylene rods spatially segregated in a 96-well microtiter plate format. A library of 208 overlapping hexapeptides covering the entire 213 amino acid sequence of the foot-and-mouth disease virus coat protein VPI was generated and used to identify epitopes by screening the library using ELISA methods. The

rapid synthesis of libraries of hundreds of individual peptides, and organic compounds in later studies, using this and similar techniques represents a distinct advancement over the traditional linear stepwise solid-phase synthesis. In the "tea-bag" method (*3*), peptides were synthesized on resin beads contained in porous polypropylene packets that are coded and treated batchwise for the various steps of peptide synthesis. This procedure enables the production of useful quantities (>10 mg) of a much larger number of peptides (>500−1000) at a faster rate. These various types of multiple peptide synthesis, including the light-directed, spatially addressable parallel synthesis method (*31*) and the "spot" synthesis on cellulose paper (*32*), have proven to be particularly useful for mapping biological interactions. In general, these studies are based on the parallel synthesis of an array of peptides with a conserved sequence motif and defined positions of the peptide chain. Libraries generated by these methods are limited in size compared to the theoretical possible numbers, and they are not universally random or truly combinatorial.

In general, the peptide mixture in the library is screened or assayed while attached to the solid support. However, one can design the synthesis so that the peptide mixtures on solid supports will be cleaved and assayed as free peptides in solution (*33*). The use of solution peptide libraries overcomes the constraint of the immobilized peptide on the interactions with the ligands in the mobile phase. Han et al. (*5*) developed liquid-phase combinatorial synthesis, which employs a soluble polymer poly(ethylene glycol) monomethyl ester (MeO-PEG) instead of a conventional solid phase as support material for combinatorial assembly. The polymer is soluble in a variety of aqueous and organic solvents and can be precipitated out of solution by crystallization for purification purposes. This approach makes it possible to construct more diverse libraries without the need to use large amounts of solid polymers. Both solution- and liquid-phase combinatorial libraries may eliminate certain limitations that solid-phase synthesis presents, such as nonlinear kinetic behavior, uneven distribution and access to the chemical reaction, and the use of insoluble reagents or catalysts (*34*). However, all of these approaches result in the library synthesized as a single reaction pool, and it is necessary to extract individual members of the library for direct chemical analysis (*35*) or to indirectly identify the active compound of interest by a recursive deconvolution strategy (*36*). The one-pot synthesis, in which a single-batch resin is allowed to react with mixtures of the combinatorial amino acids without partitioning, also falls into this category (*37*).

The size of a library that could be synthesized was immensely expanded to include millions of individual peptides by the development of the portioning−mixing method (*4*). The basic idea of this method, also known as split-and-mix synthesis, is to divide the solid support into equal portions before the coupling reaction (portioning), couple a different amino acid to each portion (coupling), and mix the portions (mixing), followed by repeating cycles of portioning, coupling, and mixing. **Figure 2** illustrates the principle, using a three-amino-acid three-cycle process. The size of the library increases exponentially with increasing number of amino acids and cycles (**Figure 3**) (*38*). For a pentapeptide library randomizing all 20 amino acids, the number of peptides synthesized is 3.2 million. This method has distinct advantages because (a) the library is truly random,consisting of all combinations of sequences, (b) the peptides are formed in an equimolar mixture, and (3) each bead carries one unique peptide sequence. A disadvantage of this technique is that diverse libraries require large amounts of resin. For example, a hexapeptide library containing 19 amino acids, with 4 copies of each peptide, synthesized on a 200 m bead (∼160 000 beads/g) requires 2.94 kg of resin (*37*).

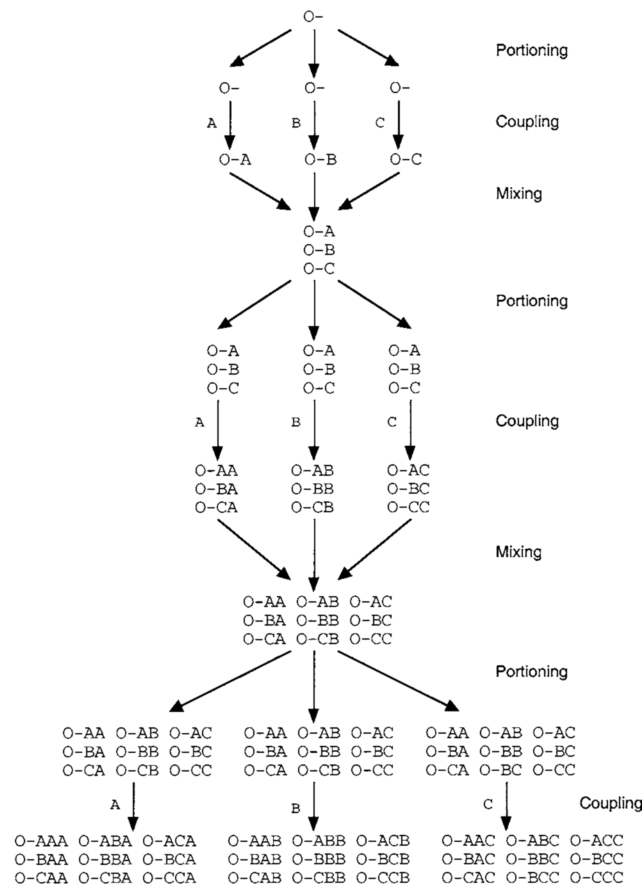

Figure 2. Portioning–mixing synthesis illustrated by a three-amino-acid three-cycle scheme (*38*).

# Residues	Number of Peptides	Number of Coupling Cycles	
		Portioning-Mixing	One by One
2	400	40	800
3	8,000	60	24,000
4	160,000	80	640,000
5	3,200,000	100	16,000,000
6	64,000,000	120	384,000,000
7	1,280,000,000	140	8,960,000,000
8	25,600,000,000	160	248,000,000,000
9	512,000,000,000	180	4,608,000,000,000
10	10,240,000,000,000	200	102,200,000,000,000

Figure 3. Number of coupling cycles in the synthesis of complete oligopeptide libraries (*38*).

The synthesis of peptide libraries has been automated by various procedures (*39*−*41*). The techniques in the initial phase of the development of combinatorial peptide libraries have been supplemented by various modifications to enhance the versatility of the methods, such as the Selectide method (*15*, *42*), positional scanning (*43*), encoded or tagged libraries (*44*), recursive deconvolution (*36*), and libraries from libraries (*45*).

A large number of publications have appeared using combinatorial peptide libraries to identify active peptides for novel diagnostic and therapeutic applications. Several recent investigations in this area are food-related.

Antimicrobial Peptides. Houghten et al. (*46*) screened a synthetic peptide library of 34 × 10^6 hexapeptides for new antimicrobial peptides against *Staphylococcus aureus* and *Staphylococcus sanguis* (Gram-positive bacteria), *Escherichia coli*

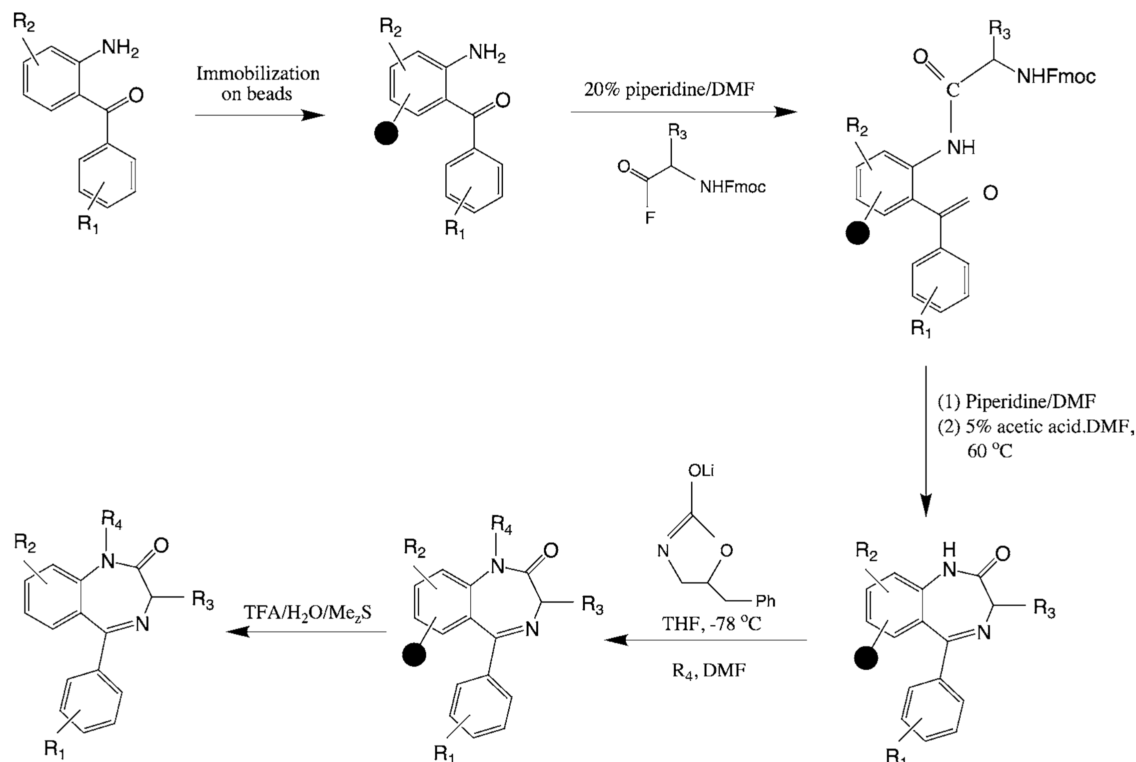

Figure 4. General and expedient solid-phase synthesis of 1,4-benzodiazepine derivatives (*58*).

and *Pseudomons aeruginosa* (Gram-negative bacteria), and the yeast *Candida albicans*. A series of peptides in the form of Ac-RRWWCX-NH_2 was found to exhibit high activity, showing the cationic character common to the major families of bioactive peptides (*47*). Kundu et al. (*48*) isolated a novel antifungal hexapeptide RwfIfH-NH_2 from a library by randomization of the 1-, 4-, and 6-positions of a lead peptide HwfFfK-NH_2. Libraries were then designed by increasing the chain length to nonapeptides with positions 7−9 subjected to randomization. Screening of these combinatorial libraries resulted in the identification of two nonapeptides, RwfifHKKR-NH_2 (IC_{50} = 1.64 μM) and RwfifHKKI-NH_2 (IC_{50} = 1.65 μM), 17 times more active than the lead peptide (IC_{50} = 28.65 μM) against *C. albicans* and *Crytococcus neoformans* (*49*).

Aflatoxin-Binding Peptides. Another recent application of combinatorial peptide synthesis was directed to obtaining affinity compounds that bind to aflatoxins. Aflatoxins are a group of carcinogenic metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus*, and contaminantion of these toxins in cereals and nuts has been a continuous health and safety concern. Tozzi et al. (*50*) created a dipeptide library using eight amino acids as building blocks, and screened the pool for the affinity binding. A tetrapeptide library was then designed based on the lead peptide identified, and a tetrapeptide, LLAR-NH_2, was selected with binding constants $K = 1.2 \times 10^4$ and 2.9×10^4 M^{-1} for aflatoxins B_1 and B_2, respectively.

Antioxidative Peptides. Peptides with antioxidative activities have been isolated from protein hydrolysates, including soybean proteins, Alaska pollack skin gelatin, and animal skeletal muscles (*51*). On the basis of the sequence information on six antioxidative peptide fragments isolated from the digestion of β-conglycinin, a soybean protein, Chen et al. (*52*, *53*) chemically synthesized 28 peptides structurally related to the lead compound HHPLL-NH_2 and identified HHP-NH_2 to be the most antioxidative. Secondary libraries of peptides structurally related to HHP-NH_2 and containing varying Tyr subtitutions were constructed to examine the sequence−activity relationships and to elucidate the mechanisms of radical scavengers, metal chelation, and singlet oxygen removal (*54*).

Synthetic Libraries of Small Organic Molecules. Peptides and oligonucleotides are important biopolymers, but have limited bioavailability because they are rapidly degraded when administered in vivo. To circumvent this negative effect, methods to incorporate unnatural building blocks into a peptide sequence have received considerable attention (*55*, *56*). Increasing interest has also been shifted to the creation of libraries of small organic molecules, by attaching various organic building blocks (relative substituents), other than amino acids and nucleotides, on a core structure (also variously known as templates, scaffolds, backbones), using diverse chemical reactions. High-diversity libraries of complex structures can be constructed using combinations of a variety of core molecules and building blocks. A recent comprehensive survey (*57*) records a total of 388 chemical libraries in that one year!

Bunin and Ellman (*58*) first demonstrated the extension of solid-phase synthesis methods to the synthesis of non-natural organic molecules. 1,4-Benzodiazepine derivatives, a class of bioavailable therapeutic agents, were constructed by attaching 2-aminobenzophenones to polystyrene solid supports through an acid-cleavable linker (**Figure 4**). The synthesis of benzodiazepine derivatives on the solid support was carried out by removal of the Fmoc protecting group, coupling to an α-N-Fmoc-amino acid, cyclization into a diazepine ring, and alkylation. A library of 192 structurally diverse 1,4-benzodiazepine derivatives was prepared by using Geysen's pin method, and screened by binding assay for cholecystokinin A receptor (*59*). Since this first demonstration, numerous core molecules and building blocks have been derived, with all possible organic reactions utilized in the synthesis of libraries in a volumetric scale. For a review of the recent development on the chemistry of small molecule combinatorial libraries, see Burke et al. (*60*) and Weber (*61*).

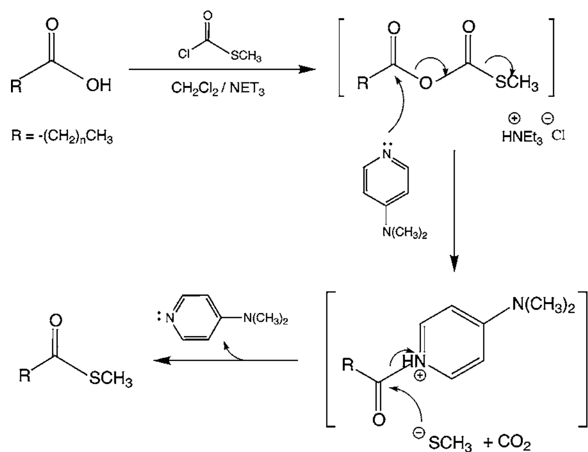

Figure 5. Synthesis of *S*-methyl thioesters from carboxylic acids and methyl chlorothiolformate (*68*).

It is noteworthy that a major objective of small molecule development is to mimic the complex molecular interactions of natural peptides and proteins of potential therapeutic values. The structure–activity relationship and conformation properties of a peptide structure provide the lead for the design of non-peptide analogues with equal or often enhanced bioactivity (*62*). Strategies in the development of peptidomimetics include peptide backbone modifications, β-turn-based architecture, constrained cyclic structures, and others (*63*). In one of the studies, Lowe's group has illustrated the utilization of structure–activity information derived by X-ray crystallography and computer modeling to create focused libraries of peptidomimics for the refinement and optimization of the active analogue (*64*). On the basis of the conformation analysis of the hydrophobic core dipeptide Phe132–Tyr133 of staphylococcal protein A involved in the binding interaction with IgG Fc fragment, analogues were designed using a triazine ring as the framework for the two mimetic groups. One of the analogues was shown to bind IgG with a binding constant of 10^5–10^6 M^{-1} and used as the lead compound to create a combinatorial library of triazine ring with various amino substituents for activity optimization (*65*). The same group utilized similar strategies to identify a triazine–histamine/tryptamine ligand for glycoproteins. This involves detailed investigation of protein–carbohydrate interactions to identify key residues for binding specificity, followed by library synthesis of mimetic analogues (*66*). In essence, this is a minimization approach to reduce the binding complex of two macromolecules to the few residues at the recognition site at the interface.

Libraries of Flavor Compounds. Two research groups have constructed flavor libraries in the past few years. Volfson's group prepared a library of *S*-methyl thioesters and screened it for sensory unique components (*67*, *68*). Thioesters are known to form in maturing cheese and contribute to the characteristic aroma with very low thresholds. Library synthesis of *S*-methyl thioesters involves a reaction between methyl chlorothiolformate and carboxylic acids with various carbon chain lengths (**Figure 5**). Sensory evaluation of the synthetic library using GC–olfactometry identified *S*-methyl thiopropionate as the most possible characteristic aroma of Camembert cheese.

Collin's group synthesized libraries of mercaptoketones by simple addition of hydrogen sulfide to α,β-unsaturated ketones with various substituents and subsequent reduction of the mixture to mercaptoalcohols (**Figure 6**) (*69*). Two flavor compounds, 4-mercapto-4-methylpentan-2-one and 4-mercapto-3-methylpentan-2-ol, were identified with very low detection limits of 0.004 and 0.012 ng, respectively. Combinatorial synthesis of mercaptoaldehydes was created by a similar approach and also by the reaction of α,β-unsaturated aldehydes with thioacetic acid (*70*). Three 3-mercapto-2-methylaldehydes (propanal, butanal, and pentanal) were identified to possess meaty aroma at GC–sniffing levels. The same group also synthesized primary mercaptoalcohols and analogues and identified a number of characteristic flavor compounds (*71*, *72*).

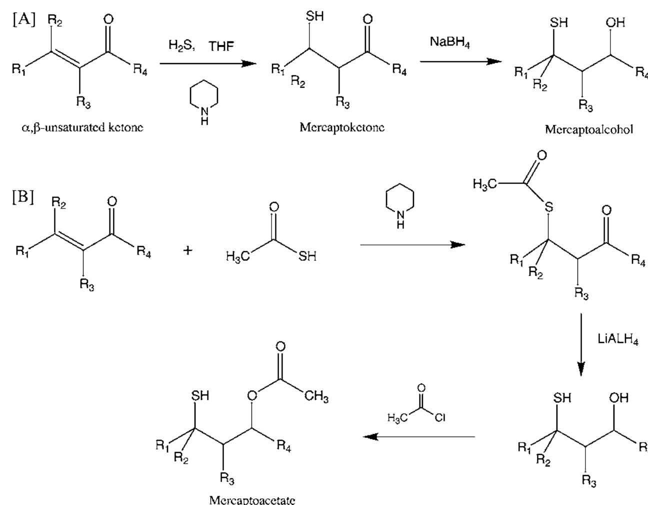

Figure 6. Synthetic pathways of mercaptoalcohols for α,β-unsaturated ketones (*71*).

Food Enzyme Inhibitors. Identification of enzyme inhibitors is of interest to the industry. For example, inhibitors of polyphenol oxidase are used to prevent enzymatic browning in fruits and vegetables, and α-amylase inhibitors are therapeutics for diabetes, obesity, and hyperlipemia. In one recent investigation, polyphenol oxidase inhibitors were selected from a combinatorial library of compounds generated by lipase-catalyzed random acylation of four commercial phenols with six vinyl esters (*73*). It was found that benzoylation of the 4-hydroxybenzyl alcohol by vinyl benzoate produced the best inhibitor, 4-hydroxybenzyl benzoate, with a K_i of 40 μM. This value compares favorably with the K_i of the starting phenol (400 μM) and is less competitive with the commercial antibrowning agent, 4-hexyl resorcinol ($K_i = 2$ μM). In another investigation, a library was prepared by coupling 11 aromatic amines with 14 sugars, in a Maillard-type reaction, in 96-well plates. It was found that the products fructose and 4-hydroxyaniline showed a strong inhibition of polyphenol oxidase (*74*).

BIOLOGICAL APPROACH

The precursors of this type of librarry are genetic codes, and the approach is to randomize the nucleotide sequence of the protein/peptide of interest in vitro, resulting in a vast population of millions of variants that can be expressed in various biological systems. One of the early versatile systems developed to express random libraries of peptides and proteins employs a derivative of filamentous bacteriophage.

Phage and Cell Display. The technique of phage display was first described by Smith (*6*), with subsequent refinements (*75*, *76*). The method employs a specially constructed filamentous fusion-phage system that enables the expression of peptide variants displayed on the surface of the phage particles by gene fusion (**Figure 7**) (*22*). The general scheme involves the synthesis of a combinatorial library of DNA sequences encoding the peptide of interest. The DNA sequences are then inserted downstream of the signal sequence of gene *III* or *VIII* (of the

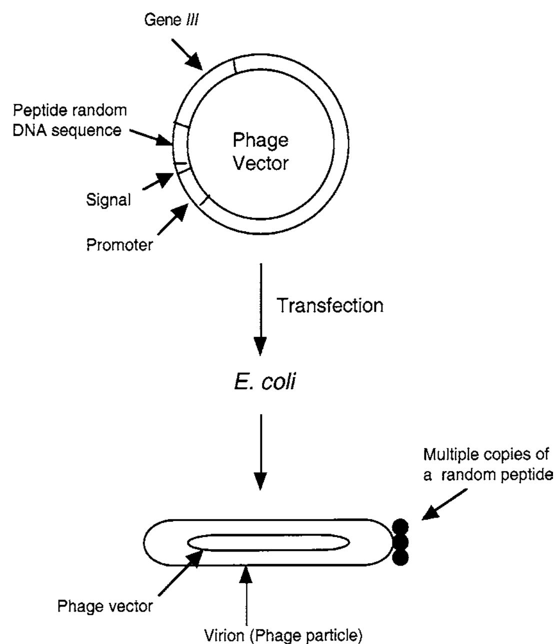

Figure 7. Construction of peptide libraries displayed by filamentous phage (*22*).

bacteriophage major coat proteins) in the phage vector. Infection of *E. coli* with the resulting recombinant phage vector will produce progeny phage particles each displaying a single peptide variant. The phage library can be selected by various methods depending on the experimental objective. By far, the most common selection imposed on phage displayed peptide libraries is affinity for a target protein such as receptor proteins. An affinity-selection method called "biopanning" is often used (*77*). The target protein is immobilized to a solid support, such as a polystyrene Petri plate, and incubated with the phage mixture. Phage particles carrying displayed peptides that bind to the target protein are captured on the plate, with the unbound phages removed by repeated washing. Finally, the bound phages are eluted with acidic buffer, yielding an enriched population of high-affinity clones. These eluted phages can be used for another round of infection and affinity selection. The amino acid sequence of the peptide that shows high affinity for the target protein can be deduced from the coding sequence in the viral vector.

Phage display has been successfully used in various research and development applications. These include mapping and mimicking of epitopes, identifying new receptors and natural ligands, identifying high-affinity antibodies and analogues, isolating specific antigens that bind to bioactive compounds, producing novel enzyme inhibitors and DNA-binding proteins, probing cellular and tissue-specific processes, and numerous other examples (*78*). The phage display technology has also been extended to expressing peptides and proteins on bacterial and yeast cell surfaces (*79*, *80*).

Phage-Displayed Botulinum Antibodies. Phage display offers a high-throughput method of generating antibodies in vitro and has been a major source of diagnostic and therapeutic antibodies in clinical development (*81*). The principles are identical to that of displaying peptides and proteins except, in this case, the *V* genes that encode antibody variable domains (the antigen-binding fragments, Fab) are targeted. Emanuel et al. (*82*) demonstrated the use of phage display to isolate antibodies with high specificity toward botulinum neurotoxin serotype B. The combinatorial antibody library was constructed by isolating IgG sequences via cDNA synthesis and PCR amplification of the mRNA of the spleen cells of immunized mice. Amersdorfer et al. (*83*) constructed phage antibody libraries from the IgG *V* genes isolated from mice immunized with the botulinum neurotoxin type A binding domain (the region that binds to presynaptic neuronal receptors leading to toxin internalization and toxicity). Phage-displayed antibodies binding four non-overlapping epitopes of BoNT/A were isolated with equilibrium constants of 7.3×10^{-8} to 1.1×10^{-9} M. A combination of antibodies binding epitopes 1 and 2 prolonged the time to neuroparalysis by 389% compared to the control. The sequences and structures of these epitopes were later determined by mapping the locations using phage library techniques (*84*). The neutralization of botulinum neurotoxin A by these antibodies was evaluated using in vivo systems (*85*), and a human antibody for botulism prevention was developed (*86*).

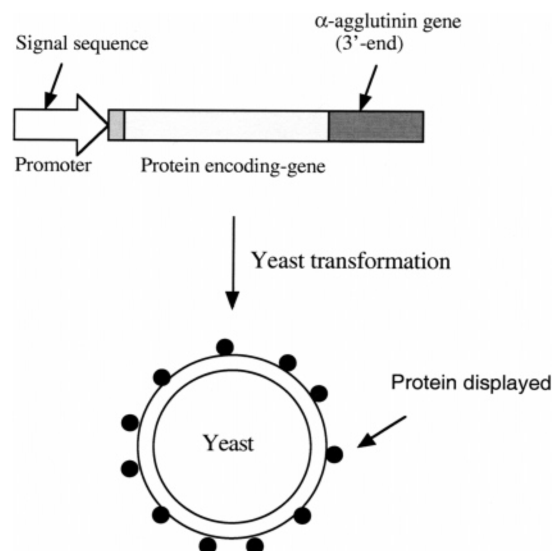

Figure 8. Display of proteins on the cell surface of yeast using the α-agglutinin gene sequence (*87*).

Displayed Enzymes as Whole-Cell Bioreactors. In general, the phage display technique has not been widely applied to the field of protein engineering. Expression of proteins/enzymes on the yeast cell surface of *Saccharomyces cerevisiae* offers more advantages than phage and other microbial systems, particularly if the system is intended for food and pharmaceutical production. *Saccharomyces* is a generally recognized as safe (GRAS) organism. Yeast with enzymes displayed on the cell surface can be used as whole-cell bioreactors. Yeast can be cultivated to a high cell density and commonly results in high fidelity of folding and glycosylation of eukaryotic heterologous protein expression. Schreuder et al. (*80*) first developed the yeast display system by fusing the α-galactosidase gene (from *Cyamopsis tetragonoloba* seeds) to the gene encoding the C-terminal half of α-agglutinin, inserted downstream of the phosphoglycerate kinase (PGK) promoter and the invertase signal sequence (**Figure 8**) (*87*). The fusion protein was expressed and anchored onto the surface of the yeast, due to the tight binding (by the formation of covalent linkage) between the truncated α-agglutinin and the cell wall structural glucans.

Using a similar approach, Tanaka's group immobilized *Bacillus stearothermophilus* α-amylase and *Rhizopus oryzae* glucoamylase coexpressed in active forms on the surface of yeast cells, and observed enhanced degradation of starch compared to a single-enzyme-displaying strain (*88*, *89*).

The same group displayed on yeast cell surface another industrially important enzyme, lipase from *R. oryzae*, active toward soluble 2,3-dimercaptopropanol tributyl ester and in-

soluble triolein. Because the *Rhizopus* lipase active site is at the C-terminal region, a flexible peptide spacer was placed between the *R. oryzae* lipase and the α-agglutinin to preserve the conformation of the active region of the enzyme (*90*). A novel display system was developed that enables the fusion of the N terminus of the lipase enzyme with Flo1p, a lectin-like cell wall protein in *S. cerevisiae*. The yeast cells were used as a whole-cell bioreactor to synthesize methyl esters from triglyceride and methanol (*91*). Cell surface-engineered yeast displaying a hexahistidine oligopeptide has been demonstrated to enhance the adsorption of copper and, hence, its potential use for bioremediation of pollutants (*92*).

Combinatorial Biosynthesis. Metabolic pathways often employ multienzyme cascades to catalyze sequential reactions to synthesize natural biopolymers. These reactions can be manipulated, shuffled, and recombined in different ways in an in vitro system. Applying the concept of combinatorial chemistry to metabolic engineering therefore results in a structurally diverse library of products with novel functional properties.

Polyketide Biosynthesis. In the biosynthesis of polyketides, the multifunctional polyketide synthase enzyme complex catalyzes repeated Claisen condensations between acylthioesters (acetyl, propionyl, malonyl, or methylmalonyl). Each condensation cycle results in the formation of a β-keto group on a growing polyketide chain that may undergo all, some, or none of a series of reduction cycles of ketoreduction, dehydration, and enoylreduction. The start unit and chain length as well as the degree of reduction and the type of elongation can be varied by combinatorially cloning the genes encoding the various PKS subunits, yielding a large repertoire of unnatural polyketides. Engineered biosynthesis of the polyketide pathway has been harnessed to design medicinally active compounds, such as antibiotics and anticancer (*93*, *94*).

Biosynthesis of Novel Carotenoids. More relevant to agricultural and food science research is the recent focus on the design of novel carotenoids by combinatorial biosynthesis. A variety of structurally different carotenoids have been successfully biosynthesized by noncarotenogenic microorganisms, such as *E. coli* and yeast, by manipulation of the terpenoid pathway. A majority of carotenoids are C_{40} molecules derived from a head-to-tail condensation of diterpene geranylgeranyl diphosphates (GGDP) to form a colorless carotenoid, phytoene. Subsequent desaturation creates the conjugation system to produce neurosporene or lycopene, which are further branched into acyclic and cyclic carotenoids. More than 150 genes encoding 27 different carotenoid enzymes (*crt* genes) have been cloned from bacteria, plants, and fungi, including various synthases, desaturases, cyclases, hydrolases, methyltransferases, epoxidases, oxygenases, glucosylases, and elongases (*95*). Combinations of *crt* genes from different species (mostly *Erwinia uredovora* and *Rhodobacter species*) have been used to engineer new pathways in heterologous hosts for the synthesis of novel carotenoids not found in nature. For example, acyclic and cyclic hydroxycarotenoids with enhanced oxidative properties were produced by the combination of several carotenogenic genes encoding various desaturases, hydratases, cyclases, and hydroxylase (*96*, *97*). Two new carotenoid glucosides, astaxanthin β-D-diglucoside and astaxanthin 3′-β-D-glucosidediglucoside, were produced by *E. coli* engineered with a combination of seven *crt* genes (*98*). Using gene-shuffling techniques, Schmidt-Dannert et al. (*99*) created diverse libraries of phytoene desaturase (*crtI*) and lycopene cyclase (*crtY*) mutants by in vitro recombination, and transformed them into *E. coli* harboring a combination of other carotenogenic genes. The approach results in a six-step desaturation and 3,4-cyclization to produce 3,4,3′,4′-tetradehydrolycopene and the cyclic carotenoid torulene.

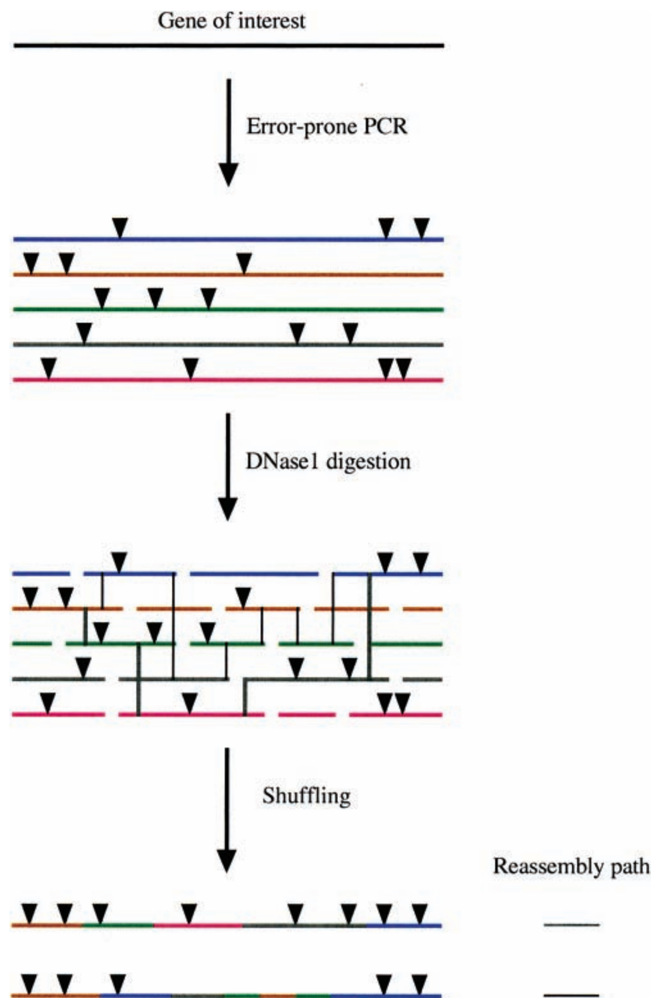

Figure 9. DNA shuffling by fragmentation and PCR reassembly (*8*).

Directed Evolution. The method of directed (molecular) evolution was initially developed for creating RNA with novel binding and catalytic activities, through cycles of in vitro mutation, selection, and amplification, of RNA (*100*). The same approach was later extended to evolve DNA enzymes that can cleave RNA and enzymes with Mg^{2+}-dependent RNA phosphoesterase activity (*101*, *102*). The application of this concept for evolving proteins was expanded in capability by the development of DNA-shuffling techniques (*8*, *103*). Subsequent modification, refining, and expansion of the technique in the past decade has resulted in a proliferation of evolutionary methods as a major tool for protein design and engineering (*104*). The general scheme of the method involves random mutation of the gene encoding the protein of interest, using error-prone PCR. The resulting pool of gene mutants is digested with DNase I to a mixture of small DNA fragments. These DNA fragments are shuffled by PCR, relying on self-priming of the overlapping end sequences, resulting in crossover of the templates (**Figure 9**) (*105*). The reassembled gene library is subjected to selection for individual protein variants that best fit the desired properties. The candidate molecules selected can further undergo a new cycle of mutation, shuffling, and amplification to generate a new population of variants for selection. In each of the repeated cycles, the selection constraint applied to screen the library will effectively concentrate a combination of beneficial mutations to evolve the best-fit protein molecules. From the original single-gene shuffling, the method

has been extended to include the shuffling of a family of genes from diverse species (*106*) and genome shuffling (*107*).

The techniques of directed evolution have been successfully employed in numerous investigations to engineer various aspects of structure−function of proteins and enzymes. Although there are ∼4000 known enzymes, <100 of them are considered to be food-related, and only a handful of them are currently used in the food-processing industry (*108*). The future expansion of enzyme technology for food industry use depends on the continuous discovery and development of novel enzymes for better utilization of our food resources. Combinatorial chemistry will be essential for realizing the potential of this relatively unexplored territory. Several recent studies report on the use of directed evolution to create new and improve existing properties of food-related enzymes.

Amylolytic Enzymes. α-Amylase is used in many industrial applications and is one of the major enzymes in starch hydrolysis for the production of high-fructose corn syrup and modified starch. Progress in engineering α-amylases has been traditionally focused on bacterial enzymes and improvement of thermostability and pH activity using rational design (*109*, *110*). Recently, Richardson et al. (*111*) used high-throughput screening of microbial libraries to identify bacterial α-amylases with improved thermostability at low pH in the absence of added calcium. The result is particularly attractive to starch processing, because, in the current scheme, the pH of the starch slurry after liquefaction (at pH ∼6.5) has to be adjusted to pH 4.2 for saccharification. The development of an α-amylase with a low-pH optimum without added calcium would eliminate the chemical cost of pH adjustment and calcium addition. Kim et al. (*112*) generated thermostable maltogenic α-amylases from *Thermus* species by random mutation and DNA shuffling. The highly thermostable mutant enzyme had an optimum reaction temperature 15 °C higher than that of the wild type, with a half-life of 172 min at 80 °C compared with <1 min for the native enzyme. In another study, a high-performance *Bacillus licheniformis* α-amylase mutant containing five amino acid replacements, L13P, W194R, S197P, E356D, and N414S, was identified by directed evolution (*113*). The mutant exhibited a shift in the pH optimum from pH 6 to 7 and was active over a broader pH range than the wild type with a 5 times increase in specific activity at pH 10.

In our recent investigation, directed evolution was used to create α-amylases targeted for cold hydrolysis of starch and other unique properties. The gene encoding barley α-amylase was cloned into and constitutively secreted by *S. cervisiae* (*114*, *115*). The barley gene was subjected to error-prone PCR and gene shuffling, and the mutant library was screened by halo formation on starch agar plates, followed by high-throughput liquid assay and chemiluminescent detection method (*116*). After three rounds of mutation and shuffling, a mutant enzyme was isolated with >100 times the total activity and considerable increase in the specific activity of the wild-type enzyme (*117*). Comparison of the amino acid sequence of this mutant with the wild type revealed five substitutions. Two of these mutations resulted in amino acids highly conserved in cereal α-amylases, and the others were located in the raw starch-binding fragment of the enzyme molecule (*118*, *119*). This investigation, together with others, substantiates the hypothesis that native α-amylases, and enzymes in general, that have been optimized in vivo for specific biological and physiological requirements can be altered and further optimized to meet particular industrial demands.

Lipases. Lipases are another group of enzymes that have wide applications in the food industry, including lipid hydrolysis and modifications of fat and oil, manufacture of cheese and confectionery, and development of flavors in processed foods. To expand the functionality of lipase B from *Candida antarctica*, Zhang et al. (*120*) used directed evolution to create mutants with improved resistance toward irreversible thermal inactivation. Two mutants were generated with 20 times increases in half-life at 70 °C compared with the wild type. The increase in half-life was attributed to a lower propensity of the mutants to aggregate in the unfolded state and to improved refolding. Amino acid substitutions at positions 221 and 281 were determined to be critical for the lipase stability. Liebeton et al. (*121*) created a novel mutant from *Pseudomonas aeruginosa* lipase with high enantioselectivity by directed evolution to catalyze the hydrolysis of the chiral substrate, 2-methyldecanoic acid *p*-nitrophenyl ester. The best variant contained five amino acid substitutions that resulted in increased flexibility of distinct loops in the enzyme molecule. Kauffmann and Schmidt-Dannert (*122*) converted *Bacillus thermocatenulatus* lipase into phospholipase with a 10−12 times increase in the hydrolysis of the *sn*-1 acyl ester bond of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine. Song et al. (*123*) created libraries of DNA-shuffled and incrementally truncated genes to successfully alter the substrate specificity of a *Serratia* species phospholipase to that of a lipase. The same group also engineered the phospholipase to enhance thermostability with an 11 °C increase in the melting temperature (*124*) and to increase stability in an organic solvent, dimethyl sulfoxide (*125*). The half-life of the wild type in 50% DMSO was 90 min compared to 5−7 h for the mutant enzymes.

Peroxidases. Peroxidases are distributed ubiquitously in plants and animals, and cause irreversible damage to most foods if they are not inactivated before processing. Inactivation of peroxidases is widely used as an indicator for the blanching of vegetables and in life sciences as a reporter for various diagnostic assays. A peroxidase from the ink cap mushroom *Coprinus cinereus* was subjected to multiple rounds of directed evolution to generate a mutant with 110 times the thermostability in high-alkaline pH of 8.5−10 and 2.8 times the oxidative stability of the wild-type enzyme (*126*). In another study, Lin et al. (*127*) screened libraries of horseradish peroxidase mutants produced by error-prone PCR and shuffling to identify enzyme variants with measurable improvements in folding and expression of the active form in *E. coli*. The same group also isolated mutants that were more stable and active in the presence of H_2O_2, SDS, NaCl, and urea (*128*).

CONCLUSION

Combinatorial chemistry has revolutionized the discovery and development of therapeutic and diagnostic compounds by the pharmaceutical industry. Recent studies have begun to demonstrate that the incorporation of combinatorial techniques in food research could open a large area of novel applications with huge potential benefits to the food industry. Traditional rational design requires prior knowledge of structure−function to guide the manipulation of the starting molecule for specific changes in chemical or biochemical properties. Combinatorial chemistry employs a reverse strategy by which a large number of structurally distinct molecules are created and subjected to screening for the target molecule. Once a “hit” is registered in the search, one then goes back to determine its structure and elucidate the mechanistic bases for the functional change.

Combinatorial synthesis can provide a greatly expanded collection of libraries for lead identification of active compounds for a defined criterion in food application. This means accelera-

tion in the discovery and development of novel antioxidants, antimicrobial agents, sweeteners, flavors, and other functional additives that do not presently exist. The lead compounds can be further improved with refinement of the structure−activity by the synthesis and screening of knowledge-based, small focused libraries of families of related targets through design strategies. The latter step can be applied directly to existing food compounds that are already in use with considerable information available for predicting the structure and function relationships. In this case, the aim is then to improve and expand the capability of an existing functional ingredient by focusing on a particular property, instead of creating an entirely new additive. Some examples include increasing the intensity and stability of existing sweeteners and antioxidants, eliminating undesirable side effects of antinutrients in foods, identifying structure−activity and changing the characteristics of flavor compounds, and many other potential applications. With the tremendous number of functional additives used in the food industry, this approach is the more appropriate route, because it is relatively inexpensive, target-specific, and result-oriented and can be easily implemented into existing research programs.

The same strategies of screening density-driven libraries versus more focused libraries are also applicable to macromolecules, which require biological systems to create and screen DNA or peptide/protein libraries. These libraries are genetically coded, and the hit molecules can be amplified and mutated in successive cycles for further screening. This concentration of beneficial mutations under selection pressure is uniquely found only in the biological approach, which is not possible by the chemical methods. However, this advantage also implies that the development of assays for functional screening is critically important so that the mutations accumulated in each successive cycle are directed specifically toward the defined property. In general, more elaborate manipulations are required in this approach, but this is the only appropriate system for working with processes involving biocatalysis. With the increasing number of enzymes being used in food processing, the demand for specialty enzymes that can function optimally in a defined set of processing conditions is on the rise. Characteristics such as improved stability, efficiency, substrate specificity, and cofactor requirements for existing food enzymes are targets that can be attained by the use of combinatorial chemistry. Because many of these enzymes have been extensively investigated and the three-dimensional structural information is available, the synthetic efforts could be focused on the specific parts of the molecule or motifs that are most likely to effect changes in the target property. The combination of structure-based design and combinatorial chemistry will enhance the success rate, whether the chemical or biological approach is employed.

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