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Perspective

Mixture-Based Synthetic Combinatorial Libraries

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Introduction

In the 15 years since their first description, the conceptual and technical approaches that encompass combinatorial chemistry have gained wide acceptance across virtually all scientific disciplines. More than 1000 articles have been published on the subject, including numerous books and journals, and a range of "combichem" symposia are held each year. Also, college courses have been introduced on the subject, and Internet sites are available (www.5z.com is one of the most complete). The journal *Science* recognized combinatorial chemistry as one of nine breakthroughs in scientific research for 1998,[1] and a unique perspective on the history of this growing field was recently presented.[2]

The emphasis in this Perspective will be on the power of combinatorial chemistry, more specifically mixture-based libraries, to accelerate chemical information acquisition for basic research and drug discovery. While a number of innovative approaches have been presented, the majority of combinatorial chemistry programs still prefer the time-tested but slow and expensive preparation of large numbers of individual compounds by parallel synthesis methods using either solid- or solution-phase approaches. In contrast, the synthesis and screening of combinatorial libraries made up of mixtures of compounds, which was one of the first combinatorial approaches presented, is still underutilized and often regarded with skepticism. This Perspective illustrates the inherent strengths of mixture-based combinatorial libraries and reviews the successful applications of this efficient and powerful technology.

Combinatorial techniques have their origins in Merrifield's seminal solid-phase synthesis of peptides in 1963[3,4] and have been greatly accelerated by the parallel solid-phase synthesis methods developed during the mid 1980s. These parallel synthetic approaches were initially used for the synthesis of peptides as first illustrated by the "pin",[5] "tea bag",[6] and "spot"[7] approaches. Such approaches enabled hundreds of individual compounds to be prepared in a fraction of the time and cost of earlier linear "one at a time" solid-phase methods. The original mixture-based combinatorial concepts involved the generation of all possible sequence combinations for peptides of a given length (e.g., $20^6 =$ 64 million hexapeptides when the 20 proteogenic amino acids are used), hence the origin of the term "combinatorial". Since peptides are known to have limitations in their later development as pharmaceuticals, often due to poor bioavailability and/or rapid enzymatic degradation, the focus of combinatorial chemistry in recent years has shifted to libraries of small acyclic and heterocyclic compounds.

Two different approaches for the generation of immense mixtures (ranging from 10^6–10^{12} compounds) have been developed and used successfully. Recombinant DNA techniques have been used to generate millions of peptides expressed randomly in a fusion phage or other vector system.[8−11] This method has proved popular with laboratories already familiar with molecular biology techniques; however, it is restricted

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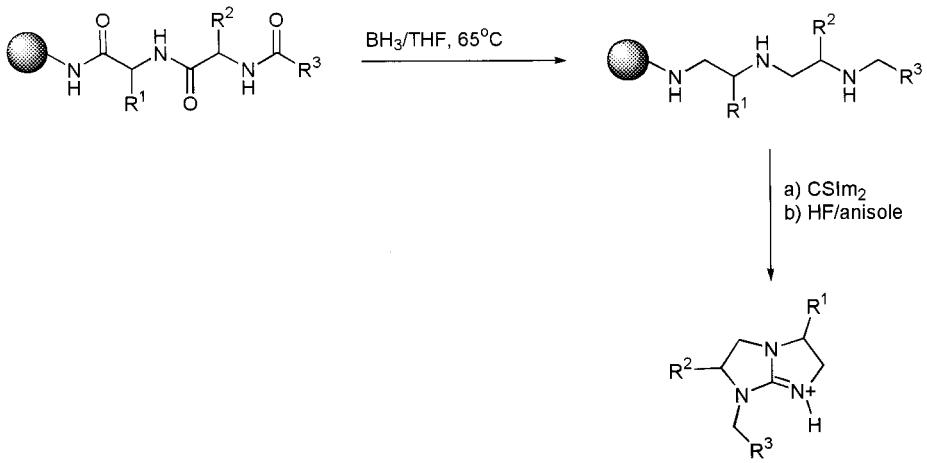
Scheme 1



to the generation of peptides displayed in the context of an expressed protein using the 20 proteogenic amino acids as building blocks. In contrast, solid-phase synthetic methods can incorporate virtually any building block of interest to produce combinatorial libraries of an explosively increasing range and number.

Due to their versatility, amino acids and short peptides have been used as starting materials for the synthesis of low-molecular-weight organic compounds.[12,13] A wide range of protected amino acids, possessing a variety of functional groups, has greatly facilitated synthetic operations. Their activation, protection, and deprotection are well-documented, and they are commercially available in enantiomerically pure forms. Postsynthetic chemical modification of peptide libraries using a "libraries from libraries" approach enables the generation of peptidomimetic libraries[14] and low-molecular-weight, organic compound libraries.[15] A greater understanding of heterocyclic chemistry on the solid phase has expanded the strategies for the synthesis of such compounds. The rapidly increasing number of organic reactions being adapted for use in solid-phase chemistry[15,16] is testament to the escalating importance of synthetic combinatorial libraries. Heterocyclic and small molecule combinatorial libraries synthesized to date have been prepared as individual compound arrays, in resin-bound one-bead−one-compound or "tagged" arrays, and as non-support-bound mixtures. The principal differences between the various solid-phase synthetic combinatorial technologies are the (1) solid support, (2) synthetic strategy for the incorporation of building blocks to generate mixtures, (3) screening conditions (support-bound or in solution), and (4) methods used to identify individual compounds (deconvolution).

In this Perspective, the synthesis and screening of a specific heterocyclic mixture-based library is described to familiarize the reader with the principles and terminology used in this field. This is followed by a review of the recent solid-phase chemistry used to prepare small molecule and heterocyclic mixture-based libraries. A number of synthetic approaches for the generation of combinatorial libraries as parallel arrays of individual compounds have been extensively reviewed elsewhere[15,17−20] and will not be included here. One-bead−one-compound libraries[21] (including various strategies for the coding of nonpeptide compounds)[22−24] are not viewed as mixture-based libraries since each individual compound is physically separated from the others by its attachment to an individual resin bead. We have only included one-bead−one-compound libraries in those instances in which the library has been cleaved from the bead and screened as a mixture of compounds. The published identification of compounds from mixture-based libraries for receptors, antibodies, major histocompatibility complex (MHC) molecules, T-cells, enzymes, antimicrobials, and other targets is arranged in five different tables. The most commonly used deconvolution techniques are described, followed by a discussion on the practical aspects required for the screening and analysis of data generated from mixture-based synthetic combinatorial libraries. This Perspective closes with a discussion on the advantages of using mixture-based libraries, including answers to frequently asked questions regarding this technology.

Identification of Individual Active Bicyclic Guanidines from a Mixture-Based Positional Scanning Synthetic Combinatorial Library

A heterocyclic combinatorial library composed of trisubstituted bicyclic guanidines is used here to illustrate that the concepts central to the use of mixture-based libraries apply regardless of the compound chemical class used. This example is intended to give the reader an understanding of the methods employed for the synthesis and use of a positional scanning library for the identification of active compounds. General methods for the synthesis of mixture-based libraries, deconvolution strategies, and library-screening conditions and requirements that are used in this example are described in more detail in later sections of this Perspective.

A. Synthesis. Scheme 1 shows the synthetic pathway for the solid-phase synthesis of bicyclic guanidines. The first step requires the exhaustive reduction of a resin-bound N-acylated dipeptide using borane in THF.[25] Following treatment with thiocarbonyldiimidazole, the presence of three secondary amines allows the reaction to proceed via highly reactive intermediates to the positively charged resin-bound bicyclic guanidine. HF cleavage yields protonated trisubstituted bicyclic guanidines in good yield and high purity.[25]

Although the reaction shown in Scheme 1 appears straightforward, extensive optimization of reaction con-

Table 1. 206 Individual Building Blocks Were Tested for Use in the Positional Scanning Bicyclic Guanidine Combinatorial Library[a]

Parent	Position 1 L-phenylalanine	Position 2 L-phenylalanine	Position 3 acetic acid
	L-alanine	L-alanine	*1-phenyl-1-cyclopropanecarboxylic acid*
	L-cysteine(MeoBzl)	*L-cysteine(MeoBzl)*	*2-phenylbutyric acid*
	L-aspartic acid(Bzl)	*L-aspartic acid(Bzl)*	3-phenylbutyric acid
	L-glutamic acid(Bzl)	*L-glutamic acid(Bzl)*	*m*-tolylacetic acid
	L-phenylalanine	L-phenylalanine	3-fluorophenylacetic acid
	glycine	glycine	*3-bromophenylacetic acid*
	L-histidine(DNP)	*L-histidine(DNP)*	*α,α,α-trifluoro-m-tolylacetic acid*
	L-isoleucine	L-isoleucine	*p*-tolylacetic acid
	L-lysine(ClZ)	L-lysine(ClZ)	4-fluorophenylacetic acid
	L-leucine	L-leucine	3-methoxyphenylacetic acid
	L-methionine sulfoxide	L-methionine sulfoxide	*4-bromophenylacetic acid*
	L-asparagine	*L-asparagine*	4-methoxyphenylacetic acid
	L-glutamine	*L-glutamine*	4-ethoxyphenylacetic acid
	L-arginine(tosyl)	L-arginine(tosyl)	*4-isobutyl-α-methylphenylacetic acid*
	L-serine(Bzl)	*L-serine(Bzl)*	*3,4-dichlorophenylacetic acid*
	L-threonine(Bzl)	*L-threonine(Bzl)*	*3,5-bis(trifluoromethyl)-phenylacetic acid*
	L-valine	L-valine	3-(3,4-dimethoxyphenyl)-propionic acid
	L-tryptophan(formyl)	*L-tryptophan(formyl)*	4-biphenylacetic acid
	L-tyrosine(BrZ)	L-tyrosine(BrZ)	3,4-dimethoxyphenylacetic acid
	D-alanine	D-alanine	phenylacetic acid
	D-cysteine(MeoBzl)	*D-cysteine(MeoBzl)*	hydrocinnamic acid
	D-aspartic acid(Bzl)	*D-aspartic acid(Bzl)*	4-phenylbutyric acid
	D-glutamic acid(Bzl)	*D-glutamic acid(Bzl)*	butyric acid
	D-phenylalanine	D-phenylalanine	heptanoic acid
	D-histidine(DNP)	*D-histidine(DNP)*	isobutyric acid
	D-isoleucine	D-isoleucine	(+/-)-2-methylbutyric acid
	D-lysine(ClZ)	D-lysine(ClZ)	isovaleric acid
	D-leucine	D-leucine	3-methylvaleric acid
	D-methionine	*D-methionine*	4-methylvaleric acid
	D-arginine(tosyl)	D-arginine	*2-ethylhexanoic acid*
	D-valine	D-valine	*trimethylacetic*
	D-tryptophan(formyl)	*D-tryptophan(formyl)*	*t*-butylacetic acid
	D-tyrosine(BrZ)	D-tyrosine(BrZ)	cyclohexanecarboxylic acid
	L-α-aminobutyric acid	L-α-aminobutyric acid	cyclohexylacetic acid
	α-aminoisobutyric acid	*α-aminoisobutyric acid*	*dicyclohexylacetic acid*
	L-norvaline	L-norvaline	cyclohexanebutyric acid
	D-norvaline	D-norvaline	cycloheptanecarboxylic acid
	L-norleucine	L-norleucine	lactic acid
	D-norleucine	D-norleucine	acetic acid
	L-ornithine(Z)	L-ornithine(Z)	*3-hydroxybutyric acid*
	L-2-naphthyl-alanine	L-2-naphthyl-alanine	*2-methylcyclopropanecarboxylic acid*
	D-2-naphthyl-alanine	D-2-naphthyl-alanine	cyclobutanecarboxylic acid
	L-cyclohexylalanine	L-cyclohexylalanine	cyclopentanecarboxylic acid
	D-cyclohexylalanine	D-cyclohexylalanine	3-cyclopentylpropionic acid
	L-methionine sulfone	L-methionine sulfone	3-cyclohexanepropionic acid
	L-*p*-nitro-phenylalanine	L-*p*-nitro-phenylalanine	4-methyl-1-cyclohexanecarboxylic acid
	D-*p*-nitro-phenylalanine	D-*p*-nitro-phenylalanine	4-*t*-butyl-cyclohexanecarboxylic acid
	L-*p*-chloro-phenylalanine	L-*p*-chloro-phenylalanine	*1-adamantanecarboxylic acid*
	D-*p*-chloro-phenylalanine	D-*p*-chloro-phenylalanine	*4-methylcyclohexaneacetic acid*
	L-*p*-fluoro-phenylalanine	L-*p*-fluoro-phenylalanine	2-norbornaneacetic acid
	D-*p*-fluoro-phenylalanine	D-*p*-fluoro-phenylalanine	1-adamantaneacetic acid
	L-ϵ-acetyl-lysine	L-ϵ-acetyl-lysine	2-ethylbutyric acid
	L-3-pyridyl-alanine	L-3-pyridyl-alanine	3,3-diphenylpropionic acid
	D-3-pyridyl-alanine	D-3-pyridyl-alanine	2-methyl-4-nitro-imidazolepropionic acid
	L-2-thienyl-alanine	*L-2-thienyl-alanine*	cyclopentylacetic acid
	D-2-thienyl-alanine	*D-2-thienyl-alanine*	*trans-styrylacetic acid*
	L-cyclohexylglycine	L-cyclohexylglycine	*trans-2,4-pentadienoic acid*
	D-cyclohexylglycine	D-cyclohexylglycine	indole-3-acetic acid
	L-*t*-butyl-glycine	L-*t*-butyl-glycine	*2-furoic acid*
	p-Fmoc-amino-L-phenylalanine	*p*-Fmoc-amino-L-phenylalanine	*2-thiophenecarboxylic acid*
	p-Fmoc-amino-D-phenylalanine	*p*-Fmoc-amino-D-phenylalanine	
	L-tryptophan	*L-tryptophan*	
	D-tryptophan	*D-tryptophan*	
	O-ethyl-L-tyrosine	O-ethyl-L-tyrosine	
	O-ethyl-D-tyrosine	O-ethyl-D-tyrosine	
	L-aspartic acid(β-Fm)	L-aspartic acid(β-Fm)	

Table 1 (Continued)

Parent	Position 1 L-phenylalanine	Position 2 L-phenylalanine	Position 3 acetic acid
	D-aspartic acid(β-Fm)	D-aspartic acid(β-Fm)	
	p-iodo-L-phenylalanine	*p*-iodo-L-phenylalanine	
	p-iodo-D-phenylalanine	*p*-iodo-D-phenylalanine	
	L-citruline	*L-citruline*	
	D-citruline	*D-citruline*	
	O-methyl-L-tyrosine	O-methyl-L-tyrosine	
	O-methyl-D-tyrosine	O-methyl-D-tyrosine	

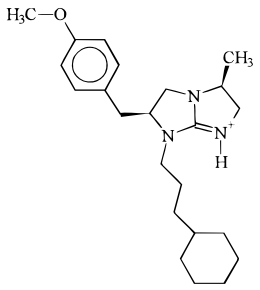
[a] Individual control compounds were prepared by individually substituting each of the building blocks into a guanidine control synthesized from L-phenylalanine at position 1, L-phenylalanine at position 2, and acetic acid at position 3. Those individual control compounds derived from the italicized building blocks did not meet the criteria for inclusion in the library and these building blocks were excluded when generating the library.

ditions was necessary prior to the synthesis of the library. Optimization of the chemistry involved is required for any library synthesis to ensure a broad range of functional group incorporation. Reaction conditions for the exhaustive reduction of peptide libraries have been determined.[25−28] The conditions necessary for the cyclization in the second step were determined by the synthesis of approximately 100 individual control bicyclic guanidines through the variation of the temperature, choice of solvent, and reaction time.

The next step was to determine the breadth of the reaction by synthesizing individual controls encompassing all proposed individual building blocks to be used in the library. Table 1 lists the 206 individual building blocks tested for use in this bicyclic guanidine combinatorial library. Using the parallel synthesis approach, commonly referred to as the "tea bag" method,[6] individual compounds were synthesized as controls for each of the three positions of diversity. Three sets of controls were prepared, in which one position was varied with each of the available building blocks while the other two positions were fixed with a single specific building block. Starting with the parent bicyclic guanidine described in Table 1, 73 individual controls were synthesized for position 1, 73 controls for position 2, and 60 controls for position 3. Following analysis of the 206 compounds by LC−MS and RP-HPLC, 49 different building blocks for position 1, 51 for position 2, and 41 for position 3 were found to have both yields and purities greater than 80% and showing a single major product by RP-HPLC. We have found that selecting only building blocks in which the individual control compounds give yields and purities greater than 80% greatly simplifies the deconvolution process.

On the basis of the synthesis of the controls described above, a positional scanning synthetic combinatorial library (PS-SCL) having three positions of diversity and composed of 102 459 bicyclic guanidines was prepared. The mixtures within the library are listed in Table 2. It should be noted that three separate sublibraries were synthesized, each differing from the others solely by the location of the defined position. Sublibrary 1 is made up of mixtures 1−49, in which the first position of each mixture is defined with an individual functionality. The same diversity is found in sublibraries 2 and 3 (mixtures 50−100 and 101−141, respectively). The 141 individual control compounds corresponding to the building blocks chosen for inclusion into the library were also synthesized concurrently with the synthesis of the mixtures making up the library. These compounds serve as controls to permit the determination of the completion of all reaction steps and the reproducibility of the synthesis. In addition, these individual compounds are screened with the mixture-based library to serve as controls for activity differentiation.

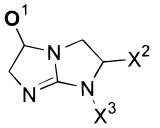
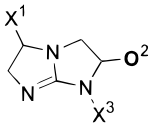
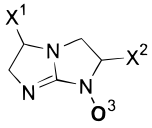
B. Screening. To illustrate the screening and deconvolution of mixture-based libraries, this trisubstituted bicyclic guanidine PS-SCL was screened at 4 μg/mL in a radioreceptor binding assay specific for the κ opioid receptor (Figure 1). The first panel represents the 49 mixtures of the first sublibrary in which the R_1 position is defined, the second panel represents R_2 (51 mixtures), and the third panel represents R_3 (41 mixtures). Mixtures exhibiting greater than 80% inhibition (total of 38 mixtures) were selected and tested in a dose−response manner. The IC_{50} values obtained for these mixtures are listed in Table 3. The most active mixtures were chosen (outlined in Table 3), and all possible combinations of these were synthesized as individual compounds. In this case, three building blocks were chosen for R_1, four for R_2, and four for R_3. Forty-eight ($3 \times 4 \times 4$) individual bicyclic guanidines were then synthesized and tested, and their activities ranged from 37 to 10 000 nM (Table 4). TPI 614-1 (**1**) was found to be the most active ($IC_{50} = 37$ nM). These results show the structure−activity relationships (SAR) of the compounds identified.



(1) TPI 614-1

This same bicyclic guanidine PS-SCL has been screened to identify potent antifungal compounds.[29] Each of the 141 mixtures was tested for its ability to inhibit *Candida albicans* growth in a standard microdilution assay. Half of the generated 32 individual compounds exhibited antifungal activity with minimum inhibitory concentrations (MIC) varying from 3 to 10 μg/mL.

Table 2. The Mixtures Contained within the Mixture-Based Positional Scanning Combinatorial Library Composed of 102 459 Bicyclic Guanidines*

	Sublibrary 1	**Sublibrary 2**	**Sublibrary 3**
			
	Mixtures 1-49 49 mixtures 2 091 compounds each	Mixtures 50-100 51 mixtures 2 009 compounds each	Mixtures 101-141 41 mixtures 2 499 compounds each

no.	**R^1**	**R^2**	**R^3**
1	S-methyl[a,b]	X^2 [c]	X^3
2	S-benzyl	X^2	X^3
3	S-2-butyl	X^2	X^3
4	S-N-methylaminobutyl	X^2	X^3
5	S-isobutyl	X^2	X^3
6	S-methylsulfinylethyl	X^2	X^3
7	S-guanidinopropyl	X^2	X^3
8	S-isopropyl	X^2	X^3
9	S-4-hydroxybenzyl	X^2	X^3
10	R-methyl	X^2	X^3
11	R-benzyl	X^2	X^3
12	R-2-butyl	X^2	X^3
13	R-N-methylaminobutyl	X^2	X^3
14	R-isobutyl	X^2	X^3
15	R-guanidinopropyl	X^2	X^3
16	R-isopropyl	X^2	X^3
17	R-4-hydroxybenzyl	X^2	X^3
18	S-ethyl	X^2	X^3
19	dimethyl	X^2	X^3
20	S-propyl	X^2	X^3
21	R-propyl	X^2	X^3
22	S-butyl	X^2	X^3
23	R-butyl	X^2	X^3
24	S-N-methylaminopropyl	X^2	X^3
25	S-2-naphthylmethyl	X^2	X^3
26	R-2-naphthylmethyl	X^2	X^3
27	S-cyclohexylmethyl	X^2	X^3
28	R-cyclohexylmethyl	X^2	X^3
29	S-methylsulfonylethyl	X^2	X^3
30	S-4-nitrobenzyl	X^2	X^3
31	R-4-nitrobenzyl	X^2	X^3
32	S-4-chlorobenzyl	X^2	X^3
33	R-4-chlorobenzyl	X^2	X^3
34	S-4-fluorobenzyl	X^2	X^3
35	R-4-fluorobenzyl	X^2	X^3
36	S-N-ethylaminobutyl	X^2	X^3
37	S-3-pyridylmethyl	X^2	X^3
38	R-3-pyridylmethyl	X^2	X^3
39	S-cyclohexyl	X^2	X^3
40	R-cyclohexyl	X^2	X^3
41	S-*t*-butyl	X^2	X^3
42	S-4-N-methylaminobenzyl	X^2	X^3
43	R-4-N-methylaminobenzyl	X^2	X^3
44	S-4-ethoxybenzyl	X^2	X^3
45	R-4-ethoxybenzyl	X^2	X^3
46	S-4-iodobenzyl	X^2	X^3
47	R-4-iodobenzyl	X^2	X^3
48	S-4-methoxybenzyl	X^2	X^3
49	R-4-methoxybenzyl	X^2	X^3
50	X^1	S-methyl	X^3
51	X^1	S-benzyl	X^3
52	X^1	hydrogen	X^3
53	X^1	S-2-butyl	X^3
54	X^1	S-N-methylaminobutyl	X^3
55	X^1	S-isobutyl	X^3

Table 2 (Continued)

no.	R^1	R^2	R^3
56	X^1	S-methylsulfinylethyl	X^3
57	X^1	S-guanidinopropyl	X^3
58	X^1	S-isopropyl	X^3
59	X^1	S-4-hydroxybenzyl	X^3
60	X^1	R-methyl	X^3
61	X^1	R-benzyl	X^3
62	X^1	R-2-butyl	X^3
63	X^1	R-N-methylaminobutyl	X^3
64	X^1	R-isobutyl	X^3
65	X^1	R-guanidinopropyl	X^3
66	X^1	R-isopropyl	X^3
67	X^1	R-4-hydroxybenzyl	X^3
68	X^1	S-ethyl	X^3
69	X^1	S-propyl	X^3
70	X^1	R-propyl	X^3
71	X^1	S-butyl	X^3
72	X^1	R-butyl	X^3
73	X^1	S-N-methylaminopropyl	X^3
74	X^1	S-2-naphthylmethyl	X^3
75	X^1	R-2-naphthylmethyl	X^3
76	X^1	S-cyclohexylmethyl	X^3
77	X^1	R-cyclohexylmethyl	X^3
78	X^1	S-methylsulfonylethyl	X^3
79	X^1	S-4-nitrobenzyl	X^3
80	X^1	R-4-nitrobenzyl	X^3
81	X^1	S-4-chlorobenzyl	X^3
82	X^1	R-4-chlorobenzyl	X^3
83	X^1	S-4-fluorobenzyl	X^3
84	X^1	R-4-fluorobenzyl	X^3
85	X^1	S-N-ethylaminobutyl	X^3
86	X^1	S-3-pyridylmethyl	X^3
87	X^1	R-3-pyridylmethyl	X^3
88	X^1	S-cyclohexyl	X^3
89	X^1	R-cyclohexyl	X^3
90	X^1	S-*t*-butyl	X^3
91	X^1	S-4-N-methylaminobenzyl	X^3
92	X^1	R-4-N-methylaminobenzyl	X^3
93	X^1	S-4-ethoxybenzyl	X^3
94	X^1	R-4-ethoxybenzyl	X^3
95	X^1	S-hydroxyethyl	X^3
96	X^1	R-hydroxyethyl	X^3
97	X^1	S-4-iodobenzyl	X^3
98	X^1	R-4-iodobenzyl	X^3
99	X^1	S-4-methoxybenzyl	X^3
100	X^1	R-4-methoxybenzyl	X^3
101	X^1	X^2	3-phenylbutyl
102	X^1	X^2	*m*-tolylethyl
103	X^1	X^2	3-fluorophenethyl
104	X^1	X^2	*p*-tolylethyl
105	X^1	X^2	4-fluorophenethyl
106	X^1	X^2	3-methoxyphenethyl
107	X^1	X^2	4-methoxyphenethyl
108	X^1	X^2	4-ethoxyphenethyl
109	X^1	X^2	3-(3,4-dimethoxyphenyl)-propyl
110	X^1	X^2	4-biphenethyl
111	X^1	X^2	3,4-dimethoxyphenethyl
112	X^1	X^2	phenethyl
113	X^1	X^2	phenylpropyl
114	X^1	X^2	4-phenylbutyl
115	X^1	X^2	butyl
116	X^1	X^2	heptyl
117	X^1	X^2	isobutyl
118	X^1	X^2	(+/-)-2-methylbutyl
119	X^1	X^2	isovaleryl
120	X^1	X^2	3-methylvaleryl
121	X^1	X^2	4-methylvaleryl

Table 2 (Continued)

no.	R^1	R^2	R^3
122	X^1	X^2	*t*-butylethyl
123	X^1	X^2	cyclohexylmethyl
124	X^1	X^2	cyclohexylethyl
125	X^1	X^2	cyclohexylbutyl
126	X^1	X^2	cycloheptylmethyl
127	X^1	X^2	2-hydroxypropyl
128	X^1	X^2	ethyl
129	X^1	X^2	cyclobutylmethyl
130	X^1	X^2	cyclopentylmethyl
131	X^1	X^2	3-cyclopentylpropyl
132	X^1	X^2	3-cyclohexylpropyl
133	X^1	X^2	4-methyl-cyclohexylmethyl
134	X^1	X^2	4-*t*-butyl-cyclohexylmethyl
135	X^1	X^2	2-norbornylethyl
136	X^1	X^2	1-adamantylethyl
137	X^1	X^2	2-ethylbutyl
138	X^1	X^2	3,3-diphenylpropyl
139	X^1	X^2	2-methyl-4-nitro-imidazolepropyl
140	X^1	X^2	cyclopentylethyl
141	X^1	X^2	3-indolylethyl

*This library is composed of three sub-libraries, each one containing the same compounds, but differing from the others in the location of the defined position. O^1-O^3 are individual defined functionalities (R^1-R^3) at a given position of diversity. X^1-X^3 are mixtures of functionalities (R^1-R^3) at a given position of diversity. [a]R functionalities were derived from the building blocks listed in Table 1. [b]Boc derivatives of the amino acids were used. [c]X^1 is derived from a mixture of the defined R^1 building blocks listed for mixtures 1 through 49. X^2 is derived from a mixture of the defined R^2 building blocks listed for mixtures 50 through 100. X^3 is derived from a mixture of the defined R^3 building blocks listed for mixtures 101 through 141.

Existing Mixture-Based Heterocyclic and Small Organic Molecule Libraries

The majority of the current literature on mixture-based libraries describes the use of peptide libraries, which is due primarily to the fact that robust solid-phase chemistries are available for peptides.[3,4] A variety of mixture-based libraries of heterocycles and small organic molecules have now been reported (Table 5). Substituted heterocyclic compounds offer a high degree of structural diversity and have proven to be broadly and economically useful as therapeutic agents.

Imines are often used as intermediates in organic synthesis and are a starting point for chemical reactions such as cycloadditions, condensation reactions, and nucleophilic addition. The formation of imines via condensation of amines with aldehydes was initially used for the reductive alkylation of resin-bound amino acids.[30−32] Imines have now been used as synthetic intermediates in the generation of a range of mixture-based heterocyclic combinatorial libraries.

For example, Murphy and co-workers reported the synthesis of pyrrolidine combinatorial libraries.[33] Starting from polystyrene resin-bound amino acids, the α-amino ester was condensed with aromatic and heteroaromatic aldehydes to afford the resin-bound arylimine. Pyrrolidine and pyrroline derivatives were obtained through cycloaddition of the 1,3-dipoles to olefin and acetylene dipolarophiles (Scheme 2). A library of 500 compounds was screened for in vivo inhibition of angiotensin-converting enzyme (ACE), leading to the identification of 1-(3′-mercapto-2′(*S*)-methyl-1′-oxopropyl)-5-phenyl-2,4-pyrrolidinedicarboxylic acid 4-methyl ester as a potent ACE inhibitor.

Imine intermediates were also used for the solid-phase synthesis of a 43 000 compound tetrahydroisoquinoline combinatorial library.[34] The resulting tetrahydroisoquinolines were obtained by treating imines with homophthalic anhydride. This tetrahydroisoquinoline library was prepared using 11 protected amino acids, 38 aldehydes, and 51 amines (Scheme 2).

An efficient method for the solid-phase synthesis of 4-amino-3,4-dihydro-2(1*H*)-quinolinones was developed by Pei and co-workers.[35] Following formation of an imine by reaction of a resin-bound amino acid with *o*-nitrobenzaldehyde, treatment with a ketene afforded a four-member ring *cis*-β-lactam intermediate. Following reduction of the nitro group, the β-lactam ring underwent an intramolecular rearrangement to afford the *trans*-3,4-dihydro-2(1*H*)-quinolinones. A library of 4 140 dihydroquinolinones derived from 69 amino acids, 6 *o*-nitrobenzaldehydes, and 5 acid chlorides was synthesized (Scheme 2).

Roussel and co-workers described the synthesis of a library of amidinonaphthols.[36] This library was screened for inhibition of tissue factor/factor VIIa complex. A compound having an IC_{50} of 4 μM (10 times more potent than the original lead compound) was identified.

The solid-phase synthesis of diketopiperazines was developed based on reductive alkylation of support-bound amino acids[32] or resin-bound dicarboxylic amino acids[37] with aldehydes (Scheme 3). The resulting secondary amines were then acylated with protected amino acids. Cyclization to the desired diketopiperazines occurred either by heating at reflux in toluene[32] or by a second reductive alkylation followed by ring closure using diisopropylcarbodiimide (DIPCDI).[37] In the second case,[37] the use of bifunctional amino acids allowed the diversity of the library to be extended through further modification.

In a different approach, a novel route to a library of 22 540 diketopiperazines has been reported using α-bromocarboxylic acids and a range of amines.[38] Following

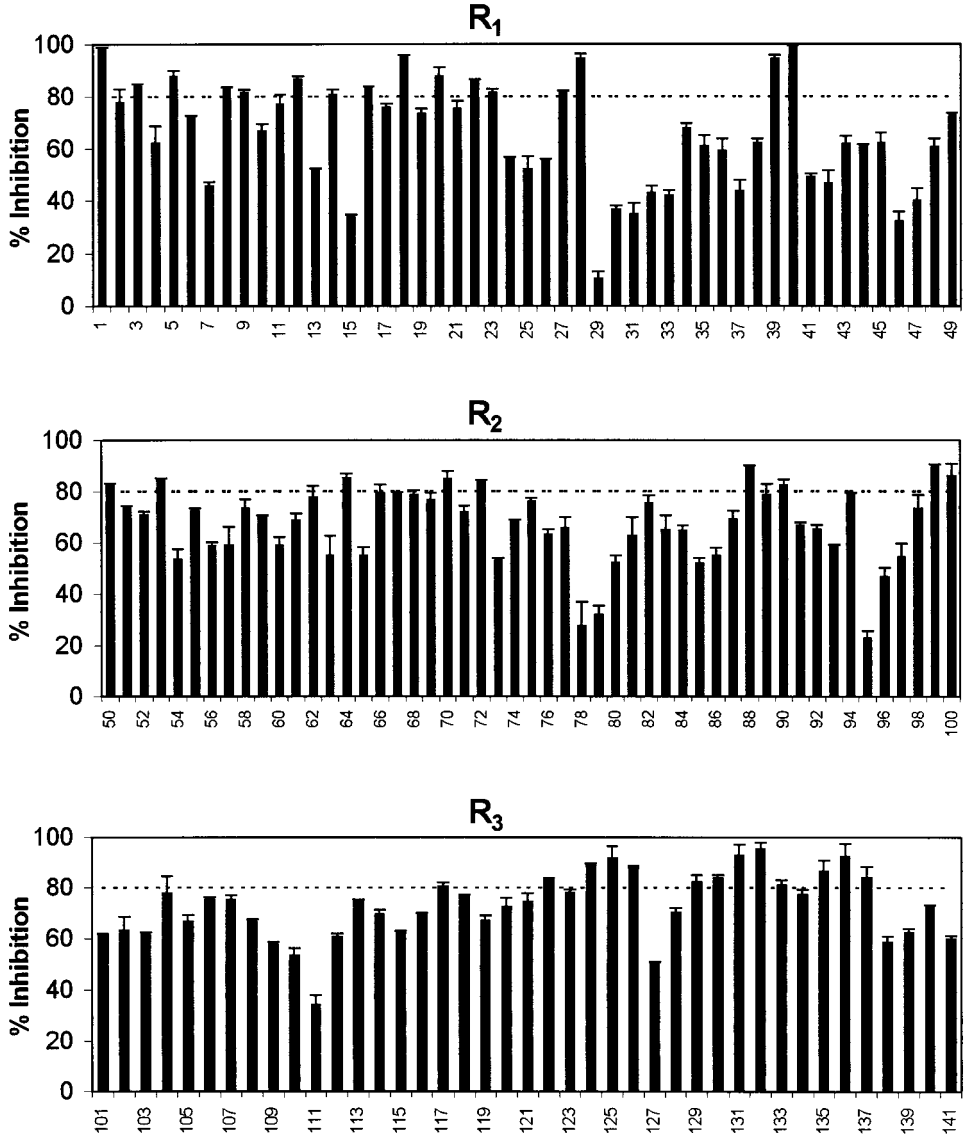


Figure 1. Screening results of the bicyclic guanidine library in a κ opioid receptor binding assay. Each graph shows the results of one of the three sublibraries. Each bar within a graph represents the percent inhibition of a given mixture. The mixtures are represented by the same numbers used in Table 2. Mixtures having activity >80% inhibition (dotted line) were further tested in a dose−response manner (see Table 3). The library was screened at 4 μg/mL; mixtures were incubated for 2.5 h at 25 °C, with 3 nM [^{3}H]U69,593 in a total volume of 0.65 mL of guinea pig brain homogenate.

esterification of Wang resin with an α-bromocarboxylic acid, reaction with a primary amine was followed by the N-acylation of the secondary amine with an additional α-bromocarboxylic acid. Treatment of the resulting resin-bound bromide with a primary amine, followed by cyclization of the resulting intermediate, afforded the desired diketopiperazine. Two routes to cyclization were observed: intramolecular cyclization could occur directly under the reaction conditions to afford the desired diketopiperazine or the cyclization could be induced with TFA. The cyclization via in situ release was influenced by the nature of the aminoalkyl groups.

Krchňák et al. reported the solid-phase synthesis of 1,4,7-trisubstituted perhydro-1,4-diazepine-2,5-diones.[39] Following attachment of an amino alcohol to chlorotrityl resin, the amino group was reductively alkylated and then N-acylated with Fmoc-Asp(OAll)-OH. Following deprotection, a second reductive alkylation was performed. The generated secondary amine was treated with TFFH-activated bromoacetic acid. The bromo functionality was displaced by a 2 M solution of a primary amine in DMSO. Following deprotection of the allyl ester with $Pd(PPh_3)_4$, an intramolecular cyclization occurred by mild activation of the carboxyl group with diphenyl phosphorazidate overnight. The desired products were obtained following treatment of the resin with TFA vapor. Using 8 secondary diamines and amino alcohols, 17 aldehydes, and 20 primary amines, a library of 2 720 compounds was prepared (Scheme 4).

Using the same approach previously described for the solid-phase synthesis of diketopiperazines via α-bromocarboxylic acids, a library of diketomorpholines has been prepared.[38] The cyclization of resin-bound bromides to diketomorpholines was induced by treatment with TFA. Initial cleavage from Wang resin afforded an acyclic bromo acid, which was followed by intramolecular displacement of the bromine by the carboxylate to afford the diketomorpholines. The diketomorpholine library consisted of 980 compounds (7 acids × 20 amines × 7 acids).

Table 3. IC_{50} Values for Active Mixtures from the Bicyclic Guanidine Library

	R^1XX	IC_{50}±SD nM		XR^2X	IC_{50} ± SD nM		XXR^3	IC_{50} ± SD nM
40	**R-cyclohexyl**	**1162 ± 27**	**99**	**S-4-methoxybenzyl**	**1474 ± 548**	**135**	**2-norbornylethyl**	**857 ± 28**
1	**S-methyl**	**1242 ± 38**	**100**	**R-4-methoxybenzyl**	**1646 ± 152**	**132**	**3-cyclohexylpropyl**	**985 ± 257**
18	S-ethyl	1445 ± 54	**88**	**S-cyclohexyl**	**3169 ± 0**	**133**	**4-methyl-cyclohexylmethyl**	**1292 ± 323**
39	**S-cyclohexyl**	**1638 ± 11**	53	S-2-butyl	3315 ± 294	**136**	**1-adamantylethyl**	**1318 ± 145**
28	R-cyclohexylmethyl	1792 ± 11	**64**	**R-isobutyl**	**3346 ± 577**	125	cyclohexylbutyl	1688 ± 485
5	S-isobutyl	2215 ± 0	70	R-propyl	3462 ± 1849	126	cycloheptylmethyl	1800 ± 348
12	R-2-butyl	2331 ± 424	50	S-methyl	3538 ± 609	122	*t*-butylethyl	1870 ± 1141
16	R-isopropyl	2623 ± 707	90	S-*t*-butyl	3700 ± 228	137	2-ethylbutyl	2069 ± 120
20	S-propyl	2969 ± 152	72	R-butyl	4723 ± 152	131	3-cyclopentylpropyl	2738 ± 196
3	S-2-butyl	3015 ± 87				130	cyclopentylmethyl	2769 ± 87
22	S-butyl	3023 ± 250				117	isobutyl	2962 ± 664
14	R-isobutyl	3131 ± 141				124	cyclohexylethyl	2977 ± 228
9	S-4-hydroxybenzyl	3169 ± 1023				129	cyclobutylmethyl	3454 ± 1012
8	S-isopropyl	3177 ± 729						
23	R-butyl	3677 ± 674						
27	S-cyclohexylmethyl	3746 ± 337						

Table 4. Individual Compounds Identified for the κ Opioid Receptor

	R^1	R^2	R^3	IC_{50} (nM)		R^1	R^2	R^3	IC_{50} (nM)
1	S-methyl	S-4-methoxybenzyl	3-cyclohexylpropyl	37	25	S-cyclohexyl	R-isobutyl	1-adamantylethyl	1206
2	S-methyl	R-4-methoxybenzyl	2-norbornylethyl	85	26	S-cyclohexyl	S-cyclohexyl	1-adamantylethyl	1492
3	S-methyl	S-4-methoxybenzyl	2-norbornylethyl	185	27	S-methyl	S-4-methoxybenzyl	1-adamantylethyl	1523
4	R-cyclohexyl	S-4-methoxybenzyl	2-norbornylethyl	219	28	S-methyl	S-4-methoxybenzyl	4-(Me)-cyclohexylmethyl	1568
5	S-methyl	R-4-methoxybenzyl	1-adamantylethyl	238	29	R-cyclohexyl	R-isobutyl	1-adamantylethyl	1747
6	R-cyclohexyl	R-4-methoxybenzyl	2-norbornylethyl	276	30	R-cyclohexyl	R-isobutyl	3-cyclohexylpropyl	1767
7	S-cyclohexyl	S-4-methoxybenzyl	4-(Me)-cyclohexylmethyl	336	31	S-methyl	S-cyclohexyl	1-adamantylethyl	1941
8	R-cyclohexyl	R-4-methoxybenzyl	1-adamantylethyl	341	32	S-methyl	R-isobutyl	2-norbornylethyl	2309
9	R-cyclohexyl	R-isobutyl	2-norbornylethyl	359	33	S-methyl	S-cyclohexyl	3-cyclohexylpropyl	2479
10	R-cyclohexyl	R-4-methoxybenzyl	4-(Me)-cyclohexylmethyl	362	34	R-cyclohexyl	S-4-methoxybenzyl	3-cyclohexylpropyl	3456
11	S-cyclohexyl	S-cyclohexyl	4-(Me)-cyclohexylmethyl	365	35	S-methyl	R-isobutyl	1-adamantylethyl	3641
12	S-cyclohexyl	R-4-methoxybenzyl	4-(Me)-cyclohexylmethyl	369	36	S-cyclohexyl	R-isobutyl	3-cyclohexylpropyl	3744
13	S-methyl	R-4-methoxybenzyl	4-(Me)-cyclohexylmethyl	425	37	S-cyclohexyl	S-4-methoxybenzyl	3-cyclohexylpropyl	3872
14	S-methyl	R-4-methoxybenzyl	3-cyclohexylpropyl	502	38	S-methyl	S-cyclohexyl	2-norbornylethyl	4424
15	R-cyclohexyl	S-cyclohexyl	2-norbornylethyl	524	39	S-cyclohexyl	S-cyclohexyl	3-cyclohexylpropyl	4482
16	R-cyclohexyl	R-4-methoxybenzyl	3-cyclohexylpropyl	547	40	S-cyclohexyl	S-4-methoxybenzyl	1-adamantylethyl	4923
17	S-cyclohexyl	S-cyclohexyl	2-norbornylethyl	560	41	S-cyclohexyl	R-4-methoxybenzyl	3-cyclohexylpropyl	5026
18	S-cyclohexyl	S-4-methoxybenzyl	2-norbornylethyl	715	42	S-methyl	S-cyclohexyl	4-(Me)-cyclohexylmethyl	5061
19	R-cyclohexyl	R-isobutyl	4-(Me)-cyclohexylmethyl	738	43	S-methyl	R-isobutyl	3-cyclohexylpropyl	5436
20	R-cyclohexyl	S-4-methoxybenzyl	4-(Me)-cyclohexylmethyl	804	44	S-methyl	R-isobutyl	4-(Me)-cyclohexylmethyl	6477
21	S-cyclohexyl	R-isobutyl	2-norbornylethyl	827	45	R-cyclohexyl	S-cyclohexyl	3-cyclohexylpropyl	>10000
22	S-cyclohexyl	R-4-methoxybenzyl	2-norbornylethyl	924	46	S-cyclohexyl	R-4-methoxybenzyl	1-adamantylethyl	>10000
23	S-cyclohexyl	R-isobutyl	4-(Me)-cyclohexylmethyl	999	47	R-cyclohexyl	S-4-methoxybenzyl	1-adamantylethyl	>10000
24	R-cyclohexyl	S-cyclohexyl	4-(Me)-cyclohexylmethyl	1140	48	R-cyclohexyl	S-cyclohexyl	1-adamantylethyl	>10000

Other small molecule mixture-based libraries include: (1) the synthesis of 4-thiazolidinones and 4-metathiazanones derived from three-component condensation of an amino acid ester or a resin-bound amino acid, an aldehyde, and an α-mercaptocarboxylic acid[40] (the identification of cyclooxygenase-1 inhibitors from 4-thiazolidinone combinatorial libraries has recently been reported by Look and co-workers[41]); (2) the synthesis of hydantoin and thiohydantoin libraries through isocyanate and thioisocyanate formation on the solid support and intramolecular cyclization;[42] (3) the synthesis of amide-based small molecules using various cyclic/acyclic amines, primary/secondary amines, and different protected bifunctional amines as nucleophiles to react with different anhydrides;[43] (4) the synthesis of phosphoramidate combinatorial libraries from oxidation with primary and secondary amines to support-bound aminodiol scaffolds;[44] (5) the synthesis of dihydropyridines, pyridines, and pyrido[2,3-*d*]pyrimidines;[45] and (6) the synthesis of a library composed of 1 296 N-acylated dipeptides with the identification of inhibitors of phosphomannose isomerase having low micromolar activity.[46]

In this laboratory, we have focused on the design and

Table 5. Mixture-Based Heterocyclic Combinatorial Libraries

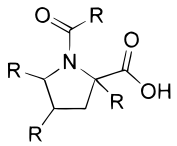
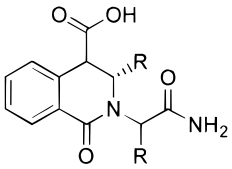
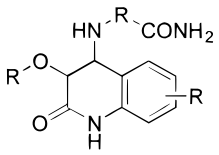
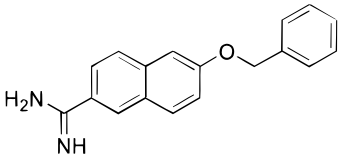
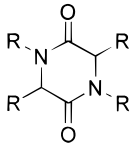
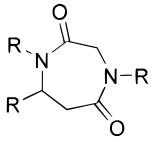
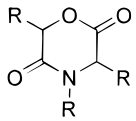
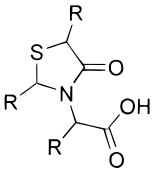
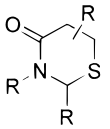
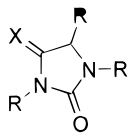
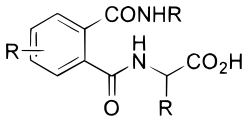
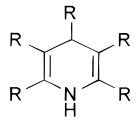
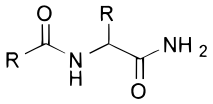
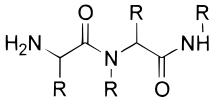
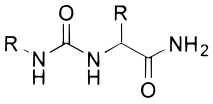
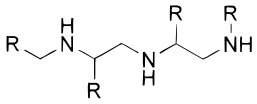
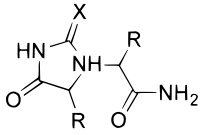
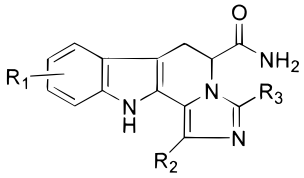
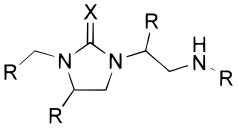
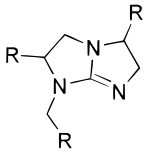
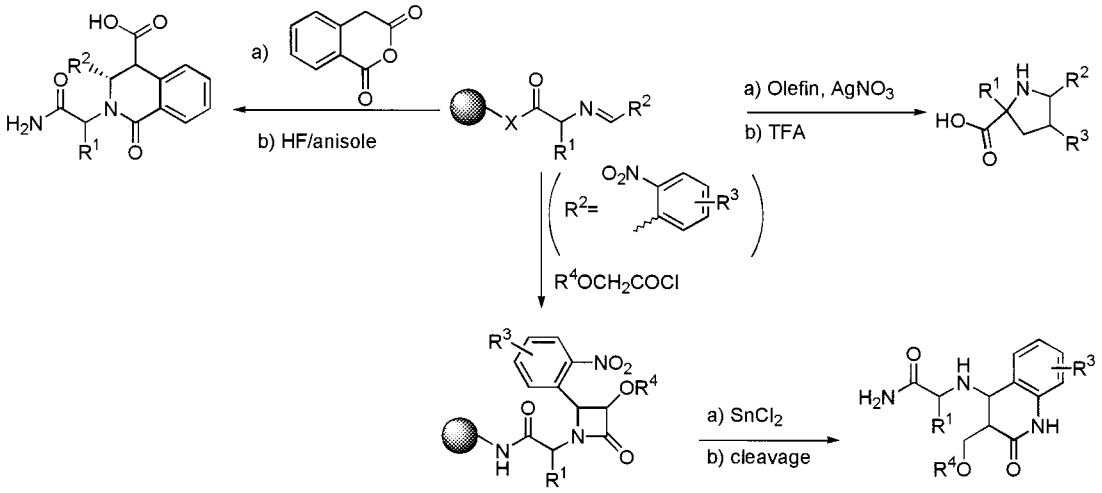
Name	Target	Structure	Number of compounds	Ref.
Pyrrolidines	Angiotensin converting enzyme		500	33
Tetrahydroisoquinolines	Sigma receptors		43,000	34
Dihydroquinolones	not reported		4,140	35
Amidinonaphthols	Tissue factor/factor VIIa complex		144	36
Diketopiperazines	not reported		1,000 not reported 22,540	32 37 38
Perhydrodiazepinediones	not reported		2,720	39
Diketomorpholines	not reported		980	38
Thiazolidinones	Cyclooxygenase-1		25 3 x 540	40 41
Metathiazanones	not reported		not reported	40
Hydantoins and thiohydantoins	not reported		not reported	42

Table 5 (Continued)

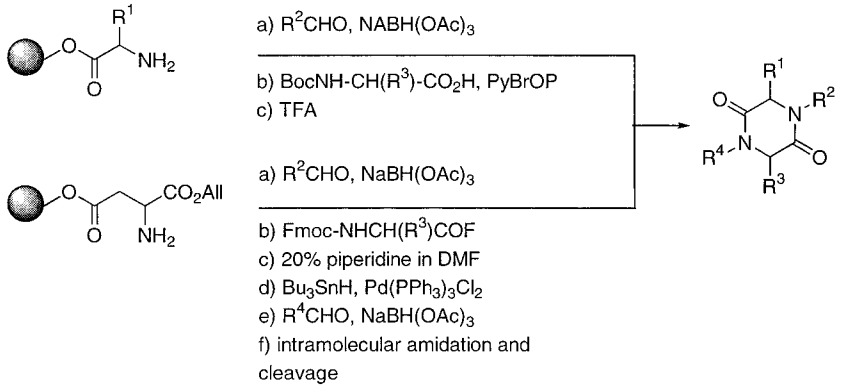
Name	Target	Structure	Number of compounds	Ref.
Anhydride templates	not reported		not reported	43
Phosphoramidates	not reported	see reference	not reported	44
Dihydropyridines	Calcium channel blocker		100	45
Acylated amino acid amides	Phosphomannose isomerase inhibitor		66	46
Dipeptomimetics	Opioid receptors (mu)		57,500	47,89
Linear ureas	Opioid (mu) and Sigma receptors		125,000	in prep.
Polyamines	Tumor cell lines		10^5-10^6	28,90
Hydantoins and thiohydantoins	Sigma receptor		38,880	13,53
Imidazol-pyrido-indoles	not reported		46,750	52
Cyclic ureas and thioureas	*Candida albicans*		118,400	50,54
Bicyclic guanidines	Opioid receptors (kappa) *Candida albicans*		102,459	25,29 and herein

synthesis of organic and heterocyclic synthetic combinatorial libraries (SCLs) using short resin-bound amino acids and peptides as starting materials.[13,25,47] Entirely new chemical diversities are generated by the chemical modification of existing libraries ("libraries from libraries" approach[14]). Thus, existing resin-bound peptide libraries have been successfully modified by per-N-alkylation[47−49] and/or exhaustive reduction[25−28] of the amide bonds (Figure 2). Linear peptide SCLs have also been used as starting materials for the generation of

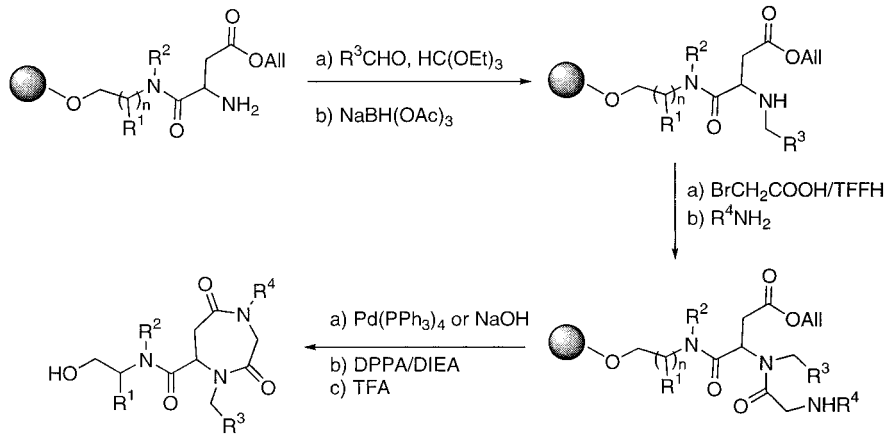
Scheme 2



Scheme 3



Scheme 4



small heterocyclic SCLs such as *N*-alkylamino cyclic urea and thiourea SCLs,[50] diazepine SCLs,[51] bicyclic guanidines,[25] imidazol-pyrido-indoles,[52] and hydantoins and thiohydantoins.[53]

For example, we have developed a straightforward synthetic route to the solid-phase synthesis of hydantoin and thiohydantoin SCLs from resin-bound dipeptide SCLs. This involves the reaction of the N-terminal amino group of a resin-bound dipeptide with phosgene or thiophosgene, leading to the formation of the intermediate isocyanate or isothiocyanate, which undergoes an intramolecular cyclization to form the five-member ring hydantoin or thiohydantoin (Scheme 5). To increase the number and range of available compounds, we first selectively alkylated the resin-bound amide. Following formation of the hydantoins, a second alkylation was carried out to generate a dialkylated hydantoin library. Using 54 amino acids for the first site of diversity, 60 amino acids for the second site of diversity, and 4 different alkylating agents, a library of 38 880 compounds ($54 \times 60 \times 3 \times 4$) was synthesized (Scheme 6). This positional scanning library was examined in a σ-specific receptor binding assay using [^{3}H]pentazocine as the σ receptor radioligand. Two of the individual dialkylated hydantoins identified following deconvolution of this library had IC_{50} values of 50−60 nM.[13]

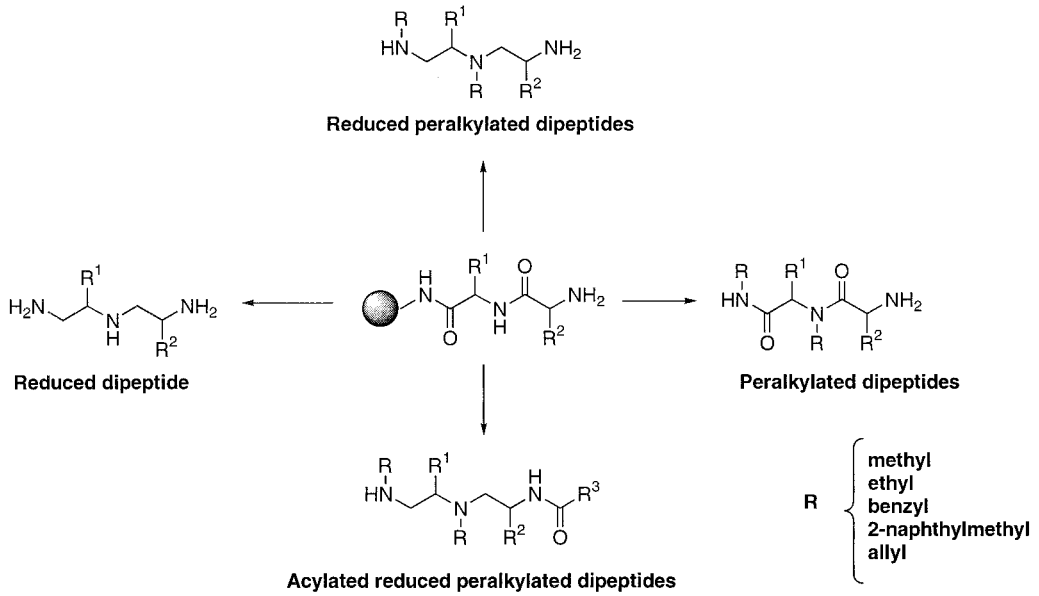
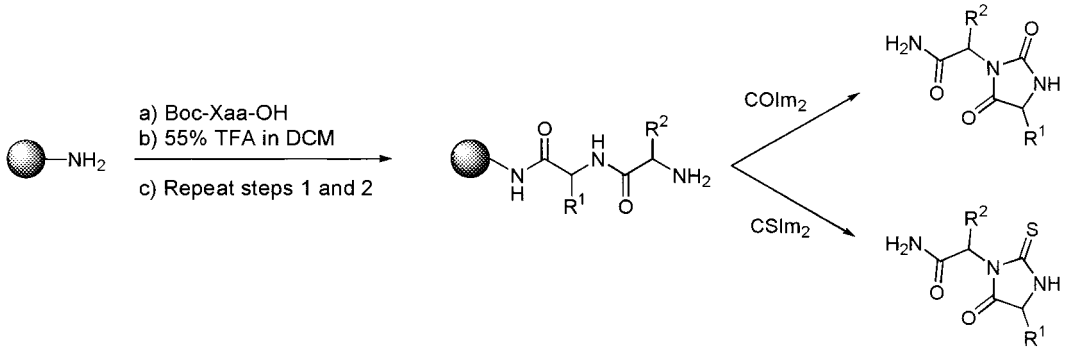
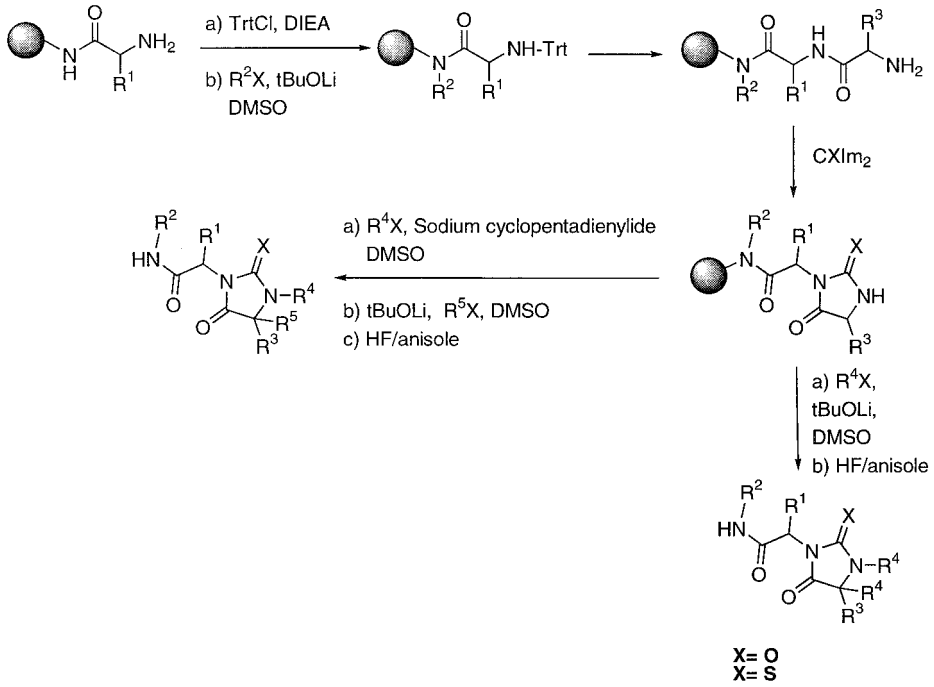


Figure 2. Generation of dipeptidomimetic libraries through chemical modifications of an existing dipepeptide library.

Scheme 5

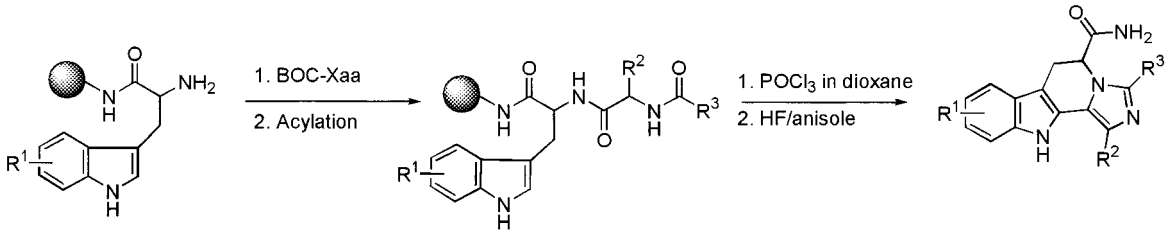


Scheme 6

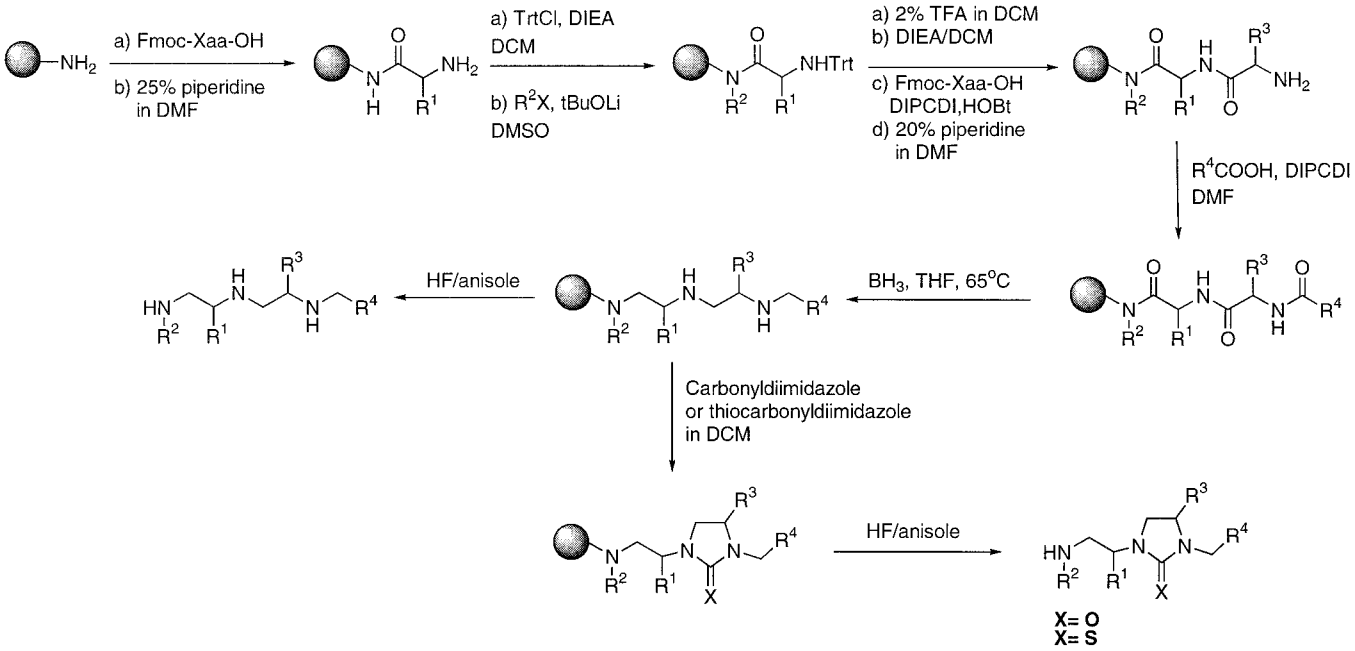


In another example, a library of 46 750 imidazol-pyrido-indoles was generated by cyclodehydration under Bischler−Napieralski conditions of resin-bound N-acylated dipeptide amides having tryptophan as the C-terminal amino acid. Starting from methylbenzhydrylamine resin, 22 tryptophan analogues were

Scheme 7



Scheme 8



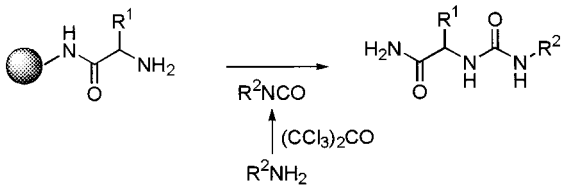
coupled. Following deprotection, 25 additional amino acids were coupled to generate a mixture of dipeptides. Upon deprotection of these and N-acylation with 85 different carboxylic acids, dehydrative cyclization was carried out by treatment with phosphorus oxychloride in dioxane. A library of 46 750 individual imidazol-pyrido-indoles was obtained in high yield and good purity following HF cleavage (Scheme 7).[52]

The "libraries from libraries" approach has also been successfully used for the generation of peralkylated peptide and polyamine SCLs derived from existing peptide SCLs. Thus, a wide range of *N*-methyl and *N*-benzyl trisubstituted diethylenetriamines were generated from *p*-methylbenzhydrylamine resin-bound dipeptides.[28] Following Boc deprotection, the initial amino acid amine salt was neutralized and the resulting primary amine protected using triphenylmethyl chloride. The resin-bound secondary amide was then selectively alkylated in the presence of lithium *tert*-butoxide and an N-alkylating reagent (methyl iodide or benzyl bromide). Following addition of a second Fmoc-protected amino acid, removal of the Fmoc group, and N-acylation of the resin bound-dipeptide, the exhaustive reduction of the amide bonds was achieved using borane in tetrahydrofuran.[25] The desired products were readily obtained following cleavage of the resin-bound triamine with anhydrous HF (Scheme 8). Such resin-bound polyamines have also been used as starting materials for the solid-phase synthesis of a range of heterocyclic compounds, such as cyclic ureas and cyclic thioureas.[50]

Similar to the synthesis previously described for bicyclic guanidines, a monoalkylated resin-bound N-acylated dipeptide library was exhaustively reduced using borane in THF, yielding a triamine SCL having two available secondary amine functionalities. Treatment of this resin-bound triamine SCL with carbonyldiimidazole or thiocarbonyldiimidazole afforded the corresponding cyclic urea and cyclic thioureas in good yield and high purity (Scheme 8). Following the initial synthesis of individual control compounds and using 37 amino acids for the first site of diversity, 40 amino acids for the second site, and 80 carboxylic acids for the third site, four separate PS-SCLs were generated from N-alkylated dipeptide SCLs. Each separate PS-SCL was composed of 118 400 ($37 \times 40 \times 80$) compounds containing either a methyl or benzyl group on the C-terminal amide and a urea or thiourea within the ring. These libraries were assayed for their ability to inhibit *C. albicans* growth, which is one of the most common opportunistic fungi responsible for infections and is the fungal infection most frequently associated with HIV-positive patients. Greater activities were found for the N-benzylated compounds relative to N-methylated compounds (MIC values of the most active compounds varied from 8 to 64 μg/mL and from 64 to 125 μg/mL, respectively).[54]

Finally, a linear urea library has been prepared by the reaction of a resin-bound amino acid with individual isocyanates, affording the linear ureas in good yields. The isocyanates were generated by slowly adding an amine to a solution of triphosgene in anhydrous DCM

Scheme 9



in the presence of diisopropylethylamine (DIEA). Condensation of the resulting isocyanates with resin-bound deprotected amino acids afforded the expected linear ureas (Scheme 9). To increase the number of available compounds, a selective N-alkylation was performed on the resin-bound amino acid, following the individual synthesis of control compounds as described earlier. A library of 125 000 linear N,N′-disubstituted ureas was prepared and has been tested for opioid activity in μ, δ, and κ opioid and σ receptor binding assays. Following deconvolution of the library, individual compounds with 1−10 nM affinities at the μ or σ receptors were found (manuscript in preparation).

Active Compounds Identified from Mixture-Based Libraries

Mixture-based SCLs have been successfully used in a wide range of bioassays by a number of groups from both pharmaceutical companies and academic institutions. Novel enzyme inhibitors, agonists, and antagonists to specific receptors, antimicrobial, antifungal, and antiviral compounds, and B- and T-cell epitopes have been identified from such libraries. The libraries used for these studies cover a large diversity of chemical structure and size (i.e., from small organic molecules to long peptide sequences). The library characteristics (i.e., chemical class and total number of compounds within the library and within a single mixture), the deconvolution method, and the structure and activities of the lead compounds are listed in Tables 6−10. The tables are categorized by screening target. Within each table, the libraries have been sorted by peptide length, followed by peptidomimetics and finally acyclic and heterocyclic molecules.

While the general utility of mixture-based combinatorial libraries is still questioned by some in the scientific community, more than 100 separate studies in which active compounds have been identified from mixture-based libraries of various classes have now been reported. These studies have been carried out by nearly 50 separate research groups. A larger number of publications have come from academic groups than from the pharmaceutical industry, and this may be due in part to pharmaceutical companies being accustomed to traditional high-throughput assay systems which utilize individual compounds.

As shown in Tables 6−10, the number of compounds present within different mixture-based libraries tested ranges from less than 10 to greater than 10^{14} individual compounds, and the number of individual compounds per mixture ranges from less than 10 to more than 2×10^{10}. It should be noted, however, that the ranges in activity of the individual compounds identified from these libraries are independent of the complexity and chemical nature of the library tested. In contrast, the ranges in activity of the final individual compounds that have been identified so far from mixture-based libraries appear to be target- and assay-dependent with picomolar to nanomolar activity for antigenic determinants specific to T-cells, nanomolar activity for receptor ligands and antigenic determinants specific to B-cells, nanomolar to micromolar activity for enzyme inhibitors, and micromolar activity for antimicrobial and antifungal compounds. This is most likely due to the nature of ligand/target specificity, i.e., mechanisms of interactions or actions of the ligands toward various target systems. For example, a given receptor binding site often requires a highly specific ligand conformation, and, therefore, may require a high-affinity ligand to trigger activity. In contrast, lysis of microorganisms may occur via a number of different mechanisms, with cell-membrane disruption being the main mechanism of the antimicrobial and antifungal compounds identified from combinatorial libraries.[55] This lysis mechanism lacks high specificity in compound sequence and structure but requires common physicochemical characteristics to disrupt the cell membrane, which may explain their micromolar activity range.

Mixture-based libraries have been used in a number of different biological assays, ranging from binding assays to whole cell-based assays. The majority of studies (33% of published reports in Tables 6−10) involving mixture-based libraries have been reported for receptor−ligand interactions (Table 6). Most of these studies belong to the class of receptors known as seven-transmembrane G-protein-linked coupled receptors, in particular the neuropeptide receptors. These include the opioid, melanocortin, bradykinin, endothelin, somatostatin, and neurokinin receptors, among others. Libraries screened include peptide, peptidomimetic, and heterocyclic organic compounds, which range in size from 25 to >50 million compounds. Individual compound activities range from 50 pM to 850 nM. It is of interest that of the individual ligands identified and further tested, 60% were found to be agonists and 40% were found to be antagonists.

A number of reports have been presented on the determination of B- and T-cell specificity using mixture-based libraries made up of thousands to millions of synthetic peptides (Table 7). The use of this approach has led to: (a) the definition of high-affinity ligands for both T-cells and antibodies, (b) alternative means for identifying immunologically relevant peptides for use as potential preventive and therapeutic vaccines, (c) new appreciation of the requirements for T-cell receptor interactions with peptide/MHC complexes in immunogenicity, and (d) new principles regarding the level of cross-reactivity in immunological recognition.[56]

Approximately 15% of the published studies have focused on the identification of enzyme inhibitors (Table 8). Enzyme specificity represents a broader range relative to receptor or antibody specificity, which may explain why the activities range from nanomolar to micromolar for the inhibitors that have been identified. The activity found will likely depend on the target enzyme rather than the library structure. For example, nanomolar activities were found for both hexapeptides and heterocyclic compounds.

In contrast to the above-mentioned targets, only four groups reported the use of mixture-based libraries for

Table 6. Mixture-Based Libraries Screened for the Identification of Receptor Ligands

Name	Receptor	Number of Compounds	Deconvolution Method	Compounds/ mixture	Activity of lead compound (nM)	Agonist/ Antagonist	Ref.
Tripeptide	Melanocortin (MC1)	221,184	Iterative	2,304	IC_{50} = 620	antagonist	91
Tripeptide	Vasopressin (V2)	15,625	Orthogonal	125	IC_{50} = 63	Not reported	67
Tetrapeptide	Opioid (mu)	6,250,000	Iterative	125,000	IC_{50} = 2	agonist	92
Tetrapeptide	Opioid (mu)	6,250,000	PosScan	125,000	K_i = 0.4	agonist	93
Tetrapeptide	Opioid (mu)	6,250,000	PosScan	125,000	K_i = 1.8	agonist	93
Tetrapeptide	Opioid (delta)	6,250,000	PosScan	125,000	K_i = 3	agonist	93
Tetrapeptide	Opioid (kappa)	6,250,000	PosScan	125,000	K_i = 1.2	agonist	93
Hexapeptide	Opioid (mu)	52,128,400	Iterative	132,321	IC_{50} = 28	agonist	94
Hexapeptide	Opioid (mu)	52,128,400	Iterative	132,321	IC_{50} = 13	agonist	95
Hexapeptide	Opioid (mu)	52,128,400	Iterative	132,321	IC_{50} = 10	agonist	95
Hexapeptide	Opioid (mu)	52,128,400	Iterative	132,321	IC_{50} = 5	antagonist	96
Hexapeptide	Opioid (mu)	52,128,400	Iterative	132,321	IC_{50} = 5	antagonist	95
Hexapeptide	Opioid (mu)	52,128,400	Iterative	132,321	IC_{50} = 33	agonist	95
Hexapeptide	Endothelin	361	Iterative	361	IC_{50} = 5	antagonist	97
Hexapeptide	Opioid (mu)	34,012,224	PosScan	1,889,568	IC_{50} = 17	agonist	98
Hexapeptide	Opioid (delta)	34,012,224	PosScan	1,889,568	IC_{50} = 61	Not reported	99
Hexapeptide	Opioid (delta)	34,012,224	Pos Scan	1,889,568	IC_{50} = 45	Not reported	99
Hexapeptide	Orphanin	52,128,400	PosScan	132,321	IC_{50} = 23	agonist	81
Hexapeptide (D-amino acids)	Opioid (mu)	52,128,400	Iterative	132,321	IC_{50} = 18	agonist	100
Hexapeptide (D-amino acids)	Somatostatin	64,000,000	Iterative	160,000	K_i = 160	antagonist	101
Heptapeptide	Bombesin	1,444	Sequencing	5,000	IC_{50} = ~50	agonist	102
Nonapeptide	Melanocortin (MC1)	31,360	Sequencing	1,500	IC_{50} = 11	antagonist	103
Cyclic pentapeptide	Endothelin	82,944	PosScan	1,728		antagonist	104
Cyclic hexapeptide	Neurokinin (NK-1)	4	HPLC	4	IC_{50} = 2	antagonist	105
Bicyclic Peptide	Neurokinin (NK-1)	60	Iterative	5-9	IC_{50} = 850	agonist	106

Table 6 (Continued)

Name	Receptor	Number of Compounds	Deconvolution Method	Compounds/ mixture	Activity of lead compound (nM)	Agonist/ Antagonist	Ref.
Hinged peptides	GnRH	3,773	None				107
Trimer Peptoid	Adrenergic	3,672	Iterative	204	K_i = 5	Not reported	108
Trimer Peptoid	Adrenergic	9,216	PosScan[a]	96	Not reported	Not reported	83
Trimer Peptoid	Opioid (mu)	3,672	Iterative	204	K_i = 6	Not reported	108
Trimer Peptoid	Endothelin	30,752	Iterative	992	IC_{50} = 2.5	antagonist	109,110
Trimer Peptoid	α-melanotropin	328,509	Iterative	4,761	K_D=1,580	Not reported	111
Trimer Peptoid	GRP/Bombesin	328,509	Iterative	4,761	K_D=3,400	Mixed	111
Dipeptidomimetic	Opioid (mu)	57,500	Iterative	230	IC_{50} = 3	agonist	89
Heptamine	Opioid (mu)	37,791,360	Pos Scan	1,889,568	IC_{50} = 14	antagonist	27
N-succinyl-3(-2-thiazolylethenyl)-anilide	Leukotriene (D_4)	700	Iterative	70	Not reported	antagonist	112
Phosphodiester	Leukotriene (B_4)	20,736	Iterative	1,728	IC_{50} = 680	Not reported	113
Hydantoins	Sigma	38,880	PosScan	135-240	IC_{50} = 62	Not reported	13
Tetrahydro-isoquinoline	Sigma	43,472	Iterative	836	IC_{50} = 56	Not reported	34
Spiroindane	Somatostatin	130,000	Iterative	1,330	K_i = 0.05	agonist	114
Non-peptide	Bradykinin	25	Mass Spec.	3-6	K_i = 80	Not reported	115
Bicyclic Guanidine	Opioid (mu)	102,459	PosScan	2,091-2,499	IC_{50} = 37	Not reported	Herein
Flavone	Benzodiazepine	36	HPLC	4	K_i = 17	Not reported	116

[a] Termed spatially arrayed mixture (SpAM) by authors.

the identification of novel antimicrobial and/or antifungal compounds (Table 9). This application is limited to the use of non-support-bound combinatorial libraries, which permit the screening of compounds free in solution in a cell suspension environment without interference from a solid support. While most of the compounds identified show broad-spectrum activity against various strains of Gram-positive and Gram-negative bacteria and fungi, N-permethylated hexapeptides were found to act specifically on Gram-positive bacteria.[14]

Finally, Table 10 illustrates the use of mixture-based libraries against a variety of other biological targets. These include the identification of enzyme substrates, inhibitors of hemolysis by melittin, compounds selected based on permeability, and compounds selected for tumor cell toxicity. The large number of successes against a wide variety of assay targets in which highly active compounds have been identified demonstrates the great potential for identifying compounds with desired properties from screening mixture-based libraries.

Synthesis of Mixture-Based Libraries

Typically, combinatorial libraries containing several positions of diversity are synthesized by the consecutive incorporation of multifunctional building blocks with orthogonal protecting groups, using Merrifield's concept of solid-phase synthesis.[3,4] In the case of the first building block added, the solid-phase support serves as a protecting group for one functionality following incorporation. Following deprotection of the orthogonal protecting group, subsequent building blocks are similarly incorporated until all positions of diversity are added. Since amide bond formation on the solid phase has been optimized, it is used extensively in combinatorial synthesis. Two synthetic approaches, involving either the mixing of multiple resins [a process known as "divide-couple-recombine" (DCR),[57] "split-synthesis",[58] or "por-

Table 7. Mixture-Based Libraries Screened for the Identification of Antigenic Determinants

Name	Assay target	Number of Compounds	Deconvolution Method	Compounds/ mixture	Activity	Ref.
	Antigen/antibody					
Hexapeptide	Peptide/19B10	34×10^6	Iterative	104,976	IC_{50} = 2 nM	57
		34×10^6	PosScan	1,889,568	IC_{50} = 2 nM	63
Hexapeptide	Peptide/125-10F3	52×10^6	Iterative	130,321	IC_{50} = 4 nM	117,118
		34×10^6	PosScan	1,889,568	IC_{50} = 12 nM	63
Hexapeptide	Peptide/222-35C8	52×10^6	Iterative	130,321	IC_{50} = 2 nM	119
Hexapeptide	Peptide/3E7	52×10^6	Iterative	130,321	IC_{50} = 3 nM	120
		34×10^6	PosScan	1,889,568	IC_{50} = 3 nM	99
Hexapeptide	Peptide/134B29	34×10^6	PosScan	1,889,568	IC_{50} = 1.5 nM	121
Hexapeptide	Peptide/155B16	52×10^6	Iterative	130,321	IC_{50} = 40 nM	121
Hexapeptide	Peptide/M1	34×10^6	PosScan	1,889,568	IC_{50} = 3 nM	122
Hexapeptide	Peptide/121-15B10	52×10^6	Iterative	130,321	IC_{50} = 9 nM	82
Hexapeptide	Peptide/172-12A4	34×10^6	PosScan	1,889,568	IC_{50} = 1.2 μM	123
Hexapeptide	Anti-MHR mAb	64×10^6	Iterative	160,000	Not reported	124
Hexapeptide	Peptide/Tab2	3.7×10^7	Amino acid clusters	73,984	K_D = 80 nM	125
Hexapeptide	TSRE/Graves's IgG	34×10^6	PosScan/Iterative [a]	1,889,568	IC_{50} = 10 μM	126
Hexapeptide	HBsAg/12	34×10^6	PosScan	1,889,568	IC_{50} = 170 nM	126
Hexapeptide (D-amino acids)	GlcNAc/ HGAC 39.G3	52×10^6	PosScan	2,476,099	IC_{50} = 200 nM	128
Octapeptide	Anti-FMDV/15	64×10^6	Iterative	160,000	Not reported	620
Octapeptide	β-factor XIIa/ 201/9	1.7×10^{10}	PosScan/Iterative	4.7×10^7	Not reported	129
Decapeptide	Peptide/17/9	4×10^{12}	PosScan	1.9×10^{11}	IC_{50} = 0.6 nM	87
Tetradecapeptide	p-24 (HIV-1)/ CB4-1	8×10^{17}	Iterative	1×10^{14}	K_i = 1.2 nM	130
	MHC binding					
Octapeptide	H-2K^b	1.7×10^{10}	PosScan	8.9×10^8	anchor residues [b]	131
Octapeptide	H-2K^k, H-2K^b, HLA-A0204	2.0×10^8	PosScan	1×10^7	anchor residues	132
Octapeptide	H-2L^d	1.7×10^{10}	PosScan	8.9×10^8	anchor residues	133
Undecapeptide	HLA-DR1	2×10^{14}	PosScan	1×10^{13}	IC_{50} = 1.2 μM	134
Nonapeptide	HLA-A2, HLA-B8, HLA-B53	3.2×10^{11}	Elution [c]	3.2×10^{11}	anchor residues	135
Nonapeptide	RT1-A^a, RT1-A^u, RT1-1A^c	3.2×10^{11}	Elution	3.2×10^{11}	anchor residues	136
	T cell clones					

Table 7 (Continued)

Name	Assay target	Number of Compounds	Deconvolution Method	Compounds/ mixture	Activity	Ref.
	T cell clones					
Octapeptide	2C	1.7×10^{10}	PosScan	8.9×10^{8}	IC_{50} = 10 pM	137
Octapeptide	269/3	1.7×10^{10}	PosScan	8.9×10^{8}	IC_{50} = 12 pM	138
Octapeptide	H3-16, H3-30 H3-35	1.7×10^{10}	PosScan	8.9×10^{8}	IC_{50} = 1 pM	139
Nonapeptide	EL4	2.7×10^{8}	Iterative	2.7×10^{8}	IC_{50} = 50 pM	72
	10F4	3.4×10^{4}	Dedicated	3.4×10^{4}	IC_{50} = 2.5 nM	140
Decapeptide	GDBP	6×10^{12}	PosScan	3.2×10^{11}	contact residues	141
Undecapeptide	TL567	2×10^{14}	PosScan	1×10^{13}	IC_{50} = 1 nM	88
Tridecapeptide [d]	2.102	1.92×10^{6}	Iterative	1.92×10^{6}	0.3 ng/mL	142
Tetradecapeptide [e]	1c10	1×10^{6}	Sequencing	1×10^{6}	5nM	143
	MT1, MT2, HG	1×10^{6}	Sequencing	1×10^{6}	10-1000 nM	144

[a] Mixture iterations were purified by HPLC and individual peptides were identified by amino acid analysis and sequencing. [b] Anchor residues were identified and compared with binding motifs obtained from previously published data. [c] MHC-bound peptides were subjected to acid elution and pool sequencing. [d] Only five positions were prepared as mixtures. [e] Three positions contained less than 19 amino acids as in other positions.

tioning-mixing"[59]] or the use of mixtures of incoming reagents,[60] are now widely used to incorporate multiple building blocks at any position within a combinatorial library. The following section discusses these two approaches, as well as the importance of optimizing new chemical reaction schemes prior to library synthesis.

A. DCR Synthesis. Two important aspects of library synthesis are the need to obtain an approximate equimolar representation of all individual compounds within the library and the need to synthesize such libraries with good reproducibility. Therefore, the various building blocks have to be incorporated into each of the diversity positions in as close to equimolar ratios as possible. The DCR process involves the incorporation of each building block to separate, equal portions of the resin followed by combining and mixing all resin portions. The resin is divided again for the next building block incorporation. This method allows the generation of resin-bound one-bead−one-compound arrays, which when cleaved yield approximately equimolar mixtures of compounds. Due to the statistical distribution of beads at each step, care must be taken in using the appropriate amount of resin in the synthesis in order to ensure appropriate statistical representation of all compounds in the library.[19,61] After assembly, the library can either be cleaved from the resin for bioassays in solution or left on the resin for solid-phase assays.

The main limitation of the DCR method is inherent in the physical nature of mixing resins. Once a position of diversity is defined in a particular reaction scheme, the work required to incorporate further mixture positions increases proportionally to the number of functionalities at the defined position, since each resin must be kept separate from the other resins during any subsequent steps. Therefore, for practical purposes, once a position of diversity is defined with individual building blocks, no additional mixture positions can be readily incorporated.

B. "Reagent Mixture" Method. The "reagent mixture" method, on the other hand, allows mixtures to be incorporated into the molecule anywhere within the reaction scheme. The "reagent mixture" method for the generation of mixtures of compounds uses a predefined ratio of reagents in excess to achieve approximately equimolar incorporation of each reagent at a position of diversity.[60−63] Reaction of incoming building blocks in molar excess over amino groups is typically used in solid-phase synthesis, due to the differences in reaction rates of the various building blocks. When preparing mixture-based libraries, the use of a mixture of incoming building blocks can lead to nonequimolar incorporation, resulting in an unequal distribution of individual compounds within the library. The use of reagent mixtures, therefore, requires a thorough knowledge of the mechanism and kinetics involved in the specific reactions being carried out. This method offers the advantage that once the conditions have been established, a mixture of reagents can be readily incorporated at any diversity position.

Two different strategies to achieve equimolar incorporation of building blocks are used. In the first, a large excess of incoming reagents is used such that pseudo-first-order reaction kinetics are observed and the ratio of building blocks within the coupling mixture is adjusted according to their different coupling rates, i.e., the higher the coupling rate of a particular building block, the lower the concentration of that building block in the mixture.[60,64] It is important that the relative reaction rates of the incoming reagents are approximately equal and relatively independent of the resin-

Table 8. Mixture-Based Libraries Screened for Enzyme Inhibitors

Name	Enzyme	Number of Compounds	Deconvolution Method	Compounds/ mixture	Activity of lead compound	Ref.
Tripeptide	Tc80 protease[a]	15,625	PosScan	125	IC_{50} = 12 μM	145
Tetrapeptide	HIV protease	244,904	Iterative	11,132	IC_{50} = 1.4 μM	146
Tetrapeptide	Thrombin	3,515,625	Iterative	390,625	IC_{50} = 1.9 μM	147
Tetrapeptide	FPTase[b]	21,381,376	PosScan	314,432	IC_{50} = 42 nM	148
Hexapeptide	Trypsin	52,128,400	Iterative	130,321	IC_{50} = 46 μM	64
Hexapeptide	HIV integrase	52,128,400	Iterative	130,321	IC_{50} = 2 μM	149
Hexapeptide	PC1[c]	52,128,400	PosScan	130,321	K_i = 3.2 nM	150
Hexapeptide	PC2	52,128,400	PosScan	130,321	K_i = 360 nM	150
Hexapeptide	GTF[d]	52,128,400	PosScan	130,321	IC_{50} = 1.4 mM	151
Nonapeptide	MLCK[e]	5,832	Iterative	324	K_i = 52 nM	152
Dodecapeptide	PTS[f]	1.024×10^{13}	Iterative	2.56×10^{10}	IC_{50} = 30 μM	153
Cyclic hexapeptide	α-Glucosidase	2,476,099	Iterative	130,321	K_i = 1.7 μM	154
Cyclic peptide template	Chymotrypsin	24,389	PosScan	841	IC_{50} = 51 μM	155
Acylated dipeptide	PMI[g]	1296	Iterative[h]	36	IC_{50} = 4 μM	46
Alkylated dipeptide	ACE[i]	361	Not reported	361	Not reported	156
Azasugar tripeptide	β-glucosidase	125	Iterative	25	K_i = 20 μM	157
Pseudotripeptide	TeNT[j]	361	Iterative	19	K_i = 5 μM	158
Phosphinic tripeptide	Neurolysin	40	Iterative	20	K_i = 4 nM	159
N-carboxyalkyl tripeptide	MMP[k]	100	Iterative	200	IC_{50} = 0.4 μM	160
Octapeptidomimetic	PTP-1B[l]	8000	Iterative[m]	400	IC_{50} = 0.7 μM	161
Peptidylphosphonate	Thermolysin	540	Iterative	90	K_i = 49 nM	162
Carbamate	Acetylcholinesterase	54	PosScan	6 or 9	IC_{50} = 67 μM	73
Pyrrolidone	ACE	240	Iterative	80	K_i ~ 160 pM	33
Diketopiperazine	MMPs	684	Iterative	19	IC_{50} = 30 nM	163
Adenosine	ADA[n]	20	Mass spec.	20	$K_a = 10^8\ M^{-1}$	78
Thiazolidinone	COX-1[o]	540	Iterative	540	IC_{50} = 3.7 μM	41

[a] *Trypanosoma cruzi* [b] Farnesyl-protein transferase. [c]Prohormone convertase. [d]Glucosyltransferase. [e] Myosin light chain kinase. [f]Bacterial phosphotransferase system. [g]Phosphomannose isomerase. [h]Library byproduct was identified by HPLC and MS analysis, and a set of analogs were made to discover the inhibitor. [i]Angiotensin converting cnzyme. [j]Tetanus toxin. [k]Matrix metalloproteinases. [l]Human protein tyrosine phosphatase. [m]Two cross-validating methods, iterative and affinity selection/mass spectrometry, were used to identify the lead compound. [n]Adenosine deaminase. [o]Cyclooxygenase-1.

bound reagents (e.g., similar nucleophilicity, no significant steric hindrance). These ratios are established by adjusting the relative concentration of each building block according to its incorporation ratio after coupling of an equimolar building block mixture, as determined by HPLC. We have found that this concept applies equally well to mixtures of incoming reagents such as aldehydes, carboxylic acids, etc., as for amino acids. The

Table 9. Mixture-Based Libraries Screened for Antimicrobial/Antifungal Activity

Name	Microorganism	Number of Compounds	Deconvolution Method	Compounds/ mixture	Activity of lead compound[a]	Ref.
Tetrapeptide	*Staphylococcus aureus*	10,185,728	Iterative	175,616	MIC = 3-4 μg/mL	164
Hexapeptide	*Staphylococcus aureus*	34,012,224	Iterative	104,976	MIC = 3-7μg/mL	57
Hexapeptide	*Staphylococcus aureus*	52,128,400	Iterative	130,321	IC_{50} = 5μg/mL	117,165
Hexapeptide	*Escherichia coli*	52,128,400	Iterative	130,321	MIC = 16-32μg/mL	166
Hexapeptide	*Candida albicans*	52,128,400	Iterative	130,321	IC_{50} = 28μg/mL	167
Hexapeptide	Fungi broad spectrum[b]	52,128,400	Iterative	130,321	MIC = 5-36μg/mL	168
Decapeptide	*Candida albicans*	40,353,607	Iterative	5,764,801	MIC = 0.78μg/mL	169
18-mer peptide	*Staphylococcus aureus*	130,321	PosScan	6,859	MIC = 2-3μg/mL	170
Permethylated hexapeptide	*Staphylococcus aureus*	34,012,224	Iterative	104,976	MIC = 11-15μg/mL	14
Alkylated tetrapeptide	*Staphylococcus aureus*	7,311,616	Not reported	140,608	Not reported	49
Pentamine	*Staphylococcus aureus*	7,311,616	Not reported	140,608	Not reported	49
N-alkyl aminocyclic urea/thiourea	*Candida albicans*	118,400	PosScan	1,480-3,200	MIC = 8-16μg/mL	54
Bicyclic guanidine	*Candida albicans*	102,459	PosScan	2,091-2,499	MIC = 3-4μg/mL	29
Polyazapyridinophane	*Escherichia coli imp⁻* *Streptococcus pyogenes*	1,500	Not reported	100	MIC = 5-10μM	77,171
Pyridinopolyamine	*Escherichia coli imp⁻* *Streptococcus pyogenes*	1,638	Iterative	126	MIC = 1-3μM	172
Oxyamine	*Escherichia coli imp⁻* *Streptococcus pyogenes*	405	Not reported	81	MIC = 1-5μM	173

[a] MIC=minimum inhibitory concentration and IC_{50}=concentration necessary to inhibit 50% growth as determined by a microdilution assay. [b] *Fusarium oxysporum, Rhizoctonia solani, Ceratocystis fagacearum, Pythium ultimum.*

reagent mixture method has also been applied to the synthesis of heterocyclic compounds, including cyclic urea, cyclic thiourea,[50] and bicyclic guanidine libraries.[25]

The second strategy uses double couplings of equimolar building block mixtures in an approximate equimolar ratio to resin-bound functional groups,[65,66] thus preventing some building blocks from being incorporated in greater amounts than others. A potential problem associated with this strategy is the fact that the coupling is a second-order reaction, whose rate is influenced by both incoming and accepting pairs. Consequently, when a mixture of building blocks is coupled to a resin-bound mixture using this method, some combinations of incoming and accepting building blocks are likely to be formed at variable ratios, although the apparent overall incorporation of building blocks is close to equimolar at both positions.

C. Chemistry Optimization. When developing a mixture-based synthetic combinatorial library, many factors must be considered. Once the reaction scheme is established, the reaction conditions need to be optimized for widest possible breadth of diversity and reproducibility. This generally means that the synthetic scheme should be tested under a variety of reaction conditions using control compounds derived from the most reactive and least reactive building blocks. Optimal conditions are those that yield acceptable products using the largest number of building blocks. In our efforts, we have defined acceptable products as those having the correct molecular weight and appearing as a single major peak in RP-HPLC with greater than 80% yield and purity. A recent paper by our group[28] details the synthesis and analysis of the control compounds used in the development of N-alkylated triamines obtained by the selective N-alkylation and exhaustive reduction of N-acylated dipeptides. After general reaction conditions have been determined, NMR should be used for structure determination of the controls synthesized. Once confidence in structural assignment is

Table 10. Mixture-Based Libraries Screened in Other Bioassays

Name	Target	Number of compounds	Deconvolution method	Compounds/ mixture	Activity of lead compound	Ref.
Tripeptide	DNA cleavage	324	PosScan	18	Affinity = 1.4×10^8 M^{-1}	174
Tetrapeptide	ICE [a]	6,480	PosScan	324	$k_{cat}/K_m = 3.3 \times 10^6$ M^{-1} s^{-1}	175
Tetrapeptide	Caspase	6,480	none	324	Not reported	176
Tetrapeptide	Ftase [b]	6498	Iterative	342	K_m = 6.2 μM	177
Hexapeptide	Inositol phosphate	50×10^6	PosScan	2×10^6	IC_{50} = 60 nM	178
Hexapeptide	Melittin	50×10^6	PosScan	2×10^6	IC_{50} = 3 μg/mL	99
Hexapeptide	Melittin	50×10^6	Iterative	2×10^6	IC_{50} = 8 μg/mL	179
Hexapeptide	NMDA [c] channel	50×10^6	PosScan	2×10^6	IC_{50} = 100 nM	180
Hexapeptide (D-amino acids)	Calmodulin	50×10^6	PosScan	2×10^6	IC_{50} = 5 μM	181
Hexapeptide (D-amino acids)	Melittin	50×10^6	PosScan	2×10^6	IC_{50} = 10 μg/mL	182
Hexapeptide (D-amino acids)	TNF-α [d]	47×10^6	Iterative	130,321	IC_{50} = 0.33 μM	183
Nonapeptide	TAP [e]	3.2×10^{11}	PosScan	1.7×10^{10}	K_D = 137 nM	184
Peptidosteroid	enkephalin	10,000	Encoded	10,000	ΔG = -1.6 kcal/mol	185
Tetraurea	DNA	2,080	Omission	2,080	5-10 μM	186
NSG-peptoid	Caco-2 [f]	144	LC/MS	24	$P_{app} = 15 \times 10^{-6}$ cm/sec	187
N-acyl triamine	Tumor cells [g]	450,000	Iterative	2,704	GI_{50} = 0.7 μM	90
Pentamine	Tumor cells	7×10^6	PosScan	140,000	GI_{50} = 0.8 μM	90
Dihydropyridine	Calcium blocker [h]	100	Iterative	10	IC_{50} = 14 nM	45
Amidinonaphthol	Factor VIIa	144	Iterative	8	IC_{50} = 4.1 μM	36

[a] Interleukin-1β converting enzyme. [b] *S*-farnesyl transferase. [c] N-methyl-D-aspartate receptor channel. [d] Tumor necrosis factor. [e] Transporter-associated protein. [f] A model system that measures intestinal permeability (P_{app}) of compounds across cell monolayers. [g] Human tumor cell lines assayed for cytotoxicity. [h] Measures calcium blockade activity using a cortex membrane binding assay.

obtained, LC−MS can be used for analyzing large numbers of compounds.

Methods for determining yields and purity are also factors. We generally measure purities by UV absorbance at 215 nm during RP-HPLC analysis. Depending on the chromophores present, wide variability in molar absorbance can be obtained. Purities can also be determined through the measurement of total ion current during RP-HPLC with mass spectral detection. However, we have found that ionization within a single class of compounds can vary widely. An alternative approach is to use light scattering detection. Again, different compounds within the class can yield different results. Thus, the analysis used should be based on the particular structures being synthesized.

During the development of general reaction conditions, it is also important to synthesize a series of controls several times to test for synthetic reproducibility. This is especially important for reactions carried out under inert conditions or those with moisture-sensitive reagents. Care should also be taken during scale-up of the reaction. Although initial experiments

may be performed on the milligram to gram of resin scale, the library synthesis may involve up to 100 g of resin.

The breadth of the synthetic approach should also be determined. Every proposed building block should be tested in the synthesis of a control before inclusion in a library. In this laboratory, once general reaction conditions have been determined, we vary a parent compound with each of the individual proposed building blocks (as described above in the synthesis section of the bicyclic guanidine library). The structure of the parent compound is chosen such that the building blocks used are of average reactivity and yield a clean product of sufficient purity. The effect of an individual building block on the reaction can then be seen. It is possible that some adverse interactions between individual building blocks can be missed with this approach. Controls to check for this type of interaction are included during the development of the general conditions.

Once the appropriate building blocks for a particular library are chosen, a set of control compounds incorporating the chosen building blocks should always be synthesized concurrently with the mixture-based library as controls for completeness of the reactions and for reproducibility. When a library is being synthesized as mixtures, the use of these controls is extremely important. Individual control compounds should also be included and cleaved at all intermediate stages of the library synthesis to test for reaction completion.

Following synthesis, mass spectral analysis of mixtures within the library is used to confirm that the expected range of masses is present. The individual control compounds synthesized both during the development and synthesis of the library can then be used in conjunction with the mixture-based library to provide an abundant amount of SAR data on the particular class of molecules.

Deconvolution Strategies of Mixture-Based Libraries

Deconvolution procedures are an essential element in the identification of active individual compounds from mixture-based libraries. A number of deconvolution approaches have been presented, the most commonly used being iterative,[57,62] positional scanning,[63] and the sequencing of resin-bound peptides or tags from one-bead−one-compound libraries.[21,24,58] While straightforward, the iterative approach is an inherently time-consuming deconvolution method due to the repetitive rounds of synthesis and testing, requiring the same number of syntheses as the original number of nondefined diversity positions in the library. Other deconvolution methods include orthogonal pooling,[67] subtractive pooling,[68−70] bogus coin pooling,[71,72] indexed pooling,[73] libraries of libraries,[74] affinity separation,[75−77] and mass spectrometry[78] based strategies. The theoretical and comparative evaluations of these methods have been reported.[70,79,80]

A. Iterative Method. An iterative synthetic combinatorial library consists of a single set of mixtures, typically having one or two positions defined. Following the screening of this initial set of mixtures, the iterative process is carried out by selecting the most active mixture and fixing the defined position with the corresponding functionality associated with this mixture. Subsequent mixture positions are then sequentially defined in a similar manner until a final set of individual compounds is obtained and tested.

The concept of iterative deconvolution is illustrated in Figure 3 with a library having three diversity positions (OXX). Three different building blocks are incorporated into each diversity position, resulting in a total of 27 (3^3) compounds. Such a small number of compounds could obviously be generated as a single compound array and is only used here to illustrate the deconvolution concept. The defined or "O" position represents one of three functionalities (Me, Et, Bzl), while the remaining two positions (X) are mixtures of three functionalities (F, Cl, Br and NH_2, OH, SH, respectively). Thus, the library is composed of three mixtures, and each mixture contains nine compounds (3^2). For this example, let us assume that only one compound is recognized by a given receptor, with all of the other compounds being inactive. In this illustration, let us define this single active compound as having an ethyl at the first position, a fluorine at the second position, and a thiol functionality at the third position (represented as Et/F/SH). When the library (OXX) is screened, only mixture 2 defined by the "Et" functionality would therefore show any activity because it is the only mixture containing Et/F/SH. We have set this example so that no other compounds are active. This mixture would therefore be selected, and a new set of three mixtures (iteration 1) would then be synthesized, each composed of a mixture of three compounds now having defined functionalities in the first two positions (Et and F or Cl or Br). Since we have defined here that only Et/F/SH is active, then only the "F" mixture would be active at the second position, and the most active mixture identified would then be mixture 1 (Et/F/X). A second and final iterative synthesis and screening step would be required to identify the active individual compound, "Et/F/SH". The majority of iterative libraries reported to date require two to four iterative steps prior to the identification of final individual compounds.

B. Methods Related to Iterative Deconvolution. A variation of the iterative approach has been presented and is referred to as subtractive pooling.[69] This approach involves the synthesis of mixtures using all building blocks, minus a defined group of building blocks at all of the diversity positions. Loss in activity indicates the importance of one or several of the missing building blocks. On the basis of the screening results, one can identify potential key building blocks (i.e., those building blocks contained in the missing groups defining the active mixtures) and generate a smaller subtractive library by separating those building blocks into smaller groups. This step is repeated until a small number of potential key building blocks are identified. While this method allows the nature of important functionalities to be determined, the position of those building blocks within the molecule remains undetermined. The final step is the synthesis of all possible combinations of these building blocks at all positions. This method may become labor-intensive if several mixtures show similar loss in activity.

The "bogus coin" strategy is also a variant of the iterative process; it involves the synthesis of a library

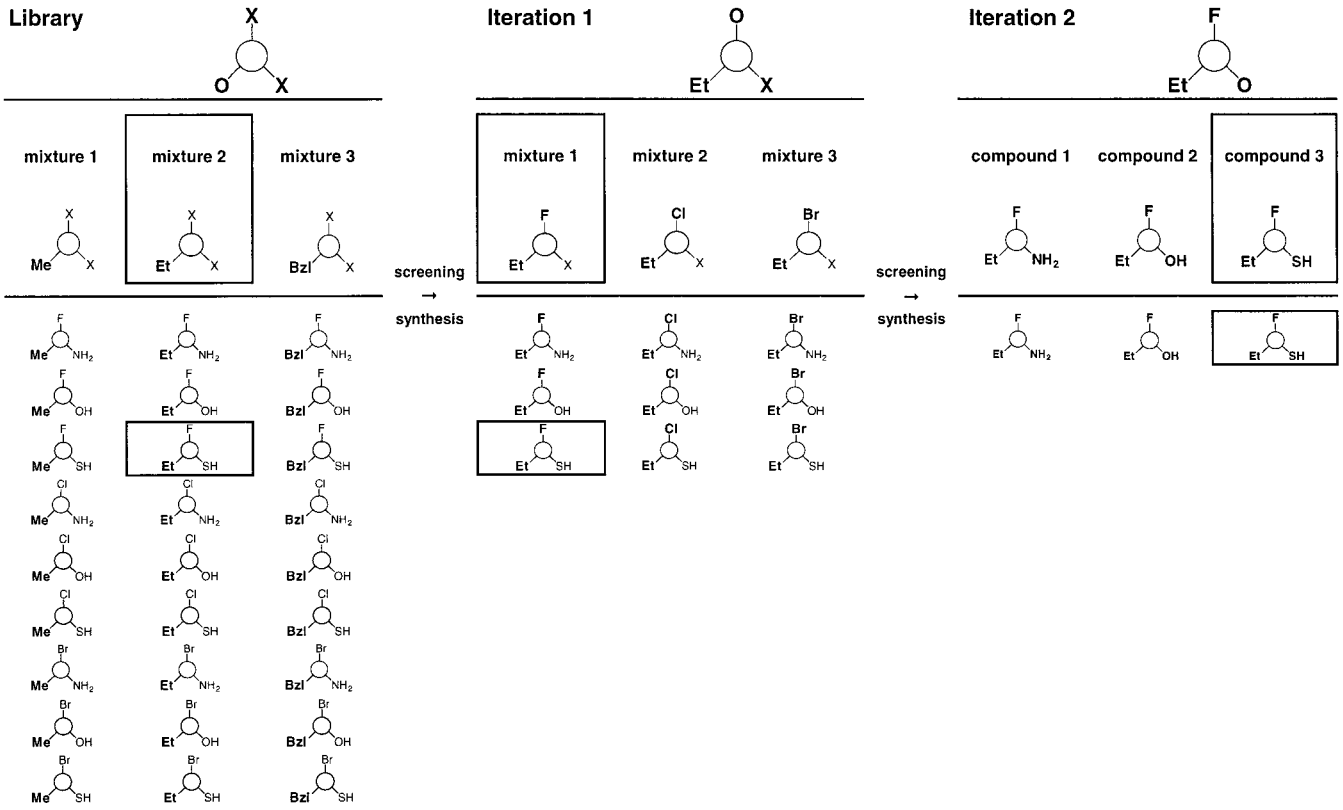


Figure 3. Illustration of an iterative trifunctional combinatorial library. The library is composed of three mixtures having one position defined with three different building blocks. The individual compounds that make up each mixture are shown below. Two rounds of screening and synthesis are required to identify individual compounds. See text for more details.

in a single mixture and the preparation of additional mixtures made up using a different ratio of the building blocks.[71,72] Variations in activity relative to the initial single mixture can then be used to indicate which group of building blocks is responsible for the activities found. For example, if a mixture is generated that has twice the proportion of defined groups at a given position and is found to show higher activity, it indicates that this group is responsible for the activity seen in the original library. In a second step, the key groups are divided into smaller subgroups, and mixtures having varying proportions of these subgroups are generated and screened to narrow down the possible building blocks responsible for activity. This step is repeated until an individual active compound is identified. Unless highly specific, an increase in proportion of an important group of functionalities is not likely to result in a proportional increase in activity relative to the original single-mixture library. This is due to the occurrence of less active, but still significantly detectable, compounds in the original single-mixture library, which reduces the potential success of this deconvolution method.[70]

C. Positional Scanning. Positional scanning synthetic combinatorial libraries (PS-SCLs) are composed of one sublibrary for each variable position.[63] In the case of single-position-defined PS-SCLs, each compound present in a given mixture has a common individual building block at a given position, while the remaining positions are composed of mixtures of all of the building blocks used to prepare the library; a common single building block defines each relevant mixture. The sublibraries for each position represent the same collection of individual compounds, and they differ only by the location of the defined position. The screening data permit the identification of key functionalities at each diversity position. It is important to note, however, that the activity found for a mixture is due to the presence of specific active compound(s) within the mixture, and not the individual functionalities as separate independent entities. The combination of all positional functional groups identified as key elements leads to active individual compound(s).

As an illustration of the PS-SCL concept, the same trifunctional combinatorial library used above to describe the iterative deconvolution will be used. When this same diversity of compounds is arranged as a PS-SCL (Figure 4), nine separate mixtures (3 building blocks × 3 positions) are synthesized. It is important to note that each of the three sublibraries of mixtures, namely OXX, XOX, and XXO, contains the same compounds, and they differ only in the location of their defined functionalities. This illustration assumes that only one compound (Et/F/SH) is active, with all of the other compounds being inactive. Since each sublibrary contains the same diversity of compounds, "Et/F/SH" is present in only one mixture in each of the three sublibraries. The only mixtures exhibiting activity will therefore be mixture 2 (Et/X/X) from sublibrary 1, mixture 4 (X/F/X) from sublibrary 2, and mixture 9 (X/X/SH) from sublibrary 3, since only these mixtures contain the active individual compound "Et/F/SH". Following screening, one need synthesize only this one compound corresponding to the combination of these three building blocks in their respective positions to yield "Et/F/SH". Testing of "Et/F/SH" in the assay of interest not only confirms that the selections made

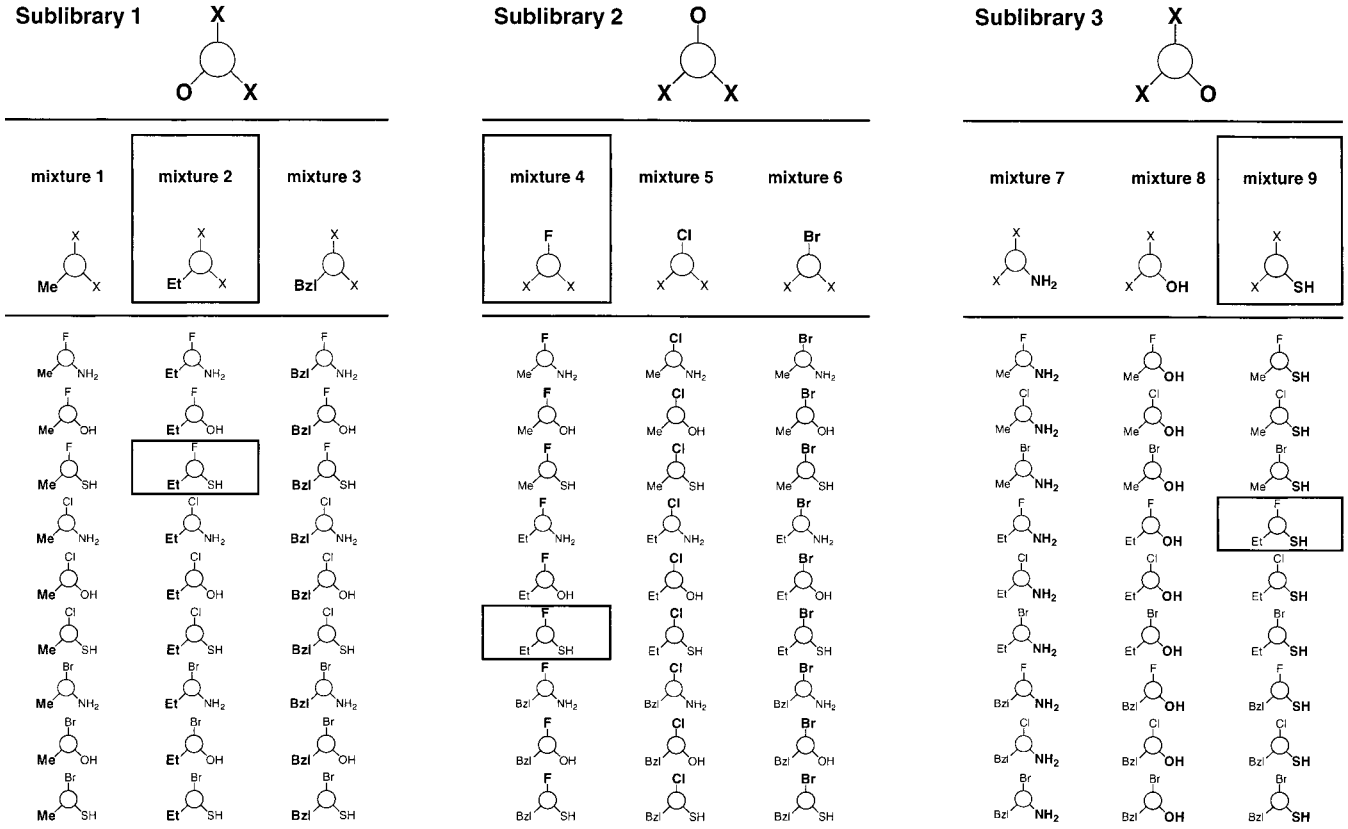


Figure 4. Illustration of a trifunctional positional scanning combinatorial library. The library is composed of nine mixtures, which are grouped into three sublibraries for each of the three diversity positions. Each sublibrary contains three mixtures having one position defined with three different building blocks. The individual compounds that make up each mixture are shown below. Each sublibrary contains the same total compounds but differs only in the location of the defined position, i.e., the grouping of those compounds. Following the library screening, the most active mixture(s) is selected from each sublibrary (boxed mixtures), and the individual compound(s) having the combination of the functionality defining those mixtures is synthesized. The individual compound responsible for the activity seen in the three active mixtures is boxed (this compound is the same in the three sublibraries). The resulting individual compounds are tested to determine their final activities. See text for more details.

actually lead to the active compound but also allows the determination of its biological activity. As stated above, the activity observed for each of the three mixtures (Et/X/X, X/F/X, and X/X/SH) is due to the presence of a single active compound "Et/F/SH" within each of these mixtures and is not independently due to the individual building blocks Et, F, and SH that occupy the relevant defined positions.

In more complex libraries, more than one mixture is often found to exhibit significant activity at each position. In the process of selecting building blocks for the synthesis of individual compounds, one first selects based on relative activity and then on differences in the chemical character of the building block. While positional scanning deconvolution is usually highly preferred over iterative deconvolution due to the speed in which individual compounds can be identified, iterative deconvolution may also be performed from any of the sublibraries making up a positional scanning library. An iterative deconvolution can therefore be initiated at any of the diversity positions of a positional scanning library and typically would be initiated with the most active mixture.

While the preceding description illustrates the single defined PS-SCL, the same concept has been applied to libraries having more than one defined position. For example, a dual-defined hexapeptide PS-SCL composed of three sublibraries, each composed of 400 mixtures (OOXXXX, XXOOXX, XXXXOO), was successfully generated and screened by this laboratory for the identification of novel peptides having high affinity for the nociceptin/orphanin FQ receptor, ORL1,[81] as well as for exploring antibody polyspecificity.[82] Another example of dual-defined positional scanning libraries, termed a Spatially Arrayed Mixture library, has been reported by Berk and Chapman.[83] This library was composed of two sublibraries of over 9 000 peptoids and was validated by the identification of an α1-adrenergic receptor agonist, known to be present within the library.

D. Methods Related to Positional Scanning Deconvolution. The indexed pooling strategy involves the generation of a positional scanning library and the screening of pools of mixtures in a matrix format, having one sublibrary screened in rows and the other sublibrary in columns.[73] Each cell of the matrix represents a defined building block from each of the two sublibraries, which upon screening allows the direct determination of the key building blocks responsible for an activity of interest. Acetylcholinesterase inhibitors were successfully identified from an indexed library of 64 carbamates grouped into 15 mixtures.[73]

A hybrid of the one-bead−one-compound and positional scanning library formats is presented by an approach termed "library of libraries"[74] directed toward the identification of pharmacophore motifs (i.e., structural motifs necessary for the bioactivity of interest),

rather than the complete structures of individual active compounds. This library format enables the identification of specific (nonreplaceable) positions in a compound having bioactivity of interest versus nonspecific positions, which can be replaced by a variety of different building blocks without loss in activity.

A deletion deconvolution approach that uses a concept similar to the positional scanning approach was reported for the identification of active heterodimeric structures.[84] In contrast to PS-SCLs, in which all compounds present within a mixture have a common building block at a given position, each mixture of a deletion library is prepared using all building blocks minus a defined building block at a given position. The activities of such mixtures are compared to a control mixture containing all of the compounds making up the library. A loss in activity observed for a specific mixture indicates that the building block omitted to build this particular mixture is a key element for the activity of interest. Combinations of these building blocks should lead to active individual compounds. While similar results are obtained when deconvoluting homooligomers using the positional scanning or deletion approaches, the deletion approach facilitates the identification of heterooligomers, where different building blocks are necessary at a given position for activity to occur. However, the number of individual compounds per mixture is greater in a deletion library than in the same diversity formatted in a PS-SCL.

E. Orthogonal Deconvolution Strategies. Two-dimensional orthogonal libraries have been reported by Déprez et al.[67] These libraries consist of two separate sublibraries, which represent the same set of compounds in different arrangements, so that each mixture of a sublibrary has a single compound in common with any mixture of the other sublibrary. Both sublibraries (A and B) of the reported two-dimensional orthogonal library were composed of separate compound mixtures, generated by coupling three separate groups of five building blocks. The building blocks were grouped differently in each sublibrary, such that each group in sublibrary A had one, and only one, building block in common with each group in sublibrary B. Thus, each of the two sublibraries was composed of 125 (5^3) separate tripeptide mixtures, and each mixture contained 125 tripeptides, for a total of 15 625 (25^3) individual tripeptides in the library. After determining the most active mixtures in both sublibraries for a given assay, individual active compounds are identified by deciphering the compounds common to the active mixtures in sublibraries A and B. If more than one mixture is active in each sublibrary, individual compounds representing the common compounds of different mixture pairs from both sublibraries must be synthesized and tested in order to identify the most active individual compounds.

F. Deconvolution by Analytical Techniques. A number of analytical techniques have been used to identify active compounds from mixtures. For example, online pulsed ultrafiltration and electrospray mass spectrometry[78] have been developed to rapidly identify solution-phase ligands that bind with moderate or high affinity to solution-phase receptors. Using this technique, ligand−receptor complexes in solution following ultrafiltration can be determined by electrospray mass spectrometry. Similar deconvolution approaches involve the separation of ligand−receptor complex from unbound compounds by size exclusion chromatography followed by identification of bound ligand by electrospray mass spectrometry[75,76] or successive fractionations by HPLC followed by mass spectrometry analysis of the library and active fractions.[77] These different analytical-based deconvolution approaches to date have been used only for relatively small libraries (i.e., containing up to 4 000 individual compounds).

Library Screening Conditions and Requirements

The screening of mixtures is not a new concept. In vivo and in vitro biochemical interactions, such as receptor−ligand interactions, routinely occur in a vast milieu of other compounds (e.g., proteins, salts, cofactors, amino acids). Furthermore, natural product extracts and bacterial broths are screened as diversity sources that are naturally composed of complex mixtures of compounds. Many of the existing approved therapeutics are directly identified from natural product mixtures or are derived from these compounds. Compared to natural product extracts or bacterial broths, mixture-based SCLs have a number of clear inherent advantages: (1) the concentration of individual compounds within the libraries is approximately equal; (2) the structures of the compounds making up the libraries are known; and (3) no synthetic hurdles have to be overcome once an active individual compound has been identified.

A. Assay Optimization. While often overlooked as a factor in screening combinatorial libraries, a thorough knowledge of the assay parameters, such as signal-to-background ratio, variability, and sensitivity, is essential for the successful use of mixture-based libraries. These parameters are inherent to every assay and are not influenced by the samples being screened (mixtures or individual compounds). The main objective in optimizing an assay for the screening of mixture-based libraries is to obtain the highest and most reproducible signal-to-background ratio possible. For inhibition assays, the signal of the reagent to be inhibited should be as high as possible, while the background should be as low as possible. Table 11 illustrates the signal ranges of various assays in which mixture-based libraries have been used. Signal-to-background ratios of 5 or higher have proven to allow clear identification of active compounds from mixture-based libraries. When screening mixture-based libraries, it is critical that the assay variability (i.e., noise of the signals) is known. Positive and negative control compounds facilitate the evaluation of the variability of an assay. Both intra- and intervariability of an assay should be determined prior to screening any library. Such information is critical for the design of the screening program to determine the number of experiments and replicates required, as well as for the interpretation of the results. It is, unfortunately, too often neglected.

B. Screening Conditions. The initial concentration used to screen a library varies from assay to assay (see Table 11). While many assays can tolerate high concentrations of compounds (1−10 mg/mL of compound or mixture), a number of cell-based assays are sensitive

Table 11. Examples of Assay Signal Ranges and Initial Screening Concentrations for Mixture-Based Libraries

Assay	Readout	Range	Ratio	Screening concentration (mg/mL)
Radioreceptor	counts/min.	200-1000	5	0.5-5
T cell proliferation	counts/min.	see footnote [a]	3	0.05-0.3
Microdilution	optical density	0.1-0.5	5	0.1-0.5
Enzyme inhibition	optical density	0.1-0.8	8	0.1-1
ELISA	optical density	0.1-2.0	20	0.1-5

[a] Range varies depending on the T cell clone.

to high concentrations of compounds, whether in individual or mixture formats. In such cell-based assays, the screening of mixtures typically starts at 0.1−0.5 mg/mL. Since many nonpeptide libraries are not highly water-soluble, solubility can often be enhanced by the presence of 1−10% DMF or DMSO or other cosolvent in order to achieve these concentrations. When stored in 100% organic solvent, it is necessary to aliquot mixture-based libraries at the highest concentration possible (~10 mg/mL), since many assays are sensitive to more than 1% organic solvent. This ensures that individual compounds within each mixture are present at a detectable concentration without having excess solvent affecting the assay. Another interesting feature of mixture-based libraries that we have found is the "self-solubilizing" nature of the related compounds making up the mixtures.

C. Data Handling. Analysis of screening data from mixture-based libraries is not fundamentally different from the analysis required using individual compounds. Discrimination among active and inactive mixtures is not dependent on the number of compounds per mixture, but rather it is dependent on the activity of the individual compound or compounds within the mixture. The large quantity of generated data points is more easily handled with a computer spreadsheet. To ensure the selection of active versus non- or less-active mixtures, one must first confirm the screening results by replicating the assay. Screening of replicates enables simple averages, within and between assays, and standard deviations to be determined, which in most cases is sufficient to differentiate among active mixtures or compounds. To compare replicate assays, it is convenient to normalize the activity of the mixtures. In competitive assays, for example, the data can be normalized and expressed as percent inhibition using both the background and total binding measurements. In noncompetitive assays or in those cases where the variability between assays is high, the activities of the mixtures can be ranked within each assay and the order between assays compared.

To further differentiate between mixtures having similar activities, serial-dilution experiments should be carried out to determine dose−response curves. This is also necessary when the distinction between active and inactive mixtures is difficult to establish at a particular concentration. The inhibitory activities of the mixtures can then be expressed as the concentration that inhibits 50% of the activity of interest (IC_{50} values).

When the activity of the most active mixtures at the screening concentration is less than 50% and assay parameters prevent the use of a higher concentration, a useful strategy is to compare the activity of a given mixture relative to the average mixture activity within the same sublibrary. The use of the average activity of all mixtures in a library is also valuable when dose−response information is required for different mixtures.

Although statistical analysis is not always necessary, it can be useful as a more rigorous strategy for evaluation of the significance of the relative activities observed (e.g., analysis of variance, ANOVA, with a Tukey or Dunnett post test). ANOVA determines if the screening results have significant differences, while a Tukey post test compares each single mixture to each of the others using a fixed confidence interval. Dunnett's post test may also be used to compare each mixture's activity to the activity of a control set (usually multiple replicates of a negative control). These statistical tests are valid only if an adequate number of replicates have been performed (i.e., >4 within and between combined assays). One example of such use of statistical analysis for mixture screening has been reported for the identification of antifungal compounds from a bicyclic guanidine library.[29] In this study, ANOVA analysis combined with Tukey post test confirmed the significance of a 2-fold difference in activity between the most active mixtures and the rest.

D. Deconvolution and Building Block Selection. The successive steps and decision junctures involved in the screening and deconvolution of mixture-based libraries are outlined as a flowchart in Figure 5. After screening a library in a given assay, there are three possible outcomes: (1) none of the mixtures are active, (2) all of the mixtures are active, or (3) some of the mixtures are active. If no activity is observed (i.e., all mixtures exhibit <10% inhibition), the library should be rescreened at a higher concentration if possible, or a different library should be tested. Not all libraries will contain active compounds in a particular assay system. If all mixtures are found to exhibit activity (>80% of mixtures having >50% activity), the library should be rescreened at a lower concentration. When a small number of mixtures (<20% of the library) are found to

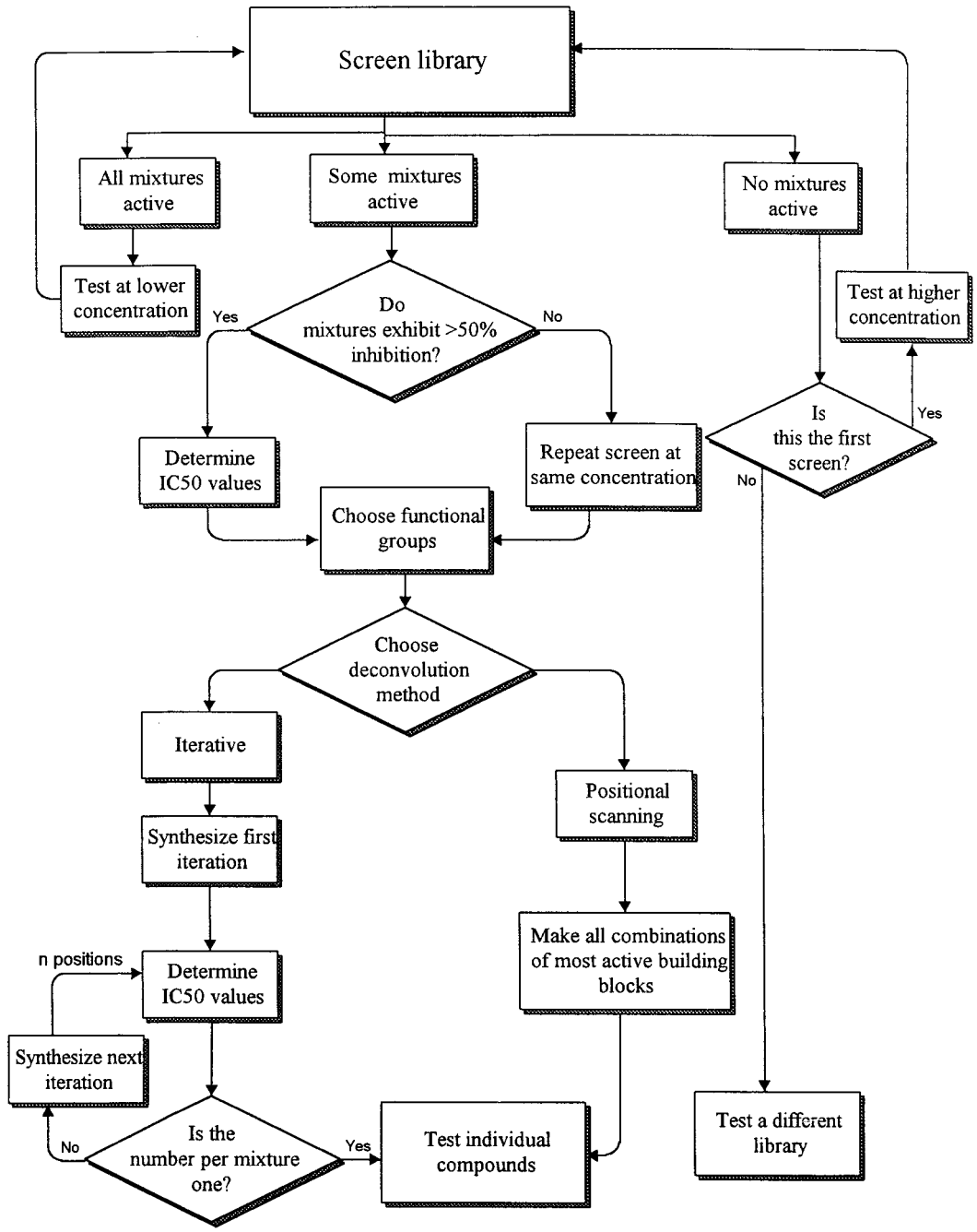


Figure 5. Flowchart of the steps taken when screening a mixture-based library to identify individual compounds. See text for more details.

exhibit activity, IC_{50} values should be determined for those mixtures (see data handling section above).

The number of individual compounds, or alternatively smaller intermediate mixtures, to be synthesized following the screening of a given library is based on several factors: (1) the library format, (2) the statistical significance of the activities, (3) the chemical nature of the defined functionalities of the active mixtures, and (4) the resources available for the given project. Often mixtures defined at a given position with functionalities of similar chemical character will have similar activities. This may indicate that a number of related analogues of the same compound are responsible for the observed activity. To reduce the number of final compounds needed to be synthesized, similar functionalities can often be excluded from the original selection. One can at a later stage iterate other mixtures, or for positional scanning libraries, synthesize analogues of the most active individual compounds containing functionalities that were originally excluded.

If iterative deconvolution is used, then the selection of mixtures should be kept to a minimum since separate syntheses will be required for each mixture chosen. However, more than one mixture should be chosen, so that the activities in the next iterative step can be compared. The active parent mixture should always be resynthesized along with its subsequent iteration to serve as an internal synthetic control. There is typically a significant improvement in activity found upon defining each additional position. This is due to a combination of the reduction in the number of compounds in the new mixture (iterations are typically tested at lower

Table 12. Increase in Mixture Activities During Iterative Deconvolution

Mixture [a]	Number of compounds	Antibody [57] Activity IC_{50} nM	Increase	Receptor [96] Activity IC_{50} nM	Increase	*S. aureus* [57] Activity IC_{50} μg/mL	Increase
Ac-OOXXXX-NH_2	130,321	250,000	-	2,524	-	450	-
Ac-OOOXXX-NH_2	6,859	41,000	6	815	3	126	4
Ac-OOOOXX-NH_2	361	4,400	9	140	6	32	4
Ac-OOOOOX-NH_2	19	380	12	27	5	25	1
Ac-OOOOOO-NH_2	1	30	13	5	5	5	5
Overall improvement			8,000		500		90

[a] O represents a defined functionality and X represents a mixture of functionalities.

concentrations than their parent mixtures) and the importance of the defined building block(s) on the activity of the individual compounds present in the active mixture. If only a single building block at a particular position is acceptable in a given active compound, then only a single mixture in an iteration will be significantly more active than the parent mixture. If all building blocks can be accommodated in the active compound at that position, the position is redundant and little or no increase in activity will be found for the iteration when compared to its parent mixture. This does not mean that further improvement will not be found for subsequent iterative steps. Iterating more than one mixture offers more assurance of finding active compounds in such cases. Table 12 illustrates the improvement found following the deconvolution processes in various assay systems.

To identify the most active individual compounds from a PS-SCL, individual compounds corresponding to the combination of the building blocks defining the most active mixtures at each position are synthesized and tested. For practical purposes, the number of the building blocks selected to prepare individual compounds is minimized whenever possible as the number of compounds to be made rises exponentially with the number of diversity positions. Since PS-SCLs are composed of separate sublibraries, in which each sublibrary contains the same compounds but differs only in its defined positions, each sublibrary can be considered to yield results independent of the others. This enables each sublibrary to be independently screened and pursued using an iterative synthesis and selection process if desired.

Advantages of Mixture-Based Libraries

There is an ongoing debate on the relative merits of the preparation and screening of libraries as individual compound arrays or as mixtures.[85,86] The central issue is the balance between the time and cost of synthesis, analysis, storage, and screening of these libraries, in addition to the chances of identifying or missing active compounds. Starting with the assumption that the same number of compounds will be tested, we believe that the two approaches that will prove the most pragmatic in the long-term are the robotic synthesis of very large individual compound arrays and the synthesis of mixtures deconvoluted using the positional scanning approach. Robotically prepared individual compound arrays and classical high-throughput screening systems have the advantage of providing more complete information since all the compounds in a given class are individually examined. On the other hand, while not providing complete information on every individual compound present within the library, mixture-based libraries combined with positional scanning deconvolution have the advantage of greatly decreasing the economics and time constraints of compound array systems. Screening of millions of compounds can be accomplished even in assays that are not formatted for conventional high-throughput.

Table 13 illustrates a comparison of the effort needed to prepare and screen a library of 27 000 compounds using 30 different building blocks on a pharmacophore having 3 diversity positions ($30 \times 30 \times 30 = 27\,000$, which is 3 orders of magnitude greater than the simple illustration shown in Figure 3). A parallel array format of this library would require the preparation, analysis, screening, and storage of 27 000 individual compounds. Alternatively, the same 27 000 compounds prepared as mixtures in either an iterative or a positional scanning format would require the preparation of 30 or 90 mixtures, respectively. A substantial cost reduction in using libraries formatted as mixtures is also found in decreased amounts of assay reagents and materials required for screening. As illustrated in Table 13, the number of 96-well microtiter plates that are required for testing this library as mixtures is much lower than testing as individual compounds. As can be seen, even with the additional deconvolution steps required, an exceptional time and labor savings is achieved with mixture-based libraries.

Frequently Asked Questions About Screening Mixture-Based Libraries

When first introduced to the concept of screening mixtures, many researchers appear to be confounded by the numbers of compounds involved, and many of the same questions arise. Brief answers to a number of the most frequently asked questions are given below.

Why do mixtures work? Virtually all biological

Table 13. Comparison of Synthetic and Screening Efforts for Three Different Formats of a Library Composed of 27 000 Compounds

Library type	Diversity positions [a] R^1	R^2	R^3	Compounds/ sample	Samples to synthesize and screen	96-well plates
Individual array	O	O	O	1	27 000	282
Iterative [b]	O	X	X	900	30	2
PosScan [c]	O	X	X	900	90	2
	X	O	X			
	X	X	O			

[a] O is defined with one of 30 different building blocks; X is a mixture of 30 different building blocks. [b] Two additional synthesis of 30 samples each would be required to identify individual compounds for each active mixture found in the library. [c] An additional synthesis is required to identify individual compounds from active mixtures.

interactions occur in a milieu of other compounds. The following simple example may help explain why individual compounds can be readily identified from mixtures of compounds. Envision a mixture of 100 different compounds, one of which is recognized by a given target and the other 99 compounds are completely inactive. This mixture can then be considered a simple 100-fold dilution of this particular active compound (i.e., 100 μg/mL of mixture will have the same activity at the target as 1 μg/mL of the individual compound). This is an essential principle behind the concept of using mixtures as a screening tool. The signal and discrimination between active and inactive mixtures are dependent on the activity of the individual compound(s) within the mixture.

How can one detect activity in mixtures when the individual compound concentrations are so low? Most mixture-based libraries are made up of compounds of similar structures and chemical characteristics. Thus, a number of analogous compounds within a mixture are typically responsible for the observed activity of the mixture. Analogues having activity within the mixture can be expected to increase the "effective" molar concentration of that particular mixture. Even extremely large mixtures such as those making up a decapeptide PS-SCL (200 mixtures, each mixture containing 2×10^{11} individual decapeptides) have been successfully deconvoluted for a peptide–antibody interaction[87] and for the identification of novel T-cell ligands.[88] Furthermore, from the same library in conjunction with a κ-specific opiate receptor binding assay, two decamers were identified (Ac-YRTRYRYRRR-NH_2, IC_{50} = 28 nM; Ac-RGWFHYKPKR-NH_2, IC_{50} = 30 nM). It would be unlikely that a single active compound within such a large mixture would be identified.

How can one be sure that the most active compound in the library has been identified? Other than physically making all of the individual compounds that make up the library and testing them individually, one can never be certain to have identified the single most active compound. Many case studies have shown that not only is it possible to identify the expected known ligands from mixture-based libraries, but also new compounds have been identified having greater or similar activities to these known compounds.

How does one distinguish between mixtures having many low-affinity compounds and those containing a few high-affinity compounds? Differentiation between mixtures having low-affinity compounds and those containing high-affinity compounds becomes apparent upon deconvolution. Although with experience one comes to recognize the warning signs for one's particular assay (e.g., when it is found that all mixtures have similar activities), each library screening should be treated on a case-to-case basis. In the end, only the individual compounds prepared from the mixture-based screening data can truly answer this question.

Will antagonists present in a given mixture cancel out other compounds having agonist activity? In functional cell-based assays, an antagonist would have to be present in a specific excess over an agonist to yield a "null" response. While the occurrence of a cancellation of activity response is unlikely, it should be considered when performing such assays. When using radioreceptor binding assays, one cannot distinguish between agonists and antagonists present within the same mixture, and both have been identified in these assays. However, functional assays have been used to selectively detect agonists or antagonists (i.e., competition with a control agonist/antagonist).

Is synergy a problem when deconvoluting mixtures? While anticipated, we have never observed synergy within mixtures. Synergism should result in a loss of activity when going from a complex to a less complex mixture or to individual compounds; such activity would be restored upon mixing all components in question. Any loss of activity reported thus far appears to have been due to a lack of reproducibility in synthetic procedures or errors made during the synthesis. In no reported cases has mixing all of the inactive components resulted in an active mixture. Synergism would be harder to identify in positional scanning libraries.

Is it better to screen the widest diversity possible or a small, directed diversity? The answer to this question is dependent on prior knowledge of the target and its assay. If little is known about the target, then a larger diversity is recommended. If prior information is available, then a more directed approach would be preferable. It has been argued that a very restricted number of building blocks having desired chemical characteristics, such as those selected based on computer modeling, should be sufficient to represent the whole spatial array of a class of building blocks.

However, our studies and others have shown that chemical dissimilarity should not be the only criterion for building block selection, since slight variations can often result in dramatic effects on a biological activity of interest.

Is it better to screen individual compound arrays or small mixtures rather than large mixtures? The decision to screen large versus small mixtures is based on individual circumstances. Obviously, if one's synthetic, analytical, storage, and assay capabilities permit all desired individual compounds to be prepared and tested, then this will result in the clearest most complete data acquisition. If one wants to screen mixtures of chemically unrelated compounds, it would seem preferable to keep the number of compounds per mixture to under 100. Not only are small mixtures easier to assemble, but where structures are unrelated, the increase in effective concentration as a result of more than one active compound present in the mixture may not be observed. Thus, individual hits may be missed in larger mixtures. If one synthesizes individual compounds and then combines them as mixtures for screening (e.g., when high-throughput is available for synthesis but the assay of interest is not amenable to large numbers of compounds), it is obviously easier to assemble small mixtures of 10−100 compounds each. In this case, mixtures can be used to shorten the screening process. A point−counterpoint discussion on screening mixtures versus individual compounds was recently reported.[85,86] We believe that if high-throughput screening facilities are not available, the preparation and screening of large mixtures is the most time- and cost-efficient technique.

Will you miss weakly active compounds? This is somewhat dependent on one's definition of "weakly active". Weakly active compounds often have a higher degree of redundancy in their positions, which will result in a number of similar analogues existing in the same mixture and their cumulative concentration will confer activity to the mixture. Weakly active compounds can be identified from mixtures. Weaker compounds in a mixture may be missed if more active compounds are present and have been identified from the same mixture, but in this case it would seem that the weaker compounds would be of lesser interest.

Why have others had trouble using mixture-based libraries? We believe that the difficulty others have had in the preparation and use of mixture-based combinatorial libraries stems from a fundamental misunderstanding of what is required for their successful synthesis and use. Each step must be more carefully planned and executed than in the synthesis of individual compounds. It is of fundamental importance to develop chemistries that afford high yields and are as clean as possible, and that are also reproducible. The reproducibility of the chemistries allows the identification of side reactions that might be responsible for a given activity during the deconvolution of individual compounds. We have encountered instances in which an activity did not reside in the major component of an individual crude compound following deconvolution, but rather in a 5−10% side reaction. In these instances, due to the reproducible nature of the chemistries involved, identifying the active side reaction was straightforward. In fact, every step in the solid-phase synthesis, cleavage, extraction, lyophilization, reconstitution, storage, and use must be carefully and reproducibly carried out. It is important to note that the assays used in conjunction with these libraries should have good signal-to-noise ratios and, of course, good reproducibility. One other factor which appears to often be misunderstood is that a single library, even if it is composed of a very large number of compounds, will not necessarily have any activity in a given assay. We have repeatedly found that our preconceived expectations for activity in a given library and assay have been incorrect. Our experience has been that when a given library appears to be inactive, the best course of action is to simply test a different library and let the assay dictate the next step.

Summary

The field of combinatorial chemistry has grown exponentially in the past decade. It can be expected that all aspects of combinatorial chemistry will continue to develop as researchers refine these methods and use them to identify relevant compounds in a variety of biological as well as nonbiological systems. Not only have automated high-throughput screening systems greatly improved in recent years, but it is also likely that the daily synthesis of thousands of compounds will seem routine in the very near future.

Mixture-based combinatorial library approaches will continue to find favor with researchers who have limited resources, limited knowledge of their biological target, and/or assays that are not amenable to classic high-throughput methods. Mixture-based libraries offer a powerful advantage in that very large diversities can be synthesized and screened in a rapid and cost-efficient manner. Mixtures also enable large numbers of compounds to be tested in "low-throughput" assays (e.g., tissue and/or in vivo systems) and in those systems in which target reagents are limited by availability or cost.

The methods encompassed by combinatorial chemistry are now 15 years old. As with virtually all new and far reaching methods, combinatorial methods have been slow to win acceptance. As discussed in this Perspective, this has been especially true for mixture-based combinatorial methods. The resistance to mixture-based methods is likely due to the distance between these approaches and the traditional "one at a time" methods the pharmaceutical industry has successfully employed for decades. As with all innovations, only those methods that prove to be practical will eventually be embraced by those who will benefit by their use. What began as a need to produce larger numbers of compounds per unit time (approaches such as Merrifield's solid-phase method[3,4] and the pin[5] and tea bag[6] parallel methods) has now evolved to permit an individual to synthesize not just hundreds of compounds per year but hundreds of thousands or even millions. Combinatorial methods include high-throughput parallel synthesis, phage display approaches, synthetic mixtures, one-bead−one-compound concepts, etc. These were first directed toward and used by the pharmaceutical industry but have now evolved to encompass all areas of research and development that benefit from increased numbers and/or rapid information gathering. As originally presented and now practiced, combinatorial chemistry results in a tremendous increase in the information

gathering capabilities across all areas of scientific exploration.

In the future, the concepts inherent in combinatorial approaches will be applied to a wide range of other disciplines and interests. Thus, new materials will be devised, synthetic chemical reactions will be readily optimized, and chemical information gathering will be greatly improved. The de novo design of highly specific receptors, new ceramic materials, and artificial catalytic compounds will also be developed by the application of combinatorial methods to these areas. The future direction of combinatorial methods has such a broad range of possibilities that the most important and greatest impact will likely be in an area that has not yet even been considered. What is clear, however, is that combinatorial methods have forever changed the expectations of chemists, biologists, immunologists, molecular biologists, and their organizations in terms of what can and must be done in a given period of time. While combinatorial methods are now becoming more and more part of the routine tools used by the scientific community, they have forged interdisciplinary collaborations that would have been inconceivable in earlier times and with earlier methods. We believe that combinatorial methods will continue to evolve and be used in other areas of basic research and applied science.

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Biographies

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Clemencia Pinilla received her Ph.D. in Microbiology from the Los Andes University in Santa Fe de Bogotá, Colombia, in 1986. She joined The Scripps Research Institute in 1987 as a postdoctoral research fellow and joined TPIMS in 1989. Her research focuses on the use of combinatorial libraries to identify B- and T-cell antigens for infectious diseases and cancer. She is one of the inventors of the patented positional scanning concept. She is an Associate Member at TPIMS and an Officer of Mixture Sciences, Inc.

Jon R. Appel received his B.S. in Biology from San Diego State University in 1986. He joined The Scripps Research Institute in 1987 and joined TPIMS in 1989. He is currently a Senior Scientist in the Immunochemistry Department. He is one of the inventors of the patented positional scanning concept.

Sylvie E. Blondelle received her Ph.D. in Organic Chemistry from the University of Montpellier, France, in 1988. She joined The Scripps Research Institute in 1988 as a postdoctoral research fellow. She joined TPIMS in 1989 and is currently an Associate Member and head of the Biochemistry/Microbiology Department. Her research focuses on the de novo design of functionalized protein-like structures, modulators of biological functions through peptide−protein interactions, and development of novel antimicrobial and antiviral compounds using combinatorial libraries.

Colette T. Dooley received her M.S. in Zoology from University College, Dublin, Ireland, in 1992. She joined TPIMS in 1990 and is currently a Senior Scientist and head of the Neuroendocrinology Department. Her research focuses on the use of combinatorial libraries for the identification of novel ligands for G-coupled receptors, such as the opioids, orphanin, substance P, and melanocortins.

Jutta Eichler received her Ph.D. in Bioorganic Chemistry from Humboldt University, Germany, in 1991. She joined TPIMS in 1991 as a postdoctoral research fellow and was an Assistant Member and head of the Bioorganic Chemistry/Enzymology Department until 1998, when she joined Graffinity Pharmaceutical Design GmbH (Heidelberg, Germany) as the Director of Combinatorial Chemistry.

Adel Nefzi received his Ph.D. in Organic Chemistry from the University of Lausanne, Switzerland, in 1995. He joined TPIMS in 1995 as a postdoctoral research fellow and is currently an Assistant Member in the Chemistry Department working on the development of synthetic and analytical methods for use in combinatorial chemistry.

John M. Ostresh received his M.S. in Chemistry from the University of California, San Diego in 1986. He worked with Dr. Houghten at both The Scripps Research Institute and MPS and joined TPIMS in 1991. As the Director of Chemistry, his research is focused on the development of heterocyclic libraries and new combinatorial chemistry methods. He is the co-inventor of the "libraries from libraries" concept.

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