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Synthetic Combinatorial Libraries of Scaffolded and Unordered Structures

MICHAL LEBL,[1]* VIKTOR KRCHŇÁK,[1] MARCEL PÁTEK,[1] and KIT S. LAM[2]

[1]Selectide Corporation, Subsidiary of Hoechst Marion Roussel, 1580 E. Hanley Boulevard, Oro Valley, Arizona 85737, USA; and [2]Arizona Cancer Center and Department of Medicine, University of Arizona College of Medicine, Tucson, Arizona 85724, USA

Split synthesis can be applied to the synthesis of nonpeptidic combinatorial libraries based on the one-bead–one-compound principle. Examples of the synthesis and screening of libraries based on (1) building a rigid scaffold in the randomization process, (2) randomization of a flexible scaffold structure, and (3) building of heterogenic organic molecules are presented in this article.

INTRODUCTION

The development of new leads for drug design and structure–function relationship studies were revolutionized by the introduction of combinatorial or "library" techniques. Some of the various approaches, including advantages and disadvantages, were described in Lam and Lebl (1996), and the interested reader is referred to a compilation of articles describing the methodologies and the results from their application in a dynamic database (Lebl, 1996). These techniques enable the generation and screening of millions of potentially active structures. Due to the well-developed and fine-tuned synthetic methodologies, peptides were the first group of compounds evaluated by this new approach. However, the challenge today is to synthesize libraries of nonpeptidic structures.

The combinatorial library approach applied at Selectide consists of three basic steps: (1) chemical synthesis based on the split synthesis method (Furka et al., 1991; Houghten et al., 1991; Lam et al., 1991), yielding a library with only one test compound structure on each bead; (2) screening of the library by using either an on-bead binding assay or a multiple-step release assay (Lam and Lebl, 1996); and (3) recovery of positive beads and determination of the structure of the compounds on those beads.

Each chemically synthesized combinatorial library represents a certain structural diversity and multiplicity. Libraries containing sequential repetition of amino acids (peptide libraries) are easy to synthesize, and the structure of compounds of interest can be easily determined by sequencing. However, such libraries do not represent a very

M. Lebl and V. Krchňák are now at Houghton Pharmaceuticals, 3550 General Atomics Court, San Diego, CA 92121 USA.

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*To whom reprint requests/correspondence should be addressed at Houghton Pharmaceuticals, 3550 General Atomics Court, San Diego, California 92121, USA.

high structural diversity because the only variable parameter is the type of side chain connected to the alpha carbons of the peptide backbone, and those side chains occupy only limited conformational space. Combining natural L-amino acids with D-amino acids creates more diversity; nevertheless, the diversity is still quite limited. The advent of nonpeptide libraries increased the diversity of conformational space filled by the test compound subunits and increased the chemical diversity due to the nature of the subunits. In this article, we will give examples of the syntheses of libraries of the following structural types: (1) libraries based on a rigid scaffold structure that is built during the randomization process (Pátek et al., 1995); (2) libraries based on a flexible scaffold that is built during the synthesis of the library and can be randomized (branched scaffold based on diamino acids and the α,β,γ,δ library; Krchnák et al., 1995b); (3) libraries of organic molecules without defined scaffold or backbone ("heterogeneous libraries"; Krchnák et al., 1996). We will not discuss libraries based on preformed scaffolds (usually multifunctional cyclic scaffold, e.g., derivatized cyclopentane or cyclohexane ring, functionalized steroid skeleton, tricarboxybenzene, and diaminobenzoic acid), which are the basis for another convenient preparation of structural diversity (Pátek et al., 1994; Kasal et al., 1995; Kocis et al., 1995; Lebl et al., 1995). Detailed discussion of presented libraries can be found in original articles (Krchnák et al., 1995b, 1996; Pátek et al., 1995). Nonpeptidic libraries designed in various laboratories were recently reviewed in several comprehensive articles (Gordon et al., 1994; Terrett et al., 1995; Ellman and Thompson, 1996; Rinnova and Lebl, 1996).

METHODOLOGY

Solid-phase syntheses were performed on TentaGel S NH_2 130 μm resin, TentaGel S OH 130 μm resin (TG, Rapp Polymere, Tübingen, Germany), polyethylene-grafted copoly(styrene–1% divinylbenzene) 220 μm resin (PEG/PS, Millipore, Bedford, MA), or chlorotrityl polystyrene resin (Advanced ChemTech, Louisville, KY; Bachem Bioscience, King of Prussia, PA). Fluorenylmethyloxycarbonyl (Fmoc) amino acids with standard side-chain protecting groups (Cys protected by acetamidomethyl group) were obtained from Advanced ChemTech or Propeptide (Vert-le-Petit, France). Aromatic hydroxy acids, aldehydes, amino alcohols, diamines, and diacids used in randomizations, diethyl azodicarboxylate (DEAD), diisopropyl azodicarboxylate (DIAD), diisopropyl carbodiimide (DIC), diisopropylethylamine (DIEA), dimethylaminopyridine (DMAP), N-hydroxybenzotriazole (HOBt), phenol, triphenylphosphine (PPh_3), piperidine, sodium triacetoxyborohydride, thioanisole, trifluoroacetic acid (TFA), and triethyl orthoformate were obtained from Aldrich Chemical (Milwaukee, WI) or Sigma (St. Louis, MO). Anhydrous tetrahydrofuran (THF) was obtained from Aldrich Chemical. High-purity solvents (Baxter, McGaw Park, IL) were used without further purification.

Analytical HPLC was carried out on a Waters 625 LC system with a Waters 490E Programmable Multiwavelength Detector (Milford, MA) using Vydac Peptide and Protein C18 analytical column (4.6 × 250 mm, 5 μm, 1 ml/min; The Separation Group, Hesperia, CA). The analytical gradient was run from water containing 0.07% TFA to 60% of acetonitrile (MeCN)/water (0.07% TFA) in 30 min. UV/VIS absorption spectra were recorded on a Hewlett Packard HP 8452A Diode-Array spectrophotometer (Palo Alto, CA) using a 1-cm quartz cuvette. Ion-spray mass spectra were obtained on a triple quadrupole PE-Sciex API III+ mass spectrometer (Per-

kin-Elmer/Sciex, Thornhill, Ontario, Canada) with an articulated ion-spray sample inlet system.

Manual solid-phase synthesis. All reactions were carried out in a plastic syringe equipped with a sintered polypropylene disc at the bottom (Krchnák and Vágner, 1990). To wash the resin, the solvent, typically 3 ml of solvent per 1 ml of swollen resin, plus a small amount of air, were drawn into the syringe: the syringe was shaken for 0.5 min and the solvent discharged. Reactions were performed using 3 ml of solution of reagents per 1 ml of swollen resin beads. The excess of reagents was always calculated with respect to the original substitution of the resin.

Library Based on a Scaffold Structure Built During Randomization (Limited N-Acetyl-Thiazolidine Library)

H-β-Ala-O-TG. A mixture of Fmoc-β-Ala-OH (218 mg, 0.70 mmol), DIC (110 μl, 0.7 mmol), HOBt (95 mg, 0.7 mmol), and DMAP (1.2 mg, 0.01 mmol) in dimethylformamide (DMF; 1 ml) was added to a suspension of TentaGel S-OH resin [500 mg, 0.14 mmol (0.28 mmol/g, 90 μm beads)] in DMF (4 ml), and the suspension was shaken overnight. The Fmoc group was removed by using 20% piperidine/DMF solution (1 + 20 min), thus yielding the modified solid support containing 85% of attached β-Ala as determined from the dibenzofulvene-piperidine absorbance at 302 nm.

H-AA-β-Ala-O-TG. The total amount of resin was split into three equal parts, followed by coupling with HOBt active esters of amino acids for 2–3 hr. Thus, Fmoc-Gly-OH (36 mg, 0.12 mmol), Fmoc-Val-OH (41 mg, 0.12 mmol), and Fmoc-Tyr(But)-OH (55 mg, 0.12 mmol) were used for coupling after activation with HOBt (16 mg, 0.12 mmol) and DIC (19 μl, 0.12 mmol) in DMF (2 ml). After a thorough washing (DMF), the Fmoc group was removed by using 20% piperidine/DMF solution (1 + 20 min), thus yielding the dipeptide resin in nearly quantitative yield as determined from the dibenzofulvene piperidine absorbance at 302 nm.

H-Cys-AA-β-Ala-O-TG. All three parts of resin from the previous step were combined, and a mixture of Fmoc-Cys(Fmoc)-OH (204 mg, 0.36 mmol), HOBt (49 mg, 0.36 mmol), and DIC (56 μl, 0.36 mmol) in DMF (2 ml) was added to the resin suspension. After 3 hr, the Fmoc group was removed with 20% piperidine/DMF solution (1 + 20 min) to give resin-bound mercaptoamines in nearly quantitative yield as determined from the dibenzofulvene piperidine absorbance at 302 nm.

AcOH•H-thiazolidine-AA-β-Ala-O-TG. The total amount of resin was split into three equal parts, followed by reaction of each resin portion with one of three aldehydes in 5% AcOH-CH_3CN:toluene (1:1, 1 ml) solution for 2 hr. Thus, benzaldehyde (36 μl, 0.36 mmol), isobutyraldehyde (33 μl, 0.36 mmol), and isovaleraldehyde (39 μl, 0.36 mmol) were used to form a thiazolidine ring on the solid support. After washing with 5% AcOH-CH_3CN:toluene (1:1), all three parts of resin were combined.

Ac-thiazolidine-AA-β-Ala-O-TG. The suspension of resin was thoroughly washed with acetonitrile (5 × 2 ml), toluene (5 × 2 ml),

and pyridine (5 × 2 ml) and treated with a mixture of acetic anhydride (2 ml) in pyridine (2 ml) for 2 hr at room temperature.

Ac-thiazolidine-AA-β-Ala-OH. After washing the resin with toluene (5 × 2 ml), acetonitrile (5 × 2 ml), and dichloromethane (DCM; 5 × 2 ml), the tert. butyl protection of Tyr was removed by treating the resin with TFA:water (95:5) for 15 min, followed by washing with DCM (5 × 2 ml), acetonitrile (5 × 2 ml), MeOH (5 × 2 ml), and MeOH:water (80:20; 5 × 2 ml). Final release of the material from the solid support with 0.1% NaOH in water:methanol (80:20) for 30 min yielded a mixture of nine thiazolidines, which were characterized by LC-MS.

Library Based on a Randomized Flexible Scaffold Built During the Synthesis (Alpha, Beta, Gamma Library)

The library was synthesized on 10 g of TG. The following procedures were used during the library synthesis.

Fmoc deprotection. The resin was washed with DMF, pretreated with 20% piperidine in DMF for 5 min, treated for 20 min, and washed with DMF (6×). All washes were collected and absorbance measured at 302 nm to calculate Fmoc release (ε_{302} = 8,100 M^{-1} cm^{-1}).

Boc deprotection. The resin was washed with DCM, pretreated with a mixture of 45% TFA, 45% DCM, and 10% anisole for 5 min, treated with the same mixture for 20 min, and washed with DCM (6×), 7% DIEA in DCM (2 × 2 min), and DCM (4×).

Alloc deprotection. The resin was washed five times with DMF, followed by addition of a mixture of DMF/acetic acid/N-methyl morpholine (5 ml/1 ml/0.5 ml), and bubbling with argon for 15 min. After addition of tetrakis(triphenylphosphine)/palladium(0), the reaction was allowed to proceed for 3 hr. The resin was then washed five times each with DMF, DCM, and DMF, followed by treatment with a 2% solution of HOBt in DMF to prevent premature loss of Fmoc-protecting group.

Coupling of carboxylic acids. Carboxylic acid or protected amino acid (3 molar excess) was activated by DIC and HOBt (molar ratio 1:1:1) in DMF. Acetic, propionic, hexanoic, isobutyric, benzoic, succinic, and glutaric acids were coupled as anhydrides. The completeness of each condensation reaction was checked with the ninhydrin test (Kaiser et al., 1969).

Benzyloxycarbonyl (Z) deprotection. The resin was washed with DMF and DCM and pretreated with 10% thioanisole in TFA (2 × 1 min). The third treatment with 10% thioanisole in TFA was performed overnight. The resin was washed with DCM, neutralized with 5% DIEA in DCM, and washed with DCM and DMF.

The library synthesis consisted of 50 synthetic steps:

1. Couple Fmoc-Lys(Alloc)-OH
2. Remove Fmoc
3. Couple Fmoc-Gly-OH
4. Remove Fmoc
5. Couple Fmoc-βAla-OH

6. Remove Fmoc
7. Couple Fmoc-Gly-OH
8. Remove Fmoc
9. Couple Boc-βAla-OH
10. Remove Alloc
11. Divide resin into six portions
12. Couple six coding pairs of Fmoc amino acids (Table 1)
13. Remove Boc
14. Couple Boc-Dap(Alloc)-OH to portions 1 and 2
    Couple Boc-Dab(Alloc)-OH to portions 3 and 4
    Couple Boc-Orn(Alloc)-OH to portions 5 and 6
15. Combine portions 1, 3, and 5
16. Remove Boc
17. Combine portions 2, 4, and 6
18. Remove Alloc
19. Combine all resin
21. Divide resin into 46 portions
22. Couple 46 acids, one acid to each portion
23. Remove Fmoc
24. Couple coding doublets of Fmoc amino acids (Table 1)
25. Combine all resin portions
26. Remove Alloc
27. Remove Boc
28. Divide resin into six portions
29. Couple Boc-Dap(Alloc)-OH to portions 1 and 2
    Couple Boc-Dab(Alloc)-OH to portions 3 and 4
    Couple Boc-Orn(Alloc)-OH to portions 5 and 6
30. Remove Fmoc
31. Couple six coding pairs of Fmoc amino acids (Table 1)
32. Combine portions 1, 3, and 5
33. Remove Boc
34. Combine portions 2, 4, and 6
35. Remove Alloc
36. Combine all resin portions
37. Divide resin into 46 portions
38. Couple 46 acids, one acid to each resin portion
39. Remove Fmoc
40. Couple coding doublets of Fmoc amino acids (Table 1)
41. Combine all resins
42. Remove Alloc
43. Remove Boc
44. Divide resin into 50 portions
45. Couple 50 acids, one acid to each resin portion
46. Remove Fmoc
47. Couple coding doublets of Fmoc amino acids (Table 1)
48. Remove Fmoc
49. Combine all resins
50. Remove Z

Library of Organic Molecules Without Defined Scaffold or Backbone (Heterogeneous Library)

Preparation of linker–resin construct. TentaGel S NH_2 (1 g, 0.25 mmol/g, 130 μm average particle size) or PEG/PS hydrochlo-

TABLE 1. Coding Scheme for the α,β,γ Library[a]

	Acids	Coding doublets	
Carboxylic			
1	Acetic acid[b]	Abu	Cha
2	Propionic acid[b]	Abu	Ile
3	Hexanoic acid[b]	Abu	Leu
4	Isobutyric acid[b]	Abu	Val
5	Trimethylacetic acid	Abu	Nval
6	Cyclopentane carboxylic acid	Abu	Chg
7	Cyclohexane carboxylic	Abu	Nal1
8	Cyclohexylacetic acid	Abu	Nal2
9	1-Adamantaneacetic acid	Abu	Nle
10	Z-Gly	Cha	Ile
11	Z-βAla	Cha	Leu
12	Z-ε-aminocaproic acid	Cha	Val
13	γ-Guanidinobutyric acid hydrochloride	Cha	Nval
14	Z-Met	Cha	Chg
15	Succinic acid[b]	Cha	Nal1
16	Glutaric acid[b]	Cha	Nal2
17	cis-1,2-Cyclohexanedicarboxylic acid	Cha	Nle
18	Succinamic acid	Ile	Leu
19	Benzoic acid[b]	Ile	Val
20	1-Naphthylacetic acid	Ile	Nval
21	Biphenylacetic acid	Ile	Chg
22	Diphenylacetic acid	Ile	Nal1
23	4-Aminobenzylacetic acid	Ile	Nal2
24	4-Dimethylaminobenzoic acid	Ile	Nle
25	4-Guanidinobenzoic acid	Leu	Val
26	4-Nitrophenylacetic acid	Leu	Nval
27	4,5-Dimethoxy-2-nitrobenzoic acid	Leu	Chg
28	4-Chlorobenzoic acid	Leu	Nal1
29	α,α,α-Trifluoro-*p*-toluic acid	Leu	Nal2
30	4-Hydroxybenzoic acid	Leu	Nle
31	4-Hydroxyphenyl acetic acid	Val	Nval
32	3-(3,4,5-Trimethoxyphenyl)propionic acid	Val	Chg
33	4-(3-Methyl-5-oxo-2-pyrazolin-1-yl)benzoic acid	Val	Nal1
34	Z-Pro	Val	Nal2
35	3-Carboxyl-1,4-dimethyl-2-pyrrole acetic acid	Val	Nle
36	2-Methyl-4-nitro-1-imidazole propionic acid	Nval	Chg
37	2-Amino-1-imidazole acetic acid	Nval	Nal1
38	3-Amino-1,2,4-triazole-5-carboxylic acid	Nval	Nal2
39	4-Imidazole acetic acid	Nval	Nle
40	Isonicotinic acid	Chg	Nal1
41	2,3-Pyridinedicarboxylic acid	Chg	Nal1
42	2-Pyrazinecarboxylic acid	Chg	Nle
43	2,3-Pyrazinedicarboxylic acid	Nal1	Nal2
44	1-Methylindole-2-carboxylic acid	Nal1	Nle
45	2-Methyl-3-indoleacetic acid	Nal2	Nle
46	Indole-4-carboxylic acid	Abu	PheCl
47	Fmoc-Ser(tBu)	not coded	
48	Fmoc-Thr(tBu)	not coded	
49	Fmoc-pipecolinic acid	not coded	
50	Fmoc-3,4-dichlorophenylalanine	not coded	
Diamino			
1	Dap(Alloc)	Ala	Gln
2	Boc-Dap	Ala	Asn
3	Dab(Alloc)	Ala	Phe
4	Boc-Dab	Gln	Asn
5	Orn(Alloc)	Gln	Phe
6	Boc-Orn	Asn	Phe

[a]Fmoc-protected amino acids were not used in the first and second randomization. Abu, α-aminobutyric acid; Cha, cyclohexylalanine; Chg, cyclohexylglycine; Nal1, 1-naphtylalanine; Nal2, 2-naphtylalanine; Nle, norleucine; Nval, norvaline; PheCl, *p*-chlorophenylalanine.
[b]Coupled as anhydride.

ride (1 g, 0.58 mmol/g, 220 μm average particle size) was swollen in DMF (swollen volume = 5 ml/1 g). (PEG/PS beads were neutralized by repeated 3-min treatment with 10% DIEA in DMF and washed five times with DMF.) The iminodiacetic acid-based double cleavable linker (IdaDC linker; structure in Fig. 1; 0.765 g, 0.75 mmol) and HOBt (0.1 g, 0.75 mmol) were dissolved in DMF, activated by DIC (75 μl, 0.75 mmol), and the solution was transferred to the resin. After overnight reaction, the resin was checked for the presence of free amino groups by the ninhydrin test (Kaiser et al., 1969) and washed five times with DMF. If the test was positive, the entire coupling procedure was repeated.

Preparation of glutaric acid-modified resin. TentaGel S NH_2 resin was swollen in DMF, a fivefold excess of glutaric anhydride in DMF was added to the resin, and the slurry was shaken on the tumbler for 1 hr. The resin was washed five times with DMF and checked for the presence of free amino groups by the ninhydrin test. If the test was positive, the acylation was repeated.

Reverse esterification by Fmoc amino alcohols. The carboxyl functionality containing resin (0.5 ml, ca. 100 mg) was washed five times with dry THF, then 0.5 ml of a 0.5-M solution of Fmoc–amino alcohol in THF and 0.25 ml of 0.5 M PPh_3 in THF was added. The syringe with the resin was chilled in the freezer for 10 min, and DIAD in THF (0.25 ml of 0.5 M solution) was added. The resin was placed again for 15 min in the freezer to cool down and the reaction was continued for 2 hr at room temperature. The resin was washed three times with THF, and the reaction was repeated overnight by using half of the volume of reagents. The resin was washed with DMF, and the Fmoc group was cleaved.

Attachment of building blocks to 2-chlorotrityl chloride resin. The syringe was filled with 100 mg of resin (Advanced ChemTech, substitution 1.7 mmol/g; or Bachem, substitution 1.15 mmol/g), a threefold excess (0.5 mmol and 0.35 mmol, respectively) of diamine, alcohol, or acid was dissolved in 1 ml of dichloroethane (DCE) and added to the resin. For the ester and ether formation, a fivefold excess of DIEA with respect to acid or alcohol was added to the DCE solution before adding the solvent to the resin. All reactions were carried out at 60°C for 2 hr. Conversion of amines was 60–95%, of alcohols 55%, and of acids 75%.

Condensation of aromatic hydroxy acids. The aromatic hydroxy acid and HOBt were dissolved in DMF (0.5 mmol in 1 ml), DIC was added, the solution transferred into the syringe, and the mixture shaken for 1 hr. The presence of free amino groups was checked (Kaiser et al., 1969). If the test was positive, the resin was washed with DMF (3×), and coupling was repeated.

Etherification of polymer-supported phenols by alcohols. The resin (ca. 0.8 ml/syringe) was washed five times with dry THF. A solution of PPh_3 in THF (1.5 ml of 1 M solution) and 3 mmol of alcohol were added and the slurry shaken. A solution of 1.5 mmol of DIAD in 0.2 ml of THF was added to the resin in four portions in 5-min intervals. The mixture was shaken overnight and then washed five times with DMF.

Reductive alkylation. The resin (0.2 ml, ca. 0.01 mmol of amino groups) was washed three times with DMF, followed by the addition of triethyl orthoformate (0.3 ml) and aldehyde (0.05 mmol), and the slurry was shaken vigorously for 5 hr. The resin was washed three times with DCM and a 0.1-M suspension of sodium triacetoxyborohydride in DCM (0.5 ml, 0.05 mmol) was added. The mixture was shaken for 16 hr. After the resin was washed with DMF (3×), both steps were repeated.

Side-chain deprotection and final washes. The resin was washed with DMF (3×) and DCM (3×), followed by treatment with TFA containing 5% *p*-cresol (1 min and 2 hr). The resin was then washed with TFA (3×), DCM (5×), and MeOH (3×) and dried on a freeze dryer overnight.

Cleavage of compounds from linkers

Double cleavable IdaDC linker. The resin was shaken in a syringe equipped with a frit in 0.1 M ammonium acetate buffer, pH 7.5–8, for 2 hr. The resin was washed three times with 80% aqueous MeCN, and the combined cleavage and wash solutions were lyophilized. The resin was then exposed to 5 ml of 0.5% NaOH for 2 hr, the solution was acidified by AcOH (pH 6–7), and the resin washed with 80% aqueous MeCN. The combined cleavage and wash solutions were lyophilized.

Single cleavable ester linker. The resin was shaken in a syringe equipped with a frit in 0.5% NaOH for 0.5 hr. The resin was then washed three times with water and three times with 80% MeCN in water. The combined cleavage and wash solutions were acidified by the addition of AcOH and lyophilized.

Single cleavable trityl linker. The resin was shaken in a syringe equipped with a frit in 95% TFA and 5% water for 0.5 hr. The resin was filtered off, the filtrate diluted by water, and the solution lyophilized.

Screening for binding to streptavidin. The fully deprotected bead library is first washed extensively with double-distilled water, then twice with phosphate buffered saline (PBS; 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl, pH 7.2) containing 0.1% gelatin (w/v) and 0.1% Tween 20 (v/v). Other blocking agents such as bovine serum albumin or other nonionic detergents may also be used. The 0.28 M salt in the 2× PBS prevents some of the nonspecific ionic interaction. The library is then incubated in the same buffer with streptavidin-alkaline phosphatase. After incubating for 1 hr, the library is washed extensively, mixed with 5 bromo-4-chloro-3-indolyl phosphate (BCIP), and plated in a dozen of polystyrene Petri dishes (100 × 20 mm). Within 1 hr, the positive bead turn turquoise. These beads are then isolated for structure determination. To eliminate false-positive beads, the positive beads are decolorized with DMF and restained in the presence of a specific ligand (biotin).

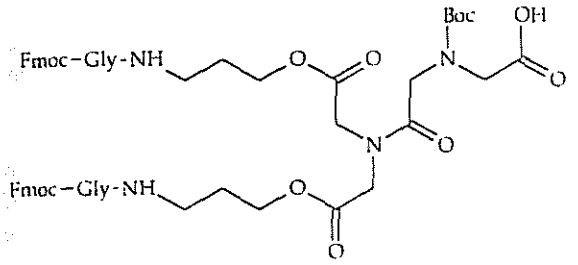

Figure 1. Structure of the linker allowing two orthogonal releases.

RESULTS AND DISCUSSION

We have designed libraries for the one-bead–one-compound strategy (Lam et al., 1991) by using the split/mix synthetic scheme (Furka et al., 1991; Houghten et al., 1991; Lam et al., 1991) in combination with cleavable linkers that allow the release of compounds into a solution, preferably in distinct multiple steps (Lebl et al., 1993; Salmon et al., 1993). We have applied three linkers in those libraries: (1) an iminodiacetic-acid-based double cleavable linker (IdaDC linker, Fig. 1; Kocis et al., 1993), (2) a single cleavable ester type linker, and (3) a trityl linker (Barlos et al., 1991). These libraries can also be screened on bead in solid-phase assays.

The IdaDC linker allows a two-stage release of defined amounts of compound into solution. Ester bond is used to attach compounds to both releasable arms. The ester bonds were cleaved in two steps by (1) the nucleophilic attack of an internal nucleophile, resulting in diketopiperazine formation, and (2) the alkaline hydrolysis. Compounds are attached to the linker via an ester bond of Fmoc-Gly-NH-$(CH_2)_3$-OH, and, when released to the aqueous solution, they contain an identical carboxy terminus (hydroxypropylamide of Gly). Diluted alkali or ammonia vapors have been used to release compounds from the singly cleavable ester linker. The trityl linker is compatible with strong nucleophiles; compounds were cleaved by acids.

Library Based on a Rigid Scaffold

A thiazolidine-structure-containing library is given here as an example of a scaffold-based library in which the scaffold is built during the library synthesis. The detailed account of the chemistry with special attention to the stability and stereochemistry of this library type was published earlier (Pátek et al., 1995). Split/mix strategy was employed to synthesize a small, limited set of nine N-acetylthiazolidines. To avoid quantitative release of construct from the resin after dipeptide assembly, a β-Ala spacer was inserted between the solid support and the first amino acid. In the first position, three amino acids (Gly, Val, Tyr(But)) were used for randomization. Coupling of Fmoc-Cys(Fmoc)-OH, followed by deprotection of cysteine with 20% piperidine in DMF, afforded resin-bound mercaptoamines. Subsequent reaction with three aldehydes (PhCHO, iPrCHO, iBuCHO) gave the corresponding thiazolidines, which were acetylated by using an excess of acetic anhydride in pyridine. TFA-mediated Boc deprotection and concomitant final release of the material from the solid support with 0.1% NaOH provided mixture of nine thiazolidines that were characterized by LC-MS. The synthesis scheme is given in Figure 2.

Library on a Flexible Randomized Scaffold

The α,β,γ library was designed to accommodate different side-chain spacing on a variable scaffold

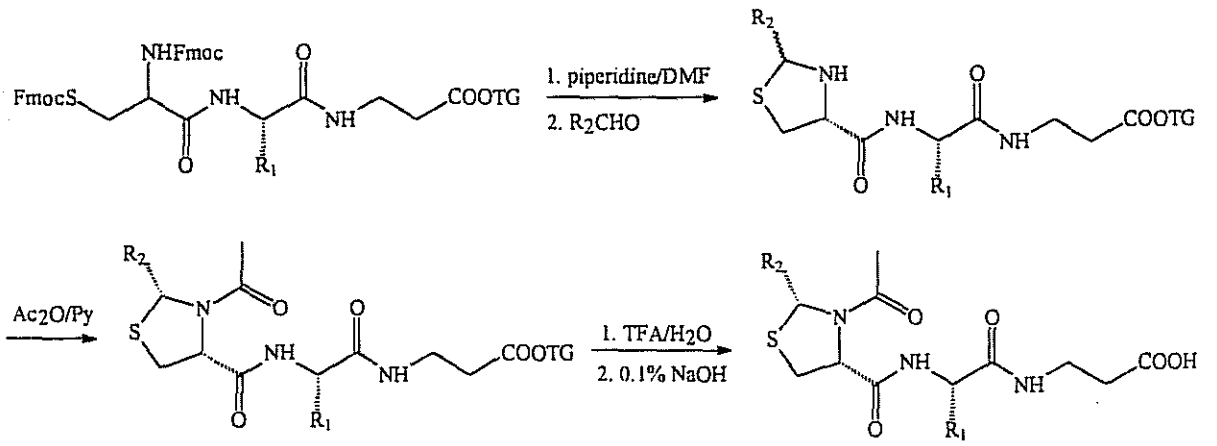

Figure 2. Synthesis scheme for the thiazolidine library.

within one library and thus provide high diversity (dissimilarity) among library members. Because there was not an adequate number of commercially available β and γ amino acids, we created the diversity by coupling carboxylic acids to the amino groups of diamino acids. The backbone (scaffold) was formed by diamino acids connected via amide bonds, and carboxylic acids ("pharmacophores") were attached to the second amino group of each diamino acid. The amino terminal diamino acid was derivatized by two carboxylic acids. Because the α,β,γ-library compounds are not sequenceable and the complexity of the library is too high to apply molecular weight determination by mass spectrometry for the structure elucidation, a history tag was independently synthesized on each bead, thus enabling the structure of each α,β,γ compound to be decoded. The coding technique has been described elsewhere (Brenner and Lerner, 1992; Kerr et al., 1993; Nikolaiev et al., 1993; Nielsen and Janda, 1994; Chabala et al., 1995; Moran et al., 1995; Nicolaou et al., 1995). We have used amino acids for the coding structure and Edman degradation as an analytical tool to determine the sequence of the coding peptide. To reduce the number of coding amino acids, we used doublets of amino acids to code for each building block; n amino acids can code for n*(n-1)/2 building blocks. With this approach, we were able to avoid three functional amino acids, and therefore we did not require any side-chain protecting groups for coding amino acids.

The synthesis of the coding peptide independently on the synthesis of the library compounds requires orthogonal combinations of amino protecting groups. We used the Fmoc-protecting group for the α-amino group of coding amino acids, Boc- and Alloc-protecting groups for two amino groups of the diamino acids (Dap, Dab, Orn), the α-amino group being protected by Boc group (the reverse protection would result in the same library format). Boc-Daa(Alloc) amino acids were prepared from commercially available Boc-Daa (Daa, diamino acid). The side chains of carboxylic acids that required protection were protected with the benzyloxycarbonyl (Z) group.

To combine α, β, γ and δ amide bonds in one library, one can use two differently protected diamino acids, e.g., Boc-Daa(Alloc) and Alloc-Daa(Boc). Boc-Daa(Alloc) can be coupled to one-half of the library beads and Alloc-Daa(Boc) to the second half. After combining both parts and removing one protecting group, e.g., Boc, from the entire library, α and β, γ, and δ amino groups are available for acylation with carboxylic acids. Alternatively, the same result could be obtained with only one derivative, Boc-Daa(Alloc). After coupling this amino acid, the resin can be split into two portions and the Boc group removed from the first portion and the Alloc group cleaved from the second portion. Both portions can then be combined and distributed into as many coupling vessels as carboxylic acids used for acylation. With this approach, each bead contains only one type of free amino group, either α, β, γ, or δ, to which the carboxylic acid in each reaction vessel is coupled. We used the second strategy.

Both synthetic strategies required coding for the carboxylic acid and the diamino acid and for the position to which the carboxylic acid was coupled. This was achieved by using two different coding doublets for the same amino acid (e.g., Val/Ile and Val/Nval doublets for Boc-Dap(Alloc); see Table 1) and exposing the part with the first coding doublet to TFA (cleaving Boc and liberating the α-amino group for subsequent acylation), whereas the Alloc group was cleaved from the second part, which was coded by the second doublet. The synthetic strategy is illustrated in Figure 3.

Structurally Heterogeneous Libraries

High diversity of compounds in structurally heterogeneous libraries can be achieved by connecting bifunctional building blocks that do not have a fixed position of these two functional groups. This is in contrast to peptides, where the building blocks, amino acids, have always the amino group in the alpha position with respect to the carboxyl group. We selected five types of functional groups to be present on those building blocks: amines, acids, aldehydes, alcohols, and phenols. Fifteen different bifunctional reagents can be formed out of these five functionalities. We have chosen six types of the most commonly available bifunctional building blocks: aromatic hydroxy acids, aromatic hydroxy aldehydes, amino alcohols, diamines, diacids, and amino acids.

We have used the following reactions to connect two functional groups in our model libraries: (1) amide bond formation, (2) reductive alkylation, (3) esterification, and (4) aryl ether formation. All building blocks have been tested in the respective reactions on solid phase and provided good-to-excellent yield of the expected products. The reaction conditions for each condensation are described in the Methodology section.

There is a variety of ways to combine the described building blocks for library synthesis. We illustrate the feasibility of the described approach on examples of three library designs. The library examples include three randomization steps. Three

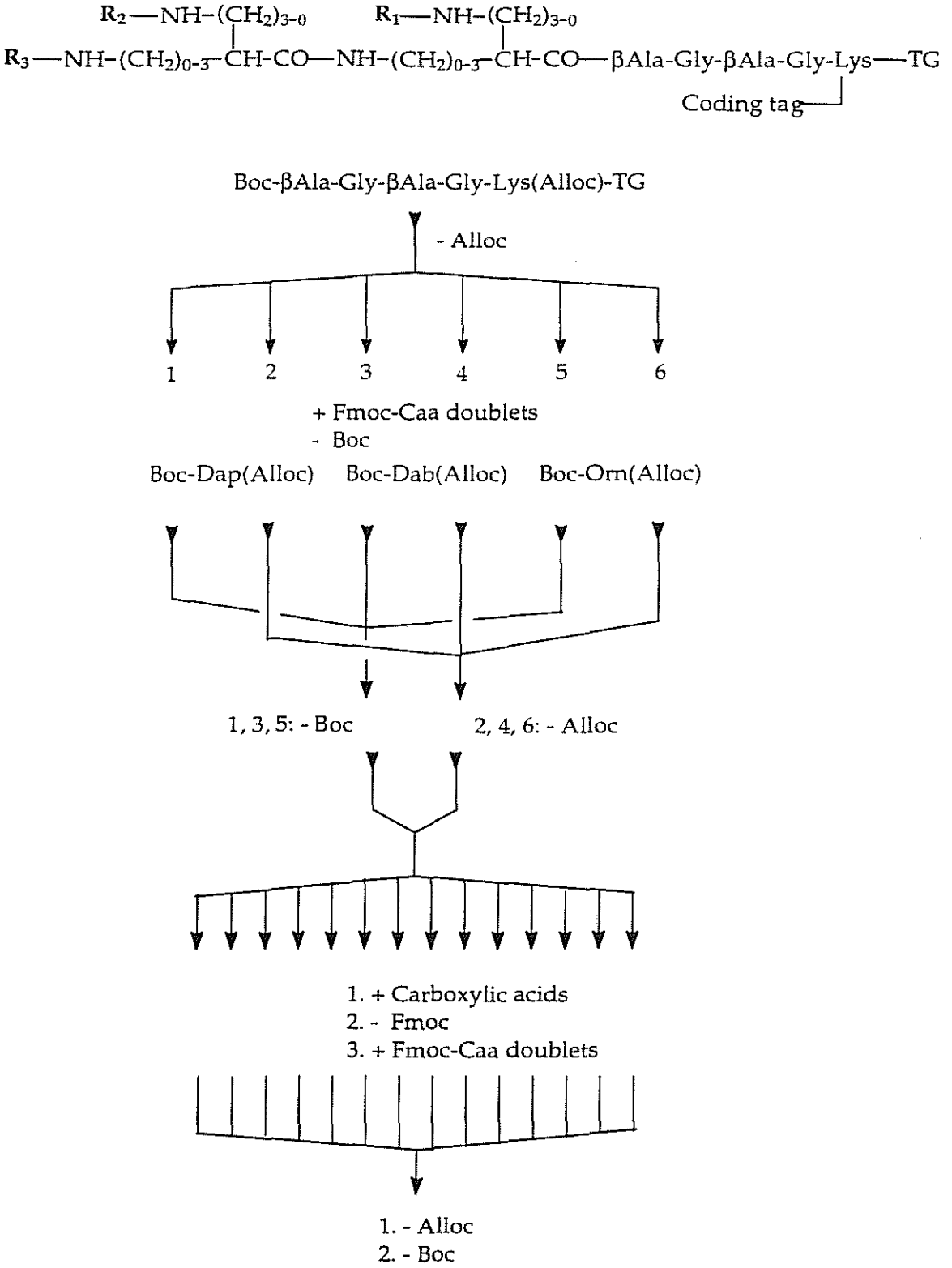

Figure 3. Synthesis scheme for the flexible library with a randomized scaffold.

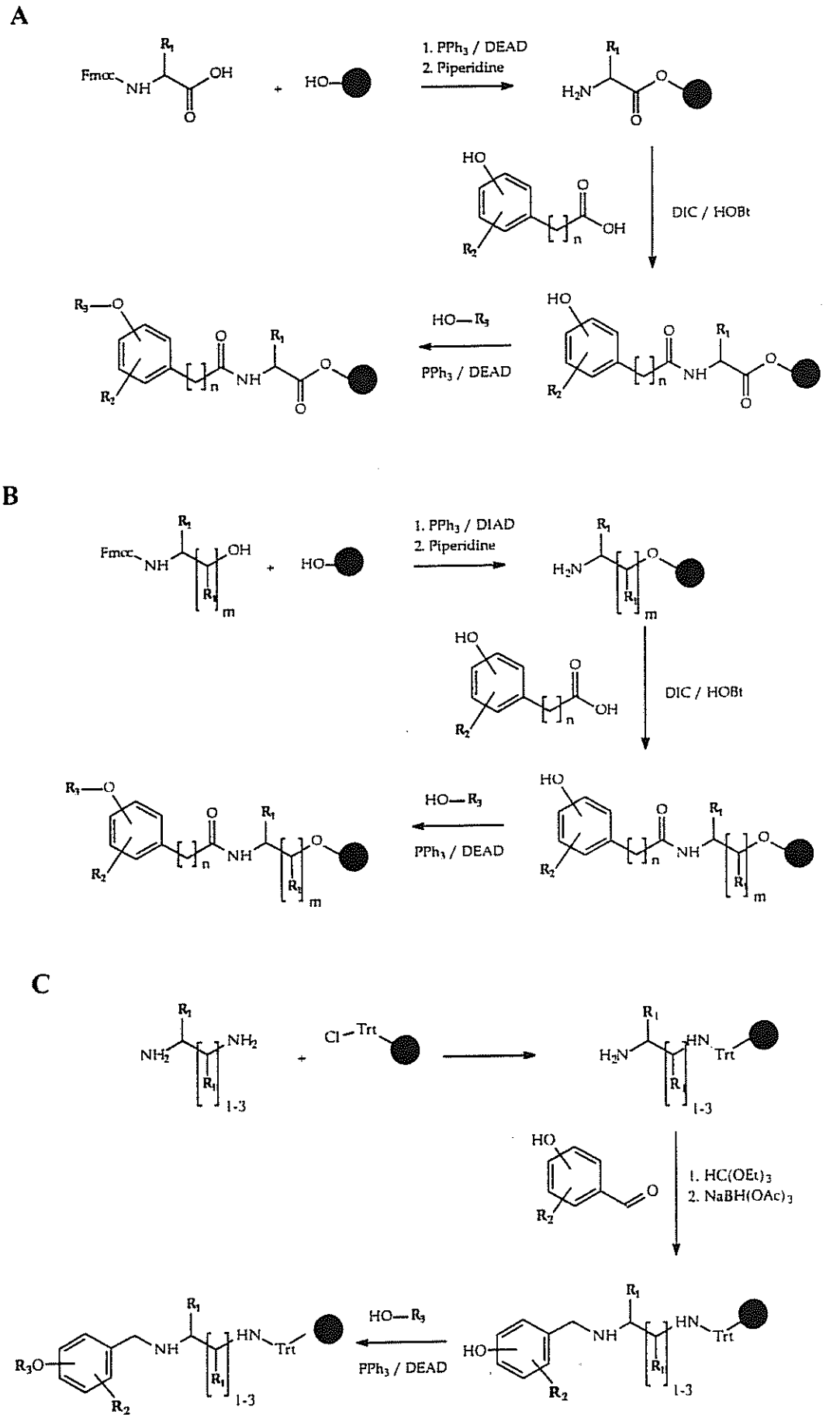
A
Fmoc
NH
OH
HO
1. PPh3 / DEAD
2. Piperidine
H2N
DIC / HOBt
HO—R3
PPh3 / DEAD
B
1. PPh3 / DIAD
2. Piperidine
C
NH2
Cl—Trt
HN
Trt
1-3
1. HC(OEt)3
2. NaBH(OAc)3

Figure 4.

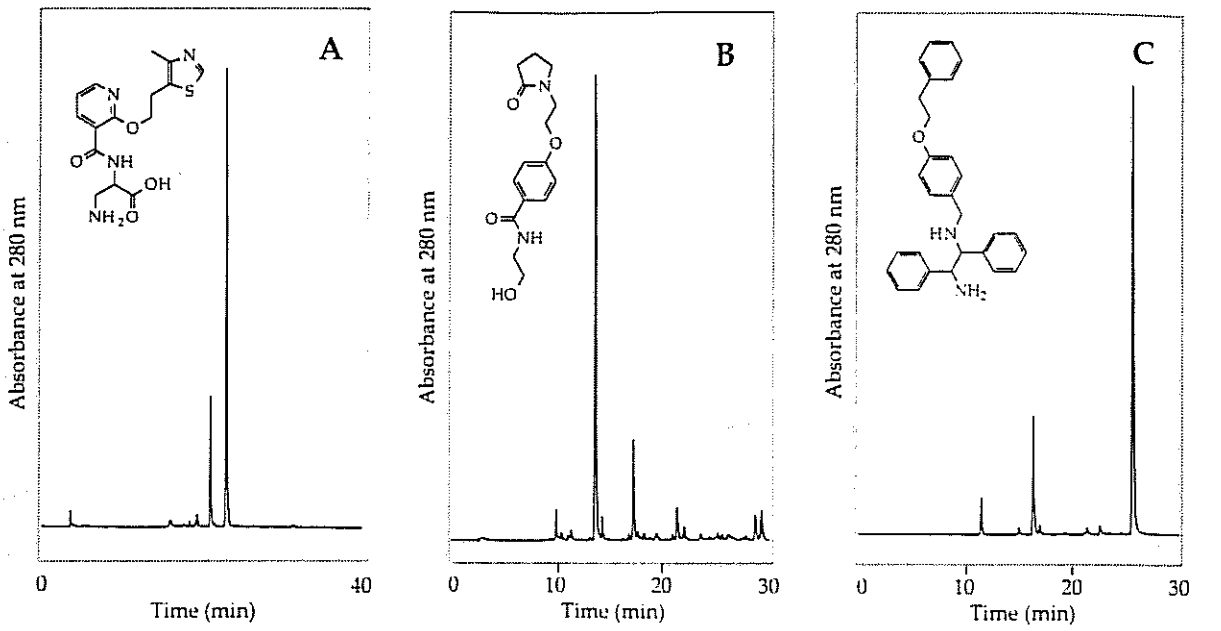

Figure 5. **A–C:** HPLC traces of crude model compounds from individual libraries represented in Figure 4.

randomizations create a reasonable complexity of libraries and still keep the molecular weight in a low range. The libraries selected as examples illustrate all the described reactions. In all libraries, the last step used monofunctional building blocks that reacted with the particular functional group present on the resin beads.

N-(Alkoxyacyl) Amino Acids

The library was designed to be synthesized on the IdaDC linker and on hydroxy TentaGel by direct esterification of the hydroxyl groups with N-protected amino acids. The synthesis of the library included three randomization steps (Fig. 4A): (1) attachment amino acids to the amino group of IdaDC linker or hydroxyl group of resin beads and (2) coupling of aromatic hydroxy acids to the amino group of the amino acid, during which no acylation of unprotected hydroxyl groups was detected under acylation conditions employed (DIC/HOBt) (Krchnak et al., 1995a). However, only mild activation of carboxyl should be used here to avoid undesirable acylation of phenols; (3) etherification of the phenolic hydroxyl group with alcohols by Mitsunobu ether formation (Mitsunobu et al., 1967; Mitsunobu, 1981). The formation of aryl ethers on insoluble carrier has been described (Krchnák et al., 1995a; Rano and Chapman, 1995).

N-(Alkoxyacyl) Amino Alcohols

This library provides compounds with free hydroxyl groups. It was synthesized on a single cleavable ester linker; however, polymer bearing the carboxyl group was esterified by the alcohol in solution. The synthesis involved three randomization steps (Fig. 4B): (1) esterification of polymer-supported carboxyl groups with Fmoc-protected amino alcohols, (2) coupling of aromatic hydroxy acids to the amino groups, and (3) etherification of phenolic hydroxyl groups by alcohols. Mitsunobu ether formation using DEAD and PPh_3 was found to be the best method for attachment of alcohols to the resin. The last step of the library synthesis was the deprotection of side chains by TFA.

N-(Alkoxyaryl) Diamines

The library was synthesized on the trityl linker, from which the compounds are cleaved with acids. The library synthesis consisted of three randomization steps (Fig. 4C): (1) reaction of diamines with chlorotrityl resin and (2) reductive alkylation of amino groups with phenol aldehydes. We originally used sodium borohydride as a reducing agent (Stanková et al., 1994; Flegelová et al., 1995). Later, we formed the Schiff base by using triethyl

Figure 4. Synthesis scheme for heterogeneous libraries. **A:** Library of alkoxyacyl amino acids. **B:** Library of alkoxyacyl aminoalcoholes. **C:** Library of alkoxyaryl diamines.

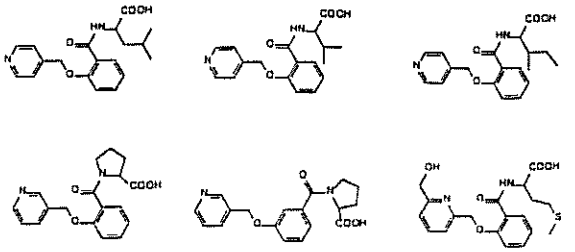

Figure 6. Streptavidin binders found in heterogeneous libraries.

orthoformate (Look et al., 1995) and reduced it with sodium triacetoxyborohydride (Gordon and Steele, 1995); (3) Mitsunobu aryl ether formation was performed with alcohols.

To document the quality of compounds synthesized in the heterogeneous libraries, the analytical HPLC profiles of representative model compounds from each library type are shown in Figure 5.

Screening of these libraries provided hits in several bioassays. As an example, the hits found in screening for binding to streptavidin in heterogeneous library are given in Figure 6.

CONCLUSION

The technique of solid-phase split synthesis can be used for the convenient preparation of both peptide and nonpeptide libraries. Libraries of both types can be screened on-bead and in solution, and the identified structures can be used as the starting point for structure activity studies and design of new families of drugs.

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