Peptide Research

Linear Presentation of Variable Side-Chain Spacing in a Highly Diverse Combinatorial Library

Viktor Krchňák, Aleksandra S. Weichsel, Dasha Cabel and Michal Lebl
Selectide Corporation, Tucson, AZ, USA

ABSTRACT

A synthetic library that presents potential pharmacophores in a linear fashion with variable spacing was designed (α,β,γ-library). To prove the concept, we synthesized a number of individual compounds as well as a model library. Diamino acids connected by amide bonds via their α- or side-chain amino groups were used to form the backbone (scaffold) of this library. The remaining amino group of the diamino acids were acylated by a variety of carboxylic acids, generating an appreciable diversity of compounds in this library. The compositions of compounds in the library were identified by reading a peptide tag synthesized concurrently with the library structures. This code contained the information regarding the carboxylic acid coupled, and the diamino acid and amino group to which the acid was coupled.

INTRODUCTION

There is no doubt that linear and cyclic peptide libraries have been and still are a valuable tool in a drug discovery program. However, there are several reasons to depart from this structural format of peptide libraries. Peptides are susceptible to degradation by a large number of proteases, and oral availability is generally poor. These adverse properties are related at least in part to the amide bond backbone of peptides (20). A separate reason for the design and synthesis of non-peptide libraries (which prompted us to design the library described in this communication) is to introduce structural diversity not present in peptide libraries. As addressed by a number of investigators (7,9,10,18,19,21,22), limited structural diversity, or dissimilarity, is caused by the monotony of the peptide backbone, where the only changing structural feature is the side chain connected to the α-carbons of the backbone.

Since Merrifield's solid-phase chemistry of amide bond formation (12) has been fine-tuned for years, we decided to design a library that still uses amide chemistry but generates considerably higher diversity among members of the library. For example, a library containing only β-amino acids will present the side chains in a different way when compared with a peptide library. Nevertheless, the diversity will again reflect a monotonous repetition of side-chain placement. However, the combination of α, β and γ amide bonds in one library increases the diversity substantially. Only a limited number of β- and γ-amino acids are available for library construction. To avoid synthesis of these building blocks, we decided to use readily available diamino acids as building blocks and acylate the second amino group (the one not involved in the backbone formation) by a variety of carboxylic acids.

We have already described the use of readily available building blocks, such as carboxylic acids, amines, alcohols etc., for diversity generation in recent years (9–11). In this article we report in detail the synthesis of one particular library format utilizing this principle. The use of diamino acids for the scaffold and carboxylic acids for randomization has recently been described by Eichler et al. (5).

MATERIALS AND METHODS

Library synthesis was performed on TentaGel S NH_2 130-μm resin (TG; Rapp Polymere, Tubingen, Germany) (2). Fluorenylmethyloxycarbonyl (Fmoc) amino acids with standard side-chain-protecting groups were obtained from Advanced ChemTech (Louisville, KY, USA) or Propeptide (Vert-le-Petit, France). *tert*-Butyloxycarbonyl (Boc) derivatives of diaminopropionic (Dap) and diaminobutyric (Dab) acids and ornithine (Orn) were purchased from Bachem BioScience (King of Prussia, PA, USA). SCAL linker (17) was a generous gift of CSPS (Tucson, AZ, USA). Allyloxycarbonyl (Alloc) chloride, anisole, diisopropylcarbodiimide (DIC), diisopropylethylamine (DIEA), *N*-hydroxybenzotriazole (HOBt), piperidine, *N*-methyl morpholine, tetrakis(triphenylphosphine)-palladium(0), thioanisole, trifluoroacetic acid (TFA), trimethyl-chlorosilane, triphenylphosphine and carboxylic acids used as building blocks were obtained from Aldrich Chemical (Milwaukee, WI, USA) or Sigma Chemical (St. Louis, MO, USA). Commercial-grade solvents were used without further purification.

Analytical HPLC was carried out on a Waters 625 LC system and their 490E Programmable Multiwavelength Detector (Milford, MA, USA) using a Vydac Peptide and Protein C18 analytical column (0.46 × 250 mm, 5 μm, 1

mL/min) (The Separation Group, Hesperia, CA, USA). The analytical gradient was run from water containing 0.07% TFA to 60% of acetonitrile (ACN)/water in 60 min. UV/VIS absorption spectra were recorded on a Hewlett Packard HP 8452A Diode-Array spectrophotometer (Palo Alto, CA, USA) using a 1-cm quartz cuvette. Ionspray mass spectra were obtained on a triple quadrupole PE-Sciex API III+ mass spectrometer (PE-Sciex, Thornhill, Ontario, Canada) with an articulated ion spray sample inlet system. Sequencing of the amino acid code was performed on an Applied Biosystems 477A Protein Sequencer (currently Perkin-Elmer/Applied Bio-systems Division, Foster City, CA, USA).

Boc-Dap(Alloc)-OH

A solution of Alloc chloride (50 mmol, 5.28 mL) in dioxane was added dropwise to a solution of Boc-Dap-OH (40 mmol, 8.4 g) in 40 mL 2 M NaOH, and the pH of the reaction mixture was kept above 12 by the addition of 2 M NaOH. The reaction mixture was stirred overnight at RT and extracted 3 times with petroleum ether; the aqueous layer was acidified by 0.5 M $KHSO_4$ (pH 3.0), and the product was extracted with ethyl acetate (4 times). The ethyl acetate extracts were washed with a saturated solution of NaCl, dried with $MgSO_4$ and the ethyl acetate was evaporated in vacuo to yield 8.4 g (70%) of oil, characterized by TLC (methanol/chloroform/acetic acid, 10/90/1, Rf = 0.51), retention time in gradient HPLC of 34.0 min and correct molecular peak on mass spectrum.

Boc-Dab(Alloc)-OH (Rf = 0.54, Rt = 35.5 min) and Boc-Orn(Alloc)-OH (Rf = 0.56, Rt = 37.0 min) were prepared analogously, and each product provided the correct molecular peak on mass spectrocopy (MS).

Library Synthesis

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 dimethylformamide (DMF), pretreated with 20% piperidine in DMF for 5 min, treated for 20 min and washed with DMF 6 times. All washes were collected and absorbance was measured at 302 nm to calculate

Protocol 1. Synthesis of Model α,β,γ-Library

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 6 aliquots
12.	Couple 6 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 into 46 portions
22.	Couple 46 acids
23.	Remove Fmoc
24.	Couple coding doublets of Fmoc amino acids (Table 1)
25.	Mix the resin
26.	Remove Alloc
27.	Remove Boc
28.	Divide resin into 6 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 6 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
37.	Divide into 46 portions
38.	Couple 46 acids
39.	Remove Fmoc
40.	Couple coding doublets of Fmoc amino acids (Table 1)
41.	Mix resin
42.	Remove Alloc
43.	Remove Boc
44.	Divide resin into 50 portions
45.	Couple 50 acids
46.	Remove Fmoc
47.	Couple coding doublets of Fmoc amino acids (Table 1)
48.	Remove Fmoc
49.	Mix resin
50.	Remove Z

Fmoc release ($\varepsilon_{302} = 8100$).

Boc deprotection: The resin was washed with dichloromethane (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 times.

Alloc deprotection: The resin was washed 5 times with DMF, a mixture of DMF/acetic acid/*N*-methyl morpholine (5 mL/1 mL/0.5 mL) was added, argon was bubbled for 15 min, tetrakis(triphenylphosphine)-palladium(0) was added and the reaction was allowed to proceed for 3 h. Then the resin was washed with DMF, DCM and DMF (five times each).

Coupling of carboxylic acids: Three molar excess of protected amino acid 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 using the ninhydrin test.

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

The library synthesis consisted of 50 synthetic steps, which are shown in Protocol 1.

Models for Fmoc Cleavage

Four protected diamino acids [Fmoc-Lys(Boc), Boc-Lys(Fmoc), Fmoc-Orn(Boc) and Boc-Orn(Fmoc)] were coupled to TentaGel S NH_2 resin (HOBt/DIC activation, 3 molar excess, 2 h). After washing with DMF (3 times) and DCM (5 times), we removed the Boc groups by 30 min treatment with 50% TFA in DCM; the resin was washed with DCM and dried in vacuum. A sample of the resin was neutralized by 5% DIEA in DCM; washed with DMF (5 times) and the resin was incubated in DMF. Absorbance of the solution was measured at 302 nm and the quantity of Fmoc release to the solution was calculated.

Synthesis of Individual Compounds

The synthesis of discrete α,β,γ-compounds were performed in a polypropylene syringe equipped with a polypropylene frit. TentaGel S NH_2 (150 mg, 0.2 mmol NH_2/g) was swollen in DMF, and SCAL linker (17) was coupled (3 molar excess, DIC/HOBt activation) overnight. The synthesis of R_2-CO-Daa_2(R_3-CO-Daa_3(R_4-CO))-Daa_1(R_1-CO)-NH_2 consisted of the steps shown in Protocol 2 (Daa_1, Daa_2, Daa_3 are diamino acids and R_1, R_2, R_3, R_4 are side-chains of carboxylic acids coupled to amino groups of diamino acids).

Protocol 2. Synthesis of R_2-CO-Daa_2 (R_3-CO-Daa_3 (R_4-CO))-Daa_1 (R_1-CO)-NH_2

1.	Couple Fmoc-Daa_1(Boc)
2.	Remove Boc
3.	Couple R_1-COOH
4.	Remove Fmoc
5.	Couple Fmoc-Daa_2(Boc)
6.	Remove Fmoc
7.	Couple R_2-COOH
8.	Remove Boc
9.	Couple Fmoc-Daa_3(Boc)
10.	Remove Fmoc
11.	Couple R_3-COOH
12.	Remove Boc
13.	Couple R_4-COOH

The resin was washed with DMF and DCM, and SCAL linker was reduced in the following way: *(i)* the resin was washed with DCM; *(ii)* 1 M solution of triphenylphosphine/trimethylchlorosilane/DCM was added and the mixture was shaken for 30 min; *(iii)* steps 1 and 2 were repeated 4 times. Free carboxamides were obtained after treatment with 95% TFA plus 5% water for 1 hour and precipitation with ether. The product was washed with ether 3 times, dried, dissolved in 5% acetic acid and lyophilized.

The following model compounds were synthesized: guanidinobutyryl-Dap(guanidinobutyryl-Dap(adamantylacetyl))-Orn(4-hydroxybenzoyl)-NH_2 (I), yield 37 mg, MW 853.5, Rt 32.1 min; benzoyl-Dap(guanidinobutyryl-Orn((4-trifluoromethyl)benzoyl))-Dap-(acetyl)-NH_2 (II), yield 31 mg, MW 762.3, Rt 35.1 min; guanidinobutyryl-Orn(guanidinobutyryl-Orn(4-hydroxybenzoyl-Dap(4-(dimethylamino)benzoyl))-NH_2 (III), yield 22 mg, MW 852.5, Rt 24.5 min. Mass spectra of all products provided the correct molecular peaks.

RESULTS AND DISCUSSION

The α,β,γ-library was designed to accommodate different side-chain spacing on a variable scaffold within one library and thus provide high diversity (dissimilarity) among library members. Since there was not an adequate number of commercially available β- and γ-amino acids, we created diversity by coupling carboxylic acids to the amino group 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. From the four most common diamino acids, Lys, Orn, Dab and Dap, we omitted Lys, the diamino acid with the longest side chain, for two reasons: *(i)* α,β,γ-Compounds are conformationally flexible, and use of Lys as a scaffold residue would produce the most floppy compound, due to Lys having the longest side-chain, and *(ii)* the complexity of the library is very high, due to randomization of both "pharmacophores" (carboxylic acids) as well as the scaffold (diamino acids). Thus, omitting certain diamino acids had little effect on the practical number of compounds that could be synthesized. The use of Orn introduced a δ-amide bond, but we kept the name α,β,γ-library for the general description of a library containing bonds other than only α-amide.

The synthesis of the α,β,γ-library was designed for Lam's "one-bead–one-compound" strategy (8). This combinatorial technique does not track the history of discrete compounds during the synthesis, but determines the structure only after positive beads are identified (for review see, for example, References 7 and 10). Since 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 spectometry for elucidation of the structure, a history tag was independently synthesized on each bead, enabling the structure of each α,β,γ-compound to be decoded. The coding technique was described previously (6,13–16). 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 (cf. Reference 16). By 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.

Synthesis of the coding peptide independently of 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 and the Boc- and Alloc-protecting groups for two amino groups of diamino acids (Dap, Dab and Orn), the α-amino group being protected by the 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 by the 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, Alloc-Daa(Boc) to the second half. After combining both parts and removing one protecting group, e.g., Boc, from the entire library, there would be both α- as well β-, γ- and δ-amino groups available for acylation with carboxylic acids. Alternatively, the same result could be achieved with only one derivative, Boc-Daa(Alloc). After coupling this amino acid, the resin can be split into two portions: the Boc group removed from the first part and the Alloc group cleaved from the second portion. Then both portions can be combined and distributed into as many coupling vessels as there are carboxylic acids used for acylation. By 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 this second strategy.

Both synthetic strategies required coding for the carboxylic acid, 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., Ala/Gln and Ala/Asn

Table 1. Coding Scheme for the α, β, γ-Library

	Carboxylic Acids	Coding Doublets
1	Acetic acid[a]	Abu Cha
2	Propionic acid[a]	Abu Ile
3	Hexanoic acid[a]	Abu Leu
4	Isobutyric acid[a]	Abu Val
5	Trimethylacetic acid	Abu Nval
6	Cyclopentanecarboxylic acid	Abu Chg
7	Cyclohexanecarboxylic acid	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[a]	Cha Nal1
16	Glutaric acid[a]	Cha Nal2
17	cis-1,2-Cyclohexanedicarboxylic acid	Cha Nle
18	Succinamic acid	Ile Leu
19	Benzoic acid[a]	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-Hydroxyphenylacetic 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-pyrroleacetic acid	Val Nle
36	2-Methyl-4-nitro-1-imidazolepropionic acid	Nval Chg
37	2-Amino-1-imidazoleacetic acid	Nval Nal1
38	3-Amino-1,2,4-triazole-5-carboxylic acid	Nval Nal2
39	4-Imidazoleacetic acid	Nval Nle
40	Isonicotinic acid	Chg Nal1
41	2,3-Pyridinedicarboxylic acid	Chg Nal2
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 Acids	Coding Doublet
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

Note: Fmoc-protected amino acids were not used in the first and the second randomizations. Abbreviations: Abu, α-aminobutyric acid; Cha, cyclohexylalanine; Chg, cyclohexylglycine; Nal1, 1-naphthylalanine; Nal2, 2-naphthylalanine; Nle, norleucine; Nval, norvaline; PheCl, *p*-chlorophenylalanine.

[a]Coupled as anhydride.

doublets for Boc-Dap(Alloc); see Table 1) and exposing the part having 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 coded by the second doublet.

To prove the synthetic concept, we synthesized individual library compounds without a coding arm and then a model library. The backbone of model α,β,γ-compounds consisted of three diamino acids, and for their synthesis we used TentaGel S NH_2 and SCAL linker (17). SCAL linker is a safety-

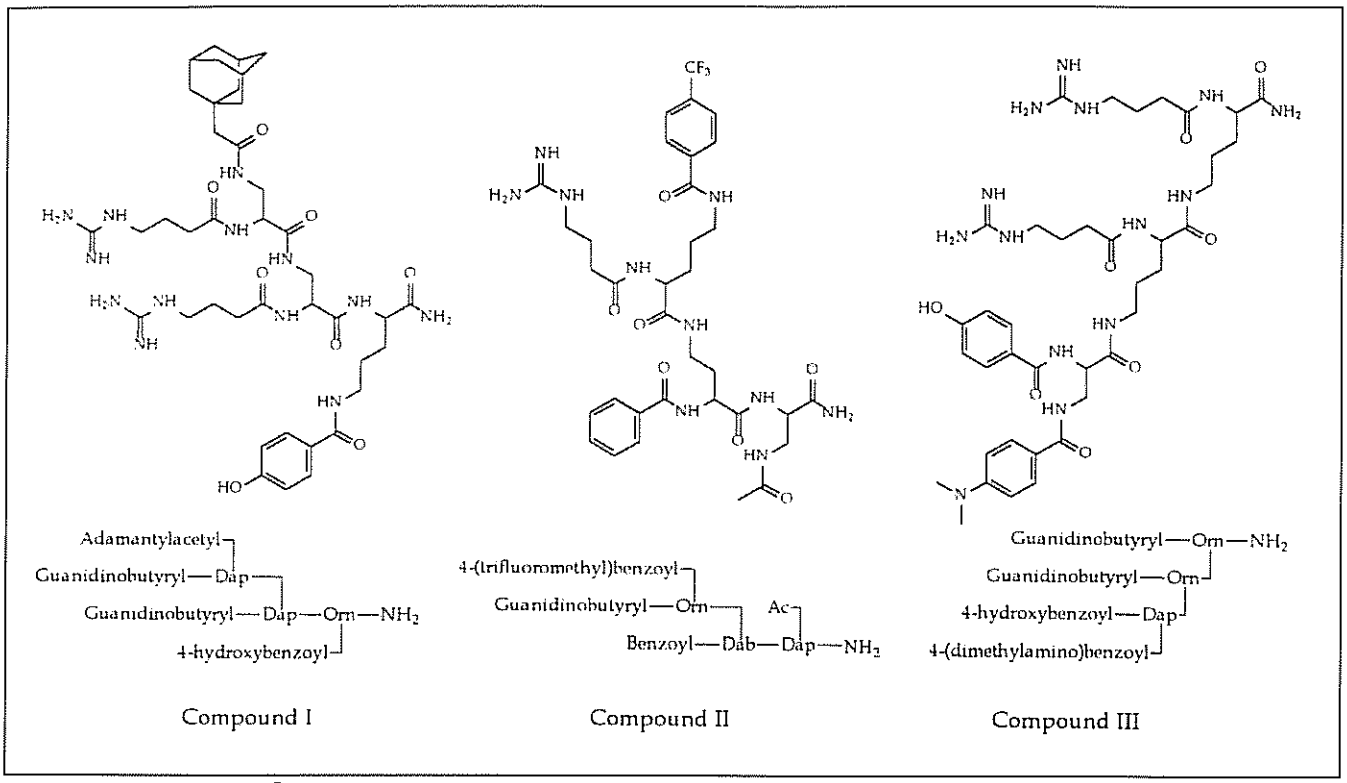

Figure 1. Structure of three α,β,γ-compounds synthesized as models.

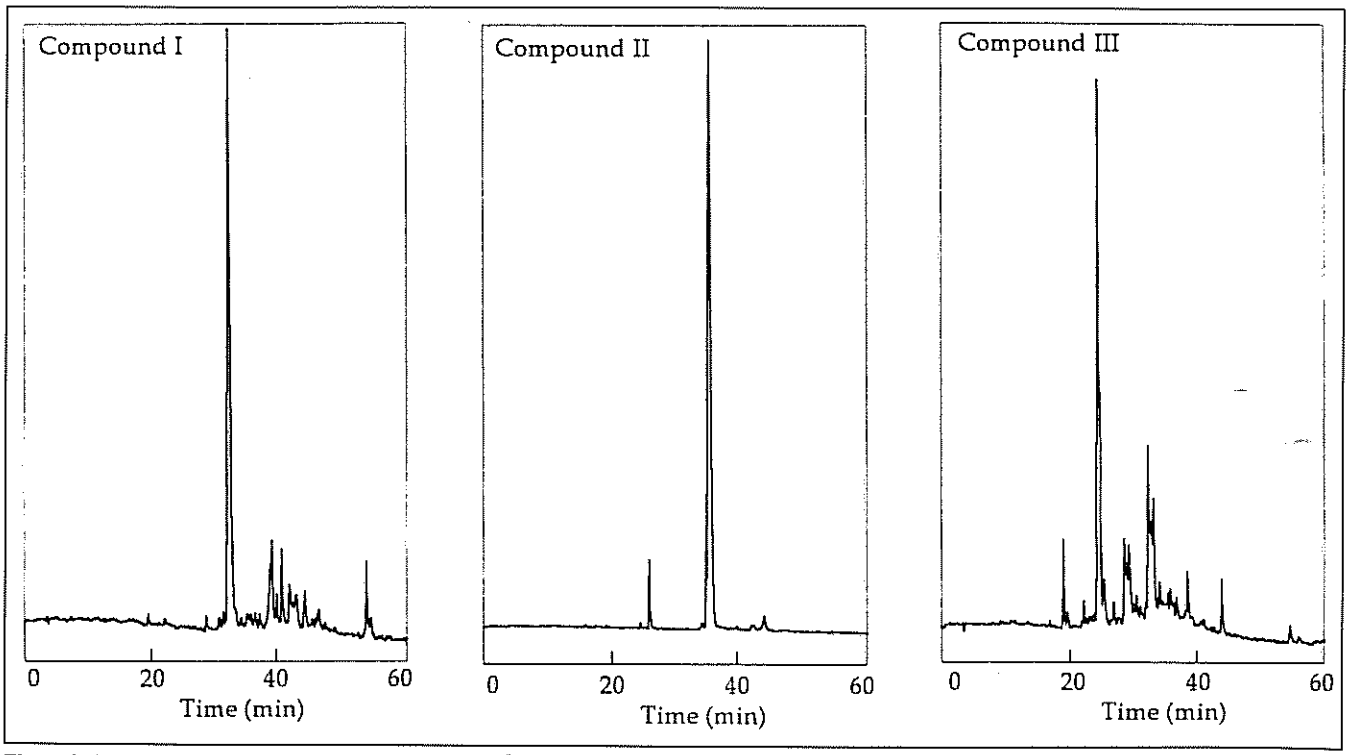

Figure 2. Analytical gradient HPLC profiles of crude α,β,γ-compounds.

catch benzhydrylamine-type linker providing carboxamides. Because there was no need to build a coding arm for the synthesis of individual α,β,γ-compounds, we used Fmoc-Daa(Boc)-protected diamino acids (commercially available). Otherwise, the synthetic strategy was the same. We synthesized three individual compounds (for their structures, see Figure 1). Guanidinobutyric acid was purposely included in those models (to mimic the most difficult scenario), since we used this acid without protection of the guanidino group and its coupling was sluggish.

The purity of the crude compounds is shown on gradient HPLC profiles (Figure 2), and the correct molecular weight of the product was confirmed by mass spectrometry.

The number of compounds in the library is obviously influenced not only by the number of carboxylic acids used in randomizations but also by the length of the backbone. Supposing that three to four interactions between the ligand and target molecule result in acceptable specific binding, the backbone of the α,β,γ-library should be composed of two to three diamino acids, resulting in three to four randomization points. In the case of a shorter backbone, one can use a larger number of carboxylic acids in each randomization step and still synthesize a library containing a large number of compounds. Longer backbone structures map larger conformational space; however, the space will be mapped by the individual compounds in a library less densely than in shorter backbone libraries.

Out of those two possibilities, we have synthesized the first model library with the backbone composed of two diamino acids. The synthesis of the model library was carried out in the following way. Fmoc-Lys(Alloc) was coupled to the resin and its α-amino group was used to synthesize the screening arm. The sequencing arm was synthesized on the ε-amino group. The linker βAla-Gly-βAla-Gly was assembled on the α-amino group to separate the screening arm from the coding structure and the bead using Fmoc chemistry (except for the amino terminal βAla, which was coupled as Boc protected). After removal of the Alloc ε-amino protecting group, the resin was divided into six parts, and six different doublets of coding amino acids were coupled to the coding arm (Table 1). The Boc group from the linker was then cleaved, and Boc-Dap(Alloc) was coupled to parts 1 and 2, Boc-Dab(Alloc) to parts 3 and 4 and Boc-Orn(Alloc) to the remaining two parts. In the next step, parts 1, 3 and 5 were combined and the Boc group was removed. Parts 2, 4, and 6 were also combined and the Alloc group was removed. Both parts were combined, the beads were thoroughly mixed and distributed into 46 vessels. Forty-six carboxylic acids were coupled, followed by removing the Fmoc group from the coding arm and coupling using 46 doublets of coding amino acids.

The entire coding arm consisted of a mixture of pentapeptides that coded for three randomizations of carboxylic acids (sequencing cycle Nos. 1, 2 and 4) and the diamino acid and position where the carboxylic acid was coupled (cycle Nos. 3 and 5). Fifty carboxylic acids were used in the last randomization and included amino acids with Fmoc protection that could not be used in the previous steps. The scheme of the first cycle of the library synthesis is summarized in Figure 3.

After finishing the library synthesis,

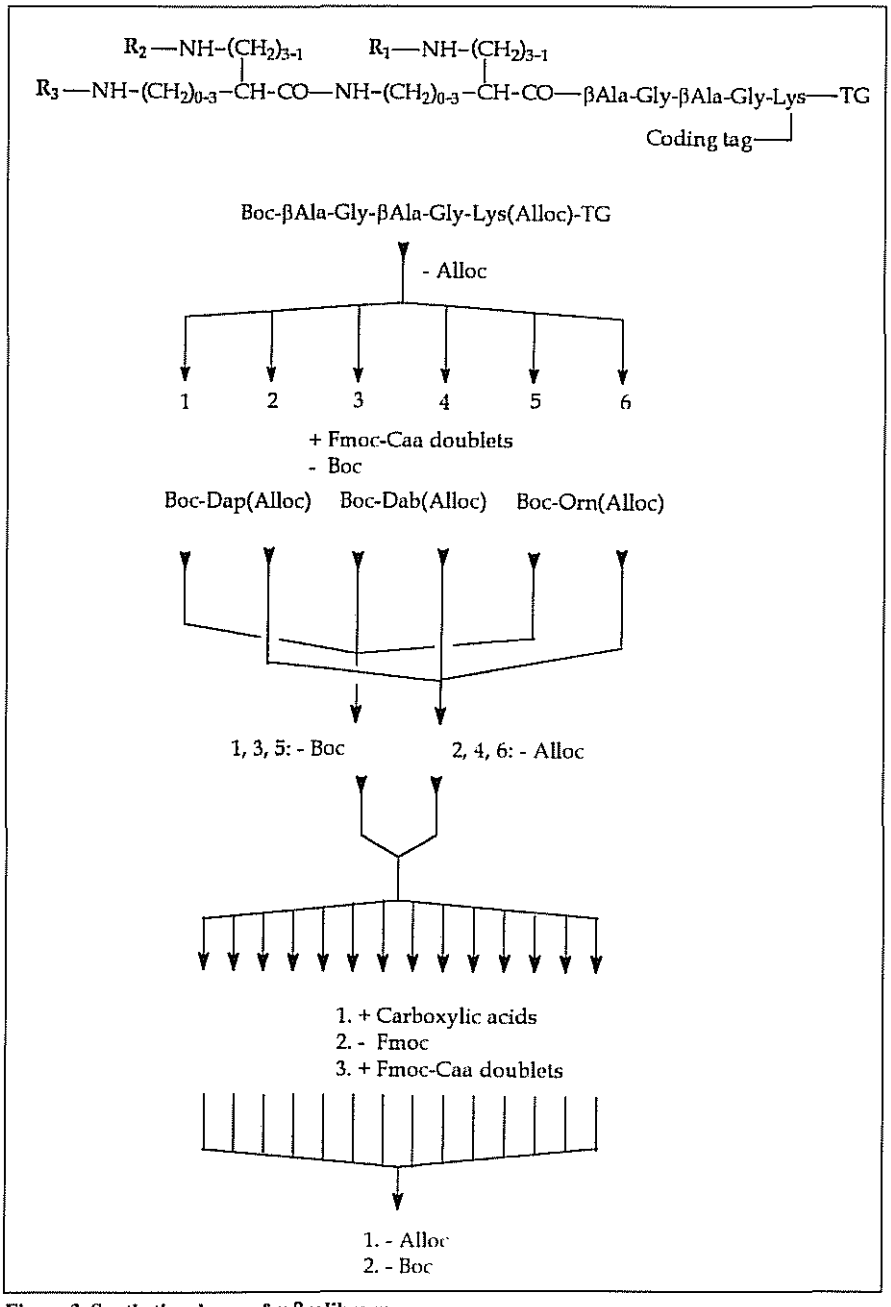

Figure 3. Synthetic scheme of α,β,γ-library.

Table 2. Extent of Fmoc Cleavage by Free Amino Group After 12 Hours

Amino Acid	Fmoc Release
Fmoc-Lys-TG	81%
Fmoc-Orn-TG	80%
Lys(Fmoc)-TG	75%
Orn(Fmoc)-TG	30%

we sequenced randomly selected beads and found that the amount of amino acid cleaved from the coding arm was unacceptably low (below 10 pmol). We simulated the library synthesis on model compounds and found that the Fmoc groups were cleaved from the coding arm by the free amino groups of the diamino acids on the screening arm. Cleavage of the Fmoc group by the free α-amino group was studied but never found substantial (1,3,4). In the case of the synthesis of the α,β,γ-library, the more basic side-chain amino groups are left free for an appreciable period of time. It takes approximately one hour to distribute the library beads into appropriate reaction vessels and add the activated carboxylic acids. During this period of time, the side-chain amino groups of Dap, Dab and Orn started to cleave the Fmoc group from the coding arm. Subsequent coupling of activated carboxylic acids acylated the free amino groups, thereby lowering the amount of amino groups available for the coding peptide.

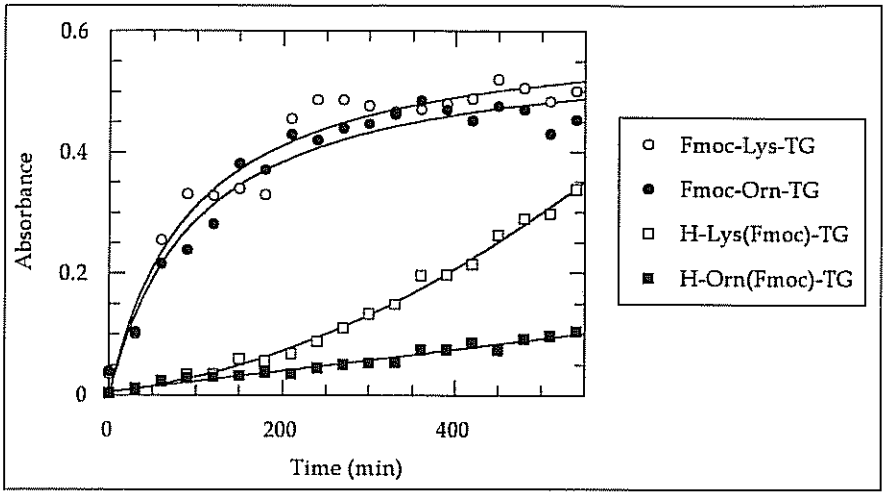

Figure 4. Kinetics of Fmoc group cleavage on four model compounds.

To address the cleavage of the Fmoc group by free amino groups on the resin, we followed the Fmoc release on four model compounds, two of them having a free α-amino group (H-Lys(Fmoc)-TG and H-Orn(Fmoc)-TG) and two models with a free side-chain amino group (Fmoc-Lys-TG and Fmoc-Orn-TG). The kinetics of Fmoc cleavage in DMF at room temperature is shown in Figure 4, and the extent of Fmoc cleavage after 12 h in Table 2. The α-amino group cleaves Fmoc very slowly, and under the standard conditions of solid-phase peptide synthesis it is negligible. More basic side-chain amino groups cause substantial release of Fmoc groups, which is particularly critical in the case of the library synthesis, since the handling time (distribution of resin and activated carboxylic acids) is sufficiently long to observe substantial Fmoc cleavage. The kinetics of H-Lys(Fmoc)-TG decomposition speaks in favor of autocatalytic mechanisms: The difference between lysine and ornithine derivatives points to the importance of steric arrangement.

To avoid this undesirable side reaction, we found that washing with a solution of HOBt in DMF sufficiently protects the Fmoc amino groups by protonating the free amino groups. We did not observe any significant Fmoc cleavage when the Fmoc-Lys-TG was exposed overnight to a solution of HOBt in DMF.

CONCLUSION

We have designed a highly diverse combinatorial library, the α,β,γ-library,

whose characteristic feature is variable side-chain spacing. This was accomplished by randomization of backbone ("scaffold") to which carboxylic acids ("pharmacophores") were attached. The synthesis involved amide bond chemistry and we did not face any particular problems in the preparation of individual library compounds. However, when a model library with a coding arm was synthesized, we faced substantial Fmoc group cleavage caused by the extended exposure of Fmoc groups to free amino groups present on the resin. Protonation of those groups by HOBt prevents undesirable loss of the Fmoc group.

REFERENCES

1. **Atherton, E., C.J. Logan and R.C. Sheppard.** 1981. Peptide synthesis. Part 2. Procedures for solid-phase synthesis using *N*-alpha-fluorenylmethoxycarbonylamino-acids on polyamide supports. Synthesis of substance P and of acyl carrier protein 65-74 decapeptide. J. Chem. Soc., Perkin Trans. *II*:538-546.
2. **Bayer, E. and W. Rapp.** 1992. Polystyrene-immobilized PEG chains. Dynamics and application in peptide synthesis, immunology, and chromatography, p. 325-345. *In* J.M. Harris (Ed.), Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications. Plenum Press, New York.
3. **Bodanszky, M., S.S. Deshmane and J. Martinez.** 1979. Side reactions in peptide synthesis. 11. Possible removal of the 9-fluorenylmethyloxycarbonyl group by the amino components during coupling. J. Org. Chem. *44*:1622-1625.
4. **Chan, C.D., M. Waki, M. Ahmad, J. Meienhofer, E.O. Lundell and J.D. Haug.** 1980. Preparation and properties of N^{α}-9-fluorenylmethyloxycarbonylamino acids bearing tert-butyl side chain protection. Int. J. Pept. Protein Res. *15*:59-66.
5. **Eichler, J., A.W. Lucka and R.A. Houghten.** 1994. Cyclic peptide template combinatorial libraries: synthesis and identification of chymotrypsin inhibitors. Pept. Res. *7*:300-307.
6. **Kerr, J.M., S.C. Banville and R.N. Zuckermann.** 1993. Encoded combinatorial peptide libraries containing non-natural amino acids. J. Am. Chem. Soc. *115*:2529-2531.
7. **Krchňák, V., N.F. Sepetov, P. Kočiš, M. Patek, K.S. Lam and M. Lebl.** Combinatorial libraries of synthetic structures: Synthesis screening and structure determination. *In* R. Cortese (Ed.), Molecular Repertoirs. Walter de Gruyter, Berlin (In press).
8. **Lam, K.S., S.E. Salmon, E.M. Hersh, V.J. Hruby, W.M. Kazmierski and R.J. Knapp.** 1991. A new type of synthetic peptide library for identifying ligand-binding activity. Nature *354*:82-84.
9. **Lebl, M., V. Krchňák, P. Safář, A. Stierandová, N.F. Sepetov, P. Kočiš and K.S. Lam.** 1994. Construction and screening of libraries of peptide and non-peptide structures. Techniques in Protein Chemistry *5*:541-548.
10. **Lebl, M., V. Krchňák, N.F. Sepetov, B. Seligmann, P. Strop, S. Felder and K.S. Lam.** 1995. One-bead-one-structure combinatorial libraries. Biopolymers *37*:177-198.
11. **Lebl, M., V. Krchňák, A. Stierandová, P. Safář, P. Kočiš, V. Nikolaev, N.F. Sepetov, R. Ferguson, B. Seligmann, K.S. Lam and S.E. Salmon.** 1994. Nonsequenceable and/or non-peptide libraries, p. 1007-1008. *In* R.S. Hodges and J.A. Smith (Eds.), Peptides. Proceedings of the Thirteenth American Peptide Symposium. ESCOM, Leiden.
12. **Merrifield, R.B.** 1963. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. J. Am. Chem. Soc. *85*:2149-2154.
13. **Nestler, H.P., P.A. Bartlett and W.C. Still.** 1994. A general method for molecular tagging of encoded combinatorial chemistry libraries. J. Org. Chem. *59*:4723-4724.
14. **Nielsen, J., S. Brenner and K.D. Janda.** 1993. Synthetic methods for the implementation of encoded combinatorial chemistry. J. Am. Chem. Soc. *115*:9812-9813.
15. **Nikolaev, V., A. Stierandová, V. Krchňák, B. Seligmann, K.S. Lam, S.E. Salmon and M. Lebl.** 1993. Peptide-encoding for structure determination of nonsequenceable polymers within libraries synthesized and tested on solid-phase supports. Pept. Res. *6*:161-170.
16. **Ohlmeyer, M.H.J., R.N. Swanson, L.W. Dillard, J.C. Reader, G. Asouline, R. Kobayashi, M. Wigler and W.C. Still.** 1993. Complex synthetic chemical libraries indexed with molecular tags. Proc. Natl. Acad. Sci. USA *90*:10922-10926.
17. **Pátek, M. and M. Lebl.** 1991. Safety-catch anchoring linkage for synthesis of peptide amides by Boc/Fmoc strategy. Tetrahedron Lett. 3891-3894.
18. **Simon, R.J., R.S. Kaina, R.N. Zuckermann, V.D. Huebner, D.A. Jewell, S. Banville, N.G. Simon, L. Wang, S. Rosenberg, C.K. Marlowe, D.C. Spellmeyer, R. Tan, A.D. Frankel, D.V. Santi, F.E. Cohen and P.A. Bartlett.** 1992. Peptoids: a modular approach to drug discovery. Proc. Natl. Acad. Sci. USA *89*:9367-9371.
19. **Simon, R.J., E.J. Martin, S.M. Miller, R.N. Zuckermann, J.M. Blaney and W.H. Moos.** 1994. Using peptoid libraries [oligo N-substituted glycines] for drug discovery, p. 533-539. *In* J.W. Crabb (Ed.), Techniques in Protein Chemistry. Academic Press, San Diego.
20. **Taylor, M.D. and G.L. Amidon.** 1995. Peptide-based drug design: controlling transport and metabolism. American Chemical Society, Washington.
21. **Zuckermann, R.N.** 1993. The Chemical synthesis of peptidomimetic libraries. Curr. Opin. Struct. Biol. *3*:580-584.
22. **Zuckermann, R.N., E.J. Martin, D.C. Spellmeyer, G.B. Stauber, K.R. Shoemaker, J.M. Kerr, G.M. Figliozzi, D.A. Goff, M.A. Siani, R.J. Simon, S.C. Banville, E.G. Brown, L. Wang, L.S. Richter and W.H. Moos.** 1994. Discovery of nanomolar ligands for 7-transmembrane G- protein-coupled receptors from a diverse N- (substituted) glycine peptoid library. J. Med. Chem. *37*:2678-2685.

Received 12 May 1995; accepted 19 June 1995.

Address correspondence to:
Viktor Krchňák
Selectide Corporation
1580 East Hanley Boulevard
Tucson, AZ 85737, USA