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The Synthesis of Peptidomimetic Combinatorial Libraries Through Successive Amide Alkylations

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Abstract—A soluble peptidomimetic combinatorial library of 57,500 compounds was prepared. This library has a dipeptide scaffold with each amide hydrogen replaced with five different alkyl groups (methyl, ethyl, allyl, benzyl, or naphthylmethyl). Solid-phase methodology in combination with N-alkylation were used to synthesize the library, which incorporated 50 different L-, D-, and unnatural amino acids. Repetitive amide alkylations were carried out on the solid support following each amino acid coupling step. Individual model compounds were synthesized in order to optimize the alkylation conditions, to study potential amino acid side chain modifications, to determine the extent of racemization, and to provide analytical controls during the library synthesis. Copyright © 1996 Elsevier Science Ltd

Introduction

The development and successful application of soluble peptide combinatorial libraries have demonstrated the power of combinatorial approaches for basic research and for the identification of highly active compounds having therapeutic potential. The synthetic approaches for the generation of combinatorial libraries were first developed for peptides[1–3] (see review[4]). The bioavailability and biostability of L-amino acid peptides are known to be limited and, therefore, decrease their potential value as therapeutic agents. The use of D- and unusual amino acid building blocks in the synthesis of peptide libraries increases the applicability of such libraries,[5,6] as does the use of cyclic templates to conformationally restrict the compounds within a library.[7] Furthermore, the generation of peptidomimetic and organic combinatorial libraries using a 'libraries from libraries' concept has recently been presented.[8] This concept involves the chemical transformation of tens to hundreds of millions of compounds in existing library formats (i.e. permethylation,[8,9] reduction,[10] etc.) to prepare new libraries having very different physical, chemical, and biological properties when compared to their starting combinatorial libraries. Similar combinatorial libraries of N-substituted glycines also mimic some of the structural features of peptides while having different properties.[11] The synthesis of libraries of small organic compounds using solid phase technology has now been successfully carried out by a number of laboratories (see review[12]). We present here a method for the stepwise synthesis of a peptidomimetic library containing 57,500 low-molecular weight peptidomimetics through successive alkylation of the amide functionalities present within the compounds.

Key words: alkylation, combinatorial library, peptidomimetic, solid phase synthesis.

Results and Discussion

Solid-phase methodology[13] was the basic technology used to synthesize and design this peptidomimetic library (average molecular weight of approximately 450 Daltons). A solid-phase-based synthetic method was developed to successively alkylate each amide bond following its formation. In this initial library, five different alkylating agents were used to create increased molecular diversity and to eliminate the hydrogen bonding potential of the amide functionality. Cleavage from the solid support led to peptidomimetics of the general formula 1 (Fig. 1), each having four diversity positions (two amino acid side chains and two amide alkyl groups).

Although a number of methods for the permethylation of peptides in solution[14–16] are known, the permethylation of resin-bound peptides, using sodium hydride for the formation of amide anions, was reported only recently.[8] For the purpose of a stepwise alkylation following each amino acid coupling on the solid support, lithium *t*-butoxide was found to be more effective for the successive formation of the amide anions.

As an important prerequisite for the synthesis of this library, reproducible conditions for the N-amide alkylations had to be established for the base treatment of solid-phase-bound amino acids or peptides. The reactions were carried out under an anhydrous nitrogen atmosphere, and the amino acid or peptide resin of interest was treated with excess lithium *t*-butoxide in tetrahydrofuran. Following removal of excess base, the alkylating agent in dimethyl sulfoxide was reacted with the resin-bound compound. The alkylation reaction mixture was then removed and the base and alkylation treatments were repeated to drive the alkylation reaction to completion. Potential racemi-

zation during alkylation was studied using analytical reversed-phase high performance liquid chromatography (RP-HPLC); the four possible permethylated stereoisomers of Phe-Leu-NH_2 were used as reference standards.[17] The maximum percentage of racemization found following repeated base and methylation treatments was <1% (Fig. 2).

Individual model dipeptides were used to study the modification of amino acid side chains during the alkylation conditions. Fifty *N*-trityl (triphenylmethyl; Trt) dipeptide resins, designated Trt-O-Leu-MBHA resin (MBHA = *p*-methylbenzhydrylamine) were alkylated where O represents a single proteinogenic L-amino acid, their D-counterparts, or 11 other individual 'unnatural' amino acids. Aspartic acid was excluded from the 20 proteinogenic amino acids, since multiple products were formed following base treatment and alkylation. Methyl iodide, allyl bromide, and benzyl bromide were used initially as alkylating agents.

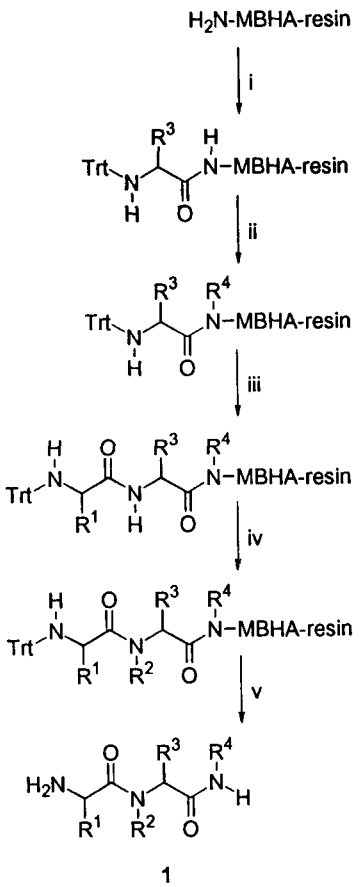

Figure 1. Scheme for the synthesis of the peptidomimetic combinatorial library. Reagents: i. Fmoc-CHR^3-OH/HOBt/DIC in DMF, 20% piperidine in DMF, TrtCl/DIEA in DCM(DCM/DMF). ii. Lithium *t*-butoxide in THF, alkyl halide (R^4X) in DMSO. iii. 2% TFA in DCM, Fmoc-CHR^1-OH/HOBt/DIC in DMF, 20% piperidine in DMF, TrtCl/DIEA in DCM (DCM/DMF). iv. Lithium *t*-butoxide in THF, alkyl halide (R^2X) in DMSO. v. HF cleavage (anisole as scavenger). For abbreviations see Experimental section. R^1: 50 L-, D- and unnatural amino acids; R^2: five alkyl groups (methyl, ethyl, allyl, benzyl, and naphthylmethyl); R^3: 46 L-, D- and unnatural amino acids; R^4: five alkyl groups (methyl, ethyl, allyl, benzyl, and naphthylmethyl).

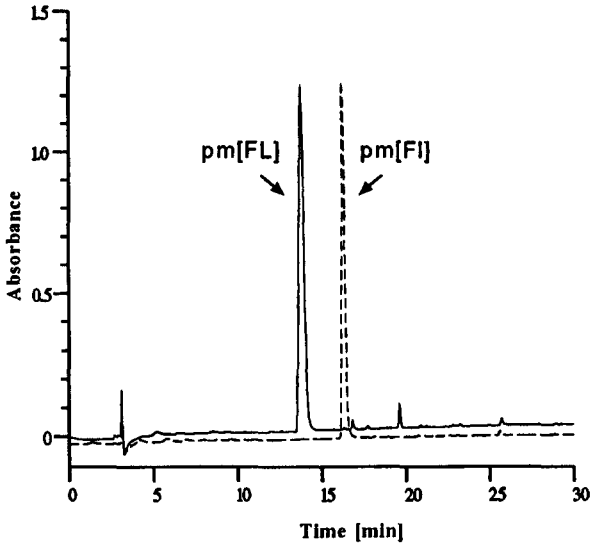

Figure 2. RP-HPLC analysis of permethylated Phe-Leu-NH_2 isomers (pmFL, pmFl) using a linear gradient 5–65% B (0.05% TFA in acetonitrile) for 30 min. The HPLC of permethylated Phe-D-Leu-NH_2 (pmFl) is shown for comparison of retention times.

The individual crude alkylated products were analyzed by RP-HPLC and matrix assisted laser desorption ionization-mass spectroscopy (MALDI-MS) to determine their purity (Fig. 3) and identity.[8] During the alkylation procedure, the functional groups of the amino acid side chains were reproducibly modified. Based on this analytical evidence the following modifications are proposed: (1) the ε-monoalkyl amine was formed during alkylation when the ε-amino group of lysine was protected with Boc; (2) the unprotected amide functionality of the side chains of L-asparagine and L-glutamine, when alkylated with any of the three alkylating agents, yielded dialkyl amides, whereas allylation, and benzylation of the D-isomers led to mono and dialkyl amides, indicating stereochemical hindrance of the diastereomers; (3) the 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) protected arginine side chain yielded the trimethyl derivative following permethylation and di- and triallyl derivatives following perallylation, but was negligibly alkylated following perbenzylation; (4) when unprotected, the reactive

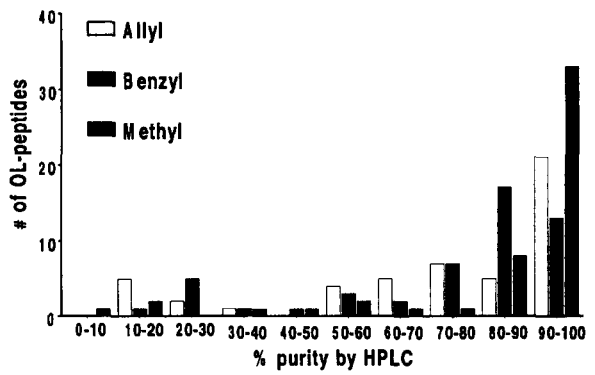

Figure 3. HPLC purity of 50 crude peralkylated OL-dipeptides. Low HPLC purities were found for compounds containing glutamic acid, cysteine, and histidine.

indole nitrogen of tryptophan was alkylated; (5) the use of 2-bromo-Cbz protection for tyrosine resulted in formation of the methyl and allyl ether analogues and any O-benzyl products formed using benzyl bromide in the alkylation were cleaved during the hydrogen fluoride treatment; and (6) when tyrosine hydroxyl was *t*-Bu protected, the side chain was unmodified. Although not studied in detail, glutamic acid *t*-Bu ester led to multiple products following repeated alkylations. Other amino acid derivatives having side chains with potentially reactive functionalities, including serine, threonine, hydroxyproline (all protected as their *t*-Bu ether), methionine (sulfoxide), and tryptophan (Boc), did not undergo any modification during the alkylation step. Repetitive alkylations of trityl-protected N-terminal glycine and β-alanine led to side products containing additional alkyl groups as detected by MALDI-MS.

The peptidomimetic combinatorial library of compounds with the general formula 1 (Fig. 1) has an OOXX format, where O represents a defined position and X represents a mixture position. Forty-six different amino acids (cysteine and histidine were excluded since analogues containing these amino acids were found to have significant side reactions and/or incomplete reaction during the alkylation procedure) were incorporated into the first X position (R^3), and 50 different amino acids were incorporated into the first O position (R^1). The amide alkyl groups in the second X (R^4) and second O positions (R^2) were: methyl, ethyl, allyl, benzyl, or naphthylmethyl. This combinatorial library consists of 250 mixtures (50 amino acids × 5 alkyl groups), each of which is composed of 230 compounds (46 amino acids × 5 alkyl groups), and was prepared applying the divide, couple, and recombine process.[1] (DCR; Fig. 4. Also independently reported as the 'mixing and portioning' and 'split synthesis' approaches[3,18]). The stepwise synthesis was carried out on the solid-phase by alternating amino acid attachment and alkylation of the previously formed amide bond as outlined in Figure 1. Standard Fmoc (9-fluorenylmethoxycarbonyl) chemistry for the incorporation of amino acids was used with MBHA resin as the solid support. Alkylation of the amide bond between the C-terminal amino acid and the MBHA linker was found to significantly decrease the stability of the amide-resin linkage to acidolytic conditions.[19] The α-amino groups were protected with the bulky trityl group to avoid modification of the N-terminal amine during the manipulation of the amide groups of the resin-bound compounds. The five alkyl halides [methyl iodide, ethyl iodide, allyl bromide, benzyl bromide, and 2-(bromomethyl)naphthalene] were reacted with the previously formed amide anions using repeated treatments of the alkylation method described above. Replicates of control resins Trt-Leu-MBHA and Trt-Trp-MBHA were added during each of the five separate alkylation treatments on the solid-phase resins. The R^4 residues were introduced at the same time, enabling the completeness of each of these reactions to be determined. A second amino acid derivative was then coupled to these control resins following

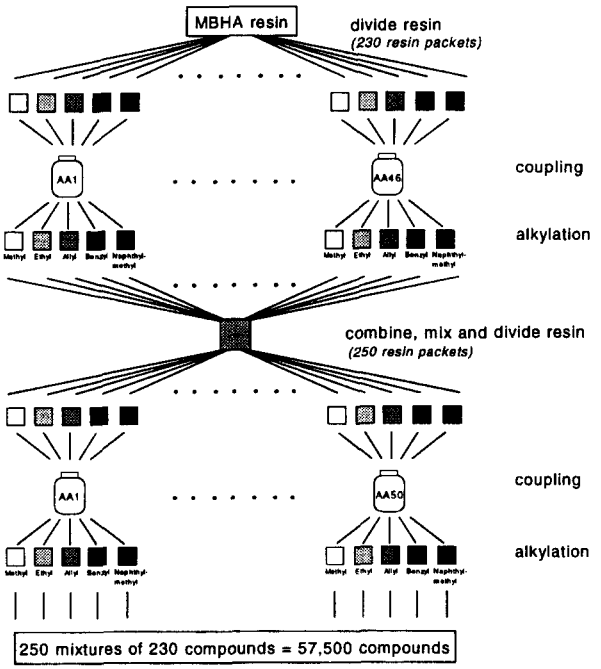

Figure 4. The DCR method[1] was used to prepare this peptidomimetic library, which consists of 250 mixtures, each composed of 230 compounds. After assembling the first two library positions, the resin packets were opened and mixed, then the resin was divided into 250 resin packets to continue the synthesis with the attachment of the two defined library positions.

removal of the trityl group with 2% trifluoroacetic acid in dichloromethane. This resulted in the generation of individual compounds having the formulas H_2N-Phe-Leu-NHR and H_2N-Ala-Trp(R)-NHR (R = methyl, ethyl, allyl, benzyl, or naphthylmethyl). No starting material was detected by RP-HPLC for the crude compounds following three treatments with methyl iodide and ethyl iodide. Allylation, benzylation, and naphthylmethylation required six repetitions of the alkylation procedure, with generally less than 10% starting material remaining (as determined by RP-HPLC). In case of the Ala-Trp controls up to 40% of monoalkylated material was seen (using RP-HPLC). The 230 resins containing the first two library positions were then combined, thoroughly mixed and divided into 250 equal portions (50 × 5 library resin packets). Following trityl removal, the second group of protected amino acids was added (cysteine and histidine included), the Fmoc group was removed, and the free amino groups were again reacted with trityl chloride. The newly formed amide bond was then alkylated as described above, with the exception that five repetitions of the alkylation procedure were carried out. For this second alkylation step, control resins were prepared having the formula Trt-Phe-Leu-NMe-MBHA and Trt-Ala-Trp-NMe-MBHA. These control resins were permethylated at the first amide position to determine the completeness of the second alkylation. Following trityl removal, starting material was not detected by RP-HPLC (Fig. 5) or MALDI-MS for any of the five crude alkylation control products. The highly acid labile amide linkage between the peptidomimetic and the MBHA resin linker does not permit

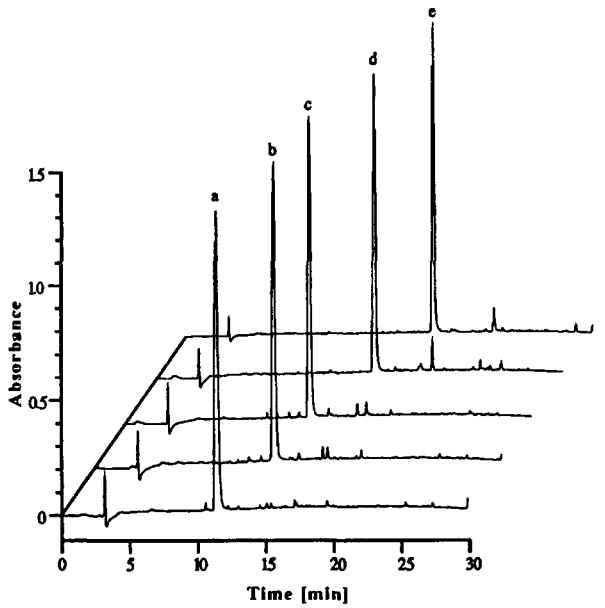

Figure 5. RP-HPLC chromatograms of the five crude control compounds H_2N-Phe-N(R)-Leu-NMe [R = methyl (**a**), ethyl (**b**), allyl (**c**), benzyl (**d**), or naphthylmethyl (**e**)] used to test the completeness of the second alkylation step during the library synthesis. A linear gradient of 5–95% B (0.05% TFA in acetonitrile) was used.

the acid labile side chain protecting groups to be removed prior to final cleavage from the resin. Thus, the peptidomimetic mixtures were cleaved from the resin under standard high hydrogen fluoride cleavage conditions[20] and obtained as lyophilized powders following extraction with 50% aqueous acetonitrile. The yields of some of the crude control compounds were found to be sequence-dependent. During the final acidic Trt removal, compounds having bulky alkyl residues in position R^2 were partially cleaved from the resin.[21]

Peptidomimetics of the formula H_2N-Phe-N(R)-Leu-NHMe (R = methyl, ethyl, allyl, benzyl, or naphthylmethyl) were individually synthesized using the described method to provide material as analytical controls. Following purification by preparative RP-HPLC, the identity of each compound was confirmed by RP-HPLC, MALDI-MS, HR-MS, microanalysis, and NMR.

The nonsupport-bound peptidomimetic library mixtures were screened in solution in radio-receptor, antimicrobial, and enzyme inhibition assays. Deconvolution of the highly active mixtures is currently in progress.

The concept of the peptidomimetic library presented can be readily extended to include other alkyl groups, longer sequences, etc. A range of other chemical reactions (reduction of the carbonyl groups, formation of thioamides, etc.) will yield new diversities having very different physicochemical characteristics. Other reactions, such as reductive alkylation of the free N-terminal amino group using a wide variety of aldehydes, readily leads to a further expanding range of unique peptidomimetic and small molecule organic combinatorial libraries. The concepts described, and the ease with which they can be carried out, greatly extend the molecular diversities available for drug discovery and basic research.

Experimental

Materials and methods

Fmoc amino acid derivatives were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA), Bachem Bioscience Inc. (Philadelphia, PA), and Bachem California (Torrance, CA). MBHA resin, 1% divinylbenzene, 100–200 mesh, 0.9 mmol/g substitution, was received from Peninsula Laboratories, Inc (Belmont, CA). N,N′-Diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBT) were purchased from Chem Impex International (Wood Dale, IL), trifluoroacetic acid from Halocarbon (River Edge, NJ) and hydrogen fluoride from Air Products (San Marcos, CA). All other reagents and anhydrous solvents (DMSO and THF) were purchased from Aldrich Chemical Company (Milwaukee, WI). The solvents dichloromethane (DCM), DMF, isopropanol (IPA), and MeOH were obtained from Fisher Scientific (Fair Lawn, NJ). All reagents and solvents were used without further purification. MALDI-MS analyses were carried out on a Kratos Analytical Compact MALDI II (Ramsey, NJ). HR-FABMS were recorded at the University of California, Riverside Mass Spectrometry Facility, Department of Chemistry (Riverside, CA) on a ZAB mass spectrometer. Analytical RP-HPLC was performed on a Beckman System Gold instrument (Fullerton, CA). Samples were analyzed using Vydac 218TP54 C_{18} columns (0.46 × 25 cm). Preparative RP-HPLC purification was performed on a Waters Delta Prep 3000 instrument (San Francisco, CA). Samples were purified using Waters Delta-Pak C_{18} columns (2.5 × 10 cm). All gradients reported were linear in eluent A (0.05% TFA aq) and eluent B (0.05% TFA in acetonitrile); flow rates were 1 mL/min (analytical) and 20 mL/ min (preparative); the eluent was monitored at 214 nm. Routine ^1H and ^{13}C NMR spectra were recorded on a Varian Gemini 200 (Palo Alto, CA). Microanalyses were performed at Galbraith Laboratories, Inc. (Knoxville, TN).

Library synthesis

Amino acid derivatives. The following amino acid derivatives were used in the X and O positions of the library: Fmoc-Ala-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Fmoc-Met(O)-OH, Fmoc-Asn-OH, Fmoc-Pro-OH, Fmoc-Gln-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Ser(*t*-Bu)-OH, Fmoc-Thr(*t*-Bu)-OH, Fmoc-Val-OH, Fmoc-Trp(Boc)-OH, Fmoc-Trp-OH, Fmoc-Tyr(2BrCbz)-OH, Fmoc-Tyr(*t*-Bu)-OH, Fmoc-D-Ala-OH, Fmoc-D-Phe-OH, Fmoc-D-Ile-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-D-Leu-OH, Fmoc-D-Asn-OH, Fmoc-D-Pro-OH, Fmoc-D-Gln-OH, Fmoc-D-Ser(*t*-Bu)-OH, Fmoc-D-Thr(*t*-Bu)-OH, Fmoc-D-Val-OH, Fmoc-D-Trp(Boc)-OH, Fmoc-

D-Trp-OH, Fmoc-D-Tyr(*t*-Bu)-OH, Fmoc-D-Arg(Pmc)-OH, Fmoc-L-Nle-OH, Fmoc-D-Nle-OH, Fmoc-L-Nve-OH, Fmoc-D-Nve-OH, Fmoc-L-Nal-OH, Fmoc-D-Nal-OH, Fmoc-L-Phg-OH, Fmoc-L-Glu(*t*-Bu)-OH, Fmoc-D-Glu(*t*-Bu)-OH, Fmoc-β-Ala-OH, Fmoc-L-Cha-OH, Fmoc-D-Cha-OH, and Fmoc-Hyp(*t*-Bu)-OH. Fmoc-Cys(MeOBn)-OH (MeOBn = 4-methoxybenzyl), Fmoc-Cys(MeBn)-OH (MeBn = 4-methylbenzyl), Fmoc-His(Trt)-OH, and Fmoc-D-His(Trt)-OH were also used in the O positions of the library.

Assembly of the peptidomimetic combinatorial library

Coupling of the first amino acid derivative. The peptidomimetic library presented here was synthesized using simultaneous multiple peptide synthesis.[22] The solid support (MBHA resin) was contained in 230 polypropylene mesh packets (250 mg resin per packet; packet size 5 × 5 cm). For use in the synthesis of control compounds, 40 additional polypropylene mesh packets were prepared containing MBHA resin (100 mg).

After the common wash and neutralization steps were carried out (1 × DCM, 2 × 5% DIEA, 2 × DCM, 2 × DMF; *ca.* 8 mL per packet; all resin packets were completely covered with solvent) on all of the resin packets, the individual resin packets were separated into 46 groups, each containing five packets for the addition of the 46 amino acid derivatives used in the first coupling step. Fmoc-Leu-OH and Fmoc-Trp-OH were added to two groups of 20 control resin packets. Amino acid couplings were carried out on each of the 46 groups of five library resin packets by vigorously shaking 44 groups in a solution (67.5 mL) of 0.1 M Fmoc amino acid derivative (6.75 mmol)/DIC/HOBt in DMF overnight (Fmoc-Gly coupling required only 75 min); for the other two groups, library resin packets and control packets were vigorously shaken in a solution (175.5 mL) of 0.1 M Fmoc-L-Leu (17.55 mmol)/DIC/HOBt and 0.1 M of Fmoc-L-Trp (17.55 mmol)/DIC/HOBT in DMF overnight. The resin packets were washed (2 × DMF, 1 DCM, 1 × MeOH; approximately 8 mL per packet) and the completeness of amino acid coupling was verified using the ninhydrin test.[23] The only amino acids which required repetitive couplings were Fmoc-L-Gln-OH, Fmoc-D-Gln-OH, Fmoc-L-Arg(Pmc)-OH, and Fmoc-D-Lys(Boc)-OH. Removal of the Fmoc protecting group was accomplished by shaking the resin packets in 20% piperidine/DMF (1 × 3 min, 1 × 10 min; 2 L) followed by a wash cycle (5 × DMF, 2 × IPA, 3 × DCM; approximately 8 mL per packet).

Tritylation of the N-terminal amino group. Following removal of the Fmoc group, the 270 resin packets (a total of 55 mmol of free N-α-amino groups) were shaken for 3 h in a 0.077 M solution of trityl chloride (276.75 mmol) in DCM/DMF (9:1, 3.6 L) containing diisopropylethylamine (DIEA, 1.6 mol, 280 mL). After a short wash procedure (1 × DMF, 1 × 5% DIEA, 1 × DCM; approximately 8 mL per packet), the tritylation procedure was repeated twice more by shaking overnight in a 0.05 M solution of trityl chloride in DCM (5.5 L) containing the same amount of base and washed (2 × DMF, 1 × 5% DIEA, 3 × DCM, 1 × MeOH; approximately 8 mL per packet). The completeness of the trityl coupling was verified for each of the 46 different amino acid resins using the bromophenol blue color test.[24]

Alkylation of the first amide position. All manipulations were performed under a N_2 atmosphere and strictly anhydrous conditions. The 270 resin packets were dried overnight at 50 mTorr. Each of five groups, containing 46 amino acid resin packets plus control resin packets (including four Trt-Leu-MBHA resin packets and four Trt-Trp-MBHA resin packets), were placed in one of five separate round-bottom flasks, one for each of the five alkylation reactions. Each flask contained the same amount of available amide groups (11.07 mmol each). Lithium *t*-butoxide (1 M) in THF (220 mmol, 220 mL) and THF (220 mL) were added to each of the five reaction vessels and shaken at room temperature for 15 min. Excess base solution was removed by cannulation. Following addition of DMSO (440 mL), the individual alkylating agent was added (665 mmol, i.e. 41.4 mL methyl iodide; 53.1 mL ethyl iodide; 57.5 mL allyl bromide; 79.0 mL benzyl bromide). 2-(Bromomethyl)naphthalene (665 mmol, 147 g) was dissolved in DMSO (440 mL) and transferred as a solution to the reaction vessel. The reaction mixture was vigorously shaken for 2 h at room temperature. The alkylation solution was removed by cannulation and the entire procedure repeated twice more. The resulting resin packets were washed (3 × DMF, 2 × IPA, 3 × DCM, 1 × MeOH; approximately 8 mL per packet) and dried. Following complete drying of the resin packets overnight at 50 mTorr, the process described above was repeated three times for allylation, benzylation, and naphthylmethylation (each alkylation, 2 × 2 h and 1 × 5 h).

Recombine, mix and divide the resin. The resin of the 230 library packets was combined, mixed in DCM (2 L; 15 h shaking), and dried. The resin was divided into 250 polypropylene mesh packets (packet size 5 cm × 5 cm; each containing 310 mg resin).

Removal of the trityl protecting group. The resin packets, prepared as described above, were washed (1 × DCM; approximately 8 mL per packet), treated twice with 2% TFA in DCM (1 × 2 min, 1 × 30 min; 2 L), and washed (1 × DCM, 2 × IPA, 2 × DCM, 1 × MeOH; approximately 8 mL per packet).

Coupling of the second amino acid derivative and second alkylation. The amino acid coupling (using the 50 different amino acid derivatives), Fmoc removal, tritylation of the free amino groups, alkylation of the previously formed amide bond, and trityl removal were performed as described above. Trt-Phe-Leu-NMe-MBHA resin packets and Trt-Ala-Trp(Me)-NMe-MBHA resin packets were added as control resins during alkylation. The second amide position was

treated five times for alkylation (methylation and ethylation, each 5 × 2 h; allylation, benzylation and naphthylmethylation, each 3 × 2 h and 2 × 3 h).

HF cleavage. The 250 mixture resin packets were cleaved 24 at a time with hydrogen fluoride (5 mL per resin packet with 0.35 mL anisole added as scavenger) using a multiple vessel cleavage apparatus.[20] The resulting mixtures were extracted by sonicating with 50% aq acetonitrile (3 × 5 mL). The resulting solutions were lyophilized and relyophilized twice more from 50% aq acetonitrile.

Individual compounds. Individual compounds were prepared in the same manner as described for the library synthesis. The alkylations were generally performed with repetitions. Following HF cleavage, the crude individual compounds were purified by prep HPLC (C18, 25–55% B, in 30 min).

Phenylalanyl-*N*-methyl-leucinemethylamide. Yield after prep HPLC (TFA salt): 59.8%. ^{1}H NMR (200 MHz, $CDCl_3$; mixture of conformers; selected data for the major conformer; ratio 78:22): δ 0.85 (m, 6H), 1.23–1.57 (m, 2H), 1.71–1.85 (m, 1H), 2.65 (s, 3H), 2.7 (d, 3H), 3.01–3.28 (m, 2H), 4.51–4.58 (m, 2H), 6.85 (m, 1H), 7.15–7.28 (m, 5H), 8.5 (br, 2H); ^{13}C NMR (200 MHz, $CDCl_3$; selected data for major conformer): δ 22.0, 22.7, 24.7, 26.1, 32.6, 36.8, 37.4, 51.7, 57.8, 128.1, 129.1, 129.4, 133.4, 168.9, 170.1; MALDI-MS: 307 [M+2], 329 [M+Na]; Anal. calcd for $C_{19}H_{28}F_3N_3O_4$ (TFA salt): C, 54.39; H, 6.73; N, 10.02; Found: C, 54.19; H, 7.03; N, 9.99; HR-FABMS calcd for $C_{17}H_{28}N_3O_2$ [MH$^+$] 306.2175, found 306.2165.

Phenylalanyl-*N*-ethyl-leucinemethylamide. Yield after prep HPLC (TFA salt): 18.8%. ^{1}H NMR (200 MHz, $CDCl_3$; selected data for major conformer; ratio 72:28): δ 0.75–1.15 (m; 9H), 1.35–1.60 (m, 2H), 1.95–2.20 (m, 1H), 2.66 (d, 3H), 2.85–3.45 (m, 4H), 3.97 (m, 1H), 4.41 (m, 1H), 6.99 (m, 1H), 7.18–7.40 (m, 5H), 8.2–9.2 (br, 2H); ^{13}C NMR (200 MHz, $CDCl_3$; selected data for major conformer): δ 14.3, 22.9, 23.1, 25.7, 26.6, 38.3, 43.5, 52.2, 58.3, 128.6, 129.6, 130.1, 134.1, 169.3, 171.2; MALDI-MS: 321 [M+1], 343 [M+Na]; Anal. calcd for $C_{20}H_{30}F_3N_3O_4$ (TFA salt): C, 55.399; H, 6.9789; N, 9.697; Found: C, 54.39; H, 6.97; N, 9.47; HR-FABMS calcd for $C_{18}H_{30}N_3O_2$ [MH$^+$] m/z = 320.2331, found m/z = 320.2335.

Phenylalanyl-*N*-allyl-leucinemethylamide. Yield after prep HPLC (TFA salt): 20.05%. ^{1}H NMR (200 MHz, $CDCl_3$; selected data for major conformer; ratio 71:29): δ 0.76–0.90 (m, 6H), 1.27–1.50 (m, 2H), 1.98–2.08 (m, 1H), 2.68 (d, 3H), 3.02–3.60 (m, 4H), 4.18 (m, 1H), 4.41–4.49 (m, 1H), 5.12–5.24 (m, 2H), 5.56–5.78 (m, 1H), 6.99 (m, 1H), 7.21–7.36 (m, aromatic protons), 8.35–9.35 (br, 2H); ^{13}C NMR (200 MHz, $CDCl_3$; selected data for major conformer): δ 22.3, 25.0, 26.0, 37.8, 49.9, 51.8, 58.0, 119.9, 128.1, 129.1, 129.5, 132.1, 133.5, 169.1, 170.4; MALDI-MS: 333 [M+1], 355 [M+Na]; Anal. calcd for $C_{21}H_{30}F_3N_3O_4$ (TFA salt): C, 56.60; H, 6.79; N, 9.44; Found: C, 56.00; H, 6.83; N, 9.23; HR-FABMS calcd for $C_{19}H_{30}N_3O_2$ [MH$^+$] m/z = 332.2331, found m/z = 332.2335.

Phenylalanyl-*N*-benzyl-leucinemethylamide. Yield after prep HPLC (TFA salt): 20.34%. ^{1}H NMR (200 MHz, $CDCl_3$; selected data for major conformer; ratio 65:35): δ 0.72–0.89 (m; 6H), 1.02–1.53 (m, 2H), 1.87–2.11 (m, 1H), 2.52 (d, 3H), 2.98–3.45 (m, 2H), 4.05–4.79 (m, 4H), 6.92 (m, 1H), 7.08–7.38 (m, 10H), 8.20–9.20 (br, 2H); ^{13}C NMR (200 MHz, $CDCl_3$; selected data for major conformer): δ 22.0, 22.9, 25.5, 26.0, 37.7, 38.1, 47.3, 52.5, 57.9, 127.2, 127.7, 128.2, 129.3, 129.8, 133.5, 134.9, 169.7, 170.6; MALDI-MS: 383 [M+1], 405 [M+Na]; Anal. calcd for $C_{25}H_{32}F_3N_3O_4$ (TFA salt): C, 60.58; H, 6.512; N, 8.48; Found: C, 60.33; H, 6.41; N, 8.43; HR-FABMS calcd for $C_{23}H_{32}N_3O_2$ [MH$^+$] m/z = 382.2487, found m/z = 382.2511.

Phenylalanyl-*N*-naphthylmethyl-leucinemethylamide. Yield after prep HPLC (TFA salt): 18.54%. ^{1}H NMR (200 MHz, $CDCl_3$; selected data for major conformer; ratio 67:33): δ 0.70–0.90 (m, 6H), 1.15–1.58 (m, 2H), 1.94–2.13 (m, 1H), 2.44 (d, 3H), 3.04–3.49 (m, 2H), 4.20–4.90 (m, 4H), 6.90–6.99 (m; 1H), 7.08–7.89 (m, 12H), 8.1–9.5 (br, 2H); ^{13}C NMR (200 MHz, $CDCl_3$; selected data for major conformer): δ 22.0, 23.0, 25.5, 25.7, 37.7, 38.1, 52.6, 57.7, 124.6–134.2 (aromatic carbons), 169.8, 170.5; MALDI-MS: 433 [M+1], 455 [M+Na]; Anal. calcd for $C_{29}H_{34}F_3N_3O_4$ (TFA salt): C, 63.82; H, 6.28; N, 7.70; Found: C, 63.96; H, 6.27; N, 7.76; HR-FABMS calcd for $C_{27}H_{34}N_3O_2$ [MH$^+$] m/z = 432.2643, found m/z = 432.2663.

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