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Screening of Completely Random One-Bead One-Peptide Libraries for Activities in Solution

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A method for the synthesis and screening of polymeric libraries for biologic activities in solution is described. Libraries are constructed on a completely random basis with one structure on one particle of solid support (bead). The screened structure is then released into solution in several distinct steps, allowing screening of aliquots of the library in the first step to identify a mixture containing a structure of interest. Biological evaluation of individual compounds released from the polymeric carrier is performed in the second step. Multiple release of equimolar amounts of the randomized compounds is achieved by use of a construct capable of intramolecular closure of the diketopiperazine ring under neutral conditions in the first stage release. The second release is accomplished by alkaline hydrolysis at higher pH or by ammonolysis by ammonia vapors.

The Selectide technology (1), described in the previous article (2), is limited to the screening of soluble targets. Even though in principle it is possible to screen for the interaction of bead-immobilized ligands with macroscopic, insoluble ligates (such as bacteria and living cells), there are practical limitations to the effective implementation of such an approach, including nonspecific binding. Moreover, the observation of the interaction between the ligand and, e.g., the cell surface may not be the most relevant information needed for the design of a new drug. On the other hand, there are a number of biologically relevant large-scale screening assays based on growth inhibition or the proliferation of cell lines, microorganisms, fungi, etc. To employ our technology of multiplicity generation and screening, the essential aspect of which is the synthesis of one compound (peptide) on one bead, we developed a technique of multiple release of a defined portion of the species from one bead in several distinct steps.

To speed up the screening of large collections of unrelated compounds, one may prepare simple mixtures of 5 to 100 of these compounds and test them concurrently for the biological activity of interest. The rationale of this approach is based on the likelihood that a compound with sought-after activity will be identified in a mixture containing only a small percentage of the active component, while it is unlikely that the activity of one compound will be masked by that of another component of the mixture. All efforts to isolate biologically active compounds from natural sources have used this principle. For example, the isolation and structure elucidation of oxytocin were accomplished from an original homogenate containing only 0.02% of the active substance (3). Recently, Jung *et al.* (4) have studied the problem of the activity of a particular peptide in a mixture and found that even though the activity was modulated by other components of the mixture, it was still clearly detectable.

Another approach to screening of peptide mixtures in solution was introduced by Houghten *et al.* (5). With this iterative method (first applied to screening techniques by Geysen (6)), families of similar peptides with one or more defined positions are built, and the most active mixture is selected as the base for building the next generation of libraries. This approach does not require the elucidation of the structure of the active compound, as its identity is defined by the synthetic algorithm. On the other hand, this iterative method cannot simultaneously explore all motifs present in the library since one must limit the search to that subset identified in the first steps of library synthesis and screening; that is, the identified motif may be influenced by the strategy of library construction (defined positions in the first-round libraries—on amino terminus, carboxy terminus, in the middle of the peptide chain, etc.).

The strategy we chose for large library screening in solution is based on a random approach to compound synthesis that allows the identification of multiple hits. The generation of several millions structures based on the split solid-phase synthesis (1, 5, 7) is well described (for discussion of the synthetic strategy see the article by Lam *et al.* (2)). The difficulty arises in the next steps—release of the library compounds into solution and functional testing. Since it is not possible to test all compounds separately, we have built the same structure on each solid-phase support in three copies attached via differentially

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cleavable linkers. Release can then be carried out in sequential steps using different mechanisms of cleavage. The screening strategy is illustrated in Fig. 1. The random library is distributed into the wells of microtiter filtration plates, and the release of the first portion of the compound attached to the solid support is performed under very mild conditions. In this step several hundred to several thousand beads are placed in each well, and therefore a mixture of a substantial number of unrelated compounds is generated in approximately equimolar quantities. The relationship between individual components of the mixture is based on only the statistics and structure of the library. These small aliquots of soluble compounds (sublibraries) are filtered to the testing microtiter plate, and an appropriate biological assay is then performed. Biologically active mixtures are identified, and beads from the well responsible for the activity are recovered from the active well of the master filtration plate and redistributed at one to a few beads per well. The second portion of each compound is then released, and the biological test is repeated. In the second step the filtration may not be necessary, provided that a bead can be retrieved from a well after the test assay is completed. When dealing with soluble targets, the binding assay can be combined with the release assay, or alternatively active compounds identified in the binding assay can be verified by biological testing of the released compound.

Limitations of this approach relate to the concentration of the tested compound after its release from the single solid-phase carrier bead. One 130-μm-diameter bead of polyoxyethylene-modified polystyrene (TentaGel) contains about 100 pmol of the synthetic molecule. Release of this amount into 100 μl of solution generates a 1 μM solution. Therefore, identification of compounds with an affinity less than 1 μM is unlikely. Higher concentrations of released compound can be achieved using beads of greater substitution and/or size. However, the bead material must be compatible with the synthetic and release conditions used in the screening process. Beads that contain high nanomolar levels of compound (capable of generating millimolar concentrations of the tested species), however, cannot be extracted effectively using aqueous buffers. Additional steps requiring organic solvent extraction and solvent evaporation may be required for effective extraction. Another very important factor in this screening method is that both the substitution level and the size distribution of the beads should be homogeneous. Beads with diameters in the range 80 to 120 μm would provide up to three times the variation in quantity of compound from individual beads. For beads with diameters in the range 90 to 110 μm, this variance would be nearly two times. The release process may also be complicated by varying solubility properties of the generated compounds and their affinities to surfaces (well walls, filter). However, since the goal of this broad screening is to identify the initial hits, this imprecision in concentration definition is acceptable.

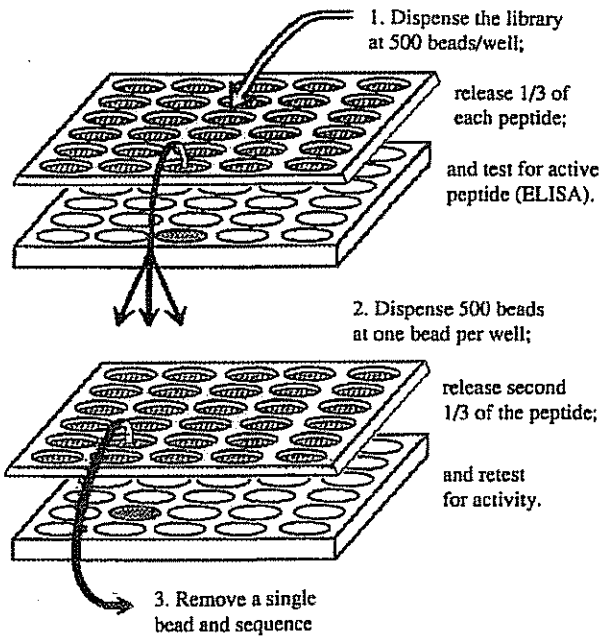


FIG. 1. Scheme of screening the libraries of structures attached to solid support by multiply cleavable linker.

It is also important to take into account the so-called "expected hit rate," i.e., the number of structures in the library providing activity higher than the sensitivity threshold of the given assay. If the expected hit rate is higher than 1 in 1000, it does not make any sense to screen the library in the multiple release format since the probability of having at least one positively reacting particle in every well is too high. At a lower expected hit rate, such as 1 in 160,000 (which is the hit rate expected for peptides that have an activity defined by four "critical" amino acid residues) the chances for success are good.

The bead identified as the source of biologically active compound is then submitted for structural determination. We routinely use Edman degradation of the peptide synthesized on the bead either as the active test species or as a tag encoding the nonsequenceable structure synthesized in parallel on the same bead (8). Mass spectrometric techniques may also be used (9) as an alternative sequencing method for shorter peptide sequences. Chemical sequencing of peptides is reliable at low picomolar levels; therefore for larger, highly substituted beads, only a fraction of the synthesized peptide is sufficient for sequencing. This arrangement can be achieved by coupling the first amino acid in the peptide sequence to be used for sequencing, protected by an orthogonally cleavable protecting group, in subequimolar amounts to the amino group containing the carrier before attaching the construct. This allows multiple release of the peptide or non-

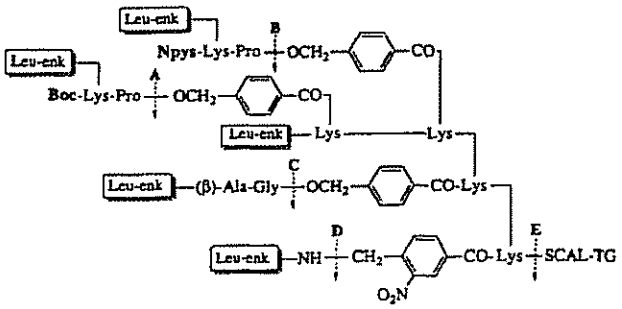


FIG. 2. Structure of quadruply cleavable linker assembly used in the evaluation of the technology.

peptide structure. However, we use equimolar construction of releasable and nonreleasable parts as the standard technique.

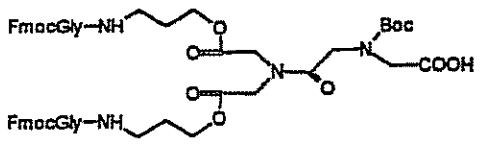
As a model for the demonstration of multiple release of an equimolar amount of peptide from a single bead, we constructed a molecule on which five copies of the same peptide were prepared (10) (see Fig. 2). The model peptide was released in four distinct steps using different stabilities of protecting groups followed by intramolecular cyclization, hydrolysis at higher pH, and photolysis. After the final release, we were able to determine the peptide sequence from the peptide remaining attached to the bead by noncleavable bond. Due to the construction of the scaffold holding the releasable molecule, the peptide content released at every step was equivalent to that which would be obtained from classical synthesis on this type of carrier. Since we did not require four discrete stages of release for functional screening, we selected the two release methods yielding the best results, intramolecular cyclization and hydrolysis, for construction of releasable libraries. Intramolecular cyclization is a very mild method for release and was used earlier by Geysen's group for release in the case of multiple peptide synthesis on polyethylene pins (11–13). The disadvantage of the original method is that the diketopiperazine (DKP) structure remained in the released peptide molecule. We overcame this limitation by redesigning the cleavable linker (10). In all the above-mentioned experiments, we used alpha amino acids for synthesis of the releasable construct.

However, we recently developed a more convenient and substantially less expensive linker construct (14). The dipeptide motif Ida–Ida was particularly suitable for designing double cleavable linkers. The Ida–Ida dipeptide is prone to DKP formation; it provides three carboxyl groups, one on the amino terminal Ida and two on the carboxy terminal. To construct the double cleavable linker, two carboxyl groups are needed for derivatization and subsequent peptide building. There are two possible ways to attach two Fmoc–Gly–PAs (PA = propanolamide) to carboxyl groups. Fmoc–Gly–PAs are coupled to both carboxyls of carboxy terminal Ida (linker I), or each Ida bears one Fmoc–Gly–PA (linker II) (see Fig. 3). In any case there is one free carboxyl group that serves to connect the linker to the solid support (e.g., via Lys, which provides one extra amino group for the third copy of the peptide, nonreleasable, used to read its sequence by Edman degradation).

The whole linker **I** was synthesized in solution. Both carboxyl groups of Boc-Ida were activated by diisopropylcarbodiimide (DIC) and *N*-hydroxybenzotriazole (HOBt) and allowed to react with Fmoc–Gly–PA in the presence of the catalyst dimethylaminopyridine. After the Boc protecting group was removed by trifluoroacetic acid (TFA), the free base was used to open preformed cyclic anhydride of Boc-Ida. In this case both Fmoc–Gly–PAs are chemically indistinguishable, and the first peptide is cleaved via DKP formation from either of the two arms. The DKP formed bears on one nitrogen the second copy of the peptide and it is bound to the support via the second nitrogen.

The structures shown in the Fig. 3 can be attached to the polymeric carrier onto which diaminocarboxylic acid (e.g., lysine) was already coupled. Peptide synthesized directly on the lysine side chain is permanently linked to the bead and serves as the "reference" for structural determination. It can be identical to the peptides synthesized on the branches of the cleavable linker, or it can be a peptide coding for the nonpeptide structure on the releasable linker (8).

After synthesis on all points of attachment, the constructed library is deprotected and the Boc group protecting the second molecule of iminodiacetic acid is re-



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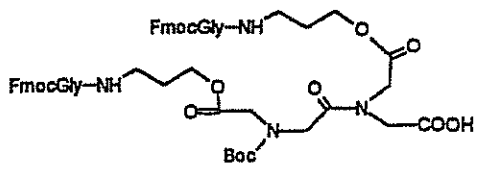

II

FIG. 3. Structure of iminodiacetic acid (Ida) based doubly cleavable linkers.

moved. This structure is stable at acidic pH (3–4), but removal of the proton from the secondary amino group (pH 7–9) leads to the rapid spontaneous formation of a diketopiperazine structure (see Fig. 4) and release of the first part of the peptide as hydroxypropylamide (overnight incubation in buffer pH 7–9, 50–100 μl per well). The release of the second part of the peptide is achieved by the use of higher pH (3–12 h in 10–50 μl of 0.05–0.2% NaOH per well) or by exposure to gaseous ammonia (12–24 h incubation in a desiccator).

Synthesis of the multicleavable linker is shown in Fig. 4. N-protected iminodiacetic acid is esterified by hydroxypropylamide of Fmoc–Gly using carbodiimide condensation catalyzed by dimethylaminopyridine. The Boc group is removed by trifluoroacetic acid, and the amino group is acylated by anhydride derived from protected iminodiacetic acid. To test the performance of this linker, we have attached it to the polymeric carrier by a carbodiimide reaction. To check the release kinetics, we have coupled tryptophan to both arms and exposed the construct to the conditions of both releases. Rapid and quantitative release of tryptophan was observed at each step.

This new linker is now used routinely in the preparation of multireleasable libraries. We have published the application of multicleavable linker in finding ligands to our model target, anti-β-endorphin monoclonal antibodies (15). In this case, we have found the expected ligands, YGGF, YGVF, and YGAF. We were also able to combine the release assay with the bead binding assay, giving us the same results. Another target screened with the releasable technique was the platelet IIb/IIIa receptor for fibrinogen. In addition to the known motif RGD, we have found several unrelated motifs, the structures of which will be presented later. We are applying the release assay method to a variety of targets of pharmacologic interest, including human cell lines in culture. The practical performance of an assay is illustrated below.

MATERIALS AND METHODS

Instrumentation

Melting points were determined on a Kofler block and are uncorrected. ^{1}H NMR spectra were recorded on a General Electric QE 300 instrument. All spectra are reported in parts per million relative to tetramethylsilane (δ) using either $CDCl_3$ or CD_3SOCD_3 as solvents. UV/VIS absorption spectra were recorded on a Hewlett–Packard HP 8452A diode-array spectrophotometer using a 1-cm quartz cuvette. Amino acid analyses were carried out on a D-500 (Durrum Corp., U.S.A.) system. Both analytical HPLC and preparative HPLC were carried out on a modular Spectra Physics system using Vydac (0.46

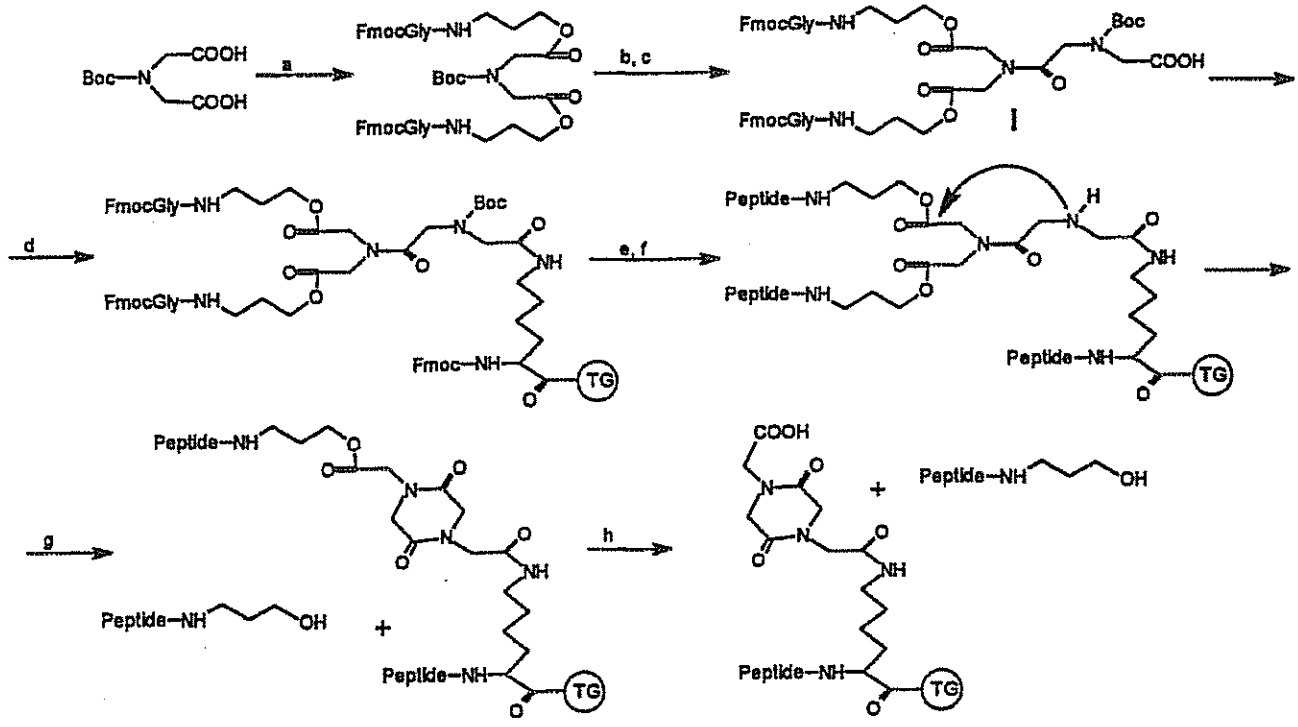

FIG. 4. Scheme of the synthesis and double release of peptide structure from the symmetrical linker I.

× 250 mm, 5 μm, flow 1 ml/min) and Vydac (10 × 250 mm, 10 μm, flow 3 ml/min) C-18 columns, respectively.

General Procedures

Flash chromatography was performed with Merck silica gel 60 (40–63 μm). Thin-layer chromatography was performed either on Silufol UV 254 (Kavalier, Czechoslovakia) or on Merck DC-Alufolien kieselgel 60. Preparative thin-layer chromatography was performed on Whatman silica gel 60A plates (1-mm thickness). The spots were detected by fluorescence quenching or by spraying with a dilute ethanolic ninhydrin solution. Reaction solutions were concentrated using a rotary evaporator (at 2.0–2.6 kPa).

Materials

Unless indicated otherwise, commercial-grade solvents were used without further purification. TentaGel (TG) resin (0.21 mmol/g) was received from Rapp-Polymere (Tübingen). Protected amino acids were obtained from Bachem (Torrance, CA), Advanced ChemTech (Louisville, KY) and Propeptide (Vert-le-Petit, France).

Synthesis of Multicleavable Linker I

tert.-Butyloxycarbonyliminodiacetic acid (Boc-Ida). A solution of iminodiacetic acid 30.0 g (225 mmol) in 1 M NaOH (225 ml) and dioxane (200 ml) was stirred and cooled in an ice-water bath. Di-*tert.*-butyl pyrocarbonate (53.9 g, 247 mmol) was added in several portions, and stirring continued at room temperature for 1 h. Dioxane was evaporated *in vacuo*, covered with a layer of ethyl acetate (100 ml), and acidified with a saturated solution of $KHSO_4$ to pH 2–3. The aqueous phase was extracted with ethyl acetate (3 × 150 ml). Combined acetate extracts were washed with water (100 ml), dried over anhydrous $MgSO_4$, and evaporated *in vacuo*. The product was crystallized from a solvent mixture of ethyl acetate and petroleum ether. Yield 47.0 g (90%).

$HN(CH_2$-CO-O-$(CH_2)_3$-NH<-Gly<-Fmoc$)_2 \cdot$ HCl. A solution of Fmoc–Gly–PA (7.08 g, 20 mmol) in 30 ml dimethylformamide (DMF) was added to the solution of Boc-Ida (2.33 g, 10 mmol), HOBt (2.7 g, 20 mmol), and DIC (3.14 ml, 20 mmol) in 30 ml DMF, dimethylaminopyridine (0.48 g, 4 mmol) was added, and the reaction mixture was stirred at room temperature (RT) for 5 h. DMF was evaporated under reduced pressure, and the oily residue was dissolved in 100 ml of AcOEt, filtrated, and extracted three times with water, 5% aqueous HCl, water, a saturated solution of $NaHCO_3$, water, and a saturated solution of NaCl in water. The organic layer was dried by anhydrous $MgSO_4$, and AcOEt was evaporated under reduced pressure. Yield 7.2 g (80%) of crispy foam, R_f 0.55 (contains two impurities with R_f 0.37 and 0.62). The product was dissolved in 30 ml of DCM, 30 ml of TFA was added, and the reaction mixture was kept for 30 min, at RT. DCM and TFA were evaporated under reduced pressure, and the oily residue was dissolved in AcOEt and washed three times with water and a saturated solution of $NaHCO_3$. After extraction with solution of Na_2CO_3, three layers were formed. The bottom layer containing the product was separated, acidified by shaking with 5% HCl, dissolved in chloroform, dried by anhydrous $MgSO_4$, concentrated to a small volume, and poured into a large excess of ether. Precipitate was collected, washed with ether, and dried. Yield 5.4 g (64%) of crispy foam, single spot on TLC, R_f 0.28 in $CHCl_3$:MeOH:AcOH (90:9:1). ^{1}H NMR (300 MHz, DMSO, 27°C) δ: 1.78 (2H, PA CβH_2), 3.17 (2H, PA CαH_2), 3.60 (2H, Gly CH_2), 4.01 (4H, Ida CH_2), 4.18 (2H, PA CγH2), 4.19–4.34 (3H, Fmoc CH_2 and CH), 7.52 (1H, Gly NH), 7.33, 7.43, 7.72, and 7.90 (8H, Fmoc aromatic H), 7.96 (1H, PA NH).

Boc-N(CH_2-COOH)-CH_2-CON(CH_2-COO-$(CH_2)_3$-NH<-Gly<-Fmoc$)_2$. Boc-Ida (2.33 g, 10 mmol) was dissolved in 50 ml of DCM:DMF (10:1) mixture, DIC (1.57 ml, 10 mmol) was added, and the reaction mixture was stirred for 30 min. Then the solution of HN(CH_2-CO-O-$(CH_2)_3$-NH<-Gly<-Fmoc$)_2 \cdot$ HCl (8.4 g, 10 mmol) in 50 ml DMF was added, the pH was brought to ca. 8 by DIEA, and the reaction mixture was stirred at RT for 1 h. DMF and DCM were evaporated under reduced pressure, and the oily residue was dissolved in 100 ml of AcOEt and extracted three times with water, 5% aqueous HCl, water, and saturated solution of $NaHCO_3$. $NaHCO_3$ extracts were combined and acidified with aqueous HCl, and the product was extracted three times with AcOEt and dried with anhydrous $MgSO_4$, and AcOEt was evaporated under reduced pressure. The oily residue was triturated with ether and the product crystalized. Yield 6.8 g (67%). Linker (300 mg) was dissolved in 6 ml of acetonitrite (ACN) and diluted by 6 ml of water. A solution of linker (3 ml) was applied on an RP column equilibrated with 100 ml of 40% ACN in water. The column was washed with 100 ml of 40% ACN, 100 ml of 45% ACN, 200 ml of 50% ACN, and 200 ml of 70% ACN. Fractions (10 ml) were collected and their purity was checked by TLC in the system chloroform:toluene:MeOH:water (10:10:10:1). Fractions containing homogeneous linker were combined, ACN was evaporated under reduced pressure, and the solution was lyophilized. Yield 188 mg, single spot on TLC, R_f 0.28 in $CHCl_3$:MeOH:AcOH (90:9:1). ^{1}H NMR (300 MHz, DMSO, 27°C) δ: 1.36 (9H, Boc), 1.75 (2H, PA CβH_2), 3.16 (2H, PA CαH_2), 3.60 (2H, Gly CH_2), 3.76–4.23 (8H, Ida CH_2), 4.11 (2H, PA CβH_2), 4.19–4.34 (3H, Fmoc CH_2 and CH), 7.49 (1H, Gly NH), 7.34, 7.42, 7.72, and 7.89 (8H, Fmoc aromatic H).

Synthesis of the Library for Two-Stage Solution Testing

Polymer carrier (TentaGel, Rapp Polymere, Tübingen, Germany, 5 g, 0.23 mmol/g, 130-μm average particle size) was swollen in DMF (swollen volume 25 ml), and Fmoc-Lys (Boc) (3 eq) was coupled by DIC (3 eq) in the presence of HOBt (3 eq) in DMF. After 2 h the resin was washed

(5× DMF and 1× dichloromethane (DCM)) and the Boc group was cleaved with 50% TFA in DCM (1 plus 20 min). After washing with DCM (5×) and DMF (4×), the resin was neutralized by washing with 2% diisopropylethylamine in DMF and washed by DMF (3×), and linker I (3 eq) was coupled by the action of DIC and HOBt (3 eq each) in DMF (overnight). The resin was washed with DMF (5×) and the Fmoc group was cleaved by 20% piperidine in DMF (20 min). After an additional washing with DMF (3×) and division of the resin into m reaction vessels, m individual Fmoc protected amino acids were coupled to each part of the resin using DIC and HOBt (3 eq each). The reaction was monitored by the bromophenol blue method (16). After complete coupling was observed in all of the reaction vessels (all resin particles were decolorized), completeness of the coupling was verified by the ninhydrin test (17). The resin was then combined and washed with DMF (5×), and the Fmoc group was deprotected as above. This procedure (separated coupling and recombined deprotection) was repeated n times for a library of n peptides. The library was then deprotected by application of the mixture of TFA (82.5%), *p*-cresole (5%), thioanisole (5%), water (5%), and ethanedithiol (2.5%) (mixture K (18)) for 2 h and washed with TFA (3×), DCM (5×), DMF containing 0.1% HCl (4×), and 0.1% HCl in water (5×). The library must be stored in an acidic solution to prevent premature loss of the synthesized peptides.

Quality Control of the Double Releasable Library

Quality control was performed by analytical release. Dried resin (5–10 mg) was shaken overnight with 2–5 ml of buffer pH 8.5 (0.1 M Hepes) in a polypropylene syringe equipped with a polypropylene or Teflon frit at the bottom and a polypropylene plunger. Absorbance of the solution (diluted if necessary) at 280 nm was measured and the amount of the peptide released was calculated according to the formula

$$\text{Release (mmol/g)} = \frac{\text{Absorbance} * \text{Volume} * \text{Dilution}}{(n * 1197/x + m * 5559/y) * \text{Mass}},$$

where Mass is amount of resin in g, x is number of amino acids used for randomization at positions where tyrosine was used, y is the number of amino acids used for randomization at positions where tryptophan was used, n is the number of positions in the library at which tyrosine was used for randomization, and m is the number of positions in the library at which tryptophan was used for randomization.

A solution of 0.2% NaOH was introduced into the syringe with the test resin followed by shaking for another 4 h. The solution was then expelled from the syringe, and absorbance was measured at 280 nm. The same calculation was performed using the formula shown above with the coefficients 1507 and 5377 instead of 1197 and 5559. Results of releases in both steps should not differ by more than 10% from the theoretical values, which can be calculated with the formula

$$\text{Theor. Release (mmol/g)} = \frac{\text{Subst.}}{1 + \text{Subst.} * (3 * \text{MW} + 686)/1000},$$

where Subst. is the starting substitution of the resin (in mmol/g), MW is the average molecular weight of one library structure, and 686 is the molecular weight of Ida linker (without Fmoc groups) plus one Lys residue minus one molecule of water. In the case of peptide library, the average molecular weight of one natural amino acid is 119.7 (19 amino acids, Cys excluded). The average molecular weight of one structure in a pentapeptide library is therefore 598.5. For the resin substitution level of 0.2 mmol/g we should expect 0.134 mmol of pentapeptide released from one arm per gram of library beads.

Two-Stage Release Assay for Peptide Library Testing in 96-Well Microassay Plates

For the first release, library beads were transferred into pH 4.5 buffer containing 1.0% carboxymethyl cellulose (to retard sedimentation), shaken, and rapidly pipetted into the upper chambers of a vacuum-control 96-well filtration manifold (Model 09601, Millipore Corp., South San Francisco, CA). Approximately 500 beads were placed in each filtration well, so that each plate contained approximately 48,500 unique peptides. The filtration plates therefore serve as "master" plates for retaining subsets of peptides in unique locations. After vacuum filtration removal of the transfer buffer, the first-stage release of peptides was accomplished by dispensing the appropriate buffer or tissue culture medium (neutral pH) to each well, followed by overnight incubation to cleave the first part of the linker via diketopiperazine formation, and the released peptides were then vacuum filtered into 96-well microassay test plates to be used for determination of peptide biological activity. In some experiments the released peptides were aliquoted into several replicate plates for multiple simultaneous assay against different molecular targets. Subsequent to the first-stage bioassay, wells identified as "positive" were marked and the beads of origin were recovered from the corresponding well(s) of the filtration master plate with the aid of a low-power stereomicroscope. The recovered beads were then transferred one by one (one bead per well) into individual microwells in the required number of 96-well filtration plates. Cleavage of the ester (second) linker was then accomplished by addition of 0.2% NaOH and overnight incubation followed by pH adjustment. Alternatively, the second-stage release may be achieved by overnight incubation in ammonia vapors in a desiccator. After drying, the appropriate buffer was then added and the plates were gently shaken for several hours. Thereafter, the peptide-containing buffer was filtered into the test plates for bioassay.

The individual peptide beads corresponding to each positive well in the second-stage assay were then recovered and submitted for microsequencing.

Example of Application of Two-Stage Release Assay (15)

A releasable cyclic library of the structure H–Cys–Xxx–Xxx–Xxx–Cys–linker, where at positions Xxx all natural amino acids and their D-antipodes (besides cysteine) were randomized, was screened for binding to the gpIIb/IIIa receptor. Peptides that would compete with fibrinogen for binding to the gpIIb/IIIa receptor were sought using an ELISA-based assay (19). Approximately 100,000 beads were distributed into 500 wells of microtiter filter plates (200 beads/well), and the first part of the peptide was released and filtered into the test plates coated with fibrinogen. In the first stage of release, two wells were found that inhibited the binding of gpIIb/IIIa to immobilized fibrinogen by 3 standard deviations from the mean of each plate (automated densitometry plot of all wells). The beads from these wells were divided one bead/well to a second set of microtiter filter plates, and the second stage linker was cleaved with NaOH. Following neutralization and filtration into the fibrinogen-coated plates, the competitive binding assay revealed that 3 of a total of 406 wells on seven plates tested (58 wells used per plate) had significantly reduced absorbance and were the sites of inhibitory activities exceeding the threshold value of 3 standard deviations. The 3 individual beads from these 3 wells were recovered and subjected to microsequencing. The two most inhibitory beads contained CRGDC, and the third, weaker bead had the sequence CARYC.

As both L- and D-amino acids had been used to randomize the cyclic peptide library, both RGD and ARY cyclic peptides were synthesized using L- and D-amino acids in all possible combinations (except for the flanking cysteines, where only L-cysteine was used). The relative potency of each peptide was measured using the fibrinogen binding assay. Cyclic CRGDC had an IC_{50} of 1 μM and hence was the active stereoisomer identified in the screen. Of the other three stereoisomers, CrGDC, CRGdC, and CrGdC (G has no chirality), only CRGdC showed any activity in our assay, with an IC_{50} of 100 μM. The cyclic ARY peptide, which was found in the least active well of the series, represents a false positive "hit." Generally, the frequency of false positives is less than the frequency suggested by this experiment of 1/406 wells. The two RGD "hits" inhibited binding to the degree expected—about 50%. Under the conditions used in this assay, a released peptide should have a final concentration of about 1 μM, which coincides with the IC_{50} for cyclic RGD.

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