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Combinatorial Libraries

Synthesis, Screening and Application Potential

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2 Combinatorial Libraries of Synthetic Structures: Synthesis, Screening, and Structure Determination

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2.1 Introduction

One of the basic questions for which modern science continues to seek an answer is the nature of intermolecular communication. Many biological effects are triggered by the interaction of a ligand with its signaling counterpart, and many diseases are caused by the malfunction of this signaling mechanism. Once the role of a particular signaling mechanism is recognized, one may deliberately intercede in this process in an attempt to obviate pathological activity. If there is no *a priori* knowledge of structure activity relationships between ligand and receptor, or one would like to discover a novel structural motif, the only practical approach is to test a multitude of compounds for the desired activity. The success of this random screening approach depends on both the number and the spread of diversity among compounds available for testing.

Of the approaches to the generation of molecular diversity, multiple synthesis – in which a multitude of compounds are separately though simultaneously synthesized – was the first to accelerate dramatically the speed of compound synthesis (Geysen et al., 1984; Houghten et al., 1985; Frank et al., 1988; Krchňák and Vagner, 1990; Fodor et al., 1991; DeWitt et al., 1993; Kramer et al., 1993; Meldal et al., 1993; Bunin et al., 1994; Cass et al., 1994). However, it was the advent of combinatorial techniques (Geysen et al., 1986) that have revolutionized high throughput screening and, as such, can be considered one of the most exciting recent developments in medicinal chemistry. The first breakthrough in generating vast numbers of compounds in a format suitable for high throughput assays, in this case peptides, was the filamentous phage technique (Parmley and Smith, 1989; Cwirla et al., 1990; Devlin et al., 1990; Scott and Smith, 1990; Felici et al., 1991; Cull et al., 1992; O'Neil et al., 1994). In contrast to multiple synthesis techniques,

in which the structure of the compound to be synthesized is known throughout the process, the phage technique represents a true "library," in which the structure of a tested compound is not determined until its biological relevance has been established. Soon thereafter, complex synthetic combinatorial libraries emerged, including Lam's one-bead one-structure concept (Lam et al., 1991) (based on Furka's ingenious split/mix method (Furka et al., 1988a; Furka et al., 1988b; Furka et al., 1991)) and Houghten's iterative combinatorial library strategy (Houghten et al., 1991) (analogous to Geysen's mimotope strategy (Geysen et al., 1986)). The advent of synthetic combinatorial library techniques has paved the way for the ever expanding diversity of compounds available for screening, increasing the chances of discovering novel molecules of known function and expanding fundamental knowledge of biological mechanisms and potentially the discovery of effective disease treatments.

Adding to a growing number of reviews on the field of combinatorial libraries (Scott, 1992; Houghten et al., 1992; Dooley et al., 1993; Houghten, 1993; Moos et al., 1993; Pavia et al., 1993; Scott and Craig, 1994; Sebestyen et al., 1993; Gallop et al., 1994; Gordon et al., 1994; Houghten, 1994) and the one-bead one-structure strategy (Lebl et al., 1995), this contribution will describe our recent developments in synthetic combinatorial libraries based on the one-bead one structure concept (Lam et al., 1991), with a focus on the design and synthesis of non-peptide libraries, the chemistry of releasable linkers as applied to solution phase assays and structure determination using codes for non-sequencable compounds.

2.2 One-Bead One-Structure Concept

The one-bead one-structure combinatorial library approach consists of three basic steps: (i) chemical synthesis yielding a library with a unique structure on each bead; (ii) screening the library using an on-bead binding assay or multi-step release assay; and (iii) structure determination for beads of interest. Library synthesis follows the mix and split method, first described by Furka (Furka et al., 1991), and illustrated in Figure 2.1. For the on-bead binding assay, beads containing active compounds are identified using an ELISA-type assay (Lam et al., 1991; Lam et al., 1993; Ohlmeyer et al., 1993; Lam and Lebl, 1992), or a fluorescent labeled probe (Chen et al., 1993; Needels et al., 1993; Meldal et al., 1994), and then physically segregated from the inactive compounds. Since not all projects are amenable to the on-bead assay, we have developed chemistries for stepwise release of compounds from library beads and extended the one-bead one-structure technique to solution phase assays (Lebl et al., 1993, Kocis et al., 1993; Salmon et al., 1993). The final step of the initial screen is structure determination of active

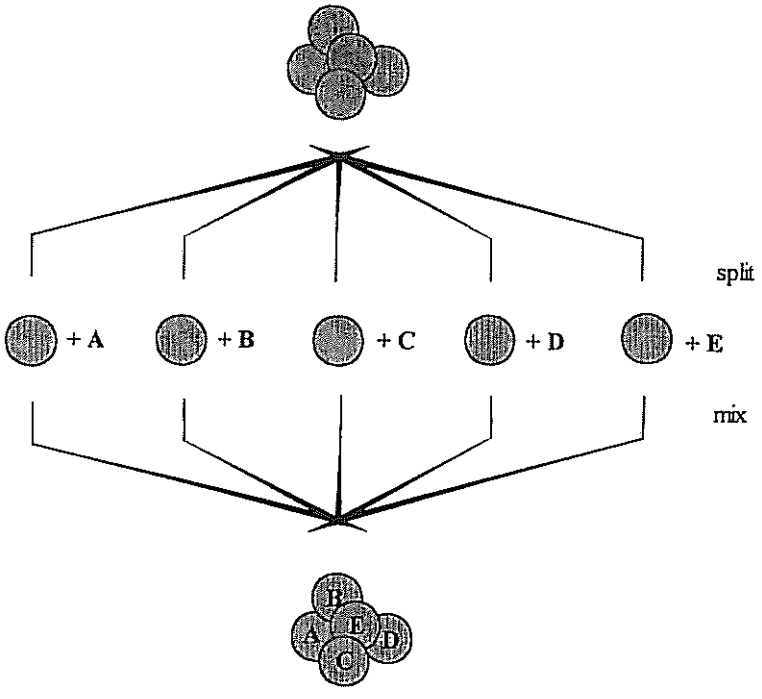


Figure 2.1: Schematic representation of Furka's split/mix method. Beads are equally split into a number (five in this Figure) of portions and in each portion a reaction with different building block (A, B, etc.) is performed. Then the beads are mixed and the process can be repeated.

compounds, using either direct methods, such as Edman degradation (Edman, 1950; Edman and Begg, 1967) and mass spectroscopy, or indirect coding approaches.

2.3 Design and Synthesis of Non-Peptide Libraries

The first synthetic combinatorial libraries were composed of peptides for obvious and pragmatic reasons. The chemistry used for synthesis on solid phase was well described (for review see e.g. (Atherton and Sheppard, 1989; Fields and Noble, 1990) and references cited therein), as were methods for structure determination

with small amounts of material. Although peptides represent an important class of organic compound, numerous groups, including us, have embarked on an effort to create combinatorial libraries of non-peptidic small molecules (DeWitt et al., 1993; Zuckermann et al., 1992; Simon et al., 1992; Nikolaiev et al., 1993; Cho et al., 1993; Simon et al., 1994; Chen et al., 1994; Stankova et al., 1994; Lebl et al., 1994a; Lebl et al., 1994b, Krchňák et al. 1995a). The rational for this effort is two fold. First, the structural diversity of peptides is limited by the character of the peptide backbone. In addition, other classes of compounds have been shown to be structurally and chemically diverse and have characteristics not present in peptides that are important to drug candidates – such as oral bioavailability and resistance to protease degradation.

2.3.1 Diverse and Complex Libraries

To effectively create a large chemical diversity one needs reliable and high yield chemical reactions that can be performed on solid phase, as well as a sizable collection of building blocks that afford the possibility of different types of molecular interactions. Peptide libraries can be extremely complex, in that they contain large numbers of compounds of differing sequence – although these may be similar in structure. N amino acids used in each of x randomization steps create n^x structures. Using only 20 natural amino acids one can easily synthesize millions of peptides (e.g. all 3.2 million possible pentapeptides). However, while this is quite an achievement never before possible, mere numbers do not necessarily

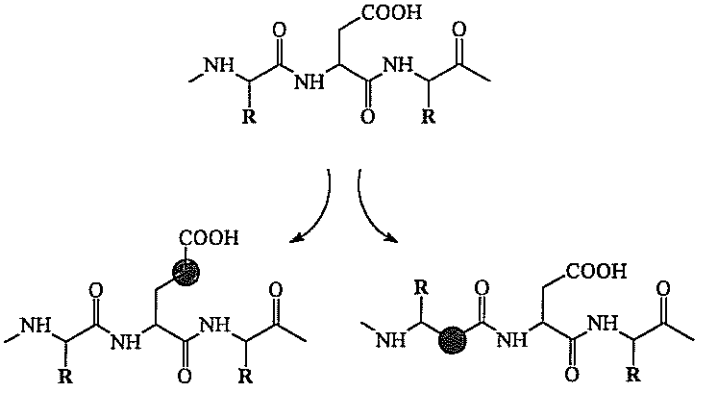


Figure 2.2: Changing the diversity of libraries by insertion of one methylene group.

increase the probability of discovering a desirable compound. Instead, the key to success in many instances may be the number of structurally unique compounds in a library; that is, the diversity within a library. Libraries of peptides do not represent a great diversity of structure, since the only changing parameter is the type of side-chain connected to the α-carbon of peptide backbone, and those side-chains can occupy only a predetermined conformational space. Combining L amino acids with D achieves greater diversity by enlarging the conformational space (Ramachandran plot (Ramachandran and Sassisekharan, 1968)), nevertheless, it is still quite limited. As an example, if a methylene group is inserted into the side chain, replacing an aspartyl residue with glutamyl (a typical scenario in peptide libraries), the number of components will double, but the diversity will not change substantially since it results in only the extension of one side chain. However, if the methylene group is inserted into the backbone, forming a β-amino acid, the complexity will also be doubled, but the diversity will be enhanced compared with the former case, since this insert will influence the relative spacing of the side chains. Figure 2.2 illustrates this point.

The differing characteristics of diverse and complex libraries can be used to one's advantage depending on the application. For example, a large number of building blocks of the same type (α-amino acids) coupled in the same format (peptide) will map a given conformational space very densely; however, the breadth of space covered by this library may be relatively small. If this space can accommodate crucial structural elements of compounds known to interact with the target molecule, a dense library may yield high affinity ligands. However, if the structural requirements for binding are not known, increasing the number of components in such a library will not necessarily enhance the probability of finding a hit. In this case, a more fruitful tactic may be to screen a diverse library that maps a large conformational space with lesser density. Hits identified from this library may not have the desired level of activity, but may be taken as a lead for further optimization. Subsequent libraries, containing a structural bias based on the initial lead can be designed providing maximum complexity in order to fine-tune the activity of the first hit.

2.3.2 Flexible and Rigid Libraries

In a simplified model, there are two primary determinants of whether a compound will bind specifically to a target molecule. The ligand should have the critical structural elements (functional groups) and present them in positions predetermined by the target. The first condition is readily met by selecting building blocks which represent all major sources of interactions known to play a significant role in molecular recognition. The second condition is closely related to the conformational space that is covered by individual library members. To enhance

the probability that critical functional groups will be located properly without *a priori* knowing where they must be oriented, the library should cover a large conformational space. The one bead one structure concept presents a single chemical entity on each bead, but each individual is presented in a number of different conformations, depending on its flexibility. The higher the flexibility of structures, the larger the conformational space covered by individual members of a library. However, since there will be present many different conformers of one structure, the correct one (if present) will not be highly populated at any time. The trade off in this approach is that one may have to settle for relatively low affinity interactions as a result of an adverse entropy factor. Conversely, one may have a lower probability of identifying a high affinity interaction in screening libraries of more rigid structures. At present, there is no definitive means to quantify changes in the free energy of binding between the target molecule and ligand as a function of conformational freedom. An interim solution to this dilemma can be found by using the intuition and experience of the chemist, and knowledge of the target of interest. Future data should enable a more definitive answer regarding the optimal degree of flexibility. In the meantime, however, the speed with which libraries can be synthesized and screened enables one to try multiple approaches.

2.3.3 Examples of Libraries

Every combinatorial library is biased by the selection of building blocks, coupling chemistries and the linkers or "scaffolds" to which the building blocks are attached. The selection criteria are most likely based on a number of factors, including knowledge of the target molecule and availability of compatible build-

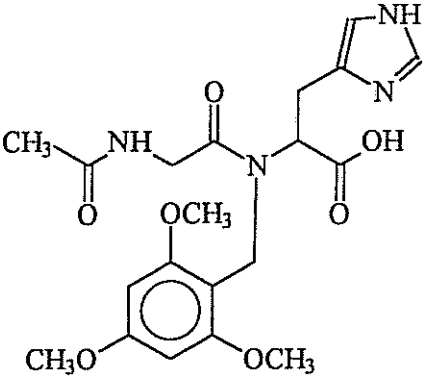


Figure 2.3: Structure of streptavidin binder from the library of alkylated and acylated amino acids.

ing blocks and coupling chemistries. The following is a list of various libraries we have synthesized and screened:

(i) Libraries of small, compact, and relatively rigid structures (e. g. N-acyl-N-alkyl amino acids (Stankova et al., 1994)).

(ii) Libraries based on a scaffold structure with variable rigidity (usually a multifunctional cyclic scaffold, e.g. a derivatized cyclopentane or cyclohexane ring, benzenetricarboxylic and diaminobenzoic acids (Lebl et al., 1994b; Kocis et al., 1994)).

(iii) Libraries based on a flexible scaffold that is built during the synthesis of the library and can be randomized (branched scaffold based on diamino acids, α,β,γ -library; Krchňák et al. 1995b).

To elaborate further on a representative non-peptidic small molecule library we have synthesized, we describe here the synthesis of a library of acylated and alkylated amino acids. Twenty α-amino acids were coupled to the resin beads. The amino protecting group was removed and the liberated amino group was reacted with a set of 20 aldehydes. The resulting Schiff base was protonated and reduced by sodium cyanoborohydride and the secondary amino group was acy-

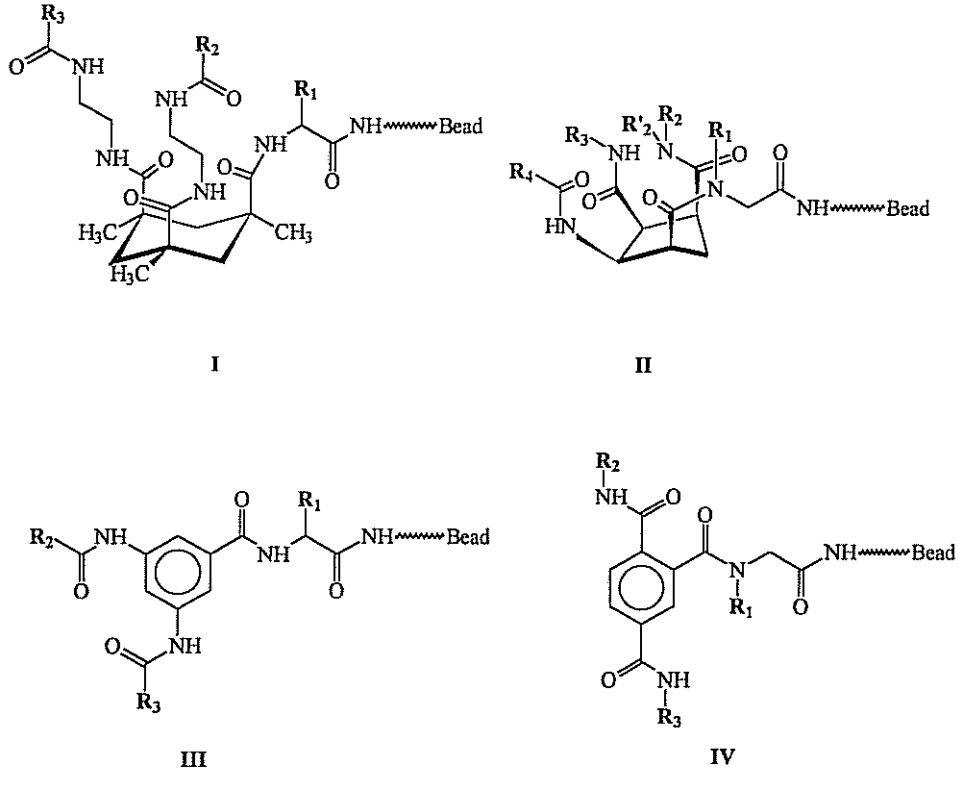
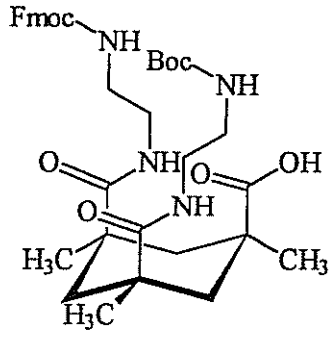


Figure 2.4: Structure of scaffold based libraries.



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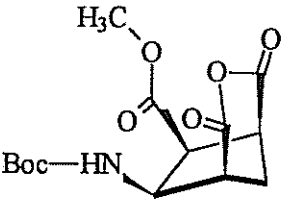

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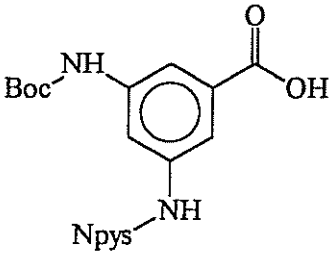

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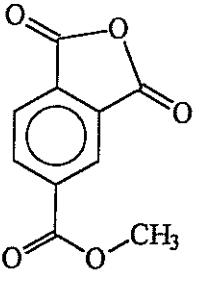

IV

Figure 2.5: Structure of protected scaffolds used in library synthesis. The following protecting group abbreviations were used: fluorenylmethyloxycarbonyl, Fmoc; t-butyloxycarbonyl, Boc; nitrophenylsulphenyl, Npys.

lated by 20 different carboxylic acids. We have developed a technique by which to identify the structure of these compounds using mass spectroscopy, avoiding the need to provide a sequencable coding tag (Stankova et al., 1994). Screening this library against streptavidin yielded three positive beads, each containing the same structure (see Figure 2.3). The compound was resynthesized and binding was confirmed in solution and found to be higher than that of previously identified peptide ligands (Lam et al., 1991; Lam and Lebl, 1992).

In order to constrain the presentation of a given set of functional groups, we have synthesized several libraries based on scaffolds with variable rigidity. Examples of scaffold based libraries are represented by four different cyclic skeletons (Figure 2.4): trimethylcyclohexanetricarboxylic acid (Kemp's triacid, structure I, Kocis et al. 1995), aminocyclopentanetricarboxylic acid (II, Patek et al. 1994), diaminobenzoic acid (III) and benzenetricarboxylic acid (IV). In all cases

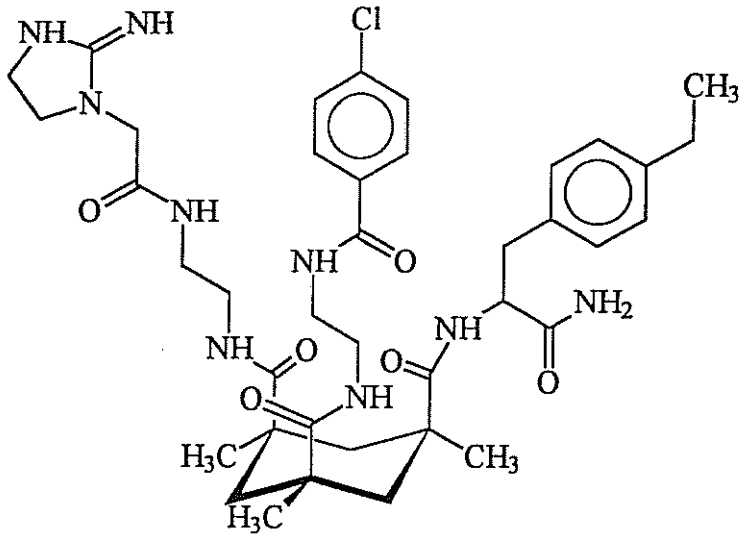

Figure 2.6: Structure of a thrombin inhibitor identified from a library based on a cyclohexane tricarboxylic acid scaffold.

suitably protected scaffolds have been synthesized (Figure 2.5) and coupled via a carboxyl group to an amino acid on the resin bead. The protecting groups are then removed one by one; each deprotection followed by coupling a set of building blocks – carboxylic acids when a liberated amino group is present on the resin, and amines when a free carboxyl group is available on the scaffold. The library based on the Kemp's triacid scaffold was tested against thrombin and five hits were found, the best of which (Figure 2.6) exhibited a Ki of 4μM (Kocis, P., et al., in preparation), higher than the classical active site peptide inhibitor fPRPG (20μM) which is the active site pharmacophore of Hirulog, currently in clinical trials.

As an example of a flexible library with randomized backbone, we synthesized a library with variable side chain spacing, having an α, β or γ amide bond between each subunit. The synthesis of this library will be outlined in the section describing coding.

2.4 Release Assay

The one-bead one-structure concept is inherently suitable for performing the on-bead binding assay. However, not all assays are amenable to on-bead screening, and for some important targets relevant assays must be performed in solution. In

principle it is possible to distribute beads one by one, cleave the compound from each bead, test solutions and then recover those beads that released compounds displaying activity in given test and determine their structures. However, this technique is applicable in small complexity libraries only. To be able to screen libraries containing millions of different compounds we have developed a two step releasable assay. In the first step, beads are distributed into a 96 well format filtration plate, each well containing ca 500 beads, one third of the compound is released from each bead into solution and the mixture of compounds is tested. Beads from positive wells are re-distributed into individual wells and the second portion of the compound is released. Each bead corresponding to an active solution is recovered, and the compound (or coding tag) remaining on the bead is used for structure determination (Lebl et al., 1993; Kocis et al., 1993; Salmon et al., 1993). An alternative approach to performing the releasable assay, based on repeated partial release from one type of linker, was introduced recently by Jayawickreme et al. (Jayawickreme et al., 1994).

2.4.1 Chemistry of Releasable Linkers

Releasable libraries have been constructed according to the scheme depicted in Figure 2.7. Functional groups (amino groups) on the resin were branched to produce three independent branches, two of which were used for attaching the test compound. The third branch linked the compound used for identification, which can be the same compound or a tag encoding the structure of any non sequencable compound. Test compounds were attached to the releasable arms via an ester bond; however, the ester bond was cleaved by two unique mechanisms – entropically favored cyclization resulting in diketopiperazine (DKP) formation and alkaline hydrolysis. The release of a peptide based on DKP formation was described (Bray et al., 1993; Bray et al., 1994; Maeji et al., 1992; Bray et al., 1991a; Bray et al., 1991b; Maeji et al., 1990); however the DKP moiety was cleaved from

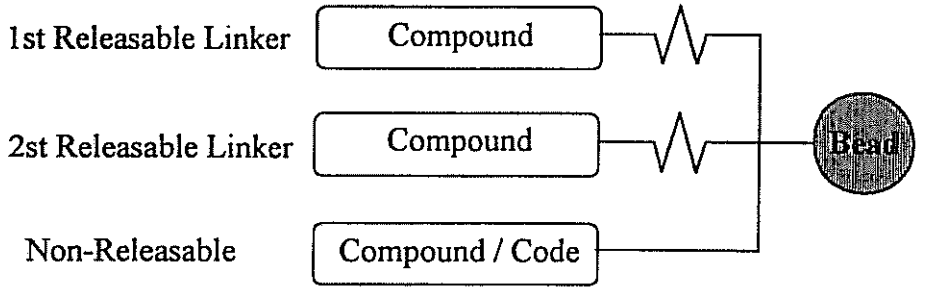

Figure 2.7: Design of double cleavable libraries.

the resin and stayed with the released compound. To release compounds with the same terminus in both stages and not containing DKP, we designed a "reverse" DKP linker, in which the DKP stays on the resin. Compounds are attached to the linker via an ester bond of Fmoc-Gly-NH-$(CH_2)_3$-OH (Fmoc-Gly-HOPA) and when released to the aqueous solution they contain an identical carboxy terminus, the hydroxypropylamide of glycine (Gly-HOPA).

The first generation of linkers was based on the Glu-Pro motif (Lebl et al., 1993) (Glu provides a side chain function and Pro enhances the tendency to cyclize). The recognition of iminodiacetic acid (Ida) as the α-amino acid involved in diketopiperazine formation allowed us to design novel double cleavable linkers. Iminodiacetic acid was found suitable for several reasons: (i) The imino group is in the α-position relative to the carboxyl groups; (ii) both carboxyl groups are chemically equivalent; (iii) as an N-substituted amino acid it is prone to cyclization via DKP formation with practically any other α-amino acid; (iv) it is not chiral; (v) it is inexpensive. In general, there are three variations of the Ida-based linker. They can be schematically depicted as dipeptides containing Aaa-Ida, Ida-Aaa, or Ida-Ida, where Aaa is any α-amino acid, preferably one that is prone to cyclization via DKP formation. We found that the position of Ida in such a dipeptide was not important. This is not true for the combination of Glu and Pro. The dipeptide Glu-Pro provides satisfactory kinetics of DKP formation unlike the dipeptide Pro-Glu, the cyclization of which takes more than 24 hours.

The dipeptide motif Ida-Ida was found particularly suitable for designing double cleavable linkers (Kocis et al., 1993). 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 synthesis of test compounds, one for attaching the linker to the resin beads. Two Fmoc-Gly-HOPA's are either coupled to both carboxyls of the carboxy terminal Ida (Figure 2.8, linker I), or each Ida bears one Fmoc-Gly-HOPA (linker II). In either case there is one free carboxyl group that serves for connecting the linker to the solid support, e.g. via Lys which provides one extra amino group for a third, nonreleas-able copy of the compound or the code. The chemistry of both releases is shown on Figure 2.9.

Using peptide libraries built on double cleavable linker, we have identified ligands for the anti-β-endorphin antibody and the glycoprotein IIb/IIIa receptor (Salmon et al., 1993).

The released compounds from both double releasable linkers I and II contained the Gly-HOPA. Since it may be desirable in some instances to release a compound without the Gly-HOPA, but having a free carboxyl group instead, we designed a modified linker that incorporates an additional ester linkage (Figure 2.10). The appended ester bond is introduced into the linker by attaching a hydroxy acid (e.g. 3-hydroxypropylamide of glutaric acid) to both arms of the linker. During the first release at pH8 the DKP is formed and compound with Gly-HOPA is released to the solution. The beads are then separated from the

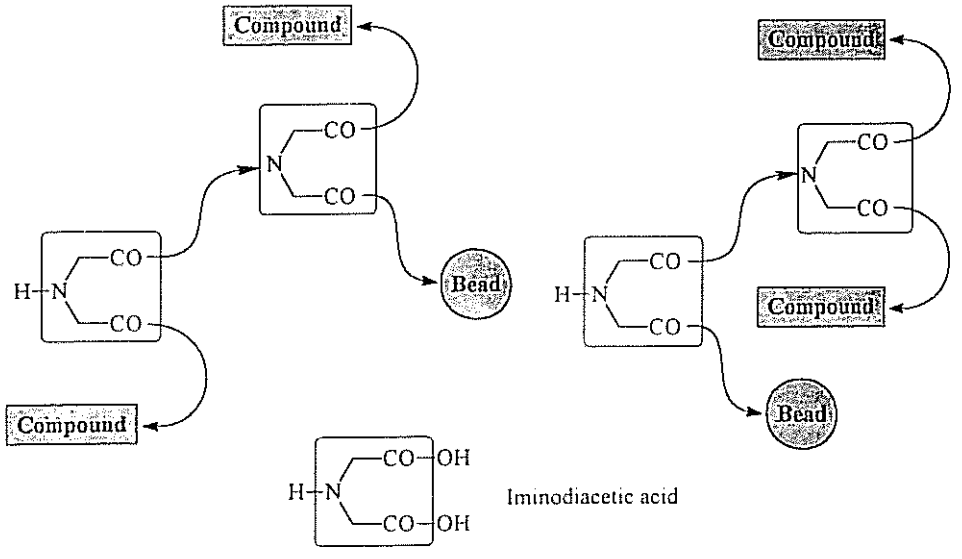

Figure 2.8: Scheme of two imminodiacetic acid based linkers.

solution by filtration, the pH is brought to ca 13 using NaOH, and after incubation to permit ester hydrolysis (typically ca 30 min) the solution is adjusted to physiologic pH for biological screening. The second release is performed using NaOH as previously described and yields the desired compound with a free carboxyl group.

2.4.2 Sensitivity of the Assay

The on-bead binding assay is very sensitive, due to the existence of a high local concentration of test compound on the surface area of the resin bead. One gram of TentaGel resin (polyethylene glycol grafted polystyrene crosslinked with 1 % divinylbenzene) of average size 130 µm and with a substitution at 0.2 mmol of amino groups per gram of resin contains ca 1 million beads. A simple calculation reveals that the local concentration of test compound at the surface area of each bead is approximately 20 mM. However, if the test compound from the same bead is released to solution, the final concentration is approximately 2 µM, assuming a 100 µl assay volume and quantitative release of test compound.

This number is critical from the assay point of view, since the weakest binder that can be detected will have an IC_{50} in the micromolar range. To detect weaker interactions, the volume of assay can be decreased (we have been able to decrease the vol-

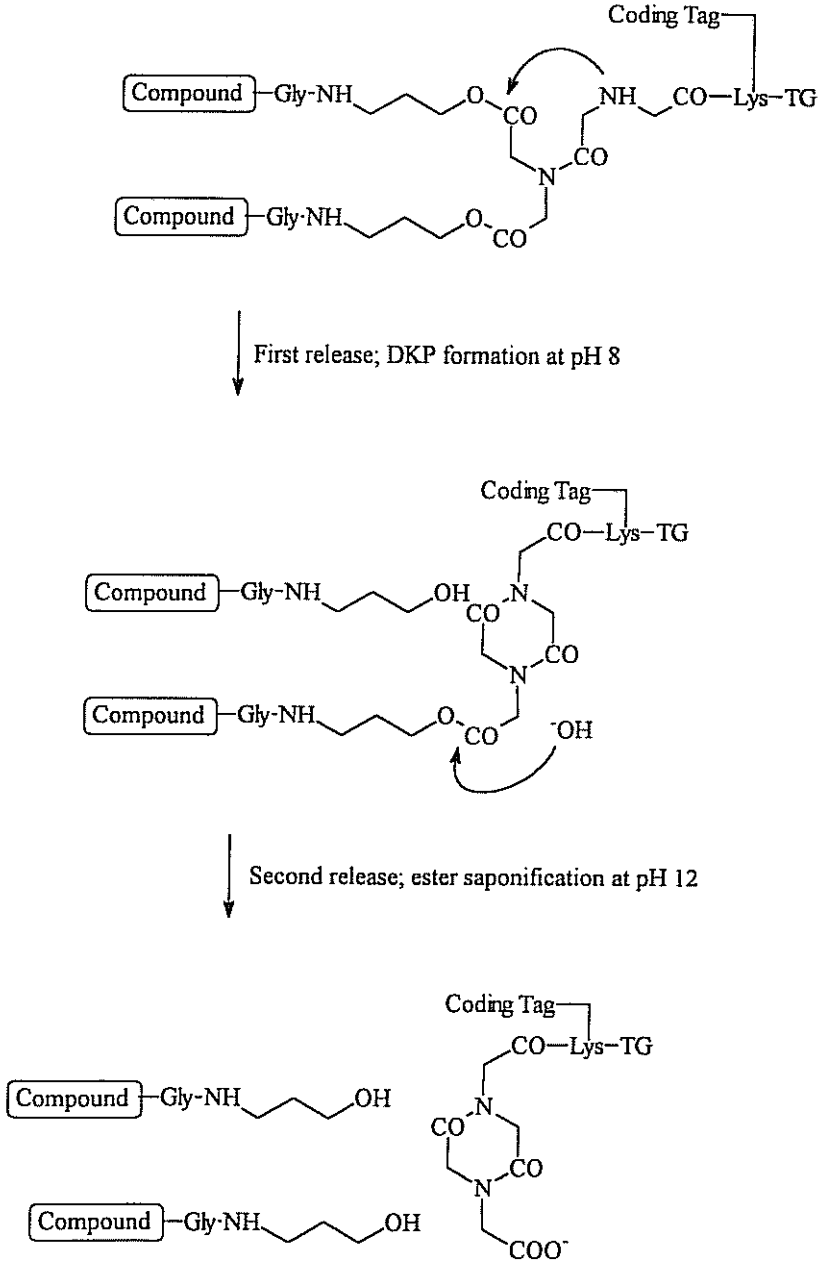

Figure 2.9: Chemistry of two stage release of a test compound from the iminodiacetic acid based linker.

ume three-fold). Alternatively, one can increase the amount of compound released from each bead. This can be achieved in three different ways: (i) increase the substitution of beads, maintaining the same size of beads, (ii) increasing the size of the beads, or (iii) using an alternative solid support, e.g. cotton thread or plastic membrane, that can be cut into pieces of almost any size, modulating the

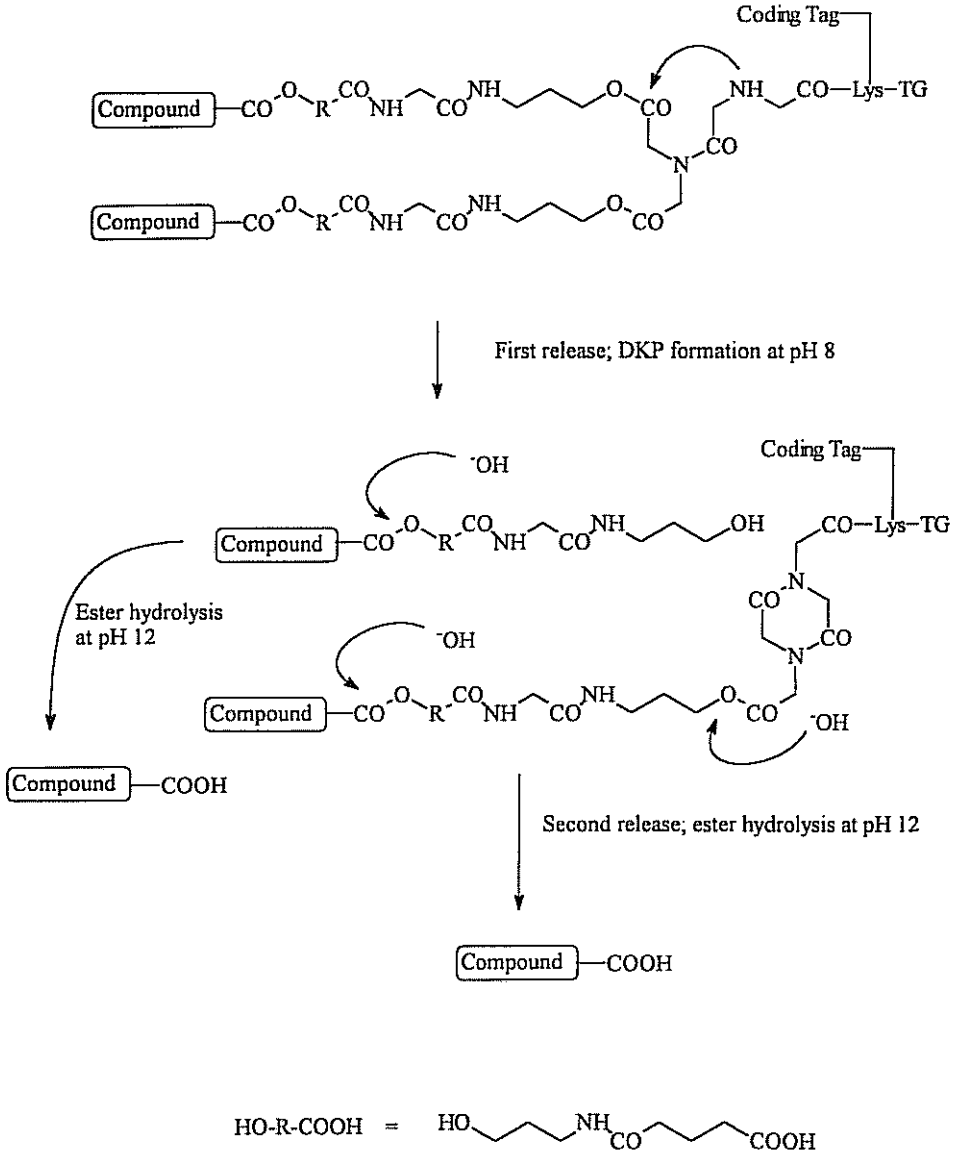

Figure 2.10: Chemistry of two stage release of a test compound with free carboxyl group.

releasable quantity by length or area of support. We have increased substitution up to 1 mmol of amino groups per gram of resin with the same size of beads with good success, but found that any further increase in substitution leads to slow reaction rates and incomplete coupling. We have used custom made 220μm beads constructed of polystyrene grafted with polyethylene glycol that bring the release to the nmol range. Finally, synthesis on cotton thread or fabric is feasible (Eichler et al., 1991; Lebl et al., 1992; Rinnova et al., 1993), although difficult to handle in a library format.

2.5 Structure Determination

The final step in the one-bead one-structure approach is structure identification for selected beads. A limiting factor in this respect is the amount of compound available for analysis, typically in the range of 100pmol. This is not sufficient to apply classical structure determination techniques (elemental analysis, spectroscopic methods, etc.). In the case of peptide libraries the problem can be solved easily since there is an established technique for the determination of the sequence of peptides, Edman degradation (Edman, 1950; Edman and Begg, 1967). This method has been automated and its sensitivity (2–3pmol in our hands) is well below the amount available on one bead.

Determination of the structure of organic molecules in diverse combinatorial libraries requires alternative methods. We have applied two different techniques: direct structure determination using mass spectroscopy and an indirect coding method.

2.5.1 Mass Spectroscopy

There is enough material on one resin bead for analysis by mass spectroscopy. Due to the large number of individual components in one library, determination of molecular weight is not sufficient information to resolve the structure. Therefore we have applied MS/MS techniques, which provide information regarding fragmentation. Since the molecular weight of all building blocks is known, in the case of informative fragmentation it is in principle possible to recall the composition of individual library compounds. The success of this method depends on knowing the fragmentation pattern and we have been able to use this technique in a number of cases, including the N-alkyl-N-acylamino acid library (Stankova et al., 1994), the Kemp's triacid library, and libraries based on side chain substituted diamino acids assembled into a peptide chain. We have also applied deuterium exchange of labile protons to increase the reliability of structure determination (Sepetov et al., 1993).

2.5.2 Coding Principle

If direct structure determination is not feasible we apply, similarly as the others, the coding strategy (Ohlmeyer et al., 1993; Needels et al., 1993; Nikolaiev et al., 1993; Brenner and Lerner, 1992; Nielsen et al., 1993; Kerr et al., 1993), just as mother nature has been doing for ages by coding of protein sequences with nucleic acids. The chemistry of encoded library synthesis is more difficult and time consuming, since there must be two compatible chemistries available to build a screening arm and a coding arm at the same time. In the case of peptides as coding tags, each chemical reaction in a randomization step is coded by a corresponding amino acid (Figure 2.11). The structure of the resulting peptide code is resolved using Edman degradation (Edman, 1950; Edman and Begg, 1967). If a different set of coding amino acids is used in each randomization step, each amino acid codes for not only the building block, but also the randomization step. However, taken individually, the number of different amino acids would be too high (e.g. four randomizations, each performed with 45 building blocks would require 180 amino acids). To reduce the number of coding amino acids we have applied a binary code, in which each chemical reaction is coded by a doublet of amino acids (Sepetov et al., in preparation; binary coding using different chemistry has recently been described (Ohlmeyer et al., 1993)). In this arrangement n amino acids can code for $n(n-1)/2$ building blocks, substantially reducing the necessary number of coding amino acids. In addition, since both the encoded building block and the round of synthesis can be determined, it is possible to design the structure of the coding tag to permit cleavage of all the doublets simultaneously, substantially reducing the time for structure determination.

To have a battery of α-amino acids with desirable properties (suitable reten ion time of their phenylthiohydantoins on HPLC and compatibility with chemistries applied during library synthesis) we acylated the side chain amino group of

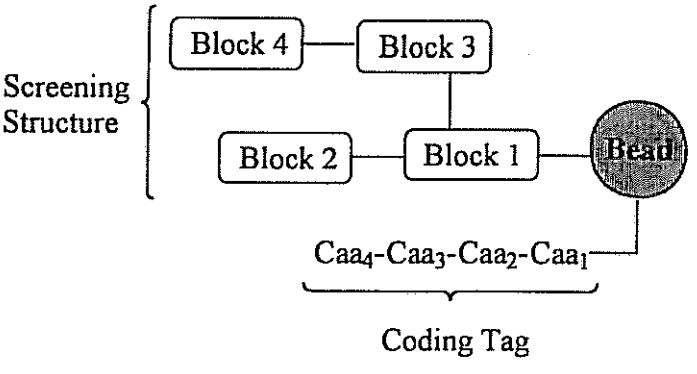

Figure 2.11: Principle of coding

diamino acids (lysine, ornithine, diaminobutyric, and diaminopropionic acids), evaluated retention time of their phenylthiohydantoins, and selected those that provided baseline separation.

2.5.3 Alternative Coding Techniques

When the library is synthesized in a releasable format, all components of the library are tested in solution, separated from the bead of origin. However, when the on-bead binding assay is employed, the coding structure may interact with the target molecule. There are at least three possibilities to prevent unwanted interaction of the coding structure: (i) present the coding structure in very low concentration, decreasing the probability of interaction; (ii) make the code inaccessible to the target molecule; (iii) code using a mixture of coding structures rather than by only one coding compound.

The first method can be achieved using an oligonucleotide sequence as the code (Needels et al., 1993; Brenner and Lerner, 1992; Nielsen et al., 1993). The high sensitivity of this method results from the use of PCR amplification. The relative abundance of coding tag can thus be minimized and the probability of interaction is diminished. An alternative and elegant scheme was recently described by Ohlmeyer et al. (Ohlmeyer et al., 1993), in which high sensitivity was gained by using halogenated aromatic acids analyzed by gas chromatography.

We have developed the second strategy, based on physical separation of the surface of the bead from its interior (Vagner et al., 1994a; Vagner et al., 1994b). TentaGel resin used for this concept is not penetrable by high molecular weight compounds, therefore the interaction with the macromolecular target occurs only on the surface. Several methods to separate reactive functional groups (amino groups) on the surface from those located inside beads were tested. The most promising results were obtained by enzymatic shaving (Figure 2.12). An enzyme

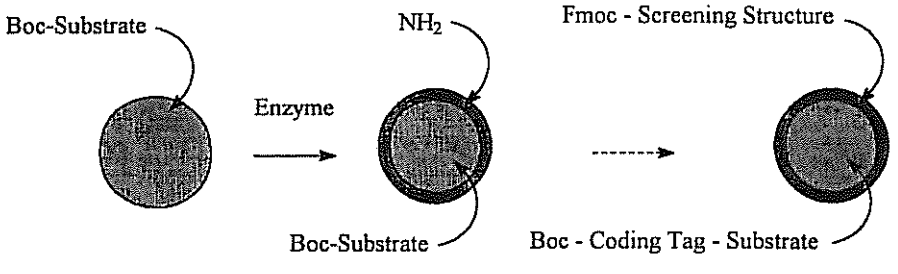

Figure 2.12: Enzymatic shaving of beads.

substrate, Boc-Phe-Gly, was synthesized on all available amino groups and then cleaved by chymotrypsin. Since the enzyme cannot penetrate inside the bead, only the substrate on the surface was cleaved (shaved). To show that the surface was efficiently depleted, we synthesized the streptavidin ligand LHPQF and the thrombin inhibitor fPRPG inside separate batches of beads. Neither ligand was detected by the corresponding assay, although both peptides were successfully sequenced. Both peptides were also synthesized on the shaved surface of beads and were then detectable in the corresponding assay.

In the third technique coding is not achieved by a single structure, but rather by a set of different structures having one common feature, thereby providing unambiguous identification. We explored three different strategies for design and synthesis of the coding arm. In the first approach (Figure 2.13, panel B), each chemical reaction performed on the screening arm is followed by deprotection of the amino group on the coding arm, coupling a mixture of two coding amino acids, protected so that the protecting group can be removed after the next chemical reaction on the screening arm is complete. The next mixture of two coding amino acids are then coupled to the amino groups of the previous coding amino acids. After finishing the synthesis, the coding arm represents a mixture of peptides, in the case of four coded chemical reactions the coding structures comprise 16 different tetrapeptides. Since four cycles of Edman degradation (Edman, 1950; Edman and Begg, 1967) are needed to determine the structure, the same set of coding amino acids can be used in each step, even though they can code for different reactions or building blocks.

Another approach uses only a portion of available amino groups for attaching the coding amino acids (Figure 2.13, panel A). After liberating the amino groups on the coding arm, approximately half of the equimolar amount of mixture of the two coding amino acids is coupled. The remaining 50% of free amino groups on the coding arm is then reacted with a protected diamino acid. In this way the number of remaining amino groups is doubled, reaching the original level of substitution, since only 50% of amino groups were doubled. In this arrangement a different set of acids must be used for each coded step because the sequencing will reveal all coding amino acids in one step of Edman degradation and the coding amino acids have to bear not only the information about the chemistry but also code for the step number.

In the last version of coding strategy all coding amino acids are linked to one amino group (Figure 2.13, panel C). The protecting group from the coding arm is removed and the first doublet of coding amino acids is coupled to a portion (e.g. 25% in the case of four coding steps) of available amino groups. The rest (75%) of the amino groups are blocked by a suitable protecting group, that can be removed before the second coding step. The limiting factor in this scheme is the amount of each coding amino acid needed to see a reasonable signal upon sequencing.

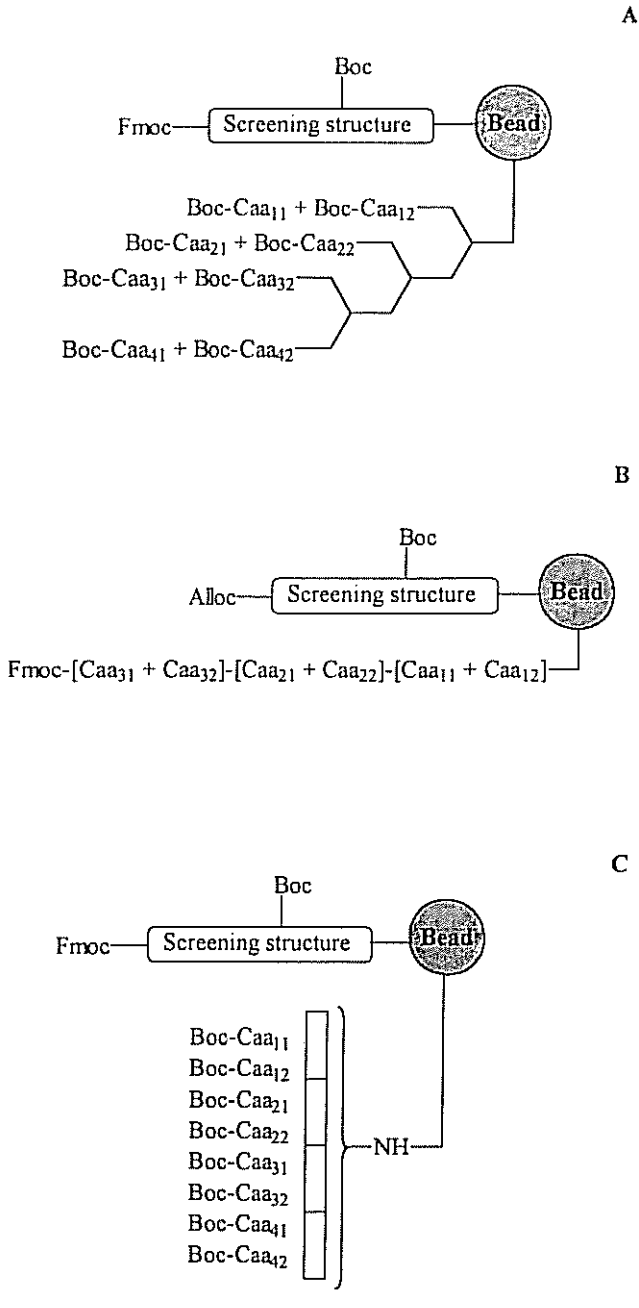

Figure 2.13: Three strategies for design of the digital coding tag. Orthogonal combination of protecting groups was used for the construction of the screening structure (Fmoc and Boc in the panel A and C, Alloc and Boc in the panel B) and coding tag (Boc in panels A and C, Fmoc in panel B).

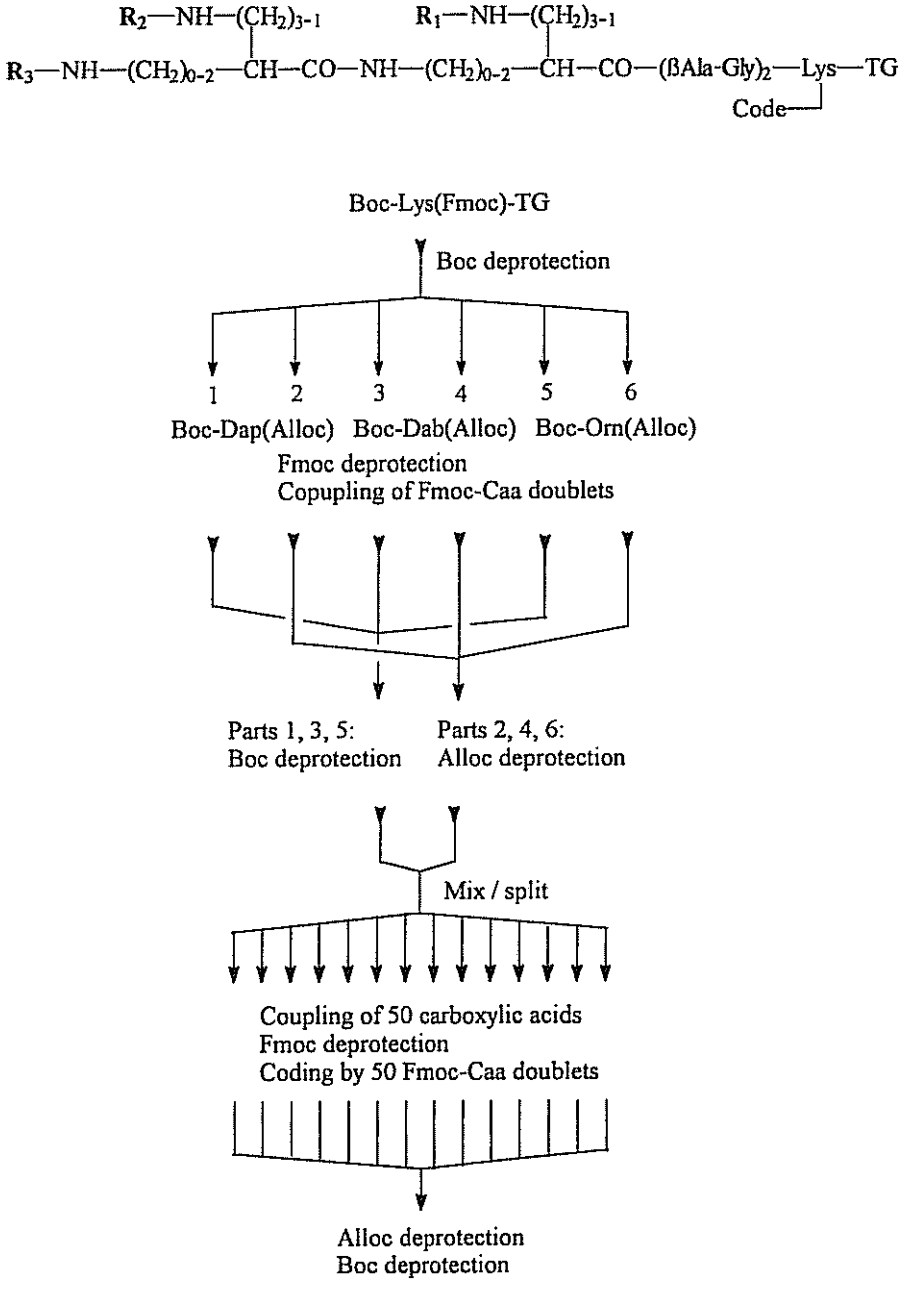

Figure 2.14: Synthetic scheme of coded library having variable side-chain spacing.

Coding in a more general sense tracks the history of synthesis performed on the resin beads. The coding tag does not necessarily need to include that which was coupled to the resin, but can be used to store, for example, the information what was removed from the resin. This type of coding is illustrated by the synthesis of a library with variable side-chain spacing (α,β,γ-library; Krchňák et al. 1995b). In this library (Figure 2.14) we attached a set of building blocks (carboxylic acids) to one amino group of a diamino acid (diaminopropionic acid, diaminobutyric acid, and ornithine) and used the second amino group for the extension of the backbone by coupling another diamino acid. Half of the library had the carboxylic acid coupled via the α-amino group, the second half had coupled the acids to the side chain amino group.

Synthesis was performed using N^{α} Boc, N^{ω} Alloc protected diamino acids. Coding amino acids were protected by an Fmoc group. Boc-Lys(Fmoc) was coupled to the resin and its α-amino group was used to build 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. After removing the Boc protecting group the resin was divided into 6 parts and diaminopropionic acid was coupled to parts 1 and 2, diaminobutyric acid to parts 3 and 4 and ornithine to the remaining two parts. The Fmoc group from the coding arm was then cleaved and 6 different doublets of coding amino acids were coupled to the coding arm. 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 50 parts and a set of 50 carboxylic acids was coupled, followed by coding using 50 doublets of coding amino acids (requiring only 11 coding subunits). After finishing the library synthesis the entire coding arm consisted of a mixture of heptapeptides that coded for four randomizations of carboxylic acids (sequencing cycles No 1, 2, 4, and 6) and the type of diamino acid and, at the same time, coded for the protecting group which was removed from the diamino acid (cycles No 3, 5, and 7).

2.6 Conclusion

This contribution has focused exclusively on the one-bead one-structure technique applied at Selectide, illustrating developments in three areas that we consider critical to the application of the process. Several examples were documented in which interesting results have been obtained. The structural and chemical diversity represented in combinatorial libraries through the use of building blocks other than amino acids and the use of scaffolds rather than a peptide backbone were given. Notable are the inhibitors of thrombin identified from the

Kemp's library. Not only was the most active compound higher affinity than the classical active site peptide inhibitor fPRPG, it is not a linear molecule. This is a clear validation of success in increasing library chemical and structural diversity in the Selectide process. The second point, addressing aspects relating to the release and screening of compounds in solution, focused on the need to be able to apply the Selectide Process to all target not just select few purified proteins. The third point focused on methods which have been developed to identify the composition of active compound in the case where they are not peptides. These examples serve to demonstrate that our approach, along with those of several other groups represent an increasingly important set of tools which can be applied to the discovery of new pharmaceuticals and to the increased understanding of molecular bioscience.

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