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Limitations of the coupling of amino acid mixtures for the preparation of equimolar peptide libraries

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Summary

The standard method of peptide library synthesis involves coupling steps in which a single amino acid is reacted with a mixture of resin-bound amino acids. The more recently described positional scanning strategy (in which each position in the peptide sequence is occupied in turn by a single residue) is different since it involves the coupling of mixtures of amino acids to mixtures of resin-bound amino acids. In the present study, we analyze the compounds produced under these conditions measuring coupling rates and amounts of formed products, using mainly UV, HPLC, LC/MS and MS/MS techniques. Our data do not permit to conclude that the resulting libraries are complete. Indeed, our analytical data indicate that a large part of the di-, tri- and tetrapeptides synthesized with this method are not present in the final mixture. Although chemical compensation (in which poor coupling kinetics is compensated by a larger excess of the incoming amino acid) has been thought to counterbalance these biases, our experiments show that the compensation method does not take into account the crucial influence of the resin-bound amino acid and that even the dipeptide libraries obtained in this way are far from completeness. The present work provides strong evidence that the coupling of mixtures of amino acids to resin-bound residues, which is required by the positional scanning strategy, results in incomplete and/or non-equimolar libraries. It also clearly confirms that coupling rates in solid-phase peptide synthesis are dependent on the nature of both the incoming and the immobilized amino acid.

Abbreviations: DCCI, *N*,*N*′-dicyclohexylcarbodiimide; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; DTT, dithiothreitol; ESI, electrospray; HOBt, hydroxybenzotriazole; MS, mass spectrometry; MS/MS, tandem mass spectrometry; Pmc, 2,2,5,7,8-pentamethyl-chroman-6-sulfonyl; TFA, trifluoroacetic acid; Trt, triphenylmethyl

Introduction

The successful synthesis of the first combinatorial libraries [1,2] demonstrated that peptides could be obtained by simple methods in numbers several orders of magnitude greater than by conventional means. The access to peptide libraries has provided in the last years (see Refs. 3–5 for reviews) a series of new tools to address problems that were out of reach before, such as the systematic study of protein kinase substrates [6,7], or of the nature of their SH2 and SH3 sequences [8,9]. Alternatively, these techniques provided new ways to speed up the screening and discovery of pharmacological leads (see the comment by Service [10]) including anticancer agents [11,12].

Although standard peptide libraries may contain very large numbers of compounds, such as 64 million hexapeptides made up of 20 amino acid residues, their

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synthesis is simple, because it can be conducted as an iterative process. At any step and in each reactor (split synthesis), a single amino acid in 3–4 M excess is reacted with a mixture of resin-bound growing peptide chains [13]. In this way, no competition between the incoming amino acids occurs, and coupling can be expected to reach completion, which insures an almost equimolar representation of the individual peptides in the resulting sublibraries. Furthermore, due to the same synthetic history of all the peptides on a single bead, split synthesis (which implies a mix-and-divide sequence) results in only one peptide type on each bead (one-bead–one-peptide approach [14,15]).

Some limitations of this procedure have been recognized, such as the rapidly increasing amounts of resin with library size and sequence length [16,17]. Another kind of problem arises from the fact that the success of synthesis is taken for granted and that the content of product mixtures is difficult to be assessed analytically (see Ref. 18 for a review). However, peptide libraries made up of natural amino acids which do not exceed the hexapeptide size can be obtained on gram amounts of resin, in good yields and in nearly equimolar ratios [15,19].

Positional scanning libraries are different from these standard libraries because they provide sets of sublibraries where the fixed residue is not necessarily at the N-terminus but occupies in turn any position in the peptide sequence [20–22]. For instance, a set of tetrapeptide sublibraries built in view of a positional scanning will comprise four subsets as follows: O1X2X3X4, X1O2X3X4, X1X2O3X4 and X1X2X3O4, where O is a fixed residue and X denotes any possible amino acid in the given position. In order to synthesize these subsets, one has to modify the coupling steps of the previous mix-and-divide technique. Indeed, to obtain, e.g., all the possible oligomers X1X2O3X4, the coupling between the residues in positions 2 and 3 must be done between a *mixture* of incoming amino acids and a single immobilized one (O3). In the next step of synthesis, the coupling of a mixture of amino acids has to be done with a mixture of the immobilized ones (positions 1 and 2). It follows that standard one-bead–one-peptide libraries are replaced by a complete sublibrary on the same bead. The positional scanning strategy requires the synthesis of all the subsets with a single building block in each position, from which, after the biological test, the best sequence(s) will be selected.

Although described as a time-saving technique, positional scanning implies the same number of syntheses as the standard method. This method avoids combinatorial steps, which are not available in most of the automated apparatus for parallel synthesis. The sublibraries comprising all the possible fixed amino acids at all the positions can be synthesized simultaneously and the screening of all the sublibraries can be done in parallel. This technique also avoids the deconvolution process, since the best molecule is constructed by choosing the best candidate amino acid at each individual position, implying that the contributions of the positions to biological activity are independent (see a contradictory point of view in Refs. 23 and 24). In fact, the peptide made up of the 'best' residue at each scanned position is not necessarily active (or the most active, see Ref. 12) and a number of parent analogues are generally synthesized to obtain a good lead.

The present study focuses on the critical step(s) required by positional scanning, which involves the coupling of mixtures to single or to other mixtures of amino acids. These steps are crucial for proper identification of the most active oligomers, which relies upon complete and equimolar representation of the postulated products.

Very few studies are available on the coupling of amino acid mixtures, since in classical solid-phase peptide synthesis a molar excess of a single N-protected residue is reacted with a single resin-bound amino acid [25,26]. Exceptions are the study of Volkmer-Engert et al. [27] on the synthesis of peptide using mixtures of incoming amino acids onto a unique immobilized amino acid and the early investigations of the effect of protecting groups on coupling efficiency performed with mixtures of four components in order to increase the number of data in a single experiment [28,29]. These data are either not conclusive or in favor of a dependence of the coupling on both the incoming and immobilized amino acid. A strong dependence of the relative coupling rates upon the amino component was also observed by Mutter [30] in competition experiments in liquid-phase peptide synthesis. Furthermore, a set of studies deals with the so-called chemical compensation, in which the concentration of the incoming amino acids is inversely proportional to their coupling efficiency [31]. While the authors demonstrate that adjusted concentrations in mixtures of building blocks reacting on a single amino acyl-resin produce nearly equimolar incorporation, they fail to report data on the coupling of mixtures to mixtures of free and resin-bound residues, respectively, and use a biased model peptide (Gly^1-AA^2-Ala^3-AA^4-Gly^5), with a constant alanyl residue (Ala^3) between the two

positions (AA^2 and AA^4) where competitive coupling takes place. Similarly, the variation of a single position each time of the decapeptide HIV-1 p-14 epitope using double coupling of substoichiometric amounts of the acylating amino acid in mixture, which resulted in approximately equimolar incorporation of the 19 residues in this position [27], or the synthesis of the H-2Kd binding motif [32] or of magainin-2 analogues, using six amino acid mixtures [33], neither resulting in equimolar mixtures, are not relevant to the coupling of mixtures to mixtures required by the general positional scanning strategy.

The present work investigates the coupling kinetics of single to single, mixture to single and mixture to mixture of amino acids, using N^{α}-Fmoc-protection and DCCI, HOBt activation. Contrary to published reports, we choose to analytically identify the products of the reactions, using mainly UV, MS and LC/MS/MS techniques instead of amino acid analysis. Extending our studies to di-, tri- and tetrapeptide libraries, we show that large fractions of the expected peptides are not obtained when the coupling has been performed once or several times with mixtures of reacting residues.

Materials and Methods

Synthesis

N^{α}-Fmoc-protected amino acids and resin-bound N^{α}-free amino acids were purchased from Propeptide (Vert-le-Petit, France). Substitution on the *p*-benzyloxybenzyl alcohol-resin, the standard 1% cross-linked polystyrene-resin for the preparation of peptides by the Fmoc strategy [34], varied from 0.50 to 0.65 mmol per gram of derivatized resin. *Mixture-on-mixture synthesis.* Synthesis on the solid phase [26] of the peptide libraries was carried out in a robotic device able to perform parallel synthesis in 25 reactors and to insert the mix-and-divide procedure between each peptide elongation step [35]. Protecting groups of the *tert*-butyl family were used for the side chains, except for Arg(Pmc), Asn(Trt) and Gln(Trt). HOBt and DCCI were used for coupling and 20% piperidine in DMF was used for N^{α}-deprotection [36]. The protocol for a typical cycle was as follows: (i) DMF washing twice for 2 min (10 ml solvent per g resin); (ii) 20% piperidine in DMF for 5 min, and a second time for 15 min; (iii) DMF washings thrice for 2 min; (iv) DCM washings thrice for 3 min; (v) protected incoming amino acid single or as a mixture in DMF/DCM (20/1) in a volume of about 30 ml solvent per g resin, for 2–3 h at room temperature in the presence of HOBt and DCCI (1 equiv with respect to the carboxy component(s)); (vi) DMF washing twice for 2 min; (vii) alternatively DMF and isopropanol, thrice, 2 min; and (viii) DCM washings thrice for 2 min. Final deprotection and cleavage from the resin were achieved in 100% TFA in the presence of 10 mm of the thiol scavenger DTT for 3 h at room temperature. The resulting TFA solution was evaporated under vacuum, and the analysis took place on the resulting residue. A number of different ratios of the coupling components were used throughout the present work, as indicated in each case together with the corresponding results.

Classical conditions were a single incoming amino acid (3 equiv) reacting on a single or on a mixture of resin-bound residues in the presence of DCCI and HOBt (3 equiv each) and the mix-and-divide step as in Boutin et al. [19]. The relative amounts of the components of these classical dipeptide libraries were used as comparisons in Figures 3, 5 and 7.

Incubations

The kinetics of amino acid incorporation were done on a Model SP650 semiautomatic solid-phase Labortec AB synthesizer (Bubendorf, Switzerland) in the incubation medium DCM/DMF (1/3). Zero times were obtained by sampling the reaction medium before the addition of the resin. Sampling was done by taking an aliquot (20 μl) of the reaction mixture, after the settling down of the resin. This aliquot (total amino acid concentration between 20 and 60 mM) was diluted in 200 μl of acetonitrile, vigorously shaken and 10 μl of this solution was injected in a Waters HPLC system equipped with a Delta-Pak RP_{18} column (150×3.9 mm, 5 μm). Areas under the peaks at 264 nm were used for the quantification of the unreacted Fmoc-amino acids. Retention times were recorded in the same chromatographic system, using a known amount of the pure amino acids. Therefore, positive recognition and relative quantification (compared to the starting 100% at zero time) were easily performed under these standardized conditions. Furthermore, because of their unique absorption property, only Fmoc-amino acids were detected. In particular, despite the presence of a small amount of DMF in the injected sample (7%, i.e., 1.4 μl per injection), no amino acid was eluted in the front peak.

Analysis

Nuclear magnetic resonance (NMR) experiments were performed on an AMX500 Bruker spectrometer equipped with a 5 mm inverse probe. Samples consisted of 5–10 mg of each mixture (X1-O2) dissolved in 0.5 ml of DMSO-d_6 (SDS, France, isotopic enrichment >99.96%). 2D heteronuclear multiple quantum coherence (HMQC) experiments [37] were conducted on the four samples in the phase-sensitive way (TPPI) with 64 scans and 400 t_1 increments. Spectral widths of 20 000 Hz in the $\omega 1$ dimension and 5500 Hz in the $\omega 2$ dimension were used. A 145 Hz coupling constant value was used to adjust the delay of these experiments.

Liquid chromatography/evaporative light scattering detection was performed using a Hewlett-Packard HP 1050 system. Separation was carried out on a Kromasil C_{18} column (250×4.6 mm i.d., 5 μm). The mobile phase (1 ml min^{-1}) consisted of water-acetonitrile containing 0.1% trifluoroacetic acid. Evaporation was performed at 43 °C at an air pressure of 2.2 bar in a Sedex 45 ELSD instrument (Sedere, Alfortville, France). The isocratic conditions were acetonitrile/water 50/50, 0.1% TFA. Separations were performed on a 5 mg ml^{-1} sample dissolved in the mobile phase.

Mass spectrometry was performed with a TSQ 7000 triple quadripole mass spectrometer (Finnigan MAT, San Jose, CA, U.S.A.) equipped with an electrospray (ESI) source.

Liquid chromatography/mass spectrometry was performed on a Hewlett-Packard HP 1090 system. Separation was carried out on a Kromasil C_{18} column (250×4.6 mm i.d., 5 μm). The mobile phase consisted of water–acetonitrile (containing 0.1% of trifluoroacetic acid) and was programmed as follows: 80% water for 3 min and a gradient decrease to 20% water at 35 min. The mobile phase flow (1 ml min^{-1}) was divided at the outlet of the column by means of a zero-dead-volume Tee fitting resulting in the introduction of 30% of the eluent in the ESI interface. The capillary temperature was 230 °C and the sheath gas pressure was 80 psi. An auxiliary gas (15 ml min^{-1}) was then added in order to achieve the nebulization. The Fmoc-dipeptide library (15 mg in 1 ml, 5 μl injected) was analyzed in LC/MS/MS by recording at unit resolution the ionic current of the precursor ions of m/z 179 (9-fluorenylmethyl cation) expected to be a common product ion of all protonated Fmoc-peptides. The collision energy was 30 eV and argon (1.2×10^{-3} mT) was used as the collision gas. The N^{α}-Fmoc-protected tri- and tetrapeptide libraries were analyzed in the infusion mode (1 mg ml^{-1} in acetonitrile/water/acetic acid 1/1/0.05, 5 μl min^{-1}). The ESI operating conditions were as follows: electrospray voltage, 4.5 kV; capillary temperature, 200 °C; sheath gas (nitrogen) pressure, 60 psi.

N^{α}-Fmoc-protected amino acids, without side chain protecting groups, were purchased from Bachem (Voisin-le-Bretonneux, France, ≥98% pure). A precise amount of each of these samples was dissolved in either DMF or acetonitrile (Baker, France, >99% pure) and their molar absorbances were measured in a Perkin-Elmer $\lambda 7$ UV/visible spectrophotometer. The UV spectra of these N^{α}-Fmoc-protected amino acids were recorded. The maximal absorbance was experimentally found to be ca. 260 nm, showing similar molar intensity whatever the amino acids. Controls were done on N^{α}-free amino acids, and the results recorded were in good accordance with those reported by Wetlaufer [38].

Analyses of the dipeptide libraries were done using a Waters HPLC system. The chromatographic conditions (stationary and mobile phases) were maintained as described for the LC/MS/MS experiments. Integrations were done manually at 264 nm, a wavelength at which only the Fmoc moiety showed absorption. Estimation of the Fmoc-peptides was chosen not only for analytical convenience, but also in order to eliminate additional possible biases (Fmoc-deprotection and ether precipitation) after coupling. The maximum of absorption of the Fmoc-protected compounds was distinct of the wavelength (301 nm) currently used to quantify deprotection in automatic synthesizers, the latter corresponding to maximum absorbance of the deprotection by-product Fmoc-piperidine.

Results

Kinetic data, coupling yields and presence of the expected products are reported systematically as a function of the number and the relative molarities of the reactants.

Single reactant amino acid on single resin-bound residue, 1 equiv

We tested these conditions by incubating in individual reactions various N^{α}-Fmoc-amino acids (Gly, Phe, Arg and Gln) with the same resin-immobilized amino acids. The disappearance of the Fmoc derivative was followed

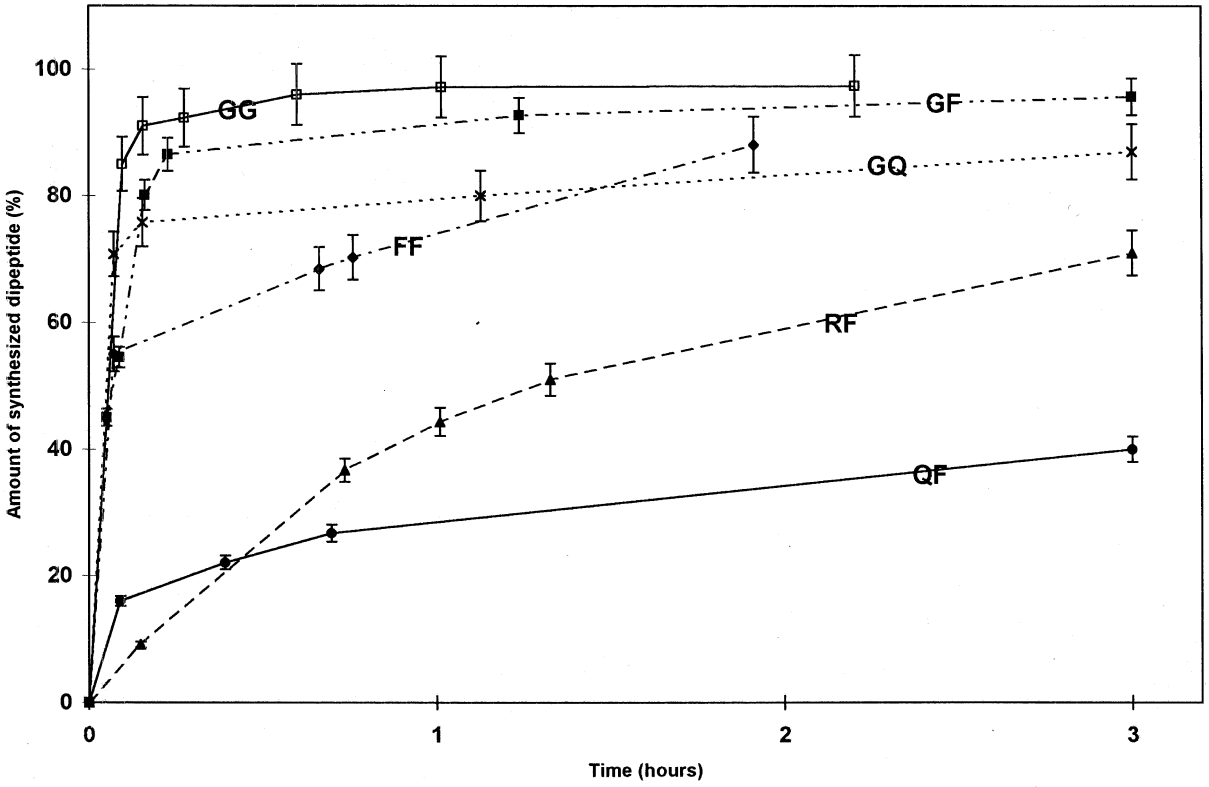

Figure 1. Coupling efficiency between equivalent free and resin-bound amino acids. The coupling was done individually, between 1 M equiv of resin-bound amino acid and 1 M equiv of N^{α}-Fmoc-protected free amino acid. The coupling agents were HOBt and DCCI. Typically, the amount of amino acid injected was 20 nmol. The extent of coupling was estimated from the disappearance of the Fmoc-protected amino acid by measuring its residual amount in solution using HPLC separation and UV detection at 264 nm (n = 3 independent experiments each).

in the reaction mixture. It can be seen from Figure 1 that the couplings occur at various rates. Ninety-five percent completion, under these conditions, was obtained only in the cases of Fmoc-glycyl-glycine dipeptide synthesis after 1 h, and Fmoc-glycyl-phenylalanine after 2 h. For all the other examples, 95% completion was not reached after 3 h of incubations. The striking example of the synthesis of Fmoc-glutaminyl-phenylalanine shows that only 38% completion was reached after 3 h.

Single reactant amino acid (3 equiv) on single resin-bound amino acid

Under these very common conditions in classical solid-phase peptide synthesis, more than 95% completion of coupling is obtained for all the proteinogenic amino acids in less than 3 h (data not shown).

Amino acids in mixture reacting on single resin-bound amino acid, 1 equiv

In the next set of experiments, we used a mixture of the four incoming amino acids in concentration equal to the available reaction sites onto the phenylalanyl-resin (i.e., 0.25 equiv each). Figure 2A shows that after 3 h of incubation, 70% of the free amino acids have disappeared from the solution when phenylalanine was on the resin, while with the glutamine resin (Figure 2B) about 85% of the free amino acids have disappeared after 6 h. Therefore, under these conditions, a large portion of the available sites did not react with any of the incoming amino acids. It is interesting to notice that although, in these two experiments, the same free amino acids were used as in Figure 1, their relative coupling rates were different, thus showing that they depend both on the incoming and the immobilized amino acid. These data are similar to those reported by Ragnarsson et al. [28,29].

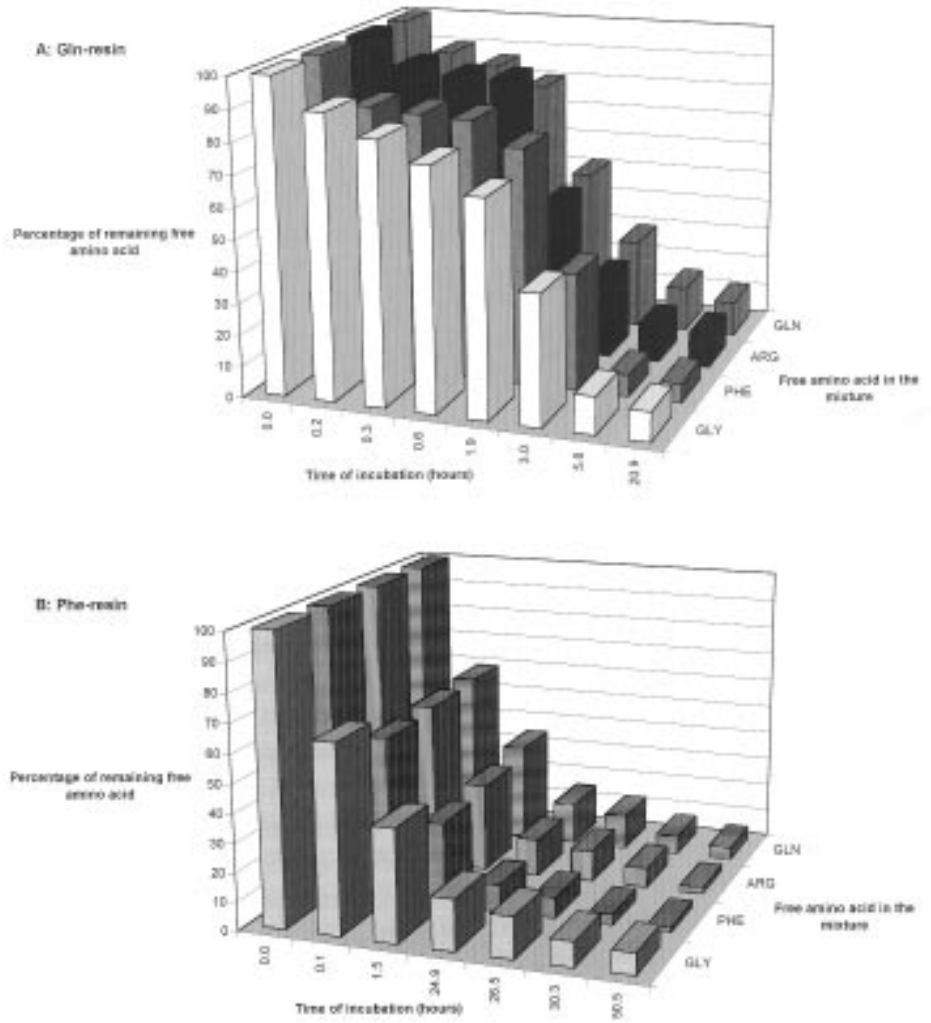

Figure 2. Coupling efficiency between free and resin-bound amino acids: (A) glutaminyl-resin and (B) phenylalanyl-resin. The couplings were done in mixtures, between 1 M equiv of resin-bound amino acid and 0.25 M equiv of each of the N^{α}-Fmoc-protected free amino acids (Arg, Gln, Gly, Phe) in the presence of HOBt and DCCI. Typically, the amount of amino acid injected was 5 nmol. The amount of free Fmoc-protected amino acid was estimated by UV detection at 264 nm during HPLC separation.

Amino acids in mixture (3 equiv) on single resin-bound amino acid

Coupling rates. The experiment was repeated in four different sets in which a single amino acyl-resin was used with an incoming mixture of four amino acids, at a total concentration of 3 times the available sites on the resins (i.e., 0.75 equiv each). Completion was obtained within less than 1 h of reaction (data not shown). Interestingly enough, the order of the reaction rates of the free amino acids was Arg<Gly<Phe<Gln, again different from that observed in the previous experiments.

Analysis of the products formed. For all the experiments reported here, the influence of the amino acid immobilized on the resin was noteworthy and different from one amino acid to another. For the four different resins (glutaminyl-, arginyl-, phenylalanyl- and glycyl-resins), the dipeptides formed were cleaved from the resins and analyzed. Because the mixtures contained only four components, the relative amounts of each dipeptide within the mixture could be estimated by NMR, thus providing an additional analytical tool which avoids the necessary HPLC calibration with individually synthesized peptides. This was done for two of the mixtures: X1-Phe and X1-Gly. Integrations of HMQC correlations were done on the basis of the intensity of the $C\alpha/H^{\alpha}$ spot of the pheny-

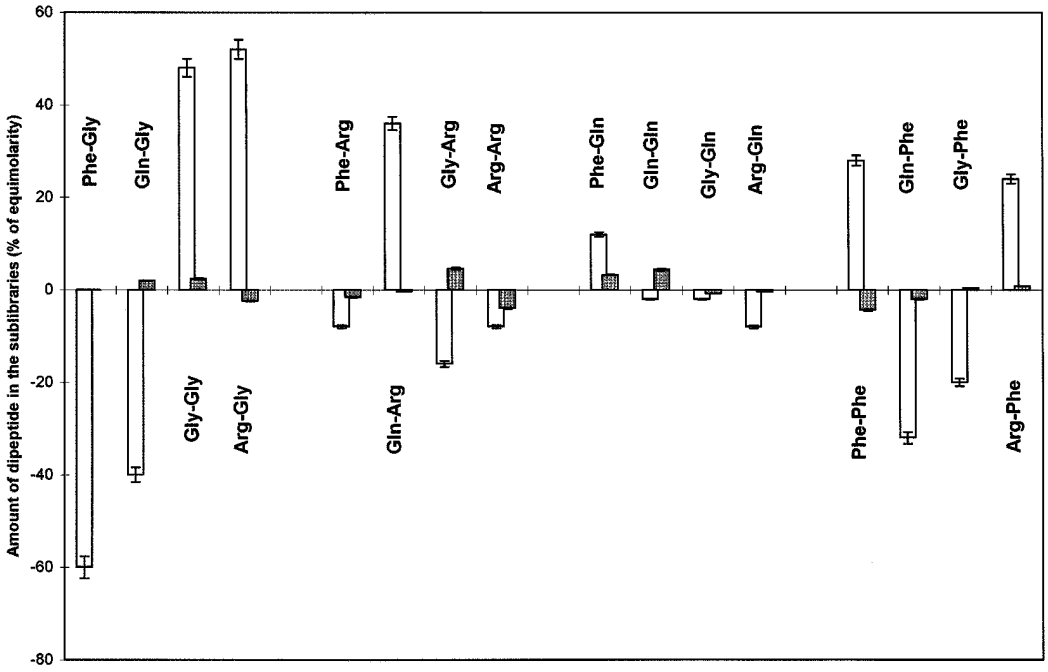

Figure 3. Amounts of the dipeptides formed by coupling mixtures of free amino acids with a unique resin-bound residue. The couplings were done in four different reactors containing 3 equiv of amino acids (Arg, Gln, Gly, Phe) in mixtures, each in the presence of 1 equiv of a different and unique resin-bound amino acid, HOBt and DCCI (coupling time: 1 h). Fmoc was then removed by 20% piperidine/DMF and the resulting dipeptides were cleaved from the resin (TFA, DTT). The four resulting mixtures (without ether-precipitation) were analyzed and quantified by ^{1}H NMR. This was confirmed by HPLC using the evaporative light scattering detection method. The synthesis and analyses were independently done thrice. The white bars represent the experiments and the dark ones reflect the synthesis of dipeptides under mix-and-divide conditions (see the Materials and Methods section).

lalanine (or glycine) in position 2 (relative intensity = 4/8). It was possible to differentiate between phenylalanine (or glycine) at positions 1 and 2. On this basis, specific correlation of each of the four amino acids was established. The dipeptide relative concentrations were as follows: Phe-Phe 32%, Arg-Phe 30%, Gly-Phe 20%, Gln-Phe 18% for the phenylalanine series and Phe-Gly 13%, Arg-Gly 33%, Gln-Gly 27%, Gly-Gly 27% for the glycine series (compared to the expected ratio of 25%). These results were confirmed after HPLC analysis using the evaporative light scattering detection method [39], thus suppressing the generally high variation of the native dipeptide absorption in UV for Fmoc-free samples. The results are reported in Figure 3. Each of the sublibraries, comprising a common C-terminal amino acid, was obtained separately. The theoretical equimolarity (25% of each of the four dipeptides in the mixtures) was not reached. The dipeptides ranged from –60% to +50% of their theoretical representation. Only six dipeptides out of 16 were represented in an amount less than 10% apart from the theory (Phe-Arg, Phe-Gln, Gln-Gln, Gly-Gln, Arg-Arg and Arg-Gln). To the contrary, when dipeptides were synthesized using a single amino acid reacting on a mixture of resin-bound amino acids in separated vessels, the dipeptides were all present in the final mixture, and ranged within less than 5% from equimolarity (Figure 3). These results show that the reactivities of individual amino acids are widely different and that, although all coupling reactions apparently take place, some dipeptides are formed in relative small amounts. This seems to be due to the competition between the incoming amino acids. Indeed, comparison between the various experiments shows, for instance, that Phe reacts better with phenylalanyl-resin and less well with glycyl-resin, while the opposite is true for Gly reacting on the same resins.

Amino acids in mixture on amino acid-bound resins in mixture, 3 equiv

Coupling rates. To further clarify the limitations linked to the synthesis of libraries using mixtures of amino

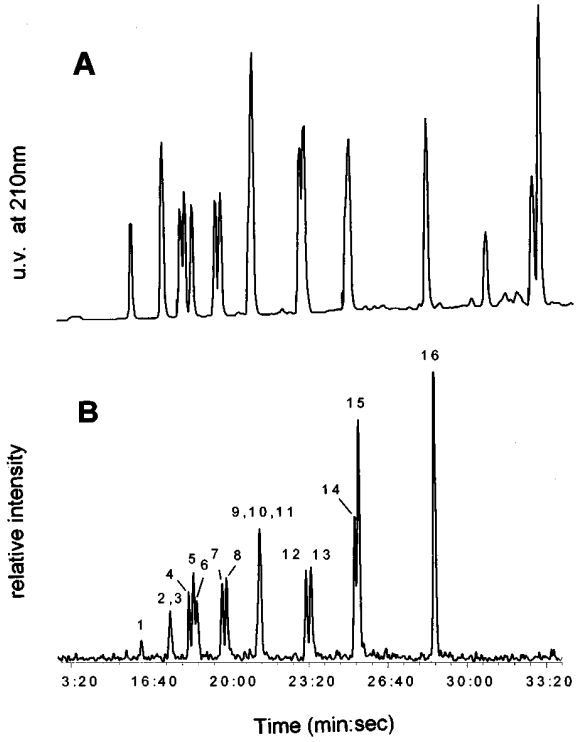

Figure 4. LC/MS/MS of the Fmoc-dipeptide library. The coupling was done between 3 equiv (total) of amino acids (Arg, Gln, Gly, Phe) in mixtures with 1 equiv of a mixture of the same resin-bound amino acids, HOBt and DCCI (coupling time: 1 h). Cleavage from the resin was done in TFA supplemented with DTT. The final mixture was analyzed by LC/MS/MS. (A) UV profile at 210 nm; (B) LC/ESI/MS/MS mass chromatogram: total ionic current of the precursor ions of m/z 179. Each peak corresponds to the 16 dipeptides of the library: 1, Fmoc-Arg-Arg; 2,3, Fmoc-Gln-Arg, Fmoc-Arg-Gln; 4, Fmoc-Gly-Arg; 5, Fmoc-Arg-Gly; 6, Fmoc-Gln-Gln; 7, Fmoc-Gly-Gln; 8, Fmoc-Gln-Gly; 9,11, Fmoc-Arg-Phe, Fmoc-Phe-Arg; 10, Fmoc-Gly-Gly; 12, Fmoc-Phe-Gln; 13, Fmoc-Gln-Phe; 14, Fmoc-Gly-Phe; 15, Fmoc-Phe-Gly; 16, Fmoc-Phe-Phe.

acids, a dipeptide library of 16 compounds was synthesized by coupling four amino acids as a threefold excess mixture to the mixture of the corresponding amino acyl-resins. While following the disappearance of the individual Fmoc-amino acids from the solution, important variations in their coupling rates were noticed (data not shown). These reaction rates not only depended on the incoming amino acids but also on the nature of those immobilized on the resin.

Analysis of the products formed. The dipeptide library was kept in its Fmoc-protected form in order to facilitate identification and quantification. The dipeptides were not ether-precipitated, to minimize any biases due to possible differential solubility of the individual compounds. LC/MS/MS permitted us to positively identify (Figure 4A) every compound in the mixture. Tandem mass spectrometry used in the parent mode permitted us to detect selectively all the precursor ions of m/z 179 (characteristic of the 9-fluorenylmethyl moiety), originating from the N^{α}-Fmoc-protected compounds of the sample (Figure 4B). The last three peaks appearing on the UV profile (Figure 4A) were not Fmoc-comprising compounds (Figure 4B) and, because of their late retention times, are not likely to be unprotected peptides. The relationship between retention times and molecular masses of N^{α}-Fmoc-dipeptides was unambiguously established and all the dipeptides were detected in the library. Because MS/MS in the absence of internal standard is not a good quantitative method, we designed a second analytical step in which all the dipeptides were quantified by the measurement of their absorption of the Fmoc-dipeptides at 264 nm. Indeed, at this wavelength, absorption of the peptides was only due to the Fmoc moiety as assessed in a separate experiment. The absorption of the individual N^{α}-Fmoc-amino acids, without side chain protection, was measured and their respective ϵ(264 nm) values ranged from 16 559 M^{-1} l cm^{-1} for Fmoc-Gln-OH to 18 895 M^{-1} l cm^{-1} for Fmoc-Asp-OH (see Table 1). Incidentally, at this wavelength, the molecular absorbance of Gly, Asn and Arg was barely measurable, while for Phe it was about 160 M^{-1} l cm^{-1}, which is below 1% of the absorptivity of the Fmoc moiety. A contrario, the ϵ(264 nm) for Trp was 5620 M^{-1} l cm^{-1}, a value likely to interfere with the Fmoc absorbance measured with the alternative library reported in Figure 6.

Table 1. Experimental measurement of ϵ(264 nm) of Fmoc-protected amino acids

Type	ϵ(264) M^{-1} l cm^{-1}	
	Acetonitrile	DMF
Fmoc-Trp-OH	17 856	n.d.
Fmoc-Phe-OH	18 233	n.d.
Fmoc-Arg-OH	Insol.	17 421
Fmoc-Lys-OH	Part. insol.	17 120
Fmoc-Gly-OH	17 833	n.d.
Fmoc-Ala-OH	17 419	n.d.
Fmoc-Gln-OH	Part. insol.	16 559
Fmoc-Asp-OH	18 895	17 421
Average	18 047±554	17 270±103

Commercial samples were solubilized in DMF or acetonitrile (UV grade).
n.d.: not determined.

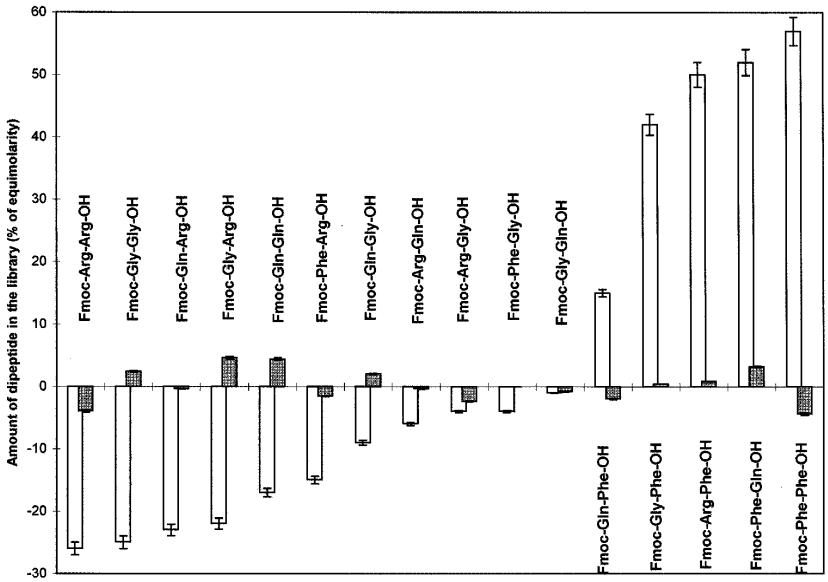

Figure 5. Variations of the amounts of the dipeptides formed by coupling a mixture of free amino acids (Arg, Gln, Gly, Phe) with a mixture of resin-bound residues. The coupling was done between 3 equiv (total) of the amino acids in mixtures with 1 equiv of a mixture of the same resin-bound amino acids, HOBt and DCCI (coupling time: 1 h). Cleavage from the resin was done in TFA supplemented with DTT. The resulting solution was analyzed as such by HPLC. UV detection was set at 264 nm and the areas under the peaks were measured. The amount of each peak is presented as a ratio of the total and expressed as a percentage of the equimolarity. The synthesis and analyses were independently done thrice. The white bars represent the experiments and the dark ones reflect the synthesis of dipeptides under mix-and-divide conditions (see the Materials and Methods section).

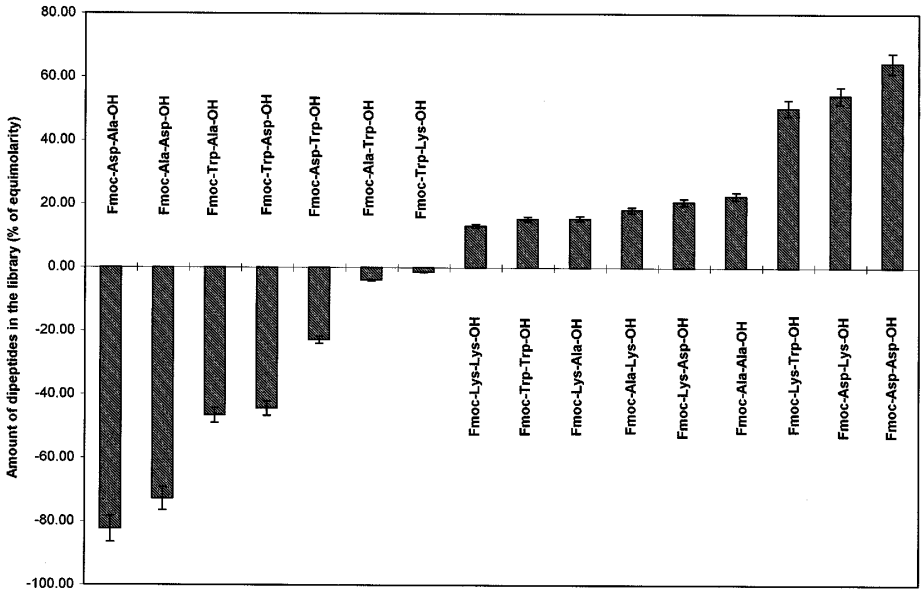

Figure 6. Variations of the amounts of the dipeptides formed by coupling a mixture of free amino acids (Ala, Asp, Lys, Trp) with a mixture of resin-bound residues. The coupling was done between 3 equiv (total) of the amino acids in mixtures with 1 equiv of a mixture of the same resin-bound amino acids, HOBt and DCCI (coupling time: 1 h). Cleavage from the resin was done in TFA supplemented with DTT. The resulting solution was analyzed by LC/MS/MS for positive identification. Quantification was done by UV detection (264 nm) after HPLC separation. The amount of each peak is presented as a ratio of the total and expressed as a percentage of the equimolarity.

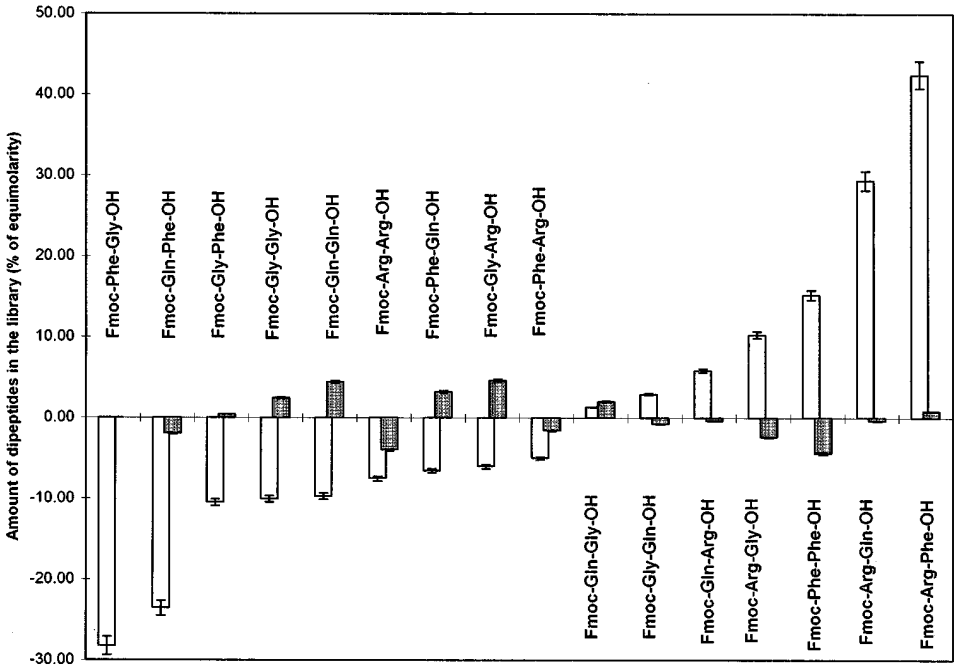

Figure 7. Variations of the amounts of the dipeptides formed by coupling a mixture of free amino acids (Arg, Gln, Gly, Phe) with a mixture of the same resin-bound residues. The coupling was done using chemical compensation: the respective molar concentrations of each amino acid in the mixture were Arg/Gln/Gly/Phe = 1.3/1.0/0.6/1.0 compared to 1.0 of the resin-bound amino acids. Coupling reactions occurred with HOBt and DCCI (coupling time: 1 h). Cleavage from the resin was done in TFA supplemented with DTT. The resulting solution was analyzed as such by HPLC and UV detection at 264 nm. The amount of each peak was calculated as a ratio of the total and expressed as a percentage of the equimolarity. The synthesis and analyses were independently done thrice. The white bars represent the experiments and the dark ones reflect the synthesis of dipeptides under mix-and-divide conditions (see the Materials and Methods section).

Figure 5 shows the variation of the individual dipeptide amounts in the mixture, compared to the expected equimolarity. These variations ranged from –25% (Arg-Arg) to +58% (Phe-Phe) when compared to theory. We tried to somehow generalize the observation by selecting another set of amino acids (Asp, Ala, Trp and Lys) and repeating the same experiments (Figure 6). The variation ranged from –80% (Asp-Ala) to +70% (Asp-Asp) compared to the expected equimolar repartition. As stated above, the amounts recorded whenever Trp was present are likely to be slightly overestimated (<20%), due to the absorption at 264 nm of this amino acid. Furthermore, we tested the first set of amino acids on a mixture of the second set of amino acids linked to the resin. In this case, the rates of disappearance of the Fmoc-protected amino acids (Arg, Gln, Gly and Phe) were different. In particular, Arg was reacting about three times faster than the other incoming amino acids, as measured after 1 h incubation (not shown).

Chemical compensation

The originators of the positional scanning strategy [21] systematically checked the coupling rates of the amino acids on a single resin. Ranging the amino acids according to their rates of coupling, they came up with the idea that these differences could be compensated by coupling the amino acids at concentrations inversely proportional to the coupling rates. We calculated a theoretical concentration based on the coupling rates obtained using a mixture of resins (see Figure 5), and incubated these amino acids under these conditions (mixtures on mixtures). The following concentration ratios were used: Arg/Gln/Gly/Phe, 1.3/1.0/0.6/1.0. The resins were cleaved after 1 h coupling [21], without Fmoc-deprotection. The Fmoc-dipeptides were analyzed by HPLC and the absorbance of the various peaks was measured at 264 nm (Figure 7). The variation to the equimolarity of the dipeptide concentrations ranged from +45% (Arg-Phe) to –28% (Phe-Gly). No other concentration ratios could be found which reduced the variations from equimolarity to less than 25%.

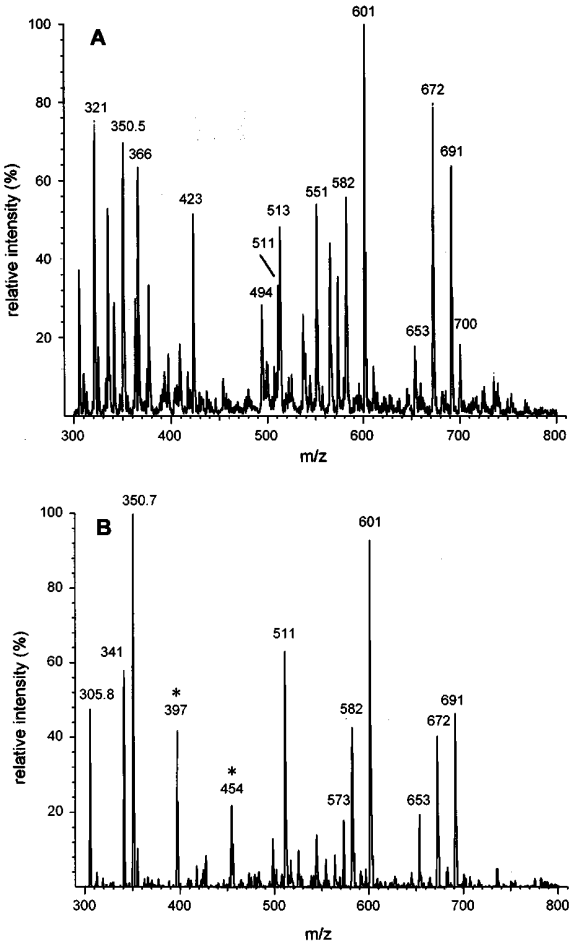

Figure 8. ESI/MS (A) and ESI/MS/MS (B) spectra of the library theoretically containing 64 tripeptides. The library was synthesized using the mixture-on-mixture technique. The dipeptide library containing 16 peptides reacted after deprotection with a mixture (3 equiv total) of the same four amino acids: Arg, Gln, Gly and Phe. The final N^{α}-Fmoc-protected tripeptide library was cleaved from the resin by TFA supplemented with DTT. (A) The mixture was analyzed as such. (B) ESI/MS/MS of the precursor ions of m/z 179 positively identifies all the Fmoc-containing compounds except as noted. (∗): unidentified ions.

Tri- and tetrapeptide library formation

Tri- and tetrapeptide libraries were obtained by incubating at each step the deprotected library with the mixture of incoming amino acids at a total concentration of 3 equiv, without chemical compensation.

Analysis of the tripeptide library. We studied the actual composition of the tripeptide library obtained with four amino acids (Gly, Asn, Phe and Arg) coupled in mixture using an excess of 3 equiv of the available sites on the resin. Two types of data could be obtained using the MS technique: (i) the analysis of the ions showed that 10 of the 20 families of ions (comprising 22 of the 64 different tripeptides of the library) were undetectable in the library (Figure 8 and Table 2); and (ii) the dramatic differences between the observed peaks and theory (Table 2) suggested an uneven representation of those species in the mixture, as predicted by our previous experiments.

Analysis of the tetrapeptide library. Similarly, an MS study of the tetrapeptide library constructed with Arg, Gln, Gly and Phe was conducted. The results (Figure 9 and Table 3) indicated that this tetrapeptide library was even more lacunary than the tripeptide library. More than one half of the species were not detected in the mixture. In Table 3, 65 peptides (out of 256) were not detected at all by MS/MS. The most abundant ion ($[M+H]^+$ m/z 729, corresponding to the general formula Fmoc-[GQFR]+H^+) according to theory was also the most abundant ion recorded. Furthermore, several of the other main families of ions were equally well represented in the mixture.

As a control, we randomly chose nine peptides (Arg-Gln-Gln-Gln, Gln-Arg-Phe-Arg, Gln-Gln-Arg-Phe, Gly-Phe-Gly-Arg, Gly-Phe-Gly-Gly, Gly-Phe-Gly-Phe, Gly-Phe-Phe-Arg, Phe-Gln-Phe-Gln and Phe-Phe-Gly-Phe) which were either absent or underrepresented according to the results presented in Figure 9. They were individually synthesized and kept in their N^{α}-Fmoc-protected forms. The resulting peptides were mixed in an equimolar fashion and analyzed by ESI/MS (Figure 10). The relative intensities of the ESI-produced ions widely differed, but remained in the classical MS response scale of 1–10. These observations showed that suppression effects cannot be invoked to explain the absence of some of the expected ions. This strongly suggests that the corresponding tetrapeptides were indeed not synthesized during the mixture-on-mixture coupling process.

Discussion and Conclusions

The present study demonstrates in several ways the impossibility to preserve equimolarity in synthetic peptide libraries as soon as, in at least one synthesis step, a *mixture* of the incoming synthons (carboxy components in amide formation) is reacted with one or several resin-immobilized residues (amino components). This result can be expected since (i) it is well known from classical solid-phase peptide synthesis that a manifold

Table 2. Tripeptide library analysis by ESI mass spectrometry[a]

No.	$[M+H]^+$ m/z	$[M+2H]^{2+}$ m/z	Number of peptides	Theoretical relative proportions[b]	Measured relative proportions[b]	Amino acid composition
1	412		1	16.7	n.d.[c]	GGG
2	483		3	50	n.d.	GGQ
3	502		3	50	n.d.	GGF
4	511		3	50	33	GGR
5	554		3	50	n.d.	GQQ
6	573		6	100	35	GQF
7	582		6	100	56	GQR
8	592		3	50	n.d.	GFF
9	601		6	100	100	GFR
10	610	305.5	3	50	13 + 38 = 51	GRR
11	625		1	16.7	n.d.	QQQ
12	644		3	50	n.d.	QQF
13	653		3	50	17	QQR
14	663		3	50	n.d.	QFF
15	672		6	100	78	QFR
16		341	3	50	29	QRR
17	682		1	16.7	n.d.	FFF
18	691		3	50	64	FFR
19	700	350.5	3	50	18 + 69 = 87	FRR
20	709		1	16.7	n.d.	RRR

[a] All the peptides in the library are in their N^{α}-Fmoc-protected forms.
[b] Proportions are relative to the most abundant ion family: m/z 601.
[c] n.d.: not detected.

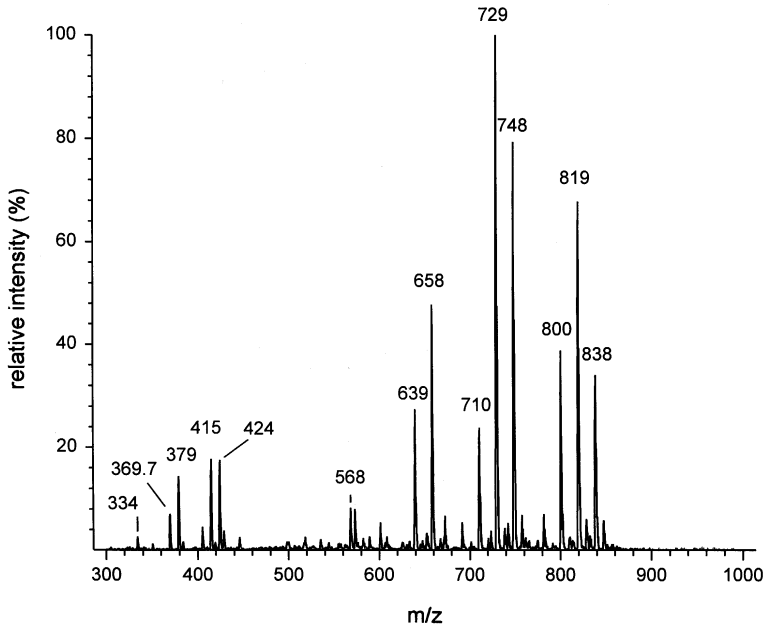

Figure 9. ESI/MS spectrum of the library theoretically containing 256 tetrapeptides. The library was synthesized using the mixture-on-mixture technique. The tripeptide library containing 64 peptides (Figure 8) was reacted, after deprotection, with a mixture (3 equiv total) of the same four amino acids: Arg, Gln, Gly and Phe. The final tetrapeptide library was cleaved from the resin by TFA supplemented with DTT. The mixture was analyzed as such.

Table 3. Tetrapeptide library analysis by ESI mass spectrometry[a]

No.	$[M+H]^+$ m/z	$[M+2H]^{2+}$ m/z	Number of peptides	Theoretical relative proportions[b]	Measured relative proportions[b]	Amino acid composition
1	469		1	4.2	n.d.[c]	GGGG
2	540		4	16.7	n.d.	GGGQ
3	559		4	16.7	n.d.	GGGF
4	568		4	16.7	8	GGGR
5	611		6	25	n.d.	GGQQ
6	630		12	50	n.d.	GGQF
7	639		12	50	27	GGQR
8	649		6	25	n.d.	GGFF
9	658		12	50	48	GGFR
10		334	6	25	3	GGRR
11	682		4	16.7	n.d.	GQQQ
12	701		12	50	2	GQQF
13	710		12	50	24	GQQR
14	720		12	50	2	GQFF
15	729		24	100	100	GQFR
16	738	369.5	12	50	4 + 7 = 11	GQRR
17	739		4	16.7	n.d.	GFFF
18	748		12	50	79	GFFR
19	753		1	4.2	n.d.	QQQQ
20	757	379	12	50	7 + 14 = 21	GFRR
21	766		4	16.7	n.d.	GRRR
22	772		4	16.7	n.d.	QQQF
23	781		4	16.7	7	QQQR
24	791		6	25	n.d.	QQFF
25	800		12	50	39	QQFR
26		405	6	25	4	QQRR
27	810		4	16.7	n.d.	QFFF
28	819		12	50	45	QFFR
29	828		12	50	6	QFRR
30		415	1	4.2	18	FFFF
31	837		4	16.7	n.d.	QRRR
32	838		4	16.7	34	FFFR
33	847	424	6	25	6 + 18 = 24	FFRR
34		428.5	4	16.7	4	FRRR
35	865		1	4.2	n.d.	RRRR

[a] All the peptides in the library are in their N^{α}-Fmoc-protected forms.
[b] Proportions are relative to the most abundant ion family: m/z 729.
[c] n.d.: not detected.

excess of the carboxy component is necessary to drive coupling to completion [25,26]; and (ii) it has been observed in several studies that the reactivity of the carboxy components (generally, protected amino acids) is highly variable [30]. Attempts to chemically compensate differences in reactivity by varying the concentration ratios of the reactants are partially successful for a single amino acid resin partner [1,27,31–33] but fail for a mixture of them. The present investigations confirm that the resulting libraries are far from complete, and that they may contain the individual products in relative ratios far from equimolarity.

Despite a real need for new techniques for a close analysis of finished libraries or mixtures of compounds (such as those described in Refs. 40–42), the results reported here cannot be due to some analytical flaws. Libraries obtained with the standard mix-and-divide procedure and analyzed with the same methods proved

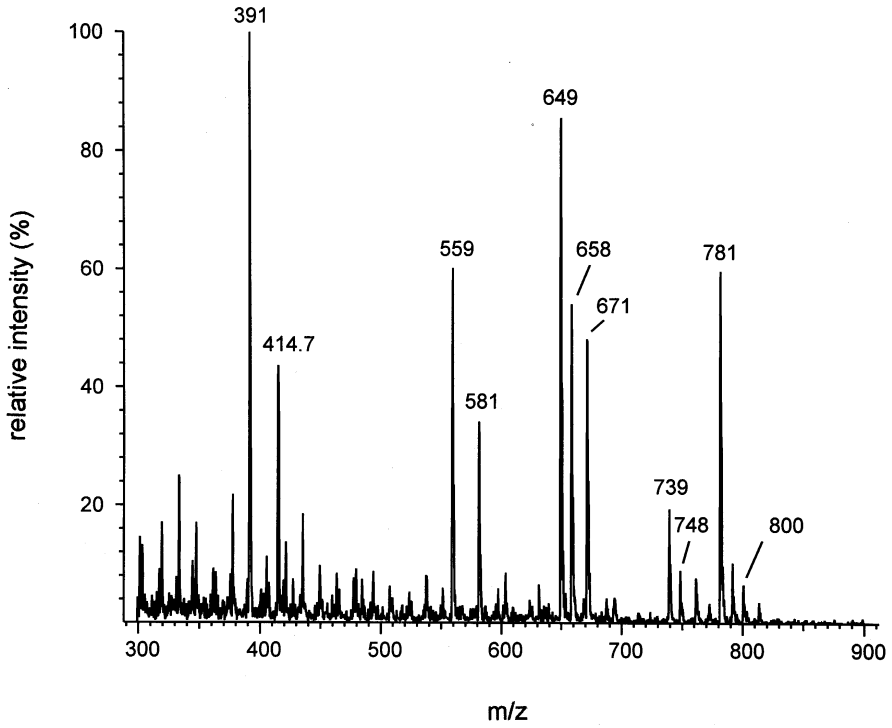

Figure 10. MS analysis of an equimolar mixture of nine randomly chosen Fmoc-tetrapeptides synthesized separately under standard conditions. The tetrapeptides were synthesized using the classical method: 3 equiv of a unique incoming amino acid. The three cycles of synthesis were done in separate reactors, on 1 g of resin, using DCCI and HOBt (3 equiv each). The Fmoc-N^{α}-protected forms of the tetrapeptides were cleaved from the resin using TFA supplemented with DTT. The compounds were precipitated by diethyl ether. An equimolar mixture of these nine products was prepared and analyzed by MS. Increasing m/z: [Fmoc-Arg-Gln-Gln-Gln-OH+2H]$^{2+}$ 391; [Fmoc-Gln-Arg-Phe-Arg-OH+2H]$^{2+}$ 415; [Fmoc-Gly-Phe-Gly-Gly-OH+H]$^{+}$ 559; [Fmoc-Gly-Phe-Gly-Gly-OH+Na]$^{+}$ 581; [Fmoc-Gly-Phe-Gly-Phe-OH+H]$^{+}$ 649; [Fmoc-Gly-Phe-Gly-Arg-OH+H]$^{+}$ 658; [Fmoc-Gly-Phe-Gly-Phe-OH+Na]$^{+}$ 671; [Fmoc-Phe-Phe-Gly-Phe-OH+H]$^{+}$ 739; [Fmoc-Gly-Phe-Phe-Arg-OH+H]$^{+}$ 748; [Fmoc-Arg-Gln-Gln-Gln-OH+H]$^{+}$ 781; [Fmoc-Phe-Gln-Phe-Gln-OH+H]$^{+}$ 791; [Fmoc-Gln-Gln-Arg-Phe-OH+H]$^{+}$ 800.

to contain nearly complete sets of products in nearly equimolar amounts. The direct comparison of the data in Figures 3, 5 and 7 also leads to the same conclusion. This is in contrast to the analytical assessment of equimolarity using amino acid analysis of the final libraries [21,31,43]. Equimolarity in the final hydrolysates does not prove equimolarity of the present peptides, an averaging effect surprisingly misregarded in these studies, since amino acid analysis of the mixture of the four peptides RRRR, GGGG, FFFF and QQQQ would give the same result as a tetrapeptide library XXXX constructed from the same four residues, R, G, F and Q.

Our results are in agreement with those of Dooley and Houghten [21], not with those of Ostresh et al. [43] from the same research group. They do not contradict those of Geysen et al. [1], Volkmer-Engert et al. [27], Ivanetich and Santi [31], Tjoeng et al. [32] and Quesnel et al. [33], who obtained approximately equimolar mixtures of products when in a *single* step with one mixture coupling to a *single* resin-bound residue.

It may be argued that changes in the resin type, the coupling reagents or of other reaction conditions may alter the reaction rates and the ratio of the products in the resulting libraries. We describe classical experiments using the most common resin type for the preparation of peptides by the standard Fmoc strategy and the most common coupling reagent conditions, as they were used for mixture coupling in a number of libraries. In addition, if changes in the reaction parameters may improve the coupling kinetics or even change the ratio of the products, they are not likely to restore equimolarity of the products, since the latter still depends on the reaction partners.

Our results are relevant to the libraries obtained with the positional scanning techniques [8,9,12,44–

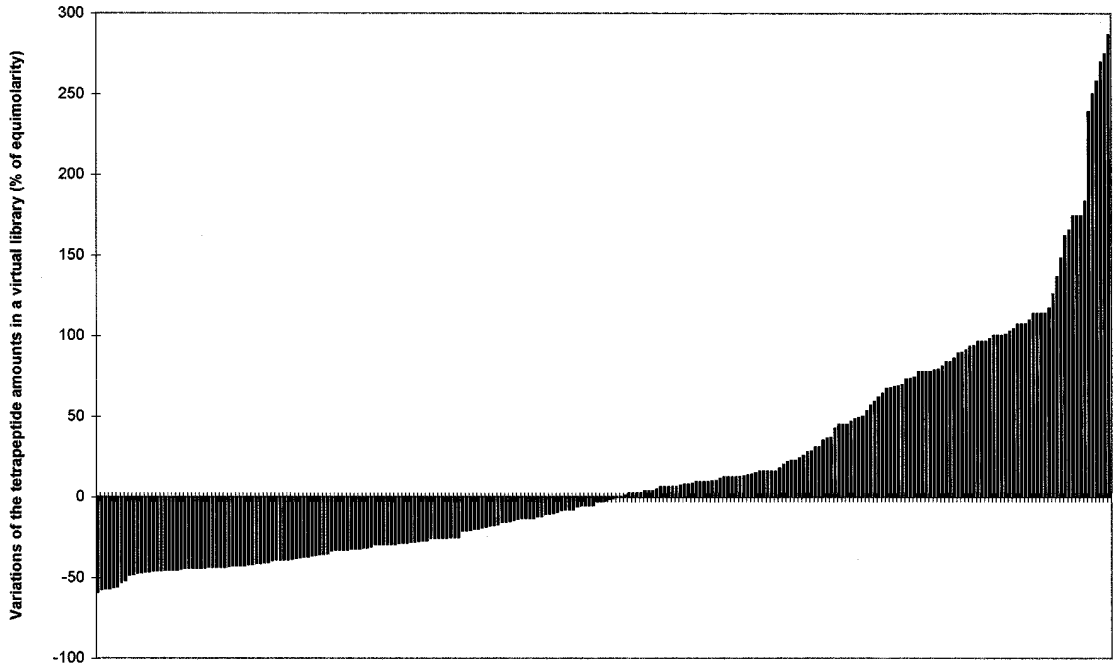

Figure 11. Theoretical deviations (%) from equimolarity of the 256 members of a virtual tetrapeptide library. The tetrapeptide library made of Gln, Gly, Arg and Phe was constructed by calculation, extrapolating the yields in each step from the dipeptide coupling rates measured in Figure 5 and assuming constant rates for the coupling between two defined partners, e.g. Fmoc-Gln-OH on H-Gly-resin, H-Gly-Xa1-resin and H-Gly-Xa1-Xa2-resin (Xa1,Xa2 = defined amino acid residues on the resin).

50] which are still commonly used, because they avoid both the combinatorial step in the automated synthesis and the iterative resynthesis and deconvolution. While the proponents of this strategy recognized that the coupling of mixtures to pools of amino acyl-resins may distort the equimolar representation of the peptide products, they came to conflicting conclusions about the difficulty [21] or the possibility [43] of compensating the differential coupling kinetics of the reaction partners. In addition, it is clear that only few efforts have been put into the analytical assessment of this approach, a situation already observed for 'standard' amino acid libraries (see Refs. 18 and 19 and references therein). From a theoretical point of view, positional scanning provides a way to synthesize much larger peptide libraries, with sequences of hepta- to decamers. For many biological applications, e.g., the search for ligands of orphan receptors or the search of G-protein-coupled receptor antagonists, larger soluble libraries are desirable. There is no way to produce such large mixtures by the mix-and-divide technique, which would require up to several tons of resins in order to insure the completeness of the libraries, even when using the newly available microbeads. By synthesizing the whole libraries on a single bead, much more sites are freely available and 10^7–10^{10} compound libraries may become reachable. Nevertheless, the preparation of nearly equimolar individual peptides is one of the crucial conditions for screening to be reliable.

The virtual tetrapeptide library extrapolated from the fractions of dipeptides formed in experiment 5 (Figure 5), and assuming that the rate of coupling of the same two components (for example, Fmoc-Gly-OH on H-Phe-resin, H-Phe-Xa1-resin or H-Phe-Xa1-Xa2-resin) is constant, leads to ratios of the final compounds of 1 to 10 (Figure 11), which are hardly compatible with reliable screening and deconvolution. Even if this badly equimolar representation of the library components is a fact, the number of the compounds is so high that biological testing was often reported to produce a hit. However, reproducibility may be a problem and it remains questionable whether a given target should be screened on incomplete and/or non-equimolar libraries.

Nevertheless, it should be pointed out that the use of the positional scanning technique can be successful in certain particular situations [51]. Theoretically, for a given sublibrary XiXjOXn, the synthesis of Xn and

O can be conducted under mix-and-divide conditions, while for Xj a single step of mixture on a single position would be needed and the literature provides examples according to which it can be reasonably successful [1,27,31–33]. For Xi, though, a mixture-on-mixture coupling synthetic step would have to be performed leading to uncontrolled composition of the sublibraries as demonstrated in the present work.

The split synthesis of peptide libraries (one-bead–one-peptide approach [14]) followed by a SURF deconvolution [13] is a robust screening procedure able to produce new pharmacological leads [Refs. 3, 8, 9, 12 and 52 amongst others) for chemical optimization. Provided the synthetic procedure is automated (due to the number of synthetic steps) [19,35,53], and the amounts of resin and the oligomer size are adjusted to reasonably fulfill the expected equimolarity of the products [16], it seems to be more reliable than positional scanning, since it can be unequivocally deconvoluted (cf. Ref. 23 for polynucleotides) and since it avoids coupling of mixtures of the soluble components. The mix-and-divide strategy appears to be a reliable method for the preparation of nearly equimolar libraries up to the hexapeptide size (gram amounts of resin in each reactor using up to 20 building blocks [16]) while mixture coupling should be avoided for more than one step, one step being acceptable when using chemical compensation and coupling of the mixture of amino acids on a *single* resin-bound residue [27,31–33].

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