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Using a Noncovalent Protection Strategy to Enhance Solid-Phase Synthesis

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1. Introduction

Since the introduction of solid-phase peptide synthesis by Merrifield ***(1)*** nearly forty years ago, solid-phase techniques have been applied to the construction of a variety of biopolymers and extended into the field of small molecule synthesis. The last decade has seen the emergence of solid-phase synthesis as the leading technique in the development and production of combinatorial libraries of diverse compounds of varying sizes and properties. Combinatorial libraries can be classified as biopolymer based (e.g., peptides, peptidomimetics, polyureas, and others ***[2,3]***) or small molecule based (e.g., heterocycles ***[4]***, natural product derivatives ***[5]***, and inorganic complexes ***[6,7]***). Libraries synthesized by solid-phase techniques mainly use polystyrene-divinylbenzene (PS) derived solid supports. Owing to physical and chemical limitations of PS-derived resins, other resins have been developed ***(8,9)***. Most of these resins are prepared from PS by functionalizing the resin beads with oligomers to improve solvent compatibility and physical stability ***(8,9)***.

Solid-phase synthesis offers several attractive features over solution-phase synthesis: (1) Molecules are synthesized while covalently linked to the solid support, facilitating the removal of excess reagents and solvents. (2) The solid-supported reaction can be driven to completion through the use of excess, soluble reagents. (3) Mechanical losses are minimized as the compound–polymer beads remain in single-reaction vessels throughout the synthesis. (4) Physical manipulations are easy, rapid, and amenable to automation. (5) The physical separation of the reaction centers on resin furnishes a "pseudo-dilution" (physi-

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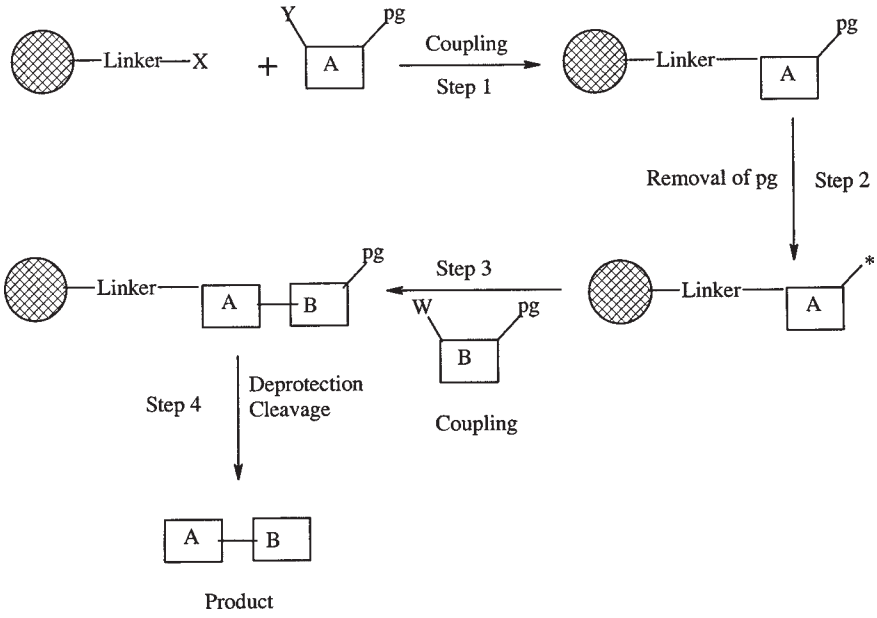


Fig. 1. Linear solid-phase synthesis of biopolymer-like peptides and polynucleotides.

cal separation in space minimizes or eliminates contact between resin-bound reacting sites), which makes certain transformations more successful when compared to solution-phase synthesis. A general schematic representation of the steps involved in a linear synthesis of compounds on solid phase is outlined in **Fig. 1**.

In linear solid-phase synthesis, the building blocks (i.e., A and B in **Fig. 1**) are covalently attached to the solid support via a linker ***(10)***. In the case of peptide synthesis, the building blocks are protected amino acids. Usually the N^{α}-group is protected by an acid-sensitive *tert*-butyloxycarbonyl (Boc) group, a base-sensitive 9-fluorenylmethyloxycarbonyl (Fmoc) group, or Pd(0)-sensitive allyloxycarbonyl (Alloc) group. The use of protecting groups (pg in **Fig. 1**) prevents side reactions and complications arising from the incorporation of multiple building blocks in the desired product. The presence of a protecting group requires additional chemical step(s) for deprotection and exposure of the functional group (in the present example, an amino group). Only then can further coupling with other amino acids be performed. Similar strategies are used in the construction of peptide nucleic acid oligomers using Boc or Fmoc protection ***(11,12)***.

It was envisaged that instead of using covalently linked protecting groups that require chemical synthesis and removal, a transient protection scheme

could be used to facilitate the same overall chemical transformation. Noncovalent protection was first used in peptide synthesis under solution- and solid-phase protocols ***(13–17)*** to prevent double coupling and other side reactions. One approach is based on the fact that crown ethers can form stable complexes with ammonium ions ***(18–20)***. Because crown ethers selectively sequester potassium ions, solutions containing potassium salts can be used to remove the crown ether from the ammonium group. Similarly, it was found that the noncovalent nature of the protection afforded by the crown ether entity allowed its mild and rapid removal from resin-bound peptides by treatment with 1% *N,N*-diisopropylethylamine (DIEA) solutions ***(16)***.

1.1. Noncovalent Protection in Solid-Phase Peptide Synthesis

The use of crown ethers for protection of the amino group of amino acids offers, in principle, several advantages over the more commonly used protecting groups *tert*-Boc and Fmoc. The noncovalent nature of the interaction between crown ethers and ammonium ions, coupled with the high affinity of crown ethers for inorganic ions ***(21)***, provides the basis for a rapid but mild protection and deprotection scheme. The crown ether protection of N^{α}-amino acids in solution ***(13–15)*** and solid-phase syntheses ***(16,17)*** has been extensively studied.

Mascagni and co-workers ***(13–17,22)*** have investigated conditions under which peptide synthesis by the fragment condensation approach in the solid phase can be carried out using crown ethers as noncovalent protecting groups for the N^{α}-amino group. As a model system, the syntheses of tripeptides was performed by coupling the 18-crown-6 complex of the dipeptide Gly-Gly-OH (III and IV, **Fig. 2**) with either resin-bound Tyr or Pro amino acids while varying the solvent choice between *N,N*-dimethylformamide (DMF) and dichloromethane (DCM). Each coupling was carried out with a fourfold excess of the activated dipeptide–crown ether complex using 1,3-dicyclohexylcarbodiimide (DCC, **Fig. 2**) and 1-hydroxybenzotriazole (HOBt, **Fig. 2**) as activating reagents. The couplings were run for 30–45 min at room temperature. In these experiments the goal was to evaluate the effect of solvent, counter ion, the nature of the carboxy-(C)-terminal amino acid, and the viability of noncovalent protection in fragment condensation. Synthetic performance of the syntheses was judged by the level of the desired peptides vs the presence of double-coupled side products (**Table 1**). It should be noted that preliminary experiments found that a polyacrylamide-based support performed poorly in comparison to a PS support (i.e., Wang resin). The ability to control the reaction was found to vary as a function of solvent and the C-terminal amino acid. The identity of the counter ion appeared to have no effect. The best results were obtained

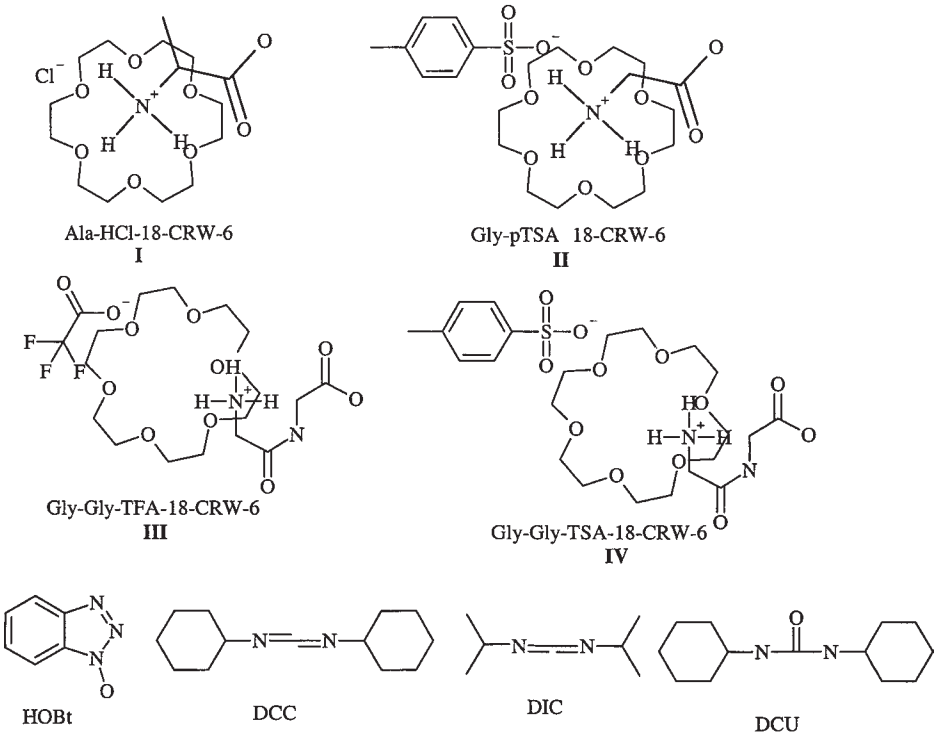
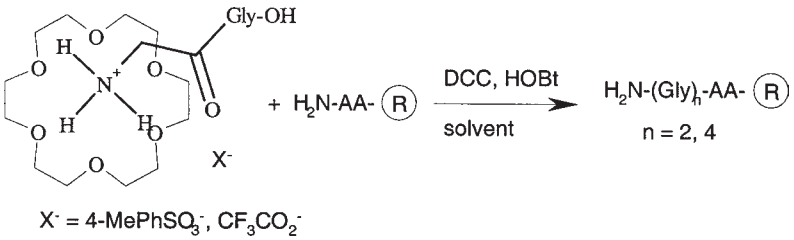


Fig. 2. Chemical structures of reagents and building blocks for peptide synthesis using noncovalent protection.

using Wang resin functionalized with Pro and DCM as a solvent. Interestingly, reactions involving Tyr as the C-terminal amino acid tended not to go to completion. Detailed studies established that the crown ether protection was transferred from the terminal Gly of the activated dipeptide to the resin-bound amino- (N)-terminus, a likely cause for the observation of double-coupled products and unreacted, resin-bound amines. That Pro was not affected by this same circumstance is in accord with the observation that 18-crown-6 selectively forms a complex with primary ammonium salts in preference to secondary ammonium salts. The use of a secondary amine as the C-terminal group in noncovalent protection was investigated as well ***(16)***. The observed solvent effect is believed to be related to the greater solvating ability of DMF for the ammonium salt relative to DCM. It is postulated that a competition is established between DMF and the crown ether for solvation of the ammonium ion. The authors also found that this protection scheme is not applicable to single amino acid condensation, as polymerization results immediately after activation ***(22)***.

Table 1
Peptide Sequences Synthesized by
Non-Covalent Protection on a Solid Phase *(16)*



Entry	C-Terminal amino acid	Solvent	Product ratio (n = 2:n = 4)
1	Tyr	DMF	1:1
2	Tyr	DCM	5:2
3	Pro	DCM	96:4

The use of crown ethers for noncovalent protection of N^{α}-amino acids and for protection of side chains of Lys or Arg residues has found the most successful utility in the fragment condensation approach to solid- and solution-phase peptide synthesis ***(15–17)***.

1.2. Noncovalent Protection in Solid-Phase Rhodamine-Labeled Peptide Nucleic Acid Synthesis

Another investigation employing noncovalent protection was the labeling of peptide nucleic acids (PNAs) with fluorophores as probes for characterizing nucleic acid sequences by *in situ* hybridization ***(23)***. Cellular uptake of PNAs was monitored using fluorescent microscopy ***(24)***. Non-bonded interactions between the lipophilic resin backbone and the fluorophore reagent carboxytetramethylrhodium succinimidyl ester (CTRSE) hindered full incorporation of the fluorophore on the PNAs ***(25)***. To improve efficiency, noncovalent protection was employed by addition of an analog (sulforhodamine sodium [CTRS]) of the intended fluorophore prior to the coupling of CTRSE to the resin-bound PNAs. CTRS served to noncovalently block the interfering lipophilic sites on the resin. The incorporation of CTRSE was improved by more than fivefold relative to the reaction in the absence of CTRS. The result was that a cheap reagent was used to improve efficiency and reduce the amount needed of a more expensive building block (e.g., CTRSE).

Based on these findings on noncovalent protections, similar approaches could be proposed in cases where either temporary protection is needed for chemical transformation or where resin–reagent compatibility is an issue ***(8,9)***.

The potential of noncovalent protection schemes to address these kinds of issues has not been fully explored.

2. Materials

2.1. Preparation of 18-Crown-6 Ether Complexes of Peptides and Amino Acids

1. Solvents: *N*,*N*-Dimethylformamide (DMF), dichloromethane (DCM).
2. Fmoc-Tyr(OtBu)-Wang (0.59 mmol/g) from Calbiochem-Novabiochem (San Diego, CA).
3. Coupling reagents: *N*-Hydroxybenzotriazole (HOBt), dicyclohexylcarbodiimide (DCC), and diisopropylcarbodiimide (DIC) from Aldrich (Wisconsin).
4. Gly-Gly-OH dipeptide from Sigma Biochemicals (St. Louis, MO).
5. 18-Crown-6 from Aldrich.
6. Trifluoroacetic acid (TFA) and piperidine from Aldrich Chemical.

2.2. Preparation of Fluorescein-Labeled PNAs on a Solid Support

1. Fmoc-PNA monomers (**Fig. 3**) protected nucleic acid bases from Applied Biosystems (http://www.appliedbiosystems.com/ds/pna/) ***(26)*** (*see* **Note 1**).
2. Dry DMF (Sigma, St. Louis, MO) (*see* **Note 2**).
3. Fluorescein tags (**Fig. 3**) Carboxytetramethylrhodamine succinimidyl ester from Molecular Probes (Eugene, OR and Leiden, The Netherlands) and sulforhodamine from Sigma-Aldrich, St. Louis, MO.
4. Coupling reagent HATU ([*O*-(7-aza-benzo-triazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate]) (**Fig. 3**) from PerSeptive Biosystem (Framingham, MA).
5. PEG-PS resin functionalized with XAL linker (9-Fmoc-aminoxanthen-3-yloxymethyl) (**Fig. 3**) from Applied Biosystem (Foster City, CA) (*see* **Note 3**).
6. PE (Perkin-Elmer) Biosystems Expedite 8909 automated synthesizer.

3. Methods

3.1. Preparation of Amino Acid and Peptide Complexes with 18-Crown-6 (see Note 4)

1. Alanine hydrochloride-18-crown-6 complex: Dissolve alanine (1 Eq) in aqueous hydrochloric acid (1.1 Eq) and lyophilize to dryness to give alanine hydrochloride in quantitative yield. Suspend alanine hydrochloride (1 Eq) with 1 Eq of 18-crown-6 in chloroform and stir the mixture at room temperature to give a clear solution. Evaporate chloroform to dryness to give the title compound as a powder (*see* **Note 5**).
2. Alanine tosylate-18-crown-6 complex: Lyophilize alanine (1 Eq) from 5 mL of water containing p-toluenesulfonic acid monohydrate (1.1 Eq). The alanine–tosylate salt is added to a chloroform solution of 18-crown-6 (1 Eq) and the mixture stirred until homogeneous. Evaporation of chloroform and crystallization of the residue from methanol–ethyl acetate (*see* **Note 6**) yields the solid alanine–crown ether complex with a melting point of 123–125°C.

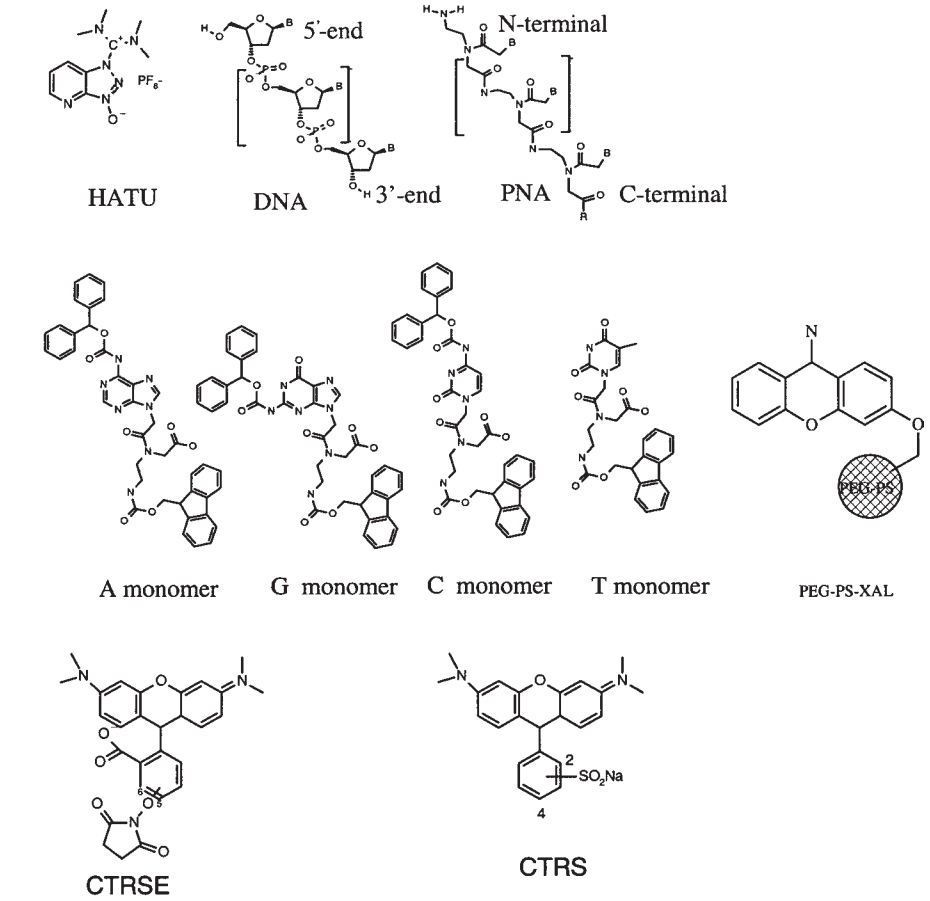


Fig. 3. Chemical structures of reagents and building blocks for synthesis of rhodamine-labeled PNA oligomers.

3. Gly-Gly trifluoroacetate crown ether complex (**III** in **Fig. 2**): To a solution of Gly-Gly trifluoroacetate in water (1 Eq) is added 18-crown-6 (1 Eq) with stirring. Lyophilize the reaction solution. Dissolve in water, and lyophilize again. This process is repeated until all traces of acid are eliminated (monitored by pH paper). The complex is used without further purification.
4. Gly-Gly tosylate crown ether complex (**IV** in **Fig. 2**): Gly-Gly (5 g, 38 mmol) is added to a solution of *p*-toluenesulfonic acid (7.2 g, 38 mmol) in water–ethanol (50 mL, 1:1). Stir the reaction mixture at room temperature for 1–2 h and then evaporate to dryness. Suspend the residual dipeptide salt in 50 mL of ethanol (*see* **Note 7**) and add 18-crown-6 (10 g, 38 mmol). Stir the reaction mixture with

warming to give a clear solution. Cool the solution to room temperature and add dry ethyl acetate dropwise until the solution becomes turbid. Leave the suspension at room temperature for 6–8 h and filter the precipitated crystals to give 20 g (93%) of compound **IV** (**Fig. 2**).

5. Gly-Gly hydrochloride crown ether complex: Prepare as described in **step 4**. Use similar equivalents as in the synthesis of **IV**. The yield is 80% of glycylglycine hydrochloride–18-crown-6 complex.

3.2. Solid-Phase Synthesis of NH_2-Phe-Gly-Gly-Pro-Asp-Leu-Tyr-OH Heptapeptide by the Fragment Condensation Approach Using Noncovalent Protection of Dipeptide Glycylglycine (IV, Fig. 2, see Note 8)

1. Add 1.5 mL of 50% piperidine in DMF to 100 mg of Fmoc-Tyr(OtBu)-Wang resin (loading 0.52 mmol/g). Agitate the resin for 1 h at room temperature. Filter the resin and wash with DMF (1.5 mL ×6).
2. Add a solution of Fmoc-Leu (73.5 mg, 208 μmol), HOBt (28.1 mg, 208 μmol), and DIC (26.2 mg, 208 μmol) in 1 mL of dry DMF to the resin from the above step. Agitate the suspension at room temperature for 45 min. Monitor the completion of coupling with the ninhydrin test. Wash the fully coupled resin with DMF (1.5 mL ×6). Remove the protecting group by adding 1.5 mL of 50% piperidine in DMF and shaking at room temperature for 10 min. Wash the resin with DMF (1.5 mL ×8) and use in the next step.
3. Repeat **step 2** using Fmoc-Asp(OtBu) (85.6 mg, 208 μmol) with equivalent amounts of DIC and HOBt in 1.5 mL of DMF. Continue coupling for 45 min at room temperature. Treat the resin as in **step 2** and use in the next step.
4. Repeat **step 2** using Fmoc-Pro (70.1 mg, 208 μmol). After completion of the coupling, remove the protecting group with 50% piperidine in DMF and wash with DMF (1.5 mL ×8), DCM (1.5 mL ×6). Suspend the product in DCM.
5. In a separate vial dissolve 106 mg (208 μmol) of Gly-Gly trifluoroacetate–crown ether complex (prepared as described in **Subheading 3.1.**, **step 3**, compound **III** in **Fig. 2**), in 2 mL of dry DCM (*see* **Note 9**). To the solution add sequentially 28 mg of HOBt (208 μmol) and 42.6 mg of DCC (208 μmol). Stir the mixture at room temperature for 12 min and then filter the precipitated DCU (*see* **Fig. 2**). Transfer the clear solution to the reactor containing the filtered tetrapeptide Pro-Asp (OtBu)-Leu-Tyr (OtBu)-Wang resin from **step 4** (*see* **Note 10**). Add more DCM to facilitate the suspension of the resin (about 300 μL) and agitate the reaction mixture for 45 min (*see* **Note 11**). Test for completion of coupling by placing a few resin beads into a small test tube and running the ninhydrin test. On completion of the coupling, filter the resin and wash with DCM (3×), DMF (2×), and then treat with 1% DIEA in DMF 2× (3 min each) to remove the crown ether protecting group.
6. Suspend the resin from step 5 in DMF (1.7 mL) and add Fmoc-Phe-Pfp activated ester (115.1 mg, 208 μmol). Agitate the suspended resin at room temperature for 1 h and monitor for completion of the coupling by ninhydrin

analysis. Filter the reagents and solvent, wash the resin with DMF (2 mL ×4), and then suspend in 2 mL of 50% piperidine in DMF for 20 min to remove the Fmoc protecting group. Wash the deprotected resin with DMF (2 mL ×8) and DCM (2 mL ×8). Dry the finished resin in a desiccator over anhydrous potassium carbonate for 2 h.

7. Transfer the dried resin from **step 6** to a glass vial with a screw cap and add 2 mL of a trifluoroacetic acid–water mixture (95% TFA, 5% H_2O). Close the vial and allow the cleavage reaction to proceed at room temperature for 1 h. Filter the cleavage mixture, wash the resin with additional TFA–water, and combine the filtrates. Evaporate TFA at room temperature using a rotary evaporator or acid-resistant centrifugal vacuum system. Triturate the residual product with anhydrous ether and separate the white solid product by decantation or centrifugation. Dry the crude peptide over potassium hydroxide pellets under vacuum for 1 h.
8. Take a sample of the dried, crude peptide made in **step 7** (0.05–0.1 mg) and dissolve in a water–methanol mixture. Add acetonitrile until the solution clears. Analyze by high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC–MS) to verify the purity and identity of the synthesized peptide. For Phe-Gly-Gly-Pro-Asp-Leu-Tyr, MS: Expected 768.8 or 769 for M+1 by electrospray mass spectrometry.

3.3. Solid-Phase Synthesis of Rhodamine Labeled Peptide Nucleic Acids using Noncovalent Protection

1. Fmoc-Gly-CCCTAACCCTTACCCTAA-Lys(Boc)-RAM-PS: Synthesis of the protected PNA on a small scale (0.05 mmol) can be achieved by the Fmoc strategy ***(12,25,27)*** on PE Biosystems Expedite 8909 automated synthesizer using the protocol supplied by the manufacturer (http://www.appliedbiosystems.com/ds/pna/) (*see* **Notes 12–14**)
2. Suspend the resin-bound, protected PNA synthesized in **step 1** in DMF containing 20% piperidine in a reaction tube (500 µL). Agitate the resin for 20 min, filter the reagent and the solvent, and wash the resin with DMF (500 µL ×8).
3. Connect the reaction tube containing the resin from **step 2** to two 1-mL syringes. Dissolve 70 m*M* of sulforhodamine in 300 µL of 1:30 mixture of DIEA–DMF in one syringe. Keep the other syringe empty. Pass the sulforhodamine solution over the PNA resin in the reaction tube for 20 min using the two syringes. Wash the resin with DMF–DCM (1:1) 8×.
4. Connect the reaction tube of the resin from **step 3** with two 1-mL syringes. In one syringe load 300 µL of a 10 m*M* solution of tetramethylrhodamine succinimydyl ester in DIEA–DMF (1:30) and pass the solution over the resin using the dual syringes for 20 min. Wash the resin with DMF (0.5 mL ×8), DCM (0.5 mL ×8), and dry under vacuum for 2 h.
5. Suspend the dry resin made in **step 4** in 1 mL of TFA containing 25% *m*-cresol for 45 min at room temperature (*see* **Note 15**). Filter the cleavage mixture, wash the resin with the same cleavage solution and combine the filtrates. Evaporate the TFA solution under vacuum and triturate the residual product with dry ether at

0°C. Centrifugation of the crude rhodamine–PNA will give a pellet that can be purified by RP C_{18} HPLC using acetonitrile and 0.1% aqueous TFA buffer as solvents. HPLC will give two peaks corresponding to the two isomers of carboxytetramethyrhodamine. The calculated molecular weight is 5326.46 and M+1 = 5327.46

4. Notes

1. PNA monomers should be stored under dry, cold conditions. If the physical appearance of the monomers changes from a free-flowing powder form to aggregates, then the monomers should be dried *in vacuo* overnight before use.
2. Dry DMF is required in the synthesis of PNAs to dissolve the monomers and the activating reagent (HATU) under anhydrous conditions. The presence of moisture interferes with the purity and yield of the final products especially in the case of long PNAs (18-mers and longer). Dry DMF should be stored under nitrogen over dry 4 Å molecular sieves.
3. All resins should be stored under dry, cool conditions until their use.
4. Crown ether complexes with amines, N^{α}-amino acids, peptides of varying size, and side chain amino group of Lys and Arg have been prepared ***(15–23)***. The examples given here are only representative.
5. Evaporation of chloroform solutions is best accomplished by placing the solution in a round-bottom flask and use of a rotary evaporator.
6. **Recrystallization should be done in a fume hood away from sources of ignition, as both methanol and ethyl acetate are highly flammable.** Recovery of the crystals is most easily accomplished by filtration through a sintered glass funnel.
7. Absolute ethanol (100%, 200 proof) is the best choice.
8. Noncovalent protection of N^{α}-amino acids and the side chain amino group of Lys or Arg residues with crown ethers has most successfully been applied in the synthesis of peptides by the fragment condensation approach. This is illustrated here by the synthesis of NH_2-Phe-Gly-Gly-Pro-Asp-Leu-Tyr-OH. Single amino acid condensation in linear peptide synthesis often leads to undesirable oligomerization resulting from ineffective protection.
9. The optimal protocol requires the use of DCM as solvent for all the coupling reactions involving the crown ether complexes. The crown ether complexes are unstable in polar solvents such as DMF or DMSO. Consequently, use of DMF or DMSO as solvent in coupling reactions involving the crown ether complexes results in extensive oligomerization and other side product formation.
10. The efficiency of coupling to the crown ether complex is dependent on the nature of the amino acid in the N-terminus of the resin bound peptide. Competition for the crown ether molecule by primary amino groups compromises efficiency of coupling. Thus, the best results are obtained when the N-terminus amino acid is proline or other secondary amino acids.
11. Peptides larger than diglycine may require extension of coupling reaction time to 24 h.

12. In the case of PNAs containing consecutive identical bases, double coupling after the incorporation of the second base is necessary; otherwise a truncated product will be present.
13. Purine-rich PNA sequences require double coupling to improve the purity and yield of the final compound.
14. For analysis of PNA and PNA conjugates, an analytical HPLC equipped with a C18 300 Å reverse-phase column at a flow rate of 1.0 mL/min is recommended.
15. **Caution:** TFA is a highly corrosive irritant. Wearing proper protection for the hands and eyes is required. All operations involving TFA solutions should be performed in a well ventilated hood. Caution should also be exercised in making the TFA–*m*-cresol (4:1) solution for cleavage of the final product.

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2

Quality Control of Solid-Phase Synthesis by Mass Spectrometry

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1. Introduction

Combinatorial chemistry ***(1–7)*** has drastically modified the drug discovery process by allowing the rapid simultaneous preparation of numerous organic molecules to feed bioassays. Most of the time, syntheses are carried out using solid-phase methodology ***(8)***. The target compounds are built on an insoluble support (resins, plastic pins, etc). Reactions are driven to completion by the use of excess reagents. Purification is performed by extensive washing of the support. Finally, the molecules are released in solution upon appropriate chemical treatments.

Such a procedure is well established in the case of peptides, but solid-phase organic chemistry (SPOC) is more difficult. Optimization of the chemistry is required prior to library generation most of the time. Compound identification is complicated by the insolubility of the support. Release of the anchored structure in solution followed by standard spectroscopic analyses may impart delay and/or affect product integrity ***(9)***. A direct monitoring of supported organic reactions is thus preferable to the "cleave and analyze" methodology. Nevertheless, it presents several constraints. A common resin bead loaded at 0.8 mmol/g commonly produces nanomole quantities of the desired compound, and only 1% of the molecules are located at the outer surface of the bead ***(10)***. Very few materials, covalently bound to the insoluble support, are thus available for the analysis, which should ideally be nondestructive.

The relevance of mass spectrometry in the rehearsal phase of a combinatorial program is demonstrated through the control of various peptide syntheses. Fourier transform infra red (FTIR) ***(11)*** and cross polarization-magic angle

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spinning nuclear magnetic resonnance (CP-MAS-NMR) spectroscopies are also suitable techniques ***(12)***, but they lack the specificity or the sensitivity achievable by mass spectrometry.

Solid samples can be analyzed by mass spectrometry with techniques providing ionization by desorption ***(13)*** such as MALDI (matrix assisted laser desorption ionization) ***(14)*** and S-SIMS (static-secondary ion mass spectrometry) ***(15)***. Ions are produced by energy deposition on the sample surface. The analysis can be performed at the bead level. Most of all, chemical images can be produced to localize specific compounds on the studied surfaces.

S-SIMS was found to be superior to MALDI for following supported organic synthesis for many reasons. First, cocrystallization of the solid sample with a matrix is required for MALDI experiments, which is not the case in S-SIMS (no sample conditioning). Second, libraries of organic molecules contain mostly low-molecular-weight compounds, which are not suitable for MALDI analysis owing to possible interference with the matrix ions. Finally, a specific photolabile linkage between the support and the built molecules is necessary to release the desired molecular ions in the gas phase upon laser irradiation. Standard resins allowing linkage of the compounds through an ester or an amide bond are directly amenable to S-SIMS analysis.

Characteristic ions of peptide chains (*see* **Note 1**) have been obtained by S-SIMS whatever the nature of the polymeric support ***(16–18)***. N-Boc–protected peptides were synthesized on polystyrene resins ***(16)***. Fmoc-protected peptides anchored to polyamide resins ***(17)*** were also studied, and a wide range of dipeptides were loaded on plastic pins ***(18)***. All protecting groups (Boc, Fmoc, tBu, Z, Bn, Pht) gave characteristic ions in the positive mode, except Boc and tBu, which were not differentiated (*see* **Note 2**). The amino acids were evidenced by their corresponding immonium ions in the positive mode. These informative product ions were more abundant than ions related to the polymer, which require at least the rupture of two bonds ***(19)***. Peptide synthesis was thus easily followed step-by-step. Coupling reactions were monitored by detection of the incoming residue immonium ion and of the N-protecting group ion. The deprotection reaction was evidenced by the absence of the latter ion. Nevertheless, the identification of a peptide at any stage of the preparation required that the whole peptide sequence, and not fragments, was released in the gas phase. In other words, orthogonality between the peptide-resin linkage and the internal peptide bonds was compulsory. The ester linkage was found suitable since the peptide carboxylate ion was identified in the negative mode. This bond was thus termed "SIMS-cleavable." The amide linkage was broken simultaneously with the internal peptide amide bond and so was not adequate for such studies (*see* **Note 3**).

The recourse to a "SIMS cleavable" bond allowed direct identification of support-bound peptides. Several results have illustrated this concept. As an

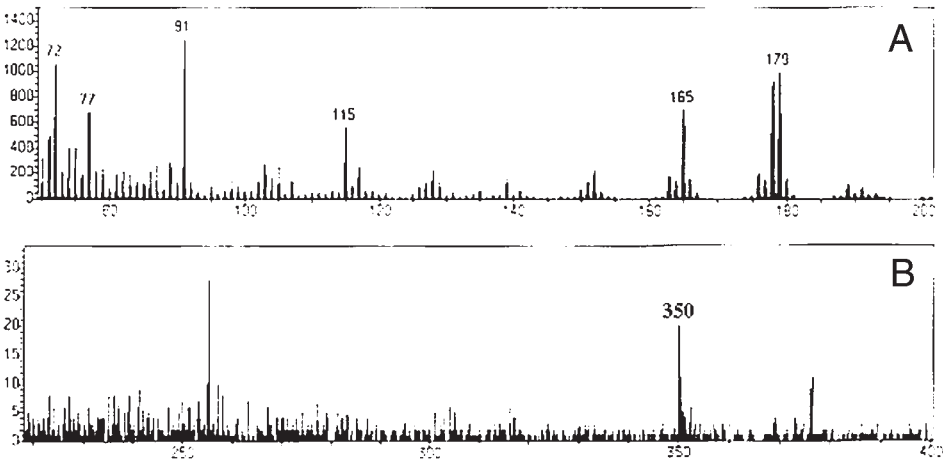


Fig. 1. **(A)** Positive S-SIMS spectrum of Fmoc-Met$(O)_2$-Ala-Val anchored to Wang resin: immonium ion of valine at m/z 72, Fmoc protection at m/z 165/178/179, polystyrene at m/z 77/91/115; **(B)** Negative S-SIMS spectrum of Fmoc-Met(O_2)-Val-Ala anchored to Wang resin: carboxylate ion H-Met(O_2)-Val-Ala-O^- at m/z 350.

example, a tripeptide bearing an oxidized methionine, Fmoc-Met(O_2)-Ala-Val anchored to Wang resin, was subjected to S-SIMS bombardment and the spectra were recorded in both positive and negative modes (**Fig. 1**). Some immonium ions were present in the positive spectrum as expected (valine at *m/z* 72), but there was no information about the methionine residue. The negative spectrum provided the carboxylate ion of the whole peptide sequence (*m/z* 350), which showed, without any ambiguity, that methionine was completely oxidized.

The S-SIMS technique was found specific through the use of a S-SIMS cleavable bond. The technique was sensitive because fentomoles of growing peptides were analyzed in each experiment, and it was nondestructive ***(20)***. Indeed, only 1% of the molecules were located at the surface, and small areas of 20×20 μm^2 were selected and bombarded to generate a spectrum. So, the bead can be reused after the analysis.

Any organic molecule is suitable for S-SIMS analysis provided that stable ions could be produced. The domain of SPOC can now be explored. Different linkers are currently investigated to determine the specific lability of the molecule-support bond under S-SIMS bombardment whatever the compound and the type of insoluble support.

Imaging studies were also performed to identify mixtures of peptides in a single analysis in the search of a high-throughput process adapted to combina-

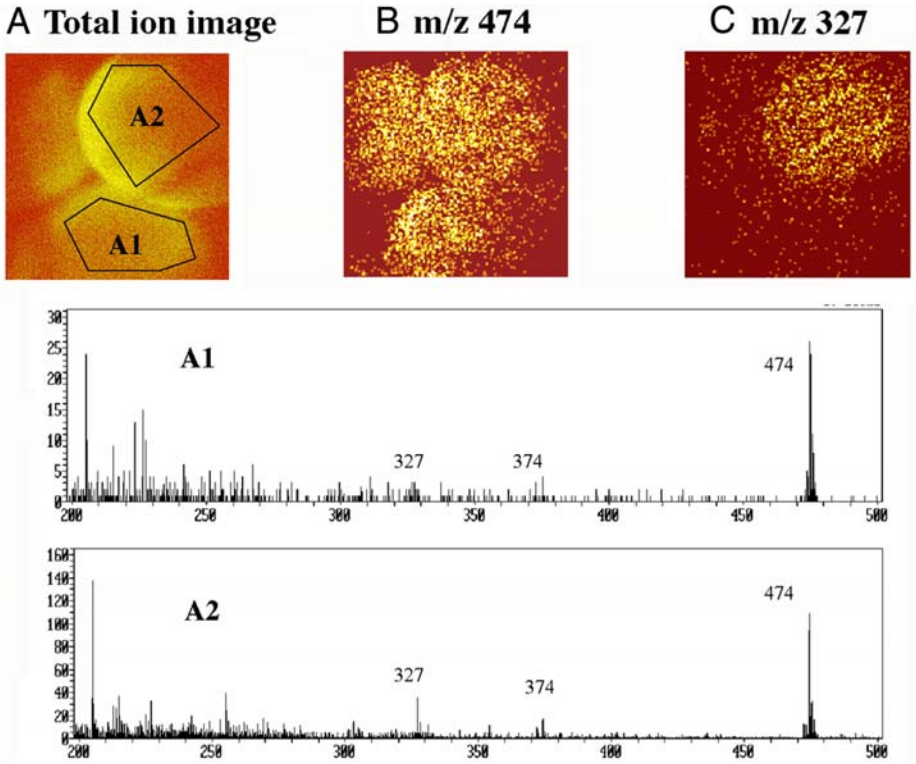


Fig. 2. **(A)** Total ion image showing two selected areas (*A1* and *A2*) each corresponding to one bead. The negative S-SIMS spectra generated from these two surfaces are given underneath. **(B)** Negative S-SIMS image of Boc-Pro-Phe-Leu (carboxylate ion at *m/z* 474); **(C)** Negative S-SIMS image of the deleted sequence Boc-Pro-Leu (carboxylate ion at *m/z* 327).

torial library profiling ***(21)***. Two types of mixtures can be envisaged. Beads, which were each loaded by the same molecules, were pooled or the beads could themselves bear different components (starting material, byproducts). For instance, the unwanted intramolecular cyclization of glutamic acid into pyroglutamic acid was evidenced by S-SIMS down to a level of only 15% of side-reaction ***(22)***. Incomplete coupling leading to truncated chains was also detected ***(23)***, and clear images were produced with only 9% of deleted sequences as displayed in **Fig. 2**.

2. Materials

2.1. Solid-Phase Peptide Synthesis

2.1.1. Synthesis of Boc-Protected Peptides

1. Carry out peptide syntheses on hydroxymethylpolystyrene resin loaded at 0.93 or 2.8 mmol/g (Novabiochem, Meudon, France).

2. L-configuration Boc-protected amino acids available from Senn Chemicals (Gentilly, France) and Propeptide (Vert le Petit, France).
3. Load first Boc-protected amino acid onto the resin according to the symmetrical anhydride procedure (dissolve 10 Eq of the residue in a minimum of dichloromethane).
4. Cool this solution in an ice-water bath and add 5 Eq of diisopropylcarbodiimide.
5. Stir the solution for 30 min at 4°C, filter, and concentrate under vacuum.
6. Dissolve the resulting symmetrical anhydride in dimethylformamide (DMF) and add to the resin with 0.1 Eq of dimethylaminopyridine.
7. Release the Boc protection by treatment with trifluoroacetic acid in dichloromethane.
8. Couple the second residue by 2 Eq of (benzotriazol-1-yloxy)tris(dimethylamino) phosphonium hexafluorophosphate (BOP) and diisopropylethylamine in dimethylformamide for 2 h.

2.1.2. Synthesis of Fmoc-Protected Peptides

1. Fmoc-protected amino acids available from Senn Chemicals (Gentilly, France).
2. 4-Methylbenz-hydrylamine (*MBHA*) *resin*: Carry out peptide syntheses on MBHA resin loaded at 0.8 mmol/g (Novabiochem, Meudon, France). Couple the amino acids by two equivalents of (BOP) and diisopropylethylamine in dimethylformamide for 2 h. Remove Fmoc protection with two treatments (3 and 15 min) of the resin with a solution of piperidine in DMF (20%, v/v).
3. *Wang resin*: Anchor the first amino acid to the resin (0.93 mmol/g, Novabiochem, Meudon, France) according to the symmetrical anhydride method. (The standard above-mentioned procedure was applied to build the sequence.)
4. *Chlorotrityl resin*: React the first amino acid overnight with the resin (1.5 mmol/g, Senn Chemicals, Gentilly, France) in the presence of N,N-diisopropylethylamine (DIEA). (The standard above-mentioned procedure was applied to build the sequence.)

2.1.3. Peptide Characterization

1. Check all syntheses prior to S-SIMS experiments by treating a few resin beads with hydrofluoric acid (HF) to release the built sequences in solution.
2. Identify the peptides with high performance liquid chromatography (HPLC) on an Alliance 2690 from Waters (Milford, MA) and electrospray mass spectrometry (ESI-MS) on a Platform II from Micromass (Manchester, UK).

2.2. Mass Spectrometry Instrumentation

1. Perform S-SIMS measurements on a TRIFT I spectrometer from the PHI-Evans Company (Eden Prairie, MN) equipped with a time-of-flight (TOF) analyzer.
2. Record spectra using a pulse (1 ns, 12 kHz) liquid metal source (^{69}Ga, 15 keV) operating in the bunched mode to provide good mass resolution (m/Δm = 2000 measured at *m/z* 43).
3. Perform charge compensation for all samples using a pulsing electron flood (Ek = 20 eV) at a rate of one electron pulse per five ion pulses (*see* **Note 1**).
4. Analyze surfaces in squares of 20×20 μm^2 to produce a S-SIMS spectrum.
5. Acquire all positive and negative spectra within 1–10 min with a fluence of less than 10^{12} ions/cm^2 ensuring static conditions on the sample.

6. For imaging studies, raster the primary ion beam on 400 × 400 μm^2 during 30 min to generate a complete mass spectrum at each pixel, and record a chemical image.
7. Use the "scatter" raster type, which is the one designed to be used for insulating samples: each pixel point is located as far from the previous and next pixel so as to spread the primary beam charge homogeneously.
8. Obtain mass spectra in an image from different selected areas by using simple drawing tools.

3. Methods

3.1 Sample Conditioning

1. At the end of the synthesis wash the resin beads with dichloromethane, ethanol, water, ethanol, and dichloromethane. Repeat this procedure three times.
2. Dry the resin beads overnight in a dessicator.
3. Fix an adhesive aluminum tape on a nonmagnetic stainless grid and place it in the cavity of the TOF-S-SIMS sample holder (the metallic grid prevents large variations in the extraction field over a large area insulator; it is possible, therefore, to move from one grid "window" to any of the other "windows" without any concern for retuning).
4. Sprinkle a few beads on the adhesive aluminum tape. (Do not touch the beads but manipulate them with tweezers.) The resin in excess is removed by an inert gas stream, and the remaining beads are well attached to the tape.
5. Insert the holder in the load lock of the mass spectrometer and pump it down until the required vacuum is reached.
6. Visualize the resin beads by a camera and select an area that contains well-defined beads of spherical appearance that are all roughly in the same plane. Record mass spectrometric data from this area.

3.2. Acquisition of a S-SIMS Spectrum

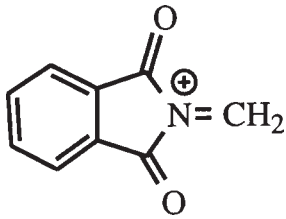
1. Choose one bead in the selected area, and define a surface of 20 × 20 μm^2 on the bead surface.
2. Trigger the primary bombardment. Examine the emitted secondary ions from the selected surface to modify the mass spectrometer tuning if required.
3. Start the acquisition. It should last 5 min.

3.3. Acquisition of a S-SIMS Image

1. Choose a surface in the selected area of 400 × 400 μm^2 containing a few beads.
2. Trigger the primary bombardment. Examine the emitted secondary ions from the selected surface to modify the mass spectrometer tuning if required.
3. Start the acquisition. It should last 30 min.
4. Generate the chemical images from the total ions (total image) or from various selected ions.
5. From any recorded image, select an area of interest in the bombarded surface (for instance one specific bead) and the corresponding S-SIMS spectrum will be displayed.

4. Notes

1. Owing to large charge effects on such insulating materials, charge compensation is required for all samples.
2. We have observed many similarities between the two desorption techniques: fast atom bombardment (FAB) and S-SIMS. The recorded ions in both positive and negative modes in S-SIMS could be deduced from the well-documented behavior of molecules in FAB. The amino acids that exhibited immonium ions were the same as the ones reported in the literature in FAB experiments ***(24)***. Fragmentations leading to ions characterizing the protecting groups were also identical ***(25,26)***.
3. The studied protecting groups and the corresponding recorded ions were as follows: Boc and tBu at *m/z* 57 ($C_4H_9^+$), Fmoc at *m/z* 165 ($C_{13}H_9^+$, $C_{13}H_9^-$), and *m/z* 179 ($C_{14}H_{13}^+$), Z at *m/z* 91 ($C_7H_7^+$), and Pht at *m/z* 160 as shown below.



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