Preface

The continued successes of large- and small-scale genome sequencing projects are increasing the number of genomic targets available for drug discovery at an exponential rate. In addition, a better understanding of molecular mechanisms—such as apoptosis, signal transduction, telomere control of chromosomes, cytoskeletal development, modulation of stress-related proteins, and cell surface display of antigens by the major histocompatibility complex molecules—has improved the probability of identifying the most promising genomic targets to counteract disease. As a result, developing and optimizing lead candidates for these targets and rapidly moving them into clinical trials is now a critical juncture in pharmaceutical research. Recent advances in combinatorial library synthesis, purification, and analysis techniques are not only increasing the numbers of compounds that can be tested against each specific genomic target, but are also speeding and improving the overall processes of lead discovery and optimization.

There are two main approaches to combinatorial library production: parallel chemical synthesis and split-and-mix chemical synthesis. These approaches can utilize solid- or solution-based synthetic methods, alone or in combination, although the majority of combinatorial library synthesis is still done on solid support. In a parallel synthesis, all the products are assembled separately in their own reaction vessels or microtiter plates. The array of rows and columns enables researchers to organize the building blocks to be combined, and provides an easy way to identify compounds in a particular well. In contrast, the split-and-mix approach relies largely on solid-based synthetic methods, and produces a mixture of related compounds in the same reaction vessel. Although most combinatorial synthesis is done on solid support, solution-based synthetic methods offer some advantages. For example, solution-based synthesis offers the flexibility to use a larger number of chemical reactions; however, one classic problem of this approach is keeping track of which building blocks are added to which reaction vessel or microtiter plate well. In addition, because the compounds are not attached to a solid support, it is difficult to isolate them. Chapters 1–12 of *Combinatorial Library Methods and Protocols* discuss a variety of strategies for combinatorial library synthesis and quality control.

A combinatorial library only brings value when screened. The way library members are screened for activity depends on the form in which they were

synthesized. For solid-based methods, the compounds are usually cleaved from the solid support on which they were made and eluted into microtiter plates with one or more compounds per well. For solution-based methods, the compounds of interest must be isolated, purified, and then distributed to microtiter plates. The exact method used to determine the activity of individual compounds is dependent on the screening assay used. Assays often involve displacement of another ligand, or release of a reporter element to give a readout signal. Most commonly, screening assays involve measuring radioactivity, fluorescence, or absorbance in each reaction well and comparing those to measurements on positive and negative controls. Chapters 13–16 of *Combinatorial Library Methods and Protocols* discuss purification and screening of combinatorial libraries.

The design, production, characterization, tracking, and screening of many combinatorial libraries in multiple biological assays presents an enormous computational and information management challenge. There is a need for integrated library specification, design, synthesis, screening, and analysis with the ability to feed back information from completed experiments iteratively during the entire process. Such integration requires a combination of computational informatics and analysis solutions. Chapters 17–21 of *Combinatorial Library Methods and Protocols* discuss a range of computational approaches to combinatorial library design.

Combinatorial chemistry has rapidly evolved from its early focus on the generation of large numbers of molecules to a powerful combinatorial design technology for the generation and optimization of pharmaceutical leads to produce drug candidates. Developing trends in combinatorial chemistry that promise to further improve drug design include the integration of combinatorial approaches with a range of design strategies, including structure-based design, physiochemical parameters, and combinatorial methods to optimize natural products. Because only a very small number of biologically active compounds have been sampled from all possible chemicals, the potential to discover new pharmaceuticals by applying combinatorial techniques has opened a new frontier in biology and medicine.

Lisa B. English

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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

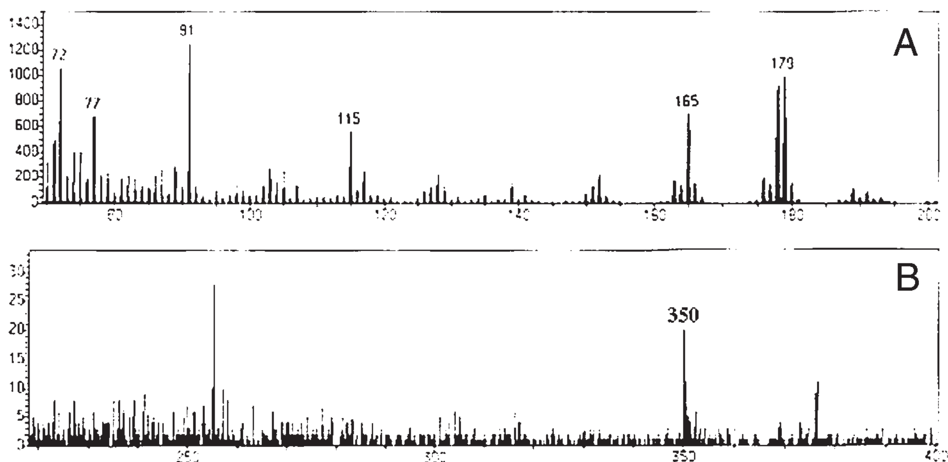


Fig. 1. **(A)** Positive S-SIMS spectrum of Fmoc-Met(O)$_2$-Ala-Val anchored to Wang resin: immonium ion of valine at m/z 72, Fmoc protection at m/z 165/178/179, polystyrene at m/z 77/91/115; **(B)** Negative S-SIMS spectrum of Fmoc-Met(O_2)-Val-Ala anchored to Wang resin: carboxylate ion H-Met(O_2)-Val-Ala-O^- 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 $20 \times 20\ \mu m^2$ 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-

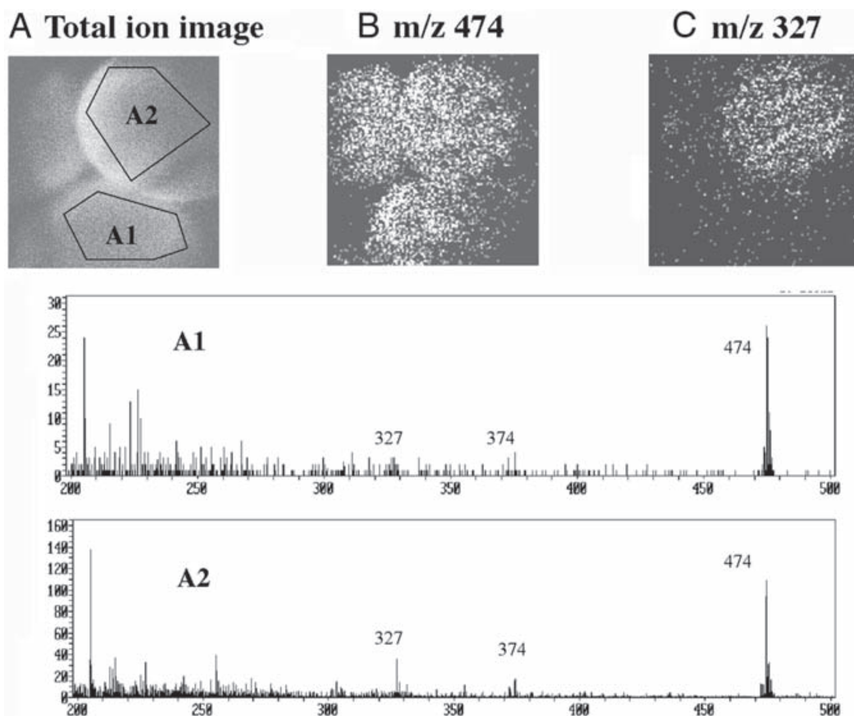


Fig. 2. **(A)** Total ion image showing two selected areas (*A1* and *A2*) each corresponding to one bead. The negative S-SIMS spectra generated from these two surfaces are given underneath. **(B)** Negative S-SIMS image of Boc-Pro-Phe-Leu (carboxylate ion at *m/z* 474); **(C)** Negative S-SIMS image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

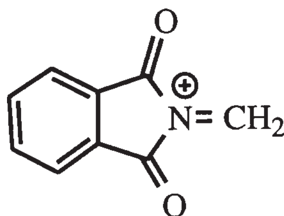
1. Choose one bead in the selected area, and define a surface of 20 × 20 μm^2 on the bead surface.
2. Trigger the primary bombardment. Examine the emitted secondary ions from the selected surface to modify the mass spectrometer tuning if required.
3. Start the acquisition. It should last 5 min.

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.



References

1. Czarnik, A. W. and Dewitt, S. H. (1997) *A practical guide to combinatorial chemistry*. American Chemical Society, Washington, DC.
2. Wilson, S. R. and Czarnik, A. W. (1997*) Combinatorial Chemistry—Synthesis and Application.* Wiley, New York, NY.
3. Bunin, B. A. (1998) *The Combinatorial Index*. Academic Press, London, UK.
4. Terrett, N. K. (1998) *Combinatorial Chemistry*. Oxford University Press, Oxford, UK.
5. Gordon, E. M. and Kervin, J. F. (1998) *Combinatorial Chemistry and Molecular Diversity in Drug Discovery*. Wiley, New York, NY.
6. Obrecht, D. and Villalgordo, J. M. (1998) *Solid-Supported Combinatorial and Parallel Synthesis of Small Molecular Weight Compound Libraries*. Tetrahedron Organic Chemistry Series, Volume 17, Pergamon, Elsevier, Oxford, UK.
7. Jung, G. (1999) *Combinatorial chemistry—Synthesis, Analysis, Screening*. Wiley-VCH, Weiheim, Germany.
8. Dolle, R. (2000) Comprehensive survey of combinatorial library synthesis: 1999. *J. Comb. Chem*. **2,** 383–433.
9. Metzger, J. W., Kempter, C., Weismuller, K.-H., and Jung, G. (1994) Electrospray MS and tandem MS of synthetic multicomponent peptide mixtures: determination of composition and purity. *Anal. Chem*. **219,** 261–277.
10. Yan, B., Fell, J. B., and Kumaravel, G. (1996) Progression of organic reactions on resin supports monitored by single bead FTIR microspectroscopy. *J. Org. Chem.* **61,** 7467–7472.
11. Yan, B. (1998) Monitoring the progress and the yield of solid-phase organic reactions directly on resin supports. *Acc. Chem. Res*. **31,** 621–630.

12. Shapiro, M. J. and Gounarides, J. S. (1999) NMR methods utilized in combinatorial chemistry research. *Prog. Nucl. Magn. Res. Spectros.* **35,** 153–200.
13. Busch, K. L. (1995) Desorption ionization mass spectrometry. *J. Mass Spectrom.* **30,** 233–240.
14. Karas, M., Bachmann, D., Bahr, U., and Hillenkamp, F. (1987) Matrix-assisted ultraviolet laser desorption of non-volatile compounds. *Int. J. Mass Spectrom. Ion Proc.* **78,** 53–68.
15. Benninghoven, A., Rudenauer, F. G., and Werner, H. W. (1987) *SIMS: Basic concepts, Instrumental Aspects, Applications and Trends.* Wiley, New York, NY.
16. Drouot, C., Enjalbal, C., Fulcrand, P., et al. (1996) Step-by-step control by time-of-flight secondary ion mass spectrometry of a peptide synthesis carried out on polymer beads. *Rapid Commun. Mass Spectrom.* **10,** 1509–1511.
17. Drouot, C., Enjalbal, C., Fulcrand, P., et al. (1997) Tof-SIMS analysis of polymer bound Fmoc-protected peptides. *Tetrahedron Lett.* **38,** 2455–2458.
18. Aubagnac, J.-L., Enjalbal, C., Subra, G., et al. (1998) Application of time-of-flight Secondary ion mass spectrometry to *in situ* monitoring of solid-phase peptide synthesis on the Multipin™ system. *J. Mass Spectrom.* **33,** 1094–1103.
19. Bertrand, P. and Weng, L.-T. (1996) Time-of-flight secondary ion mass spectrometry. *Mikrochim. Acta.* **13,** 167–182.
20. Enjalbal, C., Subra, G., Combarieu, R., Martinez, J., and Aubagnac, J.-L. (2000) Use of time of flight static-secondary ion mass spectrometry in peptide synthesis on solid support. *Rec. Res. Dev. Organic Chem.* **4,** 29–52.
21. Aubagnac, J.-L., Enjalbal, C., Drouot, C., Combarieu, R., and Martinez, J. (1999) Imaging time-of-flight secondary ion mass spectrometry of solid-phase peptide synthesis. *J. Mass Spectrom.* **34,** 749–754.
22. Enjalbal, C., Maux, D., Subra, G., Martinez, J., Combarieu, R., and Aubagnac, J.-L. (1999) Monitoring and quantification on solid support of a by-product formation during peptide synthesis by Tof-SIMS. *Tetrahedron Lett.* **40,** 6217–6220.
23. Enjalbal, C., Maux, D., Combarieu, R., Martinez, J., and Aubagnac, J-L. (2000) Mass spectrometry and combinatorial chemistry: New approaches for direct support-bound compound identification. *Combinatorial Chem. High Throughput Screening* **4,** 363–373.
24. Falick, A. M., Hines, W. M., Medzihradsky, K. F., Baldwin, M. A., and Gibson, B. W. (1993) Low-mass ions produced from peptides by high energy collision-induced dissociation in tandem mass spectrometry. *J. Am. Soc. Mass Spectrom.* **4,** 882–893.
25. Garner, G. V., Gordon, D. B., Tetler, L. W., and Sedgwick, R. D. (1983) FAB MS of Boc protected amino acids. *Org. Mass Spectrom.* **18,** 486–488.
26. Grandas, A., Pedroso, E., Figueras, A., Rivera, J., and Giralt, E. (1988) Fast atom bombardment mass spectrometry of protected peptide segments. *Biomed. Environm. Mass Spectrom.* **15,** 681–684.