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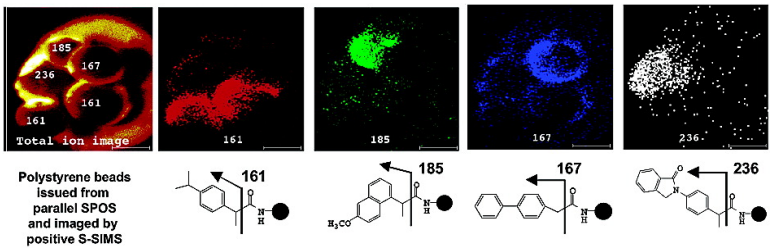

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Imaging Combinatorial Libraries by Mass Spectrometry: from Peptide to Organic-Supported Syntheses

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Supported peptide and drug-like organic molecule libraries were profiled in single nondestructive imaging static secondary ion mass spectrometric experiments. The selective rupture of the bond linking the compound and the insoluble polymeric support (resin) produced ions that were characteristic of the anchored molecules, thus allowing unambiguous resin bead assignment. Very high sensitivity and specificity were obtained with such a direct analytical method, which avoids the chemical release of the molecules from the support. Libraries issued from either mix-and-split or parallel solid-phase organic syntheses were profiled, demonstrating the usefulness of such a technique for characterization and optimization during combinatorial library development. Moreover, the fact that the control was effected at the bead level whatever the structure and quantity of the anchored molecules allows the sole identification of active beads selected from on-bead screening. Under such circumstances, the time-consuming whole-library characterization could thus be suppressed, enhancing the throughput of the analytical process.

Introduction

The discovery of hits has been considerably accelerated by the development of combinatorial chemical technologies. Design of libraries has evolved from large peptide mixtures generated by reliable well-documented reactions to arrays of organic materials presenting more structural diversity but requiring more subtle chemistries.[1,2] A significant number of reported combinatorial data are produced nowadays according to solid-phase organic synthesis strategy.[3] In that context, optimization of the supported reactions is first undertaken through the synthesis of rehearsal libraries prior to large-scale production.

Detailed library composition assessment is required to gather reliable synthetic information. A nondestructive method to achieve the direct control of supported multistep organic syntheses is required.

Indeed, any spectroscopic method that requires the release in solution of the assembled molecules (known as the "cleave and analyze" strategy) will impart sources of delay and errors. Side reactions could occur during this additional chemical treatment, hampering actual compound identification.[4] Thus, covalently bound structures must be cleaved from the support without any added chemical reagent and simultaneously identified.

Of all spectroscopic methods, mass spectrometry is particularly well suited to probe combinatorial libraries, providing rapid, specific, and sensitive measurement.[5] Only matrix-assisted laser desorption ionization (MALDI) mass spectrometry[6] and static secondary ion mass spectrometry[7] (S-SIMS) enable the performance of imaging studies.[8−12] The sample surface is mapped by targeting specific ions. The recorded image shows the spatial distributions of the selected ions related to the molecules of interest. S-SIMS was preferred to MALDI to characterize and profile low-molecular-weight synthetic drugs attached to solid support for two reasons. First, MALDI allows identification of high-molecular-weight compounds, whereas S-SIMS is most suited to low-molecular-weight structures. And second, even if MALDI has been reported for the direct analysis of resin beads, it has required either specific synthetic constraints[13] (resin bearing a UV-labile linker to release the attached molecules upon laser irradiation) or treatment of the sample on the target by a strong acid to cleave in situ the anchoring bond.[14]

The S-SIMS technique provides a surface analysis by subjecting the solid sample to an incident primary energetic beam of limited intensity to avoid substrate degradation on the impact. The emitted ions are thus attributed to the native sample and not to byproducts. It should be noted that the ion abundances are rather weak, since it requires bond breaking. The conversion of covalently attached molecules into adsorbed species by an on-target chemical clipping procedure provides more intense signals.[15] But such an in situ "cleave and analyze" strategy suffers from potential leakage of released compounds outside the surface of the support to where they were initially linked[16] and is, thus, not relevant.

The challenge is to produce sufficiently abundant ions characterizing the anchored molecules by direct analysis of the solid support without the recourse to a chemical treatment. This means that only the bond linking the growing molecule to the support must be cleaved during the S-SIMS bombardment to suppress formation of fragment ions. Iden-

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tification of individual compounds in large libraries would be otherwise impossible if intact molecules were not emitted.

Solid-phase peptide syntheses were monitored first as model studies before tackling the most challenging solid-phase organic synthesis opening the field of combinatorial library profiling.

S-SIMS has been successfully employed to characterize peptides linked to various insoluble supports, such as polystyrene resins,[17] polyamide resins,[18] and plastic pins.[19] We have developed the so-called "SIMS-cleavable bond" approach.[20] The peptide was anchored to the insoluble polymer via an ester bond that is orthogonal to the peptide amide bond. A selective point of rupture is created in the negative mode by releasing the peptide carboxylate ion. This strategy required the use of only a hydroxyl-functionalized resin, which did not impart any synthetic restriction. Some fragmentations of urethane protections (Boc and Fmoc) were obtained in the negative mode, but they were easily identified and did not hamper product identification.

At that stage of our studies, the method was validated.[21] A wide range of peptides bearing different amino acid compositions and protecting groups and of varying lengths were successfully identified. The repeatability of the S-SIMS analysis through intra- and interday assays was checked, and the abundances of the recorded ions varied within 15%. Sensitivity was also a matter of concern due to the need to break covalent bonds to generate ions. The protecting groups exhibited the most abundant ions, since they were, first, strongly exposed to the bombardment and, second, generated by a single bond rupture, whereas internal sequence ions could be produced only by at least two bond cleavages.[22] This consideration also explains the fact that ions related to the insoluble support were scarcely produced during such SIMS analysis. The sensitivity was very good and acceptable in the positive mode and negative mode, respectively. Finally, the method was assessed to be nondestructive. Spectrum acquisition was usually effected on an area of $20 \times 20\ \mu m^2$, which destroyed 0.01% of the growing sequences.

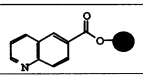
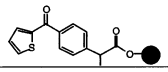
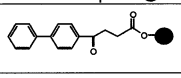
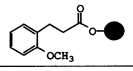
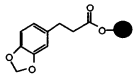
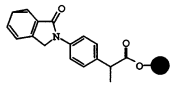
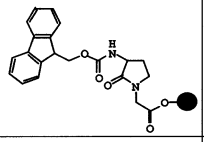
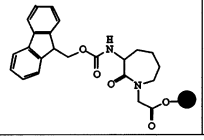
Although the direct monitoring of supported peptide syntheses was robust, reliable, sensitive, and nondestructive, further investigations were needed to apply the method to drug-like non-peptide compounds. The present stage of our work was thus to broaden the scope of the S-SIMS method to the control of supported organic syntheses.

Results and Discussion

The studied organic compounds are listed in Tables 1 and 2. Since most of the published data in solid-phase synthesis involve an ester or an amide linkage between the built molecule and the support,[23] two sets of experiments were carried out on Wang resin (ester bond, Table 1) and on Rink amide resin (amide bond, Table 2). From our preliminary studies on peptides, we knew that these anchoring bonds were "SIMS-cleavable", but their selective cleavage was required to produce "molecular ions" and not fragments. Thus, the term molecular ion represents in this discussion any ion issued from the sole rupture of the anchoring linkage, thus characterizing the whole attached molecules.

Organic molecules linked to the support by an ester bond were first considered (Table 1). As expected, most of the compounds released upon S-SIMS bombardment the carboxylate ion in the negative mode. The molecules that failed to exhibit molecular ion were fragmented readily by either the loss of the Fmoc protection, as observed for peptides,[18] or by the loss of CO_2. Decarboxylation occurred only when the resulting carbanion was stabilized by delocalization on an aromatic ring (structures 2 and 6 in Table 1). Nevertheless, the fragment ions were still characteristic of the anchored structures, and unambiguous structural assignments were effected.

Table 1. Organic Molecules Linked to the Support through an Ester Bond

		Recorded ions in the negative mode	
N°	Structure	Molecular ion $RCOO^-$	Fragment ions R^- ($RCOO^- \rightarrow R^- + CO_2$)
1		172	-
2		-	215
3		253	-
4		179	-
5		193	-
6		-	236
7		-	157 (loss of Fmoc) 165 (Fmoc)
8		-	185 (loss of Fmoc) 165 (Fmoc)

The results obtained with the same molecules attached to the support through an amide bond are presented in Table 2. Negative ion spectra were more informative than positive ion spectra, since radical molecular ions of the type $RCO\text{-}NH_2^{-\bullet}$ were generally observed. The production of such odd electron ions[24] required first protonation of the amide bond followed by its oxido−reductive rupture. The compounds that failed previously to exhibit a molecular ion when anchored through an ester bond as a result of prompt decarboxylation behaved similarly: a stabilized carbanion generated this time by the loss of the terminal moiety $CONH_2^{\bullet}$ was recovered. The competitive mechanism of molecular ion formation by direct rupture of the amide bond leading to an acylium ion was scarcely observed in the positive S-SIMS spectra. As expected, compounds bearing a benzyl group showed abundant tropylium ions in the positive mode.

All recorded ions present in S-SIMS spectra of supported organic molecules are summarized in Figure 1. For all studied supported molecules, the expected selective rupture between the compound and the insoluble support occurred in the

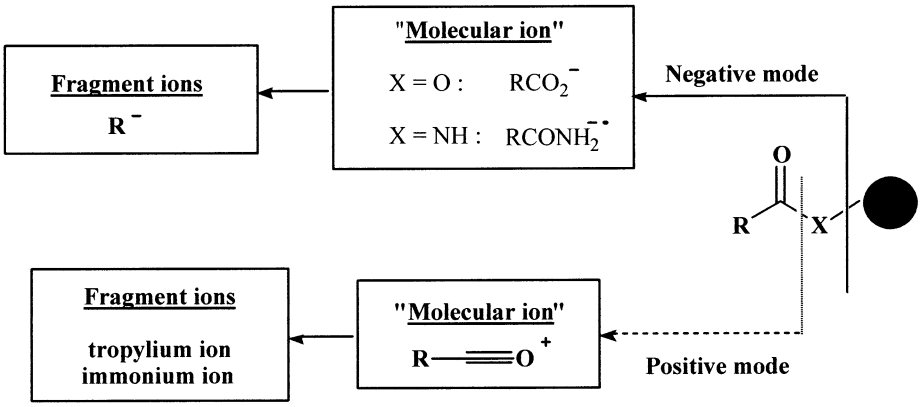
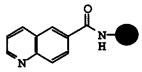
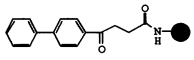
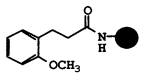
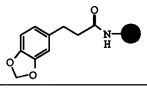
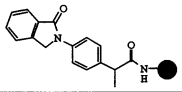
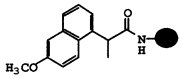
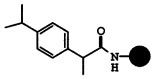
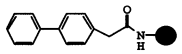
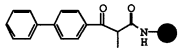
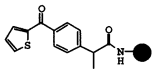
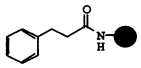
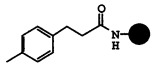
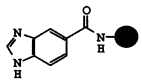


Figure 1. Ions recorded in S-SIMS from supported organic molecules ("molecular ions" represent ions issued from the sole rupture of the anchoring linkage, thus characterizing the whole attached molecules).

Table 2. Organic Molecules Linked to the Support through an Amide Bond

		Negative ions		Positive ions	
N°	Structure	Molecular ion $RCONH_2^{-\bullet}$	Carbanion R^-	Acylium ion RCO^+	Tropylium ion
1		172	-	156	-
2		-	209	-	237
3		179	-	-	-
4		193	-	-	-
5		-	236 weak	-	236
6		-	185	-	185
7		205	161	-	161
8		-	167	-	167
9		-	209	-	209 weak
10		-	215	243	215 weak
11		149	-	-	91
12		163	-	-	105
13		161 weak	117 weak	145	-

negative mode, whereas the corresponding bond breaking in the positive mode and leading to an acylium ion was rarely observed. Fragmentation of the molecular ion was evidenced when the produced carbanion was stabilized.

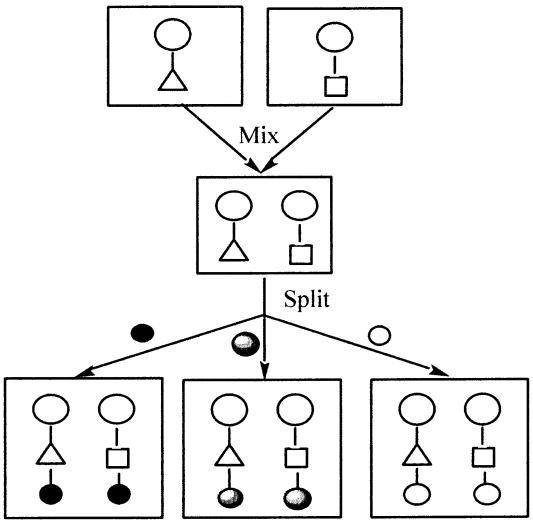


Figure 2. Schematic description of the iterative mix-and-split protocol. The resin is partitioned into two parts. Each batch (represented by O) is loaded with the first building block. The two batches are then pooled, mixed thoroughly, and split again. Each batch is reacted with the second building block. The procedure is repeated until the desired molecule is built.

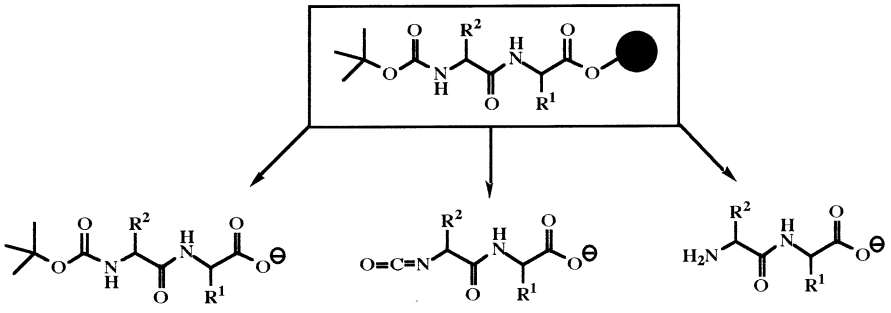
Whatever the nature of the linkage bond, two ionization pathways were demonstrated. A direct bond rupture was observed when the resulting ion was stabilized (carboxylate ion, tropylium ion, acylium ion, ...). Otherwise, a two-step mechanism involving a proton transfer, which then induced bond rupture, was evidenced. At least one of these competing pathways allowed the production of an ion related to the whole supported organic molecules (molecular ion or fragment ion unambiguously related to the molecular ion). S-SIMS was thus suitable for following any supported synthesis.

Being able to monitor any reactions on solid supports, we tackled the problem of mixtures. Two situations can be encountered in resin-supported syntheses. In the first case, various compounds loaded on the same bead constitute the mixture, whereas in the second case, different beads constitute the mixture. The latter possibility is encountered with combinatorial libraries issued from the mix-and-split synthetic strategy.[25] Such a library consists of a pool of beads, each bearing a single compound (Figure 2).

The detection of multiple compounds attached to the same bead was relatively straightforward, since it required recording the S-SIMS spectrum from a small part of the surface accessible to the bombardment ($20 \times 20\ \mu m^2$). Byproduct ions were detected, with side-reactions not exceeding 10%.[26]

Profiling combinatorial library mixtures was more challenging, since the successive analyses of each bead will impart enormous delays. Thus, the above-described punctual analysis must be substituted by imaging experiments where a large sample surface containing several beads is mapped through the detection of specific ions. Preliminary studies have shown that correct images could be obtained with peptides in the positive mode by targeting abundant immonium ions featuring the amino acid residues.[19] But these images will be useless in the case of complex peptide mixtures, since the amino acid composition will be similar in different peptides. Images of molecular ions are required to assign unambiguously the anchored structures.

To probe the feasibility of recording images in the negative mode by targeting carboxylate ions, a simple mixture of six dipeptides issued from a mix-and-split synthetic strategy was first studied (Figure 3).



Carboxylate ion $(M - H)^{\ominus}$ | Isocyanate ion $(M - 74 - H)^{\ominus}$ | Free amine ion $(M - 100 - H)^{\ominus}$

N°	Peptide sequence	Carboxylate ion	Isocyanate ion	Free amine ion
1	Boc-Phe-Ala-	335	261	235
2	Boc-Phe-Leu-	377	303	277
3	Boc-Val-Ala-	287	213	187
4	Boc-Val-Leu-	329	255	229
5	Boc-Ile-Leu-	343	269	243
6	Boc-Ile-Ala-	301	227	201

Figure 3. Ions obtained in the negative mode upon S-SIMS bombardment of the six loaded dipeptides. R^1 and R^2 represent the first and second amino acid side-chain, respectively. R^1 stands for a methyl group (Ala) or an isobutyl substituent (Leu). R^2 was a benzyl moiety (Phe), an isopropyl group (Val), or a *sec*-butyl substituent (Ile).

Statistical calculations were undertaken to find the number of beads that must be analyzed to detect all peptides.[27] The beads loaded with the sequence noted i were present in the mixture in proportion p_i. Considering that six beads were aliquoted at random, the probability that the category i was not represented in the sample is $(1 - p_i)^6$. Consequently, the probability noted, q_i, to find at least one bead i in the aliquot was $1 - (1 - p_i)$.[6] This probability was increased to $q_i = 1 - (1 - p_i)^{6n}$ in the case of n analyzed aliquots. If the synthesis is successful, equimolar amounts of the six peptides are expected, so the probability p_i corresponded to 1/6. Numerically, the probability, q_i, to find any peptide in four and in five aliquots of six beads each, equaled 0.987 and 0.996, respectively. Practically, single beads were difficult to handle, so a spatula tip was spread in one well of the SIMS target and visualized by a camera. Four different areas of $395 \times 395\ \mu m^2$ containing at least six beads were selected, which corresponded to the four aliquots of the statistical calculations. Subsequently, all required analyses were carried out during a unique S-SIMS experiment. Furthermore, up to four samples can be deposited in spatially isolated wells on the target allowing the profiling of four mix-and-split libraries in the raw. The analysis throughput could be further improved by the design of a multiposition target similar to the one used in MALDI mass spectrometry.

The surface of the deposit was visualized by a camera to locate at least four different areas ($395 \times 395\ \mu m^2$) containing each six beads that were roughly in the same plane. Clear negative images were produced provided that at least 300 counts of the ions of interest were recorded on the analyzed surfaces of $395 \times 395\ \mu m^2$. The analysis time should not exceed 30 min; otherwise, the static threshold (fluence of $<10^{13}$ ions/cm^2) is reached, inducing damage of the sample under study. Images of the six Boc-protected peptides were thus recorded by summing the abundances of the carboxylate molecular ion and the related deprotected fragments, which were the isocyanate (loss of *tert*-butyl alcohol) and the free amine (loss of Boc)[28] (Figure 3). The method was sensitive enough to produce correct images, as displayed in Figure 4. All peptide structures were found in the first three analyzed areas (A1, A2, A3), diminishing the analysis time to 3×30 min. Sixteen beads were sufficient to detect all compounds. Furthermore, the identity of the peptide anchored to any bead can be further checked by acquiring in 5 min the negative S-SIMS spectrum from a small selected surface ($20 \times 20\ \mu m^2$), as shown in Figure 5.

The relevance of S-SIMS in combinatorial chemistry has been further demonstrated through the analysis of a library constituted by small organic molecules. Positive images of a mixture of six compounds from Table 2 (nos. **5**−**10**) were recorded according to the previously described methodology. Tropylium ions featuring the whole molecules (*m*/*z* values given in Table 2) were mapped in the positive mode. Four compounds over six were identified. Among all beads visualized through the acquisition of total ion images, some were exhibiting tropylium ion images, and others were not responding. For instance, an image displaying five beads loaded by the four detected molecules (nos. **5**−**8** in Table 2) is shown in Figure 6. The fact that the beads substituted

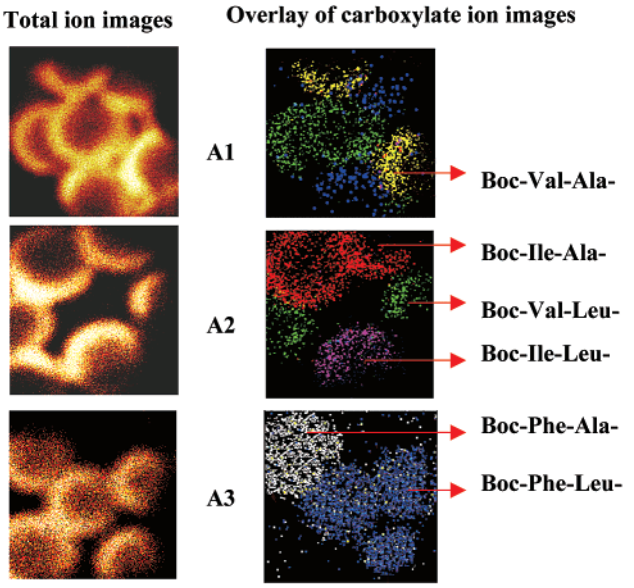


Figure 4. Polystyrene resin loaded at 0.93 mmol/g. Total ion images of three areas (A1, A2, A3). For each peptide, overlay of images of the three carboxylate ions defined in Figure 2. In each area, pixels were colored according to the detected sequence numbered in Figure 3 (1, white; 2, blue; 3, yellow; 4, green; 5, indigo; 6, red).

Table 3. Relative Abundances of the Tropylium Ion in Positive S-SIMS Spectra

	sample no. from Table 2					
	5	6	7	8	9	10
m/*z*	236	185	161	167	209	215
rel abundance (counts/10 min)	600	2500	9000	3500	120	120

by the compounds nos. **9** and **10** failed to be imaged was related to the low relative abundances of the expected tropylium ions. As a comparison, the relative abundances of the six targeted tropylium ions are gathered in Table 3.

An example of a bead seen in the total ion image but absent in all recorded tropylium images is shown in Figure 7. This nonimaged bead was necessarily related to one of the two missing compounds (no. **9** or **10**), so correlation between undetected molecules and nonidentified beads was achieved simply by acquiring a spectrum from a small surface belonging to one nonimaged bead (Figure 7). Such additional single bead analysis strategy obviously imparted some delay, as compared to a unique imaging experiment. Nevertheless, finding two compounds from few located positions in the deposit under study was very rapid and found to be more efficient than acquiring a complementary image in the negative mode, where the relative abundances of all detected ions were rather low.

Conclusion

Profiling a library by S-SIMS was more difficult than characterizing batch samples. Indeed, ions characterizing the whole anchored molecules were always detected, but their relative abundances were subjected to large variations in both positive and negative modes. Difficulties were encountered in acquiring good images of low-intensity signals. Mapping

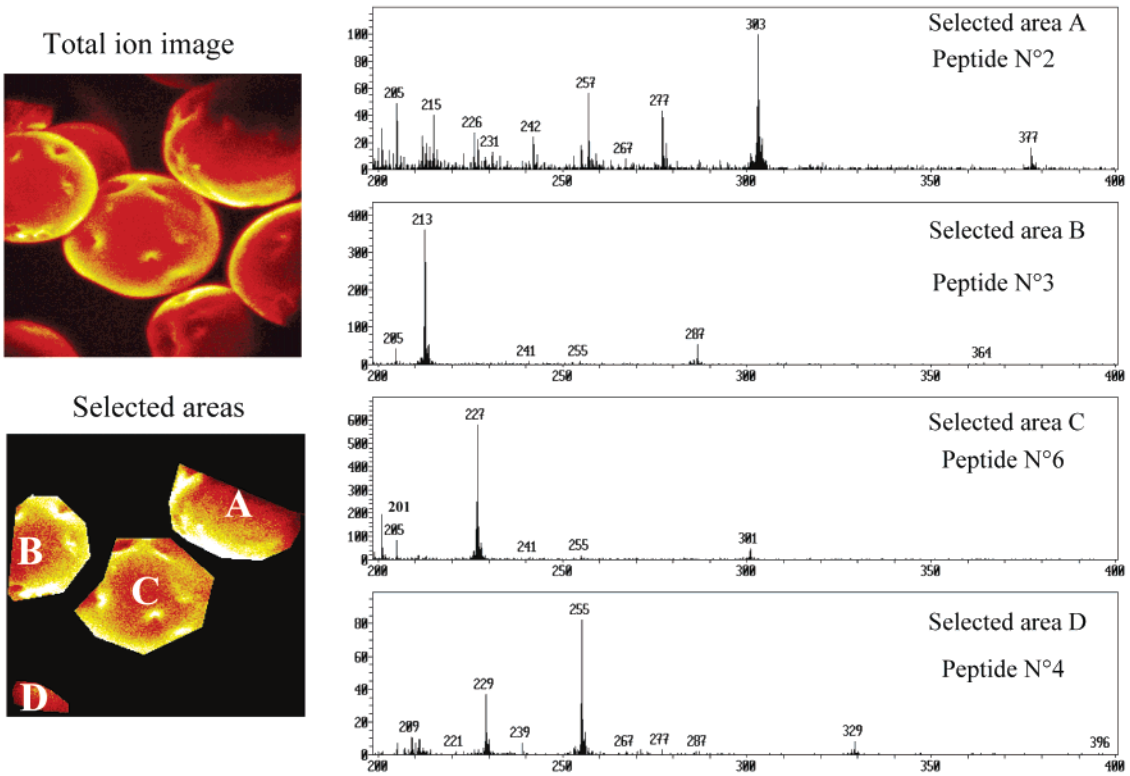


Figure 5. Polystyrene resin loaded at 2.8 mmol/g. Total ion image of six beads and four selected areas. S-SIMS spectra were recorded for each area in the negative mode to assess the sequence of the anchored peptides listed in Figure 3.

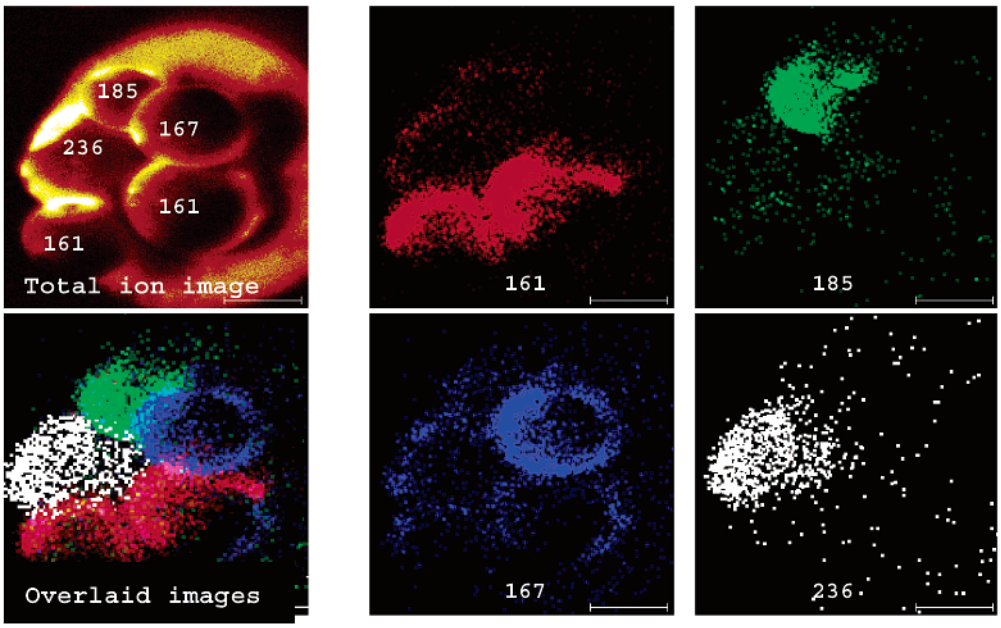


Figure 6. Randomly selected mixture of polystyrene resin beads loaded at 0.8 mmol/g by compounds nos. **5**−**10** listed in Table 2. Total ion image showing five beads and the corresponding overlaid images of four expected tropylium ions defined in Table 2 (*m*/*z* 236 in white, 185 in green, 161 in red, 167 in blue).

a sum of ions that were all related to the molecule of interest could be a solution to generate more intense images when possible. Otherwise, a two-step methodology was applied in which imaging experiments were completed by single bead analyses until all compounds have been identified.

The presence of a covalent bond in a molecule, which is selectively cleaved during the SIMS bombardment, is a prerequisite for supported compound identification. Ester and amide bonds, which are very popular linkages in supported chemistry, were broken in S-SIMS, whatever the nature of the anchored molecules and the insoluble support. Two competitive ionization mechanisms by either direct bond rupture or induced by proton transfer on a basic site produced several ions in both positive and negative modes. Mapping the molecular ion (or fragment ions easily related to the molecular ion or both), completed in some cases by a few

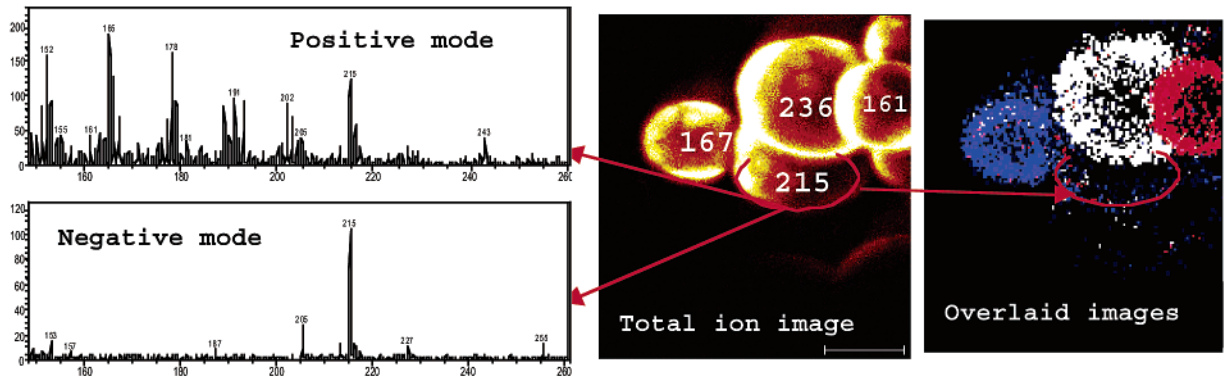


Figure 7. Randomly selected mixture of polystyrene resin beads loaded at 0.8 mmol/g by compounds nos. **5**−**10** listed in Table 2. Total ion image showing four beads and the corresponding overlaid images of all expected tropylium ions defined in Table 2 (*m*/*z* 236 in white, 185 in green (not seen in this area), 161 in red, 167 in blue, 209 not detected, 215 not detected). S-SIMS spectra were recorded in positive and negative modes from a small surface belonging to the bead circled in red that failed to be imaged.

single-bead analyses, allowed direct pooled support-bound peptide and organic molecule identification in a reliable, sensitive, and fairly rapid manner.

In addition, the control was effected down to the bead level. Thus, the sole identification of active beads from on-bead screening in which the compounds are still attached to the support could be envisaged as suppressing the time-consuming whole library profiling. Having decreased the number of samples to be analyzed, the overall analytical process throughput will be increased, even if the imaging S-SIMS protocol is rather slow as compared to standard automated ESI mass spectrometry analyses used in combinatorial technologies.

Experimental Section

Peptide Synthesis. Peptide syntheses were carried out on hydroxymethylpolystyrene resin functionalized at 0.93 mmol/g (Novabiochem, Meudon, France) or at 2.8 mmol/g (Advanced Chem Tech, Louisville, KY). Boc-protected amino acids were purchased from Senn Chemicals (Gentilly, France). The first Boc-protected amino acid was loaded onto the resin according to the symmetrical anhydride procedure. The Boc protection was subsequently released by a treatment of trifluoroacetic acid in dichloromethane. The second residue was coupled by 2 equiv of (benzotriazol-1-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate and diisopropylethylamine in dimethylformamide. All syntheses were checked prior to S-SIMS experiments by treating a few resin beads with HF to release the built sequences in solution. Identification of the peptides was effected by high performance liquid chromatography (HPLC) and electrospray mass spectrometry (ESI-MS).

Mass Spectrometry. S-SIMS measurements were performed on a TRIFT I spectrometer from the PHI-Evans Company (Eden, Prairie, MN) equipped with a time-of-flight analyzer. Charge compensation was achieved by a pulsing electron flood ($Ek = 20$ eV) at a rate of 1 electron pulse/5 ion pulses. The primary ion beam was rastered on 395 × 395 μm^2 for 30 min to generate a complete mass spectrum at each pixel. A chemical image was thus recorded. Each image was further refined by the Cadence 2.0 software, which generates the corresponding convolved image in which one pixel of the original image is replaced by the mean of the six neighboring ones. Raster type was the "scatter" type designed to be used for insulating samples: each pixel point was located as far from the previous and next pixel as possible so as to spread the primary beam charge homogeneously. Mass spectra were also obtained in an image from different selected areas by using simple drawing tools.

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