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Analysis of Combinatorial Libraries Using Electrospray Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

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Electrospray ionization coupled with Fourier transform ion cyclotron resonance (FTICR) mass spectrometry has been used to provide informtion about complete combinatorial libraries of small peptides containing 10^3–10^4 components. The fidelity of attempted synthesis steps can be ascertained rapidly, and, when the extremely high resolution FTICR mass spectra are combined with appropriate computer simulation, both diversity and degeneracy of the libraries as synthesized can be assessed.

Combinatorial library technology has facilitated rapid screening of diverse classes of bio-active compounds for drug discovery and development. It is further of great biological interest in the study of enzyme/substrate/inhibitor as well as receptor/agonist/antagonist interactions. Once limited to the natural pool and the chemical stockroom, new synthetic approaches generate large numbers of compounds bearing a high density of functionalities within short periods of time. Libraries of peptides,[1] peptoids,[2] oligonucleotides,[3] diazepines,[4,5] oligocarbamates,[6] and hydantoins[5] have already been described and new ones are constantly emerging.

Many different synthetic approaches may be involved in the production of combinatorial libraries. These include use of biological systems such as plasmids[7] or filamentous phage,[8–10] syntheses which generate compounds free in solution[2] and those which use solid supports,[11] including methods which generate one specific compound per bead.[12] Each method offers its own advantages and disadvantages. Biological syntheses have mainly concentrated on encoded libraries.[7–10] These methods do not always generate truly random libraries since genetic bias can play a role in the syntheses. However, they are capable of producing large libraries of the order of 10^{12} peptides. The production of solution-phase compounds is relatively easy, but the quantitative analysis of these libraries has presented considerable difficulties except for relatively simple compounds. In addition, free solution chemistry increases the possibility for error since coupling reactions are directed towards a group of compounds, and not just a single compound as in one-bead-one-structure libraries.[13]

As synthesis methods become more efficient, the need for new analytical techniques to characterize combinatorial libraries is heightened. Depending on the chemical nature of the library under consideration, current analytical methods of analysis include amino acid analysis,[14,15] Edman degradation,[16] ^{13}C NMR[17] and conventional mass spectrometric methods.[18–23] These techniques can, in an extremely limited fashion, help determine whether the functionality of the intended library was created (diversity) and also the degree to which each individual compound was successfully made (degeneracy). As the complexity of combinatorial libraries increases it becomes more difficult to isolate or characterize individual components. Without proper analytical methods, considerable time and effort can be expended on experiments involving incorrect or incomplete libraries.

In this paper, we will demonstrate the utility of Fourier transform ion cyclotron resonance (FTICR) mass spectrometry for analysis of selected small-peptide combinatorial libraries containing 10^2 to 10^4 individual components. The FTICR technique[24,25] offers many advantages over conventional mass spectrometric methods. These include ultra-high mass resolution, high mass accuracy, multi-stage tandem mass spectrometry (MS^n) and the ability to trap ions for extended periods of time. In particular, high-mass-resolution spectra obtained by FTICR mass spectrometry can reveal details about libraries that would otherwise be obscured in data from lower resolution mass spectrometric techniques. Furthermore, the FTICR technique can be combined with collisionally activated dissociation (CAD)[26,27] or infrared multiphoton dissociation (IRMPD)[28] of isolated species to yield a range of information about the structures of individual components.

EXPERIMENTAL

Preliminary experiments were performed at the University of Florida on a Bruker 47e external-source FTICR mass spectrometer (Bruker Instruments Inc., Billerica, MA, USA) equipped with a shielded, horizontal, room-temperature, 15 cm i.d., 4.7 T superconducting magnet (Magnex Scientific Ltd., Abingdon, UK). Ions produced external to the magnet were guided into the 170 mm^3 cylindrical RF-shimmed Infinity™ analyzer cell[29] using electrostatic ion optics. The standard external-source FTICR instrument has been previously described[30] and only relevant details are discussed further. The FTICR mass spectrometer was equipped with an external electrospray source which utilizes a hexapole ion guide (Analytica of Branford, Branford, CT, USA). The ESI source was modified in our laboratory to use a heated metal capillary similar in design to that reported by Kebarle,[31] instead of a quartz capillary and counter-current

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drying gas.

The Bruker FTICR external-source design has two stages of differential pumping to achieve a factor of 10^4 pressure reduction between the source and the analyzer regions. The mass spectrometer was pumped by a series of cryopumps: an 800 L/s cryopump (nitrogen) pumped the FTMS external source housing, a 400 L/s cryopump evacuated the regions between the first and second conductance limits that contain the electrostatic transfer optics, and an additional 400 L/s cryopump was used to maintain high vacuum in the analyzer region. After exiting the ESI source, ions were accelerated by a 3 kV potential difference and focused through two conductance limits before being decelerated, accumulated and trapped in the FTICR analyzer cell.

A dual-stage ESI source equipped with one 500 L/min mechanical pump on the first stage and a turbo-drag pump on the second stage was used to reduce the pressure through the ESI source from atmospheric pressure to *ca.* 2×10^{-3} mbar. Desolvation of the charged droplets occurred in a metal capillary heated to 120 °C and biased at *ca.* 80 V relative to ground. The stainless steel capillary was 500 μm i.d. × 170 mm long with a capillary–needle distance of *ca.* 5 mm. The pressure after the hexapole conductance limit, in the normal FTICR external source housing, was 3.6×10^{-6} mbar when pumped by a 800 L/s cryopump.

Subsequent experiments were carried out at the National High Magnetic Field Laboratory (NHMFL) in Tallahassee, Florida, using an FTICR mass spectrometer equipped with a shielded 9.4 T Oxford magnet and an external electrospray source.[32]

Sample synthesis and preparation

H-Xxx-Xxx-Lys-OH. These peptide libraries were synthesized on 9-fluorenylmethyloxycarbonyl(Fmoc)-Lys-butyloxycarbonyl(Boc)-Wang-resin by 2-(1-H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/N-hydroxybenzotriazole (HOBT)/diisopropylethylamine (DIEA) activation in dimethylformamide (DMF). A penta-coupling procedure with one equivalent of *in situ* activated Fmoc-amino acid mixture was performed for each cycle. The amino acid side chains were protected as follows: Fmoc-Gly-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Met-OH, Fmoc-Pro-OH, Fmoc-Phe-OH, Fmoc-Val-OH, Fmoc-Arg-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl(Pbf)-OH, Fmoc-Asp-t-butyl ester(OtBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-His-trityl-(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Trp(Boc)-OH. Cysteine was excluded. The Fmoc-deprotection was performed with 25% piperidine/DMF in 15 min. The peptide library was cleaved and deprotected in trifluoroacetic acid (TFA)/thioanisole/ethanedithiol/H_2O/triisopropylsilane 90/2.5/2.5/2.5/2.5 for 3 h. Precipitation with diethyl ether yielded the crude library.

H-Xxx-Xxx-Xxx-Xxx-pTyr-Cys-OH. These peptide libraries were synthesized on Boc-Cys-acetamidomethyl(Acm)-oxymethyl-phenylacetaminomethyl-resin by HBTU/HOBT/DIEA activation in DMF. After removal of the Boc-protecting group with 50% TFA/CH_2Cl_2 for 15 min, the amino acids were coupled by a double coupling procedure with 2.5 equivalents Fmoc-amino acid each. Fmoc-Tyr-OPO_3H_2-OH was introduced without side chain protection. The amino acid side chains were protected as follows: Fmoc-Gly-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Met-OH, Fmoc-Pro-OH, Fmoc-Phe-OH, Fmoc-Val-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-His(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Lys(Boc)-OH. Cysteine and tryptophan were excluded. The Fmoc-deprotection was performed with 25% piperidine/DMF in 15 min. The peptide library was deprotected on the resin in TFA/thioanisole/ethanedithiol/H_2O/triisopropylsilane 90/2.5/2.5/2.5/2.5. The Acm-cleavage was performed with 1.2 equiv. $Tl(OAc)_3$ at 0 °C for 80 min. After overnight reduction of formed disulfide bonds with excess ethanedithiol the unprotected peptide was cleaved with HF at 0 °C for 1 h.

H-Gly-pTyr-Xxx-Xxx-Xxx-Cys-OH. Synthesis was done using the Fmoc-Cys(Trt)-Wang-resin approach, without Trp and Cys according to the procedure used for the H-Xxx-Xxx-Lys-OH library discussed above.

Solutions of the amino acid combinatorial libraries were prepared by dissolving 0.2 mg of library peptides in 1 mL of 49/49/2 water+methanol+acetic acid (v:v:v). Concentrations of individual components were estimated to be in the range of 40 to 1000 femtomol/μL. Samples were introduced into the ESI source at a flow rate of 1 μL/s using a syringe pump and a high-performance liquid chromatographic (HPLC) injection valve with a 20 μL loop.

Computer simulation of combinatorial library mass spectra

Several computer programs have been developed to simulate FTICR mass spectra of combinatorial libraries, thus allowing information to be extracted about the degeneracy and diversity of the libraries. For a library of length x, the molecular formulae for all of the possible n^x (n=the number of possible amino acids at each of the x positions) individual components were calculated. The isotopic pattern for each molecular ion was calculated based on the algorithm developed by Rockwood *et al.*[33] The masses and relative intensities for the naturally occurring isotopic peaks for each component above a threshold of 0.05% of the calculated base-peak intensity were used to generate a time-domain transient. This was accomplished by summing a series of sine functions where the amplitude factors and frequencies were assigned as the relative isotopic intensities and the corresponding cyclotron frequencies, respectively. Equal concentrations for each component in the library were assumed. The simulated transient was apodized and zero-filled prior to Fourier transformation to mimic, as closely as possible, the conditions used to acquire actual mass spectra.

RESULTS AND DISCUSSION

In principle, mass spectrometry should be an ideal technique for monitoring combinatorial library syntheses, especially in confirming that the expected diversity and degeneracy are present. One important library feature that mass spectrometry can verify is whether the expected synthesis proceeded to completion. Specifically, mass

spectrometry can provide a rapid check of changes (desired or otherwise) produced by new steps in the synthetic route, cross reactions occurring between compounds in the library, problems occurring with modified amino acids (e.g. loss of a phosphoryl group), the presence of various blocking or terminal groups or differing purity of chemicals from various vendors. For example, the electrospray ionization (ESI) FTICR mass spectrum obtained for the peptide library assumed to be H-Gly-pTyr-Xxx-Xxx-Xxx-cys-OH (pTyr-=phosphorylated tyrosine, Xxx=all amino acids excluding cysteine and tryptophan) and a simulated spectrum for this library are shown in Fig. 1(a) and (b), respectively. A comparison of these two spectra indicates gross disagreement between the measured and expected peak distribution, suggesting problems with the synthesis. Upon examination it was determined that no coupling with pTyr had occurred. The experimental spectrum more closely resembles the simulated mass spectrum for H-Xxx-Xxx-Xxx-Cys-OH shown in Fig. 1(c). While several traditional techniques could have detected this problem, ESI-FTICR provided a quick, convenient, and unambiguous analysis.

The FTICR technique offers the advantage that moderate to high resolution and high mass accuracy are readily obtained, allowing the degeneracy of combinatorial libraries to be studied in detail. An expanded portion from a moderately high resolution ($m/\Delta m_{1/2}$=20 000) mass spectrum obtained on the peptide library H-Xxx-Xxx-Lys-OH (Xxx=all amino acids excluding cysteine) and the corresponding portion of the simulated spectrum are shown in Fig. 2(a) and (b), respectively. The expanded portion shown in Fig. 2(a) is from a mass spectrum acquired in broadband mode covering a range of m/z 188 to 10 000 (384 kHz bandwidth, 4.7 T, 170 ms acquisition time).

Detailed comparison of mass spectra and simulated

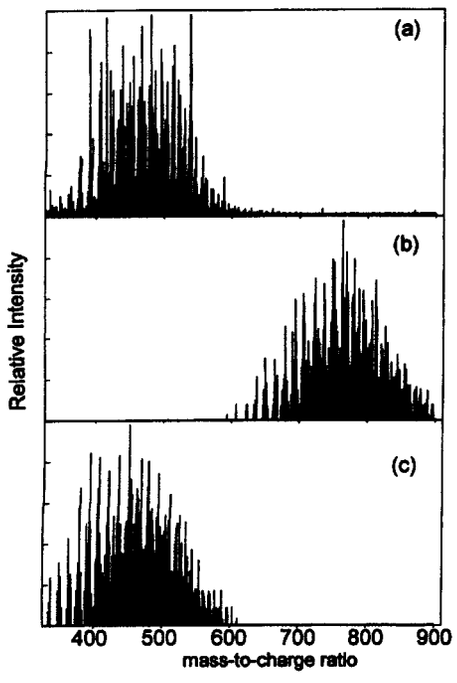

Figure 1. (a) ESI-FTICR broadband mass spectrum of a library thought to be H-Gly-pTyr-Xxx-Xxx-Xxx-Cys-OH where Xxx can be any one of the naturally-occurring amino acids with the exception of Cys and Trp. (b) A simulated mass spectrum of H-Gly-pTyr-Xxx-Xxx-Xxx-Cys-OH. (c) A computer simulated spectrum of library H-Xxx-Xxx-Xxx-Cys-OH where Xxx can be any one of the naturally occurring amino acids with the exception of Cys and Trp.

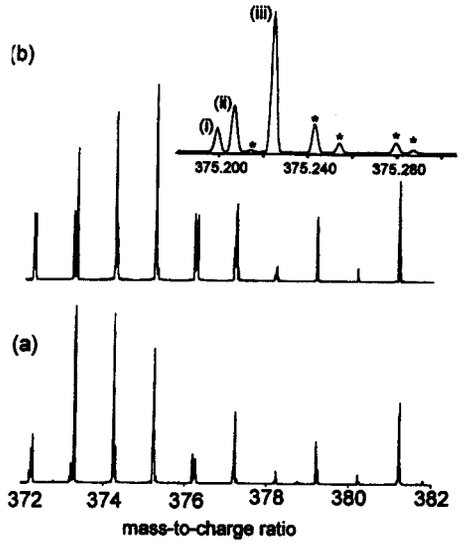

Figure 2. (a) An expanded region of the ESI-FTICR mass spectrum of the library H-Xxx-Xxx-Lys-OH where Xxx can be any one of the naturally-occurring amino acids with the exception of Cys. (b) A simulated spectrum for the same mass range as in (a) of the library H-Xxx-Xxx-Lys-OH. The inset shows a simulation with higher resolution (130 000 FWHM) of the peak at m/z 375.2 (see discussion in text).

results can reveal cases where discrimination during synthesis leads to incomplete libraries. Even at this relatively low resolution, by FTICR standards, an extremely detailed analysis of the library is possible. Consider the obvious triplet of nominal isobars observed at m/z 376 in Fig. 2. From the simulation, these peaks are assigned an Asn-Asp-Lys (m/z 376.183, redundancy=2), Thr-Gln-Lys (m/z 376.220, redundancy=2) and Thr-Lys-Lys (m/z 376.256, redundancy=2), respectively, and are observed to be in roughly equal abundance, as predicted.

Careful inspection of spectra simulated with very high mass resolution reveals that almost every m/z value in the fairly narrow m/z window (m/z 372 to 382) displayed in Fig. 2 contains multiple peaks. This is not apparent from either the actual or simulated data shown, due to overlap of very closely spaced peaks (not resolved at the lower resolution of the experiment and of the simulation, which was carried out at a resolution comparable to that of the experiment) and some loss of detail due to the compression algorithm used to plot the data. For example, consider the inset in Fig. 2(b), showing an expansion around m/z 375 for the simulated spectrum at a resolution of 130 000 full width at half maximum (FWHM) that is higher by a factor of 6.5 than that of the wider mass ranges shown in Figs. 2(a) and (b). The peaks labeled i, ii, and iii are due to four peptides, Asn-Asn-Lys (m/z 375.1992, redundancy=1), Met-Pro-Lys (m/z 375.2066, redundancy=2), Leu/Ile-Asp-Lys (m/z 375.2243, redundancy=4) and Val-Glu-Lys (m/z 375.2243, redundancy=2). The other peaks marked with a star are due to contributions from the naturally-occurring heavier minor isotopes of ions with lower m/z values. The ability to resolve such closely spaced peaks was hampered somewhat in our experiments by a lack of digital resolution (384 kHz bandwidth/128k data points) in the broadband detection mode. Obviously, this situation could be improved by working with smaller bandwidths (corresponding to narrower m/z ranges) or by digitizing more data points (assuming the time-domain transient signal is not pressure damped during the detection event).

As libraries become larger and more diverse, it will be

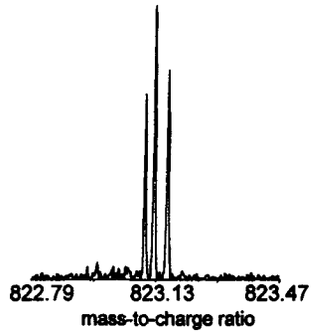

Figure 3. A high resolution spectrum obtained at 9.4 T showing a small region from a H-Xxx-Xxx-Xxx-Xxx-pTyr-Cys-OH library (Cys and Trp excluded) that exhibits baseline resolution between peaks of the same nominal mass.

more and more difficult to separate individual peaks with the same nominal mass-to-charge ratio. However, with the high mass resolution afforded by the FTICR technique it is possible to mass resolve individual isobaric species of similar nominal mass. As the field strength of the magnet increases, the mass resolution increases linearly. The high resolution ($m\Delta m_{1/2}$=120 000) mass spectrum for one nominal mass peak from a H-Asp-Xxx-Xxx-Xxx-Xxx-pTyr-Cys-OH library shown in Fig. 3 was obtained using an FTICR mass spectrometer based on a 9.4 T magnet at the NHMFL.[32] This mass spectrum was obtained in heterodyne mode, covering a range of 750 to 950 m/z, with a 3s acquisition time. The time-domain transient (128k data points) was apodized and zero-filled once prior to Fourier transformation and magnitude calculation. An increase from 4.7 to 9.4 T provided twice the mass resolving power, as expected for non-pressure-limited measurements.

Even though high mass accuracy is important for the determination of amino acid composition, it alone cannot offer complete insight into the sequence of the peptides under investigation. However, at higher magnetic field strength, CAD approaches for obtaining information about the structures of individual components are also facilitated, since the maximum kinetic energy imparted to an ion prior to collision with a target gas molecule increases as the square of the magnetic field strength.

CONCLUSIONS

The primary shortcoming of conventional combinatorial chemistry is that the degeneracy and the diversity are rarely, if ever, truly tested in libraries larger than *ca.* 100 compounds. Rather, the degeneracy and diversity of the libraries are inferred from the route by which these libraries are synthesized, assuming that all of the synthetic chemistry worked as anticipated. While this assumption is often valid for peptide synthesis, it is rarely valid for other syntheses, especially on solid supports. This report shows that the ESI-FTICR technique is capable of producing semi-quantitative analytical data for libraries of 10^4 molecules. Comparison of the mass spectra with those predicted by simulations can show if there were problems during synthesis and whether ESI-FTICR is inherently biased against certain compounds.

Additional studies of these combinatorial libraries are currently underway in our laboratory including further assessment of library purity, measurement of relative binding rates with receptor molecules, and MS^n of target molecules to identify those with the highest binding efficiency.

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