NPR

HIGHLIGHT

NP/MS since 1970: from the basement to the bench top

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This *Highlight* explores the evolution of applications of mass spectrometric technologies in the context of natural products research since the 1970's. The central themes are the analysis of mixtures, dereplication (identification) and structure determination. The ascension of HPLC as the method of choice for the analysis of pharmaceuticals was a driving force for the development of interfaces for coupling of HPLC and MS. An example of sequential analysis of fragment ions or MS/MS or MS^n methods to provide detailed structural information on muraymycins, a family of uridyl-peptide antibiotics, is presented.

1 Introduction

Mass spectrometry (MS) has had a central role in the advancement of natural products (NP) research over the last 40 years. MS has provided a starting point for the identification or structure determination of the vast majority of NPs – the molecular weight. In the 1970's MS was still very much a specialist's technique requiring careful attention to experimental parameters and knowledge of vacuum systems and electronics. No place for your typical natural products chemist. However, that situation was about to change in dramatic fashion. From the high-voltage–high-vacuum world of diffusion pumps, massive electromagnets, 70 eV electron impact ionization and McLafferty's fragmentation rules[1] – MS has emerged from the mass spectrometrist's basement lab to become a hands-on technology whose applications span the range encompassed by NP chemistry and well beyond. Perhaps the most stunning aspect of the evolution of the technology is the breadth of applications amenable to study by MS, many of which are covered in this special issue of Natural Product Reports.

In the early 1970's, the predominant method used to analyze natural products was electron-impact ionization (EI) under high vacuum conditions. Samples were introduced by direct insertion into the electron beam *via* heated glass capillaries bearing solid samples. Volatility and thermal stability were the major limiting factors, restricting the molecular weight and polarity of compounds that could be successfully ionized. For mixture analysis, directly coupled gas chromatography and MS (GC-MS) was a powerful technique but is less broadly applicable owing to more severe requirements for volatility. GC/EI-MS remains unsurpassed in its power for chromatographic resolution, sensitivity and information content from MS fragmentation. The state-of-the-art in the early 1970's is revealed in a series of books by Budzikiewicz, Djerassi and Williams that codified MS fragmentations of all classes of organic compounds and several NP classes.[2]

A benchmark of where the field of NP/MS was in the early 1970's is found in the work of Rinehart and Pandey on the structures of polyene antibiotics. Structural studies on chainin, a pentaene antibiotic are depicted in Fig. 1.[3] For the determination of molecular formula, chainin (**1**) was hydrogenated to yield the decahydro-derivative (**2**). In order to observe molecular ions, each was per-acetylated yielding octa-acetates (**3** $M^{+\bullet} = m/z$ 946) and (**4** $M^{+\bullet} = m/z$ 956), respectively. High resolution EIMS yielded a sufficiently accurate value for **4** that its formula could

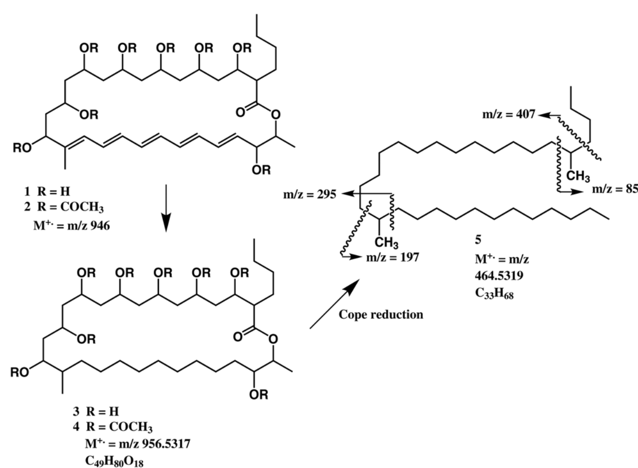

Fig. 1 Electron-impact mass spectrometric characterization of chainin (**1**) and derivatives for determination of molecular formula and carbon skeleton.

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be predicted as $C_{49}H_{80}O_{18}$. EIMS of both **3** and **4** showed ions corresponding to losses for one to eight acetic acid units. Therefore the molecular weight of chainin was determined to be 610 amu corresponding to a formula of $C_{33}H_{54}O_{10}$. The carbon skeleton of chainin was also established by EIMS. Cope reduction yielded the hydrocarbon derivative **5** ($M^{+\bullet} = m/z$ 464.5319) corresponding to $C_{33}H_{68}$. Branching was established through the EIMS fragmentation behavior of **5** that showed pairs of abundant ions at *m*/*z* 85, 407 and *m*/*z* 197/205 indicative of the one-carbon branches at 5 and 19. The completed structure required additional chemical degradation experiments and spectroscopic analysis and an abundance of chemical intuition.

In the ensuing four decades the drive to expand the role of MS in NP research was predicated upon the development of more general ionization methods that were also less energetic so as to minimize decomposition of the sample. Concomitantly interfacing high-pressure (now performance) liquid chromatography (HPLC), the new standard for analytical separation and quantification, became a priority. Selected accounts of these developments from a natural products chemist's perspective will be highlighted in the following sections, as will key developments in MS for structure determination.

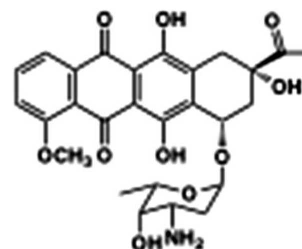
2 "Soft" ionization for the masses

Although chemical ionization (CI) methods were selectively employed in NP work, they were quite finicky, requiring optimization for each new sample type. The major limitation of CI for complex NPs was that the sample still had to be sufficiently volatile to be vaporized in the MS source. Desorption chemical ionization (DCI) somewhat mitigated the need to use high temperatures and was successfully applied for the analysis of antibiotics, such as anthracyclines (*e.g.* daunomycin).[4] In a 1985 review of MS applications in antibiotic research covering 1977–1984, Borders *et al.* asserted:

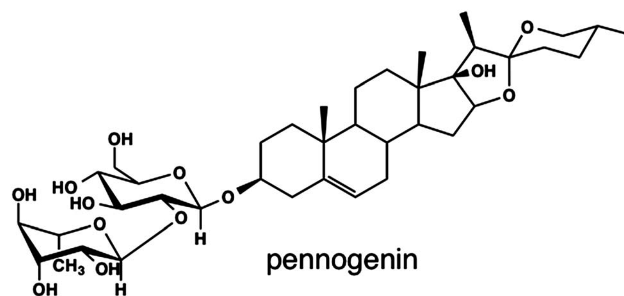
The last several years have seen the proliferation of a number of "soft" ionization methods – those which produce predominantly molecular ions or conjugated molecular ions of the type $(M + H)^+$ or $(M + metal\ ion)^+$. In some of these newer methods of ionization, the sample is ionized directly from the solid phase, thereby circumventing a major problem with EIMS, the need to volatilize the sample without causing extensive degradation or the necessity for chemical derivatization.[5]

Guy Carter obtained a bachelors degree in Chemistry at Lehigh University and a Ph.D. in Biochemistry from the University of Wisconsin. He then pursued marine natural products in the laboratory of the late Kenneth Rinehart at the University of Illinois, before beginning a career in the pharmaceutical industry in 1978. Most of these years were spent in Pearl River, NY – first with Lederle Laboratories and continuing with Wyeth Research where he headed the natural products discovery program. In 2010 he began a new career as a private consultant to advance and promote natural products R & D. He currently is the Chief Scientific Officer of Biosortia Pharmaceuticals.

At the time of the Borders review the two most promising new ionization methods for non-volatile NPs were field desorption (FD) and fast atom bombardment (FAB).



FD-MS was a useful technique that extended the range of metabolites that could be effectively analyzed by MS without chemical derivatization.[6] Technically challenging, owing to the need to create "emitters" from which compounds were desorbed at high fields, this method was practiced by only a few NP researchers. This requirement for special expertise was an example of how an MS technology was limited to "gurus" with dedicated practitioners. Prior to the widespread availability of FAB sources, however, FD-MS yielded results that enabled the study of such difficult compounds as saponins (*e.g.* pennogenin)[7] and several classes of antibiotics, including peptides and polyene macrolides (*e.g.* chainin (**1**)).[8,9]



FAB-MS made a big splash at the American Society for Mass Spectrometry Annual Conference in Minneapolis in 1981. This was the same year that the seminal paper by Barber *et al.* highlighted the utility of the method, including the successful analysis of several classes of NPs.[10] FAB-MS was widely used throughout the 1980's, leading to the publication of high-profile papers that touted its ability to analyze highly polar non-volatile compounds.[11–13] FAB-MS received more widespread usage from the NP community owing to its relative ease of operation. The study of vancomycin and related glycopeptide antibiotics was greatly facilitated by FAB-MS analysis, which was uniquely able to provide reliable values for their molecular ions.[14–16]

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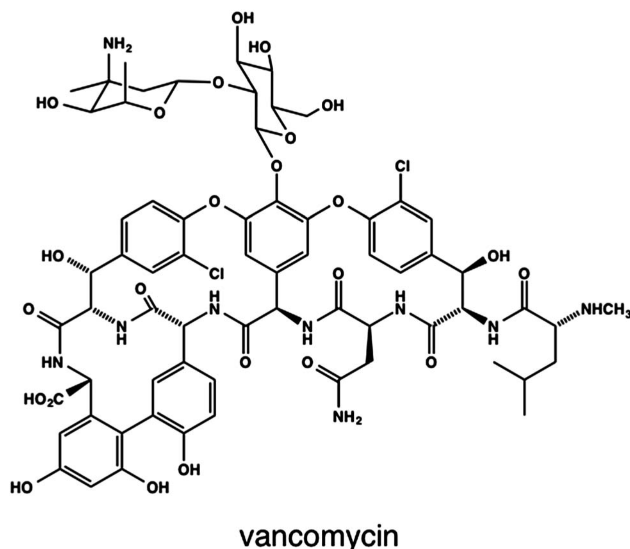

Laser desorption methods were also under development in the 1980's as an alternate to way create ions for MS analysis without the need for significant volatility. These techniques eventually evolved into MALDI (matrix-assisted laser desorption ionization), which has become a major tool for the investigation of natural products *in situ*.[17] Although all of these new ionization methods provided some advantages, none had as significant an impact on the fundamental analytical chemistry of NPs as the "spray" techniques that enabled the coupling of HPLC with MS.

3 Coupling HPLC with MS: marriage in a vacuum

As HPLC gradually replaced GC as the method of choice for pharmaceutical analysis and was becoming a routine tool in NP chemistry labs, there was a great race to couple the chromatography with MS analysis. As was the case in the development of soft ionization techniques, there were numerous approaches aimed at achieving this goal. The challenges of introducing the effluent from HPLC at about 1 mL min^{-1} into an MS ion source under high vacuum were indeed quite daunting.[18] Of the first commercially available LC/MS instruments, those employing a thermospray (TSP) interface between the LC and MS source were the most user-friendly.[19] Natural products chemists embraced TSP-MS and the technique was quite widely employed.[20] A serious limitation to the TSP technique for NP research was its variable ionization efficiencies for different classes of compounds, which limited its utility for the discovery of new metabolites.[21] Lagging a bit behind the commercial development of TSP, electrospray ionization (ESI) and its applications to biomolecules – including NPs - were being developed in the Fenn laboratory at Yale.[22,23] Fenn shared the 2002 Nobel Prize in Chemistry for his contribution to the development of ESI, and his Nobel lecture "Electrospray Wings for Molecular Elephants" contains a delightful account of this saga.[24]

3.1 LC/ESI-MS and natural products

The utility of LC/ESI-MS for NP research quickly became abundantly clear and the technique was essentially universally adopted. The generality and robustness of the ESI interface made this a logical choice for non-experts. Continuous improvements in instrumental design and data handling capabilities in this highly competitive market have created a range of excellent products – the majority of which are suitable for use by NP chemists. Without overstating the obvious, LC/MS is now an essential tool in NP research.

Dereplication, the process of identifying known NPs, was enormously facilitated by LC/MS because of the molecular weight information that is generated. Identification of known NPs by combination of molecular weight plus some secondary information, such as UV/vis absorption spectra, was highly effective and limited only by the scope of the available databases. A schematic diagram of the LC/ESI-MS dereplication system used at Wyeth (Fig. 2) is typical of systems implemented by NP groups around the world. This simple system correlated the retention time on LC with biological activity, UV/vis spectrum and mass spectra. The crucial link between biological activity and active principle was made by correlating the zones of activity, in wells of a 96-well plate, with retention time. Abstracting the corresponding UV/vis data from the photodiode-array (PDA) detector and the molecular weight data from the MS data system provided the information required to search for the compound's identity. The results represented a great leap forward in speed for NP identification. There have been numerous enhancements to this dereplication system, including higher resolution chromatography, high resolution MS as well as statistical analysis of whole metabolomes.[25]

4 MS for structure determination

The study of MS fragmentation processes for NP structure determination was of much greater significance during the 1970's and 1980's, before the full impact of 2-D NMR was realized. Often, complete EI-MS fragmentation patterns of complex NPs were only compiled once the full structure of the compound had been determined by chemical degradation, NMR and other spectroscopic methods or through X-ray crystallography. Nonetheless, the knowledge of fragmentation behavior in a structural class provided a shortcut to the structures of related compounds. An excellent example of the detailed analysis of EIMS fragmentation data was reported by Albers-Schonberg *et al.*, from Merck Research Laboratories for the antiparasitic agent avermectin.[26] Avermectin B2a produces the structurally diagnostic fragments designated a–d in Fig. 3. Each of these fragment ions in essence represents a degradation product of the parent macrolide. Fragment a is the aglycone portion (minus H_2O) and the disaccharide ion (not shown) was also observed. Ions b, c and d arise through elimination of the ester group and various carbon–carbon bond cleavages. Changes in the substituents on the macrolide ring would be observed in a ions and could be attributed to the appropriate regions of the

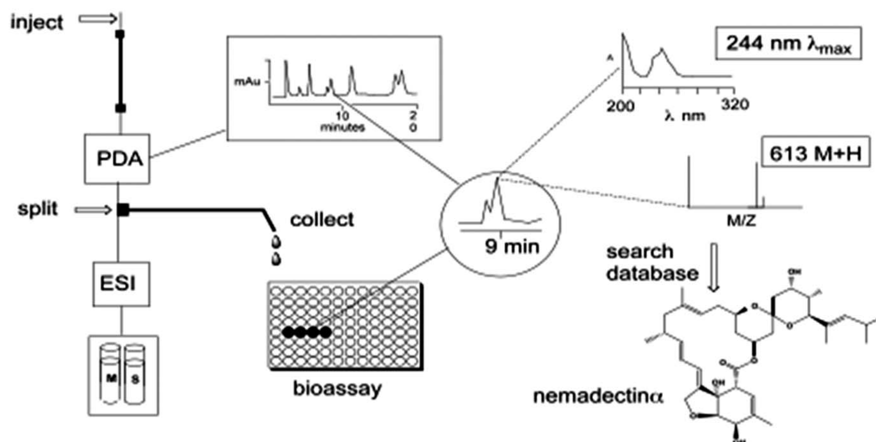

Fig. 2 HPLC/PDA/MS dereplication system for the correlation of biological activity with components of interest.

ring based upon corresponding shifts in the *m*/*z* values for b, c and d.

The information contained in this well-defined set of ions enabled structural characterization of related NPs as well as providing a highly valuable tool for the characterization of synthetic analogs, by-products and mammalian metabolites.

Perhaps the most daunting NP-MS problem that we encountered during my tenure at Lederle/Wyeth was the analysis of antibiotic LL-C19004, also known as saccharomicin. Saccharomicin represents a unique structural class of antibiotics and is composed of seventeen glycosidic residues linked to a substituted cinnamoyl chromophore. Early attempts were made to establish the molecular weight and formula for saccharomicins A and B, however, none – including FAB-MS – were totally successful. The heroic structure determination of the antibiotics was eventually solved by traditional methods of degradation and detailed spectroscopic analysis by Fangming Kong.[27] Shortly after the completion of this work we collaborated with the Marshall group at the National Magnet Lab on an FTICR-MS analysis of saccharomicins.[28] The results were so striking, including unambiguous molecular ion measurements and nearly complete sequencing of the monosaccharide units, that Wyeth purchased a 9.4 T FTMS unit, the first of its scale in an industrial setting.

MS/MS methods whereby ions are sequentially fragmented, daughter ions captured and then re-fragmented represent a powerful tool for structure determination. Defining the structures of the units lost in each step of the fragmentation enables the reconstruction of the parent molecule. This is often feasible since the lost units are typically simpler moieties (*e.g.* sugars, amino acids, fatty acids) relative to the parent compound. MS/MS methods have also evolved dramatically over the years. Before the advent of multi-analyzer instruments evidence for transitions from one ion to another were supported by the analysis of metastable ions. One method for these studies was *via* a technique known as "defocused analysis of daughter ions" (DADI).[29] Tandem MS provided the required capability of capturing daughter ions, inducing further fragmentation and analysis, which enabled fragmentation pathways to be established.[30] The culmination of this type of analysis is the ability to

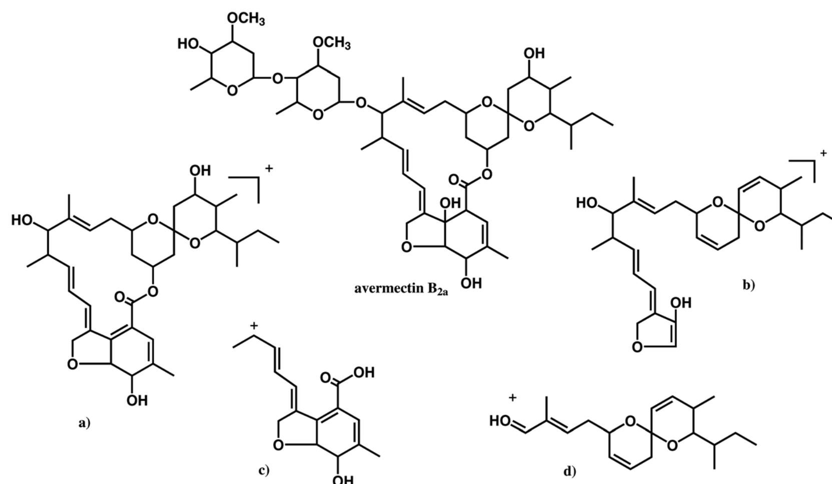

Fig. 3 Electron-impact induced fragmentation of avermectin B2a.

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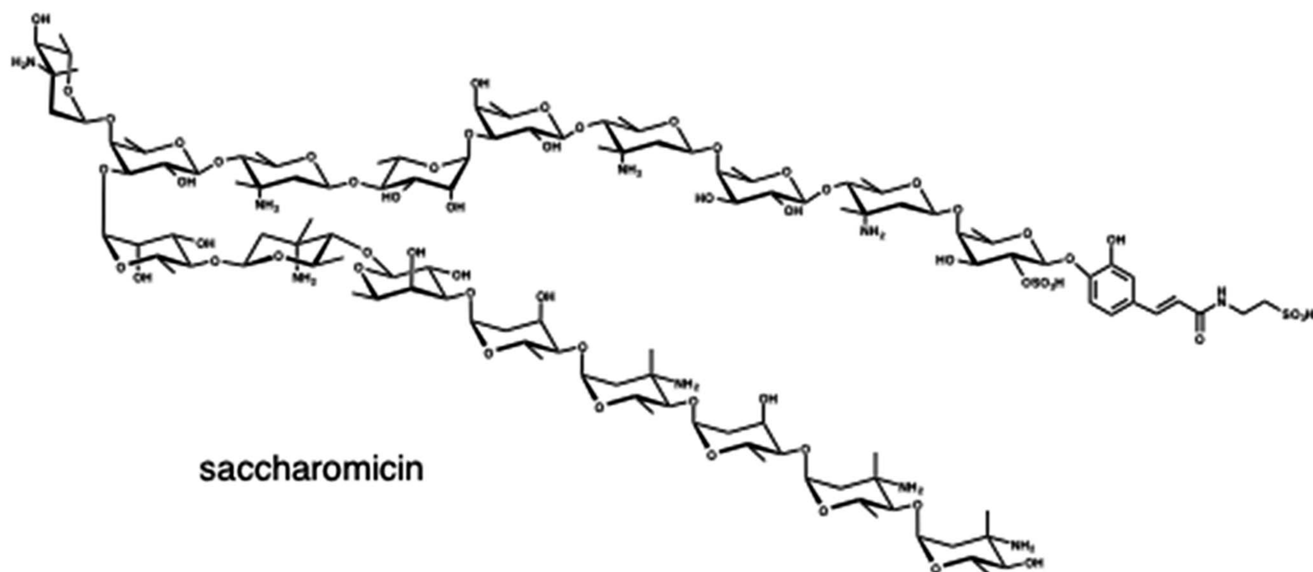

conduct MS/MS experiments sequentially over several cycles of fragmentation and capture – all done with high-resolution accurate mass measurement.

An example of the power of a high-resolution (HR) MS/MS experiment for NP characterization is the FTICR-HRMS/MS of the antibiotic muraymycin.[31] This work demonstrated two fundamental aspects required for structure determination of a new NP: determination of the molecular formula and connectivity of the constituent subunits – essentially the planar structure. Muraymycin is a uridyl peptide antibiotic that contains an aminoribose moiety, fatty acyl substituent and a unique peptidic portion (Fig. 4). Owing to its relatively high molecular weight (900–1100 amu), narrowing down the number of possible molecular formulas – even with HRFT-MS data – was challenging. By sequentially fragmenting molecular and daughter ions over the course of five MS/MS cycles (MS^5) and calculating accurate mass values for each neutral loss, an unambiguous molecular formula was reconstructed by summing the individual compositions, as shown in Fig. 4.

The sub-structural fragments of muraymycins were largely determined by NMR analysis and their linkage also proposed *via* 2D NMR experiments.[32] The MS/MS data helped to confirm this structure and enabled structure proposals for more than a dozen congeners by LC/MS/MS.

As illustrated for muraymycin, HRMS has typically been the method of choice for determining molecular formulas. In the days prior to the development of soft ionization methods, the challenge was often the low abundance of the molecular ion.

This limitation has been greatly reduced with soft ionization, such as ESI, and HR mass measurements are possible for daughter ions in MS/MS experiments.

With current time of flight mass analyzers HRMS data are routinely obtainable with sufficient accuracy to assign molecular formulas for NPs of moderate complexity. Furthermore, the ongoing development of ultra-high resolution mass spectrometers, such as the 15 T FTICR-MS, makes the unambiguous assignment of molecular formulas for NP over 1000 amu feasible by precise evaluation of the isotopic patterns of molecular ions.

5 Perspective

Recently the scientific community marked 100 years of mass spectrometry with articles and symposia celebrating Sir J. J. Thompson's book "*Rays of Positive Electricity and Their Application to Chemical Analyses*", published in 1913.

The last four decades of that century have witnessed the transformation of MS technologies to the extent that essentially every class of natural product, no matter how polar, non-volatile or massive, is now amenable to analysis – without the need for chemical modification. For the most part the original mission of NP/MS, the determination of molecular weight, prediction of molecular formula and structure analysis has been advanced. The argument can be made, however, that we have lost some of the insights derived from the high-energy fragmentation induced in EI-MS. EI-MS fragmentation patterns, which are so highly predictive of structure that they can be used to map out carbon skeletons, as in the case of the avermectins, are not duplicated by softer ionization methods. Of course now we have 2D NMR and MS/MS – albeit with less energetic fragmentation, which in most cases cover the deficit.

It is quite remarkable how mass spectrometers have made the transition from the basement lab to the bench-top. The new generation of MS detectors for HPLC is a perfect example of how

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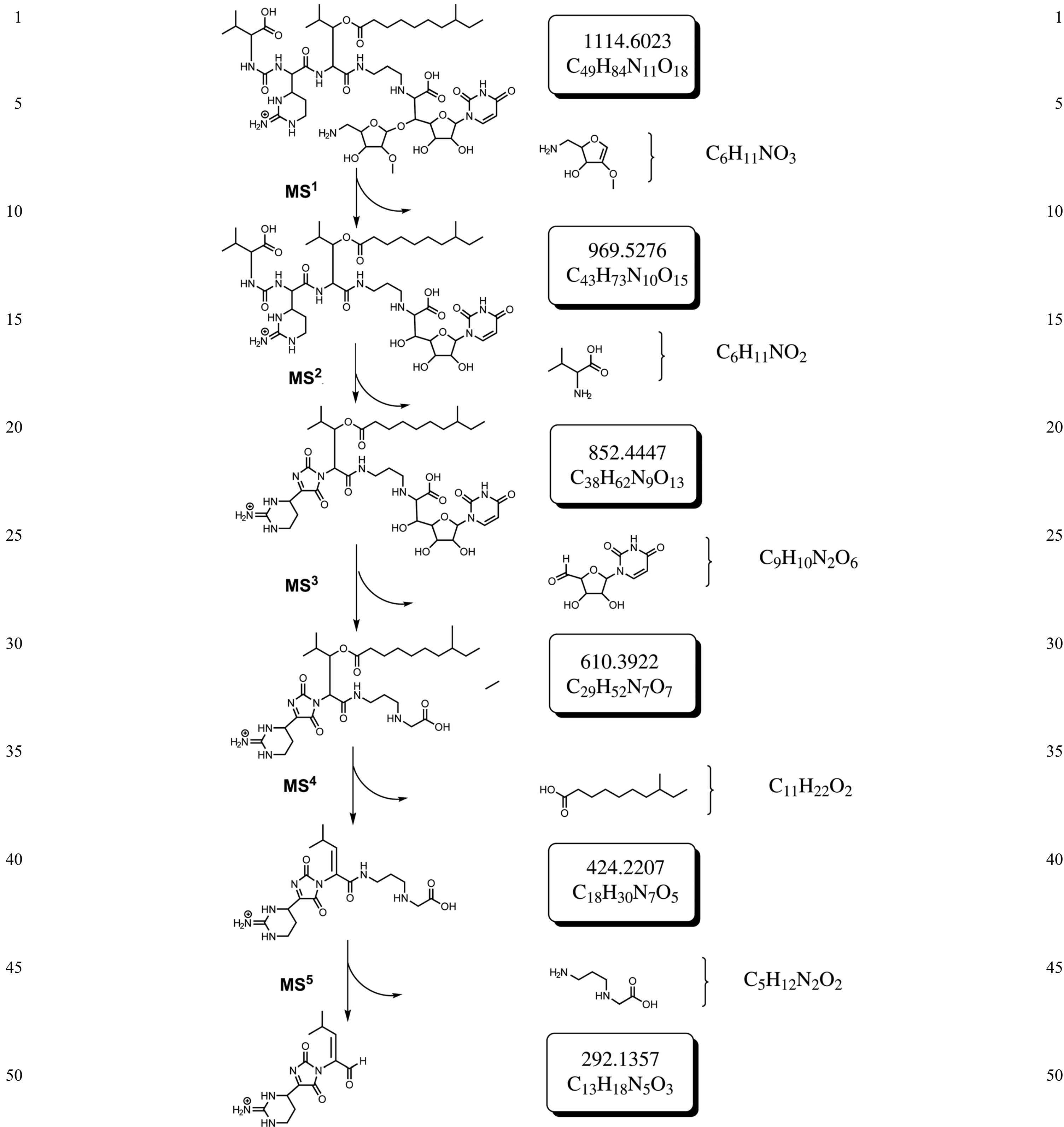

Fig. 4 MS^5 of muraymycin by FTICR-MS.

well the technology has been engineered to routine lab operation.

As the collected articles in this issue demonstrate, the application of mass spectrometry to the study of natural

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products continues to grow and evolve – driven by scientists with creative vision.

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