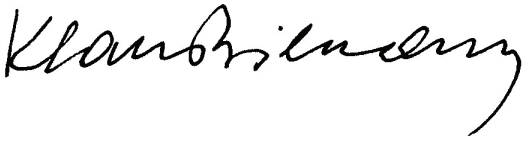

Structure Determination of Natural Products by Mass Spectrometry

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peptide and protein sequencing, alkaloids, heparin, sulfated glycosaminoglycans, Moon, Mars

Abstract

I review laboratory research on the development of mass spectrometric methodology for the determination of the structure of natural products of biological and medical interest, which I conducted from 1958 to the end of the twentieth century. The methodology was developed by converting small peptides to their corresponding polyamino alcohols to make them amenable to mass spectrometry, thereby making it applicable to whole proteins. The structures of alkaloids were determined by analyzing the fragmentation of a known alkaloid and then using the results to deduce the structures of related compounds. Heparin-like structures were investigated by determining their molecular weights from the mass of protonated molecular ions of complexes with highly basic, synthetic peptides. Mass spectrometry was also employed in the analysis of lunar material returned by the Apollo missions. A miniaturized gas chromatograph mass spectrometer was sent to Mars on board of the two Viking 1976 spacecrafts.

1. INTRODUCTION

In the fall of 1945, I entered the University of Innsbruck (Austria), the city I was born in nineteen years earlier, to study pharmacy, a tradition in my family. By the time I finished with a master's degree in February 1948, I had realized that I did not want to spend my life in an apothecary. I had become interested in organic chemistry, so I continued in that field, which was quite easy, because for both disciplines the same laboratory courses, and lectures taught in the same classroom, were required. So I had to take only a few additional courses and exams, and then continue with graduate work.

I was the only student in that category, because men my age and older either did not survive World War II or were still in prisoner-of-war camps, a fate I had avoided at my own great risk. At that time, women rarely studied chemistry; they generally went into pharmacy. When I graduated in February 1951 with a PhD in organic chemistry, I was appointed Instructor and taught a course in the analysis of pharmaceuticals; I was also charged with running the organic chemistry laboratory for the chemistry and pharmacy students.

My PhD thesis was carried out under the direction of Professor Hermann Bretschneider, an organic chemist in the pharmaceutical industry, first in Hungary and then in Germany. He moved to Innsbruck after World War II to head the organic chemistry department at the university there. He himself had studied in Vienna under Professor Ernst Späth. His research was in the design and synthesis of organic molecules that could be of therapeutic use. Consequently, my graduate work was in the same field, and, following the hierarchical principles of academia at that time, continued so after my graduation.

Although this work was interesting and productive, and I learned a great deal of organic synthesis, I became restless after a few years. By chance, I noticed one day at the dean's office an announcement for a summer program at the Massachusetts Institute of Technology (MIT) in Cambridge that was offering to host young scientists and engineers over the summer of 1954. The application process was simple, just a personal letter outlining a prospective research project, publications, if any, and proof of an adequate command of the English language. I already had six papers with H. Bretschneider, and, fortunately, had five years of English in high school. Additionally, I was at that time in the process, per Bretschneider's request, of translating an organic chemistry textbook from English into German.

My application was approved and at the end of May 1954 I was on my way, by boat from Rotterdam (The Netherlands), to Boston and Cambridge, via New York. The program at MIT had been conceived and was run by a group of undergraduate students who had spent the final years of World War II in the armed forces. They had witnessed the devastation of many cities and damage to universities, and thus wanted to help by providing facilities to carry out work that we could not do at home. I was assigned to the research group of Professor George Büchi, an organic chemist trained at the Eidgenössische Technische Hochschule, Zurich (Switzerland) and whose research interest was the structure and synthesis of natural products. During this time, I learned about modern instrumentation, such as ultraviolet (UV) and infrared spectroscopy, and about topics like reaction mechanisms. While still in Innsbruck, I discovered the 1954 Annual Congress of the American Pharmaceutical Association would be held in Boston the following August. So I took a chance and submitted an abstract about my work on the synthesis of a pyridine analog of the antibiotic chloramphenicol (1). To my surprise, it was accepted and I presented my work on August 26.

When the MIT program officially ended September 15th, Professor Büchi supported me as a postdoctoral fellow until the end of November, when my visa expired. Once home, I realized that my brief stay in America had not had the blessing of Professor Bretschneider, and he practically

ignored me after my return. I was never asked what I did or learned; I had to continue to put my nose to the grindstone and just keep quiet.

The next step in my academic career would be what was referred to as the Habilitation, a process through which one has to demonstrate independent research. After a while, Bretschneider would permit me to publish some work under my own name, to fulfil these requirements. During my brief stay at MIT, I had learned about the American way of academic life, so different from the autocratic system prevailing at that time in Austria (and Germany). It took me only a few months to decide that I would be better off in the former than in the latter.

When George Büchi heard about my desire to continue to study in the US, he offered me a new postdoctoral position, which I accepted and started October 1, 1955, in the by then familiar environment at MIT. The major project I worked on was the synthesis of muscopyridine for the purpose of proving a structure that George had proposed on biogenetic grounds. This compound had been isolated previously from the perfume gland of the musk deer. Such a proof of structure must begin with a compound of known structure and must involve reactions of well-established mechanisms. The synthesis George had designed involved eleven steps, of which six had been carried out by a graduate student, until he ran out of material. It was enough to earn him a PhD. It was now my job to repeat his work on a larger scale and carry out the remaining five reactions. At each step, the product of the reaction has to be isolated, purified, and fully characterized by taking melting points and infrared and UV spectra and burning a few precious milligrams for elemental analysis. The product of the final step was indeed identical to the natural product, thus proving the proposed structure (2).

In the fall of 1956, while I was still working on this synthesis, the head of the Department of Chemistry at MIT, Professor Arthur C. Cope, an eminent organic chemist of his day, decided to add an organic chemist to the analytical division. He knew that I had taught a course in qualitative analysis of pharmaceuticals at the University of Innsbruck, and apparently was quite impressed with my work in Büchi's lab. He offered me a position as an instructor—at the time the lowest rung of the academic ladder—starting July 1, 1957. Although it meant a rather dramatic change from the synthetic chemistry I was trained in, I accepted. I now had to think of an area of research that could benefit from that training but involved analytical chemistry.

2. PEPTIDES, PROTEINS, AND MASS SPECTROMETRY

It was just three years since Fred Sanger at the University of Cambridge (United Kingdom) had determined the first structure of a protein, insulin (3). This was accomplished by separating the two disulfide-linked chains, cleaving them by partial acid hydrolysis into a mixture of small peptides, and separating them by paper chromatography. Sanger had developed a method for marking the N-terminal amino group of peptides by reaction with 2,4-dinitro-fluorobenzene. Upon complete acid hydrolysis, followed by paper chromatography, the marked and unmarked amino acids can be identified. For a dipeptide, the sequence *A-B is unique; for a tripeptide, there are two possibilities, *A-B-C or *A-C-B, which still can be used for overlaps.

Clearly, marking also the C-terminal amino acid would greatly facilitate protein sequencing and simultaneously combine synthesis with analysis. It so happened that my final work in Innsbruck involved the synthesis of 3-amino-1,2,4-triazoles from carboxylic acid hydrazides (4). Carrying out this reaction on a peptide hydrazide, produced by partial hydrazinolysis of a protein, would label the C-terminal amino acid with a very stable marker. Combined with Sanger's reagent, a tripeptide, *A-B-C**, would be uniquely identified, whereas for a tetrapeptide two possible sequences would remain. I prepared an application for a research grant from the NIH on the basis of this proposal.

Then something happened that would dramatically change my future career. Firmenich & Cie., a preeminent Swiss firm active in flavor and fragrances that also funded my position in Büchi's group, asked me to attend a conference on food flavors to be held in Chicago in late spring 1957 and to provide a report. Although I was not interested in that topic, attending provided me with the opportunity to fly to Chicago. Of the papers presented, one in particular caught my attention: that by William H. Stahl of the research laboratory of the US Army Quartermaster Corps in Natick, MA, not far from Cambridge. He reported on the identification of simple, small molecules, such as acetone, ethyl butyrate, butyl acetate, etc., in fruit extracts using a method I had never heard of: mass spectrometry (MS). It was done by comparing the mass spectra of the compounds isolated with the spectra of authentic compounds.

Once I had sent off my report to Firmenich, I looked up what was known about the mass spectra of organic molecules. Most of it was about hydrocarbon analysis, because the method was widely used in the petroleum industry. However, there were also papers on aliphatic alcohols (5), aldehydes (6), ketones (7), methyl esters (8), and amines (9), to name the more important ones. They all dealt with the correlation between the known structure of reference compounds and their mass spectra to establish fragmentation rules. It soon became clear to me that mass spectrometry is particularly informative about the structure of linear molecules containing heteroatoms—and peptides are such molecules! But I also had learned that to obtain a mass spectrum, the compound has to be vaporized into the ion source, usually held at 250°C at low pressure. However, peptides decompose rather than vaporize upon heating due to their zwitterionic character, which is caused by the presence of an acidic carboxyl group and a basic amino group in the same molecule, as well as hydrogen bonds between the carbonyl groups and amide hydrogens. Here my training in synthetic organic chemistry came in handy. The carboxyl group can be converted to an ester, the amino group can be acylated, and the carbonyl groups can be reduced to CH_2 by lithium aluminum hydride, producing a polyamino alcohol that retains the backbone and position of the side chains of the original peptide (**Figure 1**).

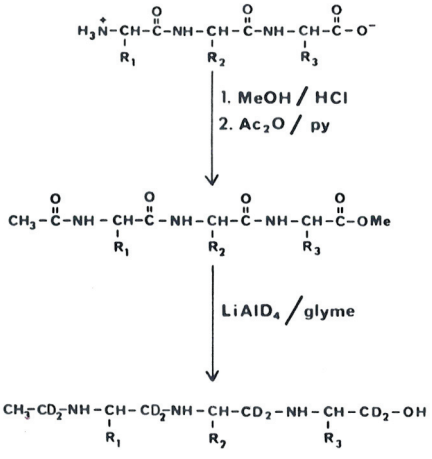


Figure 1

Reaction scheme for the reduction of peptides to polyamino alcohols. $LiAlD_4$ was used to prevent the sidechains of aspartic acid and serine, and of glutamic acid and threonine, from becoming isobaric. Reprinted from Reference 46.

A more formidable obstacle was the fact that MIT did not have a mass spectrometer (MS), nor did any academic chemistry department of a university in the United States at that time. When I asked Professor Cope why we did not have one, he replied, "It takes a full-time electrical engineer to keep it running." I remarked that I could do this myself and explained my plans. He listened carefully and responded, "If you promise the instrument will not collect dust, I promise to provide the money." We both kept our word. At that time, federal agencies did not easily fund expensive instruments like mass spectrometers, which cost more than $50,000. What I did not know was that Cope had recently negotiated with MIT's president James R. Killian a fund of $250,000 to be spent over the next ten years to upgrade the chemistry department. In a letter dated July 22, 1964, to the then president of MIT Julius A. Stratton, professor Cope stated that the purchase of my mass spectrometer was one of the best uses of the funds: "Subsequently he has become recognized as the foremost person in the world in the application of mass spectrometry to the determination of structures of organic compounds. . ." (10). In addition to the $50,000 for the mass spectrometer, Firmenich & Cie. provided $10,000 with the understanding that I would provide mass spectra of some of its research products, including their interpretation.

I ordered a CEC 21-103C mass spectrometer, the type routinely used in the petroleum industry, from Consolidated Electrodynamics Corporation (CEC) in Pasadena, CA, which was delivered early May 1958. In the meantime, the NIH grant I had applied for was approved. It allowed changing the approach to a more promising one, so I was permitted to use the funds for the mass spectrometric sequencing of peptides. It also provided funds for a postdoctoral associate, a position I offered to Josef ("Sepp") Seibl, who had recently obtained his PhD in Bretschneider's group and whom I knew well. He accepted and arrived one day before CEC's engineer Hank DeQuasie began installing the instrument. It took six weeks, including two weeks of instruction for operation and maintenance and for running an acceptance test. This was to be the quantitative analysis of a mixture of low–molecular weight hydrocarbons. As I was not interested in hydrocarbons, or in quantitative analyses, I asked that it be a mixture of the corresponding alcohols. CEC management at first refused my request, but Hank persuaded them. The experiment worked satisfactorily and we took official possession of the mass spectrometer.

A National Science Foundation (NSF) grant I had been approved for also provided a postdoctoral position. I offered it to another recent graduate from Bretschneider's group, Fritz Gapp, who arrived a few months later, at which point the three of us started our work on the peptide project. As I had predicted, the polyamino alcohols produced very good mass spectra and sequence information due to the preferential cleavage at the NHCH(R)-CH_2NH bonds (**Figure 2**).

This feature was very important, because we could not use the common method of identifying the unknown by comparison with the mass spectrum of a standard reference compound. There are 20 different amino acids in mammalian proteins; as such, there are 400 different dipeptides, 8,000 different tripeptides, etc. Therefore, it was practically impossible to compile a library of authentic di-, tri-, etc., amino alcohols for comparison, and we had to interpret these spectra from scratch. A subsequent short communication (11) was the first paper reporting the use of mass spectrometry in peptide (and protein) chemistry. These derivatives also were sufficiently volatile to be amenable to gas chromatography (GC) (12), thus allowing the separation of the complex mixtures expected from partial hydrolyses of proteins. Over time, the chemical procedure was improved with the use of trifluoroacetylation (13) and silylation (14), which allowed its extension to penta- or even hexapeptides. With the interfacing of the GC to the MS (15) and to an IBM 1800 computer (16), a powerful system was created for determining, finally, the first primary structure of a protein, subunit I of monellin, entirely by mass spectrometry (17). It was then also used for osteocalcin from chicken bone, a fifty amino acid long protein, which contains three γ-carboxy-glutamic acids which were converted to γ-dideutero-glutamic acid before acid or enzymatic hydrolysis (18).

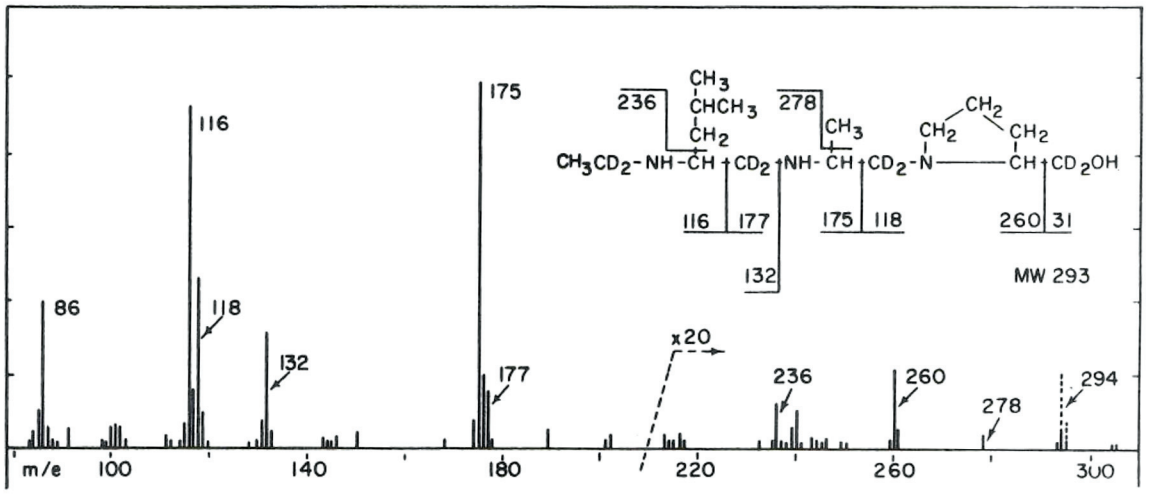


Figure 2

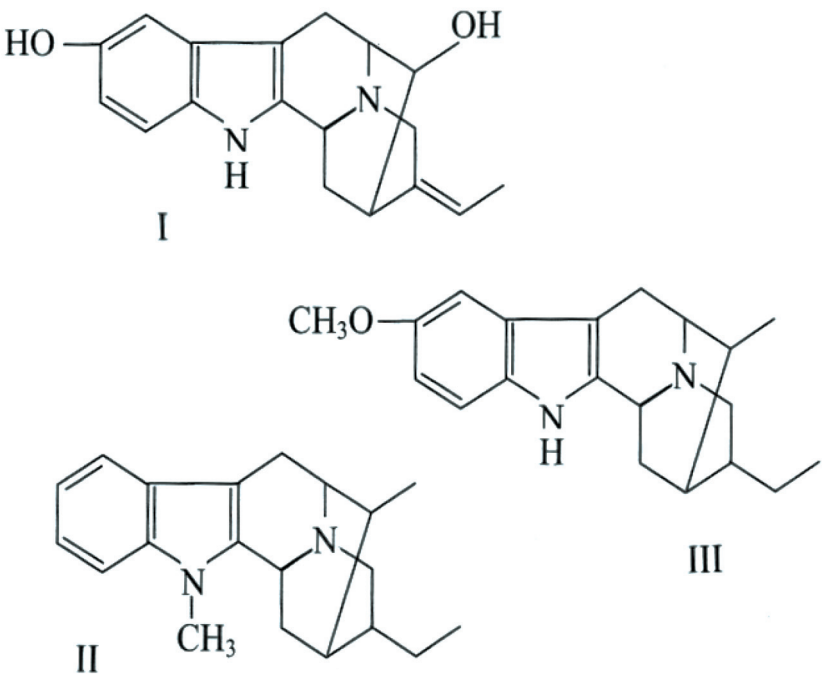
Mass spectrum of the derivative of a tripeptide (Leu-Ala-Pro). Figure reprinted from Reference 12.

3. ALKALOIDS

Naturally occurring substances, mainly from tropical plants, had been used for centuries in folk medicine and later in academic studies. Atropine, morphine, and yohimbine are good examples. In the first half of the twentieth century, the pharmaceutical industry began the systematic search for active substances. An early success was an alkaloid isolated in 1932 from the roots of *Rauwolfia serpentina* and named reserpine. Its structure was not determined until 12 years later. Reserpine was one of the first antihypertensive drugs and became a huge financial success for CIBA (Basel, Switzerland) in the early 1950s. Research laboratories in both the pharmaceutical industry and academia raced to duplicate this achievement, and shelves began to fill with alkaloids, which had names and melting points, but determining their structures was a laborious, time-consuming process.

In the course of my work on muscopyridine I had learned much about alkaloids and the problems involved in the determination of their molecular structures. I remembered one case, where three independent laboratories had proposed **Structure I** for the alkaloid sarpagine, but only one of them suggested proving it by correlation with a degradation product (**Structure II**) of another alkaloid of known structure, ajmaline. However, three years had gone by without success. This was probably the case because for such a correlation, the sarpagine molecule has to be converted to a compound *identical* to the one it should be compared with, but this involves several experimentally difficult steps. It now occurred to me that by using mass spectra for the comparison, one needed to prepare only a *similar* comparison product. The reasoning was that because these indole alkaloids consist of an aromatic system and a polycyclic unit, only the latter will fragment; the former will remain relatively intact. Therefore, the mass spectra of molecules with identical polycyclic structures, but differently substituted aromatic portions, will display the same patterns, except that some peaks will be shifted by the mass difference of the substituents on the aromatic portions of the molecules.

According to this reasoning, it was only necessary to convert sarpagine (**Structure I**) to a compound of **Structure III**, which involved only three very simple chemical reactions. Checking the product of each step by mass spectrometry eliminated the need for conventional



Structures I–III

characterization, as described for the muscopyridine work. The structure of ajmaline had been determined by Professor Robert B. Woodward at Harvard University (Cambridge, MA), just up the river from MIT. In the course of that work he had prepared compound II. I knew him very well and he gladly provided me with a sample. Woodward, the preeminent natural products chemist of the time (and a 1965 Nobel laureate) was curious about how my novel approach to structure proof would work out. The mass spectra of compounds II and III indeed showed almost identical patterns with the predicted shift in mass (**Figure 3**) (19). This method then became known as the mass spectrometric shift technique.

I presented this work at the International Symposium on the Chemistry of Natural Products in August 1960 in Melbourne, Australia. Apparently, word of my work had gotten around and the room was so crowded that the eminent Bob Woodward had to sit on the steps of the lecture room. After my talk, Professor Carl Djerassi of Stanford University (Stanford, CA) came over and invited me to come to his laboratory to help set up his mass spectrometer, which was already on order, and teach his students and postdocs how to interpret the spectra. I agreed and spent January and February of 1961 at Stanford. Later, some of my colleagues commented that it was a great mistake to help a powerful, well-funded man like Djerassi become my competitor. However, I had thought that if one develops a useful new methodology, one should also help to spread it around. Carl indeed competed with me in the alkaloid field, but we became good friends in later years. In a retrospective article (20) he paid tribute to my contributions, referring to a seminar talk I gave while at Stanford in 1961: "It was the elegant rationalization by Biemann et al. of the mass spectral fragmentation behavior of alkaloids of the aspidospermine class that stimulated a serious effort at Stanford on organic chemical applications of mass spectrometry" (p. 1341). A

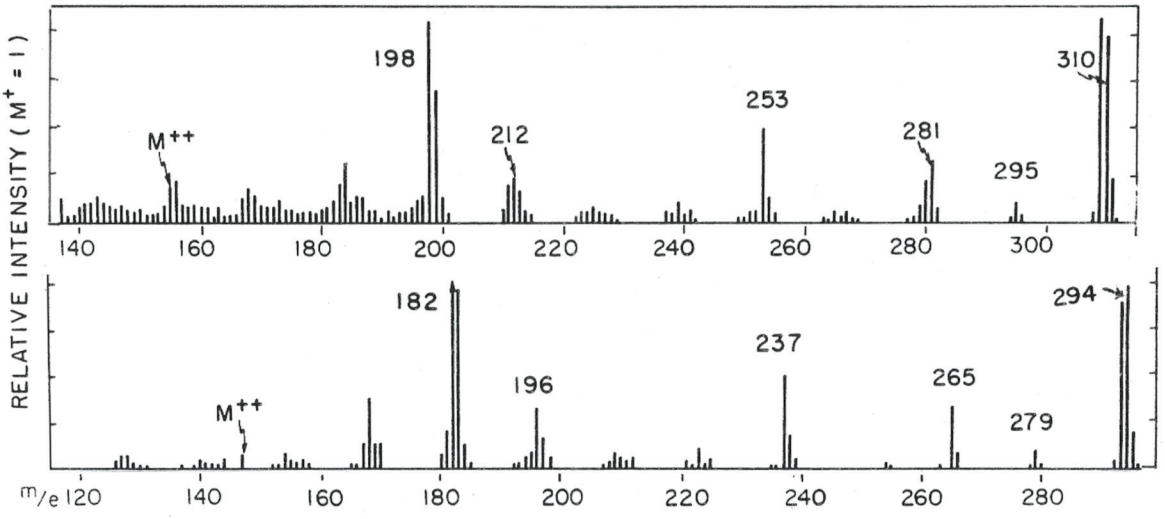


Figure 3

Mass spectra of the conversion product III (*top*) of sarpagine and that (Structure II) of ajmaline (*bottom*). Reprinted from Reference 19.

book I wrote soon thereafter (21) helped spread this information much more effectively. It was later considered so seminally significant that it was republished in 1998 by the American Society for Mass Spectrometry.

4. HIGH-RESOLUTION MASS SPECTROMETRY

By the mid-1950s, John Beynon at Imperial Chemical Industries (Manchester, UK) had built a double-focusing mass spectrometer of the Nier-Johnson geometry, with which he demonstrated the value of high-resolution mass spectrometry for organic compounds (22). This approach appealed to me for my work on alkaloids, because of the ability to determine the elemental composition of molecules and their fragments. CEC had built a double-focusing mass spectrometer of the Mattauch-Herzog geometry with a spark ion source for inorganic analyses. It used a photographic plate to record the ion beams, which is possible with the Mattauch-Herzog geometry given its focal plane, not just one focal point. Inspired by Beynon's work, they also fitted it with an electron ionization source for organic materials. With support from the NIH and NSF, I purchased the CEC 21-110B high-resolution mass spectrometer in 1962. The photoplate appealed to me, because one could record a complete mass spectrum in a minute or less at high resolution and measure it later at millimass accuracy, independent of the spectrometer. For this purpose, we adapted a commercially available microdensitometer to semiautomatically scan the photoplate and punch line position and density onto IBM cards, which then were processed to yield elemental composition and abundance at MIT's computer center. This was a great advantage over Beynon's method of carefully measuring the ratio of accelerating voltages necessary to successively focus a single ion and then a reference ion onto the collector slit (peak matching), or of scanning a very short segment of the spectrum and recording it with an oscillograph recorder. When we reported our method and associated computer algorithms for the interpretation of the data at the E-14 meeting in Montreal, Canada, in 1964 (23), it caused a scramble for any method that could accomplish the same with the Nier-Johnson geometry of Beynon's instrument, which Associated Electronics

Industries of Manchester, UK, had commercialized as the MS-9. Recording onto magnetic tapes was attempted, but interfacing the spectrometer with a computer was accomplished only years later.

The ability to record a complete mass spectrum within a minute or less enabled us also to interface the gas chromatograph to the mass spectrometer in an efficient and practical way (15). Previous efforts were hampered by the lack of a suitable way of recording the spectra. Gohlke (24) had used a time-of-flight mass spectrometer (TOF MS) and an oscilloscope, the screen of which was photographed with a Polaroid® camera. This limited it to quite small molecules eluting at relatively large time intervals. Ryhage (25) interfaced the GC with a conventional mass spectrometer, the slow scan speed of which limited the system to the recording of data in the single-ion monitoring mode. Using the photoplate, we were able to record the high-resolution mass spectra of 16 alkaloids eluting from a single injection of the crude extract from the bark of *Aspidosperma quebracho blanco*. Three of these were known alkaloids; the molecular structure of the remaining 13 we determined from their high-resolution mass spectra (26). Within two decades, we elucidated the structures of more than 40 indole alkaloids, culminating—in collaboration with chemists from the Lilly Research Laboratories of Indianapolis, IN—in the structures of the dimeric indole alkaloids vinblastine and vincristine (27), the first anticancer drugs, which are still being used today in chemotherapy.

By the mid-1980s the search for a natural product for medical use subsided. Thanks, at least in part, to mass spectrometry, most major plant alkaloids were known and had been pharmacologically tested. Rather than laboriously isolating biologically active compounds from natural sources, it became general practice to generate complex mixtures—libraries—of molecules by chemical reactions of a mixture of related molecules with one or a few reactants, followed by the isolation of biologically active products, if any. Mass spectrometry also plays an important role in this area of biopharmaceutical research.

5. LARGE PROTEINS

In the meantime, our work on the primary structure of proteins had continued, but so had other methodologies. The by far most useful technique was the stepwise removal and identification of the N-terminal amino acid developed by Pehr Edman (28), soon automated (29) and commercialized. It had some drawbacks: It did not work with proteins that are acylated at the N-terminus, which many mammalian proteins are, and it had difficulties if the C-terminal end consisted of a number of nonpolar, hydrophobic amino acids. Because these two situations do not interfere—in fact were even an advantage with our mass spectrometric approach—it was logical to combine the two. This was done in collaboration with Gobind H. Khorana, professor of biology at MIT, who was working on the structure and activity of bacteriorhodopsin from *Halobacterium halobium*, a protein that loops seven times through the cell wall and thus has long stretches of hydrophobic amino acids. This protein turned out to be 248 amino acids long (30).

By the late 1970s, DNA sequencing methods had progressed to the point that it became feasible to determine the nucleotide sequence of the DNA coding for a protein and translate it using the genetic code into the corresponding amino acid sequence of the protein. Paul Schimmel, professor of biology at MIT at the time, had isolated from *Escherichia coli* alanyl-tRNA synthetase, a very large protein, and was interested in its structure and active site. He was planning to use the method of Maxam and Gilbert (31) to determine the DNA sequence of the strand coding for the protein. He realized the potential problems that could lead to errors: The electrophoresis strips had to be read manually and repeatedly; a single missed or erroneously inserted nucleotide would result in an entirely wrong amino acid sequence beyond that point; two compensating errors would lead to a protein sequence correct at both ends, but with an incorrect stretch in the middle; and even

if the nucleotide sequence is entirely correct, it can be read in three different reading frames, of which only one represents the correct protein. When Paul discussed these problems with me, it occurred to me that all these errors can be easily detected and corrected by determining the amino acid sequence of a number of relatively small peptides scattered across the entire protein. For this, our GCMS technique was very well suited. Using a simple computer program, we generated the hypothetical amino acid sequence corresponding to each of the three reading frames and then matched these with the di- to pentapeptides we had identified in a partial acid hydrolysate of the protein. When some peptides matched in one reading frame, and some in another, the error could be identified and corrected by reinspection of the electrophoresis strip corresponding to that region. In this way, the primary structure of the alanyl-tRNA synthetase was determined. It turned out to be 875 amino acids long (32).

Word about this novel approach got around quickly, and even before this work was published we started a collaboration with Peter Söll, professor of biology at Yale University, on glutaminyl-tRNA synthetase from *E. coli*. While we were working on this protein with the GCMS methodology, a paper by Michael Barber (33) appeared that drastically changed the mass spectrometric approach to peptide and protein sequencing. Bombarding a large peptide dissolved in glycerol with a beam of argon atoms at keV energy produced protonated molecules $(M+H)^+$. Thus, fast atom bombardment (FAB) made it possible to measure the molecular weight of sizeable peptides, as demonstrated with met-lys-bradykinin (mol. wt. 1318). We immediately implemented this ionization method on our mass spectrometers and continued the work on glutaminyl-tRNA synthetase with the use of a tryptic digest of the protein and matched the molecular weights of the resulting peptides to the tryptic peptides expected for the proteins corresponding to the three reading frames of the nucleotide sequence. Errors were detected and corrected as done earlier with the smaller GCMS peptides, except that we now could cover larger stretches of the DNA sequences. This was the reverse of the approach now termed peptide mass fingerprinting in proteomics (34) for the identification of a protein using the now known human or other genomes. The primary structure of Gln-tRNA synthetase was quickly determined by this approach; it turned out to be 550 amino acids long (35). Four more aminoacyl-tRNA synthetases, ranging in size from 324 to 990 amino acids, followed soon thereafter (36–39).

6. TANDEM MASS SPECTROMETRY

Although FAB mass spectra exhibited very abundant $(M+H)^+$ signals, they showed little fragmentation, unless a large sample of pure material was used. This was an advantage, because one could measure the molecular weights of peptides in simple mixtures without being confused by fragment ions, but one could not deduce their amino acid sequences. To induce fragmentation, additional energy had to be imparted onto the protonated molecular ion to cause it to fragment. This is accomplished by collision with an inert gas, such as helium, in a tandem mass spectrometer (MS/MS). The ion to be fragmented is isolated by the first mass spectrometer (MS-1), which passes it through a cell filled with the collision gas and mass-analyzes the resulting fragment ions in the second mass spectrometer (MS-2). To achieve good resolution and sensitivity, two high-resolution spectrometers need to be used. Fred McLafferty at Cornell University of Ithaca, NY, had constructed such an instrument by 1980 (40), and in 1983 a similar MS/MS (ZAB 4F) from VG Analytical of Manchester, UK, was installed at the National Institutes of Health and Environmental Sciences (Research Triangle Park, NC) (41). I had seen that instrument in operation and decided that it would be very useful for our ongoing work on proteins. I also knew that JEOL of Akishima, Japan, had just come out with a new design for a double-focusing mass spectrometer (HX 110) (42) and made arrangements with that company to build an MS/MS, using these new ion optics,

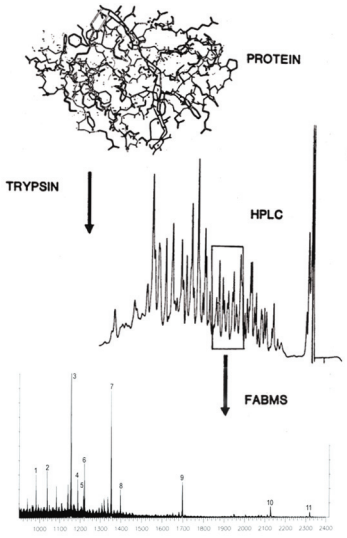


Figure 4

Schematic illustration of the determination of the molecular weights of the components of a tryptic digest. The mass spectrum (*bottom*) indicated the molecular weights of 11 peptides in the molecular weight range of 900 to 2,400 in the HPLC fraction (box) collected. Reprinted from Reference 46. FABMS, fast atom bombardment mass spectrometry; HPLC, high-performance liquid chromatography.

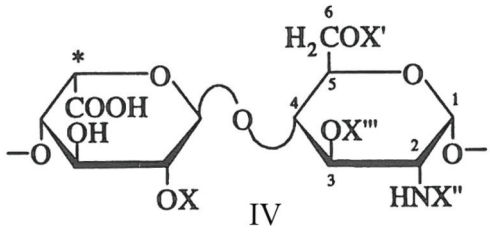
according to my performance specifications, to be installed in my laboratory at MIT. Its design, construction, and testing received top priority at JEOL and was delivered at the end of July 1985.

We then developed a strategy to determine the primary structure of medium-sized proteins solely by tandem mass spectrometry. The protein was digested using amino-acid-specific proteases, such as trypsin, α-chymotrypsin, thermolysin, etc., separating the resulting mixture of peptides by high-performance liquid chromatography (HPLC), collecting a few wide fractions containing up to a dozen peptides (**Figure 4**) (43). Each $(M+H)^+$ ion in these fractions was then sequenced by MS/MS. This strategy was then used to sequence four thioredoxins (see 44 and references therein) and three glutaredoxins (see 45 and references therein). These are proteins, 100–120 amino acids long, which catalyze various important redox reactions in the cell.

Much of this work on the sequencing of peptides and proteins, including some of the algorithms developed, contributed to the origin of a new field, proteomics, in the early 1990s (46).

7. HEPARIN AND RELATED SULFATED OLIGO- AND POLYSACCHARIDES

In 1989, another very important development occurred. Franz Hillenkamp and Michael Karas at the University of Münster, Germany, discovered that intact proteins can be ionized to $(M+H)^+$ ions when imbedded in an excess of certain aromatic acids and irradiated with a laser beam (matrix-assisted laser desorption, MALDI). Because of the pulsed nature of the ionization, a modified, commercial TOF MS was used (47). This new method was, of course, of great interest for our work and I included a request for the purchase of such an instrument in the budget of my next renewal of the NIH Research Resource grant (RR00317). This was approved, but I waited a bit to see whether there would be a commercial version available. A few months later, Marvin Vestal,



IV

Structure IV

founder and president of VESTEC Corp. of Houston, TX, called me to find out whether I had ordered one. He had been on the NIH site visit group that reviewed my application and, therefore, knew that I had the funds. When he heard that I still had not decided on one yet, he offered to build a MALDI TOF MS for me, using the design of Brian Chait of Rockefeller University (New York, NY), who had just built one (48, 49). I gladly agreed, because I knew that VESTEC would do a good job and deliver quickly. Marvin would make sure that the company's new flagship worked well and produced interesting results.

To measure the molecular weights of large peptides and medium-sized proteins, one had to run these unknowns in admixture with a standard of known molecular weight. One day, Peter Juhasz, a postdoc who had recently arrived from his native Hungary, tested this approach by measuring the molecular weight of bovine insulin using the oxidized A-chain of that protein as the standard. Surprisingly, there was no signal at all at m/z 2532.7 for the $(M+H)^+$ ion of the standard, but a very large one at m/z 8264.9 in addition to the one at m/z 5734.5 expected for the insulin. Puzzled at first, we soon realized that the signal at high mass was the monoprotonated complex formed between the insulin and the oxidized A-chain that contains four sulfonic acid groups in the form of cysteic acid (50). This complexing phenomenon observed entirely by chance turned out to be quite general, and we used it for the development of the structural analysis of heparin-derived oligosaccharides (51). The strategy was similar to that which we had developed for protein sequencing. Heparin and other heparin-like sulfated glycosaminoglycans are linear molecules consisting of consecutive disaccharides made up of one uronic acid and one glycosamine, differing in the number and position of sulfate groups or N-acetylation (**Structure IV**, where X, X′ and $X''' = H$ or SO_3H, and $X'' = SO_3H$ or $COCH_3$; both epimers can occur at *, i.e., L-iduronic or D-glucuronic acid). These long chains of disaccharides can be specifically cleaved with enzymes (heparinases I and II) or by certain oxidation reactions.

To efficiently carry out these complexing reactions, it is necessary to use a reactant containing an excess of basic groups over the acidic ones present in the unknown. We therefore used highly basic synthetic peptides of the type $(Arg\text{-}Gly)_n$ (**Figure 5**). From the m/z values of the resulting protonated complexes, one can calculate the number of uronic acids, glucosamines, and sulfate groups present, and whether the amino group is sulfonated or acetylated.

In collaboration with Ram Sasisekharan, professor of biological engineering at MIT, we applied this methodology to the study of the enzymatic mechanism of the depolymerization of heparin-like glycosaminoglycans by heparinases and related topics (52–54). It allowed us to also elucidate the antithrombin III binding site in heparin (55, 56).

8. METEORITES, THE MOON, AND MARS

One day in February 1961, while I was in Carl Djerassi's lab, Joshua Lederberg, professor of biology at Stanford University, stopped by. In the course of conversation about mass spectrometry, he asked

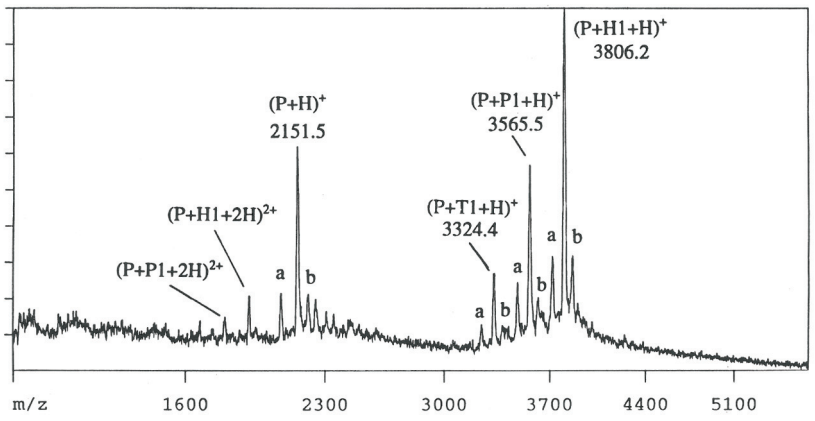


Figure 5

Matrix-assisted laser desorption mass spectrum of a mixture of three heparin-derived oligosaccharides: tetrasaccharide T1 (M_r = 1173.0), pentasaccharide P1 (M_r = 1414.2), and hexasaccharide H1 (M_r = 1655.4) with $(Arg\text{-}Gly)_{10}$ (M_r = 2150.4). The satellite peaks a and b are due to impurities in the synthetic peptide. Figure reprinted from Reference 51.

me whether I thought that this method could be used to detect and identify organic compounds in the surface material of Mars. I thought about it briefly and then replied confidently in the affirmative; thus began my involvement in space research. I began to think about how one could obtain mass spectra of traces of organic compounds embedded in very dry soil, remotely on another planet. Heating the sample directly into the ion source was the simplest method. The ruggedness of a TOF MS lent itself best to such experiments. I quickly wrote an application for a research grant from the National Aeronautics and Space Administration (NASA) asking for a Bendix TOF MS; it was approved and the instrument was installed early 1962.

The Bendix TOF MS had a vacuum lock through which a heatable metal strip bearing a sample could be introduced into the ion source housing. This was designed for pyrolysis, but we modified it so that one could heat a sample, placed in a short piece of melting point capillary, to variable temperatures. With this direct insertion probe we were able to obtain the mass spectra of nucleosides (57), free amino acids (58), and carbohydrates (59). A similar probe was constructed for use with the CEC 21-110B high-resolution mass spectrometer, and we used it to measure the organic compounds in the Murray and Holbrook meteorites (60). This work resulted in my appointment as the principal investigator for the search for organic compounds in the samples brought back from the Moon during the Apollo missions. That assignment involved two separate tasks. The first required the installation and operation of a mass spectrometer (Hitachi RMU 6E) behind the biological barrier at the Lunar Receiving Laboratory at Johnson Space Flight Center (Houston, TX). This was because there was the need to ascertain that the lunar material did not harbor any microorganisms that could be detrimental to life on Earth. A mass spectrometer is, of course, not a life detector, but the presence of any organic compounds would raise concerns. It took approximately three weeks to ascertain that the quarantined astronauts could be released and the samples could be distributed to the various research laboratories around the country for more detailed investigations. One of those was mine at MIT, where we used the CEC 21-110B high-resolution mass spectrometer to analyze samples returned during the Apollo 11, 12, and 14 missions (61–63). All of these experiments indicated the absence of organic material indigenous

to the Moon. It may be remembered that Apollo 13 was ill-fated because of the rupture of the oxygen tank and had to return to Earth in a dramatic maneuver without landing on the Moon.

In the mid-1960s NASA began planning a mission to Mars which would consist of an orbiter and a lander, to be launched in 1973. On the surface it would carry out several experiments, including three biology instruments and one to search for organic compounds in the soil. For that, a GCMS was to be used, the MS part to measure also the composition of the atmosphere at the surface of the planet. Because we had developed the original system and had written all the software to acquire, process, and interpret the resulting data, I proposed carrying out this task and in 1969 was chosen to be the principal investigator and leader of the Molecular Analysis Team. This team was put together by NASA to represent experts in the fields to which the GCMS experiment was expected to contribute: biology (John Oro, University of Houston, TX), origin of life (Leslie Orgel, Salk Institute, San Diego, CA), inorganic chemistry (Priestley Toulmin III, US Geological Service, Reston, VA), permafrost (Duwayne Anderson, Division of Polar Programs, NSF, Washington, DC), atmospheric composition (Tobias Owen, University of New York, Stony Brook, NY), and organic geochemistry [Peter Simmonds, Jet Propulsion Laboratory (JPL), Pasadena, CA; soon University of Bristol, UK]. Much later, in 1973, Alfred O. Nier (University of Minnesota, Minneapolis, MN) was added to represent physics and instrumentation. He also was the leader of the Upper Atmosphere team on Viking.

The instrument was built under a NASA contract to the JPL of Pasadena, CA, together with the Perkin-Elmer Aerospace Division of Pomona, Litton Guidance and Control Systems of Woodland Hills, and Beckman Instruments Inc. Advanced Technology Operations of Anaheim (all of California) for the mass spectrometer, the electronics, and the gas chromatograph, respectively. The unique environment expected on the surface of Mars and the weight, volume, and power limitation of the spacecraft led to the use of a miniaturized ion getter pump using the magnetic field of the mass spectrometer of the Nier-Johnson geometry. Because of the need to maintain the low pressure required within the vacuum envelope of the MS with this very limited pumping capacity, it was not possible to use the conventional helium carrier gas. Instead, we used hydrogen, which was removed with the use of an electrolytic hydrogen separator located between the GC and the MS. To further avoid overloading the pump by large amounts of effluents from the GC, such as water or carbon dioxide from hydrated carbonates, an effluent divider was incorporated, which vented such excess materials in a controlled manner (**Figure 6**). This design evolved (64, 65) over a few additional years, as the launch of the mission was postponed to 1975, given the budgetary squeezes of the early 1970s. Details of the design and construction of the Viking GCMS were published shortly after completion of the mission (66). To increase the chances of success and to investigate different spots on the planet, two identical spacecrafts were launched in the summer of 1975, two months apart. Viking Lander (VL) 1 arrived at the surface of Mars on July 20, 1976; VL-2 landed September 3.

For the analysis, a sample of the Martian soil (~100 mg) was heated to 200°, 350°, and 500°C and the effluent was analyzed by the GCMS. None of the two samples analyzed at each of the two Viking landing sites revealed the presence of indigenous organic materials at a part-per-billion detection level (67), a surprising result, because we had expected to find at least the organic material deposited at the surface of the planet by meteoritic infall. Their absence was finally explained by the oxidizing properties of the surface material, and UV and cosmic radiation.

The mass spectrometer subsystem of the instrument was also used to determine the composition of the atmosphere at the surface of the planet. The major components, CO_2, CO, N_2, and O_2, were measured directly, while the noble gases Ne, Ar, Kr, and Xe and their isotope ratios were determined after enrichment by the repeated chemical removal of the major component, CO_2, by

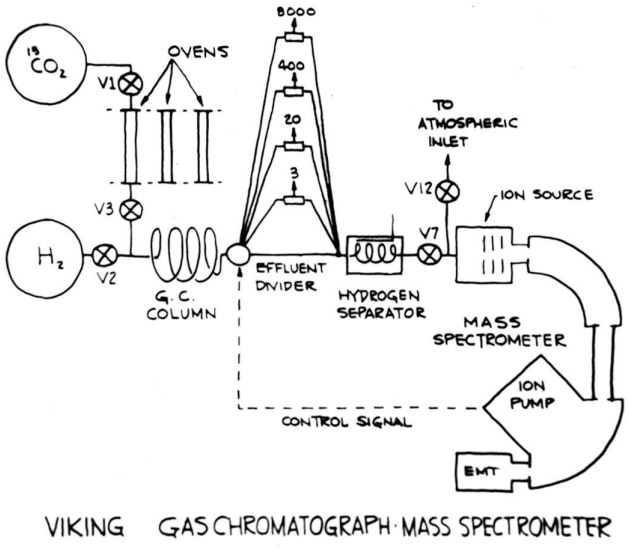


Figure 6

Schematic of the Viking GCMS instrument depicting the sample ovens, GC column, effluent divider, hydrogen separator, and electric and magnetic sectors of the mass spectrometer. Figure reprinted from Reference 67.

lithium hydroxide (68). The mixing ratio and isotope ratios were later used by others to identify certain meteorites to be of Martian origin.

The astrobiology community was not happy with the absence of organic compounds, because this indicated the absence of any living system on Mars. More than 25 years after our results had been published (67), several suggestions were made, implying flaws in the interpretation of the Viking GCMS experiments, most of which were based on a lack of understanding how the instrument and the experiments worked. For example, Navarro-Gonzalez et al. (69) claimed that the instrument was not sensitive enough. All of these assertions had to be rebutted (70). Then, in 2008, the Phoenix mission, also utilizing pyrolysis(py)-MS (without GC), also did not detect organic material in the northern region of Mars, but found evidence for the presence of perchlorate (71). This again was used as an indication that heating of a soil sample containing perchlorate destroys any organic material present (72), but the experimental evidence again was flawed and had to be rebutted (73). The Curiosity rover, presently operating at Gale Crater of Mars, still uses py-GCMS to search for organic compounds on the surface of the planet, although thus far without success. Thus the Viking findings still stand.

My involvement in what is commonly called space research was quite unrelated to the organic and biochemical work discussed in the earlier sections and, at times, was a distraction. However, it required expertise in organic mass spectrometry and I felt that I should get involved. Sometimes I ironically called it scientific charity. However, the Viking mission in particular was an unforgettable experience because of the unique mutual understanding and mutual appreciation that developed over the years of planning and then being together at JPL during the mission among the scientists of various disciplines, all working toward a common goal, the understanding of our neighboring planet.

9. SUMMARY

Over these past more than four decades, mass spectrometry has developed from a purely qualitative and quantitative tool for the analysis of relatively small molecules of known structure to an indispensable method for the structure determination of compounds of biological interest, such as alkaloids, peptides, proteins, and sulfated oligosaccharides. Vast improvements in sample processing, liquid chromatography MS instrumentation, and algorithms for data analysis have greatly added to our ability to structurally characterize biomolecules. Nowhere has this impact been felt more than in the field of protein research and the newly spawned field of proteomics. Although performance and capabilities have greatly increased since the early days, the foundational methods and principles I describe here are still largely those employed today (74).

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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During my academic career at MIT, I have enjoyed working with numerous excellent graduate students, postdoctoral associates, visiting scientists, and technical assistants. It is not possible to name them all here, but this has been done already elsewhere (75). During most of that time, my work was supported by grants from the NIH (GM05472 from 1958 to 1998 and RR00317 from 1965 to 1996, as well as a Training Grant GM01523 from 1964 to 1973), from NASA (NAS-22-009-102) from 1962 to 1979 and, in the earlier years, from the NSF.

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