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Disclosure of new and recurrent microbial metabolites by mass spectrometric methods

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The application of modern mass spectrometry methods (SI-CID-MS/MS; MS^n) in the disclosure of new and recurrent microbial metabolites is discussed. Spray ion (SI) sources coupled to different kinds of mass analyzers enable the determination of molecular weights and chemical formulas of given samples even in mixtures. Diagnostic fragment formation by collision-induced dissociation (CID-MS/MS) and MS^n experiments using ion trap mass analyzers are shown as another indispensable source of structural information. Due to the development of benchtop-type mass spectrometers coupled to high-performance liquid chromatography (HPLC), MS can be practised in almost every laboratory as a powerful tool in natural product analysis. Examples are given for special MS applications in identification of bioactive metabolites from screening strains. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 136–143.

Keywords: mass spectrometry; disclosure of metabolites; MS methods; CID-MS/MS; MS^n; MS applications in natural products screening

Introduction

Due to the huge number (more than 22,000) of known microbial metabolites [1,6], the frequency of recurrent metabolite isolation has increased dramatically. There is an increasing demand for novel and chemically diverse structures from nature launched by industrial high-throughput screening which operates concomitantly various assay systems [7]. In the future, the industrial requirements for new structures will be difficult to provide from natural sources if we are not capable of improving their discovery. Hence, the introduction and broad application of new and powerful tools into natural product screening and discovery appear to be an increasingly urgent task. This article will review instrumental configurations for mass spectrometry (MS) applications in the disclosure of natural products and structural information which can be furnished by mass spectrometric methods. Examples will be given suggesting the value of diagnostic fragmentation for the identification of structures.

The mass spectrometric hardware

The principle of MS implies the separation of gaseous, positively or negatively charged ions by a mass analyzer according to the ratio of their mass to electric charge (m/z). For general information on MS, the reader is referred to special literature reviewing the physico-chemical background in detail [2,4,17–19,20,26]. Traditional MS used purified samples in solid, liquid or gaseous state which were subjected to high-energy ionization under ultra-high vacuum conditions ("hard" ionization; e.g., electron impact [EI] and fast atom bombardment ionization [FAB]).

The advent of spray ionization (SI) sources coupled to tandem-type mass analyzer systems (CID-MS/MS; MS^n) marked a milestone in natural product analysis. SI sources such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) generate a "soft" ionization of molecules which are applied to the ion source as solutions. Due to this, the sample requirement for mass analysis is lower than with classical methods, and SI sources can be coupled directly to high-performance liquid chromatography (HPLC) [15,24]. Thus, the mass analyzer can be considered as a special kind of chromatographic detector furnishing information about the molecular weight, the chemical formula and the chemical structure of every analyte component via the formation of diagnostic daughter ions. Thus, SI source-coupled MS is one of the most promising tools in natural product screening, improving the recognition of new and recurrent metabolites at an early stage in discovery.

In contrast to the classical ionization methods (e.g., EI or FAB), the sample is sprayed into the ion source as a solution together with a gas stream. In the SI source, the solvent is evaporated under atmospheric pressure by the gas stream in the presence of an electric potential of several kilovolts. Thereby, positively or negatively charged ions are generated, which are transferred via a small capillary tube to the high-vacuum chamber of the mass analyzer which will separate them according to their mass to electric charge ratio (m/z) [2,4,17–19,20,26].

Four types of mass analyzers are presently used for the analysis of low-molecular weight compounds (<2000 mw): sector field analyzer composed of a magnetic and an electric sector field, quadrupole, time-of-flight (TOF) and ion trap analyzers. For details of the physical principles, the reader is referred to background references [2,4,17–19,20,26]. The sector field analyzer forms the constitutive part of so-called "high-resolution" instruments which are suitable for the precise determination of molecular weight for a given ion with an accuracy of ±0.001 mass units by comparison with reference molecules with molecular mass that is well known ("peak matching"). From the accurately determined molecular mass, the chemical formula of a sample ion and the number of double bonds and ring structures can be

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suggested [12]. With the TOF analyzer, a relatively accurate suggestion of the molecular weight is possible although the precision is lower than sector field instruments. In contrast, the accuracy of mass determinations by quadrupole and simple ion trap analyzers is insufficient for calculation of the chemical formula. However, two ions differing in one mass unit can be reliably discriminated. The quadrupole, TOF and ion trap analyzers are used frequently as part of the so-called benchtop mass spectrometers which can be operated in almost every laboratory without special technical prerequisites.

The principle of the tandem MS [4] supplying molecular fragments will be described briefly here: a single ion is separated, e.g., by a quadrupole mass analyzer and is not transferred to the detector but to a second quadrupole as a collision chamber where the ion collides with argon gas. The kinetic energy of the ion causes the cleavage of its chemical bonds during the collision (collision-induced dissociation, CID-MS/MS). Thereby, fragments ("daughter ions") of the initial "parent" ion are generated, which can be analyzed subsequently by a second mass analyzer (e.g., a quadrupole in "triple–quadrupole" mass spectrometers). The fragments are specific characteristics of the chemical structure of a given molecule. Hence, tandem MS furnishes additional structural ion formations via diagnostic fragment formation (see below).

Similarly, "daughter ions" can be generated by an ion trap analyzer. In the ion trap, the ions are maintained for few milliseconds on circular levels by electric and magnetic fields [18]. Determination of molecular weight is possible if special radiofrequencies are applied, causing the susceptible ion to leave the ion trap towards the detector (determination of the molecular mass of the ion from the m/z value). Alternatively, the energy can be used to induce collision of the pertinent ion with helium gas whereby fragments are formed within the ion trap analyzer which can be analyzed subsequently. In contrast to CID-MS/MS affording fragments as a mixture of "daughters," "grand daughters", etc., the ion trap fragmentation enables visualization of the "genealogy" of daughter ion formation and in this way furnishes additional possibilities for structural elucidation. Thus, the first collision of a given ion will yield its "daughters" (MS^2). Every one of these "daughters" can be fragmented separately, again, to furnish the "grand daughters" (MS^3) and so on. Up to 10 generations of fragment formation can be generated theoretically, depending on intensity and amount of the parent ion ($MS^2 \ldots MS^{10}$).

Recognition of structural information from the mass spectra

Determination of the molecular weight

A further advantage of the "soft" SI methods in comparison to classical ionization is the rare formation of fragments. Entire molecules are separated as "pseudomolecular" ions (Figure 1) which owe their positive charge to the capture of cations such as H^+, Na^+, K^+ and others. Negative pseudomolecular ions (e.g., $[M-H]^-$) are formed via proton abstraction. In addition to the singly charged ions, there can occur doubly or multiply charged entities. Singly and doubly charged pseudomolecular ions such as $[M+Na]^+$ and $[M+2Na]^{2+}$ are readily distinguishable by "zoom scanning" which renders visible the difference between the isotopic satellites and the main intensity peak ($m/z = \pm 1$ in $[M+Na]^+$ but $m/z = \pm 0.5$ in $[M+2Na]^{2+}$). Furthermore, dimeric and trimeric "cluster" ions such as $[2M+Na]^+$ and $[3M+Na]^+$ frequently occur in addition to $[M+Na]^+$, suggesting, on the first look, that the pertinent compound is a mixture (Figure 2). Due to the possible occurrence of different pseudomolecular ions of the same molecule, there will be a need for several MS experiments to unravel the true molecular weight of a given compound. For instance, by the addition of NH_4^+ ions to the analyte solution, the $[M+Na]^+$ ions can be converted to the $[M+H]^+$ and $[M+NH_4]^+$ ions. Additional recording of the negative pseudomolecular ions ($[M-H]^-$) supplies supporting evidence for the true molecular weight of a given sample molecule and thus enables reliable database searches about novelty. Conversion of the $[M+Na]^+$ ions to the $[M+H]^+$ or $[M+NH_4]^+$ ions is a prerequisite, too, for

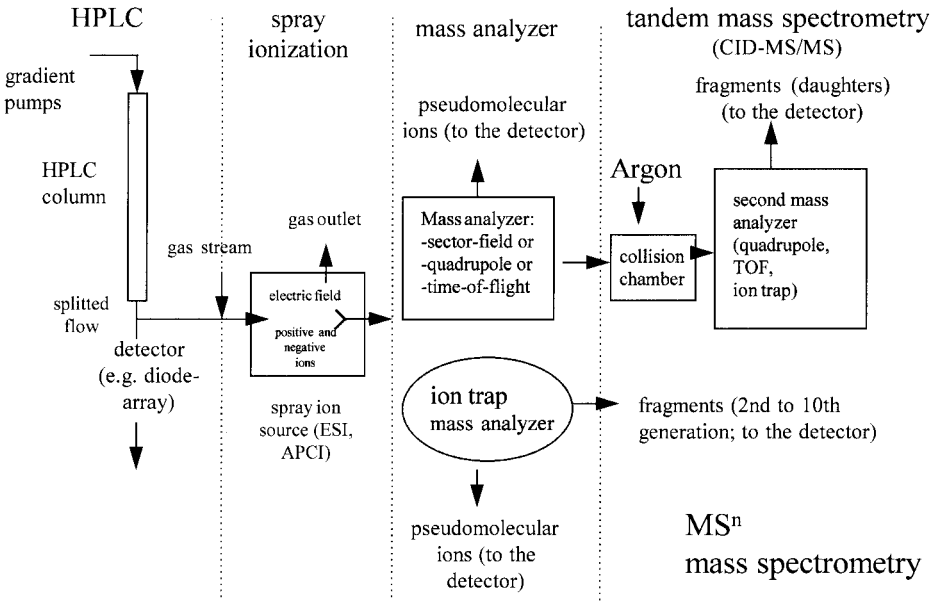

Figure 1 Operation scheme of HPLC-coupled MS.

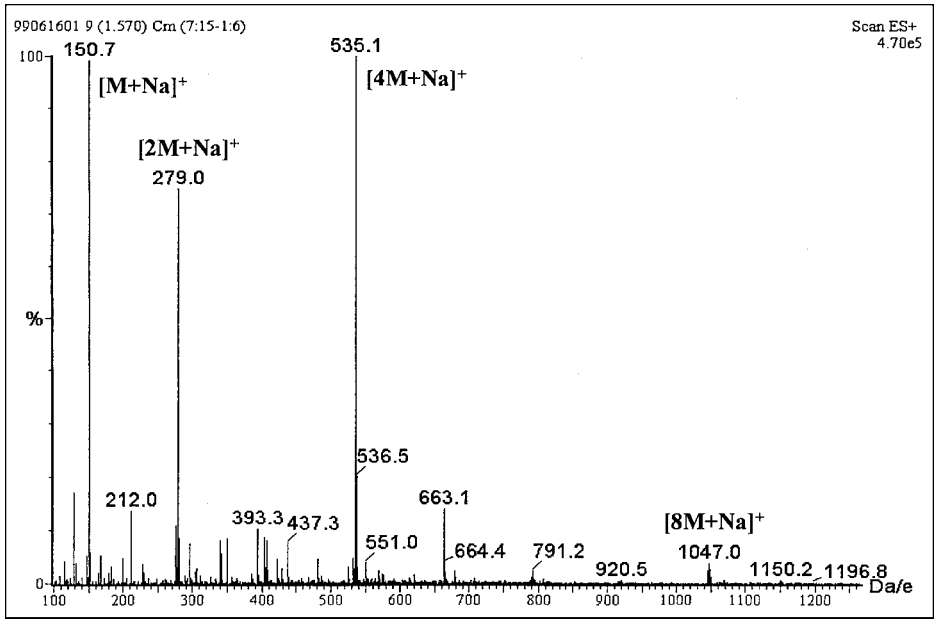

Figure 2 Positive ion mode ESI mass spectrum of 4-thiouracil showing oligomeric singly charged cluster pseudomolecular ions.

fragmentation analysis by CID-MS/MS with a triple–quadrupole mass spectrometer. However, with an ion trap analyzer, the MS^n experiments can be carried out even with the $[M+Na]^+$ ion due to the higher available collision energy.

Another problem of SI-MS concerns the observable peak intensities of the ions. Theoretically, "soft" ionization conditions should enable the ionization of every component of a mixture without concomitant fragment formation to yield a series of individual pseudomolecular ions. However, under practical conditions, the peak intensity of the pseudomolecular ions is strongly dependent on the chemical structure of the given pertinent molecules (Figure 3). In positive ion mode, the high-intensity ions are generated preferably by nitrogen-containing molecules that stabilize positive charges and bind protons. Otherwise, anion-forming compounds, such as carboxylic acids and phenols, are better detected in the negative ion mode [12]. A series of nonpolar compounds such as aliphatic or alicyclic structures, missing polar groups, usually displays low-intensity pseudomolecular ions and thus can be overlooked during SI-MS analysis. In this case, the application of classical ionization methods (EI-MS, FAB-MS)

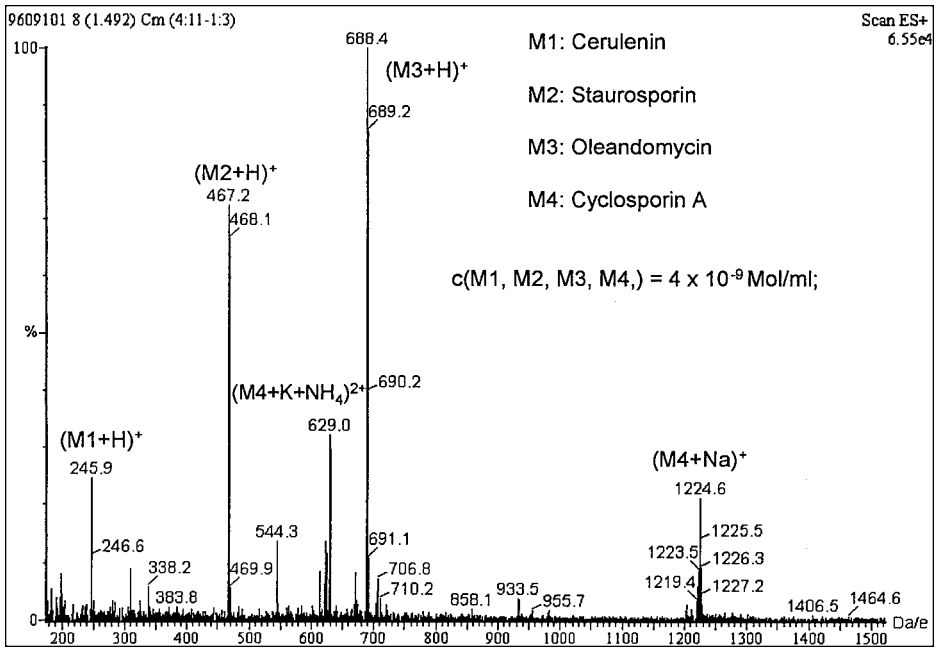

Figure 3 Comparison of the peak intensities of equimolar concentrations of cerulenin (M1), staurosporin (M2), oleanodomycin (M3) and cyclosporin A (M4) during positive ion ESI MS.

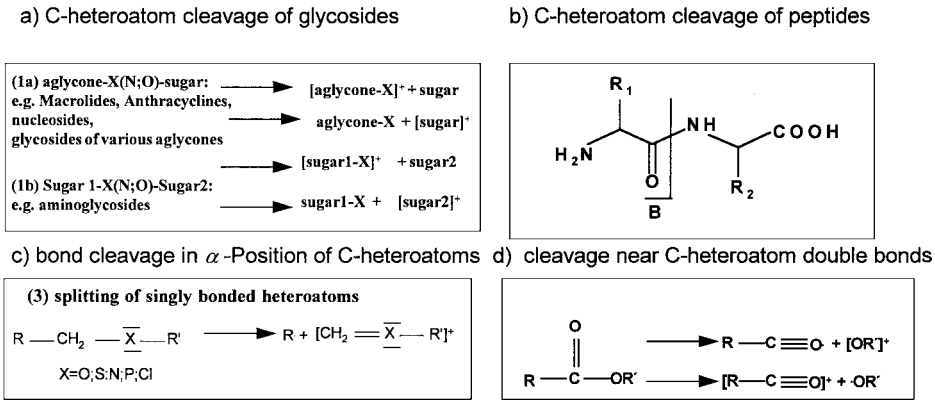

Figure 4 Frequently observed fragmentation rules during CID-MS/MS and MS^n.

appears indispensable. Due to these facts, the ion intensity during SI-MS cannot be taken as a measure of concentration for chemically different molecules in a mixture.

Fragmentation analysis

If ion intensity is too low, fragmentation analysis by CID-MS/MS or MS^n experiments will not be possible. As was discussed above, these latter MS techniques are capable of furnishing additional structural information via diagnostic fragment formation. They are also comparably "soft" while generating relatively few fragments in comparison with classical EI or FAB ionization. During collision of a given pseudomolecular ion with argon or helium gas, the collision energy of the ion is used to cleave preferably polar chemical bonds such as carbon–heteroatom bonds occurring, e.g., in glycosides, esters and peptides. Thus, these structures will generate diagnostically useful fragments (Figure 4) such as sugar fragments, aglycones and *N*-terminal peptide fragments, respectively. Another frequently observed fragmentation type involves cleavage of the bond in α-position to a carbon–heteroatom bond (see below the example of pamamycin). These and some other rules of fragment formation [21] enable some predictions about diagnostically useful fragment formation for more than 50% of the presently known microbial metabolites [12]. However, there are a large number of microbial metabolites, such as polyether-type compounds, that do not display characteristic patterns. Fragment formation as a "molecular fingerprint" cannot be explained, on the first glance, by the above rules of fragment formation. Molecules containing stable carbon–carbon bonds such as polycyclic aromatic structures (e.g., resistomycin-type compounds) display a rather low tendency for fragment formation during low-energy collision (CID-MS/MS). However, even this specific feature could be taken as a diagnostically useful hint confirming the presence of the pertinent molecule.

HPLC-MS

Determination of the molecular weight *via* the pseudomolecular ion(s) and the observation of diagnostic fragmentations (CID-MS/MS; MS^n) can be carried out routinely even with an HPLC-coupled MS system [24]. Thereby, every HPLC peak from the outflow of a HPLC column is sprayed directly into the SI source with a flow rate $\ll$ 0.5 ml min^{-1}. The appearance of a HPLC sample peak will thus increase ion formation in the ion source, and an ion chromatogram can be recorded in parallel to optical detection. Every peak of the ion chromatogram can be characterized by its molecular weight, formula and fragmentation. However, the results of HPLC-MS are strongly dependent upon the sample quality. Non-polar basic compounds, yielding high ion intensities such as aliphatic and alicyclic amines, may often persist on the HPLC column and thus be detected during subsequent runs. Due to the high intensity of these type of compounds, the true sample ion could be overlooked. Cleaning up of the sample and extended washings of the HPLC column after the run are suitable ways to circumvent this occasional problem.

Applications of SI-CID-MS/MS and MS^n in natural products analysis

TLC-MS

Due to the usually high sensitivity of SI-CID-MS/MS, the molecular weight can be determined in many cases even with single spot samples scraped off and eluted from thin-layer chromatograms (TLC). TLC-MS on normal and reverse phase silica gel layers is an alternative to HPLC-MS due to the simplicity of its operation. Thus, in screening 150 freshly isolated *Actinomyces* strains, we used the extract of one agar plate surface culture of each strain, for TLC separation as visualized by UV light and staining and subsequent SI-CID-MS/MS analysis of the main constituents. By this way, more than 20 known metabolites and one new structure were identified.

Disclosure of halogenated compounds

Due to the special isotopic composition of naturally occurring chlorine and bromine, satellite peaks appear around the pseudomolecular ion of a halogenated compound, and thus, MS can be used for identification. From the intensity and the number of the satellite peaks appearing in a distance of m/z ±2, ±4 and sometimes ±6 from the main peak, the identity and the number of halogen(s) in the molecule can readily be determined. Unfortunately, this principle works well only with smaller molecules (<500 mw). The more complex peak structure of bigger molecules renders the recognition of halogens difficult.

Disclosure of glycosides by SI-CID-MS/MS

Usually, glycosides will be cleaved at the glycosidic bond between the aglycone or the anomeric carbon and the oxygen. Thereby,

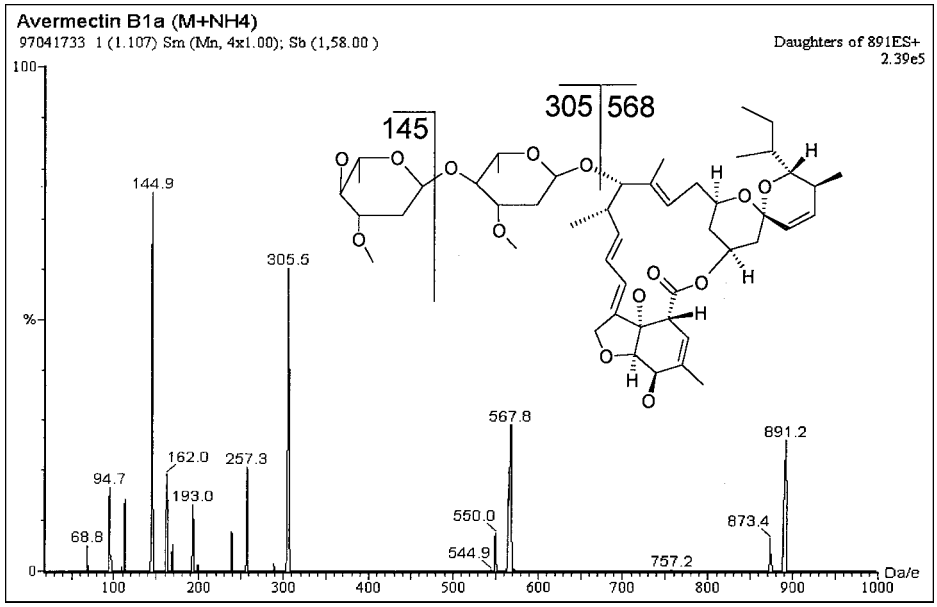

Figure 5 Positive ion CID-MS/MS of avermectin Bla as an example of the fragmentation of a glycosidic compound.

diagnostically useful sugar and aglycone fragments will be formed, which are further transformed occasionally by the elimination of water, methanol, carbon dioxide, etc. The oxygen of the glycosidic bond remains frequently with the sugar fragment. For instance, the diagnostic fragment with m/z 130 in the positive ion CID-MS/MS of daunomycin (daunosamine-O) is readily detectable in a series of other monoglycosylated anthracyclines, suggesting the presence of this aminosugar. With disaccharides of various aglycones (e.g., chartreusin, erythromycin, avermectin), the presence of disaccharide and monosaccharide sugar fragments can be observed in addition to the agly cone fragment (Figure 5). Fragmentation analysis thus appears as a useful way to disclose homologous anthracyclines and angucyclines as we demonstrated previously for related mutactimycins [27]. The same pattern of fragmentation is observable with *N*-glycosides such as nucleoside antibiotics. Fragment analysis was particularly useful for the analysis of chromatographically inseparable mixtures of related cytosine nucleosides such as blasticidin, cytomycin and their 5′-hydroxymethyl homologues [23].

Disclosure of biologically active peptides

During CID-MS/MS, the linear peptide structures are preferably cleaved between the carbonyl and amide nitrogen (B-type fragments). However, in comparison to FAB-MS, a smaller number of fragments will be formed and thus, interpretation of SI-CID-MS/MS spectra of linear peptides is simpler. In the SI-

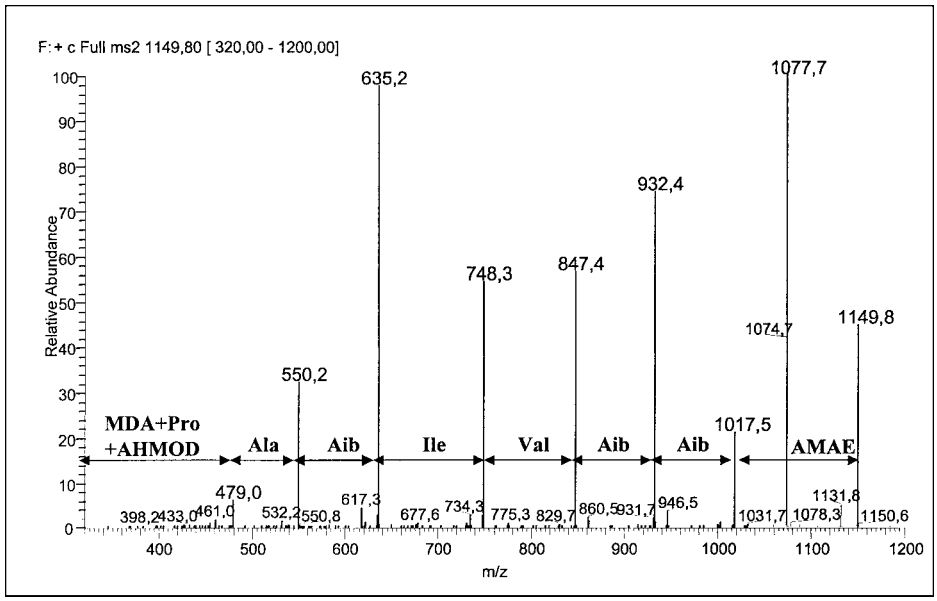

Figure 6 Diagnostic fragment formation by a linear peptide (for abbreviations, see Figure 7).

1 MOA-L-Pro-AHMOD-L-Ala-Aib-L-Ile-L-Ile-Aib-Aib-AMAE

2 2-Me-3-oxotetradecanoic acid-L-Pro-Aib-Aib-Aib-Aib-ß-Ala

3 Ac-L-Phe-Aib-L-Ser-Aib-Aib-L-Leu-L-Gln-Gly-Aib-Aib-L-Ala-Aib-L-Pro-Aib-Aib-Aib-L-Gln-L-Trpol

4 Ac-L-Trp-L-Ala-Aib-Aib-L-Leu-Aib-L-Gln-Aib-Aib-Aib-L-Gln-L-Leu-Aib-L-Gln-L-Leuol

5 Ac-L-Val-Aib-Aib-Aib-L-iVal-Gly-L-Leu-Aib-Aib-L-Hyp-L-Gln-L-Val-L-Hyp-Aib-L-Pheol

6 Ac-L-Leu-Aib-L-Leu-Aib-L-Pheol

Figure 7 Structures of new peptides detected by a mass spectrometric screening: helioferin B (1), lipohexin (2), chrosospermin A (3), ampullosporin (4), bergofungin (5), peptaibolin (6). Abbreviations: MOA: 2-methyloctadecanoic acid; AHMOD: 2-amino-4-methyl-6-hydroxy-8-oxo-decanoic acid; Aib: α-aminobutyric acid; Ac: *N*-acetyl; Trpol: tryptophanol; Pheol: phenylalaninol; Hyp: 3-hydroxypyroline; Gln; glutamine; AMAE: 2-[(2′-aminopropyl)-methylamino]-ethanol.

CID-MS/MS spectrum, positive ion peaks are visible, representing all fragments containing a nitrogen terminus. From the mass differences between two neighbouring peaks, all the pertinent amino acid residues can be calculated, which were cleaved step-by-step beginning with the C-terminal side of the original peptide structure (Figure 6). Thus, linear peptides are susceptible to sequence analysis by MS in contrast to the cyclic peptides which are only incompletely fragmented under conditions of low energy collision.

Due to their intense pseudomolecular ions, linear and cyclic peptides are readily detected by MS, sometimes even in crude extracts. This observation initiated a mass spectrometric screen in our laboratory for new peptides. We considered the strongest pseudomolecular ions of the SI-MS spectrum for molecular weight determination. Database searches suggested the novelty of the pertinent molecular mass, and fragmentation analysis by CID-MS/MS supported novelty determination.

Biologically active new peptides

As a result of the above screen, a series of new peptides and peptaibols (Figure 7), such as aurantimycins [8], chrysospermins [5], helioferins [9], roseoferins (to be published), lipohexin [11], bergofungins [3], peptaibolin [13] and ampullosporin [25], was disclosed. Aurantimycins are strongly cytotoxic peptidolactones inducing oxidative bursts of murine macrophages in very low concentration [8]. Helioferins [9] are cytostatic lipoaminopeptides conveying organic anions such as helianthate from water to organic layers. Lipohexin inhibits prolyl endopeptidase [11]. Peptaibols such as chrysospermins [5], bergofungins [3] and ampullosporin [25] form channels within artificial membranes that display different ion selectivities. Ampullosporin (*Sepedonium ampullosporum*) and chrysospermins (*Apiocrea chrysosperma*) [5,25] induce pigment formation by the fungal strain *Phoma destructiva*, suggesting that these peptaibols interfere with eukaryotic signalling pathways. Subsequently, the fungus was found to respond to some neuroleptic drugs such as chlorpromazin, which induces pigment formation in a similar manner as the peptaibols [25]. Consequently, the ampullosporin was investigated for neuroleptic activity in animals and was as active as chlorpromazin. Both in mice and rats, the characteristic features of neuroleptic activity were demonstrated with peptaibol such as reduction of body temperature and alterations of motor activities without loss of cognitive functions.

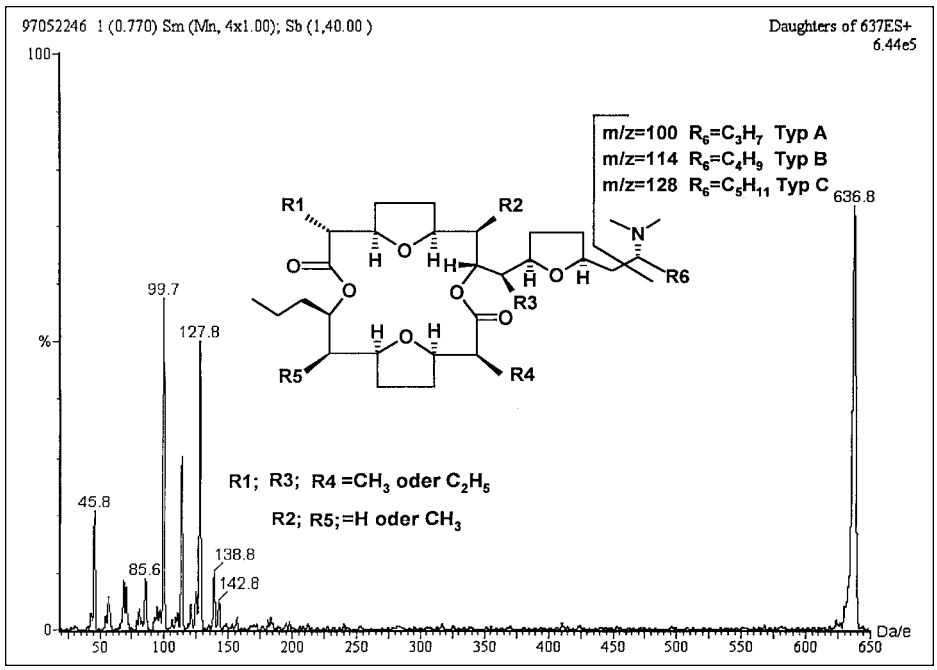

Figure 8 General formula of pamamycins and diagnostic α-cleavage of the tertiary amino side chain from the pseudomolecular ion m/z 636.8 ($[M + H]^+$) yielding fragments with m/z 99.7, 114 and 127.8.

Analysis of complex mixtures of homologous structures by CID-MS/MS

Due to the chemical similarity of homologous structures formed by an organism, their chromatographic separation will often appear as a tedious, time-consuming and sometimes non-practicable task. SI-CID-MS/MS enables the assignment of chemical constitutions even without the preceding chromatographic separation of homologues. Recently, we investigated a complex mixture of pamamycins [10] displaying seven pseudomolecular ions with m/z 607, 621, 635, 649, 663, 677 and 691 ($[M+H]^+$), suggesting initially the presence of seven homologous structures. However, every one of these $[M+H]^+$ ions, with the exception of m/z 607, afforded three diagnostic fragment ions with m/z 100, 114 and 128 due to α-cleavage of three different tertiary amino side chains in every pseudomolecular ion. This feature suggests that the above pamamycin complex is composed of not less than 21 different homologous and isomeric structures which are chromatographically inseparable (Figure 8). The pamamycins were previously shown as inducers of streptomyces morphogenesis and inhibitors of myosine light-chain kinase [16,22]. Our investigations of the biological activity of the pamamycins suggested that this macrolide structure protects certain tissues from autolysis. In a dose of >0.5 mg kg^{-1} egg, pamamycin prevented the *post-mortem* autolysis of 15-day chicken embryo chorioallantoic membrane blood vessels [10].

MS^n applications

The use of ion traps as mass spectrometric analyzers and their application to discovery of natural products are relatively new and emerging techniques. Hence, there is only a limited amount of information available. The major promise of ion trap MS concerns structural information which can be supplied by the step-by-step generation of several (exceptionally, up to 10) generations of daughter ions beginning with one selected pseudomolecular ion (MS^n). Our preliminary experiences with MS^n confirmed the excellent analytical potential of this MS method. In the case of homologous and isomeric linear peptides, the individual components are sometimes more conclusively detectable than by SI-CID-MS/MS. For instance, positional isomers of peptides (e.g., Ala–Aib instead of Aib–Ala located in the middle of a peptide sequence) are hardly recognizable by triple–quadrupole MS (SI-CID-MS/MS) because the "daughter ion" mass spectrum represents several generations of ions such as "daughters," "grand daughters," etc. However, via MS^n experiments, the formation of daughters can be analyzed in a step-by-step manner (MS^2, MS^3, MS^4...MS^{10}). This enables proper localisation of the position of isomerism in a peptide. The promising analytical potency of ion trap MS is clear in the analysis of cyclic peptides and oligolactones [14].

Conclusions

SI-coupled MS such as SI-CID-MS/MS and MS^n has been introduced in the search and discovery of new and recurrent natural products as an indispensable tool. The advent of benchtop mass spectrometers rendered MS as a method available to almost every biotechnical laboratory. Coupling of HPLC with modern MS improved the chances for a more effective disclosure of novel natural products and the recognition of recurrent metabolites at an early stage of discovery. Specific fragmentation behaviour of molecules supplies structural information which enables a more reliable identification of natural compounds. In the future, the collection of fragmentation data in special databases may improve the chances for a more rational identification of the structures of natural products.

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