An MS/MS Library on an Ion-Trap Instrument for Efficient Dereplication of Natural Products. Different Fragmentation Patterns for $[M + H]^+$ and $[M + Na]^+$ Ions†

Andreas Fredenhagen,* Caroline Derrien, and Ernst Gassmann

Syngenta Crop Protection AG, CPR Analytics, Basel, Switzerland

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The structural novelty of lead compounds is very important in the agrochemical and pharmaceutical industries, and as such, natural products can be an important source. Taking into account that the isolation of lead compounds is very time-consuming, the efficient and safe identification of compounds in microorganism and plant extracts isolated previously is essential. A suitable procedure for this task based on an HPLC system interfaced with an electrospray (ESI) source and a Thermo Finnigan LCQ deca XP plus ion-trap mass spectrometer was developed, and an extensive MS/MS spectral library of characterized natural products was built up. This report summarizes the parameters used for acquiring the library spectra and discusses current limitations of the NIST library and search algorithm. The advantages of the newly introduced Mass Frontier 4.0 for the search of MS/MS product-ion spectra are discussed. Different mechanisms for fragmentation of some $[M + H]^+$ and $[M + Na]^+$ ions that were found are proposed. Oligomycin A, a macrolide antibiotic, exhibits different fragmentation mechanisms in positive and negative ion modes. The cleavage of the ester bond is the preferred mechanism in the positive ion mode, whereas two different pathways—one showing a rare retro-Michael-addition—are observed in the negative ion mode.

Natural products are a continuous and proven source of new lead compounds for the agrochemical and pharmaceutical industries. The discovery of novel bioactive compounds is an increasingly challenging area due to successes in the past that has led to thousands of compounds being described.[1] This is especially true in the agrochemical industry, where bioassays for insecticides, herbicides, and fungicides have altered little since the beginning of natural products research about 60 years ago. Furthermore, microorganisms have a better chance of survival in the wild if they hinder the survival of other soil microorganisms by production of antibacterial or antifungal metabolites. As a consequence, the hit rate in the bioassays used to screen such microorganisms for activity tends to be high. For example, the most common secondary metabolites of *Actinomyces* are several polyene antibiotics as well as cycloheximide and antimycin.[2] These compounds are extremely active in antifungal assays.[1] It is therefore essential to detect known active compounds at a very early stage of the discovery process, preferably in the primary growth extracts. This process, called dereplication, allows the prioritization of work to concentrate on those strains that produce novel compounds.

In contrast, the pharmaceutical industry uses a high number of bioassays with changing targets in their high-throughput screens (HTS). A natural products drug discovery program is aiming at all these targets. It is unlikely that the same compounds are responsible for the biological activities observed in HTS, and there is a lesser chance that the company already knows the biologically active principle. Therefore, dereplication strategies used by pharmaceutical companies frequently search for chemical novelty in commercial databases such as Chapman & Hall[1] or Chemical Abstracts Service's NAPRALERT.[3,4]

During the last decade, LC-MS has become the method of choice for the identification of natural products in complex mixtures. Most work has been done on single-quadrupole instruments, giving information on nominal mass.[5−7] Potterat et al. used the accurate mass obtained by a TOF-MS to search literature databases for candidates.[8] The inherent danger of this approach is that compounds are often identified as false positives, especially if they do not have a distinct chromophore that would allow confirmation from characteristic UV spectra. Some papers report using spectroscopic libraries built from a collection of in-house or commercially available compounds.[9−11] Cremin and Zeng searched purified fractions using a parallel LC-MS system with a multiple-channel electrospray interface (MUX).[12]

An obvious disadvantage of the library concept is that the number of compounds detected is limited to those available in-house. Also, every compound has to be analyzed and the generated spectra entered into the library, both of which are time-consuming. However, these drawbacks are outweighed by the reliable identification of target compounds. Several application-specific parameters such as UV spectra, retention time, and mass spectrometric information can be utilized in search routines. However, as the most widely used ionization techniques are the soft atmospheric pressure ionization (API) methods, the resulting spectra provide little fragmentation and hence few characteristic patterns. Therefore, collision-induced fragmentation (CID) is employed either in the source on single-quadrupole mass spectrometers or as separate processes on triple-quadrupole and ion-trap instruments.[13] The quality of CID spectra on the latter two instruments is much higher due to the preselection of the parent ion mass, which results in spectra with much lower noise levels. The process of generating an MS/MS library based on wide-band excitation in an ion-trap MS has been described by Baumann et al. utilizing, dependent on the compounds, ESI or APCI.[14]

In contrast to the papers mentioned, this article emphasizes a practical approach to utilize an MS/MS library for a given set of compounds with the aim to identify every

† In memory of Caroline Derrien.

* To whom correspondence should be addressed. Tel: +41 61 323 66 19. Fax: +41 61 323 37 04. E-mail: andreas.fredenhagen@syngenta.com.

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single one under standardized experimental conditions. The advantages of ion-trap mass spectrometers over triple-quadrupole type instruments will be discussed, and the evaluation of experimental parameters for building an MS/MS library are presented. Emphasis has been given to search routines to highlight the degree of automation possible.

Results and Discussion

MS and MS/MS Instrument Parameters. An ion-trap instrument was chosen because it gives high yields of product ions, and by using wide-band excitation, fragment-rich MS/MS spectra are produced. On the basis of previous experience, electrospray ionization (ESI) was chosen as interface. LC-MS conditions were optimized to reduce in-source fragmentation and to favor the production of $[M + H]^+$ rather than $[M + Na]^+$ ions. Ion-trap instruments show a mass dependency of the optimal tuning parameters. Antimycin A_1 was chosen for automatic tuning of the instrument, because its molecular mass is close to the average of the library compounds and it fragments relatively readily.

The resonance excitation process in an ion-trap mass spectrometer fragments only the selected precursor ion. Therefore, hydroxylated compounds frequently show only the loss of water, which would not give rise to compound-specific library entries. Wide-band activation (or wide-band excitation) applies excitation energy in an MS/MS experiment over a mass range that extends 20 Da below the observed mass of the precursor ion, ensuring that both the precursor ion and the dehydrated precursor ion will undergo fragmentation[15] and thus yield more characteristic MS/MS spectra. However, with wide-band activation, the collision energy has to be increased, because the activation window is wider and the time that the ions are in resonance is shorter.

On ion-trap instruments, fragmentation is induced by applying a resonance excitation rf voltage. The collision energy required to induce fragmentation increases with higher *m/z* range and shows a linear correlation between the mass of the parent ion and the collision energy.[15] This mass-corrected and instrument-adjusted energy is called normalized collision energy, which theoretically should be reproducible on all ion-trap instruments from the same manufacturer.

The dependency of the fragmentation pattern on the normalized collision energy was investigated using some typical natural products. Frequently the pseudomolecular ion was still the dominant peak at a normalized collision energy of 30%. Similar MS/MS spectra were obtained between 40% and 60% normalized collision energy, whereas at higher energies lower isolation yields of fragments were observed. It is postulated that the higher collision energies lead to many more ions being ejected from the ion trap due to nonresonant excitation. A normalized collision energy of 50% was chosen, as this offers informative spectra even with some relatively stable molecular ions and a good isolation yield.

The isolation width defines the mass range taken around the parent ion for subsequent MS/MS experiments. A narrow isolation width is typically used to specifically select the precursor ion and isolate it in the ion trap with as little impurities as possible. Therefore, narrow isolation widths should theoretically be advantageous. However, this was found not to be the case for fragile ions, because the isolation waveform notch imparts some kinetic energy to the precursor ion, causing it to fragment.[16] The following example illustrates this behavior: Filipin III is a polyene antibiotic that has numerous hydroxyl functions. The mass spectrum typically shows $[M + H]^+$ and the loss of one to three H_2O molecules. Using the $[M + H - 2\ H_2O]^+$ ion as the precursor ion, a very weak intensity MS/MS spectrum can be observed even at a relatively wide isolation width of 3.3 Da. However, at 3.8 Da isolation width, the MS/MS spectrum shows two to four H_2O losses with an acceptable intensity of the fragment ions. Further increasing the isolation width did not yield any higher intensity fragment ions. An isolation width of 3.8 Da was therefore chosen to build up the library. Higher values would be desirable for masses above 1000 Da,[14,17] but the Xcalibur software does not allow a change in the isolation width within data-dependent experiments.

Table 1. Type of Precursor Ions Used in MS/MS

ionization	precursor ions	% of library entries ($n = 1020$)
ESI (+)	$[M + H]^+$	72
	$[M + 2H]^{2+}$, $[M + 3H]^{3+}$	2
	$[M + Na]^+$	6
	$[M + NH_4]^+$	3
	$[M + H - H_2O]^+$ or $[M + H - 2\ H_2O]^+$	7
	$[M + H - MeOH]^+$ or $[M + H - 2\ MeOH]^+$	1
	other fragments	3.5
ESI (−)	$[M - H]^-$	5.5

MS/MS Library. MS/MS product-ion mass spectrometric libraries were generated from commercially available and in-house stocked compounds and contained the mass spectra of 1020 compounds. Table 1 summarizes the distribution between positive and negative ion mode measurements and the precursor ion used for the MS/MS spectra creation. About 95% of the compounds were successfully recorded with positive ionization. The selection of the precursor ion was based on its intensity in full scan mode and the intensity of product ions upon fragmentation. Whenever possible, $[M + H]^+$ ions were selected as the preferred choice for precursor ion.

The quality of an MS/MS product-ion mass spectrum can be defined using the number of reasonably intense characteristic fragment ions. The larger the number of characteristic fragment ions, the higher the certainty of identifying a compound based on its MS/MS spectrum. Ion-trap MS/MS have a certain tendency for single-line spectra.[15] Wide-band activation reduces the number of MS/MS spectra with only a loss of a small neutral molecule (e.g., H_2O, NH_3) and hence reduces the number of single-line spectra.

Table 2 summarizes the quality of the MS/MS spectra recorded in positive ion mode. About 70% of the compounds gave high-quality MS/MS spectra with characteristic and intense fragment ions. Only a few compounds (~3%) gave no MS/MS spectra at all. The remaining entries were mainly compounds that produced only one single fragment ion. Exceptions are compounds with several hydroxyl functions, which show the loss of several H_2O molecules. Although these compound produce several intense fragment ions, their identification based on the MS/MS spectra is hampered by the nonspecific nature of these small losses. A similar situation arises with aliphatic methyl ethers or methyl esters, which are quite common functional groups in natural products. These compounds exhibit an intense fragment due to the loss of MeOH.

Only those compounds that gave no signal in positive ion mode were analyzed in negative ion mode. The selection of compounds in negative mode was therefore biased. About

Table 2. Quality of MS/MS Product-Ion Spectra Using ESI(+) Ionization Mode

quality of MS/MS spectra	type of pattern	neutral losses	% library entries
high	several fragment ions with high intensity	characteristic for compound	69.3
	several fragment ions originating from double charged precursor ion	characteristic for compound	1.9
	single fragment ion with high intensity[a]	neutral loss mass > 60 Da	12.0
low	single fragment ion with high intensity[a]	water, methanol, or other small molecules up to 60 Da	8.5
	several fragment ions at $\Delta_{m/z} = 18$ Da	several molecules of water	3.3
	parent plus fragment ions with low intensity	characteristic for compound	1.1
	"grassy" spectra[b]	no characteristic losses	0.9
no fragmentation	parent ion unchanged or below trapping mass range		3.1

[a] Intensity of all other fragment ions is below 20%. [b] Unspecific fragmentation yielding spectra with low-intensity ions over almost the whole mass range.

one-third of the compounds showed the loss of CO_2 as the sole fragment, due to the large number of carboxylic acids in this subset of compounds. Another third gave a more specific single fragment ion MS/MS spectrum, and only one-third gave spectra with several fragment ions. Wide-band activation was not applied, because it did not offer any advantage in the negative ion mode.

Library Search Using NIST Algorithm. MS/MS product-ion mass spectra were acquired automatically using the LCQ's data-dependent setting for the search of unknowns in extracts. The dynamic exclusion puts precursor ion masses on a temporary exclusion list after its MS/MS spectrum is acquired and allows the automatic recording of about 10 different MS/MS spectra per peak. If a compound is present only at very low concentrations, specific MS/MS methods for the appropriate precursor ion were set up. MS/MS spectra acquired in test samples were compared to the in-house-generated library using the NIST algorithm. This algorithm was originally developed for EI-MS spectral libraries and as such was found to have the following limitations for ESI-MS/MS applications.

1. Xcalibur software does not export the precursor ion, although it is known from the experimental settings, and therefore the NIST search algorithm does not make use of it.[14] As outlined above, ESI-MS/MS spectra quite often consist of a single line (Table 2); hence the search algorithm cannot differentiate between differing precursor ions that produce the same single fragment ion. For example, several nucleotide antibiotics having different precursor masses yield adenine ($m/z = 136$) as the only fragment and cannot be differentiated between by the NIST algorithm.

2. The retention time (t_R) cannot be included as an additional search parameter in the library search. The t_R is an important parameter for the safe identification of library compounds.[18] Therefore the t_R difference of likely hits has to be examined manually. With careful control of the HPLC instrument, retention time fluctuation was minimal. On a single instrument over an extended period of time with several columns in use, the retention shifted less than ±0.2 min utilizing a test mixture containing compounds distributed over the whole t_R window. On an identical analytical system, with minor modifications to match elution profiles, the retention time fluctuation was found to be in a similar range.

3. The NIST library format stores the mass information rounded to the closest integer (nominal mass). The difference between accurate and nominal mass increases with higher molecular masses, and as ESI has a broader mass range compared to EI, higher mass ions can lead to rounding errors within the library. Even with precursor ions above 700 Da some product ions have an actual mass that might be rounded up or down depending on the detector's mass assignment. The influence on the match factor was tested utilizing the spectrum shown in Figure 2a: For example, the calculated value of the fragment $m/z = 747$ is 747.39 Da. If the four major masses are rounded up and not down, they shift to the values 712, 730, 748, and 762 Da. The match factor of this modified spectrum with the NIST algorithm drops from 803 to 107. With this value the compound would not be identified. So, this mass ambiguity leads to lower match factors in automated library searches. It must be pointed out that this behavior cannot be corrected using instrument recalibration. New search algorithms are required to take this effect into account.

As a consequence of the NIST search algorithm limitations, verification of potential hits still requires time-consuming manual checking.

Library Search Using Mass Frontier. Very recently, Thermo Finnigan introduced Mass Frontier version 4.0. It was tested for its library search capabilities. The MS^n spectra are arranged as hierarchical spectral trees, and the search can be limited to the same level. Thus, most useful is to search an MS^2 spectrum in an MS^2 library. By this concept the precursor ion is fully integrated in the search. Furthermore, it is possible to import libraries in the NIST format, either as MS or MS^2 libraries. The search precision can be set according to the resolution of the instrument utilized.

An Xcalibur raw data file is opened, all MS/MS product-ion mass spectra of a single run are extracted, and, by spectra deconvolution, similar spectra are combined. Next, all MS/MS spectra are searched against mass spectrometric libraries in a process that takes several minutes for the 500−600 spectra of an HPLC run. MS/MS spectra with a match factor above a certain level are then compared manually to the library entry. As the t_R information is not utilized as a search parameter, this has to be verified case by case. The influence of mass errors due to instrument variations was tested in a similar way as with the NIST search algorithm: A mass error of plus or minus 0.5 Da was introduced to a library entry of the MS/MS spectrum of Figure 2a. When the original spectrum was searched in this library, these modified spectra were found with excellent match factors when appropriate mass precision settings were applied (settings: precision acquired from source).

In conclusion, Mass Frontier v 4.0 allows the search of all MS/MS spectra of a single run and verification of hit candidates on-screen. In an efficient process all MS/MS spectra are searched in the library. Most of the disadvantages of the NIST search algorithm are eliminated, but only a single run can be processed simultaneously and still several manual verification steps are necessary.

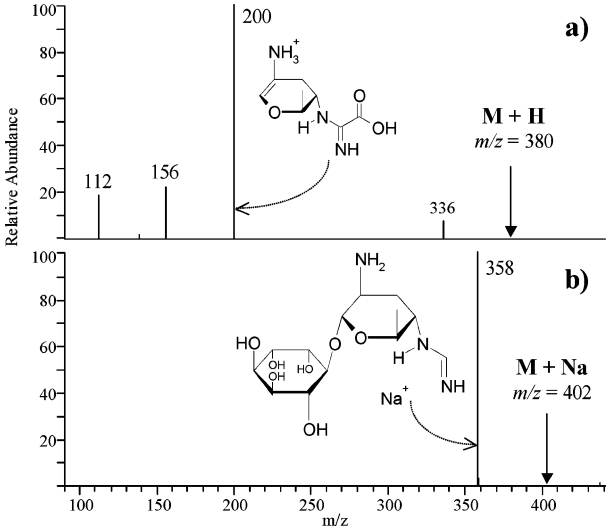


Figure 1. MS/MS of kasugamycin: (a) $[M + H]^+$, (b) $[M + Na]^+$.

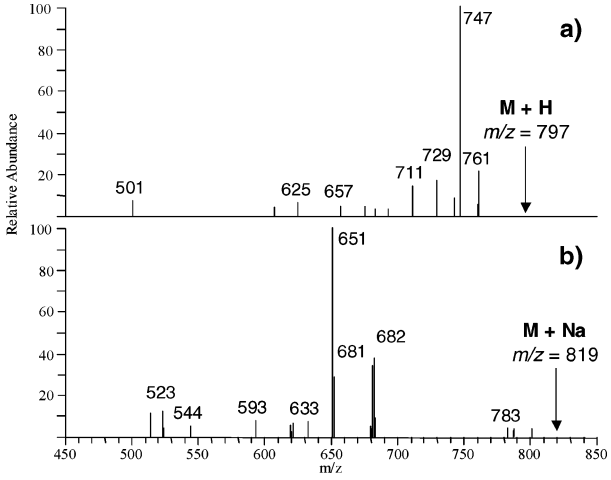


Figure 2. MS/MS of mocimycin: (a) $[M + H]^+$, (b) $[M + Na]^+$.

Different MS/MS Fragmentation Mechanisms for $[M + H]^+$ and $[M + Na]^+$. Among the molecules of our library that showed $[M + H]^+$ and $[M + Na]^+$ ions, the fragmentation patterns observed were basically the same for about one-third of the cases, although the product-ion intensities may have varied. For the remaining two-thirds, different MS/MS product-ion mass spectra were observed by fragmenting the $[M + H]^+$ or $[M + Na]^+$ as the precursor ion. The following common fragmentation patterns were observed for $[M + Na]^+$ ions:

1. Loss of CO_2 seems favored in acids, presumably due to the close spatial location of the Na^+ and the acid moiety. Hence, the MS/MS spectrum of kasugamycin, an aminoglycoside antibiotic produced by *Streptomyces kasugaensis*, shows only the loss of 44 from the $[M + Na]^+$ precursor ion, while in the MS/MS spectrum of the $[M + H]^+$ ion, the loss of inositol as well as other minor losses are observed (Figure 1).

2. Retro-aldol condensation is commonly observed. The polyene antibiotic mocimycin (Figure 2), an inhibitor of the bacterial elongation factor EF-TU, has a cyclic ketal function, which can open to form a β-dicarbonyl moiety, which chelates sodium ions.[19] The MS/MS spectrum of the sodium adduct ion shows one prominent fragment that is presumably formed by retro-aldol condensation (Scheme 1). Edwards et al.[20] observed a minor fragment at *m/z* 629 Da in the thermospray MS spectrum of mocimycin corresponding to same neutral loss. The minor product ions at *m/z* 681 and 682 Da are due to loss of a hydroxypyridone moiety from one end of the ion and a loss of $C_9H_{14}O$ from the other end (Table 3). Fragmentation of the protonated molecular ion (*m/z* = 797 Da) yields loss of MeOH and H_2O. Retro-aldol condensation is also observed upon fragmentation of the $[M + Na]^+$ of the immunosuppressive drug cyclosporin A, which has an unusual β-hydroxyamino acid, whereas the MS/MS spectrum of the $[M + H]^+$ ion described by Halket et al. shows typical fragments of amino acid losses.[14]

3. Phenolic compounds frequently show fragments corresponding to a tropylium-like ion. For example, the MS/MS spectra of the fungal metabolite ascofuranol are displayed in Figure 3. The MS/MS spectrum of the $[M + Na]^+$ ion shows a fragment at *m/z* 221 Da due to the formation of a tropylium-based ion, whereas the major

Scheme 1. Proposed MS/MS Fragmentation Mechanism of Mocimycin

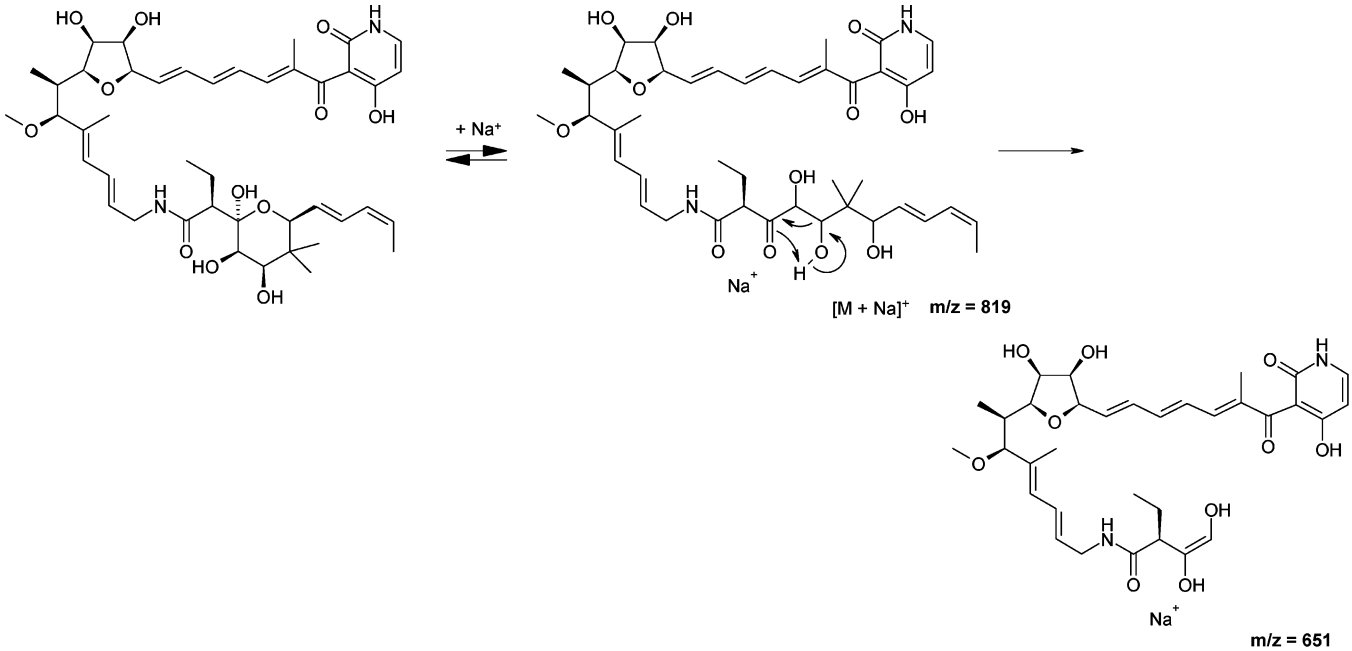


Table 3. MS^3 Data of Oligomycins A and C in Positive and Negative Mode as Well as MS^n Data of Mocimycin and Goldinodox

Mode	Compound	MS^n	Precursor ion (*m/z*)	Fragment ions (*m/z*)						
ESI(+)	**oligomycin A**	MS^2	791 [a]			663	577	549		447
		MS^3	663			→	577	549		447
		MS^3	577				→			447
		MS^3	549					→	531	447
	oligomycin C	MS^2	775	739	721	647	561	533		447
ESI(-)	**oligomycin A**	MS^2	789 [a]	687	675	635		573	533	463
		MS^3	675		→			573		
		MS^3	635			→			533	
		MS^3	573					→		463
		MS^3	533						→	463
	d_4-oligomycin A (in CH_3OD)	MS^2	793		678	638		574	534	
	oligomycin C	MS^2	773	687	659	641	619	573		
		MS^3	659		659			573		
		MS^3	619				→		533	
ESI(+)	**mocimycin**	MS^2	819 [b]	682	681	651		544	523	514
		MS^3	682	→				544		514
		MS^3	681		→	663	621			
	goldinodox [c]	MS^2	833	682	**695**	665	635		537	514

[a] See Figure 4. [b] Selected values; see Figure 2. [c] Goldinodox = *N*-methyl derivative of mocimycin.

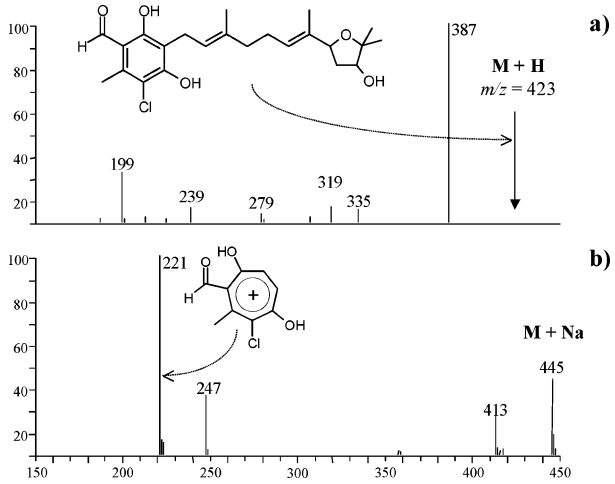


Figure 3. Structure and MS/MS of ascofuranol: (a) $[M + H]^+$, (b) $[M + Na]^+$. The spectra shown originate from the ^{35}Cl monoisotopic precursor ions.

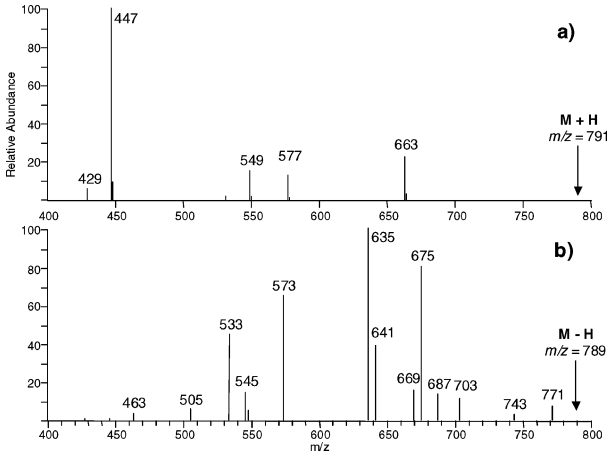


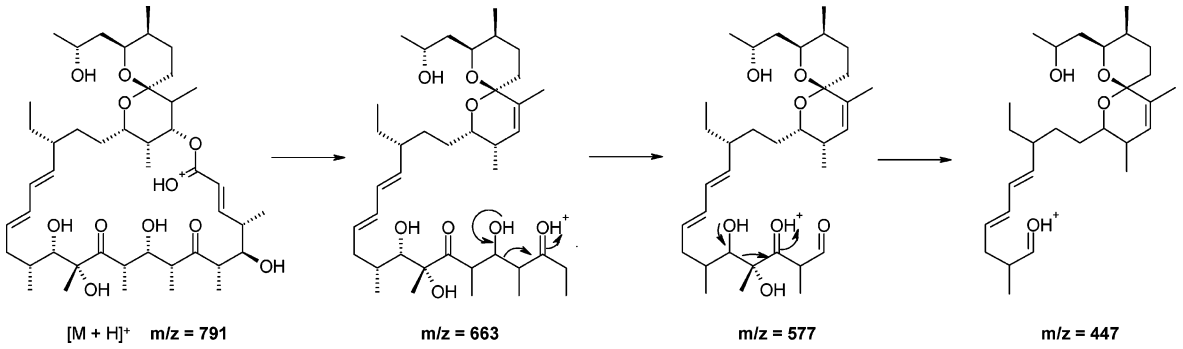
Figure 4. MS/MS of oligomycin A: (a) positive mode of $[M + H]^+$, (b) negative mode of $[M - H]^-$.

fragment ion of $[M + H]^+$ at *m/z* 387 Da corresponds to the loss of two H_2O molecules, which was shown by HR-MS/MS.

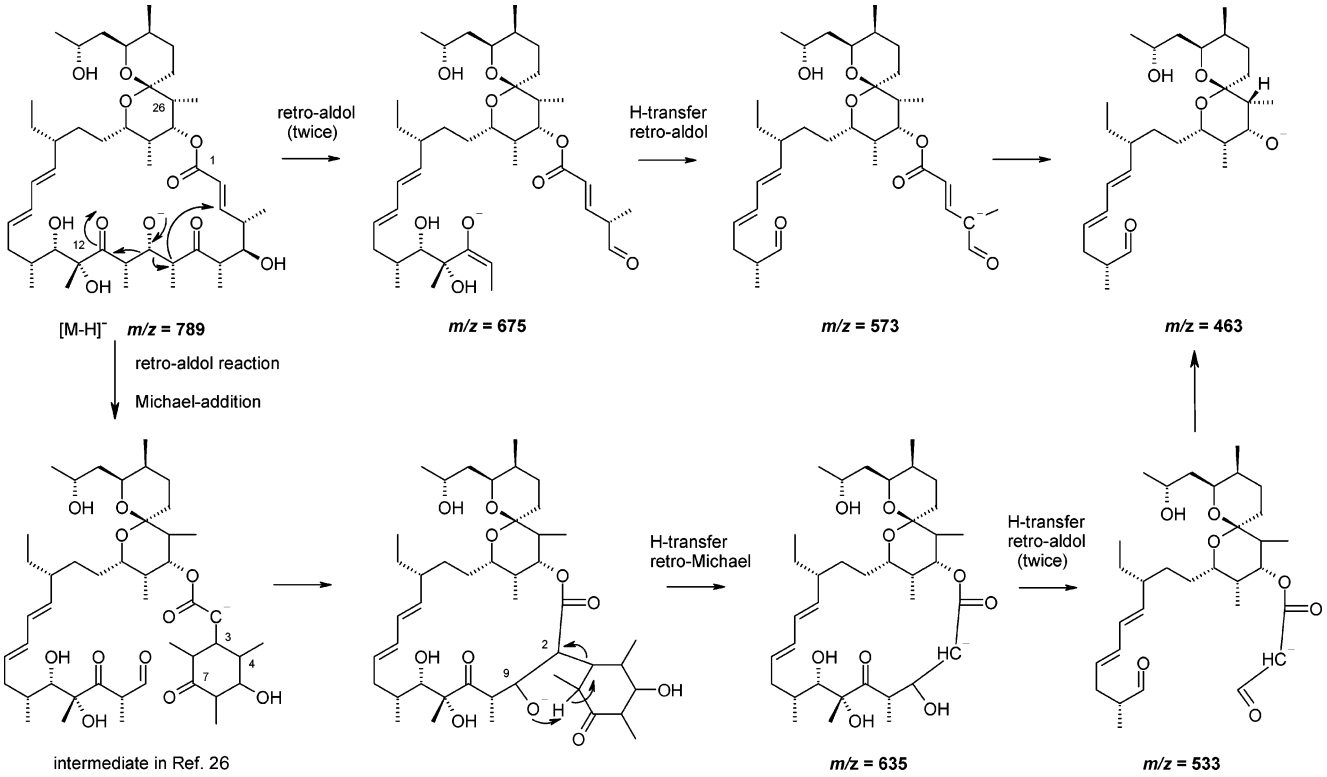
A few cases of different MS/MS fragmentation mechanisms for $[M + H]^+$ and $[M + Na]^+$ as precursor ions were described: McCrery and Gross observed that the $[M + Na]^+$ of digitoxin fragments differently than the $[M + H]^+$, presumably because the sodium ion is affiliated with the sugar moiety, whereas the H^+ is bound to the steroid part.[21] The analysis of phosphatidylcholine, a molecule with an internal positive and negative charge, by Al-Saad and coworkers with post-source decay MALDI-TOF yielded many fragment ions with the sodiated precursor ion, while the $[M + H]^+$ showed only one fragment of the headgroup.[22] Considering that MS/MS spectra of $[M + NH_4]^+$ readily lose NH_3, the following findings are also interesting. Reis et al. investigated ESI-MS/MS of oligosaccharides and observed the loss of glucuronic acid residues in the MS/MS of the $[M + Na]^+$ ion and various, but different, fragments in the MS/MS of the ammonium adduct.[23] Madhusudanan et al. suggested a retro-aldol fragmentation in the MS/MS spectra of the $[M + Na]^+$ ion of acylated nitro derivatives, whereas the ammonium adduct showed elimination of NH_3 followed by loss of HOAc.[24]

The fragmentation mechanisms of $[M + Na]^+$ ions seem to be determined by the position of the chelated sodium ion rather than the stability of individual bonds. Sometimes, MS/MS spectra of $[M + Na]^+$ ions tend to exhibit more specific fragmentation patterns than $[M + H]^+$ ions and thus allow better identification of molecules based on their MS/MS spectra.

Scheme 2. Proposed MS/MS Fragmentation Mechanism of Oligomycin A in Positive Mode



Scheme 3. Proposed MS/MS Fragmentation Mechanism of Oligomycin A in Negative Mode[a]



Oligomycin A. Different MS/MS fragmentation mechanisms were found in the positive and negative ion mode for oligomycin A, a macrolide antibiotic (Figure 4). The first fragmentation step in ESI(+) is preferentially cleavage of the ester bond, followed by retro-aldol condensations at three sites (Scheme 2).

In ESI(−) the mechanism is more complicated and two independent pathways can be observed in MS/MS experiments, which have been proven by selective MS/MS/MS (MS^3) experiments (Table 3). The fragment ion at *m/z* 675 Da is formed by two consecutive retro-aldol reactions and the one at 573 Da by a third retro-aldol condensation breaking the bond between C-12 and C-13 (Scheme 3). The observed loss of 102 Da, corresponding to $C_5H_{10}O_2$ (the C-10 to C-12 moiety), is also observed in the second pathway at the transition from *m/z* 635 Da to 533 Da. In oligomycin C, the 12-deoxyderivative of oligomycin A, the observed neutral loss is only 86 Da, or one oxygen less, clearly indicating that this part of the molecule is involved (Table 3). The following three observations indicate that the loss of 154 Da generating *m/z* = 635 Da fragment originates from a part of the macrolide moiety from carbon 1 to 12: Both fragment ions at 573 Da as well as at 533 Da decay further into 463 Da (C-13···C-23-OH) in selective MS^3 experiments; the MS/MS of oligomycin D, the 26-demethyl derivative of oligomycin A, shows all these fragments at 14 Da lower, indicating that the cyclic ketal function remains unchanged. A high-resolution MS/MS experiment suggests a molecular formula of $C_{36}H_{59}O_9$ for 635 Da (Table 4). As there is no directly accessible moiety starting from C-9 that corresponds to the loss of 154 Da, a ring opening as well as a ring closure has to take place. The proposed mechanism suggests loss of a cyclohexyl derivative which is closely related to a basic degradation product of oligomycin A[25] and is consistent with MS^3 experiments in deuterated MeOD (Table 3). The retro-Michael reaction is quite rare in fragmentation reactions and has been described for thioether conjugates of acrolein.[26]

Table 4. HR-MS/MS of Oligomycin A of Selected Fragment Ions

mode	precursor ion (*m/z*)	fragment ion found (Da)	calc formula	calc fragment ion (Da)	error (ppm)
ESI(+)	791 $[M + H]^+$	447.3528	$C_{28}H_{47}O_4$	447.3474	12
ESI(−)	789 $[M - H]^-$	675.4416	$C_{39}H_{63}O_9$	675.4472	8
		635.4258	$C_{36}H_{59}O_9$	635.4159	16
		573.3958	$C_{34}H_{53}O_7$	573.3791	29
		533.3640	$C_{31}H_{49}O_7$	533.3478	16

Conclusion

Dereplication analysis of natural products is accomplished by an LC/MS system consisting of a UV/VIS DAD detector and an ion-trap mass spectrometer operated in ESI mode. It allows the safe identification of major metabolites directly from crude extracts using data-dependent scanning methods. At the current stage, a second injection with manually designed MS/MS methods has to be utilized for identification of minor compounds. MS/MS data are searched against an in-house library using the NIST search algorithm or Mass Frontier. Presently this still includes manual steps; however the programming of an automation of the data processing steps along the lines outlined in this work is relatively straightforward.

The careful evaluation of MS/MS fragmentation patterns enabled the addition of compound-specific spectra to the library. Thus, if both the $[M + H]^+$ and the $[M + Na]^+$ were present in the MS spectrum, the precursor ion which gave a characteristic MS/MS spectrum was selected and added to the library. The higher intensity and better selectivity of fragment ion of some $[M + Na]^+$ can also be utilized in quantitative analysis on an ion-trap instrument, because the background noise is reduced, if the neutral loss is compound-specific and if a single major product ion is formed.

Experimental Section

Chemicals. Natural products were sourced from Syngenta Crop Protection's reference compounds collection. Water for chromatography, TFA (BioChemica), MeCN (gradient grade), and MeOH (p.a.) were from Fluka, Buchs, Switzerland.

HPLC. The liquid chromatograph consisted of a Waters HPLC 2690 (Waters Corp. Milford, MA), a Waters PDA 966, and a column heater module (WAT 270852). Column: Develosil RP-aqueous, 5 μm, 140 Å, 150 × 2.0 mm (Nomura, Seto, Japan); flow rate 0.4 mL/min; eluent A: H_2O/TFA 100:0.01; eluent B: MeCN/TFA 100:0.01; gradient: 0 min 0% B; 5 min 25% B; 15 min 55% B; 25−33 min 100% B; 40 °C; UV-detection: 200−600 nm, resolution 2.4 nm; injection volume 5 μL of a solution (0.5 mg/mL for reference compounds) in MeOH. The flow was split 1:5 postcolumn prior to MS analysis. For ESI(−) 0.05% (v/v) HCOOH instead of TFA was added to the eluents and a linear gradient 0% B to 100% B in 15 min at a flow rate of 0.5 mL/min was utilized.

Independently of the MS/MS library search, chromatographic data from the photodiode array detector were compared to an in-house UV/VIS library using Waters' Millennium software. About 83% of the compounds showed absorption bands in the UV/vis range and thus were added to a library. The other compounds showed either no absorption above 200 nm or only end absorption.

MS. A Thermo LCQ Deca XP plus (Thermo Finnigan, San Jose, CA) mass spectrometer equipped with electrospray interface was used. It was operated with Xcalibur software version 1.3. A sheath gas setting of 30 units was used (no auxiliary or sweep gas flows were employed) and a spray voltage of 5 kV applied. The heated metal capillary was maintained at 250 °C. The system was optimized for *m/z* 549 $[M + H]^+$ of antimycin A_1 in the positive mode. Typical parameters: capillary voltage 20 V; tube lens offset −19 V; entrance lens −95 V; mass range 120−1500 Da. MS/MS parameters: isolation width 3.8 Da; wide-band excitation activated; normalized collision energy 50%; activation time 30 ms; injection waveform: none. The mass range in the MS/MS mode is defined by the instrument. As some multiply charged precursor ions were utilized, the default charge state was set at 2. For the negative mode the system was tuned with *m/z* 167 $[M - H]^-$ of 3-methoxysalicylic acid. Typical parameters: spray voltage 2.5 kV; capillary voltage −32 V; tube lens offset −19 V; entrance lens 48 V; normalized collision energy 45% without wide-band excitation.

HR-MS/MS. If higher resolutions mass spectra were required to confirm assignments, these were recorded on a Q-TOF I (Micromass, Manchester UK) instrument equipped with an electrospray source (ESI; source temp 150 °C; desolvation temp 350 °C; cone voltage 20 V, collision energy 17−22 V) and an Agilent 1100 LC (YMC-Pak ODS-AQ 5 μm, 120 Å, 125 × 2 mm; mobile phase A: H_2O/HCOOH, 99.5:0.5; mobile phase B: MeCN/HCOOH, 99.5:0.5; 0.2 mL/min; gradient: 0 min 5% B, 2 min 5% B, 12 min 95% B; injection volume: 5 μL of a solution in MeOH).

MS/MS of cyclosporin A 1224.7 Da (ESI(+), $[M + Na]^+$): 1206.6, 1196.7, 1178.7, 1112.7, 1084.6.

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