Rapid Analysis of Antibiotic-Containing Mixtures From Fermentation Broths by Using Liquid Chromatography–Electrospray Ionization–Mass Spectrometry and Matrix-Assisted Laser Desorption Ionization–Time-of-Flight–Mass Spectrometry

Bradley L. Ackermann and Brian T. Regg
Hoechst Marion Roussel, Inc., Cincinnati, Ohio, USA

Luigi Colombo, Sergio Stella, and John E. Coutant
Lepetit Research Center, Gerenzano (VA), Italy

A crucial step in the isolation of antibiotic substances is establishing whether or not the isolated material represents a new chemical entity. Because of the importance of molecular weight to this process—known as dereplication—mass spectrometry has traditionally played an active role. In this communication a strategy for utilizing liquid chromatography–mass spectrometry (LC/MS) for novelty assessment is described. Crude extracts (20–50 μg) are chromatographed by conventional bore high-performance liquid chromatography (1 mL/min) after which a postcolumn split to divert roughly one-tenth of the sample to the mass spectrometer for molecular weight determination by electrospray ionization (ESI) mass spectrometry. The majority of the effluent is sent to a UV detector and ultimately collected as 1-min fractions for biological testing. As a secondary confirmation of molecular weight, an aliquot of each fraction ($< 5\%$) is taken for analysis by matrix-assisted laser desorption ionization (MALDI). The improved efficiency of this approach over more traditional schemes utilizing off-line fraction collection and conventional ionization methods can be explained by several factors. First, the superior sensitivity of ESI and MALDI means that less material is required for successful analysis. Second, on-line LC/MS optimizes the efficiency of sample transfer and saves both time and labor. Furthermore, the concentration dependence of ESI allows a majority of the material injected for LC/MS to be recovered for biological testing without compromising the signal available for molecular weight determination. As a validation of the above method, crude extracts containing two well-characterized antibiotics—teicoplanin and phenelfamycin—were examined. Results from these analyses are presented along with data from the analysis of a potent unknown antifungal sample.
 (J Am Soc Mass Spectrom 1996, 7, 1227–1237)

The need for new anti-infective agents is a well documented problem [1, 2]. Because of the frequent and eventual tendency of pathogenic bacteria to acquire resistance to current drugs, new antibiotics are continually being sought. In recent years this problem has been exacerbated by several factors. For instance, it has been widely speculated that the overuse of antibiotics has accelerated the rate of mutation and ultimately resistance to current therapies [3]. Other contributing factors, such as the increased number of immunocompromised patients due in part to the AIDS epidemic [4], have also contributed to a situation where novel antibiotic compounds are more in demand now than perhaps at any time in recent history.

Although several strategies exist for the discovery of antibiotics [5–7], a majority of the work still consists of screening naturally occurring sources—most commonly soil—in search of microorganisms that produce secondary metabolites inhibitory to target bacteria or fungi. Once such organisms are identified through microbiological screening [8, 9], they are subjected to large scale fermentation to obtain enough crude material for isolation and structural elucidation of the active substances.

Address reprint requests and correspondence to Dr. Bradley L. Ackermann, Hoechst Marion Roussel, Inc., 2110 E. Galbraith Road, Cincinnati, OH 45215.

1044-0305/96/$15.00
PII S1044-0305(96)00104-3

Received April 2, 1996
Revised July 3, 1996
Accepted July 6, 1996

Despite difficulties associated with structural elucidation of substances from fermentation broths, a far greater impediment to the discovery of new antibiotics is the fact that most isolated substances that show activity have previously been identified. Thus, a crucial step in the overall process is to establish whether or not a newly isolated substance indeed represents a new chemical entity. The process of establishing novelty is referred to as *dereplication*, as the aim is to avoid isolating and determining the structure of a substance that is not novel.

At the beginning of the dereplication process a crude extract from a fermentation broth is fractionated by high-performance liquid chromatography (HPLC). A common practice is to perform photodiode array detection on-line during fractionation to establish the chromophore(s) of the substances in the mixture [10, 11]. The isolated fractions are then tested for biological activity, while mass spectrometric analysis is conducted in parallel to establish molecular weights for products residing in active fractions. If molecular weight determination is not successful, a further round of purification, biological testing, and mass spectrometry must be conducted before a correlation of molecular weight and activity can be obtained.

Fortunately, it is possible to make an initial evaluation of novelty in the absence of complete structure elucidation. To aid in this determination, extensive data bases have been created that catalog bioactive molecules according to parameters such as the taxonomy of the producer strain, the observed spectrum of activity, the method used for extraction/isolation, and physical data such as molecular weights and UV absorption maxima [12, 13]. Of the various types of information assembled, molecular weight is the most critical to an effective search since it affords a level of structural specificity not found with the other search parameters. Generally speaking, once molecular weight information is obtained it is possible to make an initial evaluation of novelty or to at least suggest further experiments needed to make a pivotal decision on whether or not to proceed with the time and labor intensive process of complete structural elucidation.

Mass spectrometry has historically played an important role in the structural characterization of antibiotics [14, 15] and has been used in the process of novelty evaluation in our laboratory for a number of years. Typically, insertion probe techniques such as electron impact (EI), chemical ionization (CI), and fast atom bombardment (FAB) have been used. However, because of the limited scope and sensitivity of these methods, as well as the labor associated with processing and analyzing individual fractions, we sought to use liquid chromatography–mass spectrometry (LC/MS) for this purpose.

A variety of LC/MS methods including direct liquid introduction [16], thermospray [17], particle beam [18], and continuous flow FAB [19] have been applied to the analysis of antibiotics. However, none of these methods was considered reliable enough to be incorporated into a high throughput scheme for antibiotics dereplication. With the availability of LC/MS interfaces based on atmospheric pressure ionization (API) technology, this situation has changed dramatically. Although a number of reports have appeared in the literature that use either atmospheric pressure chemical ionization (APCI) [20, 21] or electrospray ionization (ESI) [22, 23] for the analysis of antibiotics, surprisingly little attention has been given to the use of LC/MS for dereplication.

Previously, we demonstrated the feasibility of liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI/MS) for routine characterization of fermentation broth extracts [24]. In this technique, crude extracts were fractionated by HPLC, and post-column effluent splitting was used to recover a majority of the sample for biological activity testing while ESI/MS supplied molecular weight information for components in the mixture. Since then, a similar strategy was adopted by Constant and Beecher [25] for dereplication of natural products derived from plant extracts.

In this article, a detailed account is given on the procedure developed in our laboratory for the use of LC-ESI/MS for dereplication of antibiotic substances isolated from fermentation broths. At the core of the strategy presented is the use of on-line LC/MS to dramatically enhance the efficiency of the novelty determination process. The methodology described is illustrated in the analysis of fermentation broth extracts containing two well-characterized antibiotics as well as the analysis of an unknown antifungal sample. A unique feature of the strategy presented is use of matrix-assisted laser desorption ionization (MALDI) to serve as a secondary confirmation of the molecular weights determined by ESI. The results obtained permit discussion about the role and utility of both ionization techniques in the dereplication process.

Experimental

Materials

All reagents used were of the highest quality available and were used without further purification. Acetonitrile (CH_3CN) was purchased from Burdick and Jackson (Muskegon, MI). Water used for mobile phase preparation was purified by using a Millipore (Milford, MA) Milli-Q water filtration system. Ammonium formate and ammonium bicarbonate were obtained from EM Science (Gibbstown, NJ). Formic acid (88%) was purchased from Fisher Scientific (Fair Lawn, NJ). α-Cyano-4-hydroxycinnamic acid was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Crude Antibiotics Preparation

Crude teicoplanin complex was obtained by butanol extraction from the fermentation broth of *Actinoplanes*

teichomyceticus ATCC 31121 as previously described [26]. Crude phenelfamycin complex (GE21640 F VI 45) was obtained from the fermentation broth of *Streptomyces* sp. GE21640. The filtered fermentation broth was extracted with ethyl acetate and the organic phase was concentrated under reduced pressure. The crude phenelfamycin complex precipitated upon addition of petroleum ether to yield an oily residue. Both strains belong to the Lepetit strain collection. The antifungal sample GE23845 EA II was also produced by microbial fermentation (full taxonomy not performed) and isolated by ethyl acetate extraction. To minimize antibiotic degradation all procedures used in the isolation process were performed below 40 °C. Crude extracts were stored as solids in amber vials at −20 °C prior to analysis.

Instrument Configuration for Liquid Chromatography–Mass Spectrometry

Figure 1 shows a detailed representation of the instrumental configuration used for LC/MS. The HPLC system consisted of a Waters 600MS pump and a Waters 600E system controller (Waters Chromatography, Milford, MA). Injections were made using a Rheodyne (Cotati, CA) 7725 injector equipped with a 20-μL external loop. Solutions for injection were prepared by dissolving a weighed amount of solid extract in mobile phase. Typically, 20–50 μg of crude fermentation broth extract was injected on-column and chromatographed by using gradient elution at a flow rate of 1 mL/min. The column used for all analyses was a 5-μm Nucleosil C18 (4.6 mm × 25 cm) obtained from Phenomenex

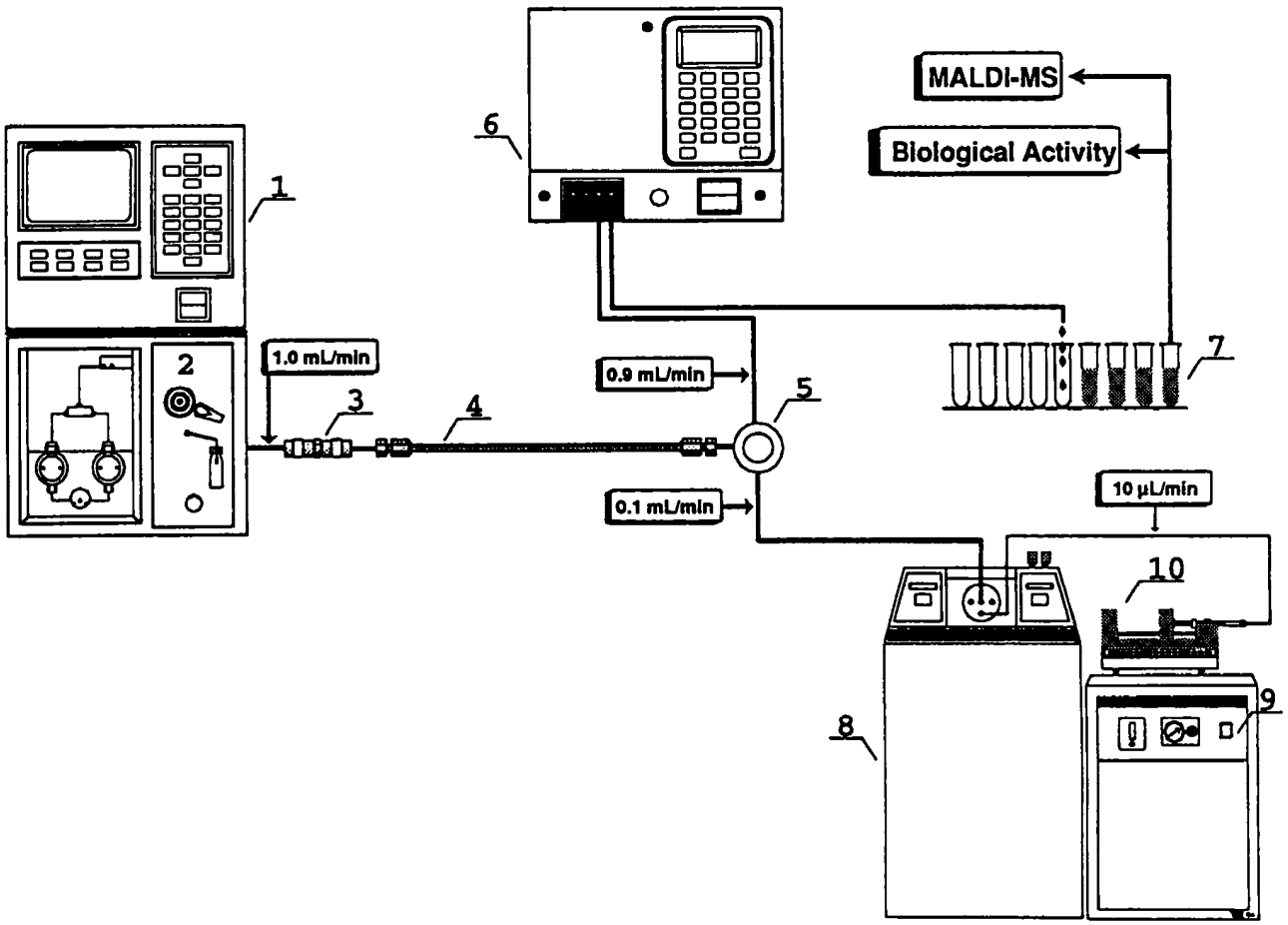


Figure 1. Diagram depicting the instrumental configuration used for mass spectrometry characterization of crude fermentation extracts. The system consists of the following components: (1) HPLC, (2) loop injector, (3) guard column, (4) 5-μm C18 column (4.6 mm × 25 cm), (5) zero dead volume tee, (6) UV detector, (7) fraction collector, (8) triple quadrupole mass spectrometer equipped with ESI interface, (9) ESI power supply and gas manifold, and (10) syringe pump. Typically, 20–50 μg of a crude extract is injected and chromatographed by using an analytical bore HPLC column (4.6-mm i.d.) at a flow rate of 1 mL/min. A low dead volume tee located postcolumn is used to split about one-tenth of the effluent to a triple quadrupole mass spectrometer for ESI analysis. The remainder of the effluent is sent to a UV detector and ultimately collected as fractions for biological activity assessment. An aliquot of each fraction (20–50 μL) is also taken for analysis by MALDI/MS serving as a second means for molecular weight determination.

(Torrance, CA) and protected by a C18 guard column. A Valco (Houston, TX) zero dead volume tee was used postcolumn to effect a 1:10 effluent split diverting a majority of the sample to a Waters 486 tunable absorbance detector by using 0.010-in. i.d. polyetheretherketone (PEEK) tubing. The remainder of the flow (~ 0.1 mL/min) was delivered to the ESI interface of a Finnigan MAT (San Jose, CA) TSQ 700 triple quadrupole mass spectrometer through 0.005-in. i.d. PEEK tubing. Prior to fraction collection the UV absorbance was monitored on-line. A wavelength of 254 nm was used for the analysis of teicoplanin, while 230 nm was used for both the analysis of phenelfamycin and GE23845 EA II. The effluent exiting the UV detector was collected as 1-min fractions into polypropylene microcentrifuge tubes. A 20–50-μL aliquot of each fraction was removed for subsequent analysis by MALDI, while the remainder was concentrated to dryness using a Speed Vac Concentrator (Savant Instruments, Farmingdale, NY) and submitted for microbiological testing.

High-Performance Liquid Chromatography Gradient Formation

All chromatography was performed by using variations of a binary solvent system composed of CH_3CN and an ammonium formate buffer. For the analysis of teicoplanin complex a 2-g/L ammonium formate buffer was prepared in water and adjusted to pH 4 by using formic acid. For all other analyses, ammonium formate was prepared in water (1 g/L) and used without pH adjustment. For teicoplanin, mobile phase A was prepared as (5/95) CH_3CN/buffer (v/v), while mobile phase B was composed as (70/30) CH_3CN/buffer (v/v). A linear gradient from 10 to 40% B was performed over 40 min.

Chromatographic separation of phenelfamycin complex and the antifungal sample GE23845 EA II were each accomplished by using the following mobile phase combination. Mobile phase A was (5/95) CH_3CN/ buffer (v/v) and mobile phase B consisted of (80/ 20) CH_3CN/ buffer (v/v). For the phenelfamycin analysis, a linear gradient from 50 to 90% B over 20 min was employed. For the antifungal sample, a linear gradient from 30 to 70% B was performed over 40 min followed by a ramp to 100% B by 45 min.

Electrospray Ionization Mass Spectrometry

The ESI interface used was provided by Finnigan MAT and used without modification. The liquid chromatography (LC) effluent (~ 0.1 mL/min) was introduced through the sample inlet port in the center of the ESI probe and exited through the stainless steel ESI needle (26 gauge) held at a potential of 5.5 kV. The ESI needle was located roughly 2 cm from the heated stainless steel capillary orifice (0.5-mm i.d.) that separated the ESI spray at atmosphere from the high vacuum condition of the mass spectrometer. The capillary was heated to 200 °C for all analyses. To further assist desolvation, a nitrogen sheath gas (60 lb/in.^{2}) was introduced through the ESI probe coaxially around the ESI needle. In the case of phenelfamycin a liquid sheath, sandwiched between the sample and gas flows, was introduced through the ESI probe. To promote cationization, a potassium bicarbonate solution prepared by using tap water (1 mg/mL) was introduced at a rate of 10 μL/min by using a Harvard syringe pump. All mass spectra were acquired by using Q3 as the scanning quadrupole. A mass range of m/z 250–2000 was covered with a scan time of 3 s. All data were collected in the positive ion mode by using an electron multiplier voltage of 1200 V and a conversion dynode potential of −15 kV.

Matrix-Assisted Laser Desorption Ionization–Time-of-Flight Mass Spectrometry

MALDI analysis was performed by using a Fisons TofSpec (Manchester, UK) linear time-of-flight (TOF) instrument equipped with a nitrogen laser (337 nm, 4-ns pulse) and a 0.7-m flight tube. An acceleration potential of 20 kV was used for all experiments. Aliquots removed from HPLC fractions were mixed 1:3 (v/v) with α-cyano-4-hydroxycinnamic acid (CHCA) prepared as a 10-mg/mL solution in 60/40 CH_3CN/0.1% trifluoroacetic acid (TFA) in water (v/v). One microliter of an aqueous solution of KCl (~ 30 nmol) was then added to this mixture. After mixing, 2 μL of this combined solution was transferred to the stainless steel MALDI target and allowed to air dry prior to analysis. For the phenelfamycin experiment, renin substrate tetradecapeptide (porcine sequence) was added to each sample as an internal mass reference standard (monoisotopic mol. wt. 1758.9) to achieve a level of 5 pmol on target.

Microbiological Testing

Teicoplanin was tested against *Staphylococcus aureus* (Isosensitest agar medium containing an inoculum of 10^6 cells/mL). Phenelfamycin complex GE21640 F VI 45 was tested against *Moraxella caviae* (Todd–Hewitt agar medium, 10^6 cells/mL). The antifungal sample GE23845 II EA was tested against *Candida albicans* (Sabouraud agar medium, 10^6 cells/mL). To facilitate microbiological testing, each HPLC fraction was reconstituted in the appropriate buffer. In each case, a 10-μL aliquot of each fraction was deposited onto the agar surface containing the target organism and incubated for 24 h at 37 °C. Biological activity was expressed as the diameter of the observed zone of inhibition in millimeters.

Results

Teicoplanin

The first sample analyzed was a crude extract obtained from a production scale fermentation of the known anti Gram positive antibiotic teicoplanin. The structure shown in Figure 2 indicates teicoplanin to be a glycopeptide whose peptide backbone is fused among an extensive network of ring structures forming the planar core of the molecule. As many as three sugar residues may be attached to the core structure. Functionally, the most important sugar is the *N*-acyl glucosamine which, as indicated in Figure 2, may be substituted with a number of fatty acids. Variation in the associated acyl chain (R) accounts for the primary source of heterogeneity in teicoplanin-related structures.

Data obtained from the analysis of 40 μg of a crude teicoplanin extract are shown in Figure 3. The upper two panels display the UV absorbance at 254 nm and the ESI total ion current (TIC) from LC/MS analysis. The lower panel is a histogram expressing the biological activity present in each HPLC fraction against the bacterium *Staphylococcus aureus*. In this example, extremely close agreement was observed between all three sets of data, making it possible to correlate observed biological activity with specific components in the teicoplanin mixture.

The peak assignments appearing on the UV trace in Figure 3 indicate that the major species present in the mixture belong to the A2 structural family of teicoplanin. To a first approximation, retention of teicoplanin-related structures under reverse phase C18 conditions is determined by the nature of the fatty acid side chain (R). For example, the isomeric species A2-4 and A2-5 are more highly retained than the corresponding pair of isomers A2-2 and A2-3, which contain one less methylene unit. This effect also explains the early elution of the A3-1 species, which lacks the *N*-acyl fatty acid-containing sugar.

A summary of the mass spectrometric data for teicoplanin appears in Table 1. In this table, assignments

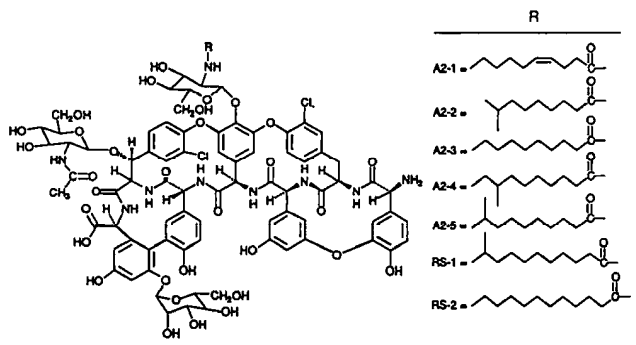


Figure 2. Structures for the major components of teicoplanin. Structural heterogeneity occurs primarily from variation in the fatty acid side chain associated with the *N*-acyl group (designated by the letter R).

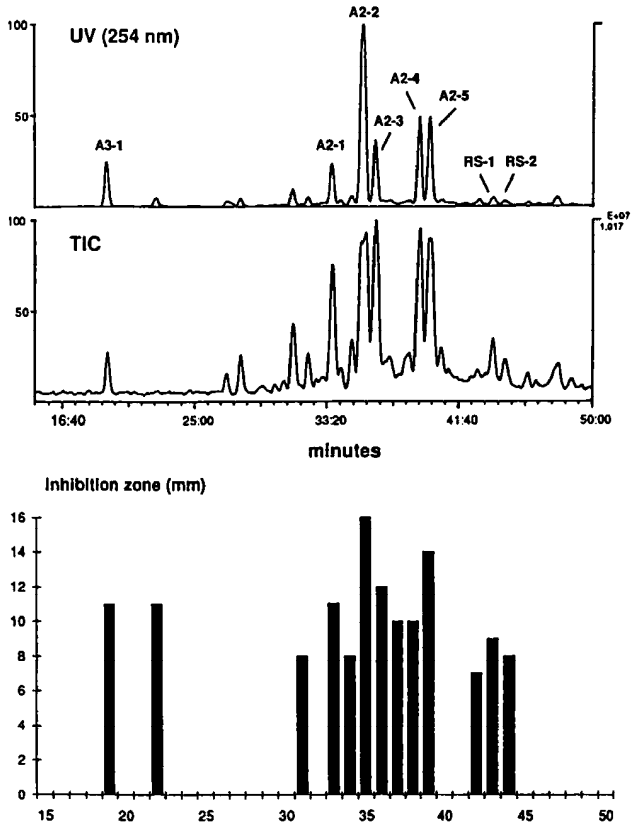


Figure 3. Chromatographic and biological activity profiles resulting from the analysis of 40 μg of a crude fermentation extract containing the Gram positive antibiotic teicoplanin. The upper and middle panels display the UV absorbance (254 nm) and ESI total ion current (TIC), respectively, from on-line LC-UV-ESI/MS analysis. The histogram in the bottom panel expresses the microbiological activity for each fraction collected from HPLC against *Staphylococcus aureus*. Activity is expressed as the inhibition zone (millimeters), which refers to the diameter of a circular area on an agar plate inoculated with a test microorganism where growth has been inhibited by the presence of an antibiotic substance.

are given for the major component present in each active fraction along with the proposed difference in elemental formula relative to A2-2, the most abundant form of teicoplanin. The assignments were based on molecular weights determined by ESI and MALDI along with the extensive knowledge available for this mixture [27. 28].

The ESI data in Table 1 are listed in terms of *monoisotopic mass*. The ESI data are represented in this fashion because the quadrupole mass analyzer used permitted resolution of the individual isotopes in the molecular ion clusters. In contrast, the comparatively limited mass resolution of time-of-flight (TOF) did not allow individual isotopes to be distinguished by MALDI. Consequently, these data appear as *average* mass. Another difference is that the MALDI molecular ions are listed as $[M + K]^+$, instead of $[M + H]^+$, reflecting the addition of KCl during MALDI sample preparation.

A representative example of the mass spectral data obtained by the two ionization methods appears in

Table 1. Mass spectrometric data summary for teicoplanin[a]

Active fraction[b]	Identity[c]	Formula relative to A2-2	$[M+H]^+$ calc. monoisotopic	$[M+H]^+$ ESI	$[M+K]^+$ calc. average	$[M+K]^+$ MALDI
19	A3-1	$-C_{16}H_{29}NO_5$	1563.4	1563.3	1603.4	1601
22	(A3-1) − HexNAc	$-C_{24}H_{42}N_2O_{10}$	1360.3	1360.1	1400.2	n.d.
31		$-CH_2$	1864.5	1864.9	1904.8	1906
33	A2-1	$-H_2$	1876.5	1876.4	1916.8	1916
34	(A2-2) + Hex	$C_6O_5H_{10}$	2040.6	2041.2	2080.9	2083
34	A2-1	$-H_2$	1876.5	1876.6	1916.8	1918
35	A2-2	—	1878.6	1878.9	1918.8	1915
36	A2-3	Isomer	1878.6	1878.8	1918.8	1918
37		Isomer	1878.6	1878.5	1918.8	1918
37	(A2-2) − Hex	$-C_6O_5H_{10}$	1716.5	1716.3	1756.7	n.d.
38	(A2-4) + Hex	$+C_7O_5H_{12}$	2054.6	2055.6	2095.0	n.d.
38	A2-4	$+CH_2$	1892.6	1892.7	1932.8	n.d.
39	A2-5	$+CH_2$	1892.6	1892.5	1932.8	1929
42		$-H_2$	1876.5	1876.8	1916.8	n.d.
43	RS-1	$+C_2H_4$	1906.6	1906.3	1946.9	1947
44	RS-2	$+C_2H_4$	1906.6	1906.2	1946.9	1945

[a] Hex = hexose; HexNAc = *N*-acetylglucosamine; n.d. = not detected.
[b] See histogram in Figure 3.
[c] For structural assignments refer to Figure 2.

Figure 4. In this figure, the ESI mass spectrum for teicoplanin component A2-5 is displayed. The inset appearing on this spectrum shows the corresponding MALDI data for A2-5 which eluted in fraction 39. Two molecular ion types were observed by ESI: the protonated molecule as well as an ammonium adduct. The protonated molecule was expected as the molecule contains a basic site located at the N-terminus. The ammonium adduct, on the other hand, can be explained by the presence of ammonium formate in the mobile phase used. Characteristic of ESI, multiple protonation of the molecule resulted in a doubly charged ion at m/z 947.5. In contrast, the MALDI data in Figure 4 indicate a series of cationized adducts produced by sodium and/or potassium. The peaks indicated were the only analyte-related ions in the mass spectrum as no fragment ions were observed. The nature of these adducts reflects a relatively high level of salt in the sample due in part to the addition of KCl during MALDI sample preparation.

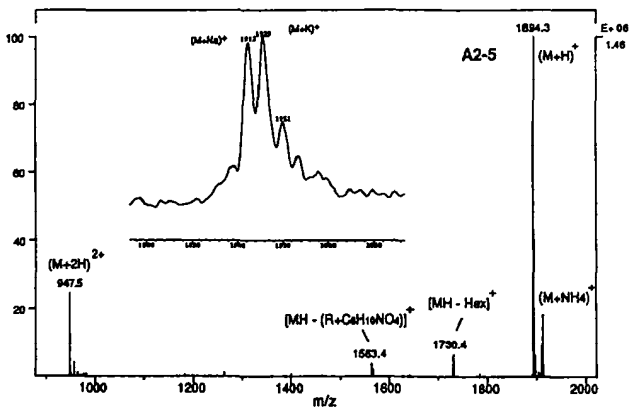


Figure 4. Mass spectral data for teicoplanin component A2-5. The ESI mass spectrum was acquired on-line by LC/MS. The MALDI data (inset) were obtained from fraction 39 (Figure 3).

The biggest difference observed between the two ionization methods was in the extent of fragmentation observed. Strictly speaking, both ESI and MALDI are extremely soft ionization methods that do not produce fragment ions. It is apparent from the mass spectrum of the A2-5 component in Figure 4 that this was not the case for ESI. This phenomenon has been traced to the heated capillary orifice of the ESI interface, which was held at a temperature of 200 °C. In the present example, two fragment ions were observed: the loss of a hexose moiety (m/z 1730) and the removal of the *N*-acyl fatty acid sugar (m/z 1563). Although these ions are structurally informative, the structural assignments given in Table 1 were based on existing structural information available for this mixture obtained by a number of methods including NMR [27, 28]. Hence, it is important to clarify that it would not be possible to make detailed structural assignments, such as the differentiation of isomers, from ESI alone.

Phenelfamycins

The second class of molecules investigated was the phenelfamycins [29], which are part of a broader family of antibiotic molecules known as elfamycins. The name elfamycin is derived from the mode of action of these molecules, which is to inhibit the elongation factor Tu involved in bacterial protein synthesis [30, 31]. The structure and nomenclature for the phenelfa-

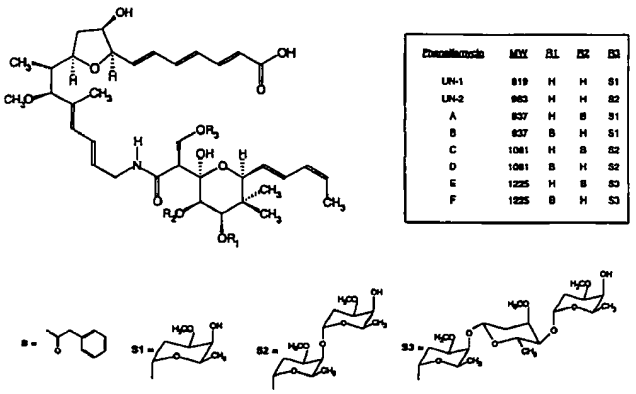


Figure 5. Structures for the phenelfamycin class of antibiotics. As indicated by the inset on the figure, phenelfamycins have been assigned letter designations according to their substitution at positions R1, R2, and R3.

mycin-related molecules identified in this study appear in Figure 5.

The sample analyzed, GE21640 F VI 45, was isolated from a 4-L fermentation of the *Streptomyces* sp. GE21640 known to produce phenelfamycin-related antibiotics. As was the case for teicoplanin, extracts from previous fermentation of this strain had been extensively characterized prior to this study. The data obtained from the analysis of 50 μg of this sample are reported in Figure 6. The upper two panels in this figure represent UV (230 nm) and ESI total ion current (TIC) from the LC/MS analysis, while the lower panel displays in histogram form the biological activity against *Moraxella caviae* for each fraction isolated. Again, a clear alignment was evident among all three sets of data.

The mass spectrometry data corresponding to the material in fraction 16, identified as phenelfamycin E, are shown in Figure 7. This figure displays the ESI mass spectrum for this product along with the molecular ion region observed by MALDI (inset). In contrast to teicoplanin, $[M + Na]^+$ was the dominant ion observed by both ionization methods. A potassium adduct was also present in each spectrum owing to the conditions used for analysis.

The failure to observe $[M + H]^+$ is attributed in part to the phenelfamycin structure which lacks a basic site. Another explanation is that $[M + H]^+$ is unstable, thus accounting for the extensive fragmentation observed in Figure 7. The ion at m/z 744 is explained by successive losses of methanol and S3-OH from the protonated molecule, where S3 stands for the trisaccharide substituent at position R3 (Figure 5). The further loss of benzoic acid from the terminus of the molecule accounts for the base peak at m/z 622. This latter fragmentation was not observed for the corresponding isomer phenelfamycin F, which instead undergoes a favorable 1,4-elimination of phenyl acetic acid B-OH to yield an ion of m/z 608 (data not shown). A similar pattern of fragmentation permitted differentiation of the other isomeric pairs in the mix-

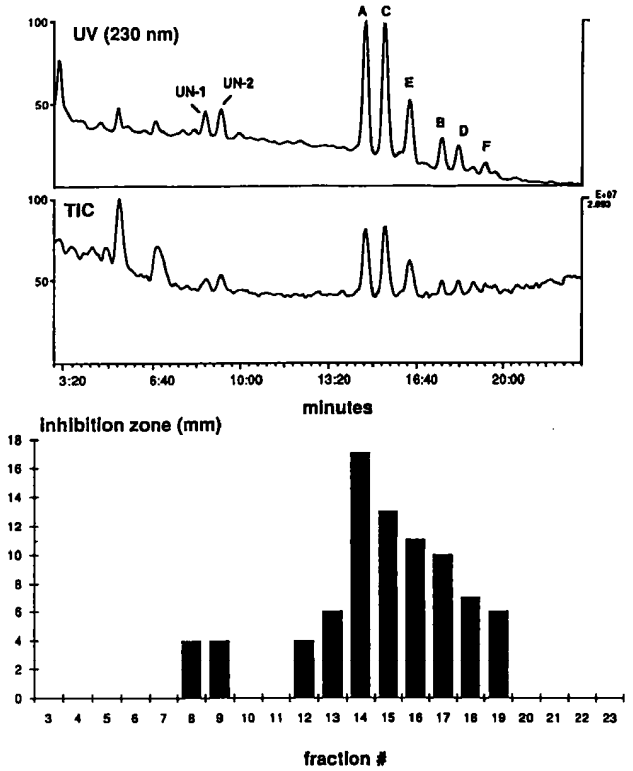


Figure 6. Chromatographic and biological activity profiles resulting from the analysis of 40 μg of a crude extract obtained from fermentation of the bacterial strain GE21640 F VI 45, known to produce phenelfamycin antibiotics. The upper and middle panels display the UV absorbance (230 nm) and ESI total ion current (TIC), respectively, from on-line LC-UV-ESI/MS analysis. The histogram in the bottom panel expresses the microbiological activity for each fraction collected from HPLC against *Moraxella caviae*. Activity is expressed as the inhibition zone (millimeters), which refers to the diameter of a circular area on an agar plate inoculated with a test microorganism where growth has been inhibited by the presence of an antibiotic substance.

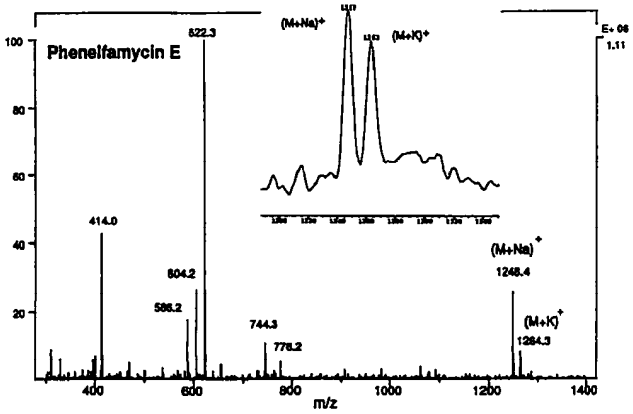


Figure 7. Mass spectral data corresponding to phenelfamycin E. The ESI mass spectrum was acquired on-line by LC/MS, while the MALDI data (inset) were obtained from fraction 16 (Figure 6).

Table 2. Mass spectrometric data summary for phenelfamycin sample GE21640 F VI 45[a]

Active fraction[b]	Ret. time (min)	Mol. wt. monoisotopic	$[M + K]^+$ ESI	$[M + K]^+$ MALDI	Identity[c]	Key fragment ions (m/z)
8	8:40	819.4	858.2	n.d.	UN-1	626, 504
9	9:15	963.5	1002.4	n.d.	UN-2	626, 504
12	n.a	n.a.	n.d.	n.d.	unk	n.a.
13	13:45	829	868.2	n.d.	unk	n.a.
14	14:30	973	1002.4	n.d.	unk	n.a.
14	14:40	937.5	976.2	976	A	744, 622
15	15:30	1081.6	1120.3	1121	C	744, 622
16	16:25	1225.6	1264.3	1263	E	744, 622
17	17:35	937.5	976.4	976	B	744, 608
18	18:20	1081.6	1120.2	1121	D	744, 608
19	19:15	1225.6	1264.4	1263	F	744, 608
19	19:40	927	966.3	n.d.	unk	n.a.

[a] n.a. = not applicable; n.d. = not detected; unk = unknown.
[b] See histogram in Figure 6.
[c] For structural assignments refer to Figure 5.

ture. The specific ions used to make these assignments are included in the mass spectrometry data summary for phenelfamycin sample GE21640F VI 45 (Table 2).

Verrucarins

The methodology described above for teicoplanin and the phenelfamycins was applied to the analysis of an unknown fermentation extract exhibiting potent antifungal activity. An injection of 40 μg of a crude extract (sample GE23845 EA II) produced the data in Figure 8. This experiment was significant to the dereplication process because the molecular weights identified (Table 3) permitted a successful search of a data base constructed in-house for novelty assessment. These data when combined with other information including knowledge about the producer strain, spectrum of activity, and UV absorbance (λ_{max} = 260 nm) suggested that the material isolated belonged to the known verrucarin family of toxic antifungal agents [32]. The structure for verrucarin A, one of the predominant members of this family, appears in Figure 9. This suspicion was corroborated by further study including a toxicological profile (unpublished results). Due to the toxicity of this substance, along with the various evidence suggesting the verrucarin family, further work toward isolation and detailed structural elucidation was not warranted.

The molecular weight of each component in the verrucarin crude was readily derived from a characteristic grouping of molecular ions, the most intense of which was the ammonium adduct. This pattern is illustrated in Figure 10 (upper panel) which displays the molecular ion region in the ESI mass spectrum corresponding to the component eluting in HPLC fraction 25. The ammonium adduct at m/z 550 is flanked by the protonated molecule at m/z 533 and the sodium

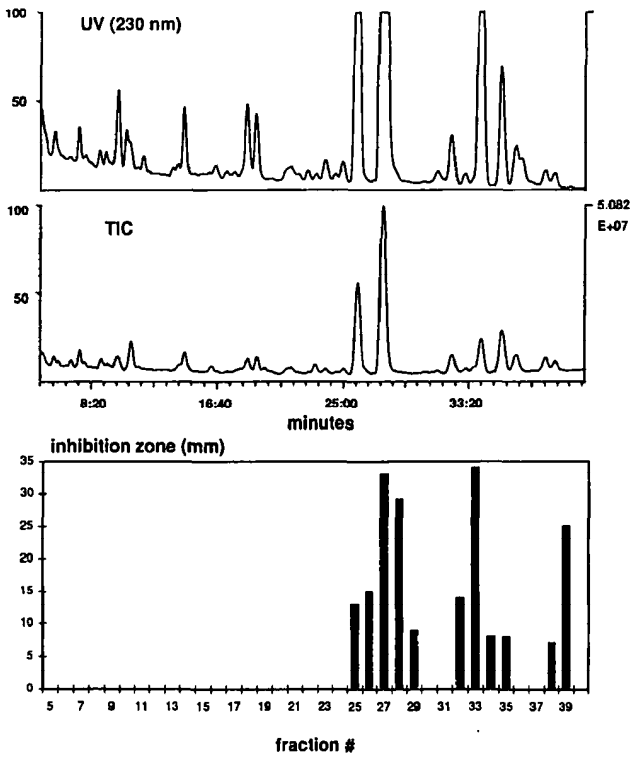
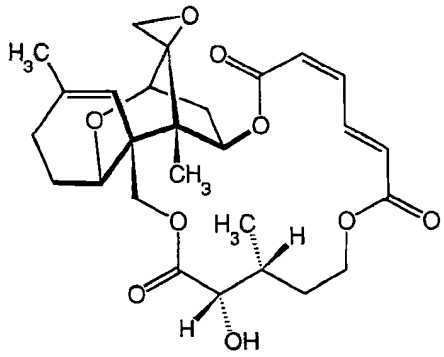


Figure 8. Chromatographic and biological activity profiles resulting from the analysis of 40 μg of a crude extract obtained from fermentation of the bacterial strain GE23845 EA II. Unknown components in this crude had previously demonstrated potent antifungal activity. The upper and middle panels display the UV absorbance (230 nm) and ESI total ion current (TIC), respectively, from on-line LC-UV-ESI/MS analysis. The histogram in the bottom panel expresses the antifungal activity for each fraction collected from HPLC against *Candida albicans*. Activity is expressed as the inhibition zone (millimeters), which refers to the diameter of a circular area on an agar plate inoculated with a test microorganism where growth has been inhibited by the presence of an antibiotic substance.

Table 3. Mass spectrometric data summary for antifungal sample GE23845 EA II[a]

Active fraction[b]	$[M + NH_4]^+$ ESI	$[M + NH_4]^+$ MALDI	Molecular weight
25	550.2	551	532
26	550.2	549	532
26	624.3	624	606
27	520.2	520	502
28	592.2	n.d.	574
29	n.d.	n.d.	
32	548.2	n.d.	530
33	546.2	544	528
33	590.2	n.d.	572
34	518.2	516	500
35	532.2	531	514
38	530.2	n.d.	512
39	532.2	n.d.	514

[a] n.d.= not detected.
[b] See histogram in Figure 8.



Verrucarin A

Figure 9. Structure of verrucarin A, one of the main members of the verrucarin family of toxic antifungal agents.

and potassium adducts at m/z 555 and 571, respectively. In contrast, only the ammonium adduct was observed by MALDI (Figure 10, lower panel). Together, these data unambiguously confirm the molecular weight assignment for the active substance in fraction 25 as 532 u.

Discussion

Mass spectrometry has long played a significant role in the dereplication of natural products isolated from fermentation broths. Typically, mass spectrometry is incorporated as part of an iterative cycle involving purification, biological activity, and molecular weight determination. This traditional approach to novelty evaluation often becomes a bottleneck step in the discovery process. Since each round of analysis involves fraction collection, sample processing, and analysis (biological and spectroscopic), the overall procedure is

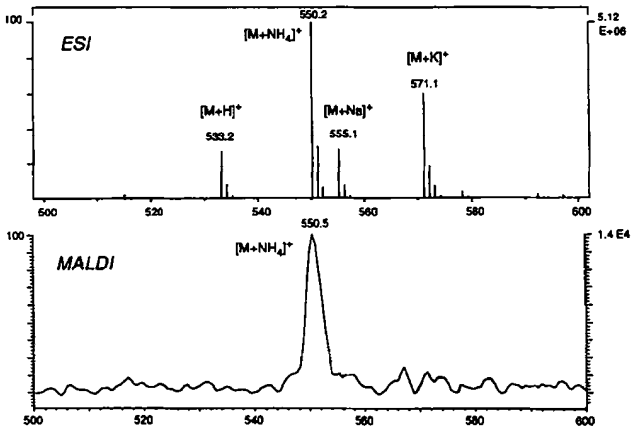


Figure 10. Comparison of ESI (top spectrum) and MALDI (lower spectrum) data for an unknown antifungal agent eluting in fraction 25 (Figure 8). The large ammonium adduct in each spectrum is attributed to the mobile phase used. Several additional molecular adducts observed in the ESI spectrum enabled the molecular weight to be unambiguously assigned as 532 u.

very time and labor intensive. The common use of insertion probe mass spectrometry methods is another source of inefficiency. One problem stems from incomplete recovery as individual fractions are concentrated and transferred to the mass spectrometer for analysis. This factor, coupled with the limited sensitivity of older ionization methods (EI, CI, or FAB), increases the total sample needed for molecular weight determination. Unfortunately, when molecular weight data are not obtained, a further iteration of the purification/analysis cycle must occur and the time taken for novelty assessment is increased accordingly.

The methodology for mass spectrometry utilization described in this paper imparts several advantages to the dereplication process. First, due to the sensitivity of ESI and MALDI, less material must be isolated for analysis. Second, the on-line nature of LC/MS reduces the labor associated with fraction collection and sample processing. LC/MS also optimizes sample transfer to the mass spectrometer and retains the chromatographic resolution lost in the process of fraction collection. A further consideration in the experimental design was the concentration dependence of ESI [33]. Because ESI/MS acts as a concentration-sensitive detector, it was possible to split the effluent postcolumn for fraction collection without unduly compromising molecular weight determination. In the present strategy, roughly 90% of the injected crude was recovered for microbiological testing.

Valid molecular weight determination is essential to a dependable dereplication strategy, as it is often the only structural information available for novelty evaluation. More sophisticated techniques are typically limited by the amount of sample available and its purity. It was for this reason that the methodology presented allows for two separate determinations of molecular weight. This feature can be extremely useful when analyzing unknown samples as it is difficult to know a

priori which type of molecular adduct ion will predominate. The intentional introduction of specific salts (e.g., KCl) was another method used in this study to ensure valid molecular weight determination.

A separate but related issue involves the reliability of molecular weight-based searches for natural products dereplication. This concern was recently addressed by Constant and Beecher [25], who demonstrated that searches of the NAPRALERT data base (over 80,000 known compounds) by using only chemotaxonomy, pharmacology, UV–visible spectroscopy, and molecular weight permitted successful dereplication of several natural product extracts.

To our knowledge, this work represents the first reported use of MALDI specifically for antibiotics dereplication. The main advantage of MALDI is that it is a rapid technique that can be used to survey individual fractions submitted for biological testing. Furthermore, because fractions may be sampled without preconcentration, a majority of the material is available for biological testing. In spite of these advantages, it is our initial assessment that MALDI should not be used as a stand alone method for characterization of crude antibiotic mixtures. The data acquired in this study (Tables 1–3) indicate a number of instances where a molecular weight was identified by ESI, but not by MALDI. One explanation for this result is inadequate sample preparation, a subject which needs to be more thoroughly investigated. However, even when adequate signal was obtained, the poor mass resolution of linear TOF frequently resulted in compromised mass accuracy. The role of MALDI is therefore probably best viewed as an adjunct to LC-ESI/MS used on an as-needed basis to corroborate assignments made by ESI. Improvements in TOF, such as delayed extraction and reflectron mass analysis [34], may overcome some limitations experienced in this study.

A topic not considered in the present experimental design was the acquisition of tandem mass spectrometry (MS/MS) data. The usefulness of this approach was recently demonstrated by Julian [35], who used ESI/MS/MS with ion trap detection for antibiotics dereplication. MS/MS is a logical extension to the work presented here as it offers a facile means to increase the certainty of novelty evaluation. In addition, it could be used with either ionization technique. Two fundamental limitations to MS/MS, however, are the lack of available reference standards and difficulty in interpreting spectra for unknown structures. For these reasons, the most appropriate use of MS/MS would be to reject or confirm structural assignments proposed from data base searches conducted using molecular weight data acquired by LC/MS.

The methodology outlined in this paper has been implemented in our laboratory for over two years. Although it is difficult to estimate, the time required to arrive at an initial determination of novelty is typically on the order of one week. By comparison, under the traditional approach involving fraction collection and classical ionization techniques, this same process could take several weeks and in some instances months. In all cases, a key objective is always to minimize the number of iterative purification/analysis steps required for novelty evaluation. The on-line efficiency of LC/MS along with the superior sensitivity of ESI and MALDI enables a situation where novelty determination can proceed with less labor and greater clarity.

References

1. Georgopapadakou, N. H.; Walsh, T. J. *Science* **1994**, *264*, 371–372.
2. Neu, H. C. *Science* **1992**, *257*, 1064–1073.
3. Davies, J. *Science* **1994**, *264*, 375–382.
4. Bates, J. H. *Am. J. Respir. Crit. Care Med.* **1995**, *151*, 942–943.
5. Omura, S. *Microbiol. Rev.* **1986**, *50*, 259–279.
6. Vandamme, E. J. *J. Biotechnol.* **1994**, *37*, 89–108.
7. Omura, S. *J. Ind. Microbiol.* **1992**, *10*, 135–156.
8. Monaghan, R. L.; Tkacz, J. S. *Ann. Rev. Microbiol.* **1990**, *44*, 271–301.
9. Yarbrough, G. G.; Taylor, D. P.; Rowlands, R. T.; Crawford, M. S.; Lasure, L. L. *J. Antibiot.* **1993**, *46*, 535–544.
10. Sedlock, D. M.; Sun, H. H.; Smith, W. F.; Kawaoka, K.; Gillum, A. M.; Cooper, R. *J. Ind. Microbiol.* **1992**, *9*, 45–52.
11. Alvi, K. A.; Peterson, J.; Hofmann, B. *J. Ind. Microbiol.* **1995**, *15*, 80–84.
12. Corley, D. G.; Durley, R. G. *J. Nat. Prod.* **1994**, *57*, 1484–1490.
13. Kinghorn, A. D.; Farnsworth, N. R.; Beecher, C. W. W.; Soejarto, D. D.; Cordell, G. A.; Pezzuto, J. M.; Wall, M. E.; Wani, M. C.; Brown, D. M. *Int. J. Pharmacogn.* **1995**, *33*, 48–58.
14. Sottani, C.; Islam, K.; Soffientini, A.; Zerilli, L. F.; Seraglia, R. *Rapid Commun. Mass Spectrom.* **1993**, *7*, 680–683.
15. Roberts, G. D.; Carr, S. A.; Rottschaefer, S.; Jeffs, P. W. *J. Antibiot.* **1985**, *38*, 713–720.
16. Edwards, D. M. F.; Selva, E.; Stella, S.; Zerilli, L. F.; Gallo, G. G. *Biol. Mass Spectrom.* **1992**, *21*, 51–59.
17. McCracken, R. J.; Blanchflower, W. J.; Rowan, C.; McCoy, M. A.; Kennedy, D. G. *Analyst* **1995**, *120*, 2347–2351.
18. Sanders, P.; Delepine, B. *Biol. Mass Spectrom.* **1994**, *23*, 369–375.
19. Coutant, J. E.; Chen, T.-M.; Ackermann, B. L. *J. Chromatogr.* **1990**, *529*, 265–275.
20. Kiehl, D. E.; Kennington, A. S. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 1297–1301.
21. Fouda, H. G.; Schnieder, R. P. *Ther. Drug. Monit.* **1995**, *17*, 179–183.
22. D'Agostino, P. A.; Hancock, J. R.; Provost, L. R. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 1038–1043.
23. Straub, R.; Linder, M.; Voyksner, R. D. *Anal. Chem.* **1994**, *66*, 3651–3658.
24. Colombo, L.; Stella, S.; Regg, B. T.; Ackermann, B. L. *Proceedings of the 42nd ASMS Conference on Mass Spectrometry and Allied Topics*; 1994, p. 613.
25. Constant, H. L.; Beecher, C. W. W. *Nat. Prod. Lett.* **1995**, *6*, 193–196.
26. Bardone, M. R.; Paternoster, M.; Coronelli, C. *J. Antibiot.* **1978**, *31*, 170–177.
27. Barna, J. C. J.; Williams, D. H.; Stone, D. J. M.; Leung, T. W. C.; Doddrell, D. M. *J. Am. Chem. Soc.* **1984**, *106*, 4895–4902.

28. Hunt, A. H.; Molloy, R. M.; Occolowitz, J. L.; Marconi, G. G.; Debeno, M. *J. Am. Chem. Soc.* **1984**, *106*, 4891–4895.
29. Hochlowski, J. E.; Buytendorp, M. H.; Whittern, D. N.; Buko, A. M.; Chen, R. H.; McAlpine, J. B. *J. Antibiot.* **1988**, *41*, 1300–1315.
30. Hall, C. C.; Watkins, J. D.; Georgopapdakou, N. H. *Antimicrob. Agents Chemother.* **1989**, *33*, 322–325.
31. Parmeggiani, A.; Swart, G. W. M. *Ann. Rev. Microbiol.* **1985**, *39*, 557–577.
32. Härri, E.; Loeffler, W.; Sigg, H. P.; Stähelin, H.; Stoll, C.; Tamm, C.; Wiesinger, D. *Helv. Chim. Acta* **1962**, *45*, 839–853.
33. Hopfgartner, G.; Bean, K.; Henion, J.; Henry, R. *J. Chromatogr.* **1993**, *647*, 51–61.
34. Vestal, M. L.; Juhasz, P.; Martin, S. A. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 1044–1050.
35. Julian, R. K., Jr. Presented at the 44th ASMS Conference on Mass Spectrometry and Allied Topics, Portland, OR, June 1996.