Matrix-Assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry of Paclitaxel and Related Taxanes

Mary E. Gimon, Gary R. Kinsel, Ricky D. Edmondson, David H. Russell, Timothy R. Prout, and Hernita A. Ewald

J. Nat. Prod., **1994**, 57 (10), 1404-1410• DOI: 10.1021/np50112a009 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 57, No. 10, pp. 1404-1410, October 1994

MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY OF PACLITAXEL AND RELATED TAXANES

MARY E. GIMON, GARY R. KINSEL, RICKY D. EDMONDSON, DAVID H. RUSSELL,*

Texas A&M University, Department of Chemistry, College Station, Texas 77843

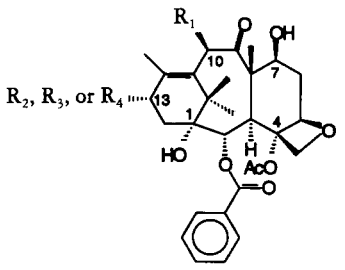
TIMOTHY R. PROUT, and HERNITA A. EWALD

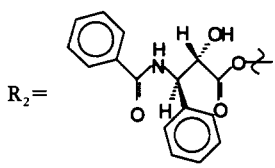
NaPro BioTherapeutics, Inc., Boulder, Colorado 80301

ABSTRACT.—Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry has been demonstrated for the analysis of *Taxus brevifolia* extracts. The detection limit for the taxanes contained therein is estimated at 1 pmol using the matrix 4-nitroaniline at a matrix-to-analyte molar ratio of 100:1. Acquisition and calibration of the mass spectral data requires less than 5 min. The $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$ ions provide mol wt confirmation and structurally significant fragment ions indicative of the various substituent groups located on the diterpene skeleton are formed.

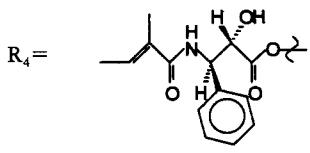
The natural product paclitaxel [**1**], commonly referred to as taxol in the chemical literature, is contained in the bark of the Pacific yew, *Taxus brevifolia* (Taxaceae), and has demonstrated reproducible anticancer activity in clinical studies (1,2) and received U.S. Food and Drug Administration (FDA) approval for treatment of ovarian cancer (3). Recently, mass spectrometric studies of **1** and the taxanes, baccatin III [**2**], and cephalomannine [**3**], using tandem mass spectrometers have appeared in the literature. McClure *et al.* (4) examined the fragmentation of **1** using electron impact (ei), chemical ionization (ci), and fast-atom bombardment (fab) mass spectrometry, thus compiling a reference data base for the mass spectral analysis of **1** and related taxanes. Cooks *et al.* (5) determined **1–3** in crude plant extracts using tandem mass spectrometry. Radical anions were produced using ammonia ci, the $[M^-]$ ion was collisionally dissociated, and the taxanes were identified by their characteristic fragments. Cooks *et al.* (6) have also demonstrated the quantitation of **1** in crude extracts to the low picomole level using tandem mass spectrometry and the method of standard addition. Electrospray ionization (esi) mass spectrometry has been used to confirm the production of **1** by the fungus *Taxomyces andreanae* isolated from the phloem of the Pacific yew (7). Analysis of **1** has also been accomplished using hplc combined with thermospray mass spectrometry (8).

A relatively new technique, matrix-assisted laser desorption/ionization (maldi) mass spectrometry has emerged as an important method for molecular weight determination





R3= HO



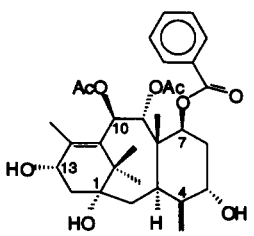
1 (Mol wt 853 daltons), R_1=AcO-, R_2
2 (Mol wt 586 daltons), R_1=AcO-, R_3
3 (Mol wt 831 daltons), R_1=AcO-, R_4
4 (Mol wt 811 daltons), R_1=HO-, R_2
5 (Mol wt 544 daltons), R_1=HO-, R_3

of peptides and proteins (9–11). Ions generated by maldi are generally detected via time-of-flight (tof) mass spectrometry or Fourier transform-ion cyclotron resonance (Ft-icr) mass spectrometry (12–14). The detection limit for maldi-tof of peptides and proteins is typically as low as several hundred fmol. This methodology has only recently been applied to the characterization of compounds other than peptides and proteins. The limited number of successful applications of maldi to such compounds includes the analysis of oligonucleotides (15–17), oligosaccharides (18), glycoconjugates (19), lignins (20), and synthetic polymers (21,22). One of the factors that has limited the success of maldi to other classes of biomolecules and natural products is our incomplete knowledge of the role of the matrix in the ionization process. The matrix, typically an aromatic compound that absorbs at the wavelength of the incident laser, seems to serve three purposes: (a) the matrix isolates the individual analyte molecules in a crystalline "solid solution"; (b) the matrix minimizes analyte decomposition by transferring energy in a controllable manner; and, (c) the matrix enhances ion production (10,23). The maldi matrices that have been used to desorb and ionize intact peptides and proteins include 3,5-dimethoxy-4-hydroxycinnamic acid (sinapic acid), 2,5-dihydroxybenzoic acid (DHB), and α-cyano-4-hydroxycinnamic acid (αCHCA). The maldi analysis of compounds such as synthetic polymers, glycolipids, and oligonucleotides has introduced a number of new matrices that work particularly well for these classes of compounds. Biemann *et al.* (22) used 2-(4-hydroxyphenylazo)benzoic acid (HABA) to obtain tof mass spectra of synthetic polymers and glycolipids. Becker *et al.* (24) and Nordhoff *et al.* (25) have demonstrated maldi of oligonucleotides using 3-hydroxypicolinic acid (at 266 nm and 355 nm) and succinic acid (at 2.94 μm), respectively. In an effort to increase the scope of maldi applications, we present a study which examines maldi-tof mass spectrometry as a method for the analysis of **1** and several related taxanes. Considering the success of non-traditional maldi matrices to the analysis of compounds other than peptide and proteins, we have examined a variety of compounds as potential matrices beyond those already proven maldi matrices.

RESULTS AND DISCUSSION

This study has examined maldi-tof mass spectrometry as a method for the analysis of **1–3** and the taxanes, 10-deacetyltaxol [**4**], 10-deacetylbaccatin III [**5**], and brevifoliol [**6**]. The diterpene skeleton is a common structural feature of each taxane. Compounds **2–5** differ slightly from **1** in the type and position of substituent groups such as the replacement of the ester side-chain at C-13 with a hydroxyl group in **2** or the modification of the ester side-chain at C-13 in **3**. The absence of the oxetane ring at C-4 and C-5 and the absence of a ketone group at C-9 make **6** unusual among the taxanes in this study.

Maldi of these natural products has not been reported previously, thus several matrices were evaluated for **1** and the optimum matrix-to-analyte ratios determined. It is important to note that no signal for the intact protonated molecule ($[M+H]^+$, 854



6 (Mol wt 566 daltons)

daltons) was observed under conditions of direct uv laser desorption; the mass spectrum was dominated by fragment ions. Samples of each matrix and **1** were examined at matrix-to-analyte ratios ranging from 50:1 to 1,000:1. In the cases of sinapic acid, DHB, 7-hydroxy-4-methylcoumarin (7HMC), and coumarin, no $[M+H]^+$ ion signal was observed. While αCHCA produced a $[M+H]^+$ ion signal, the high abundance of matrix cluster ions prevented the use of αCHCA for analysis of the small molecular weight taxanes. The best matrices for **1** in terms of ion abundance and mass spectrum reproducibility were 4-nitroaniline and 4-nitrophenol.

Figure 1 contains representative maldi-tof mass spectra of **1** obtained by using the matrix 4-nitroaniline (matrix-to-analyte molar ratio, 100:1). Approximately 100 pmol of analyte was used to obtain the example mass spectrum shown in Figure 1a. Analytically useful mass spectra are obtained with as little as 1 pmol of taxane applied to the probe (see Figure 1b). We define such mass spectra as having a resolved intact taxane ion signal at a level at least 3:1 above background noise. It is not clear that the measured detection limit was due to the efficiency of maldi; lower detection limits might be achieved with improved sample handling or by using sophisticated data acquisition techniques, such as averaging only laser shots in which the signal of the analyte is present. At the detection limit in this experiment, the maldi mass spectrum contains both the sodiated and the potassiated intact analyte molecules which can be used to confirm the mol wt. Less than 5 min were usually spent acquiring and calibrating the maldi mass spectrum. The mass spectrum of **1** illustrates salient features observed for all taxane mass spectra (see Table 1). The most abundant ions in the region between *m/z* 500–900 correspond to the cationized intact analyte molecule. Although the $[M+H]^+$ ion is observed for all taxanes with the exception of **6**, the $[M+Na]^+$ and $[M+K]^+$ ions are generally the most abundant ionic forms of the intact molecule. This observation is unusual because the $[M+H]^+$ ion is typically the most intense analyte ion formed in maldi of peptides and proteins. Na^+ and K^+ are contaminants of the sample, matrix, solvent, and stainless steel probe. Efforts to increase the observed $[M+H]^+$ ion signal with respect to the $[M+Na]^+$ ion signal by removing the sodium (sublimation of matrix, washing sample in cold solvent, ion exchange, etc.) met with little success. However, the presence of the more than one cationized form of the intact molecule is not deleterious and allows for unambiguous mol wt determination of the analyte.

In the region between *m/z* 200–850, fragment ions of the analyte were observed. The difference between the *m/z* values of these ions and the $[M+H]^+$ ion of the analyte indicates loss of one or more of the substituent groups attached to the diterpene skeleton. The fragment ions are designated using nomenclature similar to that adopted by McClure *et al.* (4). Diterpene skeletal substituents are referred to as the ester side-chain (S), acetoxyl group (AcO), benzoyl group (BzO), and OH. Fragmentation occurs by losses of small neutrals, i.e., acetic acid (AcOH), benzoic acid (BzOH), and H_2O. The larger peaks at *m/z* 509 and 696 in the maldi-tof mass spectrum of **1** are assigned to $[M-S-AcOH]^+$ and $[M+H-BzOH-2H_2O]^+$, respectively. Peaks at *m/z* 569, 551, 286, and 268 are assigned to $[M-S]^+$, $[M-S-H_2O]^+$, $[S+2H]^+$, and $[S-O]^+$, respectively. All taxanes in this study tend to fragment in a similar manner (see Table 1). The fact that these closely related taxanes fragment in similar patterns can be useful in confirming the presence of the diterpene skeletal substituents. Substituent group information, combined with the mol wt, can be useful in the structural assignment of an unknown taxane.

Fragment ions observed in the maldi-tof mass spectrum of **1** are similar to those in the fab mass spectrum reported by McClure *et al.* (4). Fragment ions $[M+H-AcOH]^+$ (*m/z* 794), $[M+H-AcOH-OH]^+$ (*m/z* 776), $[M-S]^+$ (*m/z* 569), $[M-S-H_2O]^+$ (*m/z*

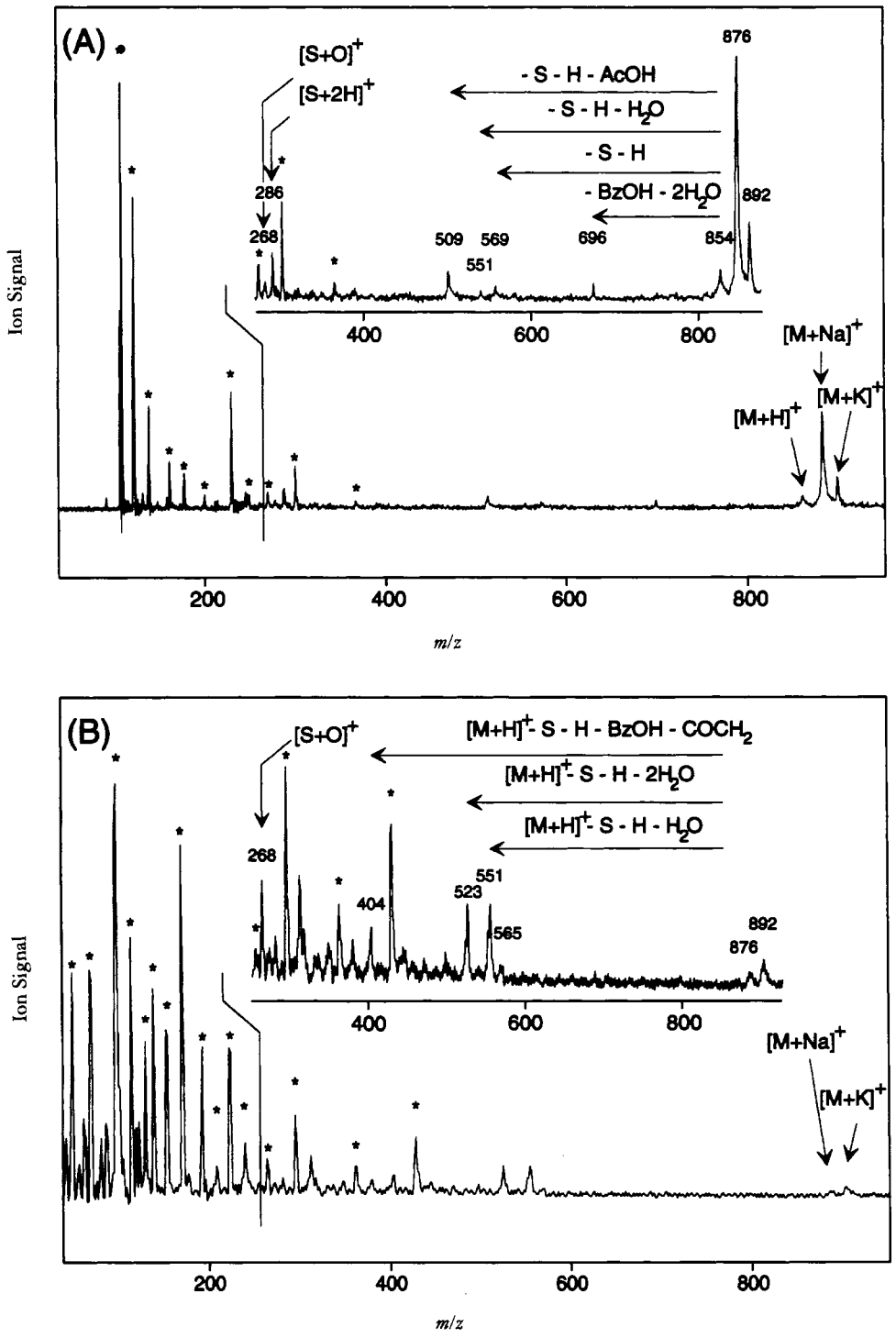


FIGURE 1. Maldi-tof mass spectra of paclitaxel [**1**] (A) 100 pmol and (B) 1 pmol (detection limit) analyte applied to the sample probe surface. See Results and Discussion for nomenclature for fragmentation. Matrix ions are marked (*).

TABLE 1. Assignment of Ions Observed in the Maldi-tof Mass Spectra of Related Taxanes Using the Matrix 4-Nitroaniline.

Analyte	*m/z*	Assignment
Baccatin III [**2**]	625	$[M+K]^+$
	609	$[M+Na]^+$
	587	$[M+H]^+$
	527	$[M+H-AcOH]^+$
	509	$[M+H-AcOH-H_2O]^+$
Cephalomannine [**3**]	870	$[M+K]^+$
	854	$[M+Na]^+$
	832	$[M+H]^+$
	572	$[M-S-H_2O]^+$
	508	$[M-S-AcOH]^+$
	265	$[S+2H]^+$
10-Deacetyltaxol [**4**]	850	$[M+K]^+$
	834	$[M+Na]^+$
	812	$[M+H]^+$
	549	$[M-S]^+$
	286	$[S+2H]^+$
10-Deacetylbaccatin III [**5**]	583	$[M+K]^+$
	567	$[M+Na]^+$
	545	$[M+H]^+$
	527	$[M+H-H_2O]^+$
	405	$[M+H-BzOH-H_2O]^+$
Brevifoliol [**6**]	595	$[M+K]^+$
	579	$[M+Na]^+$
	417	$[M+H-BzOH-H_2O]^+$

551), $[M-S-AcOH]^+$ (*m/z* 509), $[M-S-BzOH]^+$ (*m/z* 447), $[S+2H]^+$ (*m/z* 286), $[S-O]^+$ (*m/z* 268), $[S-O-CO-HCOH]^+$ (*m/z* 210), and $[BzO]^+$ (*m/z* 105) were reported as the most abundant ionic forms of **1** in the fab mass spectrum using the matrix sulfolane. A direct comparison of the type and relative abundance of the fragment ions observed in maldi-tof and fab mass spectra of **1** is made in Table 2. Fragment ions resulting from charge retention on the diterpene skeleton are observed in approximately

TABLE 2. Relative Abundances and *m/z* Ratios of Fragment Ions Observed in Maldi-tof and Fab Mass Spectra of Paclitaxel [**1**].

m/z	Assignment	Fab[a]	Maldi-tof[b]
892	$[M+K]^+$	—	19.2
876	$[M+Na]^+$	—	100
854	$[M+H]^+$	13.4	16.7
794	$[M+H-AcOH]^+$	1.9	—
696	$[M+H-BzOH-2H_2O]^+$	—	3.57
569	$[M-S]^+$	5.8	5.06
551	$[M-S-H_2O]^+$	2.4	3.6
509	$[M-S-AcOH]^+$	7.4	10.2
447	$[M-S-BzOH]^+$	2.1	—
286	$[S+2H]^+$	61.8	13.0
268	$[S-O]^+$	37.6	11.4
210	$[S-O-CO-HCOH]^+$	49.4	—
105	$[BzO]^+$	100	—

[a]Using the matrix sulfolane.
[b]Using the matrix 4-nitroaniline.

the same relative abundance for both ionization methods. These similarities between laser desorption and fab desorption techniques are not surprising and have been noted previously for other classes of compounds by Busch and Cooks (26) and Hillenkamp (27). Consider the fragment ions resulting from charge retention on the ester side-chain. In maldi the relative abundance of $[S+2H]^+$ and $[S-O]^+$ ions (13.0% and 11.4%) is significantly lower than that reported in the fab mass spectrum (61.8% and 37.6%, respectively). Perhaps this reflects fundamental differences in the method of analyte ion production and/or extent of analyte ion activation. Our research supports the idea that the maldi matrix plays a major role in analyte activation much the same as reported for fab matrices (28). The effect of matrix choice on analyte activation has recently been reported for maldi of vitamin B_{12} (29). Studies on the extent of activation of **1** as a function of a variety of maldi matrices are currently in progress.

In conclusion, the use of maldi-tof mass spectrometry for the characterization of natural products is demonstrated by this study of taxanes **1–6**. The observed $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$ ions confirmed the molecular weights. Fragment ions corresponding to loss of substituent groups also appear, providing structural information. In addition to the analysis of these known taxanes, the observed fragmentation patterns could be used to support a proposed structure of an unknown taxane.

The success of this analysis of **1–6** has prompted further research in the area of on-line liquid chromatography/maldi-tof mass spectrometry (30–32). High-resolution chromatographic separation techniques (33–36) combined with maldi-tof mass spectrometers undoubtedly will be developed in the near future. These techniques will extend the realm of possible maldi applications to an increasing number of biologically significant molecules.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The taxane samples were provided by NaPro BioTherapeutics, Inc., Boulder, CO. The matrices examined for desorption at 337 nm were sinapic acid, 4-nitroaniline, αCHCA, and 4-nitrophenol (Aldrich Chemical Co., Milwaukee, WI). DHB, 7-hydroxy-4-methylcoumarin (7HMC), and coumarin (Sigma Chemical Co., St. Louis, MO) were also used at 337 nm. The matrices and taxane samples were used as provided. An analyte sample was prepared by dissolving approximately 1 mg of the taxane in 1 ml MeOH. Approximately 15–20 mg of a matrix compound was dissolved in 1 ml MeOH. Sample solutions were prepared by mixing appropriate volumes of the matrix and analyte solutions. A 2-μl aliquot of the matrix/analyte solution was deposited onto the stainless steel probe tip of a direct insertion probe and allowed to dry at room temperature.

The dried sample containing matrix and analyte was inserted into a linear tof mass spectrometer designed and built in-house. Tof instruments have been described in detail (37), and only a brief description is given here. The ions were generated using the 337 nm output from a sealed tube nitrogen laser (Laser Photonics LN300). The laser beam was focused to a rectangular spot of ca. 0.015 cm^2 onto the sample surface of the direct insertion probe at an angle of 50° normal to the probe surface using a 1-m spherical lens to collimate the beam and a 200-mm spherical lens to achieve the final spot size. Laser power density/spot size was regulated using a mechanical iris. The nitrogen laser was operated at a repetition rate of ca. 5 Hz. Each laser shot produces a plume of desorbed ionic and neutral material. The ions generated in this manner were accelerated to 10 kV and detected using a standard dual microchannel plate detector. The transient signal from the ion detector was collected by an impedance matched (50 ohm) 300 MHz digital oscilloscope (LeCroy 9450A). The spectra presented in this paper are averages of the transient signal from 20 laser shots. The tof mass spectrum was transferred to a computer where it was calibrated and analyzed using a commercial software package (Grams 386, Galactic).

ACKNOWLEDGMENTS

Funding for laser desorption and structural mass spectrometry research by the U.S. Department of Energy, Division of Chemical Sciences, Office of Basic Energy Science (DE-FG05-89ER75502) is gratefully acknowledged. Partial funding for the construction of the linear tof mass spectrometer was provided by the Center for Macromolecular Design, Texas A&M University, the Texas A&M College of Science, and the

Department of Chemistry. M.E.G. also wishes to thank Dow Chemical USA for its generous support through an ACS Analytical Division Summer Fellowship.

LITERATURE CITED

1. W.P. McGuire, E.K. Rowinsky, and R.C. Donehower, in: "Accomplishments in Cancer Research-1990." Ed. by J.G. Fortner and J.E. Rhoads, J.B. Lippincott, Philadelphia, 1991, pp. 276–283.
2. S. Borman, *Chem. Eng. News*, 1991, September 2 issue, pp. 11–18.
3. *Federal Registry*, **58**, 3954 (1993).
4. T.D. McClure, K.H. Schram, and M.L. Reimer, *J. Am. Soc. Mass Spectrom.*, **3**, 672 (1992).
5. S.H. Hoke, J.M. Wood, R.G. Cooks, X. Li, and C. Chang, *Anal. Chem.*, **84**, 2313 (1992).
6. S.H. Hoke, R.G. Cooks, C.H. Chang, R.C. Kelly, S.J. Qualls, B. Alvarado, M.T. McGuire, and K.M. Snader, *J. Nat. Prod.*, **57**, 277 (1994).
7. A. Stierle, G. Strobel, and D. Stierle, *Science*, **260**, 214 (1993).
8. S.O.K. Auriola, A.M Lepisto, T. Naaranlahti, and S.P. Lapinjoki, *J. Chromatogr.*, **594**, 153 (1992).
9. M. Karas, D. Bachmann, U. Bahr, and F. Hillenkamp, *Int. J. Mass Spectrom. Ion Proc.*, **78**, 5368 (1987).
10. F. Hillenkamp, M. Karas, R.C. Beavis, and B.T. Chait, *Anal. Chem.*, **63**, 1193A (1991).
11. B.T. Chait and S.B.H. Kent, *Science*, **257**, 1885 (1992).
12. T. Solouki and D.H. Russell, *Appl. Spectros.*, **47**, 211 (1993).
13. T. Solouki and D.H. Russell, *Proc. Natl. Acad. Sci. USA*, **89**, 5701 (1992).
14. C. Köster, J.A. Castoro, and C.L. Wilkins, *J. Am. Chem. Soc.*, **114**, 7572 (1992).
15. F. Hillenkamp, M. Karas, A. Ingendoh, and B. Stahl, in: "Biological Mass Spectrometry." Ed. by A. Burlingame and J.A. McCloskey, Elsevier, Amsterdam, 1990, pp. 49–60.
16. R.L. Hettich and M.V. Buchanan, in: "Proceedings of the 38th ASMS Conference on Mass Spectrometry and Allied Topics." Tucson, AZ, June 3–8, 1990.
17. B. Spengler, Y. Pan, R.J. Cotter, and L.-S. Kun, *Rapid Commun. Mass Spectrom.*, **4**, 99 (1990).
18. B. Stahl, M. Steup, M. Karas, and F. Hillenkamp, *Anal. Chem.*, **63**, 1463 (1991).
19. H. Egge, J. Peter-Katalinic, M. Karas, and B. Stahl, *Pure Appl. Chem.*, **63**, 491 (1991).
20. J.O. Metzyer, C. Bicke, O. Faix, W. Tuszynski, R. Angerman, M. Karas, and K. Strupat, *Angew. Chem., Int. Ed. Eng.*, **31**, 762 (1992).
21. U. Bahr, A. Deppe, M. Karas, F. Hillenkamp, and U. Giessmann, *Anal. Chem.*, **64**, 2866 (1992).
22. P. Juhasz, C.E. Costello, and K. Biemann, *J. Am. Soc. Mass Spectrom.*, **4**, 399 (1993).
23. M.E. Gimon, L.M. Preston, M.A. White, T. Solouki, and D.H. Russell, *Org. Mass Spectrom.*, **27**, 827 (1992).
24. K.J. Wu, A. Steding, and C. Becker, *Rapid Commun. Mass Spectrom.*, **7**, 142 (1993).
25. E. Nordhoff, M. Karas, F. Hillenkamp, K. Kristiansen, and P. Roepstorff, in: "Proceedings of the 41st ASMS Conference on Mass Spectrometry and Allied Topics." San Francisco, CA, May 31–June 4, 1993.
26. K.L. Busch and R.G. Cooks, *Science*, **218**, 247 (1982).
27. F. Hillenkamp, in: "Microbeam Analysis-1989." Ed. by P.E Russell, San Francisco Press, San Francisco, 1989, p. 277.
28. M. Takayama, T. Fukai, T. Nomura, and K. Nojima, *Int. J. Mass Spectrom. Ion Proc.*, **96**, 169 (1990).
29. G.R. Kinsel, L.M. Preston, and D.H. Russell, *Biol. Mass Spectrom.*, **23**, 205 (1994).
30. L. Li, A.P.L. Wang, and L.D. Coulson, *Anal. Chem.*, **65**, 493 (1993).
31. K.K. Murray and D.H. Russell, *Anal. Chem.*, **65**, 2534 (1993).
32. K.K. Murray and D.H. Russell, *J. Am. Mass Spectrom. Soc.*, **5**, 1 (1994).
33. S.L. Richheimer, D.M. Tinnermeier, and D.W. Timmons, *Anal. Chem.*, **64**, 2323 (1992).
34. S.D. Harvey, J.A. Campbell, R.G. Kelsey, and N.C. Vance, *J. Chromatogr.*, **587**, 300 (1991).
35. K.M. Witherup, S.A. Look, M.W. Stasko, T.J. Ghiorzi, G.M. Muschik, and G.M. Cragg, *J. Nat. Prod.*, **53**, 1249 (1990).
36. K.M. Witherup, S.A. Look, M.W. Stasko, T.G. McCloud, H.J. Issaq, and G.M. Muschik, *J. Liq. Chromatogr.*, **12**, 2117 (1989).
37. R.J. Cotter, *Biomed. Environ. Mass Spectrom.*, **18**, 513 (1989).

Received 22 March 1994