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Multistep collisionally activated decomposition in an ion trap for the determination of the amino-acid sequence and gas-phase ion chemistry of lithium-coordinated valinomycin

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Abstract

Evidence from collisionally activated decomposition (CAD) of electrospray-produced ions in an ion trap shows that lithium ion binds to the backbone ester oxygen atoms of valinomycin to open the cyclodepsipeptide ring at the *D*-α-hydroxyvaleric acid and *L*-lactic acid residues. The two resulting ring-opened, linear acylium ions are sequenced by multiple stages (up to MS^{10}) of CAD. Amino-acid residues are sequentially cleaved from the acylium terminus of the peptides, one amino-acid residue at each stage of the CAD experiment, until each acylium ion is converted to a tripeptide species at MS^{10}. This method builds upon a previously published strategy for determining the amino-acid sequences of cyclic peptides and is used here for the valinomycin-based class of ionophore antibiotics. This entirely instrumental approach overcomes ambiguities encountered in assigning amino-acid sequences of cyclic peptides by other tandem mass spectrometric methods. These ambiguities arise from indiscriminate and multiple ring-opening reactions that occur during collisional activation of cyclic peptides, resulting in tandem mass spectra that are superpositions of random fragment ions. Multiple stages of CAD in an ion trap also facilitate more accurate interpretation of the tandem mass spectra of valinomycin $[M + Li]^+$ by unambiguously revealing the genealogies of the fragment ions. Furthermore, it reveals new gas-phase aldehyde elimination, proton transfer, and intramolecular ion rearrangement reactions that occur upon collisional activation of valinomycin $[M + Li]^+$. Cyclic peptides produce b_n ions upon ring opening and fragmentation. Therefore, they may serve as models for understanding the mechanisms of linear peptide fragmentation. (Int J Mass Spectrom 194 (2000) 247–259) © 2000 Elsevier Science B.V.

Keywords: MS^n; Lithium-complexed structure; Cyclic peptides; Ion trap; Sequencing

1. Introduction

The coupling of electrospray ionization (ESI) with the quadrupole ion trap has significantly extended the range of biological applications of mass spectrometry in recent years [1–4]. The virtues of the ion trap as an ion-storage device, including high full-scan sensitivity, low cost, small size, high MS/MS efficiencies, and high duty cycle make it especially suited to a wide range of biochemical applications [1–4]. In particular, use of the multiple stages of collisionally-activated decompositions (CAD) (MS^n) [5] can unravel complicated reaction pathways, yielding structural and mechanistic data of significant utility. Indeed, several

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Dedicated to Professor Jim Morrison on the occasion of his 75th birthday.

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stages of CAD experiments are needed to establish structural specificity in some analytical problems. Examples include determining linkage positions in oligosaccharides [6] and distinguishing isomeric polycyclic aromatic hydrocarbons [7], oligonucleotides [6,8], and complicated surfactants [9]. Thus, MS^n in an ion trap may permit realistic determination of certain structures by establishing fragment-ion genealogies.

Realization of the potential of MS^n, however, is often impeded by a rapid decline in the precursor-ion intensities with increasing number of stages of the CAD experiments. This comes about as a result of the partitioning of the precursor-ion intensity among its product ions. Thus, many practitioners [10] hold the view that experiments beyond MS^4 are not useful in most practical biochemical applications.

In this article, we present experimental strategies to achieve up to ten stages of CAD experiments in an ion-trap instrument for the lithium adduct of valinomycin, *m/z* 1117. Lithiated valinomycin fragments upon collisional activation to produce more than 100 significant fragment ions, resulting in a substantial partitioning of the precursor-ion intensity among its products ions. It thus acts as a realistic test of the number of stages of collisional activation that are feasible in an ion-trap instrument. To date, there are few reports of practical application of MS^{10}, and this is not surprising because MS^{10} is not necessary in solving most structural and mechanistic problems. Sequencing biopolymers, however, may be an exception.

Cooks and co-workers [7] implemented up to MS^{10} with a prototype ion-trap instrument in their studies on the fragmentation mechanisms of pyrene and anthracene. However, the highest precursor mass (pyrene, *m/z* 202) is relatively small compared to lithiated valinomycin. In another study, Gross and co-workers [11] implemented up to MS^9 in an ion-trap instrument in their studies of octaethylporphyrin and its metal complexes. As in the pyrene work [7], the highest mass of the precursor fragmented (zinc adduct at *m/z* 597) was less than that of lithiated valinomycin. In addition, the precursor ions of octaethyl porphyrin and its metal complexes produced, at most, two or three detectable fragments at each stage of collisional activation.

Valinomycin [12] is a natural ion-transport antibiotic produced by *Streptomyces fuvissimus*. It is a cyclic dodecadepsipeptide that has the structure cyclo[-(*D*-Val-*L*-Lac-*L*-Val-*D*-Hyi)-]$_3$, where *L*-Lac is *L*-lactic acid and *D*-Hyi is *D*-α-hydroxyvaleric acid. The basis of the antimicrobial activities of valinomycin and its synthetic analogs [12–16] is its ability to bind selectively to a metal ion and induce transport of the complexed metal ion through artificial and biological membranes.

Although valinomycin-based ionophores were extensively researched in the 1970s and 1980s, there is renewed interest in their structure-activity relationships following the advent of new technologies, including high throughput methods of combinatorial chemistry, proteomics, and genomics. Thus, new structural methods are needed in the search for valinomycin analogs with specific metal-binding properties and antimicrobial properties [17–19]. This effort will entail synthesizing a library of ionophores by varying their structures and conformations and screening them for antimicrobial activities. An example is the correlation of the stability constants of a library of potassium complexes with bactericidal activities [16].

A wide range of mass spectrometric methods have been utilized to study valinomycin based class of ionophores. These include electron ionization [20], laser desorption [21], field desorption [22–24], chemical ionization [25], Fourier transform mass spectrometry [26], plasma desorption [20,24,27], fast atom bombardment [28], matrix-assisted laser desorption ionization mass spectrometry [29], and electrospray ionization [20,28,30–32]. To date, no quadrupole ion-trap studies of valinomycin, its analogs, and their metal complexes have been reported; neither is there any published work on the use of MS^n to study them. Many mass spectrometric studies of metal-ion complexes of valinomycin are concerned with the measurements of the metal-ion affinities of valinomycin [20,21,29–32]. Another reason for using metal-ion attachment in mass spectrometry is to enhance structural information by fragmenting such species. In-

Table 1
Some instrumental settings for the sequencing of b^*_{12UV}

MS^n	Precursor ion (*m/z*)	Isolation width (Da)	Relative collision energy (%)	No. of scans	No. of μscans
2	1117	0.4	37	18	15
3	1018	3,2	39,34	13	20
4	946	3,3,4	39,36,34	5	20
5	847	3,3,4,4	39,36,34,30	5	20
6	747	3,3,4,4,4	39,36,34,30,30	23	20
7	648	4,4,4,4,4,4	41,37,32,30,27,27	26	20
8	576	4,4,4,4,4,4,4	41,37,32,30,27,27,25	5	20
9	477	5,5,5,5,5,5,5,5	40,37,32,30,27,27,25,23	35	20
10	377	5,5,5,5,5,5,5,5,5	40,37,32,30,27,27,25,23,20	10	20

deed, alkali-metal-ion adduction acts as a form of derivatization because it localizes the charge and directs a more coherent, sequence-specific fragmentation, resulting in more informative product-ion spectra [33,34].

2. Experimental

Valinomycin was purchased from Sigma Chemical Co. (St. Louis, MO) and was used without further purification.

Electrospray experiments were carried out with a Finnigan LCQ ion-trap mass spectrometer (San Jose, CA) equipped with an electrospray ionization source. The spray needle was at a potential of 4.2 kV. The counterelectrode was a heated (200 °C) stainless steel capillary held at a potential of 10 V. Helium gas was introduced into the center of the ion trap at a nominal pressure of 1 mTorr (measured on a remote ion gauge) to improve the trapping efficiency of the sample ions introduced into the ion trap. The background helium gas also served as the collision gas during the CAD event. A typical experimental protocol consisted of direct infusion of a sample solution (200 fmol/μL valinomycin and 50 fmol/μL lithium iodide in water) into the mass spectrometer via a 250-μL syringe at a flow rate of 2 μL/min. The percent relative collision energy was adjusted such that the relative abundance of the residual precursor ion in the product-ion spectrum was reduced to ~10%. Spectra were acquired in the profile mode. Experimental parameters for some of the CAD experiments are listed in Tables 1 and 2.

In acquiring the product-ion spectrum of the $[M + Li]^+$ (Fig. 2), the isolation width was set at 0.4 Da, which is the minimum isolation width available on the instrument. The narrow isolation width was necessary to obtain the sharpest peaks for the fragment ions, although there was a reduction in signal. Therefore, 18 scans were averaged to obtain adequate signal-to-noise ratio in the product-ion spectrum. The relative

Table 2
Some instrumental settings for the sequencing of b^*_{12XV}

MS^n	Precursor ion (*m/z*)	Isolation width (Da)	Relative collision energy (%)	No. of scans	No. of μscans
2	1117	0.4	37	18	20
3	1018	3,2	39,34	13	20
4	918	5,4,1	40,41,33	2	20
5	819	5,5,5,5	41,40,38,31	7	20
6	747	5,5,5,5,5	41,40,38,30,28	7	20
7	648	5,5,5,5,5,5	41,40,38,30,28,26	27	10
8	548	5,5,5,5,5,5,5	41,40,38,30,28,26,24	18	10
9	449	5,5,5,5,5,5,5,5	41,40,38,30,28,26,24,22	11	10
10	377	5,5,5,5,5,5,5,5,5	40,37,32,30,27,27,25,23,20	23	10

collision energy was set at 37% of maximum, which was 5 eV (laboratory frame).

Multistage CAD experiments were performed according to the following procedure. In the first stage, the lithium adduct of valinomycin $[M + Li]^+$ was subjected to collisional activation to produce the first-generation, product-ion spectrum. In the MS^3 step, the resulting $[M - Val]^+$ ion at *m/z* 1018 was selected from the product-ion spectrum and submitted to another stage of CAD. The second-generation product-ion spectrum (MS^3) contained sequence ions, from b^*_{3UV} ion at *m/z* 278 to the precursor ion (b^*_{11UV} ion, *m/z* 1018) (nomenclature [35] is discussed in the next paragraph). Next, the b^*_{10UV} ion at *m/z* 946 was selected and submitted to MS^4 to produce the third-generation product ions, dominated by the b^*_{9UV} ion at *m/z* 847. This procedure was repeated until each of the two ring-opened valinomycin acylium ion (b^*_{12UV} or b^*_{12XV}) was converted to the b^*_{3UV} or b^*_{3XV} ions at *m/z* 278 at MS^{10}.

2.1. Nomenclature for labeling fragment ions

Fragment ions arising from collisionally activated decomposition of lithiated valinomycin are labeled according to a descriptor system developed by us [35]. An ion is labeled as x_{nJZ}, where "x" is the designation for the ion (lower case "a", "b", "c"). The symbol "n" is the number of amino-acid residues in the ion, "J" and "Z" are the single letter (upper case) codes for the two amino-acid residues connecting the backbone ester or amide bond "J–Z" that was broken to form the decomposing linear acylium ion. In ring-opened valinomycin, "J" is the O-terminal amino-acid residue (*D*-α-hydroxyvaleric acid or *L*-lactic acid) and "Z" is the *C*-terminal valine residue. The amino-acid codes were defined as: *D*-α-hydroxyvaleric acid residue = U; *L*-lactic acid residue = X, valine residue = V. The lithium ion was omitted from the label for simplicity, and the lithiated fragment was designated by a superscript *, where, for example, $b^*_{nJZ} = [b_{nJZ} - H + Li]$.

3. Results and discussion

Consistent with our recent discovery that alkali metal ions direct a highly specific opening of cyclic depsipeptide rings at the backbone ester linkages [36], we find that when the lithium adduct of valinomycin is submitted to low-energy collisional activation (CA), the lithium ion, which is likely to be bound to the ester oxygen atoms of *D*-α-hydroxyvaleric acid and *L*-lactic acid, directs the cyclodepsipeptide ring to open at the two ester linkages. Thus, two primary acylium ions, b^*_{12UV} and b^*_{12XV}, are produced, and each undergoes sequence-specific fragmentation by stepwise cleavage of amino-acid residues from the acylium terminal end. The evidence for this proposal comes from MS^n (Fig. 1, Table 3, and Scheme 1).

In the MS^n of the b^*_{12UV} ion (Fig. 1), one amino-acid residue is cleaved from the acylium terminus at each stage of the experiment, until MS^{10} is reached to give b^*_{3UV} at *m/z* 278. Cleavage of amino-acid residues exclusively from the acylium terminus of linear peptides during collisional activation was also observed earlier by Gross and co-workers [37], and more recently by Glish and co-workers [33,38].

The product-ion spectrum (MS^3) of the first-generation, fragment ion at *m/z* 1018, which was formed by the loss of a valine residue from b^*_{12UV}, shows that the *m/z* 1018 ions are a mixture of two isomeric acylium ions, b^*_{11UV} and b^*_{11XV}, as is demonstrated by the MS^4 experiments. Each undergoes a sequence-specific fragmentation to produce its corresponding product ions. The b^*_{11UV} ion loses an *L*-lactic acid residue from the acylium terminus to form the b^*_{10UV} ion of *m/z* 946. Subsequent, sequential amino-acid cleavages from the acylium end, one at each stage of MS^n, yield the rest of the sequence ions (Fig. 1). Similarly, fragmentation of the b^*_{11XV} ion by acylium-terminal cleavages produces a set of sequence ions, some of which are superimposed on those of the b^*_{11UV}: b^*_{10XV}, *m/z* 918; b^*_{9XV}, *m/z* 819; b^*_{8XV}, *m/z* 747; b^*_{7XV}, *m/z* 648; b^*_{6XV}, *m/z* 548; b^*_{5XV}, *m/z* 449; and b^*_{4XV}, *m/z* 377.

An MS^4 experiment is needed to separate these two overlapping sets of fragments. The b^*_{10UV} ion of *m/z* 946 is the precursor (Panel B of Fig. 1); the

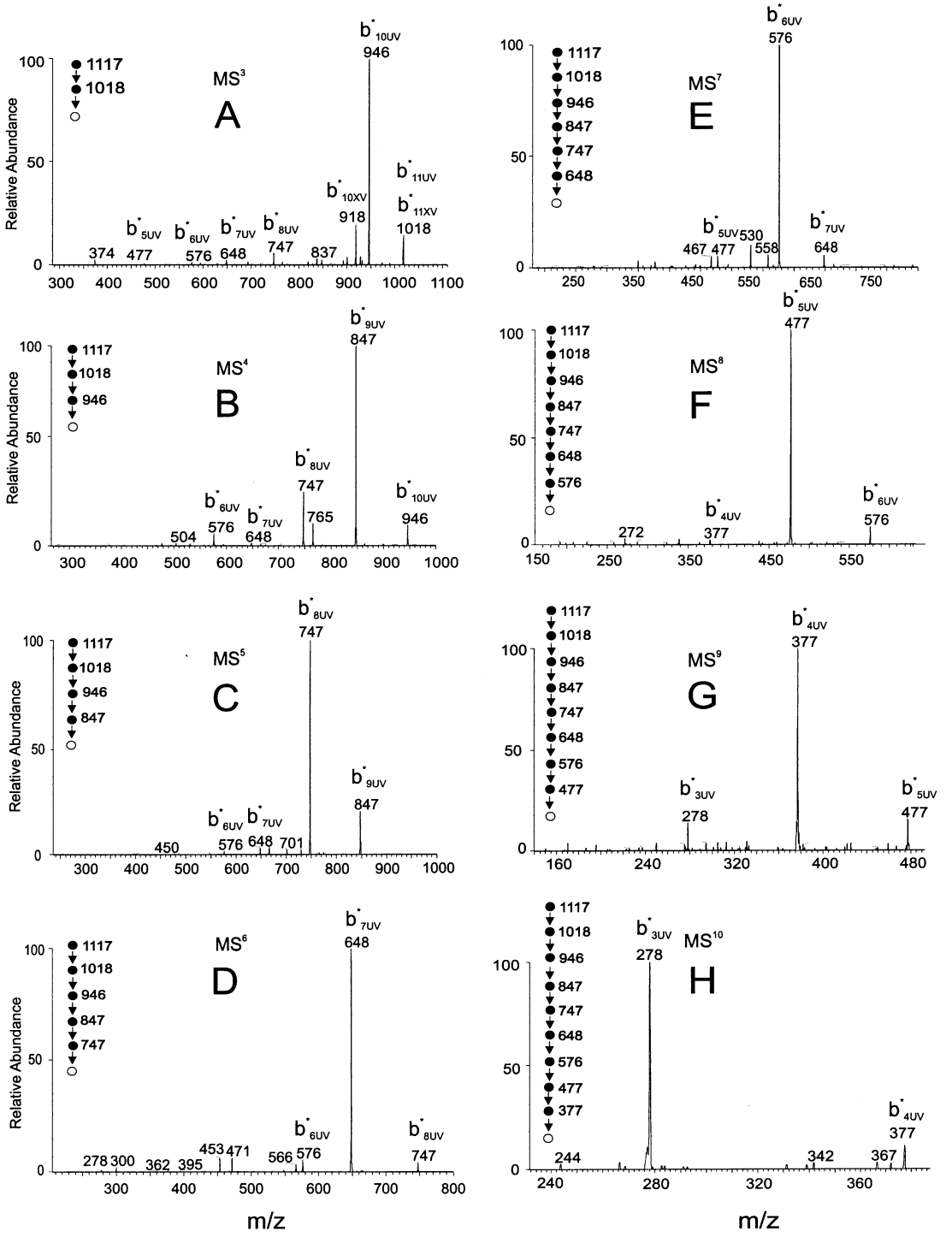


Fig. 1. MS^n of the ring-opened lithium adduct of valinomycin, b^*_{12UV}. One amino-acid residue is cleaved at each stage of CAD from the acylium terminal of b^*_{12UV}.

Table 3
MS^n of the ring-opened lithium adduct of valinomycin, b^*_{12XV}. One amino-acid residue is cleaved at each stage of CAD from the acylium terminal of b^*_{12XV}

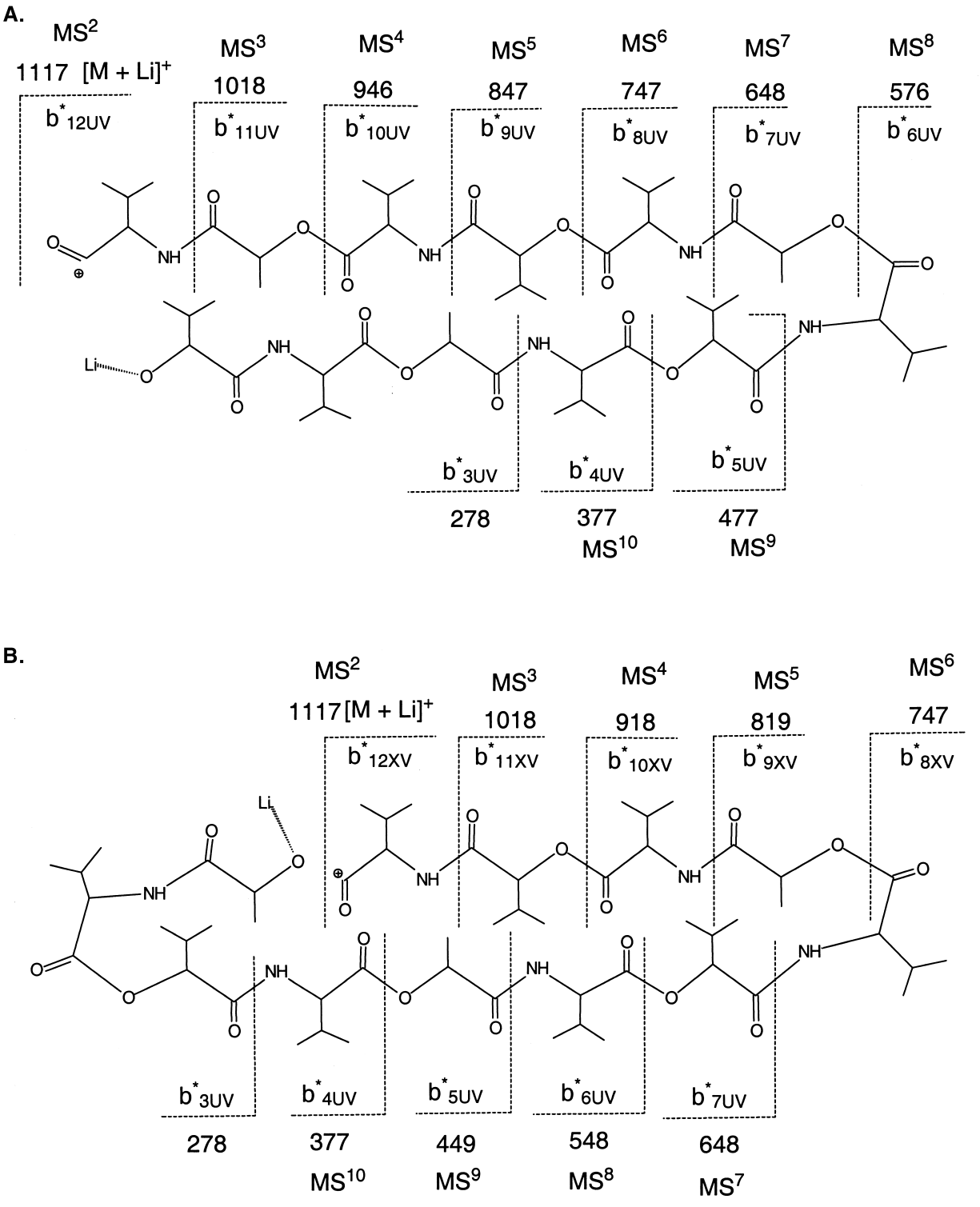
MS^n	Precursor ion (*m/z*)	Major product ions, *m/z* (% relative abundance)
2	1117	1018 (70), 918 (12), 819 (0.5), 747 (15), 648 (4), 548 (0.5), 449 (0.1)
3	1018	918 (25), 747 (6), 648 (3), 548 (1)
4	918	819 (100), 747 (10), 648 (6), 548 (8)
5	819	747 (100), 648 (9), 548 (7)
6	747	648 (100), 548 (10)
7	648	548 (100), 449 (3)
8	548	449 (100), 377 (7), 318 (13), 236 (7)
9	449	377 (100), 331 (27), 190 (17)
10	377	278 (100), 259 (7), 195 (56)

predominant reaction is a facile loss of a valine residue to produce the b^*_{9UV} ion at *m/z* 847. Other ions of low abundance, including b^*_{8UV} ion at *m/z* 747, the b^*_{7UV} ion at *m/z* 648, and the b^*_{6UV} ion at *m/z* 576, are also produced. It is, however, interesting that no losses of carbon monoxide or C_3H_8 occur from the b^*_{10UV} ion. In the MS^5 step (Panel C), with the b^*_{9UV} ion as the precursor, loss of the *D*-α-hydroxyisovaleric acid residue to form the b^*_{8UV} ion at *m/z* 747 is the predominant reaction. Panels D, E, F, G, and H show subsequent MS^n experiments, up to MS^{10}, to sequence the acylium ions (or isomeric equivalents).

The connectivity of amino-acid residues in b^*_{12XV} was also determined, starting with an MS^4 experiment (Table 3). In the MS^4 step, b^*_{10XV} ion at *m/z* 918 was selected as the precursor, thus isolating the b^*_{10XV} ion from its isomeric b^*_{10UV} ion. In this experiment, the b^*_{10XV} ion loses a valine residue to produce the b^*_{9XV} ion at *m/z* 819. Other ions of low abundance, including the b^*_{8XV} ion at *m/z* 747, the b^*_{7XV} ion at *m/z* 648, and the b^*_{6XV} ion at *m/z* 548, are also produced. As in the MS^n experiments shown in Fig. 1, no losses of carbon monoxide or C_3H_8 occur from the precursor ion. In the MS^5 step, loss of an *L*-lactic acid residue to form the b^*_{8XV} ion at *m/z* 747 is the primary reaction path. At MS^6, MS^7, MS^8, MS^9, and MS^{10}, respectively (Table 3), the b^*_{8XV} ion at *m/z* 747 was sequentially sequenced down to the tripeptide b^*_{3XV} ion at *m/z* 278 in an MS^{10} step.

The two sets of sequence ions originating from the fragmentation of b^*_{12UV} and b^*_{12XV}, respectively, can now be deciphered in the MS^2 experiments, and they are marked with arrows in the product-ion mass spectrum of the $[M + Li]^+$ shown in Fig. 2. Although this accounts for many ions, some ions require our attention. One is the ion at *m/z* 1045, which is the most abundant ion in the product-ion spectrum. It is formed by the loss of 72 u from the $[M + Li]^+$, and its source is not immediately apparent from the sets of fragments derived from b^*_{12UV} and b^*_{12XV} in Fig. 2 (marked with arrows). The same is true for the ion at *m/z* 1073, formed by loss of 44 u from the $[M + Li]^+$. MS^n experiments (Fig. 3) revealed that the ion at *m/z* 1045 is not formed by loss of Lac (72 u), but rather by loss of a neutral aldehyde, $(CH_3)_2CHCHO$, from the O-terminal *D*-α-hydroxyvaleric acid residue of b^*_{12UV}. The lithium ion is transferred to the carbonyl oxygen of the penultimate valine residue (Scheme 2). Once the aldehyde has been lost, the opportunity for additional fragmentation at the O-terminus terminates, and the fragmentation continues at the acylium terminus of b^*_{12UV}. Because it nearly has the same structure as b^*_{12UV}, it would be expected to undergo the same fragmentations as b^*_{12UV}. This sequence-specific fragmentation rules out an initial loss of Lac.

Another source of this ion, as revealed by MS^n experiments (data not shown), is the loss of carbon monoxide from the ion at *m/z* 1073. The resulting ion contains a highly conjugated structure at the acylium terminus. This conjugated moiety, $CH{\equiv}N^+COC{=}(C(CH_3)_2)OCO$, is subsequently ejected to form an abundant carbenium ion at *m/z* 892: [MS^2, *m/z* 1117 ($-$ C_3H_8)] $\rightarrow$ [MS^3, *m/z* 1073 ($-$ CO)] $\rightarrow$ [MS^4, *m/z* 1045 ($-$ $C_7H_7O_3N$)] $\rightarrow$ [*m/z*



Scheme 1. Sequencing b^*_{12UV} (**A**) and b^*_{12XV} (**B**).

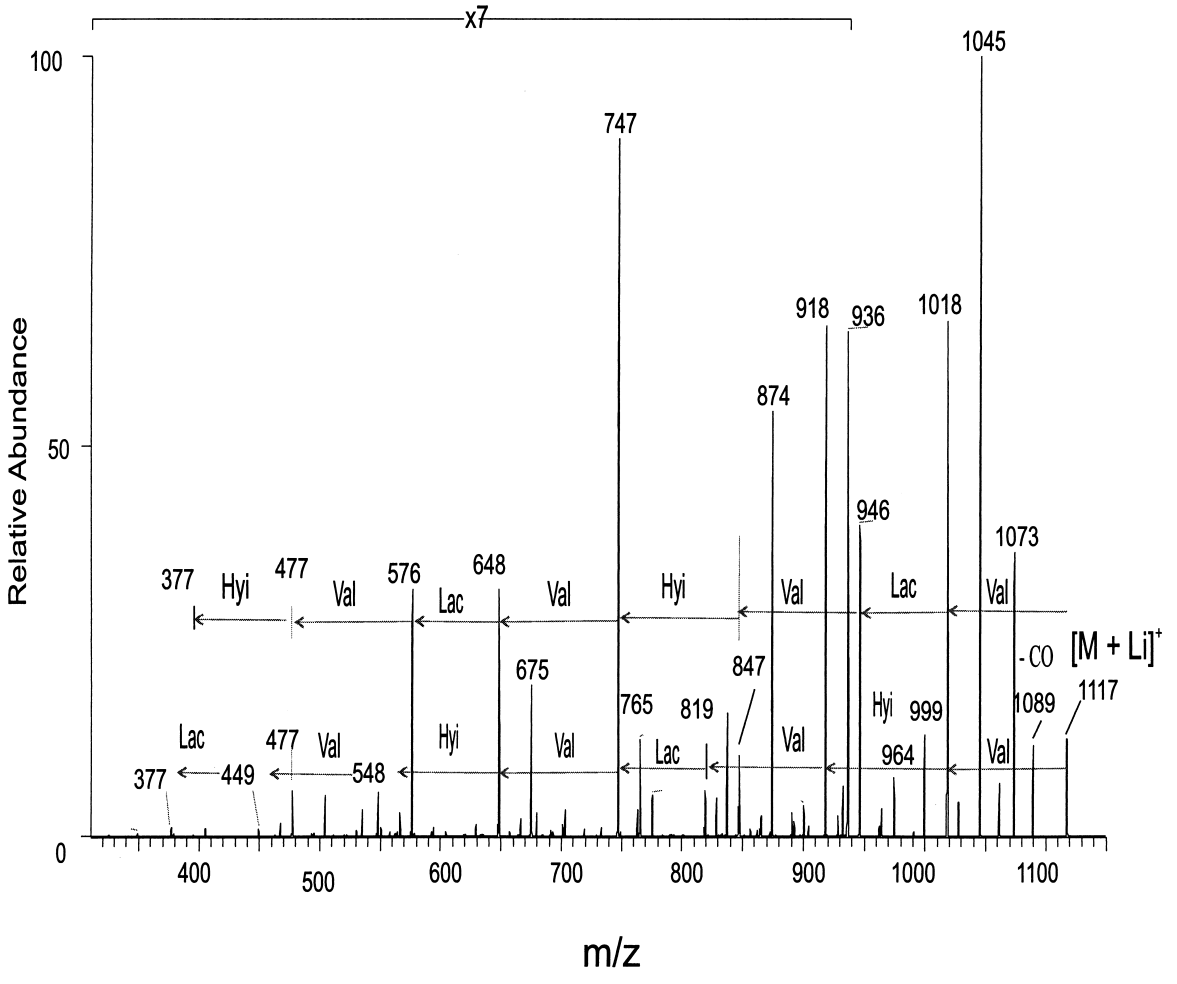


Fig. 2. Low-energy CAD mass spectrum of ESI-produced lithium adduct of valinomycin.

892, (− Val) → *m/z* 793 (− Lac) → (*m/z* 721)]. Sequential losses of Val and Lac (and Lac, Val) were also observed, presumably as a result of intramolecular rearrangements that are mediated by the carbenium ion structure.

Loss of CH_3CHO from the *O*-terminus of b^*_{12XV} to form the ion at *m/z* 1073 should occur by a mechanism similar to that for the loss of $(CH_3)_2CHCHO$ from b^*_{12UV}. It has the same structure as b^*_{12XV}, except that it is shortened at the O-terminus by the elimination of CH_3CHO. MS^n experiments (spectra not shown) showed that it undergoes the same sequential losses of amino-acid residues as b^*_{12XV}. One amino-acid residue is lost at each stage of the MS^n experiment: [MS^3, *m/z* 1073 (− Val)] → [MS^4, *m/z* 974 (− Hyi)] → [MS^5, *m/z* 874 (− Val)] → [MS^6, *m/z* 775 (− Lac)] → [MS^7, *m/z* 703 (− Val)] → [MS^8, *m/z* 604 (− Hyi)] → *m/z* 504.

Another source of the ion at *m/z* 1073 is the loss of C_3H_8 from the acylium terminal valine residue of the ring-opened $[M + Li]^+$ to form α,β-unsaturated carbonyl compounds. Thus, there would be two isomers, one arising from the loss of C_3H_8 from b^*_{12XV}, and the other from the loss of C_3H_8 from b^*_{12UV} (Schemes 3 and 4). Noteworthy is the fact that the ion at *m/z* 1073 is only ~30% of the relative abundance of the ion at

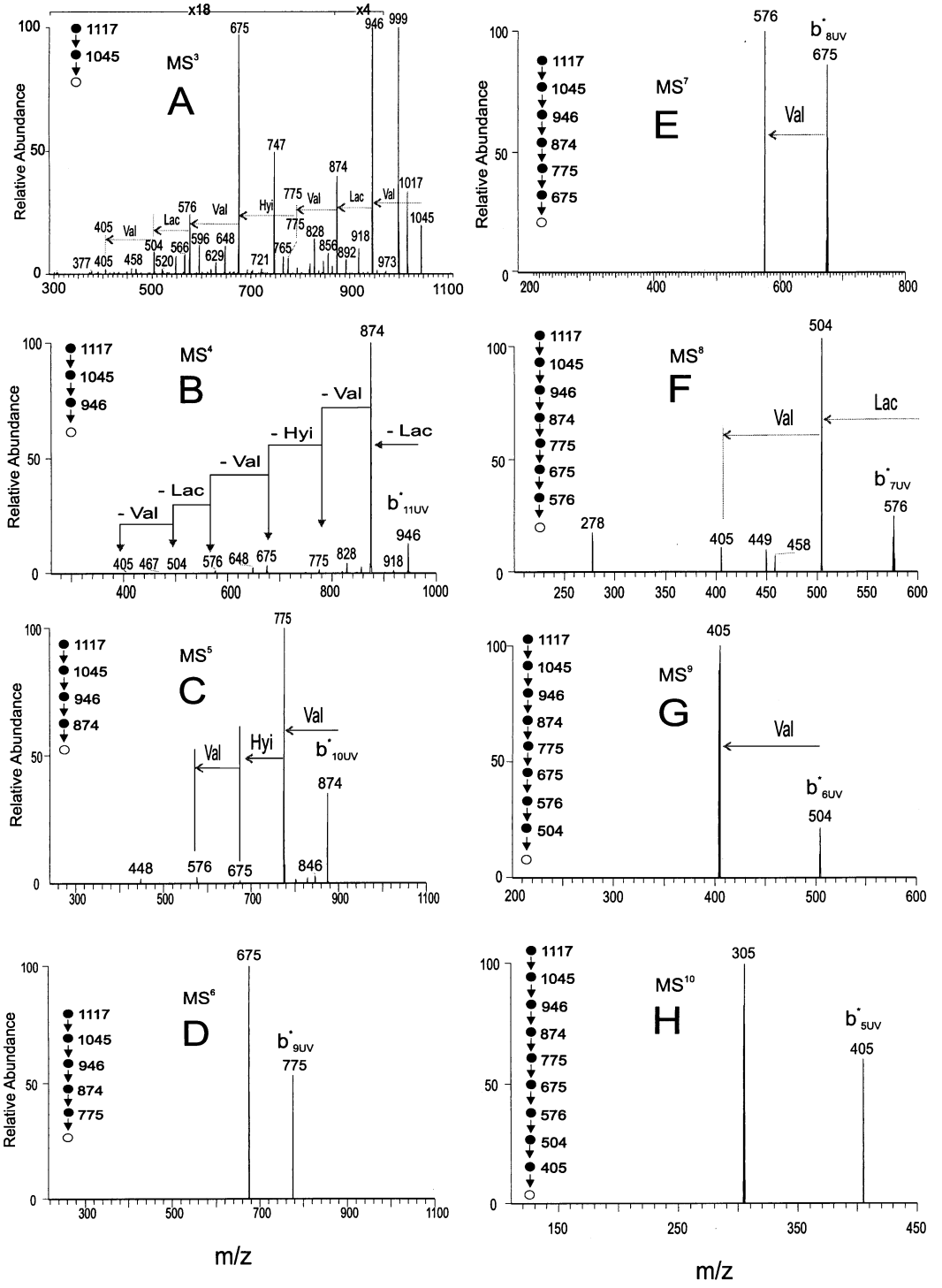
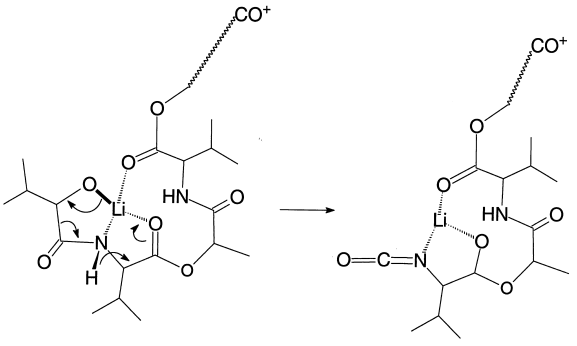


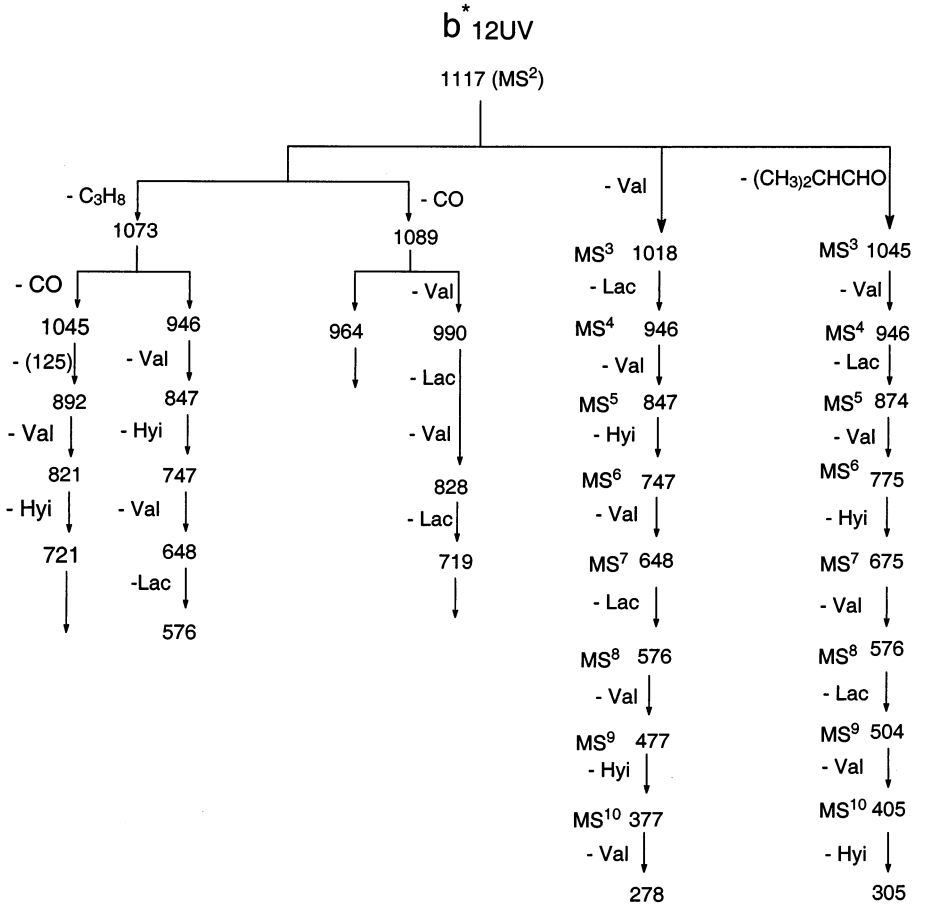
Fig. 3. MS^n of the ion at *m/z* 1045, formed by the loss of a neutral aldehyde, $(CH_3)_2CHCHO$, from the O-terminal *D*-α-hydroxyvaleric acid residue of b^*_{12UV}.



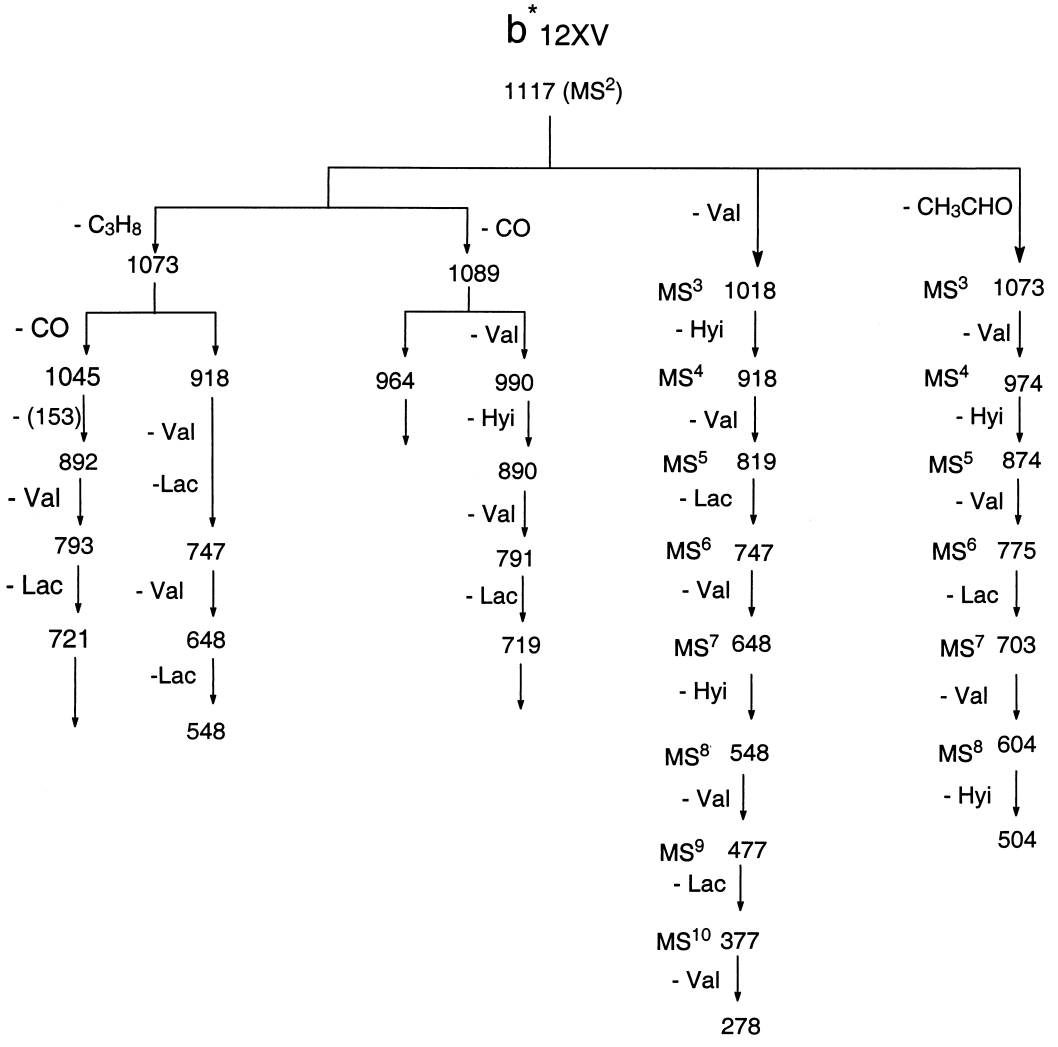
Scheme 2.

m/z 1045. This difference is consistent with the greater thermodynamic stability of $(CH_3)_2CHCHO$, compared to that of CH_3CHO.

Low-abundance ions (formed by losses of small neutral fragments such as carbon monoxide, C_3H_8, and rearranged products) are products of competing side reactions superimposed upon the primary reaction pathways discussed above. Some of these products are second- and third-generation ions arising from the decomposition of the ions at *m/z* 1089, 1073, and 1045. These include the ions of *m/z* 964 and 936 (Fig. 2), formed by the elimination of $(CH_3)_2C{=}CHNHCOCH{=}CH_2$ and $(CH_3)_2C{=}CHNHCOCH{=}C(CH_3)_2$, respectively, from the carbenium-ion termini of a^*_{12UV} and a^*_{12XV}, as revealed by MS^3 experiments (data not shown). The fact that the ion at *m/z* 936 is more abundant than the ion of *m/z* 964 (Fig. 2) is consistent with the greater stability of the symmetrically substituted alkene $(CH_3)_2C{=}CHNHCOCH{=}C(CH_3)_2$.



Scheme 3.



Scheme 4.

Several higher order product-ion spectra show ions formed by the loss of 46 Da from the precursor ions whenever a valine residue is at the C-terminal end of the ion. For example, in the MS^3 spectrum of the ion at *m/z* 1045 (Fig. 3, Panel A), the ions at *m/z* 999, 828, 629, and 458 are likely to be produced by losses of 46 Da from the ions at *m/z* 1045, 874, 675, and 504, respectively. These ions presumably arise from losses of C_3H_8 and H_2 (sequential or simultaneous reaction) to form stable, conjugated, α,β-unsaturated carbonyl compounds. This reaction may be of diagnostic value in identifying a C-terminal valine residue that is adjacent to a *D*-α-hydroxyvaleric acid or *L*-lactic acid residue.

4. Conclusions

MS^n evidence can change the interpretation of a tandem mass spectrum in ways that are impossible using any other mass spectrometric method. Several investigators [6,9,39–41] reported that the use of multistep CAD experiments in an ion trap leads to more accurate interpretation of tandem mass spec-

tra, and the results from MS^n sometimes changed otherwise convincing interpretations of product-ion spectra. For example, Harvey and co-workers [41] used MS^3 experiments to investigate formation of two isobaric ions in the product-ion spectrum of RNase B oligosaccharide $GlcNAc_2Man_5$. The study of valinomycin that we report here shows the value of MS^n to document new fragmentation and to distinguish the fragmentation of isomeric fragments.

In addition to the elucidation of reaction mechanisms, multistep CAD experiments are also useful in determining amino-acid sequences. Each spectrum shows that principally one amino-acid residue is cleaved from the acylium terminus at each stage of the MS^n experiment. The fact that this fundamental chemistry applies to both ester and amide bonds attests to the generality of this approach in sequencing peptides containing different types of amino-acid residues.

Ring opening of cyclic peptides produces exclusive b_n ions. Thus, cyclic peptides can serve as models for linear peptide fragmentation, a topic of high interest.

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