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API-Mass Spectrometry of Polyketides. I.

A Study on the Fragmentation of Triketide Lactones

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The fragmentation of δ-lactones, particularly triketide lactones, has been studied to provide information on the behavior of polyketides under atmospheric pressure ionization mass spectrometry (API-MS). The principal fragmentation patterns of triketide lactones are characterized by two sequential dehydrations followed by loss of CO to give hydrocarbon fragments. A particular goal of this study was an understanding of the origins of the two water molecules from the dehydrations. ^{18}O- and ^{2}H-isotope labeling experiments with δ-valerolactone suggest a mechanism for lactone fragmentation in which ionization by proton transfer is followed by rapid equilibration of ring-opened and ring-closed forms, which results in exchange of the ionizing proton into the hydrocarbon framework of the compound and randomization of the oxygens of the lactone. Subsequent fragmentation primarily involves sequential loss of water and CO. Similar experiments with the more complex triketide lactones show that their mass spectra share common features with that of δ-valerolactone, together with an additional water loss from the 3-hydroxyl group.

Genetic engineering of polyketide synthases now allows the production of a wide variety of new polyketides, and has advanced the concept of combinatorial biosynthesis (McDANIEL *et al.*, 1999).[1] In the preliminary analysis of genetically engineered organisms designed to produce new polyketides, liquid chromatography-mass spectrometry (LC/MS), particularly atmospheric pressure ionization mass spectrometry (API-MS) using either electrospray (ESI) or atmospheric pressure chemical ionization (APCI), plays a central role in determining which engineered organisms are worthy of further attention. Culture volumes are small at this stage, and the amounts of any new polyketides available are typically insufficient to support isolation and complete structural characterization. The API-mass spectra of complex polyketides are typically rich in fragments, and thus rich in structural information if they could be readily interpreted. It would be advantageous to glean as much structural information from the preliminary mass spectrum as possible, without having to grow large volumes of culture and isolate the products, in order to facilitate improvements to the process. To date, however, no general understanding of the fragmentation behavior of complex polyketides under API conditions is available despite effort in selected areas (GATES *et al.*, 1999; KEARNEY *et al.*, 1999; RODDIS *et al.*, 2002)[2~4].

We have undertaken experiments designed to provide some fundamental understanding of the fragmentation behavior of complex polyketides under API conditions. We herein report the results of studies on one of the simplest polyketide lactones, the triketide lactone **1** (Figure 1). The use of model compounds to study the independent behavior of the functional groups together with isotope labeling experiments provides significant information on the fundamental processes involved in the fragmentation of more complex polyketides in API-MS.

Materials and Methods

Mass spectra were obtained on a PE/Sciex API-100LC

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quadrupole spectrometer using an APCI heated nebulizer source at 325°C, with orifice and ring voltages set at 20 V and 250 V, respectively. Samples were introduced either by direct injection at a flow rate of 0.3 ml/minute using acetonitrile with 0.1% acetic acid, or as eluates from a Metachem Inertsil ODS-3 reversed-phase column (5 μm, 4.6×150 mm), using a linear gradient from 35 to 100% acetonitrile in water (both containing 0.1% acetic acid) at a flow rate of 1 ml/minute. The eluate from the HPLC column was split equally between the mass spectrometer and an evaporative light scattering detector.

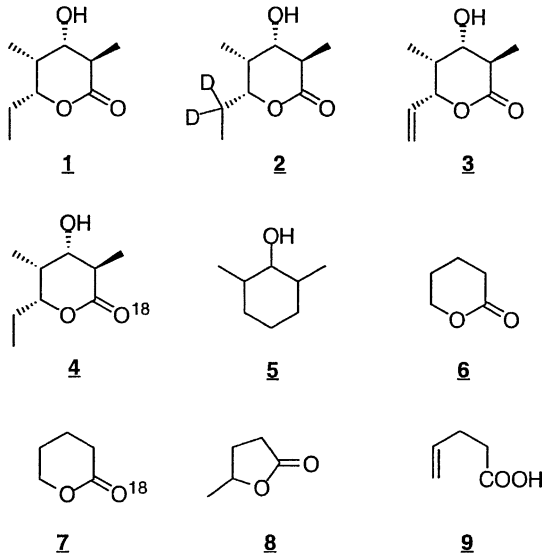
MS/MS mass spectra were obtained on an Applied Biosystems/Sciex API-3000 triple quadrupole spectrometer equipped with a TurboIonSpray source. Declustering and focusing potentials were set to 26 V and 120 V, respectively. Collision energies in the range of 10~20 eV were used. For flow inject analyses, the source was at room temperature and the analyte was infused in at 10 μl/minute from a syringe pump. For LC/MS/MS analysis the source temperature was set to 375°C. Chromatography was performed on an Agilent Eclipse XDB-C8 column (3.5 μm, 2.1×150 mm), using a linear gradient from 15 to 100% acetonitrile in water (both containing 0.1% formic acid). The column eluate was introduced directly into the source without splitting.

Triketide lactone was isolated from cultures of *Streptomyces coelicolor* CH999/pCK12 as described by KAO *et al.* (1995).[5] ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 4.136 (1H, ddd, J=2.4, 6.4, 10.4 Hz; H-5), 3.830 (1H, dd, J=4.4, 10.4 Hz; H-3), 2.478 (1H, dq, J=7.2, 10.4 Hz; H-2), 2.168 (1H, ddq, J=2.4, 4.8, 6.8 Hz; H-4), 1.811 (1H, m; H-6a), 1.570 (1H, m; H-6b), 1.398 (3H, d, J=7.2 Hz; Me-2), 0.997 (3H, t, J=7.6 Hz; H-7), 0.956 (3H, d, J=6.8 Hz; Me-4). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 174.2, 81.6, 73.9, 39.8, 36.7, 25.2, 14.2, 9.8, 4.3.

[*Carbonyl*-^{18}O]-triketide lactone was prepared by dissolving 4 mg of triketide lactone in 200 μl of [^{18}O]-H_2O (Isotec, 98 atom%-^{18}O), 50 μl of acetonitrile, and 12.5 μl of glacial acetic acid. LC/MS analysis after 3 days indicated ca. 50% incorporation of a single ^{18}O label, with no significant amount of seco-acid observed. NMR analysis confirmed the location and specificity of the labeling. ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 174.2+174.1 (C-1), 81.6, 73.9, 39.8, 36.7, 25.2, 14.2, 9.8, 4.3. This material was analyzed by MS/MS, observing the product ions from *m/z* 175.

[*Carbonyl*-^{18}O]-δ-valerolactone was prepared by dissolving 2 mg of δ-valerolactone (Aldrich Chem. Co.) in 50 μl of [^{18}O]-H_2O (Isotec, 98 atom%-^{18}O) and adding 5 μl of acetic acid. After 48 hours, the mixture was diluted into 1 ml of acetonitrile and analyzed by MS/MS, observing the products of *m/z* 103.

Fig. 1. Compounds studied.



[6-$^2H_2$]-Triketide lactone was prepared according to the method of DAYEM *et al.* (2002) by feeding 5 mM sodium [2-$^2H_2$]-propionate to two 20-ml cultures of *E. coli* expressing the first two modules of 6-deoxyerythronolide B synthase.[6] After 48 hours, the cultures were centrifuged. The supernatant adjusted to pH 4 with 1 N HCl and extracted three times with ethyl acetate. The extracts were combined, dried over $MgSO_4$, filtered, and evaporated to dryness. The residue was dissolved in 1 ml of dichloromethane and loaded onto a 5-cm column of silica gel in a Pasteur pipette equilibrated in hexane. The column was washed with one volume each of hexanes and 1 : 1 ethyl acetate - hexanes, then eluted with ethyl acetate. The ethyl acetate eluate was concentrated under vacuum, and the residue was dissolved in 200 μl of acetonitrile and analyzed by LC/MS. The labeled triketide lactone eluted at 3.8 minutes as a 1 : 1 mixture of [6-$^2H_1$]- and [6-$^2H_2$]-triketide lactones. The mass spectrum of [6-$^2H_2$]-triketide lactone was obtained by LC/MS/MS product ion analysis from *m/z* 175.

6,7-Didehydrotriketide lactone was prepared by feeding (2*S*,3*R*)-3-hydroxy-2-methyl-4-pentenoate *N*-acetylcysteamine thioester to a culture of *Streptomyces coelicolor* CH999 expressing the first two modules of 6-deoxyerythronolide B synthase having the first module ketosynthase inactivated, according to the general procedure of JACOBSEN *et al.*, 1999.[7] ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 5.837 (1H, ddd, J=5.6, 10.8, 17.0 Hz; H-6), 5.384 (1H, ddd, J=1.2, 1.2, 17.0 Hz; H-7a), 5.282 (1H, ddd,

J=1.2, 1.2, 10.8 Hz; H-7b), 4.764 (1H, m, H-5); 3.870 (1H, dd, J=4.4, 9.6 Hz; H-3), 2.515 (1H, dq, J=7.2, 9.6 Hz; H-2), 2.228 (1H, m; H-4), 1.392 (3H, d, J=7.2 Hz; Me-2), 0.958 (3H, d, J=6.8 Hz; Me-4). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 173.4, 133.6, 117.4, 80.0, 73.4, 40.0, 37.9, 14.4, 5.4.

Spectra of lactones in D_2O were obtained by preparing a solution of the lactone (0.5 mg) in 1 ml of D_2O, followed by infusing this solution into the source at a rate of 10 μl/minute.

Results

API-MS of Triketide Lactone and Analogs

A product ion scan on a triple quadrupole mass spectrometer of the pseudomolecular ion of triketide lactone (**1**) using an electrospray source is shown in Figure 2A and tabulated in Table 1. The mass spectrum of **1** resulting from atmospheric pressure chemical ionization on a single quadrupole mass spectrometer source yielded comparable fragments. The spectrum shows major fragments corresponding to $[M+H-H_2O]^+$, $[M+H-2\cdot H_2O]^+$, $[M+H-2\cdot H_2O-CO]^+$, $[M+H-102]^+$, and $[M+H-74]^+$. Minor fragments corresponding to $[M+H-H_2O-CO]^+$ and $[M+H-60]^+$ as well as others are also observed.

The spectrum of [*carbonyl*-^{18}O]-TKL (Figure 2B) shows initial loss primarily of H_2O to give $[M+H-H_2O]^+$ at *m/z* 157, although a very small amount of $[M+H-H_2{}^{18}O]^+$ is observed. The second dehydration, however, involves equal loss of labeled and unlabeled water to give *m/z* 137 and 139 of equal intensity.

An MS/MS product ion spectrum of **1** dissolved in D_2O shows initial loss of HDO rather than D_2O to give $[M+D-HDO]^+$ as the first fragment, and primarily $[M+D-HDO-H_2O]^+$ as the second fragment. Additionally, the $[M+D-HDO-H_2O]^+$ fragment (*m/z* 138) loses CO as expected to give *m/z* 110.

The API mass spectra of several analogues (Figure 1) were studied to provide additional information on the fragments formed from **1**. The spectrum of 6,7-didehydro-TKL (**3**) reveals a very similar spectrum to that of **1**, shifted by −2 amu, indicating that all the listed fragments retain the C6-C7 substituent except for *m/z* 103, which remains unshifted relative to TKL. It thus appears that *m/z* 103 does not contain the C6-C7 unit, and likely represents the C1-C3 fragment of TKL (Scheme 1). This is corroborated by the spectrum of [6-$^2H_2$]-TKL (**2**), where *m/z* 103 again remains unshifted. In agreement with the proposal of Scheme 1, MS/MS analysis shows that *m/z* 103 derives directly from $[M+H]^+$; further, the spectrum of [*carbonyl*-^{18}O]-TKL (**4**) shows full retention of the ^{18}O-label in the C1-C3-derived fragment to give *m/z* 105, despite other fragments showing only 50% retention of label, indicating retention of both lactone oxygens in this fragment. Also, the ion at *m/z* 71 in TKL (**1**) shifts appropriately in the various analogues, *e.g.*, to *m/z* 73 in [6-$^2H_2$]-TKL (**2**), suggesting that these two ions may result from the alternate charge-retention results from a stepwise fragmentation (Scheme 1). The behavior of the *m/z* ion in D_2O is complex, showing peaks of essentially equal intensity at *m/z* 103, 104, and 105, suggesting loss of the C3-C7 fragment of **1** with zero, one, or two deuterium atoms.

The $[M+H-74]^+$ fragment at *m/z* 99 in the spectrum of **1** is derived from $[M+H-H_2O]^+$ and is likely to be the result of loss of one water and the elements of acrolein (CH_2CHCHO). Previous high-resolution experiments have suggested the formula $C_6H_{11}O$ for this fragment, in agreement with this hypothesis (WEISSMAN *et al.*, 1999)[8].

The minor fragment at *m/z* 127, corresponding to $[M+H-H_2O-CO]^+$ has essentially lost the oxygen label in the spectrum of **4**, thus indicating that both oxygens lost in forming this fragment have arisen from the lactone carbonyl.

API-MS of 2,6-Dimethylcyclohexanol

The API mass spectrum of 2,6-dimethylcyclohexanol (**5**) obtained with an APCI source on a single-quadrupole mass spectrometer shows no detectible $[M+H]^+$, but rather $[M+H-H_2O]^+$ as the highest–mass peak. The base peak is *m/z* 69, corresponding to$[M+H-H_2O-C_3H_6]^+$.

API-MS of δ-Valerolactone

The API mass spectrum of δ-valerolactone (**6**) (Figure 3A; Table 2) is quite simple, showing ions corresponding to $[M+H-H_2O]^+$, $[M+H-H_2O-CO]^+$ and $[M+H-CH_2CO]^+$. The API mass spectrum of the [*carbonyl*-^{18}O]-δ-valerolactone (**7**) (Figure 3B) shows that the oxygen remaining in the $[M+H-H_2O]^+$ and $[M+H-CH_2CO]^+$ fragments is 50% labeled, suggesting formation of a symmetrical intermediate capable of losing either oxygen atom with equal probability.

The mass spectrum of (**6**) dissolved in D_2O was investigated using MS/MS. The fragmentation pattern of the deuterated ion at *m/z* 102 (Figure 3C) reveals that the initial water loss occurs primarily with loss of H_2O, with only a minor contribution from loss of HDO. The remaining fragments remain deuterated: *m/z* 74 resulting from initial loss of CO, *m/z* 60 resulting from loss of

Fig. 2. API-MS of (A) triketide lactone (B) [*carbonyl*-^{18}O]-triketide lactone (C) triketide lactone in D_2O.

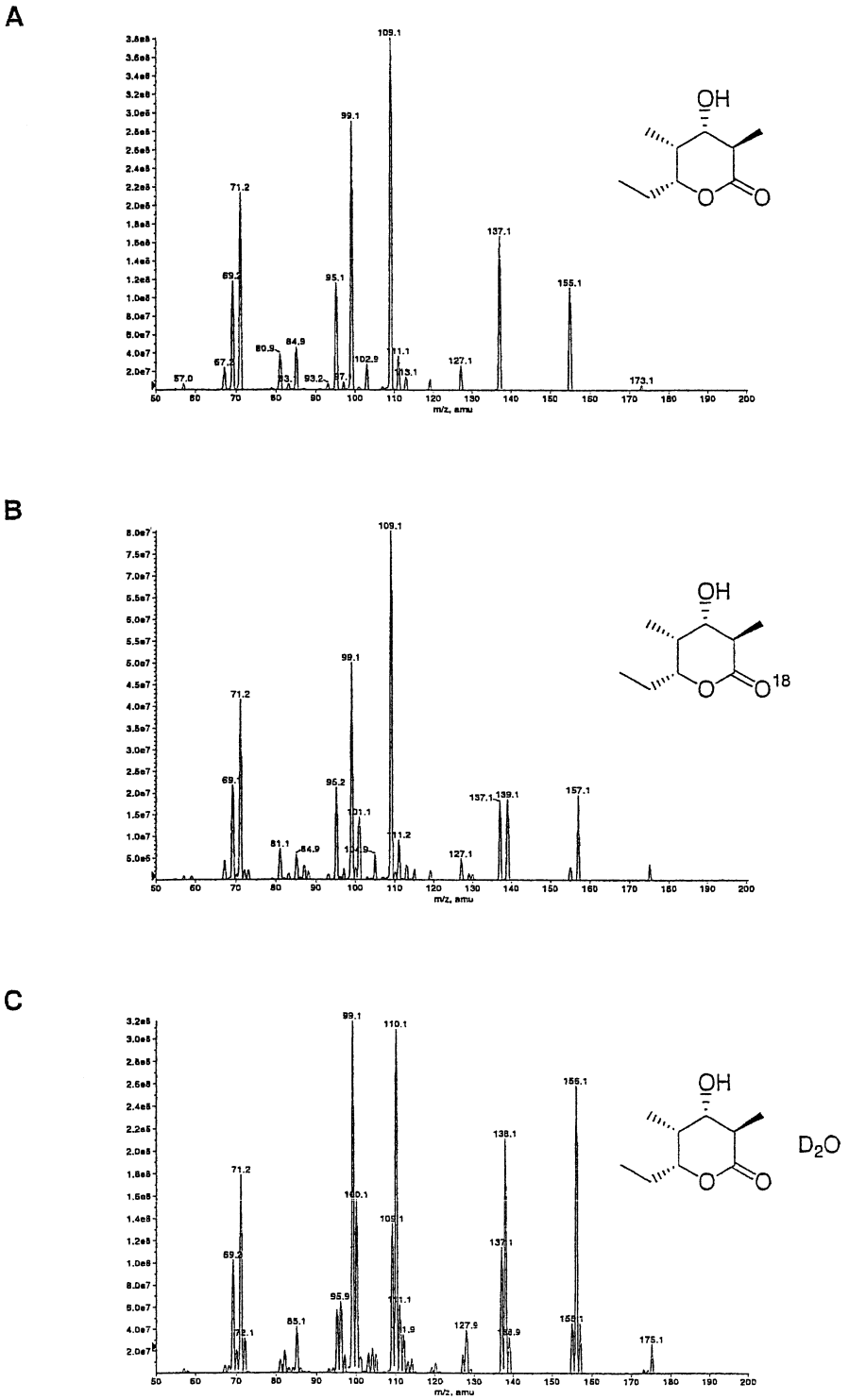


Fig. 3. API-MS of (A) δ-valerolactone (B) [*carbonyl*-^{18}O]-δ-valerolactone (C) δ-valerolactone in D_2O.

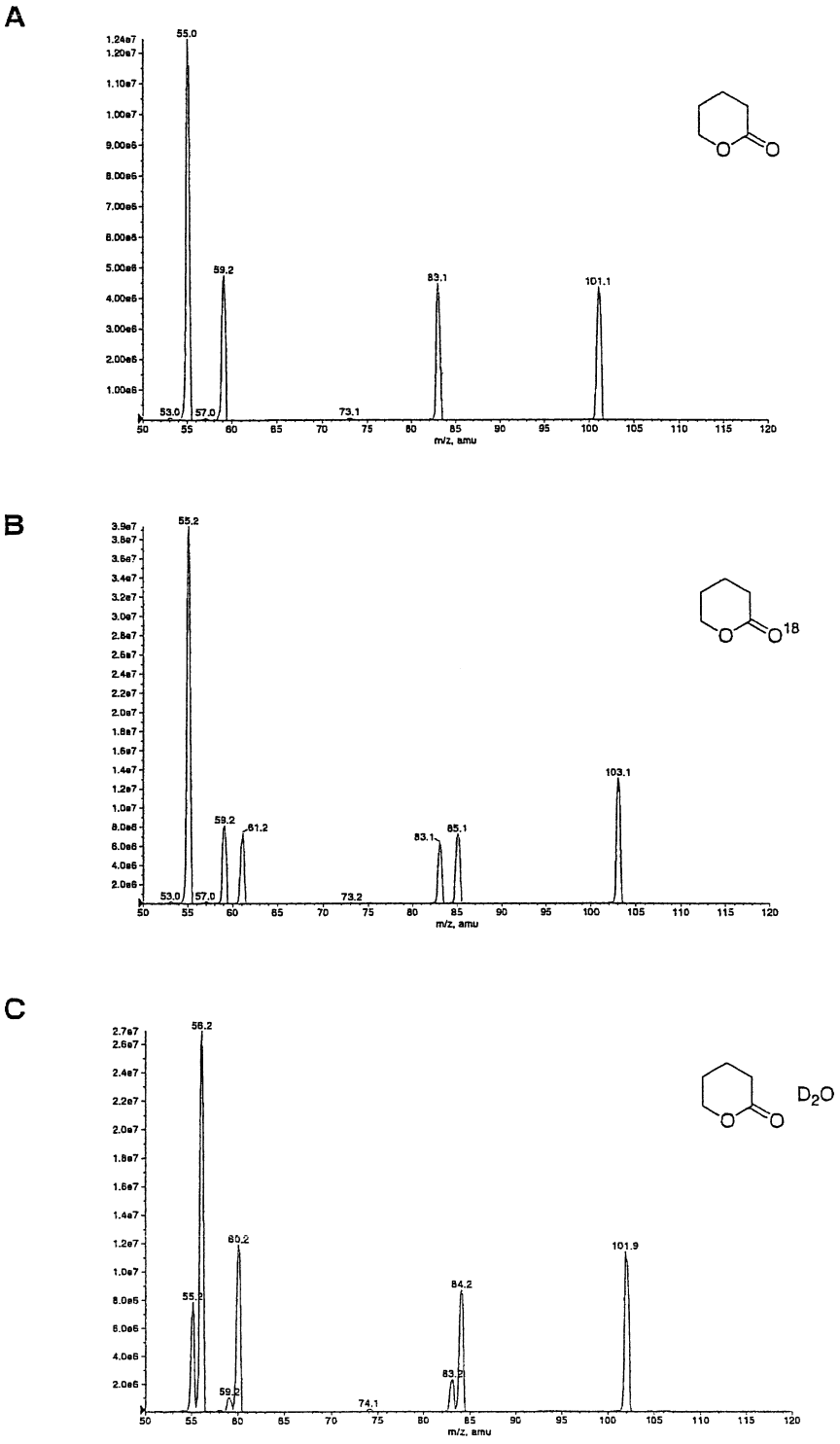


Table 1. API-MS of triketide lactones (see Fig. 1).

	$[M+H]^+$	$[M+H-H_2O]^+$	$[M+H-2•H_2O]^+$	$[M+H-H_2O-CO]^+$	$[M+H-60]^+$	$[M+H-2•H_2O-CO]^+$	$[M+H-C4:C7]^+$	$[M+H-H_2O-CH_2CHCHO]^+$	$[M+H-C1:C3]^+$
1	173	155	137	127	113	109	103	99	71
1 (D_2O)	175*	156	138^+ 137^o	128		110^+ 109^o	103 104 105	100^o 99^+	71
2	175	157	139	129	115	111	103	101	73
3	171	153	135	125	no	107	103	97	69
4	175	157 155	139 137	127	115 113	109	105	99 101	71

no = not observed (+) = major (o) = minor

*$[M+D]^+$

Table 2. API-MS of valerolactones and related compounds (see Fig. 1).

	$[M+H]^+$	$[M+H-H_2O]^+$	$[M+H-H_2O-CO]^+$	$[M+H-CH_2CO]^+$
6	101	83	55	59
6 (D_2O)	102*	84^+ 83^o	56^+ 55^o	60^+ 59^o
7	103	85 83	55	61 59
8	101	83	55	59
8 (D_2O)	102	84^+ 83^o	56^+ 55^o	60^+ 59^o
9	no	83	55	59
9 (D_2O)	no	84^+ 83^o	56^+ 55^o	

no = not observed (+) = major (o) = minor

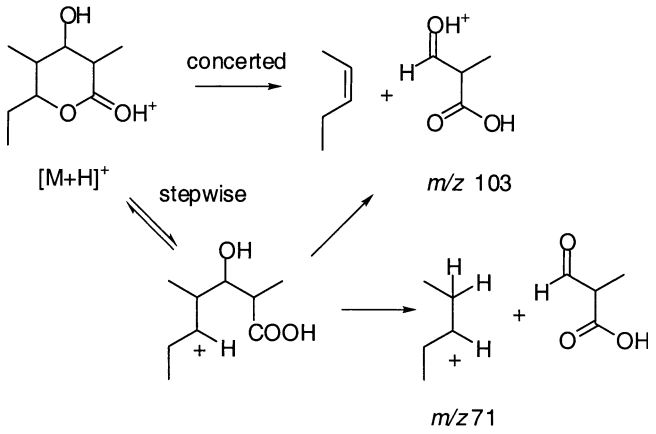
*$[M+D]^+$

CH_2CO, and *m/z* 56 resulting from loss of both H_2O and CO.

API-MS of γ-Valerolactone and 4-Pentenoic Acid

The API mass spectra of γ-valerolactone (**8**) and 4-pentenoic acid (**9**) were obtained to compare with that of **6**. The fragmentation patterns of these three molecules are very similar, each showing $[M+H-H_2O]^+$, $[M+H-H_2O-CO]^+$ and $[M+H-CH_2CO]^+$. The fragmentations of these three molecules dissolved in D_2O are also quite similar, with the fragment corresponding to $[M+D-H_2O-CO]^+$ shifting from *m/z* 55 to *m/z* 56. The dehydration fragment from **9** results from loss of HDO rather than H_2O as in the case of **6** and **8**, due to the initial dideuteration of the ionized acid in **9**.

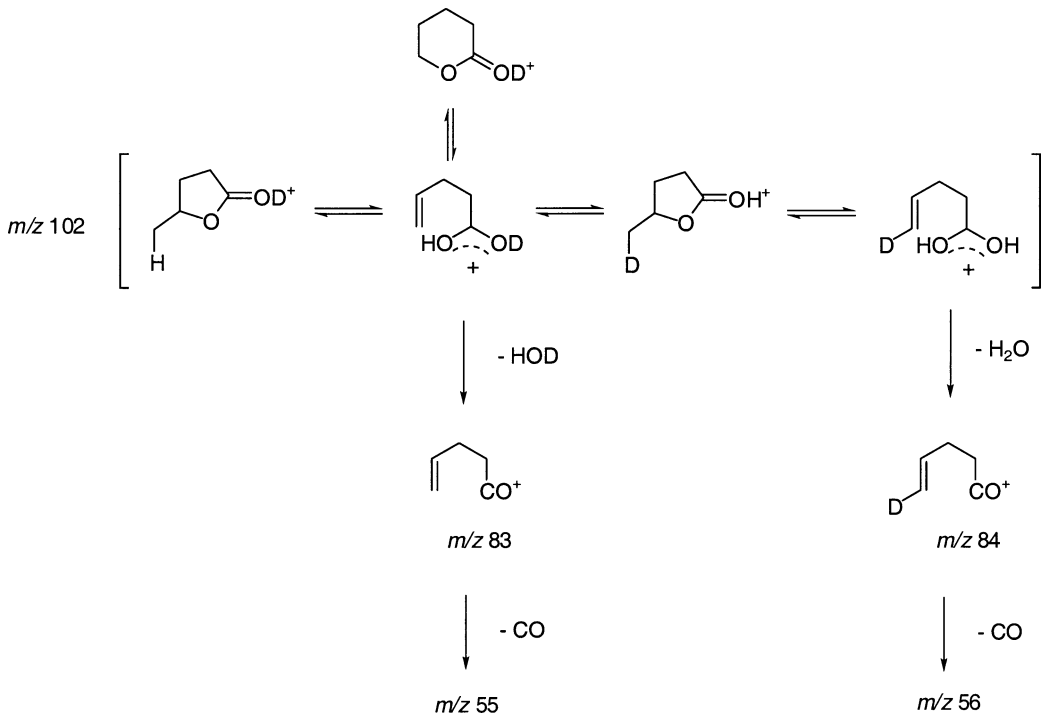
Scheme 1. Proposed formation of *m/z* 103 and *m/z* 71 in triketide lactones.



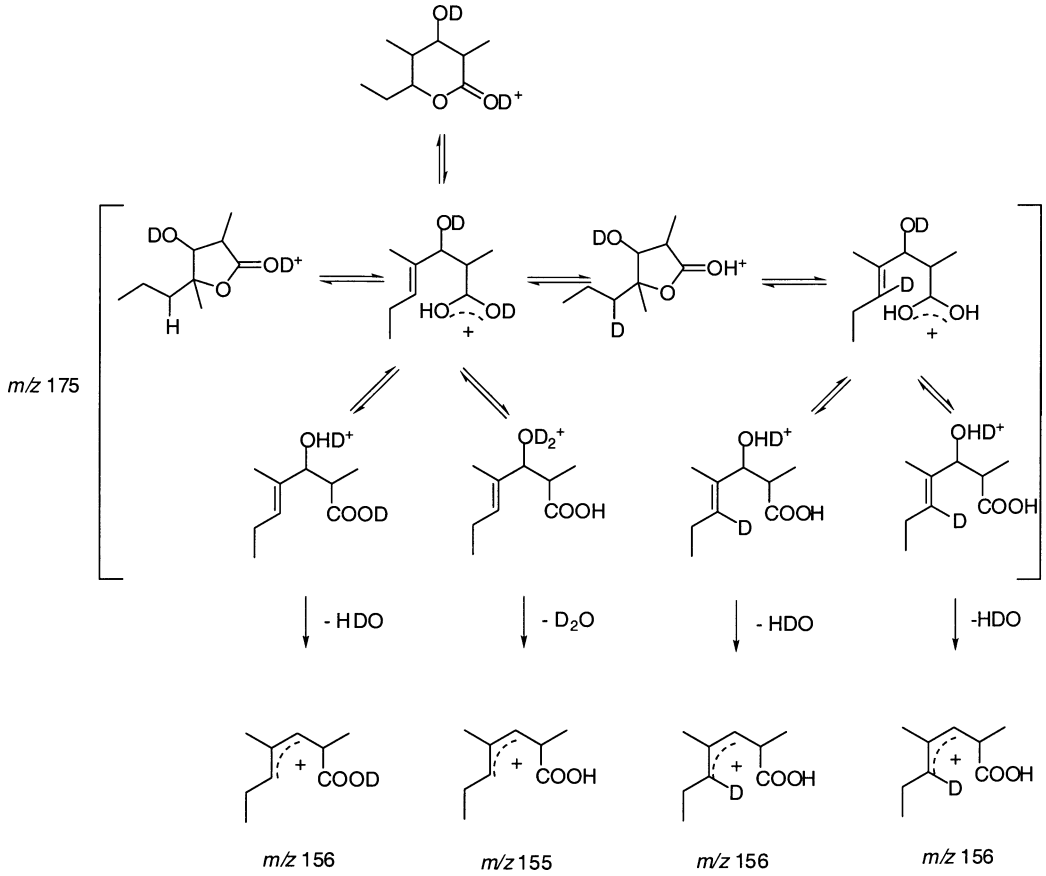
Discussion

The nature and formation of fragments resulting from loss of waters from triketide lactone (**1**) are controversial. Previous investigators (WEISSMAN *et al.*, 1999) came to the counter-intuitive conclusion that the two water losses both derive from the oxygens of the lactone group, and that the 3-hydroxyl group is not involved, although no mechanistic rationale was proposed to support this hypothesis.[8] These conclusions were based on observations that various deuterium-labeled TKLs fragmented primarily with loss of H_2O rather than HOD, that TKL dissolved in D_2O fragmented with loss of HDO rather than D_2O, and that [*carbonyl*-^{18}O]-TKL (**4**) fragmented first with loss of H_2O and then with "significant" [^{18}O]-H_2O. We have studied various TKL analogues to probe this question more

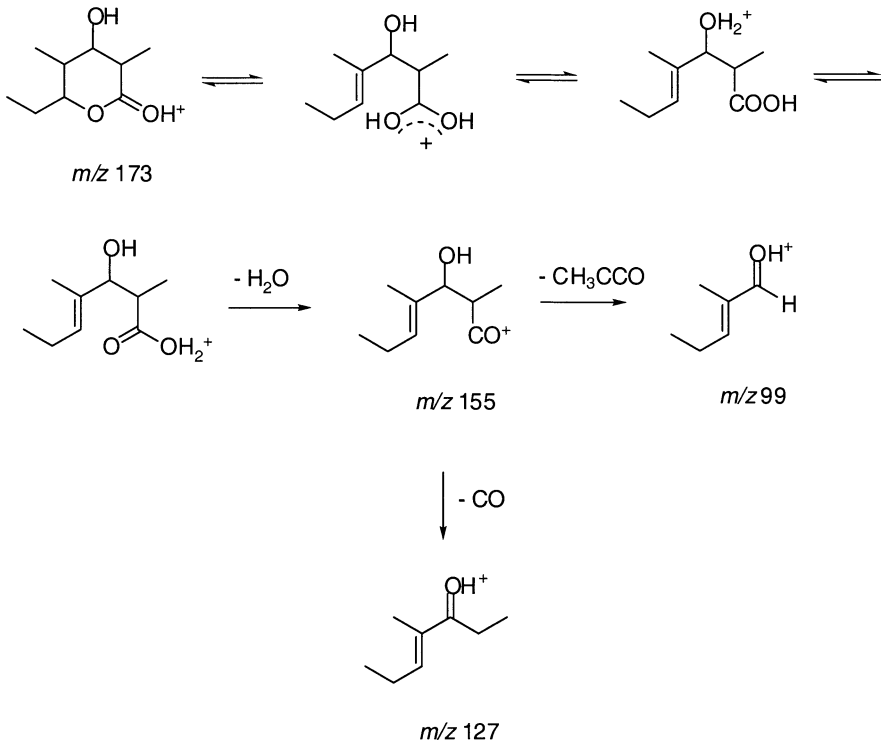
Scheme 2. Hypothetical fragmentation pathway for valerolactones.



Scheme 3. Hypothetical major fragmentation pathway for the first dehydration in triketide lactones dissolved in D_2O.



Scheme 4. Proposed minor fragmentation pathway leading to $[M+H-H_2O-CO]^+$.



systematically.

The mass spectrum of δ-valerolactone (**6**) was studied to provide information on the fragmentation of the lactone group. As shown in Figure 3A, the API mass spectrum of δ-valerolactone is quite simple, showing the expected losses of H_2O and CO along with a fragment corresponding to apparent loss of CH_2CO. To probe the mechanism of fragmentation, [*carbonyl*-^{18}O]-δ-valerolactone (**7**) was prepared by exchange with [^{18}O]-water. The API mass spectrum of the resulting labeled compound (Figure 3B) shows that the oxygen remaining in the $[M+H-H_2O]^+$ and $[M+H-CH_2CO]^+$ fragments is 50% labeled, strongly suggesting that a symmetric intermediate from which the label position can be randomized occurs prior to fragmentation. Most reasonably based on previous observations of the fragmentations of lactones to give carboxylic acids and alkenes (HERMAN & HARRISON, 1981)[9], this symmetric intermediate would be a ring-opened carboxylic acid. Scheme 2 was devised as a working model for the fragmentation of the δ-lactone functionality, illustrated with an ionizing deuteron to allow tracing of the fate of the ionizing agent.

In this scheme, initial ionization of the thermodynamically-favored carbonyl oxygen by deuteron transfer yields an $[M+D]^+$ ion that equilibrates through a series of ring opening/ring closing reactions prior to fragmentation. The ring closures are illustrated as forming the γ-lactones based on the known preference for electrophilic cyclizations; however, reclosure to the δ-lactone cannot be excluded.

To probe this further, the mass spectrum of (**6**) was obtained in D_2O, which was predicted by Scheme 2 to result in exchange of deuterium into the lactone. The fragmentation pattern of the deuterated ion at *m/z* 102 (Figure 3C) reveals that the initial water loss occurs primarily with loss of H_2O, with only a minor contribution from loss of HDO. However, the remaining fragments remain deuterated: *m/z* 74 resulting from initial loss of CO, *m/z* 60 resulting from loss of CH_2CO, and *m/z* 56 resulting from loss of both H_2O and CO. This result indicates that a deuterium atom has been incorporated with high efficiency into the hydrocarbon skeleton during fragmentation, as no oxygen atoms remain in the $[M+H-H_2O-CO]^+$ fragment, in agreement with the predictions of Scheme 2. Consistent with this hypothesis, the spectra of both γ-valerolactone (**8**) and 4-pentenoic acid (**9**) are essentially identical with that of δ-valerolactone, both in H_2O and D_2O.

The end result of this equilibration prior to lactone fragmentation is to randomize the position of the ^{18}O label in a [*carbonyl*-^{18}O]-lactone. Subsequent fragmentation by dehydration would have a 50% probability of losing the ^{18}O label as experimentally observed. With unlabeled compounds in D_2O, the mechanism of Scheme 2 will move the ionizing deuteron into the hydrocarbon skeleton, such that dehydration will occur predominantly with loss of H_2O rather than HDO if the equilibration is sufficiently complete prior to dehydration. Reversal of deuteron transfer from the methyl carbon to the acid oxygen is expected to be slow due to the competition with adjacent protons as well as any operative kinetic isotope effects.

The API-MS of 2,6-dimethylcyclohexanol (**5**) was similarly investigated to probe the behavior of the alcohol group. Consistent with observations on other simple alcohols lacking other functional groups to stabilize the $[M+H]^+$ ion, the $[M+H]^+$ ion was not observed (HARRISON, 1991); the highest-mass ion corresponds to $[M+H-H_2O]^+$. The spectrum of (**5**) dissolved in D_2O revealed only very small levels of deuterium exchange into the dehydration fragment; the observation of multiply-deuterated forms of the fragements from (**5**) again is consistent with prior observations on simple alcohols, and suggest that similar collision-mediated exchange processes occur. $[M+D-D_2O]^+$ appears to be the principal dehydration fragment from (**5**) in D_2O. It thus appears that the fragmentation pathways followed by **1** and **5** under API-MS conditions are completely different, suggesting that there may be interaction between the functional groups in **1** which is not available in **5**.

Origins of the dehydration fragments of TKL

A primary goal of the present study was to probe the origins of the dehydration fragments of TKL, given the controversial mechanisms of these fragmentations. To this end, the previously reported isotope-labeling studies have been carefully reinvestigated in light of the results with simple models.

The spectrum of [*carbonyl*-^{18}O]-TKL (**4**) (Figure 2B) shows initial fragmentation to give primarily $[M+H-H_2O]^+$, although a very small amount of $[M+H-H_2{}^{18}O]^+$ is observed. The second dehydration, in contrast, involves equal loss of labeled and unlabeled water to give *m/z* 137 and 139 of equal intensity. This again implies a symmetric intermediate in the second dehydration that is capable of losing either [^{16}O]-H_2O or [^{18}O]-H_2O with equal probability. In light of the above results with δ-valerolactone, these results strongly support a major pathway in which the first dehydration involves the 3-hydroxyl, and the second dehydration involves the lactone group according to a mechanism similar to that of Scheme 2. The behavior of model compound **7** demonstrates

that pathways involving loss of lactone oxygens are characterized by random loss of the carbonyl and ring oxygen atoms (Figure 3B). If the major pathway for fragmentation were to involve loss of a lactone oxygen, then the spectrum of **4** (Figure 2B) should show *m*/*z* 155 and 157 in nearly equal amounts. This is clearly not the case, as *m*/*z* 155 is of very minor abundance. As the only other possible source for an oxygen atom in the initial dehydration of **4** (and correspondingly **1**) is the 3-OH, logically the 3-OH is the major site of the initial dehydration as indicated in Scheme 3. Furthermore, it is clear from the high level of label retention in the second dehydration fragment that both water molecules lost during these two steps in the major pathway cannot possibly derive from the lactone moiety of TKL as previously hypothesized, as such a process would necessarily remove 100% of the oxygen label.

The present results also provide some insight to the apparently anomalous fragmentation of TKL in D_2O. As previously reported (WEISSMANN *et al.*, 1999), the mass spectra of TKL in D_2O shows initial fragmentation to yield primarily $[M+H-HDO]^+$, with small amounts of H_2O and D_2O being lost, inconsistent with direct ionization and subsequent loss of the 3-hydroxyl group to give $[M+H-D_2O]^+$. The second dehydration yields primarily $[M+H-HDO-H_2O]^+$[8]. The current experiments thus provided API mass spectra essentially identical to those previously report, indicating that the different instrumentation used did not result in different fragmentations. Taken with the results of a [*carbonyl*-^{18}O]-labeling experiment, these results were previously interpreted as indicating that both water molecules in the dehydration fragmentations must derive from the lactone oxygens, and that the 3-hydroxyl group is not lost in either of the first two dehydrations.

The initial loss of HDO rather than D_2O is not an indication that $[M+H-HDO]^+$ cannot represent loss of the 3-hydroxyl, however; if the mechanism of Scheme 2 is operative for **1** as well as for **6**, loss of the 3-hydroxyl should occur as loss of HDO, as the ionizing deuteron should be exchanged into the hydrocarbon skeleton of the molecule and thus will not be available for loss in the fragmentation. Indeed, an MS/MS product ion spectrum shows that the $[M+H-HDO-H_2O]^+$ fragment of **1** (*m*/*z* 138) loses CO as expected to give *m*/*z* 110, *i.e.*, that the fragment having no oxygen atoms still retains one deuterium atom in accord with the hypothesis of Scheme 2. The behavior of TKL is thus analogous to that discussed above for δ-valerolactone, and we hypothesize that the same fragmentation mechanisms for the lactones are occurring in each molecule. Understood in the light of the ring opening/closing equilibration of labels discussed above, the apparently anomalous result with TKL is readily explained.

The behavior of the fragment giving rise to the *m*/*z* 103 ion from **1** is also consistent with this equilibration of labels. As noted above, this fragment gives rise to ions at *m*/*z* 103, 104, and 105 in D_2O, indicating loss of the C3-C7 segment of **1** with zero, one, or two deuterium atoms. According to the mechanism of Scheme 1, only the ion at *m*/*z* 103 would be expected in the absence of label equilibration. The mechanism of Scheme 3 provides a rationale for exchange of deuterium atoms into the C3-C7 segment and the corresponding complex behavior of this fragment.

A fragmentation pathway for the initial dehydration of TKL is presented in Scheme 3, and is based on the following observations: (i) The results on the fragmentation of TKL in D_2O suggest that the equilibration of the ionizing deuteron into the hydrocarbon skeleton is essentially complete prior to fragmentation, such that fragmentation pathways having a carbon-bound deuterium predominate in D_2O. (ii) The ring-opening reaction of the lactone to give an ionized carboxylic acid must give a symmetric ion as indicated to explain the labeling results; direct dehydration from such a species is difficult as it involves a symmetry-forbidden 1,3-hydrogen shift between the oxygen atoms (MIDDLEMISS & HARRISON, 1979)[11]. It is clear from the spectrum of **6** that such proton transfer can occur in the absence of additional functionality, perhaps *via* collision. (iii) The instability of $[M+H]^+$ of 2,6-dimethylcyclohexanol **5** as compared with the relatively abundant $[M+H]^+$ of **1** indicates either that the preferred ionization site of **1** is the carbonyl oxygen of the lactone, or that intramolecular proton transfer between functional groups results in charge migration after ionization and hence stabilization of the alcohol. In either case, direct ionization and loss of the 3-hydroxyl group is apparently difficult, although it is difficult to rationalize the charge migration mechanism in the absence of ring opening and the concomitant flexibility engendered on the molecule.

The structures of polyketides are such that there are often β-hydroxycarbonyl functionalities present; such groups are expected to provide low-energy pathways for charge transfer, as proton transfer can occur through highly favored six-member transition states. In the case of **1**, we propose that proton transfer occurs readily between the protonated oxygen atoms of the ring-opened intermediate and the 3-hydroxyl group five atoms away.

Scheme 3 thus postulates that the $[M+H]^+$ for **1** is a

manifold of interconverting lactones and carboxylic acids, with free proton transfer between the protonated oxygens of the carboxylic acid and the 3-hydroxyl group. The principal first dehydration involves the loss of the 3-hydroxyl group, consistent with no significant loss of ^{18}O-label from **4** in the first fragmentation. Subsequent internal proton transfer from the resulting allylic cation to the carboxylic acid facilitates loss of the second water, this time from the oxygen atoms of the lactone moiety.

The present experiments provide no information on the identities of the hydrogen atoms lost from **1** during the two dehydrations. Investigations into this question in other systems have indicated the occurrence of multiple competing pathways, for example during cleavage of esters (BENOIT & HARRISON, 1978)[12]. The representations in Scheme 3 are provided for accounting purposes, but it is likely that the equilibrium proton transfers within the ions as well as collisionally-induced exchange together with the existence of multiple pathways may make this question difficult to answer.

Conclusions

A detailed investigation into the fragmentation behavior of δ-lactones has lead to a better understanding of the behavior of simple polyketides in API mass spectrometry. Isotope labeling experiments support a fragmentation pathway in which δ-lactones undergo a rapid equilibration between closed and open forms. This equilibration is characterized by randomization of the oxygen atoms in the lactone group and exchange of the ionizing proton into the hydrocarbon skeleton of the molecule. The current results also support a major pathway for triketide lactones in which the initial dehydration involves loss of the 3-hydroxyl group, most likely assisted by proton transfer from a ring-opened carboxylate intermediate, followed by subsequent loss of water from the carboxylate group and loss of CO. There is clear evidence for rearrangement of hydrogen atoms during the fragmentation process, but it cannot yet be determined if this is a random process or if it is mediated by specific functional groups, such as carboxylates, and so may be positionally-specific.

It seems likely that the ring-opening/closing equilibration we have observed may be general to polyketide lactones, although with macrolactones the ring-closures are more likely to generate smaller-ring lactones due to the kinetics of ring formation that tend to favor five and six-member rings. It is thus probable that the results we have observed with δ-lactones will be directly applicable to understanding the API-mass spectra of more complex polyketides. These results should thus help form a sound basis for interpretation of API-mass spectra of more complex polyketides.

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