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API-Mass Spectrometry of Polyketides. II.

Fragmentation Analysis of 6-Deoxyerythronolide B Analogs

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The API-MS spectra of 6-deoxyerythronolide B (6-dEB) and a number of its analogs have been studied to gain information into the fragmentation patterns of 6-deoxyerythronolides under atmospheric pressure ionization conditions. The API-MS spectrum of 6-dEB shows five major families of fragments. The spectra of a series of desmethyl-6-dEBs allow assignment of these fragment families to structural subunits as well as provide information regarding the fragmentation mechanisms. The spectrum of [9-^{18}O]-6-dEB is consistent with loss of the ketone oxygen during the first dehydration, and the spectra of other oxygen-modified analogs implicate the non-obligate formation of a 5,9-hemiacetal in the initial stages of fragmentation. These results taken together are used to propose a model for the fragmentation of 6-dEB and its analogs under API conditions.

Advances in genetic engineering of polyketide synthase (PKS) genes have enabled the production of a wide variety of modified polyketides. The modular nature of PKS genes provides for the replacement, inactivation, deletion, or addition of individual activities to the resulting synthase enzymes, which subsequently catalyze the biosynthesis of polyketides having defined changes in structure relative to the natural synthase product. The power of this technology has driven the development of combinatorial biosynthesis, which holds promise for the enzymatic synthesis of large libraries of new polyketides. Correspondingly powerful analytical chemistry is a necessary tool for the implementation of combinatorial biosynthesis, as large numbers of engineered organisms must typically be analyzed for production of new compounds. Atmospheric-pressure ionization mass spectrometry (API-MS) is currently a near-ideal analytical technique for this purpose. Fermentation broths can be analyzed directly and rapidly with little or no manual sample preparation, and with sufficient sensitivity to detect even low-level production. Most polyketides give complex mass spectral fragmentation patterns, and often the structural regularity of polyketides gives rise to multiple fragment structures having the same expected *m*/*z* ratio, limiting the ability to characterize new compounds by this technique despite the application of sophisticated methodology[1].

The potential of combinatorial biosynthesis has been demonstrated by the generation of a modestly-sized library of new erythronolide analogs through cassette engineering of the 6-deoxyerythronolide B synthase gene, the PKS responsible for biosynthesis of the polyketide core of the erythromycins[2,3]. As reported herein, these analogs provide a unique tool with which to study the fragmentation of erythronolides in API-MS.

Materials and Methods

General

NMR spectra were obtained using a Bruker DMX-400 spectrometer fitted with a 3-mm carbon–proton gradient probe (Z-Spec MDG400B, Nalorac) operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C measurements. Spectra were acquired in $CDCl_3$ solution with samples at 293 K unless

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otherwise noted. Resonance assignments were based on gradient ^{1}H-^{13}C-HSQC, gradient ^{1}H-^{13}C-HMBC, and gradient ^{1}H-COSY experiments.

6-Deoxyerythronolide B (**1**) and 14-desmethyl-6-deoxyerythronolide B (8,8a-deoxyoleandolide, **10**): isolated from cultures of *Streptomyces coelicolor* expressing 6-deoxyerythronolide B synthase as previously described[4].

[9-^{18}O]-6-Deoxyerythronolide B (**2**) and [9-^{18}O]-5-*O*-methyl-6-deoxy-erythronolide B (**14**): A sample of 6-dEB (**1**, 10 mg) was dissolved in acetonitrile (250 μl) and [^{18}O]-water (250 μl, Isotec, 98 atom-% ^{18}O). Glacial acetic acid (25 μl) was added, and the mixture was stirred for 48 hours at ambient temperature, at which MS analysis indicated essentially complete incorporation of one ^{18}O atom. To determine the location of the label, a 2 : 1 mixture of labeled and unlabeled 6-dEB was examined by ^{13}C-NMR. The resonance for C-9 in labeled 6-dEB was observed to be shifted upfield by 0.05 ppm relative to unlabeled 6-dEB, demonstrating incorporation of the label into the 9-ketone group.

[9-^{18}O]-5-*O*-Methyl-6-dEB (**14**) was prepared by stirring 5-*O*-methyl-6-dEB (**13**, 10 mg) in acetonitrile (200 μl), [^{18}O]-water (100 μl, Isotec, 98 atom-% ^{18}O), and acetic acid (20 μl) for 5 days, at which time MS analysis indicated 50% incorporation of label. The mixture was diluted with CH_2Cl_2, washed with saturated aq. $NaHCO_3$, dried over $MgSO_4$, filtered, and evaporated to dryness. ^{13}C-NMR revealed single peaks for all resonances except C-9, which showed resonances of equal intensity at δ 213.11 and 213.06.

2-Desmethyl-6-deoxyerythronolide B (**4**): prepared as previously described[5].

4-Desmethyl-6-deoxyerythronolide B (**5**), 8-desmethyl-6-deoxyerythronolide B (**7**), 10-desmethyl-6-deoxyerythronolide B (**8**), 12-desmethyl-6-deoxyerythronolide B (**9**), 6,11-dideoxyerythronolide B (**15**), and 5,6-dideoxyerythronolide B (**11**): prepared as previously described[2].

6-Desmethyl-6-deoxyerythronolide B (**6**): prepared as previously described[6].

15-Methylerythronolide B (**3**): prepared as previously described[7].

5-*O*-Methyl-6-deoxyerythronolide B (**13**): A mixture of 6-dEB (**1**, 11.5 mg), trimethyloxonium tetrafluoroborate (10.0 mg), and 1,8-bis(dimethylamino) naphthalene (10.0 mg, Proton-Sponge®, Sigma-Aldrich Chemical) in dichloromethane (0.5 ml) was stirred vigorously at ambient temperature for 24 hours. The mixture was filtered and applied to a 4-g column of silica gel equilibrated with 10% ethyl acetate/hexanes and eluted with a linear gradient from 10% to 50% ethyl acetate in hexanes. The product-containing fractions were pooled and evaporated to dryness, yielding the product as a colorless oil (5 mg). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 5.13 (1H, ddd, *J*=1.6, 4.0, 9.6 Hz, H-13), 3.88 (1H, d, *J*=10.4 Hz, H-3), 3.70 (1H, ddd, *J*=2.8, 4.8, 10.4 Hz, H-11), 3.51 (dd, *J*=2.4, 5.6 Hz, H-5), 3.49 (1H, s, OH), 3.38 (3H, s, 5-OMe), 2.74 (1H, qd, *J*=6.8, 10.4 Hz, H-2), 2.73 (1H, dq, *J*=2.5, 6.6 Hz, H-10), 2.61 (1H, m, H-8), 2.33 (1H, m, H-6), 1.89 (1H, dq, *J*=2.4, 7.2 Hz, H-4), 1.81 (1H, m, H-14a), 1.74 (1H, m, H-12), 1.68 (1H, m, H-7a), 1.52 (1H, m, H-14b), 1.31 (3H, d, *J*=6.8 Hz, Me-2), 1.27 (1H, m, H-7b), 1.05 (6H, d, *J*=6.4 Hz, Me-4+Me-8), 1.03 (3H, d, *J*=6.8 Hz, Me-10), 0.98 (3H, d, *J*=7.2 Hz, Me-6), 0.93 (3H, t, *J*=7.2 Hz, Me-15), 0.88 (3H, d, *J*=7.2 Hz, Me-12).

^{13}C-NMR ($CDCl_3$, 100 MHz): δ 213.1 (C-9), 178.6 (C-1), 86.1 (C-5), 79.3 (C-3), 76.1 (C-13), 70.9 (C-11), 56.4 (OMe), 44.0 (C-2), 43.7 (C-10), 40.5 (C-12), 38.6 (C-8), 37.4 (C-4), 37.3 (C-7), 29.3 (C-6), 25.4 (C-14), 15.7 (Me-6), 14.8 (Me-2), 13.1 (Me-8), 10.6 (Me-15), 9.1 (Me-12), 7.6 (Me-4), 6.1 (Me-10).

5,6-Dideoxy-15-methylerythronolide B (**12**): A 1.0 M solution of sodium bis(trimethylsilylamide) in THF (0.5 ml) was added to a −78°C solution of 15-methyl-6-deoxyerythronolide B (**3**, 200 mg, 0.5 mmol) and 1,1′-thiocarbonyldiimidazole (100 mg, 0.56 mmol) in THF (2.5 ml) and DMF (2.5 ml). After 5 minutes, the solution was warmed to ambient temperature, diluted into ethyl acetate and washed successively with 5% aqueous KH_2PO_4, water, and brine, then dried over $MgSO_4$, filtered, and evaporated. The residue was chromatographed on silica gel using a linear gradient of 10~80% ethyl acetate in hexanes to provide 15-methyl-6-dEB 3,5-cyclic thiocarbonate (85 mg) and 15-methyl-6-dEB 5-*O*-thiocarbonylimidazolide (50 mg). The 15-methyl-6-dEB 5-*O*-thiocarbonylimidazolide (50 mg, 0.1 mmol) was suspended in toluene (2.9 ml) and treated with 1,1′-azobis(cyclohexanecarbonitrile) (5 mg) and tri-*n*-butyltinhydride (0.1 ml) at 100°C for 1 hour. The resulting clear solution was cooled concentrated under vacuum. The residue was partitioned between acetonitrile (5 ml) and hexane (5 ml), and the acetonitrile phase was evaporated to dryness. The residue was chromatographed on silica gel using a linear gradient of 10~80% ethyl acetate in hexanes to provide 5,6-dideoxy-15-methylerythronolide B (25 mg). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 5.28 (1H, dd, *J*=3.3, 10.4 Hz, H-13), 3.72 (1H, d, *J*=6.8 Hz, OH), 3.66 (1H, d, *J*=10 Hz, H-11), 3.58 (1H, d, *J*=8.0 Hz, H-3), 2.77 (1H, q, *J*=6.8 Hz, H-10), 2.60 (2H, overlap, H-2+H-8), 2.29 (1H, br s, OH), 1.74 (1H, m, H-13a), 1.65 (2H, overlap, H-7a+H-12), 1.55 (1H, dq, *J*=2.7, 6.5 Hz, H-4), 1.45 (1H, m, H-6), 1.30~1.40 (4H, overlap, H-5a+H-13b+H-14a+H-

14b), 1.22 (3H, d, J=6.8 Hz, Me-2), 1.12 (1H, ddd, J=6.8, 7.0, 13.5 Hz, H-5b), 1.02 (3H, d, J=6.5 Hz, Me-8), 1.01 (1H, overlap, H-7b), 0.97 (3H, d, J=6.8 Hz, Me-10), 0.95 (3H, d, J=6.8 Hz, Me-4), 0.90 (3H, d, J=6.5 Hz, Me-6), 0.88 (3H, t, J=7.3 Hz, Me-15), 0.85 (3H, d, J=7.0 Hz, Me-12).

^{13}C-NMR ($CDCl_3$, 100 MHz): δ 215.9 (C-9), 178.0 (C-1), 76.2 (C-3), 73.8 (C-13), 70.0 (C-11), 44.4 (C-2), 43.4 (C-8), 41.9 (C-10), 41.0 (C-12 + C-5), 40.6 (C-7), 35.9 (C-4), 34.6 (C-14), 30.1 (C-6), 22.0 (Me-6), 19.4 (C-15), 15.3 (Me-2), 15.1 (Me-8), 14.5 (Me-4), 13.7 (Me-15), 9.2 (Me-12), 7.1 (Me-10).

Mass Spectrometry

APCI-mass spectra were collected using a PE/Sciex API-100LC spectrometer fitted with an APCI ion source. The heated nebulizer was thermostated at 350°C, the nebulizer current at 3～5 uA, the orifice voltage at 20 V, and the ring voltage at 250 V. Mass spectra were typically obtained over a range of 100～500 amu with a resolution of 1 amu. Higher resolution spectra were obtained with a resolution of 0.2 amu. Samples were introduced either directly into the source as solutions in acetonitrile at 0.3 ml/minute using a Beckman HPLC pump or as the eluate from a MetaChem InertSil 5 μm ODS-3 reversed-phase HPLC column run with a gradient of 35～70% acetonitrile in water, each containing 0.1% acetic acid, with a flow rate of 1.0 ml/minute split equally between the mass spectrometer and an evaporative light-scattering detector. Control experiments confirmed that the spectra resulting from each of these techniques were identical.

MS/MS mass spectra were obtained on an Applied Biosystems/Sciex API-3000 triple-quadrupole spectrometer equipped with a TurboIonSpray ion 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 ambient temperature and the analyte was infused into the source at 10 μl/minute from a syringe pump. For LC/MS/MS analyses the source 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.

Results

API-MS of 6-Deoxyerythronolide B

To simplify analysis and discussion, we characterize the API-MS spectrum of 6-dEB (**1**, Figure 1) as consisting of five major families of fragments, herein referred to as the **A**, **B**, **C**, **D**, and **E** families (Table 1, Figure 2). Each family results from a major fragmentation of the polyketide chain, and members within each family are related by dehydrations, with increasing dehydrations being indicated with higher numbers. The highest mass **A**-family fragment is thus designated "$\mathbf{A_1}$." We have investigated these five fragment families as they are relatively instrument-independent and thus most broadly applicable. The mass spectra of 6-dEB obtained using a single-quadrupole spectrometer using an atmospheric-pressure chemical ionization source and in-source fragmentation are essentially identical to those obtained using a triple-quadrupole spectrometer using an electrospray ion source and collision-induced fragmentation, although differences are observed for ions at m/z less than ～150. We note that the mass spectra obtained by Fourier transform ion cyclotron resonance mass spectrometry of sodiated 6-dEB are somewhat different, although ions analogous to some of the **A** and **B** family ions are observed[1].

The **A** family consists of four fragments ($\mathbf{A_1}$ to $\mathbf{A_4}$) resulting from successive losses of H_2O from the $[M+H]^+$ ion.

The **B** family consists of four fragments ($\mathbf{B_1}$ to $\mathbf{B_4}$) corresponding to $[\mathbf{A}_{1\sim4}-98]^+$.

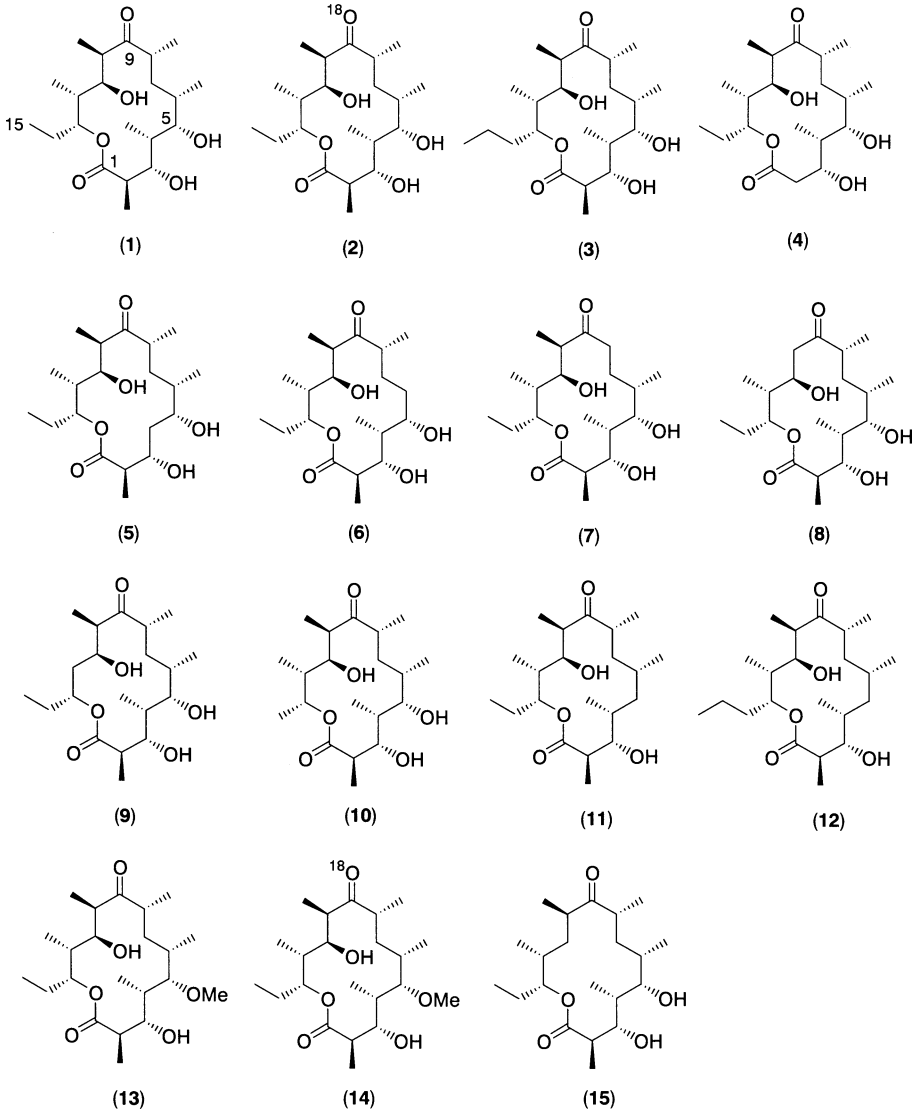
The **C** family consists of two fragments. $\mathbf{C_1}$ at $[M+H-148]^+$ is typically the most abundant ion in the spectrum at low energies.

The **D** family consists of two fragments of comparable abundance, with $\mathbf{D_1}$ observed at $[M+H-146]^+$.

The **E** family consists of a single minor fragment in the spectrum of 6-dEB corresponding to $[M+H-80]^+$, which appears to represent $[M+H-2\cdot H_2O-CO_2]^+$. As discussed below, this fragment is quite variable among the analogs examined, and is typically more abundant in APCI-MS than in ESI-MS.

The API-MS of 6-dEB labeled at the 9-ketone with ^{18}O was studied to provide information concerning the early stages of the fragmentation process. Selective labeling was readily accomplished by acid-catalyzed exchange in [^{18}O]-water, which provided essentially completely labeled material (**2**) as determined by MS and NMR analysis. Surprisingly, MS/MS product ion analysis of the fragments arising from m/z 389 reveals that none contain significant

Fig. 1. Compounds used in this study.



amounts of ^{18}O.

The spectrum of 6-dEB dissolved in D_2O provided further information concerning the fragmentation mechanisms. A product ion spectrum of the tetradeuterated $[M+D]^+$ ion at *m/z* 391 reveals that the first dehydration occurs predominantly with loss of D_2O, such that the ionizing deuteron is mostly but not entirely lost with the initial neutral fragment. The ratio of isotopic species in the **A** family fragments under these conditions suggests some rearrangement of the ionizing deuteron, and is relatively constant across the family. Subsequent dehydrations in the **A** family result from loss of HOD. Formation of the **B** family from $\mathbf{A_1}$ results from loss of 98, as is the case for formation of the **B** family in H_2O, although the relative abundances of the M and M+1 isotopic peaks appears quite different from the **A** family. $\mathbf{C_1}$ is observed principally at *m/z* 240, *i.e.*, with a single remaining deuterium atom. The **D** family shows mixed isotopic species, retaining up to 2 deuteriums in $\mathbf{D_1}$ and 1 deuterium in $\mathbf{D_2}$.

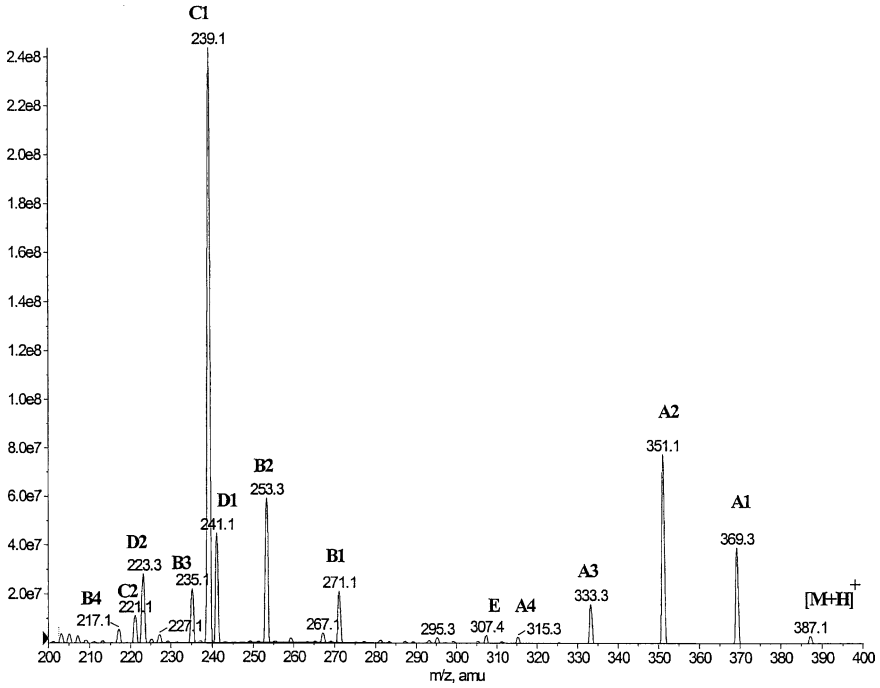
API-MS of Desmethyl-6-dEB Analogs

Correlation of the above fragment families with substructures of 6-dEB was made possible through examination of the corresponding API-MS spectra of 6-dEB analogs (**4**～**10**, Figure 1). A set of desmethyl-6-dEB analogs, obtained by fermentation of *Streptomyces coelicolor* expressing genetically engineered forms of the

Table 1. Observed *m/z* for fragments arising from compounds **1**~**15** in APCI-MS.

		Fragment *m/z*												
compound	[M+H]+	A1	A2	A3	A4	B1	B2	B3	B4	C1	C2	D1	D2	E
1	387	369	351	333	315	271	253	235	217	239	221	241	223	307
2	389	369	351	333	315	271	253	235	217	239	221	241	223	307
3	401	383	365	347	329	271	253	235	217	253	235	241	223	321
4	373	355	337	319	301	257	239	221	217	239	221	227	209	293
5	373	355	337	319	301	257	239	221	217	239	221	227	209	293
6	373	355	337	319	301	257	239	221	203	225	207	227	209	293
7	373	355	337	319	301	257	239	221	203	225	207	227	209	293
8	373	355	337	319	301	257	239	221	203	225	207	227	209	293
9	373	355	337	319	301	271	253	235	217	225	207	241	223	293
10	373	355	337	319	301	271	253	235	217	225	207	241	223	293
11	371	353	335	317	-	273	255	237	219	-	-	225	207	-
12	385	367	349	331	-	273	255	237	219	-	-	225	207	-
13	401	383	365	-	-					-	-			
		369	351	333	315	271	253	235	217					
14	403	385	369	-	-									
		371	353	335		273	255	237						
15	371	353	335	317	-	-	-	-	-	223	205	-	-	309

Fig. 2. API-MS of 6-deoxyerythronolide B.



Illustrated is the MS/MS product ion scan of *m/z* 387 ($[M+H]^+$) between *m/z* 200~400 amu, showing the observed members of the **A**, **B**, **C**, **D**, and **E** fragment families.

modular polyketide synthase 6-deoxyerythronolide B synthase, provided API-MS spectra having one or more sets of fragment families shifted by −14 amu due to the absence of the methyl group. The results for 2-, 4-, 6-, 8-, 10-, 12-, and 14-desmethyl-6-dEBs are summarized in Figure 3.

The **A** families and the **E** fragment were shifted by −14 amu in all desmethyl analogs. The **B** families were shifted by −14 amu in all families except for 12-desmethyl-6-dEB (**9**) and 14-desmethyl-6-dEB (**10**), indicating that the C-12∼C-15 fragment of 6-dEB is lost during formation of the **B** family. The **C** families were shifted by −14 amu in all families except for 2-desmethyl-6-dEB (**4**) and 4-desmethyl-6-dEB (**5**), indicating that the C-2∼C-4 fragment of 6-dEB is lost during formation of the **C** family. The **D** families were shifted by −14 amu for the 2-desmethyl (**4**), 4-desmethyl (**5**), 6-desmethyl (**6**), and 8-desmethyl (**7**) analogs, indicating that C-10∼C-15 is lost in generating the **D** family of fragments.

Further information on the $\mathbf{C_1}$ fragment was obtained from MS/MS product ion scans on desmethyl compounds (**1**), (**6**), (**7**), (**8**), and (**10**). The major daughter ions formed from $\mathbf{C_1}$ in the spectrum of (**1**) show *m/z* 123 ($\mathbf{C_1a}$), 113 ($\mathbf{C_1b}$), 99 ($\mathbf{C_1c}$), and 71 ($\mathbf{C_1d}$). Daughter ion $\mathbf{C_1a}$ is shifted by −14 amu for all these analogs except (**10**), while $\mathbf{C_1b}$ is shifted by −14 amu only for (**6**) and (**7**). Daughter ions $\mathbf{C_1c}$ and $\mathbf{C_1d}$ are shifted by −14 amu only for (**10**). These results confirm that $\mathbf{C_1}$ comprises the C-5∼C-15 segment of 6-dEB, and suggest that $\mathbf{C_1a}$ comprises C-5∼C-10, $\mathbf{C_1b}$ comprises C-5∼C-9, and $\mathbf{C_1c}$ and $\mathbf{C_1d}$ comprise carbons from the C-11∼C-15 segment of 6-dEB.

API-MS of Oxygen-Modified 6-dEB Analogs

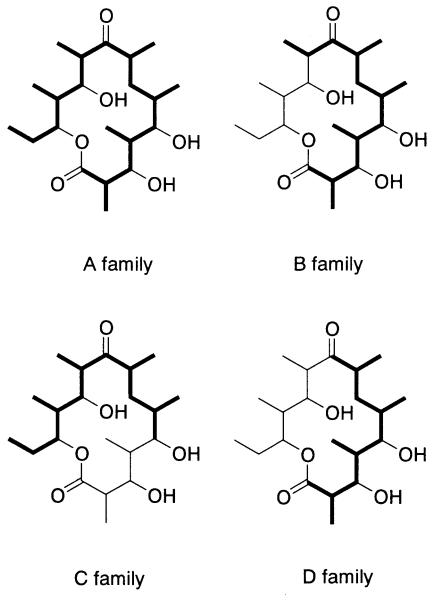
Unlike the case for desmethyl-6-dEB analogs, modification of the oxygen functionalities causes significant changes in the mass spectrum of an erythronolide. Modification of such functionality involved in fragment formation should prevent formation of selected fragments.

The spectrum of 11-deoxy-6-dEB (**15**) is particularly simple, consisting only of the **A** family, **C** family, and an **E** fragment corresponding to $[M+H-H_2O-CO_2]^+$. The **B** and **D** families are absent, indicating a role for the 11-OH group in formation of these families.

The spectrum of 5-*O*-methyl-6-dEB (**13**) was examined to provide information on the involvement of the 5-OH group in the fragmentation of 6-dEB. The spectrum of (**13**) shows an abundant $[M+H]^+$ ion together with an **A** family consisting of peaks for the unordered losses of H_2O and CH_3OH. A relatively non-abundant **B** family is observed as well, which MS/MS precursor ion analysis indicates arises directly from $[M+H]^+$ rather than from $\mathbf{A_1}$. Analysis of the spectrum of [9-^{18}O]-5-*O*-methyl-6-dEB (**14**) shows that all fragments with *m/z* greater than 150 completely retain the 9-carbonyl oxygen atom, in contrast to the observation with (**2**).

The spectrum of 5-deoxy-6-dEB (**11**) is similar to but simpler than that of (**13**), showing the expected **A** family, a **B** family that apparently arises directly by loss of 98 amu from $[M+H]^+$, and a **D** family that is 16 amu less than that observed in the spectrum of 6-dEB.

Fig. 3. Summary showing the segments of 6-deoxyerythronolide B making up fragments in the **A**, **B**, **C**, and **D** fragment families.



compound	position of demethylation	effect on fragment family			
		A	B	C	D
(4)	2	+	+	-	+
(5)	4	+	+	-	+
(6)	6	+	+	+	+
(7)	8	+	+	+	+
(8)	10	+	+	+	-
(9)	12	+	-	+	-
(10)	14	+	-	+	-

+ = shifted by -14 amu - = not shifted

Bold bonds represent the segments retained in the ions of each family in API-MS, as deduced from analysis of the API-MS of desmethyl analogs shown in the table.

Discussion

The API-MS spectra of a variety of desmethyl 6-dEB analogs have been studied to gain insight into the mechanisms of fragmentation of erythronolides under these analytical conditions. Previous studies on this class of compounds have been hampered by the structural regularity of polypropionates such as 6-dEB, which leads to ambiguities in the assignment of observed fragment ions to particular segments of the parent molecule[1]. The availability of 6-dEB analogs from genetic engineering of the polyketide synthase has provided a valuable tool with which to probe this problem.

Our working hypothesis was that the major fragmentation pathways of 6-dEB involve or are controlled by the oxygenated functionalities of that molecule. This is based on the relatively high proton affinities of carbonyls and hydroxyl groups and the lack of carbon–carbon unsaturation in the molecule. Further to this, we assume that fragmentation is controlled by the site of charge localization, with the charge serving to activate groups for reactions *via* eliminations, retro-aldol cleavages, or cyclizations. Fragmentations from different regions of the polyketide are thus assumed to occur either through differential initial protonation sites (*i.e.*, different structures for $[M+H]^+$) or through charge migration in the ions. Given the relatively dense oxygen functionality on polyketides such as erythronolides, charge migration should be facilitated by intramolecular proton transfer between appropriately spaced acidic and basic sites.

Based on these assumptions, removal of a methyl group from 6-dEB is expected to have only minor effects on the overall fragmentation pattern of the polyketide, and should result in the same families of fragment ions with one or more family shifted relative to the ions in 6-dEB due to the absence of the methyl group. When a fragment family in a spectrum of the desmethyl analog is observed to shift by 14 amu relative to the same family in the 6-dEB spectrum, the carbon bearing the methyl group is retained in the substructure that gives rise to the fragment family. Conversely, when a fragment family in a spectrum of the desmethyl analog is observed to remain unchanged relative to the same family in the 6-dEB spectrum, the carbon bearing the methyl group is lost in the substructure that gives rise to the fragment family. In agreement with this hypothesis, the spectra of the desmethyl-6-dEB analogs in general show the same families of fragment ions as 6-dEB, with some families being shifted by -14 amu relative to their counterparts in the spectrum of 6-dEB. The spectra of the desmethyl-6-dEB analogs investigated thus indicate those portions of the erythronolide skeleton that are retained or lost during the fragmentation process.

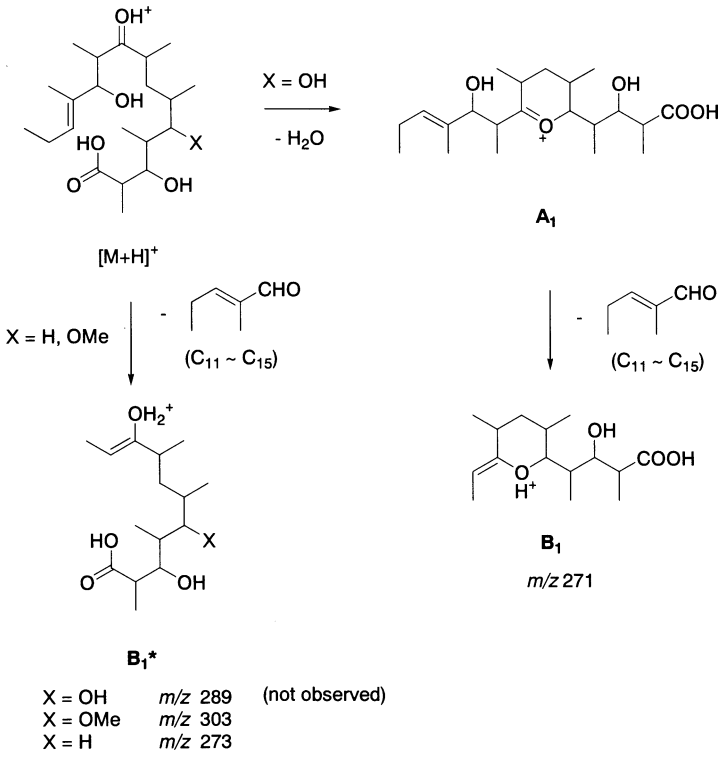
The $[M+H]^+$ ion, while weak even for 6-dEB, was observed to be typically weaker in the desmethyl-6-dEB analogs, and in many cases was observable only with difficulty. As expected, the **A** family ions, which derive from dehydrations of $[M+H]^+$, are shifted by -14 amu in all desmethyl-6-dEB analogs. For an erythronolide having n hydroxyl groups, $n+1$ ions are consistently observed in the **A** family. It was first thought that these dehydrations result from loss of each hydroxyl group, with the additional dehydration being the result of loss of water from the lactone moiety after ring opening as observed in the simple triketide lactones[8]. The actual sources of the oxygens involved in the dehydrations is more complex, however, as the spectrum of [9-^{18}O]-6-dEB (**2**) revealed that the initial dehydration must involve essentially complete loss of the 9-ketone oxygen. Nevertheless, the empirical observation of $n+1$ ions in the **A** family of an n-hydroxyl erythronolide appears to be valid. For example, 5-deoxy-6-dEB (**11**) and 11-deoxy-6-dEB (**15**) each show an **A** family with only 3 members.

The **B** family shows a more complex dependency on structure than does the **A** family, and thus provides more information regarding the analogs. The **B** family ions were shifted by -14 amu in all desmethyl-6-dEB analogs except for 12-desmethyl-6-dEB (**9**) and 14-desmethyl-6-dEB (**10**), indicating that at least the C-12～C-15 fragment of 6-dEB is lost during formation of the **B** family. Further, the **B** family remains unshifted in the spectrum of 15-methyl-6-dEB (**3**). The **B** family is not observed in the spectrum of 11-deoxy-6-dEB (**15**). These facts are consistent with a pathway through which the C-11～C-15 fragment is lost, for example through a retro-aldol type fragmentation involving the 11-OH and 9-CO groups. Thus, the **B** family of ions gives information concerning the substitution of the C-11～C-15 fragment of the erythronolide.

The observation that the **B** family fragments in the spectrum of [9-^{18}O]-6-dEB (**2**) have lost the oxygen label points to their formation from $\mathbf{A_1}$ ($[M+H-H_2O]^+$), rather than directly from $[M+H]^+$ as has been suggested[1]. Nonetheless the observation of **B** family ions in the spectra of 5-modified 6-dEB analogs does indicate that a direct pathway from $[M+H]^+$ is available. Two pathways can thus be proposed for formation of the **B** family of ions from 6-dEB depending upon the nature of the group at C-5 (Scheme 1).

In the first path, operative when X=OH at C-5, $[M+H]^+$ fragments to form $\mathbf{A_1}$ by cyclization between the 5-OH and 9-CO groups and dehydration. $\mathbf{A_1}$ subsequently fragments

Scheme 1. Two proposed pathways for the formation of **B**-family fragments.



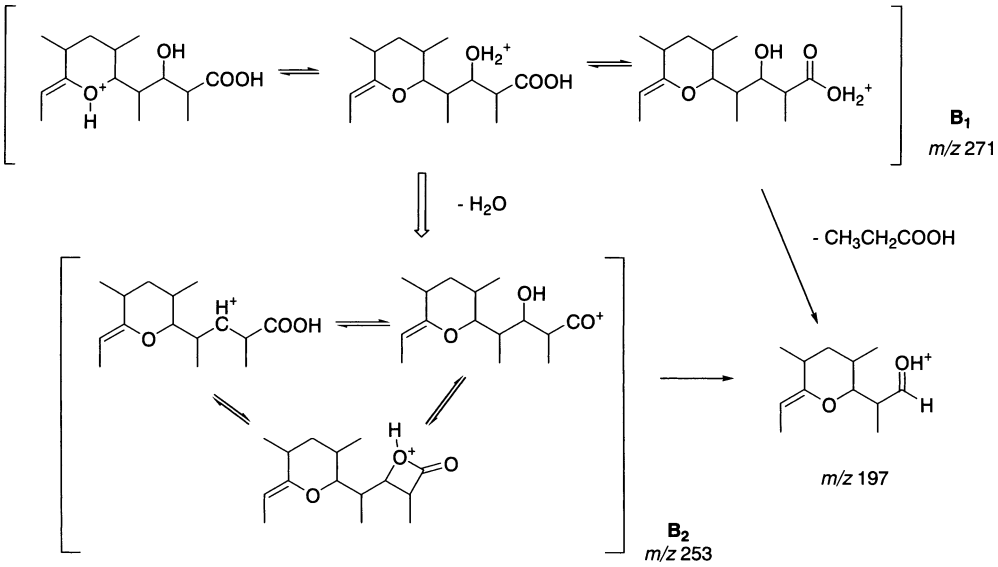
For compounds where X=OH, the pathway proceeding from $\mathbf{A_1}$ to $\mathbf{B_1}$ is observed, otherwise $\mathbf{B_1}$ arises directly from $[M+H]^+$.

by retro-aldol to eliminate the C-11~C-15 segment and provide $\mathbf{B_1}$. In the second mechanism, operative when X is not OH and possibly a minor pathway when X is OH, alternate fragment $\mathbf{B_1}$* forms by direct cleavage of the C-10~C-11 bond through a retro-aldol process. In both cases, it is assumed that eliminative delactonization is fast relative to fragmentation, as has been observed in the case of simple triketide lactones[8].

MS/MS analysis reveals that further fragmentation of $\mathbf{B_1}$ leads to $\mathbf{B_2}$ as well as formation of *m/z* 197, in keeping with the proposed structure (Scheme 2).

The spectrum of analogs modified at the 5-OH group were examined to further probe the involvement of the 5-OH group in fragmentation of 6-dEB. Methylation of the 5-OH to give (**13**) or removal as in (**11**) and (**12**) markedly changes the spectrum, pointing to the central role played by this functional group in the fragmentation of 6-dEB. The spectrum of (**13**) shows an unusually abundant $[M+H]^+$, together with an **A** family resulting from unordered losses of 18 (H_2O) and 32 (CH_3OH) amu. No ions corresponding to the **C**, **D**, or **E** families are noted. Interestingly, a low-abundance **B** family is observed, again more complex than for 6-dEB due to apparent unordered losses of 18 (H_2O) and 32 (CH_3OH) amu and differing in that $\mathbf{B_1}$ is observed at *m/z* 303, corresponding to loss of a neutral fragment of 98 amu directly from $[M+H]^+$ rather than from $[M+H-H_2O]^+$. This points to the alternate pathway for formation of **B** family fragments through direct retro-aldol cleavage from ring-opened $[M+H]^+$ to give $\mathbf{B_1}$*. This pathway is not operative in the case of 6-dEB itself (X=OH) as evidenced by the absence of an ion at *m/z* 289, presumably due to the more facile pathway proceeding through the 5,9-cyclic hemiketal. The sodiated fragment corresponding to the *m/z* 289 ion is prominent in the FT ion cyclotron resonance MS spectrum of $[M+Na]^+$, however, illustrating one difference in the fragmentations of 6-dEB between these two MS techniques[1]. Further in accord with the proposal that the 5-OH is involved in the loss of label from the fragments of [9-^{18}O]-6-dEB (**2**), the spectrum of [9-^{18}O]-5-*O*-methyl-6-dEB (**14**) shows complete retention of the ^{18}O-label in the observed **A*** and **B*** family fragments.

Scheme 2. Proposed pathway for further fragmentation of $\mathbf{B_1}$ and generation of *m/z* 197.



The **C** family of ions shows a complementary dependence upon substitution to that shown by the **B** family. The **C** family ions were shifted by −14 amu in all families except for 2-desmethyl-6-dEB (**4**) and 4-desmethyl-6-dEB (**5**), indicating that at least the C2-C4 fragment of 6-dEB is lost during formation of the **C** family. Analysis by MS/MS demonstrated that the **C** family ions can derive from $\mathbf{A_1}$, confirmed by loss of oxygen label in the **C** family in the spectrum of [9-^{18}O]-6-dEB (**2**), but cannot derive from the more highly dehydrated members of the **A** family. The **C** family is absent from the spectrum of 5-*O*-modified analogs (**11**)∼(**14**), indicating involvement of the 5-OH group in generation of the **C** family of ions. The **C** family is a dominant family in the spectrum of 11-deoxy-6-dEB (**15**). MS/MS product ion studies on the $\mathbf{C_1}$ fragment from 6-dEB (*m/z* 239) shows subsequent production of ions with *m/z* 123, 113, 99, and 71. These observations point to a working hypothesis for the formation and structure of the principal **C** fragment (Scheme 3).

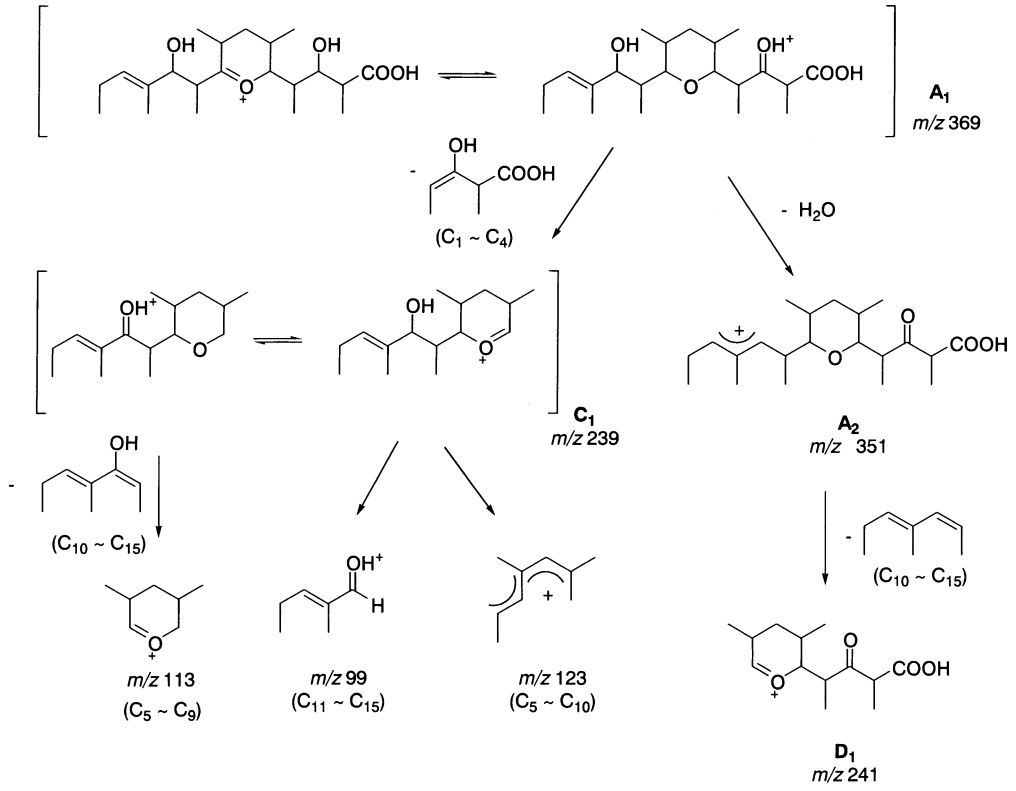
Scheme 3 postulates a [1,5]-hydride shift between the C-3-alcohol carbon and the C-9-oxonium carbon, resulting in a charge migration that allows a retro-aldol type cleavage of the C-4∼C-5 bond. Such [1,5]-hydride shifts have been previously postulated in the commonly-observed loss of water from simple aldehydes[9], and are expected to be even more facile in polyketides due to common presence of 1,5-dioxygenated carbons.

In the fragmentation of 6-dEB, the *m/z* 239 ion appears to undergo a second [1,5]-hydride shift and subsequent retro-aldol fragmentation *via* the 11-OH group, leading to an observed *m/z* 113 fragment. MS/MS product ion analysis reveals that the *m/z* 113 fragment shifts to *m/z* 99 in the spectra of desmethyl analogs (**6**) and (**7**), in agreement with the proposal of Scheme 3. This secondary fragmentation is not observed from the $\mathbf{C_1}$ ion at *m/z* 223 in the spectrum of 11-deoxy-6-dEB, as expected.

The **D** families were shifted by −14 amu only for the 2-desmethyl (**4**), 4-desmethyl (**5**), 6-desmethyl (**6**), and 8-desmethyl (**7**) analogs, indicating that at least C-10∼C-15 is lost in generating the **D** family of fragments. The absence of the **D** family in the spectra of 11-deoxy-6-dEB (**15**) together with the apparent fragmentation near C-10 points towards a role of the 11-OH in the formation of the **D** family fragments. One possible mechanism for formation of **D** family fragments is shown in Scheme 3, in which the $\mathbf{A_1}$ fragment leading to the **C** family alternatively dehydrates to form an $\mathbf{A_2}$ fragment by loss of the 11-OH group. This would allow cleavage of the C-9∼C-10 bond as indicated to give a $\mathbf{D_1}$ fragment. There are clearly other routes to the **D** family, given the observation of such fragments in the spectra of the 5-deoxyerythronolides.

The **E** fragment observed at *m/z* 307 in the API-mass spectrum of 6-dEB appears to represent $[M+H-2\cdot H_2O-CO_2]^+$. As expected, all the desmethyl-6-dEB analogs show the **E** fragment shifted by −14 amu, consistent with the loss of CO_2 from the C-1 lactone. Ions corresponding to

Scheme 3. Proposed pathways for formation of $\mathbf{C_1}$ and $\mathbf{D_1}$, and analysis of further fragmentations of $\mathbf{C_1}$ observed by MS/MS.



$[M+H-CO_2]^+$ or $[M+H-H_2O-CO_2]^+$ are not observed in the spectrum of 6-dEB. In contrast, $[M+H-H_2O-CO_2]^+$ is much more prominent in the spectrum of 11-deoxy-6-dEB (**15**) than is $[M+11H-2\cdot H_2O-CO_2]^+$. It appears that the **E** fragment may form by decarboxylation involving the 1-carboxylate and the 3-OH groups, since the 11-OH is clearly not essential for loss of the CO_2 fragment and the 5-OH is tied up by reaction with the 9-ketone as discussed above.

A summary of the portions of the 6-dEB molecule represented by the **A**, **B**, **C**, and **D** families of ions in the API-MS as deduced from the examination of the spectra of the above analogs is given in Figure 3. While the API-mass spectra of 6-dEB and analogs are quite complex, it can be seen that these families of ions represent complementary sections of the erythronolide molecule, and thus taken together provide significant structural information. Further, examination of the fragmentation mechanisms of these molecules is expected to be of more general applicability in the analysis of API-MS of other complex polyketides, particularly when simple analogs or derivatives are available. Thus, while it may prove difficult to derive extensive structural information for an individual complex polyketide based on API-MS alone, the variety of analogs made available by genetic engineering can be used to understand the complex fragmentations of these molecules and thus generate significant characterization for the members of a combinatorial genetic library.

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