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ORIGINAL ARTICLE

Biosynthesis of ebelactone A: isotopic tracer, advanced precursor and genetic studies reveal a thioesterase-independent cyclization to give a polyketide β-lactone

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Macrocyclization of polyketides generates arrays of molecular architectures that are directly linked to biological activities. The four-membered ring in oxetanones (β-lactones) is found in a variety of bioactive polyketides (for example, lipstatin, hymeglusin and ebelactone), yet details of its molecular assembly have not been extensively elucidated. Using ebelactone as a model system, and its producer *Streptomyces aburaviensis* ATCC 31860, labeling with sodium $[1\text{-}^{13}C,^{18}O_2]$propionate afforded ebelactone A that contains ^{18}O at all oxygen sites. The pattern of $^{13}C\text{–}^{18}O$ bond retention defines the steps for ebelactone biosynthesis, and demonstrates that β-lactone ring formation occurs by attack of a β-hydroxy group onto the carbonyl moiety of an acyclic precursor. Reaction of ebelactone A with *N*-acetylcysteamine (NAC) gives the β-hydroxyacyl thioester, which cyclizes quantitatively to give ebelactone A in aqueous ethanol. The putative gene cluster encoding the polyketide synthase (PKS) for biosynthesis of 1 was also identified; notably the ebelactone PKS lacks a terminal thioesterase (TE) domain and no stand alone TE was found. Thus the formation of ebelactone is not TE dependent, supporting the hypothesis that cyclization occurs on the PKS surface in a process that is modeled by the chemical cyclization of the NAC thioester.
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INTRODUCTION

A number of naturally occurring β-lactones (oxetan-2-ones) have been identified (Figure 1). Thus, natural β-lactones derived from terpenes (for example, varanisatin), amino acids (for example, SQ 26 517), fatty acids and polyketides are all well known.[1] Representative examples of the polyketide group include ebelactones A (**1**) and B (**2**) and hymeglusin (F-244, **3**), while lipstatin (**4**) is formed by degradation of fatty acids.[2–4] β-Lactones formed *via* mixed metabolic pathways include the cinnabaramides (**5**) and salinosporamides (**6**).[5,6] Many β-lactones are potent irreversible enzyme inhibitors; for example, **4** inhibits pancreatic lipase and the tetrahydro-derivative is used clinically as Orlistat in treatment of type 2 diabetes; **3** is an inhibitor of HMG-CoA synthase, an important enzyme in the mevalonate pathway to sterols; and salinosporamide A is a proteasome inhibitor that is in clinical trials.[6–8]

The ebelactones are produced by the actinomycete, *S. aburaviensis* ATCC 31860.[9] They are known inhibitors of esterases, lipases,

cutinases, homoserine transacetylase and cathepsin A, as well as modulators of the mTOR pathway.[10–14] This wide range of biological activities has triggered interest in the synthesis of these compounds.[15]

Prior studies of ebelactone biosynthesis by Uotani and co-workers with ^{13}C-labeled sodium acetate, propionate and butyrate showed that **1** is derived from a 'starter' acetate unit and six 'extender' units of propionate condensed in the typical 'head-to-tail' polyketide arrangement.[16] Likewise, **2** derives from 1 unit of acetate, 5 units of propionate and 1 terminal unit of butyrate. However, the origin of the oxygen atoms in **1** and **2** was not determined, and with it the biosynthesis of the most intriguing component of these molecules, the β-lactone ring, was left unresolved.
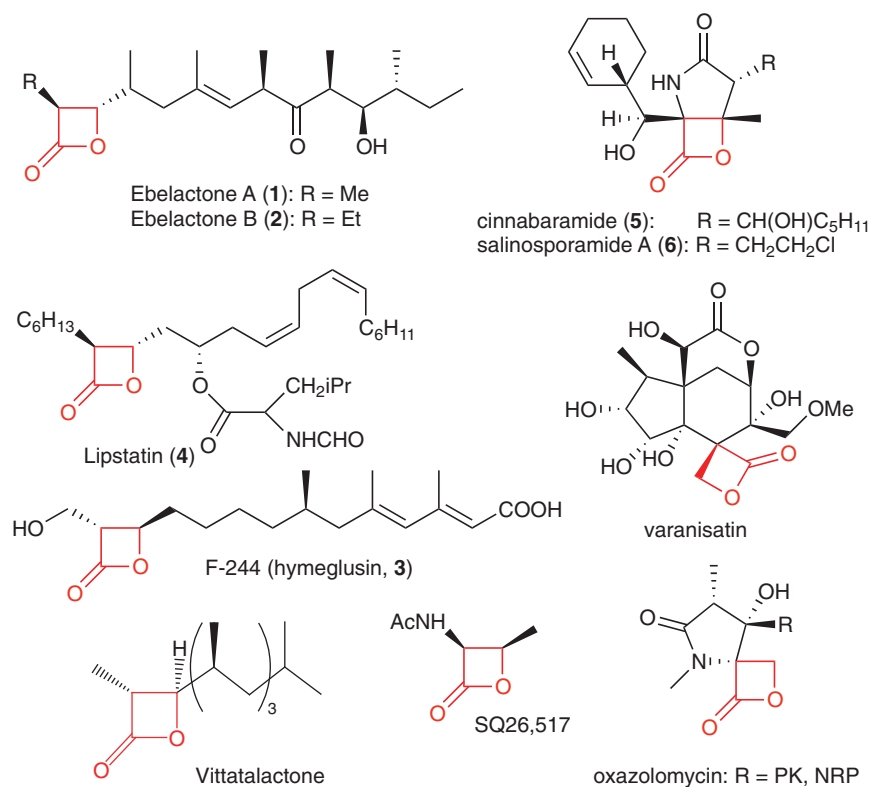
Several possible pathways for the biosynthesis of β-lactones such as **1** are feasible based on biosynthetic and/or chemical precedents (Scheme 1). In mechanisms ***a–e*** within Scheme 1, a polyketide intermediate (for example, hexaketide **7** in the case of **1**) is extended on the presumed ebelactone polyketide synthase (PKS), and the

Figure 1 Examples of naturally occurring secondary metabolites that contain a β-lactone ring, including ebelactones A and B (**1**, **2**), F-244 (**3**) and lipstatin (**4**).
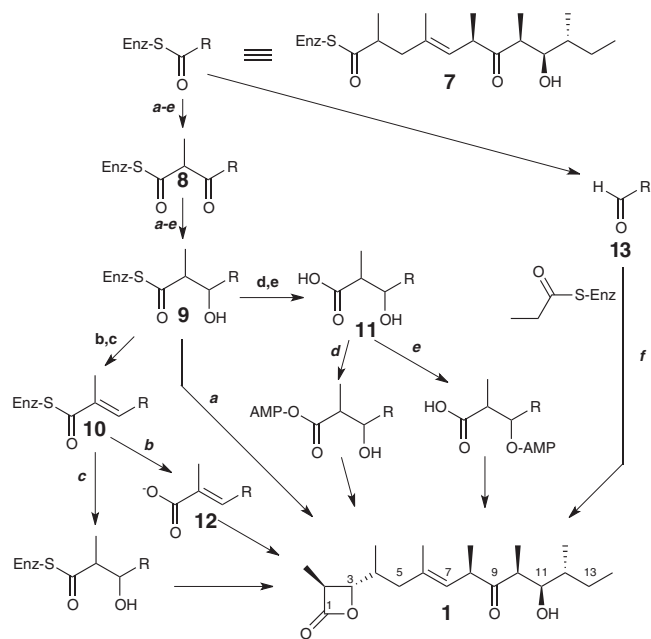
Scheme 1 Possible biosynthetic routes for lactone ring formation in β-lactones such as **1**.

resulting β-ketoacyl moiety (for example, **8**, formed from **7** and methylmalonyl-CoA for the ebelactone A case) is reduced to give the β-hydroxyacyl-enzyme (for example, **9** *en route* to **1**).

In mechanism *a*, intermediate **9** cyclizes directly by attack of the C-β hydroxy group onto the carboxylic acid derivative, whether with or without the intervention of a PKS thioesterase (TE)-dependent reaction. If a TE is present, then this mechanism gives the small ring lactone in a route that is analogous to formation of macrocyclic lactones such as erythromycin *in vivo*, and indeed TEs are found in the salinosporamide and cinnabaramide biosynthetic gene clusters, although their function remains unproven.[17–19] This route also resembles the chemical lactonization of β-hydroxy-thioesters.[20,21] In mechanism *b*, the β-hydroxyacyl-enzyme is dehydrated (for example, **9–10**), and hydrolyzed from the PKS. The resulting α,β-unsaturated acid (for example, **12**) could cyclize via protonation at C-α and nucleophilic attack by the carboxylate at C-β. This process is reminiscent of the chemical bromo-lactonization of β,γ-unsaturated acids.[20,22]

In the biosynthesis of lipstatin, a variation on pathway *b* has been proposed based on deuterium labeling experiments. A Claisen-like condensation between two esters derived from fatty acid degradation is followed by reduction of the resulting ketone. The ensuing alcohol (cf **9**, Scheme 1) then undergoes dehydration–rehydration to effect epimerization at C-β before cyclization.[23] An analogous process for ebelactone biosynthesis would correspond to mechanism *c*.

In mechanisms *d* and *e*, the β-hydroxyacyl-enzyme is hydrolyzed to give the β-hydroxy acid (for example, **9–11**), which is then activated by, for example, adenylation by ATP, either at the carboxylate (path *d*) or at the β-hydroxy group (path *e*). This activation facilitates cyclization by mechanisms analogous to the chemical synthesis of β-lactones by activation of β-hydroxyacids at carboxylate and alcohol groups, respectively.[20,24]

Lastly, by contrast, in mechanism *f*, the polyketide precursor is cleaved reductively from the enzyme to furnish an aldehyde (for example, hexaketide **7–13**).[25] This aldehyde can then undergo aldol-type condensation with an acyl donor (for example, propionyl or

methylmalonyl-CoA add to **13**), and the resulting aldol adduct can then cyclize directly in a process analogous to chemical tandem aldol-lactonization reactions.[20,21] Indeed, a tandem aldol-lactonization-like mechanism has been suggested in the biosynthesis of the salinosporamides.[18]

Mechanisms **b** and **e** require that the lactone ring oxygen atom is derived from the same biosynthetic unit as the C-1 carbonyl oxygen, and involve cleavage of the C-β-O bond during biosynthesis. In contrast, in mechanisms **a**, **d** and **f** the C-O bond at C-β remains intact from the hexaketide precursor **7**. Finally, the oxygen atom at C-β is predicted to be lost from precursor **7** in route **c**.

In order to probe the mechanism of lactone ring formation, we used the ^{18}O-induced isotopic shift method to study the origin of the oxygen atoms in F-244 (**3**) in *Fusarium* sp. ATCC 20788.[26] The results showed that the β-lactone ring is derived from an intact bond between the β-carbon and the ring oxygen atom, while the carbonyl oxygen atom is derived from an intact C=O bond in the adjacent acetate unit. Thus pathways **b**, **c** and **e** are excluded as possible mechanisms for lactone formation in this fungal metabolite.
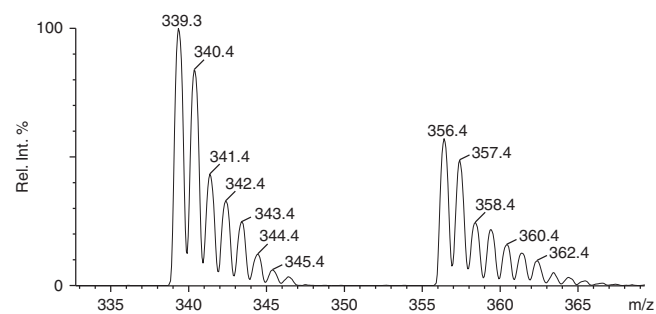
However, polyketide biosynthesis in fungi is different from that in bacteria, and we thus wished to establish whether these results still hold true in the *Streptomyces* genus, and to further discriminate between the remaining mechanisms. Here, we report the results of incorporation experiments of [1-^{13}C,^{18}O$_2$]propionate into **1**, the conversion of an acyclic precursor into **1**, and the identification of the putative biosynthetic gene cluster encoding formation of **1**.

RESULTS AND DISCUSSION

Isotope labeling experiments

Addition of sodium [1-^{13}C,^{18}O$_2$]propionate (mixed with sodium [1-^{13}C,^{16}O$_2$]- and unlabeled propionate to avoid excessive incorporation rates) to cultures of *S. aburaviensis* ATCC 31860 followed by purification gave labeled **1** (**1a**).[27] The ESI mass spectrum of this material showed a distribution of peaks corresponding to molecular ions of unlabeled **1** (MH$^+$, *m/z* 339) and labeled **1a** (MH$^+$, *m/z* 340–345) (Figure 2), along with peaks at *m/z* 356-362, due to the corresponding [M+NH$_4$]$^+$ ions. Thus, unusually high levels of isotopic enrichment were observed in this metabolite.
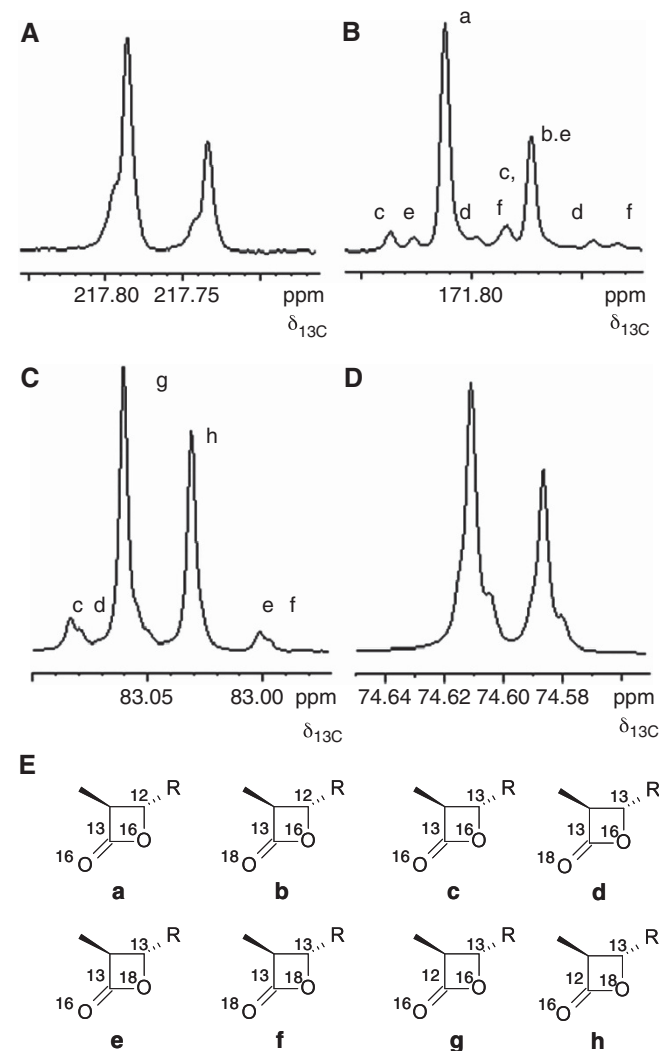
The ^{13}C NMR spectrum of this sample showed high levels of enrichment at all positions derived from C-1 of propionate, similar to the results reported by Uotani *et al.*[16] The high-resolution ^{13}C NMR spectrum exhibited isotopically shifted signals induced by the presence of ^{18}O, which were observed at all four oxygen-bearing

positions (C-1, C-3, C-9 and C-11), indicating that these carbon atoms were incorporated from propionate with the relevant C-O bonds remaining intact (Figure 3 and Table 1). Isotopic shifts of 0.053 p.p.m. and 0.039 p.p.m. were observed at C-9 and C-1, respectively (Table 1), consistent with values expected for C=O double bonds. Isotopic shifts of 0.029 p.p.m. and 0.024 p.p.m. were observed for C-3 and C-11, respectively: these fall in the range (0.010–0.035 p.p.m.) expected for C-O single bonds.[28,29]

These results demonstrate that four carbon–oxygen bonds in ebelactone A remain intact during biosynthesis from propionate (Scheme 2), showing that the ring formation takes place by attack of the hydroxyl group at C-3 of a polyketide precursor onto the terminal carbonyl group, and not by the attack of a carboxylate onto the C-3 carbon of the polyketide. Thus pathways **b** and **e** (Scheme 1) can be excluded as possible mechanisms for the formation of the β-lactone moiety. Likewise, oxygen exchange as proposed for lipstatin in

Figure 2 ESI mass spectrum of **1a** revealing the distribution of isotopomers for unlabeled and labeled ebelactone A. *m/z* 339 corresponds to MH$^+$ for unlabeled **1** and 340–346 to MH$^+$ of ^{13}C- and ^{16}O- or ^{18}O-labeled ebelactone A (**1a**). The peaks at *m/z* 356–362 are due to the corresponding [M+NH$_4$]$^+$ ions.

Figure 3 ^{13}C NMR signals for **1** from cultures of *S. aburaviensis* enriched with sodium [1-^{13}C, ^{18}O$_2$]propionate: (**A**) Signal for C-9; (**B**) Signal for C-1: a: natural abundance and ^{13}C–^{16}O-labeled molecules; b: ^{13}C–^{18}O labeled; c, d, e, f: signals with ^{13}C–^{13}C couplings due to isotopomers c, d, e, f (**E**). (**C**) Signal for C-3 (see **E** for isotopomer assignments); (**D**) Signal for C-11; (**E**) isotope labeling patterns of the β-lactone ring in **1**. Isotopomers a–h give rise to peaks marked a–h, respectively, in the ^{13}C NMR spectrum in **B**, **C**. For R, see Scheme 1.

Table 1 Isotope shifts and ^{18}O enrichments observed for 1a

$\delta^{13}C$[a]	$C\#$[b]	$\Delta\delta$[c]	$^{18}O/^{16}O$[d]
217.79	9	0.053	0.56
171.81	1	0.039	0.51
83.06	3	0.029	0.69
74.61	11	0.024	0.69

[a]Carbon-13 chemical shift, measured in $CDCl_3$ at 175 MHz.
[b]See Scheme 1 for numbering.
[c]Isotope shift due to oxygen-18.
[d]Measured by peak height ratios.
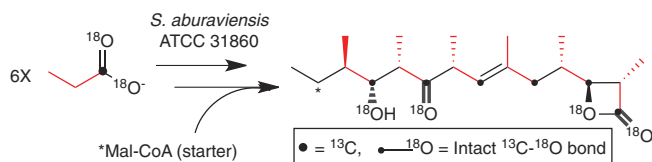
Scheme 2 Ebelactone A (**1**) showing the labeling pattern due to intact incorporation of ^{13}C and ^{18}O from propionate units.

mechanism *c* does not occur in biosynthesis of **1**.[23] This result is in agreement with our previous findings in the biosynthesis of F-244, suggesting that these fungal and bacterial β-lactone rings are made by similar mechanisms.[26]

The remaining mechanisms (*a*, *d* and *f*) are all consistent with the qualitative results obtained, as each atom has the same biosynthetic origin in all three cases.

However, an essentially equal level of oxygen-18 enrichment was observed at C-1, when compared with C-3, C-9 and C-11 (Table 1). This result indicates that a free carboxylic acid (for example, **11**) cannot be involved in the biosynthetic pathway, because hydrolysis of an intermediate acyl-enzyme species (for example, **9**) would require incorporation of unlabeled water. Cyclization of the ensuing acid would then result in loss of one-half of the labeled oxygen atoms. Hence, pathway *d* can also be excluded.

Therefore, it can be concluded that the lactone ring of **1** is either formed by direct displacement of the acyl intermediate while still attached to the PKS, thereby releasing the cyclized product (mechanism *a*) (Scheme 1); or through a route similar to the tandem aldol lactonization (*f*) proposed in the biosynthesis of salinosporamides.[18] In the former case, such a reaction might be catalyzed by a thioesterase domain that is tailored for formation of the strained four-membered ring. Mechanism *f* by contrast would require a novel enzymatic system that must still account for the equal levels of labeling in the units of the hexaketide precursor **7** and the final group that forms the ring, despite the different roles of these moieties; nonetheless, this hypothesis would account for the formation of both ebelactone A and B, as propionate and butyrate precursors are unselectively incorporated into the terminal ketide unit.

Further, the presence of oxygen-18 in both the ketone and hydroxy moieties (C-9 and C-11) of **1a** shows that ebelactone is synthesized in a manner in which the oxidation state is controlled by the PKS, and not by introduction of these oxygen atoms into a more highly reduced polyketide. These results allow a detailed prediction of the nature and sequence of the presumably modular PKS that forms ebelactone.

In addition to the large peaks shown in Figure 3, lower intensity peaks were observed at many of the ^{13}C-labeled positions in **1a**. These peaks were consistent with incorporation of multiple labeled propionate moieties into adjacent units within the same molecule, due to the high incorporation rate. Thus, long-range two-bond ^{13}C–^{13}C couplings between C-11 and C-9, C-9 and C-7, C-7 and C-5, and C-5 and C-3 account for all the observed minor peaks at these sites. In addition, a two-bond ^{13}C–^{13}C coupling of 9.3 Hz was observed between the C-1 and C-3 positions (Figures 3B and C). This unusually large coupling constant is fully consistent with literature values for $^2J_{13C-13C}$ in cyclobutanone, and can be explained by the two separate two-bond coupling paths across the lactone ring.[30] Figure 3E shows all the possible isotope labeling patterns for the lactone ring of **1a**.

As mechanisms *a* and *f* are not readily distinguishable through isotope labeling in precursors, it was clear that further understanding of the assembly of the β-lactone ring requires identification of advanced biosynthetic intermediates.

Advanced precursor studies

The formation of the β-lactone ring by cyclization through mechanism *a* implies that a β-hydroxy-acyl carrier protein (ACP)-bound thioester would be formed before cyclization. As N-acetylcysteamine (NAC) derivatives of a variety of biosynthetic intermediates have been successfully incorporated into polyketide and other natural products, we next envisioned that feedings may provide insight into the route of cyclization if the appropriate labeled N-acetylcysteamine derivative (that is, **14**) would load onto the relevant cyclizing domain of the PKS, and undergo cyclization to give **1** (Scheme 3a).[31,32] Rather than prepare **14** through total synthesis, we recognized that this N-acetylcysteamine derivative could be prepared from ebelactone A, using the unique reactivity of the lactone to effect nucleophilic attack with N-acetylcysteamine; and further that the high level of isotopic labeling observed in incorporation of ^{13}C- and/or ^{18}O-labeled propionate into **1** could be used to introduce an adequate level of isotope to act as a tracer in this biosynthetic experiment.

Therefore, the isotopically labeled **1a** from above was treated with N-acetylcysteamine and TEA in DCM, and the product isolated and rigorously purified to ensure removal of unreacted **1**. Analysis of the product revealed that the lactone ring had opened, and careful examination of the NMR spectra established that ring opening had occurred by nucleophilic attack on the carbonyl carbon, and not by attack at C-β. Direct evidence for this addition, and support for the proposed structure (**14**) came from the three-bond HMBC correlations between the methylene protons adjacent to sulfur and the thioester carbonyl carbon. Mass spectral analysis revealed that this sample of **14** had the same distribution of molecular ions and hence the same isotopic signature as described above for the ebelactone **1a**.

This sample was next used in a biosynthetic incorporation experiment in which **14** was added to growing cultures of S. aburaviensis. After growth as for the experiments above, the cultures were extracted with ethyl acetate and the organic extracts were analyzed by HPLC-MS (Figure 4). Organic extracts obtained from S. aburaviensis fed with exogenous precursors were compared directly with those not receiving precursor feedings, and with precursor added to sterile, uninoculated medium, using LC-MS. The unfed controls led to identification of ebelactone A at the expected retention time and with an isotopomer distribution corresponding to natural abundance and identical to authentic standard ebelactone (Figure 4). Culture extract derived from the incorporation of **14** gave ebelactone comprised of natural abundance isotopomers (as above), superimposed with isotopomers of higher mass that derive from the precursor (Figure 4). Therefore, **14** is cyclized to **1** under these conditions. However, to our surprise, the samples in which labeled **14** was added to uninoculated culture medium also resulted in formation
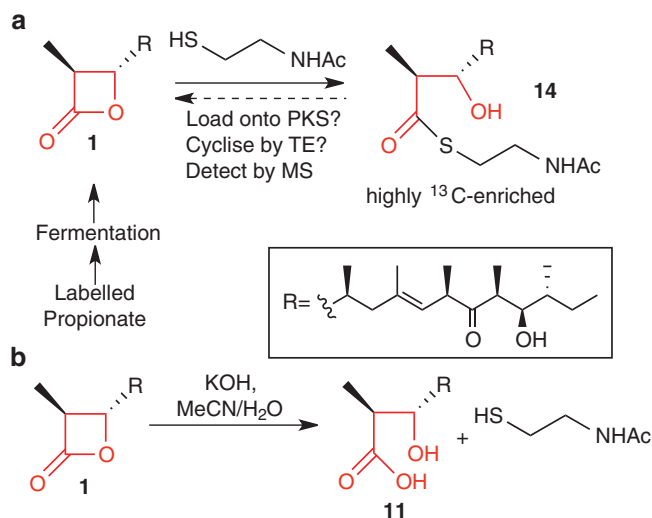
Scheme 3 (**a**) Formation of labeled ebelactone from labeled propionate, followed by reaction with *N*-acetylcysteamine, gives the thioester **14**, which acts as a putative advanced precursor for formation of **1**. For details of the cyclization, see text. Thioester synthesis: Et$_3$N, CH$_2$Cl$_2$, RT, 4 h, 60%. (**b**) Hydrolysis of **1** yields **11**.
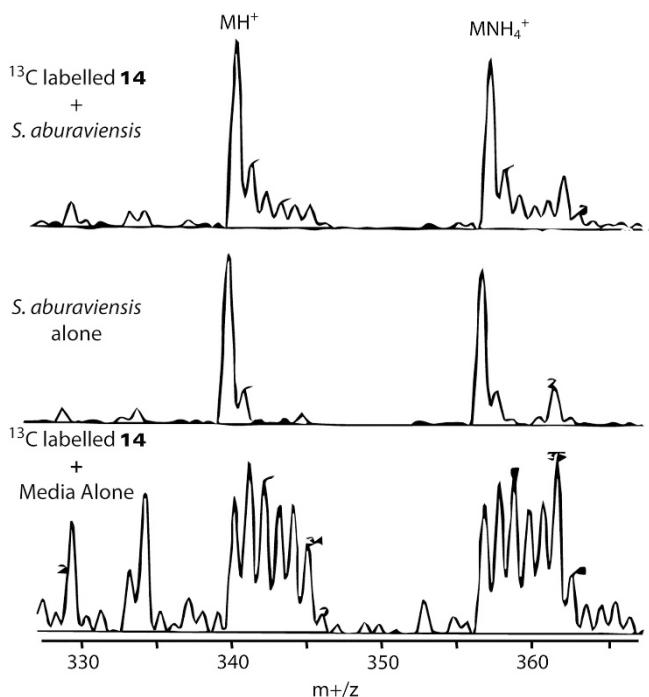
Figure 4 Cyclization of **14** by *S. aburaviensis* and media alone result in ebelactone A formation. Cyclization of ^{13}C-labeled **14** to give labeled **1** was detected by HPLC-MS, in the presence and absence of *S. aburaviensis*. Mass spectra at the retention time of ebelactone A are shown.

of the β-lactone **1**: these samples showed no unlabeled **1**, but rather ebelactone A with the same isotopomer distribution as it had before formation of the *N*-acetylcysteamine derivative (Figure 4). The level of production was, within experimental error, the same as that detected in the biosynthetic incorporation sample. Clearly, this experiment demonstrates that the *N*-acetylcysteamine derivative **14** undergoes chemical cyclization to the β-lactone without enzymatic catalysis.

This cyclization in aqueous solution is almost unprecedented: although the formation of β-lactones *via* cyclization of β-hydroxy-thioesters is used in organic synthesis, these reactions are normally performed under anhydrous conditions in organic solvents, and generally require the use of Lewis acidic cations to assist departure of the thiolate leaving group (for example, thiophenyl with mercury).[20,33] Literature precedent for this aqueous cyclization is rare, and only one example could be found where a β-lactone is formed in aqueous milieu, and this is as a transient intermediate, which is rapidly cleaved by hydrolysis in a subsequent step. Thus, hydrolysis of lactocystin proceeds through the intermediate *clasto*-lactocystin β-lactone, but only a maximum of 10% of the theoretical yield of lactone is present during this reaction.[34] Therefore, we wondered whether the *N*-acetylcysteamine thioester **14** was undergoing hydrolysis through a similar mechanism. Hence, we prepared the β-hydroxy acid **11** through hydrolysis of ebelactone A (NaOH/H$_2$O, tetrahydrofuran, RT, 8 h, 67%) and used this material as a standard for analysis of the extracts from above (Scheme 3b). In no case was there any evidence for formation of **11**, and thus the ebelactone is being formed without hydrolysis of either **14** or **1**.

The formation of ebelactone from **14** under a variety of conditions was confirmed by other results from HPLC-MS, and it was eventually found that while **14** was stable in aprotic organic solvents such as chloroform without any sign of cyclization, simply diluting into 50:50 EtOH:H$_2$O effected slow cyclization over the course of 24 h. While a concentrated solution failed to react to completion, dilute samples (*ca.* 50 mg l^{-1}) cyclized efficiently and essentially completely to furnish **1**. These observations, therefore, show that the system reaches an equilibrium position that moves toward the lactone as the rate of lactone opening falls with decreasing concentration of both lactone and *N*-acetylcysteamine.

While these results supported the TE-independent formation of the lactone, we also considered whether an alternative cyclization was occurring: if the C-11 hydroxy group attacked the thioester, then the product would be a 12-membered macrocycle, which would have the same mass as ebelactone, and could conceivably co-elute in the HPLC-MS analysis. To eliminate this possibility we performed a preparative experiment in which **14** was dissolved in EtOH:H$_2$O to a final concentration of 50 mg l^{-1}. The reaction was allowed to proceed at room temperature for 24 h, and then the mixture was quickly evaporated and separated by chromatography. The eluted **1** was identical to standard in all respects, including proton NMR.

Taken together, these results establish that the β-hydroxyacyl thioester **14** undergoes spontaneous cyclization to ebelactone A, and thus raise the possibility that an analogous process during the biosynthesis of **1** could occur to cleave the chain from the PKS. Thus a dedicated thioesterase would not necessarily be required during the formation of **1**.

To determine whether a conventional thioesterase could form a β-lactone, or if it would catalyze cyclization from the C-11 hydroxy group to form the macrocycle mentioned above, we, therefore, incubated **14** with deoxyerythronolide B (DEBS) synthase-TE, the TE responsible for formation of the 14-membered macrocyclic ring in erythromycin.[35] The reaction mixture was analyzed by HPLC-MS, along with authentic standards of **1**, **14**, and acid **11**. The results show that comparable levels of **1** are formed when **14** is incubated with or without enzyme. However, in the enzymatic sample only, extensive formation of acid **11** occurred (Figure 5a). There was no evidence for formation of the putative macrocycle. Further, kinetic characterization of DEBS-TE-mediated hydrolysis showed saturation, clearly indicating that this is an enzymatic process (Figure 5b). The kinetic parameters
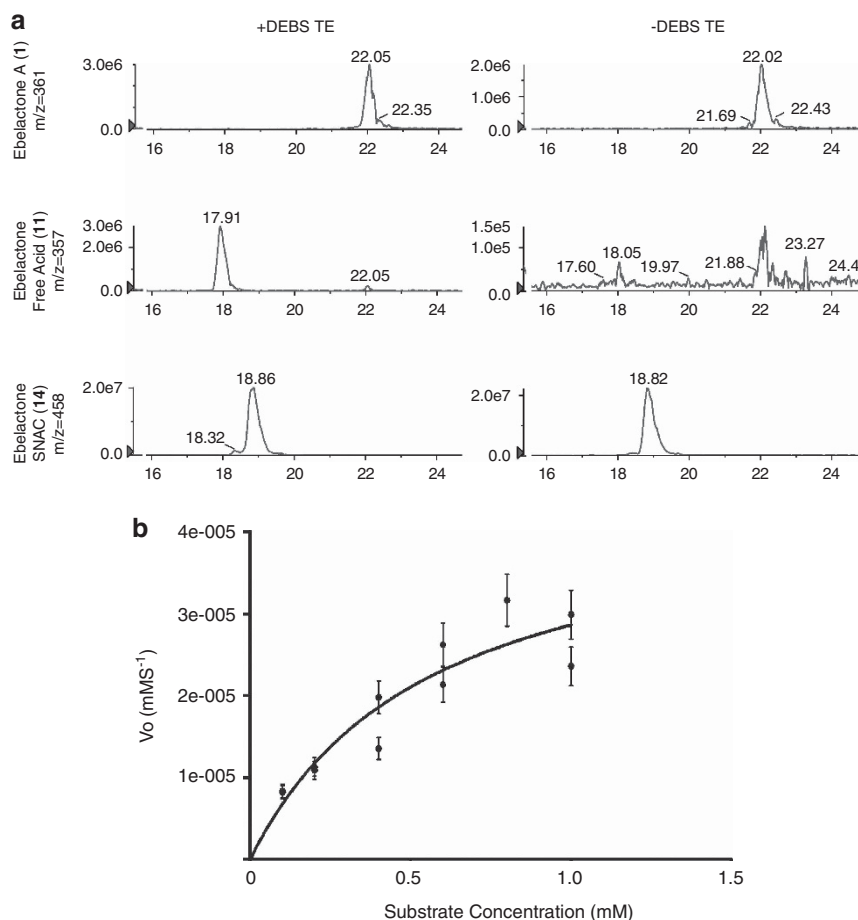
Figure 5 Hydrolysis of **14** with DEBS-TE results in hydroxy acid **11**. (**a**) Extracted ion chromatograms from HPLC-MS analyses of incubations of **14** with and without DEBS-TE. Chromatograms are, from top to bottom, for $[1+\text{Na}]^+$ ($m/z=361$), $[11+\text{H}]^+$ ($m/z=357$), and $[14+\text{H}]^+$ ($m/z=458$) ion extractions. Note that the ion extraction chromatogram for $[11+\text{H}]^+$ also produces a peak at the retention time for **1** (22.0 min). This is presumably due to hydration of the ketone in **1** during ionization. (**b**) Plot of the initial rate of hydrolysis (V_0) of **14** by DEBS-TE versus substrate concentration. The data is directly fit to the Michaelis-Menten model. Conditions: 5 μM DEBS-TE, pH 7.4. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

are in accord with those expected for hydrolysis of other *N*-acetylcysteamine derivatives with DEBS-TE ($k_{\text{cat}}=0.53\pm0.1$ min^{-1}; $K_{\text{M}}=0.5\pm0.2$ mM; $k_{\text{cat}}/K_{\text{M}}=16\pm7\,\text{M}^{-1}\text{s}^{-1}$).[36–38] Test incubations of DEBS-TE with ebelactone A failed to catalyze any hydrolysis reaction.

Putative ebelactone gene cluster

The biosynthesis of a number of macrocyclic polyketide lactones has been extensively studied and many PKS gene clusters identified. These lactones include 5-, 6-, 10-, 12-, 14- and 16-membered rings, as well as larger ring sizes. Of the gene clusters identified from which a four-membered, β-lactone ring is formed (oxazolomycin, cinnabaramide and salinosporamide), their biogenesis is not directed by canonical polyketide biosynthetic machinery.[18,19,39] Moreover, in each of these instances the exact mechanistic details remain ambiguous and as yet unproven. The cluster for oxazolomycin biosynthesis has two non-ribosomal peptide synthase-type Condensation (C) domains, one of which is suggested to cyclize the C-terminal serine residue to afford the β-lactone.[39] In the cluster for salinosporamide there is a thioesterase (*SalF*), but this has been suggested to perform an editing role and a tandem aldol-lactonization sequence has been proposed to form the β-lactone ring.[19] In the closely related cinnabaramide, a thioesterase (*CinE*) was identified, which has

sequence similarity to *SalF*, but which was suggested to make the lactone from a β-hydroxyacyl-enzyme.[18] However, this role has not been proven. Therefore, we wished to identify the PKS that effects biosynthesis of **1** and in particular to further examine the process of β-lactone ring formation.

The genome of *S. aburaviensis* was sequenced, resulting in a number of putative biosynthetic gene clusters. The cluster that encodes ebelactone biosynthesis was found on a single contig and is readily recognized through its characteristic PKS domains, that define the predicted sequence of biosynthetic reactions for ebelactone. The cluster is characterized by seven genes (*ebeA-G*)(Table 2), which are expected to encode seven proteins (EbeA-G) that assemble to make a functional PKS (Figure 6). There are six modules, each containing a ketosynthase (KS) domain to effect chain elongation. Also present in some modules are ketoreductase, dehydratase and enoylreductase domains that are largely correctly placed to correspond to the necessary processing of the growing chain to install β-hydroxy, alkene and methylene groups. The loading module contains a characteristic KS$^{\text{Q}}$ domain, which loads malonyl-CoA and decarboxylates the malonyl-enzyme to generate the starter acetate unit.[40] Specificity of the first acyltransferase domain is consistent with the loading of malonyl-CoA, whereas the remaining acyltransferase domains all have specificity for methylmalonyl-CoA, consistent with the final structure

Table 2 Ebelactone gene cluster analysis

Gene	Size, bp	Deduced role	Protein homolog	Accession number	Protein identity/similarity %/%
ebeA	3192	PKS	NP_821594 (Streptomyces avermitilis MA-4680)	NP_821594.1	58/68
ebeB	6615	PKS	BAH02268 (Streptomyces platensis)	BAH02268.1	57/67
ebeC	4761	PKS	ZP_05000638 (Streptomyces sp. MG1)	ZP_05000638.1	57/67
ebeD	2154	PKS	YP_004815656 (Streptomyces violaceusniger Tu 4113)	YP_004815656	63/75
ebeE	2130	PKS	YP_004959634 (Streptomyces bingchenggensis BCW-1)	YP_004959634.1	48/61
ebeF	7410	PKS	CAQ64687 (Streptomyces lasaliensis)	CAQ64687.1	55/65
ebeG	6537	PKS	CBA11583 (Streptomyces lydicus)	CBA11583.1	57/68
orf1	1698	Adenylation domain	ZP_10549985 (Streptomyces auratus AGR0001)	ZP_10549985.1	65/76
orf2	876	Reductase	YP_003341831 (Streptosporangium roseum DSM43021)	YP_003341831.1	81/89
orf3	807	Hypothetical protein	YP_004902238 (Kitasatospora setae KM-6054)	YP_004902238.1	62/71

Results generated by BLASTx analysis.
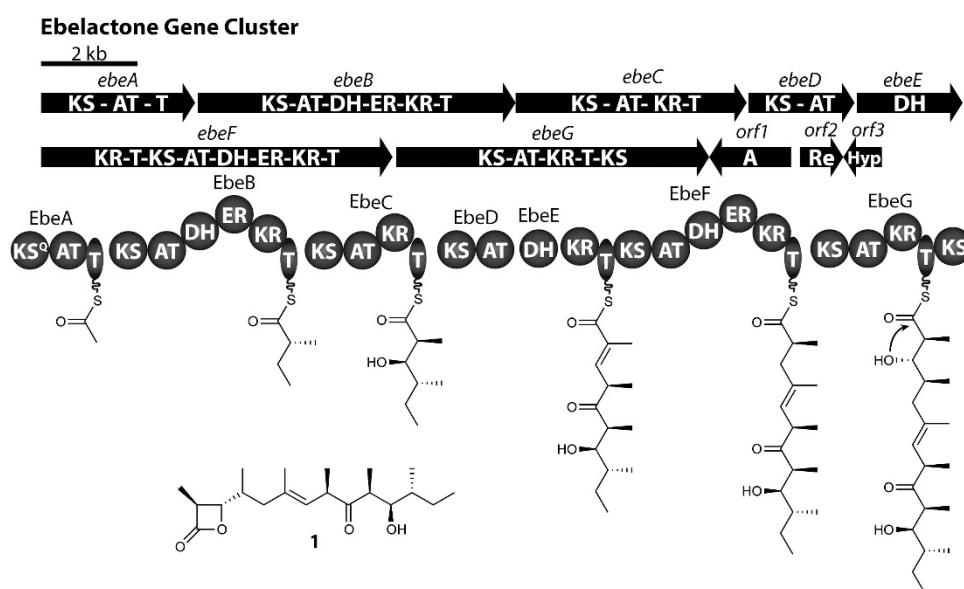
Figure 6 Ebelactone gene cluster of *S. aburaviensis* ATCC 31 860. Shown are the putative genes for ebelactone biosynthesis (*ebeA-G*) and the corresponding protein assembly-line (EbeA-G). The assembly-line contains the following polyketide synthase domains: KS, acyltransferase (AT), thiolation (T), dehydrogenase (DH), enoylreductase (ER), ketoreductase (KR). Downstream genes of possible biosynthetic relevance are also shown although their putative functions are not required in the final maturation of ebelactone (**1**). These include an adenylation domain (A), a reductase (Re), and a hypothetical protein (Hyp)(*orf1-3*). Tethered polyketide intermediates are shown on the ebelactone assembly-line. The ebelactone (**1**) release mechanism through β-hydroxy attack is shown.

of **1**. Extension on the next module using methylmalonate by the KS is followed by processing by ketoreductase, dehydratase and enoylreductase domains to generate the fully reduced diketide 2-methylbutyrate, while module three contains only KS and ketoreductase and thus produces a hydroxylated triketide. Module 4 is more complex, and may be used twice: in the first iteration, only the KS is used, leaving the required ketone intact, while in the second round we speculate that the KS is used again, but now the growing chain is processed by EbeE (dehydratase protein) and the ketoreductase domain of EbeF to generate the pentaketide intermediate shown. Normal modular chain extension continues for two further rounds to generate the acyclic, enzyme-bound precursor to ebelactone, attached to the final thiolation domain.

The cluster clearly does not contain a thioesterase to effect β-lactone ring formation. This can be understood in terms of the results described above: ebelactone can form by direct cyclization of the β-hydroxyacyl moiety on the thiolation domain in a process that is precedented by the chemical cyclization of **14**. Whether this process is accelerated by a component of the PKS is unclear, and is the subject of further investigation. Intriguingly, the PKS does contain a KS domain beyond the presumed terminal T domain. This could perform a catalytic role in cyclization, or alternatively it is possible that the 'normal' natural product of this cluster is that from further chain extension on the PKS, and the formation of ebelactone is the result of derailment through accidental cyclization on the terminal thiolation domain. Investigations to further clarify these issues are on-going.

CONCLUSION

The oxygen-18 isotopic distribution studies and the organization of the putative gene cluster establish that the biosynthetic pathway to ebelactone involves a starter malonate, which is decarboxylated by the

first KS^Q domain. Six methylmalonate units are added in the head-to-tail fashion of polyketide biosynthesis, introducing hydroxy and ketone groups with intact C–O or C=O bonds to generate a β-hydroxyacyl intermediate. This is cyclized with retention of the C(β)-O bond, consistent with nucleophilic attack of the hydroxy group onto the acyl carbon.

The putative intermediacy of a β-hydroxyacyl intermediate was then tested by synthesis of the *N*-acetylcysteamine derivative **14**, which in this unique situation was readily prepared by reaction of *N*-acetylcysteamine with ebelactone A, thereby furnishing this complex intermediate in one step without requiring extensive synthetic work. Remarkably, **14** was found to cyclize spontaneously to afford ebelactone A in virtually quantitative yield. We are not aware of any reports of corresponding cyclization of β-hydroxyacyl *N*-acetylcysteamine compounds, despite their widespread application in biosynthetic studies. We are currently continuing to investigate this process to determine whether the nature of the polyketide chain and/or the *N*-acetylcysteamine leaving group determine whether such substrates are capable of cyclization without the need for either chemical or enzymatic catalysis. Further, we were surprised that DEBS-TE, a thioesterase that might well have cyclized the β-hydroxyacyl *N*-acetylcysteamine thioester, resulted in enzymatic hydrolysis. Taken together, then, the results point away from a TE-catalyzed generation of the β-lactone ring, and suggest that cyclization might occur spontaneously on a β-hydroxyacyl moiety tethered to the PKS.

Lastly, we identified a putative gene cluster in the producing organism with the correct domain architecture for ebelactone formation. Consistent with the results from oxygen-18 labeling and advanced precursor studies, the cluster does not possess any identifiable thioesterase genes or domains, neither at the end of the ebelactone PKS nor as a distinct stand-alone TE. Taken together, this leads us to the conclusion that the β-lactone ring formation occurs on a different PKS component. This could be the ACP at the end of module 7, in a process that is simulated by the chemical cyclization of **14**. Alternatively, it is intriguing to speculate that the cluster is designed to make a different natural product, using the domain(s) found beyond the presumed terminal ACP; in this case the β-lactone could be the result of an accidental cyclization as the growing polyketide chain passes along the assembly-line, that is, premature release. Which situation occurs will demand *in vitro* experiments that install the ebelactone chain onto the terminal ACP and reveal the rates of β-lactone formation.

METHODS

General

ISP2 agar (Difco 277010, Becton Dickinson and Co., Sparks, MD, USA), fish meal (Sigma, Oakville, ON, Canada or Rothsay, Guelph, ON, Canada), CaCO$_3$ (Aldrich, Oakville, ON, Canada), glycerol (Caledon Laboratories, Georgetown, ON, Canada) and pure water were used as supplied to prepare all media. Silica gel 60 (Merck, Montreal, QC, Canada) was used for normal phase flash chromatography columns and a prepacked Merck Lobar RP-18 column (240 × 10 mm) for reverse phase chromatography. Normal phase TLC was performed on Machery Nagel alugram sil G/UV$_{254}$ or Merck plates, while reverse phase TLC used Merck silica gel RP-18 F$_{254}$ plates. HPLC was performed on a C18 column (100 mm × 2.1 mm, 3μ) with a 0.2 ml min^{-1} flow rate running a gradient from 0% B to 100% B over 30 min. A = 5% MeCN, 0.05% formic acid in H$_2$O; B = 5% H$_2$O, 0.05% formic acid in MeCN. Proton and ^{13}C NMR spectra were recorded on Bruker AV-600 (proton at 600 MHz) and AV-700 (proton at 700 MHz) spectrometers.

Growth of *S. aburaviensis* ATCC 31860 (*sp.* MG7-G1)

Cultures of *S. aburaviensis* from American Type Culture Collection (ATCC 31 860) were grown in Petri dishes at 28 °C for 5 days on ISP medium 2 agar

plates (36.6 g in 1 l pure water). The resulting spores were suspended in sterile water and transferred to liquid growth medium containing 3% glycerol, 2% fish meal and 0.2% CaCO$_3$ in 100 ml pure water in a 500-ml Erlenmeyer flask, which had been autoclaved at 121 °C for 20 min. The flask was incubated in a rotary shaker-incubator at 28 °C and 180 r.p.m. for 2 days. Aliquots of the resulting culture (2 ml) were inoculated into twenty-two 500 ml Erlenmeyer flasks containing the same sterile liquid medium as described above. The flasks were incubated in the rotary shaker-incubator at 28 °C and 180 r.p.m. for 2 days.

Extraction and isolation of ebelactones A and B

Ethyl acetate (100 ml) was added into each culture flask and the mixture was shaken on the shaker-incubator at 180 r.p.m. for 1 h. The resulting liquid was centrifuged at 4000 r.p.m. at 4 °C for 10 min. The ethyl acetate layer was removed, and the remaining aqueous layer was then extracted twice with equal volumes of ethyl acetate (4.4 l of ethyl acetate in total). The combined organic layers were concentrated to give ∼3 g of crude extract. Flash chromatography with 5:5:1 hexanes:chloroform:ethyl acetate gave fractions containing **1**, which were combined and concentrated. The residue was then filtered using a RP-18 SPE cartridge (E. Merck, adsorbex 400 mg) with 50:50 methanol-water as eluent. The filtrate was concentrated by azeotropic distillation with ethanol, and then subjected to reverse phase chromatography with a step gradient elution from 50:50 to 70:30 methanol-water to give fractions containing pure ebelactone A (**1**). Yield: 4 mg of white solid. Both one-dimensional and two-dimensional spectra were obtained by NMR; the data agreed with literature assignments made during the initial structure determination.[2]

Addition of labeled sodium [1-^{13}C, ^{18}O$_2$]propionate

A mixture of unlabeled sodium propionate (Fisher Laboratories, Ottawa, ON, Canada, 250 mg), sodium [1-^{13}C,^{18}O$_2$] propionate (150 mg) (76% ^{18}O/site) and sodium [1-^{13}C]propionate (100 mg) dissolved in sterile water (8.8 ml) was added to 22 flasks prepared as described above, at 21 and at 45 h after inoculation (200 μl per flask per feeding). Ebelactone A was isolated as above. ^{13}C NMR (CDCl$_3$, 150 MHz): as previously reported, except for: 171.1 (enhanced C-1, Δδ 0.039 p.p.m.), 83.0 (enhanced C-3, Δδ 0.029 p.p.m.), 43.4 (enhanced C-5), 126.9 (enhanced C-7), 217.1 (enhanced C-9, Δδ 0.053 p.p.m.), 74.6 (enhanced C-11, Δδ 0.029 p.p.m.); MS (ESI +ve) *m/z* 339.3 (100%), 340.4 (85%), 341.4 (45%), 342.4 (35%), 343.4 (28%), 344.4 (12%), 346.0 (3%).

Preparation of *N*-acetylcysteamine adduct 14

A mixture of ebelactone A (**1**) (12 mg, 30 μmol), *N*-acetylcysteamine (9.5 mg, 80 μmol), triethylamine (7.5 mg) and dichloromethane (0.2 ml) was stirred at room temperature for 4 h. The mixture was purified by chromatography on silica (2–4% MeOH:CHCl$_3$) to yield **14** (9.5 mg, 20 μmol, 63%) as a colorless liquid. ^{1}H NMR (CDCl$_3$, 600 MHz) δ 5.78 (1H, br s, NH), 5.00 (1H, d, *J* = 10 Hz, H-7), 3.58 (1H, dq, 8, 7 Hz, H-8), 3.49 (1H, m, H-11), 3.45 (2H, t, 6 Hz, CH$_2$-NH), 3.42 (1H, dd, *J* = 8, 4 Hz, H-3), 3.15 (1H, br s, OH), 2.95 (2H, t, 6 Hz, CH$_2$-S), 2.93 (1H, dq, 4, 7 Hz, H-2), 2.86 (1H, dq, 2, 7 Hz, H-10), 2.54 (1H, d, 7 Hz, H-4), 2.38 (1H, dd, 13, 4 Hz, H-5), 1.98 (3H, s, MeC=O), 1.79 (1H, dd, 13, 10 Hz, H-5), 1.76 (2H, dq, 8, 3 Hz, H-13), 1.72 (3H, s, 6-Me), 1.45 (1H, m, H-12), 1.28 (3H, d, 7 Hz, 2-Me), 1.19 (1H, m, H-13), 1.12 (3H, d, 7 Hz, 8-Me), 1.09 (3H, d, 7 Hz, 10-Me), 0.89 (3H, t, 7 Hz, 14-Me), 0.87 (3H, d, 7 Hz, 4-Me), 0.77 (3H, d, 7 Hz, 12-Me); ^{13}C NMR (CDCl$_3$, 150 MHz) δ 217.4 (C-9), 204.2 (C-1), 169.9 (MeC=O), 136.5 (C-6), 125.2 (C-7), 78.1 (C-3), 74.0 (C-11), 50.3 (C-2), 45.1 (C-8), 45.0 (C-10), 41.9 (C-5), 40.8 (C-4), 36.1 (CH$_2$-N), 34.0 (C-12), 28.8 (CH$_2$-S), 24.5 (C-13), 22.7 (CH$_3$-C=O), 16.1 (6-Me), 15.8 (8-Me), 15.5 (12-Me), 14.3 (2-Me), 13.6 (C-14), 10.4 (4-Me), 8.9 (10-Me); IR (CHCl$_3$, cm^{-1}) 3323, 1690, 1660; MS (ESI +ve) *m/z* 480.5 ([M + Na]$^+$, 475.5 ([M + NH$_4$]$^+$), 458.5 ([M + H]$^+$). High-resolution mass data was obtained on a Waters Q-TOF Global Ultima giving an *m/z* = 458.2930 (ESI +ve, [M + H]$^+$) with a calculated error of 2.2 p.p.m.

Preparation of hydroxy acid 11

To a solution of ebelactone A (3.1 mg, 9.2 μmol) in tetrahydrofuran (0.5 ml) was added aqueous NaOH (0.071 M, 141 μl, 10 μmol) and the mixture was stirred at room temperature for 8 h. The mixture was neutralized with HCl (1

equiv.), the THF was evaporated and MeOH (0.5 ml) was added. The mixture was loaded onto an RP-18 cartridge filter column, which was progressively eluted with MeOH:H$_2$O (50:50, 60:40, 70:30 and 100:0). Fractions containing **11** were combined and concentrated, then further purified on silica gel (CHCl$_3$:hexane: EtOAc:AcOH 33:33:33:1) to afford **11** (2.2 mg, 6.2 μmol, 67%). ^{1}H NMR (CDCl$_3$, 600 MHz) δ 5.01 (1H, d, $J = 10$ Hz, H-7), 3.55 (1H, dq, 10, 7 Hz, H-8), 3.49 (1H, dd, $J = 9$, 2 Hz, H-11), 3.41 (1H, t, $J = 5$ Hz, H-3), 2.85 (1H, dq, 7, 2.5 Hz, H-10), 2.75 (1H, dq, 5, 7 Hz, H-2), 2.33 (1H, q, 9 Hz, H-5), 1.81 (2H, m, H-5), 1.73 (1H, dq, 14, 7 Hz, H-13), 1.68 (3H, br s, 6-Me), 1.42 (1H, dq, 3, 7 Hz, H-12), 1.30 (3H, d, 7 Hz, 2-Me), 1.12 (3H, d, 7 Hz, 8-Me), 1.12 (1H, m, H-13), 1.07 (3H, d, 7 Hz, 10-Me), 0.86 (3H, t, 7 Hz, 14-Me), 0.85 (3H, d, 7 Hz, 4-Me), 0.77 (3H, d, 7 Hz, 12-Me); ^{13}C NMR (CDCl$_3$, 150 MHz) δ 217.6 (C-9), 178.0 (C-1), 137.3 (C-6), 125.7 (C-7), 78.0 (C-3), 74.5 (C-11), 45.4 (C-8), 44.9 (C-10), 42.5 (C-5), 41.7 (C-2), 36.6 (C-12), 34.7 (C-4), 24.9 (C-13), 16.5 (6-Me), 16.4 (8-Me), 16.3 (4-Me), 15.2 (2-Me), 14.8 (12-Me), 10.8 (14-Me), 9.3 (10-Me); MS (ESI + ve) m/z 374.5 ([M + NH$_4$]$^+$), 379.4 ([M + Na]$^+$). High-resolution mass data was obtained on a Waters Q-TOF Global Ultima giving an $m/z = 379.2460$ (ESI + ve, [M + Na]$^+$) with a calculated error of 0.9 p.p.m.

Cyclization of 14

A solution of **14** (0.95 mg, 2.8 μmol) in EtOH (10 ml) and water (10 ml) was stirred at room temperature for 18 h. The solvent was evaporated and the residue purified on silica gel (CHCl$_3$:hexane:EtOAc 5:5:1) to afford **1** (1 mg, ~100%), which was identical to authentic standard by proton NMR.

Enzymatic hydrolysis of 14

14 was incubated with or without recombinant purified DEBS-TE (5 μM). Each assay contained 1.2 mM substrate in 50 mM phosphate buffer (pH 7.4) at 20 °C and dimethyl sulfoxide (10% v/v). After 18 h, the assay was diluted fourfold with 1:1:0.0005 water, acetonitrile and formic acid, centrifuged to remove insoluble material and analyzed by ESI-LCMS.

ESI-positive LCMS was performed using a reverse phase BDS (base-deactivated silica) Hypersil C18 100 × 2.1 mm, particle size 3 μm (Thermo Scientific) column with mobile phase A (0.05% formic acid 5% acetonitrile in water) and mobile phase B (0.05% formic acid, 5% water in acetonitrile). A gradient from 0% B to 100% B over 30 min with a flow rate of 0.2 ml min^{-1} was used for the chemical separation. An ionization voltage of 5500V was used in the ESI TurboSpray probe interfaced with an API2000 triple quadrupole mass spectrometer.

Kinetic analysis of DEBS-TE-mediated hydrolysis

The TE-catalyzed consumption of **14** was monitored by observation of the formation of 5-thio-2-nitrobenzoate by reaction of released N-acetylcysteamine with 5,5′-dithio-2-nitrobenzoic acid. Kinetic assay mixtures consisted of 5 μM DEBS-TE, 50 mM phosphate buffer (pH 7.43), 4% (v/v) saturated solution of dithio-2-nitrobenzoic acid in 50 mM phosphate buffer (pH 7.43), substrate (at 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM) and 10% (v/v) dimethyl sulfoxide. The formation of free thiol was quantified by measuring the absorption at 412 nm using a ScanDrop 250 Nano Volume Spectrophotometer. The reactions were performed at room temperature and data points were collected every 30 s for 1.5 h. Initial velocities were determined by linear regression analysis of the data. All steady state kinetic assays were performed in duplicate. The kinetic parameters k_{cat} and K$_M$ were calculated from the direct fit of the slope of v versus [S] to the Michaelis-Menten equation.

Genome sequencing and ebelactone cluster identification

A single colony of *S. aburaviensis* ATCC 31860 was grown 3 days at 28 °C, 250 r.p.m. in DSMZ Media 65 (Deutsche Sammlung von Mikroorganismen und Zellkulturen). Genomic DNA was harvested using a GenElute Bacterial Genomic DNA Kit (Sigma). Genomic DNA was sent for library preparation and Illumina sequencing at the Farncombe Metagenomics Facility at McMaster University, using an Illumina MiSeq DNA sequencer. Contigs were assembled using the ABySS genome assembly program and with Geneious bioinformatic software. The ebelactone gene cluster was identified using BLAST with PksJ from the bacillaene biosynthetic assembly-line of *Bacillus subtilis* str. 168 as the query. The entire ebelactone cluster was found on a single contig. Gene functions and domain architectures were determined using AntiSMASH and BLAST analysis against the NCBI database.[41] The Genbank accession number for the ebelactone gene cluster is KC894072.

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