Biosynthesis of the fungal metabolite, piliformic acid (2-hexylidene-3-methylsuccinic acid)

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The biosynthesis of piliformic acid, a secondary metabolite of various xylariaceous fungi, has been studied in *Poronia piliformis* and *Xylaria mali*. The metabolite can be retro-biosynthetically cleaved to generate a C_8 and a C_3 moiety. The study reveals that the C_8 unit is derived directly from octanoate and that the octanoate in turn originates from a fatty acid synthase (FAS) rather than from a polyketide synthase (PKS). This conclusion is drawn after assaying the stereochemical course of the enoyl reductase involved in the synthesis of the octanoate unit. The C_3 unit is efficiently labelled by succinate and the citric acid cycle intermediate oxaloacetate is implicated as a key biosynthetic precursor. The location of deuterium after isotopic labelling with sodium [$^2H_{15}$]octanoate reveals a 1,3-hydrogen shift, indicative of a double-bond isomerisation, operating at a late stage in the biosynthesis. A hypothesis for piliformic acid biosynthesis is presented and discussed in the context of structurally related fungal and lichen metabolites.

Introduction

Piliformic acid (2-hexylidene-3-methylsuccinic acid) **1** was identified by Anderson *et al.*[1] in 1985 as a metabolite of several closely related fungi of the xylariaceous genera. The compound was isolated from *Hypoxylon deustum*, the slow growing dung fungus *Poronia piliformis* and from four *Xylaria* strains, *X. polymorpha*, *X. longipes*, *X. mali* and *X. hypoxylon*, fungi which grow on rotting wood. The predominant absolute stereochemistry and optical purity of piliformic acid **1** varies from different sources.[1] For example the metabolite from *P. piliformis* has an optical rotation of $[\alpha]_D^{20}$ −89, although the absolute stereochemistry has not been determined, whereas that from *X. mali* is racemic.

The structurally related saturated metabolite *erythro*-**2** has been isolated[1] from *H. illitium* and both *erythro*- and *threo*-**2** are products[2,3] of the fungus *Anthostomella avocetta*. Lactones such as **3**[1] have been isolated from *Hypoxylon serpens* and avenaciolide **4** has been isolated[4] from *Aspergillus avenaceus*. These natural products possess some structural similarities to certain lichen metabolites[5] such as protolichesterinic acid **5**, its isomer lichesterinic acid **7** and the related metabolites **6, 8** and **9**, insomuch as they all contain a linear fatty acid moiety, with a branching C–C bond at the α-position to the carboxylate making a connection to a C_3 mono- or C_4 di-carboxylate unit. Previous biosynthetic investigations on avenaciolide **4**[4] and protolichesterinic acid **5**[6] suggest a common mode of assembly by the condensation of a long-chain fatty acid (palmitic acid) with a sub-unit closely related to succinate thus implicating an intermediate of the citric acid cycle. The fungal metabolites caperatic acid **10**,[5,7] rangiformic acid **11**,[5] roccellic acid **12**[5] and chaetomellic acids -A and -B[8] **13** and **14**, could be similarly derived from the appropriate C_{14}–C_{18} fatty acids and either a C_3 or C_4 subunit. A distinction between these metabolites and piliformic acid **1** is the length of the fatty acid chain involved in the biosynthesis. Fatty acid chain lengths of C_{14}–C_{18} are clearly consistent with the incorporation of palmitate (C_{16}) into **11** and **13** and myristate (C_{14}) and oleate (Δ^9, C_{18}) into **12** and **14** respectively. Piliformic acid **1** requires an octanoate chain which could have a fatty-acid origin assembled by a fatty-acid synthase (FAS). Alternatively, however, a dedicated polyketide synthase (PKS) may deliver a short-chain carboxylic acid for secondary metabolite biosynthesis. This study aimed to establish the biosynthetic origin of both the C_3 and C_8 subunits of piliformic acid and, in particular, to resolve the issue of a FAS or PKS origin of the C_8 unit by assessing the stereochemical course of the enoyl reductase involved in the octanoate assembly. In the course of the study evidence emerged for a 1,3-sigmatropic hydrogen shift occurring during piliformic acid biosynthesis.

Results and discussion

Initial biosynthetic experiments on **1**, supplementing the *P. piliformis* medium with sodium [1,2-$^{13}C_2$]acetate and sodium [2-^{13}C,$^2H_3$]acetate, confirmed a largely acetogenic assembly for piliformic acid. The resultant ^{13}C NMR spectrum of piliformic acid, which was isolated after supplementation of the *P. piliformis* medium to a final concentration of sodium [1,2-^{13}C]-acetate at 15 mmol, is shown in Fig. 1. All of the carbon atoms are enriched to varying extents, however it is clear that the C_8 unit is significantly more labelled than the C_3 unit (~6 fold *versus* ~0.5 fold). The ^{13}C–^{13}C couplings in the ^{13}C NMR spectrum indicate the intact incorporation of five acetate units. Four of these [C(10)–C(3), C(4)–C(5), C(6)–C(7) and C(8)–C(9)] constitute the C_8 unit. The additional 'satellites' associated with the signals C-3 through to C-9 are ^{13}C–^{13}C couplings which arise due to a lower frequency incorporation of *two adjacent* intact units in given molecules of piliformic acid. The signals associated with the carbon atoms of the C_3 unit demonstrate an intact unit derived from acetate between C-1 and C-2. It is noteworthy that C-11, which is enriched (~0.5 fold) from C-2 of acetate, gives rise to the only uncoupled signal in the spectrum, indicating that it has become isolated from its original labelled partner (C-1 of acetate) during the biosynthesis. This is discussed below.

The results of the sodium [2-^{13}C,$^2H_3$]acetate experiment are summarised in Scheme 1. The final concentration of isotopically labelled acetate in the *P. piliformis* medium was 4.8 mmol in this case. Analysis of the ^{13}C NMR spectrum of the resultant piliformic acid revealed carbon-13 enrichments (~2.5 fold) at C-3, C-5, C-7 and C-9. From the expected sites derived from C-2 of acetate and from deuterium-induced α-shifts[8] in the ^{13}C NMR spectrum, it was thus possible to deduce the retention of single deuterium atoms at C-5 and C-7 and up to three deuterium atoms at C-9, the acetate starter unit. In addition there was a very low level incorporation (0.2%) of a single deuterium

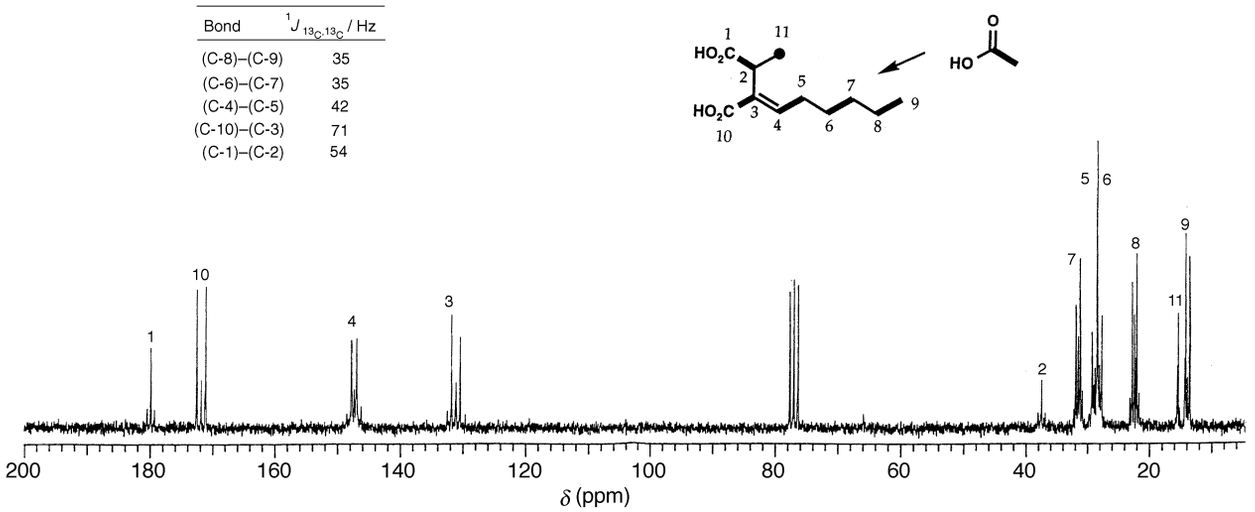


Fig. 1 ^{13}C NMR spectrum of piliformic acid **1** from *P. piliformis* after a feeding experiment with sodium [1,2-$^{13}C_2$]acetate. The resultant labelling pattern and the $^1J_{^{13}C^{13}C}$ coupling constants are summarised.

1 **2** **3** **4**

7

5 R = Et
6 R = H

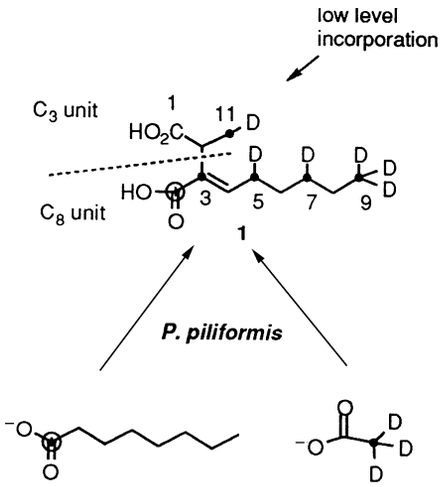
8 R = Et
9 R = H

10 R = OH
11 R = H

13 **12**

14

(^{13}C–^{2}H) into the C-11 methyl group, which was only detectable in the ^{13}C-{^{1}H,^{2}H} NMR spectrum recorded with deuterium decoupling. This latter incorporation can be rationalised by acetate processing around the citric acid cycle, possibly to oxaloacetate and contributing, after decarboxylation, to the C_3 unit. Such a process would result in the retention of a single deuterium atom, as is observed. This analysis is entirely consistent with the [1,2-^{13}C]acetate result where the C_3 unit is constructed from a $C_2 + C_1$ combination, the isolated carbon arising from decarboxylation and its subsequent divorce from



Scheme 1 Summary of the incorporation patterns into **1** from the [2-^{13}C,$^{2}H_3$]acetate and [1-^{13}C]octanoate feeding experiments

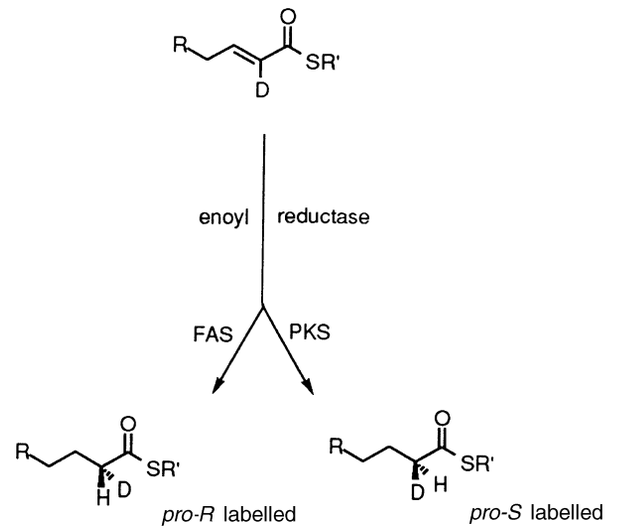
its original methyl partner. These experiments have established a disproportionate incorporation of acetate into the C_3 and C_8 units of piliformic acid, consistent with the origin of these moieties from different metabolic pools.

The C_8 unit

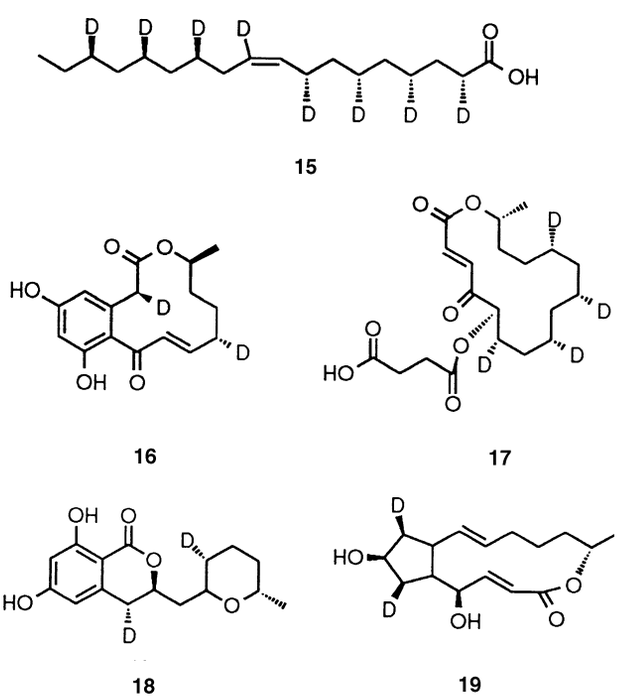
The direct involvement of an octanoate unit in the biosynthesis was demonstrated by the regiospecific incorporation of sodium [1-^{13}C]octanoate into piliformic acid from *P. piliformis*. ^{13}C NMR Analysis of the resultant piliformic acid showed an enhancement (5.5 fold) of the signal corresponding to C-10 of **1**, indicating the *intact* incorporation of octanoate into piliformic acid. Generally, microorganisms will degrade fatty acids to acetate by β-oxidation processes and recycle the carbon substrate by re-incorporation of acetyl-CoA. The signal enhancement at C-10 was indeed accompanied by lower level (2-fold) enrichments of the signals corresponding to C-4, C-6 and C-8 of piliformic acid, consistent with a background incorporation of [1-^{13}C]acetyl CoA after β-oxidation of the administered [1-^{13}C]octanoate. However, the predominant intact incorporation of octanoate reinforces its role as a biosynthetic intermediate.

Fungal FAS or PKS?

Although the [1-^{13}C]octanoate experiment has established octanoate as a *bona fide* intermediate in piliformic acid biosynthesis, the source of octanoate, from either a FAS or PKS origin, remained to be resolved. To delineate these possibilities the stereochemical course of the enoyl reductase operating during the biosynthesis of the octanoate moiety was evaluated. Vederas[10,11,13] has concluded that the stereochemical course of the enoyl reductase of fungal PKSs (secondary metabolites) is consistently opposite to that exhibited by the enoyl reductase of fatty acid synthases (FASs) *in the same organism*. These conclusions are summarised in Scheme 2. Thus, deuterium labelled acetate enriched only the 2-*pro-R* derived sites of the reduced acyl thioesters during oleic acid **15** biosynthesis in all of the cases studied but only the 2-*pro-S* derived sites in the resultant secondary metabolites. This has been established for the fungal metabolites dihydrocurvularin **16**,[10] antibiotic A26771B **17**,[10] cladosporin **18**[11] and brefeldin-A **19**,[12] and despite the perhaps limited number of samples studied to date, the phenomenon appears to be general. Interestingly, an exception is found with averufin **21**[13] where the stereochemical location of the residual deuterium in the bicycloketal ring system is consistent with the involvement of a fungal FAS enoyl reductase. However, as indicated in Scheme 3, the carbons of this ring system derive from a hexanoate starter unit[14] which is involved in norsolorinic acid **20** biosynthesis and then norsolorinic acid is converted into averufin **21**. Recent biochemical studies[15] on *Aspergillus parasiticus* suggest that this hexanoate starter unit is biosynthesised by one or possibly two fatty-acid synthases (FASs) which function closely with the polyketide synthase involved in assembling the anthraquinone moiety of norsolorinic acid **20**. Thus, the stereochemical location of the residual deuterium atoms in averufin **21** is entirely consistent with a fatty-acid origin, albeit a FAS system which may have evolved from a more classical fungal FAS to serve a unique role in secondary metabolism.

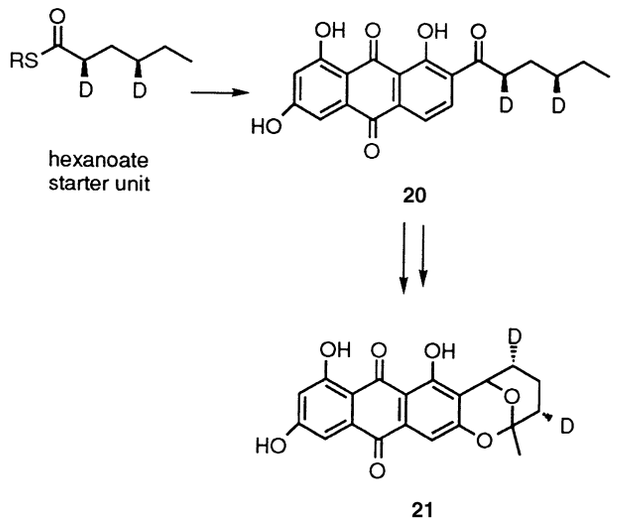


Scheme 2 Protonation at C-2 of PKS and FAS enoyl reductases occur with opposite stereochemical courses in fungi

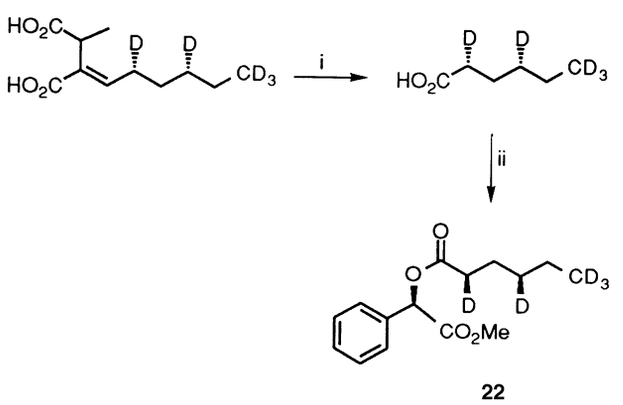


With this background we determined to assess the stereochemical location of deuterium at C-4 of piliformic acid after supplementation of *P. piliformis* with sodium [$^{2}H_3$]acetate. This was accomplished by the strategy illustrated in Scheme 4.

Oxidative cleavage[16] of the double bond in the recovered piliformic acid, with ruthenium tetroxide, furnished hexanoic acid which was shown by ^{2}H NMR analysis to be significantly enriched with deuterium at C-2, C-4 and C-6 as expected. This material was converted into its methyl (2*R*)-mandelate ester **22** for further analysis. The deuterium sited at C-2 of the hexanoate moiety was judged to occupy the 2-*pro-R* position as



Scheme 3



Scheme 4 Piliformic acid, labelled from sodium [$^2H_3$]acetate was oxidatively cleaved to hexanoic acid and derivatised for ^2H NMR analysis; i, $RuCl_3$, KIO_4, (H_2O–MeCN–CCl_4, 3:1:1), 77%; ii, DCC, DMAP, methyl (2*R*)-mandelate, 65%

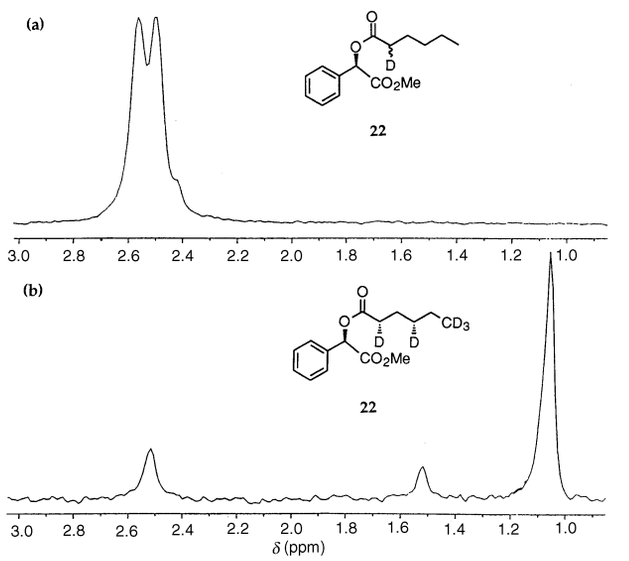


Fig. 2 Summary of the stereochemical location of deuterium after the incorporation of [$^2H_3$]acetate into various fungal metabolites and oleic acid. Only the stereochemically relevant labelled sites are shown for clarity. ^2H NMR Spectra of methyl (2*R*)-mandelate derivatives **22** of (a) racemic [2-^2H]hexanoic acid and (b) (2*S*)-[2-$^2H_1$]hexanoic acid derived from isotopically enriched piliformic acid after a sodium [$^2H_3$]-acetate feeding experiment.

determined by ^2H NMR analysis of the methyl (2*R*)-mandelate ester **22** following the assignment of Parker.[17] It can be seen in Fig. 2 that the deuterium enriched ester **22** derived from piliformic acid has a single ^2H NMR resonance at δ 2.5 which correlates only with the upfield resonance of the two ^2H NMR signals from the diastereotopic deuterium atoms in a sample of **22** prepared from racemic [2-$^2H_1$]hexanoic acid. This was further confirmed by spiking the piliformic acid-derived material with a sample of **22** derived from racemic [2-$^2H_2$]hexanoic acid. The resultant ^2H NMR spectrum of the mixture indicated a predominant upfield signal. Following from Vederas's observations, the location of deuterium in the 2-*pro-R* site suggests a FAS rather than a PKS origin for the octanoate moiety of piliformic acid. We conclude therefore that octanoate is channelled into piliformic acid biosynthesis either after synthesis by a short-chain FAS, which has evolved this particular function in secondary metabolism, or possibly octanoate is sequestered after partial β-oxidation of a higher fatty acid. The stereochemical course of the enoyl reductase is inconsistent with the operation of a fungal PKS.

The C_3 unit

The isotopically labelled acetate feeding experiments gave a strong indication that acetate had been processed around the citric acid cycle prior to incorporation into the C_3 unit in piliformic acid. To gain further insights into the origin of the C_3 unit the incorporation of DL-[3-^{13}C,$^2H_3$]alanine and sodium [2,3-$^2H_4$]succinate into piliformic acid was evaluated. DL-[3-^{13}C,-$^2H_3$]Alanine was used as a pyruvate surrogate, as transamination will generate an endogenous pool of [3-^{13}C,$^2H_3$]pyruvate. The requisite DL-[3-^{13}C,$^2H_3$]alanine was prepared following Seebach's methodology[18] from racemic 1-benzyl-2-(*tert*-butyl)-3-methylimidazolidin-4-one and using iodo[^{13}C,$^2H_3$]methane as the alkylating agent. In the event no incorporation of carbon-13 or deuterium into the C-11 methyl group of **1** was observed after supplementation of the *P. piliformis* medium to 4.5 mmol of the labelled amino acid. The method of analysis involved the computational subtraction of the ^{13}C-{^{1}H} NMR data from the deuterium decoupled ^{13}C-{^{1}H,^{2}H} NMR data and the resultant difference spectrum gave no indication of any residual ^{13}C-^2H coupling. The sensitivity of this double-labelling strategy should reveal incorporations ≤ 0.1% and this, albeit negative, result suggests that either transamination to pyruvate did not occur or that pyruvate is not involved in piliformic acid biosynthesis. On the other hand supplementation of the *P. piliformis* medium to 6.8 mmol with sodium [2,3-$^2H_4$]succinate resulted in an efficient incorporation of deuterium into the C-11 methyl group, as determined by ^2H NMR spectroscopy. Thus, the processing of succinate around the citric acid cycle, through fumarate and malate to generate [3-$^2H_1$]oxaloacetate, provides the most straightforward rationale for this observation. We conclude from these experiments and the labelled acetate incorporations discussed above that the condensation required to join the two sub-units during piliformic acid biosynthesis occurs between a C_8 octanoate moiety and a C_4 citric acid cycle intermediate, rather than to pyruvate. The most likely candidate for the C_4 intermediate is oxaloacetate.

The C-C condensation

At some stage during piliformic acid biosynthesis a carbon–carbon bond must form between C-2 and C-3. Activation of the octanoate unit at the α-carbon, facilitating nucleophilic attack to oxaloacetate, could take several forms. Three more obvious possibilities emerge.

(a) The β-keto intermediate **23** could be generated as an intermediate in the β-oxidation of fatty acids. This appeared attractive particularly as the enoyl reductase stereochemistry is consistent with a fatty acid origin for the C_8 moiety and thus the octanoate may arise from β-oxidation of a higher fatty acid. (b) Alternatively, the octanoate (thio ester) may be activated by α-carboxylation after the action of a dedicated octanoate car-

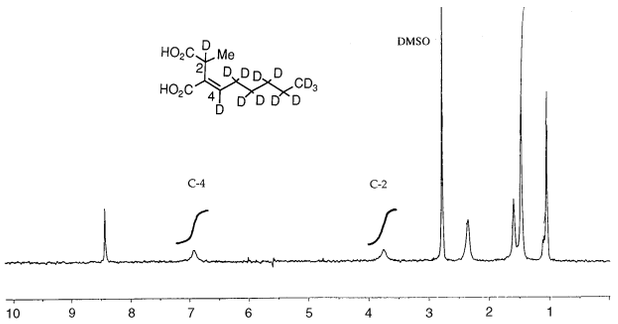
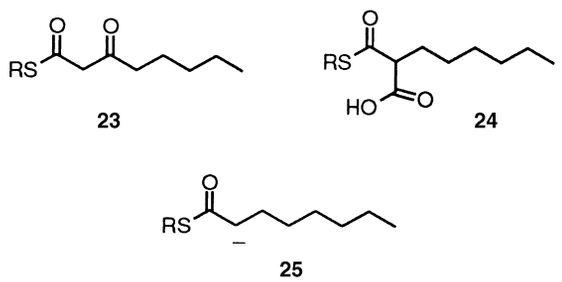
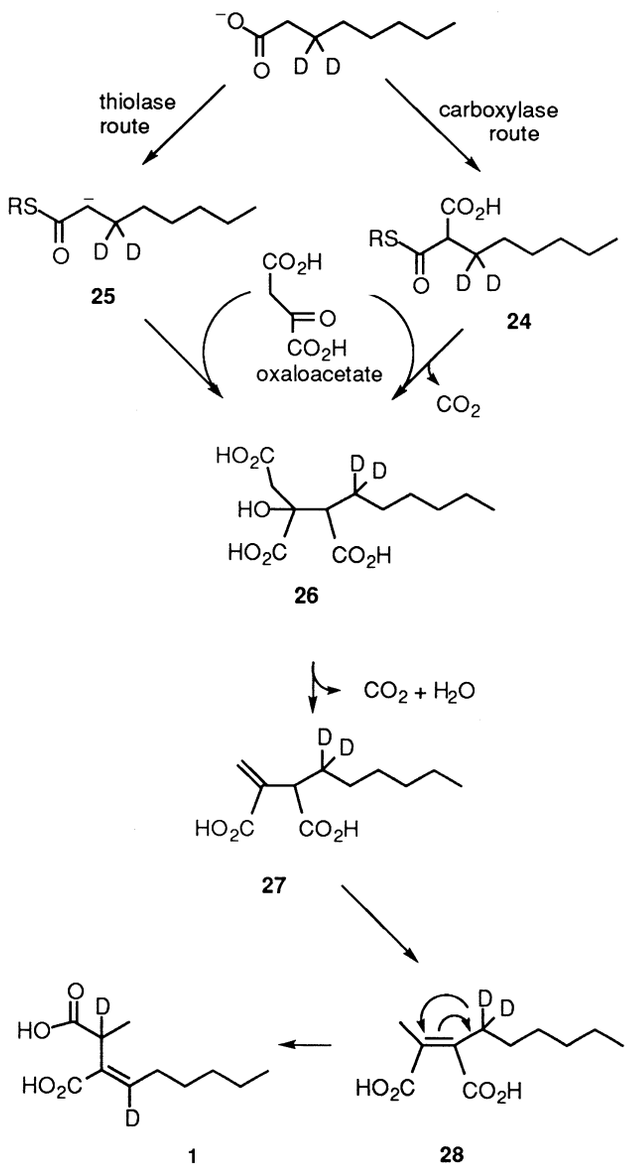


Fig. 3 ^{2}H NMR spectrum of piliformic acid isolated from *X. mali* after incorporation of sodium [$^2H_{15}$]octanoate. The level of deuterium incorporation at C-2 and C-4 is similar.



boxylase to generate **24**, and then the C–C bond would be formed with concomitant decarboxylation in a manner analogous to keto synthases in fatty acid and polyketide biosynthesis.[19] (c) Finally, the octanoate (thio ester) may be directly deprotonated to generate the anion **25** by the action of a dedicated thiolase.[20] In an effort to delineate the intermediacy of **23** from **24** and **25**, perdeuteriated sodium [$^2H_{15}$]octanoate was administered to cultures of *P. piliformis*. Clearly, if a β-keto intermediate such as **23** is involved in the biosynthesis, then the deuterium atoms at the β-carbon of the administered octanoate will necessarily be lost during metabolism. Alternatively, if the intermediates **24** or **25** are involved, then these β-deuterium atoms will be retained after activation and become incorporated into the C-4 olefinic site of piliformic acid. Accordingly, piliformic acid **1**, isolated after supplementation of the *P. piliformis* medium to 1.84 mmol with sodium [$^2H_{15}$]octanoate, was analysed by ^{2}H NMR spectroscopy. The experiment was also conducted using *X. mali* and in both cases an identical result was obtained. The resultant spectrum of piliformic acid isolated from the *X. mali* feeding experiment is shown in the ^{2}H NMR spectrum in Fig. 3. It is clear that there is significant deuterium incorporation at all of the sites along the lipophilic chain derived from the C_8 unit. This is entirely consistent with the intact incorporation of the perdeuteriated octanoate administered to the cultures. Of particular significance however is the observation that deuterium is retained at the olefinic hydrogen attached to C-4 (δ 6.9), the site which is derived from a β-hydrogen of the octanoate precursor. This result rules out β-keto octanoate **23** as an intermediate, and thus β-oxidation, if it is linked to piliformic acid biosynthesis at all, does not progress as far as **23** before octanoate is channelled towards piliformic acid biosynthesis. The retention of deuterium at C-4 does not however allow us to delineate between **24** and **25** and thus the true identity of the activated form of octanoate must remain ambiguous at present.

The feeding experiment with sodium [$^2H_{15}$]octanoate generated an additional unexpected but illuminating result. We were surprised to find that deuterium was incorporated into C-2 (δ 3.7) of piliformic acid, the methine hydrogen of the C_3 unit, at a similar level to that observed in the olefinic C-4 site. This distribution was observed in piliformic acid from both *P. piliformis* and *X. mali* and is most straightforwardly rationalised by a 1,3-hydrogen shift operating during a 2,3- to 3,4-double bond isomerisation in the later stages of piliformic acid biosynthesis (Scheme 5).



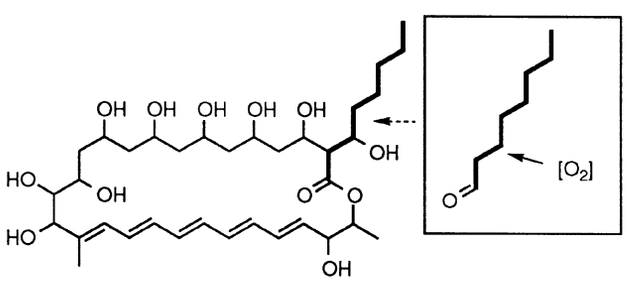
Scheme 5 A rationale for the biosynthesis of piliformic acid **1**. For clarity, deuterium atoms are attached to the β-carbon of octanoate to highlight their distribution during the biosynthesis.

Significantly in *both* experiments the ratio of deuterium at C-2 and C-4 of the resultant piliformic acid is 1:1 as judged by integration of the ^{2}H NMR signals (see Fig. 2). Thus, the migrating deuterium is not subject to dilution by any exchange processes. In order to discount any possibility that the deuterium arriving at C-2 of piliformic acid originated from the α- and not the β-position of [$^2H_{15}$]octanoate, we prepared sodium [1-^{13}C,2-$^2H_1$]octanoate and carried out a feeding experiment with *P. piliformis*. The expected high level of carbon-13 enrichment (5 fold) at C-10 of piliformic acid was obvious in the resultant ^{13}C NMR spectrum of the isolated material, indicating again the intact incorporation of octanoate. A lower level [1-^{13}C]acetate labelling pattern was also evident, as previously encountered in the [1-^{13}C]octanoate experiment, due to β-oxidation of octanoate to acetate. However ^{2}H NMR analysis revealed a labelling pattern consistent only with the incorporation of deuterium *via* [1-^{13}C,2-^{2}H]acetyl-CoA. There was no signal in the ^{2}H NMR spectrum indicating deuterium enrichment at C-2 of piliformic acid **1** and therefore the deuterium which labelled C-2 of piliformic acid in the [$^2H_{15}$]octanoate

experiment originated from the β-position of octanoate. These results force the conclusion that the observed isotopic distribution arose because of a 1,3-hydrogen shift concomitant with a double-bond migration during piliformic acid biosynthesis.

This isomerisation sets the stereogenic centre in piliformic acid and in this context it is noteworthy that piliformic acid, isolated from different *Xylaria*, can be optically active or racemic. Piliformic acid from *P. piliformis* has a specific rotation of $[\alpha]_D^{23}$ −89 whereas that from *X. mali* is racemic.[1] We have independently confirmed these values. The experiment (Fig. 3) which was initially conducted with *P. piliformis* was repeated with *X. mali* to assess if a full equivalent of deuterium at C-2 relative to the C-4 olefinic position is transferred. In each case integration of the resultant ^{2}H NMR spectra indicated a coherent transfer of deuterium from C-4 to C-2, thus racemic piliformic acid does not arise due to racemisation of piliformic acid, as half of the deuterium would have become liberated. It appears therefore that during the biosynthesis of racemic piliformic acid in *X. mali* both of the original prochiral β-hydrogens of octanoate migrate suprafacially and to similar extents to generate the C-2 labelled racemic material. If such a process is enzymatically mediated then this requires two enzymes with opposite stereospecificities operating at similar rates in *X. mali*. A satisfactory rationale for this observation must await further investigation.

The pathway proposed in Scheme 5 progresses through the putative intermediates **26**, **27** and **28**. In particular, intermediate **26** can be compared with caperatic acid **10**. The structure of **10** alone lends circumstantial support to the direct condensation of an activated fatty acid and oxaloacetate during the biosynthesis. Putative intermediate **27** and the lichen metabolites **5** and **6** have common structural features, and intermediate **28** can be compared directly with chaetomellic acids A and B, **13** and **14**. Thus the proposed intermediates on the biosynthetic pathway to piliformic acid all have close structural counterparts in other fungal and lichen metabolites.



29

The polyene antibiotic fungichromin **29**, a metabolite of *Streptomyces cellulosae*, is the only other case reported[21] where an intact octanoate unit is involved in the biosynthesis of a polyketide metabolite. It has been established[21b] for fungichromin **29** that the octanoate unit derives from β-oxidation of oleic acid and thus there may be close similarities in the origin and activation of octanoate in that system and in piliformic acid biosynthesis, despite their respective bacterial and fungal origins. Alternatively, and in accord with the emerging results on aflatoxin biosynthesis,[15] short-chain fatty acids required for fungal secondary metabolites may have unique short chain FASs. If this is the case these short chain FASs possess enoyl reductases with a common stereochemical course to the longer FAS's of that organism, unlike the PKS in fungal secondary metabolism.

Interestingly there is an oxygen atom at the β-site of the octanoate residue in fungichromin **29**, but this oxygen originates[21] from molecular oxygen rather than perhaps the more obvious acetate source. The introduction of oxygen would appear to be a late biosynthetic modification. A comparable situation is found during averufin **21** biosynthesis[22] where the hexanoate moiety of **20** is modified by oxidation, in this case at the δ position. An oxygen atom is again re-introduced into a site where a carbonyl group has been reductively removed earlier in the biosynthesis. In this context the β-oxygen atoms from the fatty-acid derived moieties of the lichen metabolites **5–9** may derive from molecular oxygen rather than from acetate, particularly if the saturated fatty acids rather than their β-oxo analogues, as is the case with piliformic acid, are true precursors.

In summary, the biosynthesis of piliformic acid **1**, and by implication the fungal metabolites **2–4**, are assembled from short (C_6-C_{12}) fatty-acid chains and citric acid cycle intermediates. Their biosynthesis mirrors that of the lichen metabolites such as **5**, insomuch as both involve the condensation between a citric acid cycle intermediate and a fatty-acid derivative. There does not appear to be a dedicated PKS associated with any of these metabolites, but rather they are assembled by a FAS system. The mechanism of C–C bond formation does not involve a β-keto intermediate, and most probably involves a decarboxylative condensation, a strategy widely employed in the synthesis of polyketide metabolites, or alternatively deprotonation of octanoate by the action of a dedicated thiolase enzyme. In the later stages of piliformic acid a 1,3-hydrogen shift occurs indicating a double-bond isomerisation.

Experimental

Materials and methods

NMR Spectra were recorded on Varian VXR-400(S) (^{1}H at 399.95 MHz, ^{13}C at 100.57 MHz) and Varian VXR-600 (^{13}C at 150.87 MHz) instruments. Chemical shifts are quoted relative to $SiMe_4$ (δ 0). [1-^{13}C]Octanoic acid and [2,3-$^2H_4$]succinic acid, were purchased from the Aldrich Chemical Co. and [$^2H_{15}$]-octanoic acid was purchased from the Sigma Chemical Co. For feeding experiments the labelled carboxylic acids were converted into their sodium salts after neutralisation of the aqueous solutions with aqueous sodium hydroxide and they were administered to the medium at final concentrations in the range 1.84–15 mmol dm^{-3}.

Piliformic acid 1 production

P. piliformis and *X. mali* were grown statically on malt extract solution at 24 °C and piliformic acid was isolated (~150 mg l^{-1} *P. piliformis* and ~25 mg l^{-1} *X. mali*) from the medium after 8 weeks, in the same manner as previously described;[1] δ_H-($CDCl_3$) 0.90 (3 H, t, *J* 6.9, CH_3), 1.28 [7 H, m, $(CH_2)_2$ and CH_3], 1.34 (2 H, m, CH_2), 2.24 (2 H, m, CH_2), 3.63 (1 H, q, *J* 7.1, CH), 7.02 (1 H, t, *J* 7.0, CH) and 10.8 (2 H, s, CO_2H); δ_C($CDCl_3$) 14.4 (C-9), 15.1 (C-11), 22.9 (C-8), 28.6 (C-6), 29.3 (C-5), 32.0 (C-7), 38.0 (C-2), 131.5 (C-3), 147.9 (C-4), 172.5 (C-10) and 180.7 (C-1). From *P. piliformis*, $[\alpha]_D$ −86 (*c* 0.067, MeOH) [lit.,[1] −89 (*c* 1.0, MeOH)]. From *X. mali*, $[\alpha]_D$ 0 (*c* 0.025, MeOH) [lit.,[1] 0 (*c* 1.0, MeOH)].

Degradation of piliformic acid 1 to hexanoic acid after the sodium [2-$^2H_3$]acetate feeding experiment

Piliformic acid (900 mg) was added to a vigorously stirred solution of potassium periodate (37.5 g, 163 mmol) and ruthenium trichloride (100 mg, 0.482 mmol) in water–acetonitrile–tetrachloromethane (3:1:1; 300 ml). The reaction mixture was stirred at 18 °C for 48 h and was then filtered through a Celite pad. 50% Aqueous HCl (100 ml) was added to the filtrate which was then exhaustively extracted with diethyl ether. The extract was evaporated under reduced pressure to afford a residue of hexanoic acid which was suspended in chloroform (20 ml) and then extracted into 0.1 M aqueous NaOH (3 × 20 ml). The alkaline solution was partitioned and acidified with 50% aqueous HCl and then re-extracted into diethyl ether (3 × 60 ml). After being dried ($MgSO_4$), the combined extracts were filtered and

evaporated under reduced pressure to give hexanoic acid (371 mg, 77%) as a yellow oil; ν_{max}/cm^{-1} 3013br, 2958, 1713 and 1414; *m/z* (EI+) 117 ($M^+ + 1$, 0.24%) and 60 ($M^+ - 56$, 100); $\delta_H(CDCl_3)$ 0.89 (3 H, *J* 6.7, t, CH_3), 1.32 [4 H, m, $(CH_2)_2$], 1.63 (2 H, m, CH_2), 2.33 (2 H, t, *J* 6.2, CH_2CO) and 11.0 (1 H, s, CO_2H); $\delta_C(CDCl_3)$ 14.3 (C-6), 22.8 (C-5), 24.8 (C-4), 31.7 (C-3), 34.5 (C-2) and 181.2 (C-1). This material was derivatised without further purification.

Methyl *O*-hexanoyl-(2*R*)-mandelate from deuterium labelled hexanoic acid

Methyl (*R*)-mandelate (221 mg, 1.33 mmol) and dicyclohexylcarbodiimide (275 mg, 1.33 mmol) were added to a stirred solution of the hexanoic acid (115 mg, 1.33 mmol) and 4-dimethylaminopyridine (5 mg, 0.04 mmol) in dichloromethane (7 ml) at −10 °C. The reaction mixture was stirred at this temperature for 3 h after which precipitated urea was filtered off. The filtrate was re-evaporated under reduced pressure and the residue suspended in dichloromethane (3 ml) and the solution refiltered. After evaporation of the filtrate the residue was purified over silica gel eluting with dichloromethane; evaporation of the active fractions afforded methyl *O*-hexanoyl-(2*R*)-mandelate (227 mg, 65%) as a colourless oil; ν_{max}/cm^{-1} 2955, 2932, 1745, 1455, 1216 and 1160; *m/z* (CI) 285 [$(M + 3)^+ + 18$, 3.1%], 284 [$(M + 2)^+ + 18$, 3.71], 283 [$(M + 1)^+ + 18$, 13.9], 282 ($M^+ + 18$, 40.9) and 166 ($M^+ - 99$, 100); $\delta_H(CDCl_3)$ 0.9 (3 H, t, *J* 6, CH_3), 1.33 [4 H, m, $(CH_2)_2$], 1.70 (2 H, m, CH_2), 2.47 (2 H, dq, *J* 7.46 and 3.94, CH_2), 3.72 (3 H, s, OMe), 5.95 (1 H, s, CH) and 7.40 (5 H, m, Ar); $\delta_D(CCl_4)$ (see Fig. 3), 0.92 (CD_3), 1.39 (CHD) and 2.46 (CHD); $\delta_C(CDCl_3)$ 14.4 (C-12), 22.8 (C-11), 25.0 (C-10), 31.7 (C-9), 34.4 (C-8), 53.1 (OMe), 74.7 (C-2), 128.1 (C-4), 129.3 (C-5), 129.6 (C-6), 134.4 (C-3), 169.8 (C-7 or C-1) and 173.7 (C-7 or C-1) [Found (from unlabelled analysis): M^+, 265.1426. $C_{15}H_{21}O_4$ requires MH^+ 265.1439].

DL-[3-^{13}C,$^2H_3$]Alanine

A solution of LDA (6.8 mmol), generated from diisopropylamine (0.69 g, 0.95 ml, 6.8 mmol) and butyllithium (1.6 M in hexanes; 4.2 ml, 6.8 mmol) in THF (10 ml), was transferred to a stirred solution of 1-benzyl-2-(*tert*-butyl)-3-methylimidazolin-4-one (1.61 g, 6.16 mmol) in THF (70 ml) at −78 °C. The reaction mixture was stirred for 1 h after which [^{13}C,$^2H_3$-*methyl*]iodomethane (1.0 g, 6.85 mmol) was added dropwise. The solution was stirred at −78 °C for 45 min and was then allowed to warm gradually to 18 °C; it was then left at ambient temperature for 12 h after which the reaction was quenched by the addition of saturated aqueous NH_4Cl (70 ml) to the mixture. The aqueous layer was extracted into diethyl ether (2 × 80 ml) and the combined organic extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure. Purification of the residue over silica gel with diethyl ether–light petroleum–methanol (60:35:5) gave 1-benzyl-2-(*tert*-butyl)-3,5-dimethyl-[5-^{13}C,$^2H_3$]imidazolidin-4-one as an amorphous white solid (0.98 g, 56%); mp 145.5–146.5 °C (lit.,[18] 145.6–146.2 °C); *m/z* (CI) 279 ($M^+ + 1$, 100%) [Found (unlabelled material): C, 69.90; H, 8.15; N, 10.01. $C_{15}H_{20}O_2N_2$ requires C, 70.04; H, 8.08; N, 10.21%].

1-Benzyl-2-(*tert*-butyl)-3,5-dimethyl[5-^{13}C,$^2H_3$]imidazolidin-4-one (0.97 g, 3.5 mmol) was heated with 6 M aqueous HCl (6 ml) to 180 °C in a sealed tube for 12 h. The solution was then removed and concentrated under reduced pressure. Purification of the residue by ion exchange chromatography (Dowex 50X2-400) gave DL-[3-^{13}C,$^2H_3$]alanine (245 mg, 70%) as an amorphous white solid, mp 289–290 °C (decomp.) [lit.,[23] 295–296 °C (decomp.)]; *m/z* (CI) 94 ($M^+ + 1$, 100%); $\delta_H(D_2O)$ 3.62 (1 H, br d, $^2J_{^1H,^{13}C}$ 4, CH); $\delta_C(D_2O)$ 18.2 (septet, $J_{^{13}C,^2H}$ 19.6, C-3), 53.13 (d, $J_{^{13}C,^{13}C}$ 34.7, C-2) and 178.63 (C-1).

[1-^{13}C,2-^{2}H]Octanoic acid

Methyl [1-^{13}C]octanoate (0.94 g, 5.9 mmol), prepared by treatment of [1-^{13}C]octanoate with diazomethane, was added dropwise to a stirred solution of lithium diisopropylamide (11.8 mmol) in THF (50 ml) at −70 °C. After 5 min the reaction mixture was allowed to warm to 18 °C at which temperature it was stirred for 1 h. The reaction was quenched by the addition of [^{1}H]methanol (5 ml) to the mixture which was then stirred for an additional 1 h. It was then poured into saturated aqueous ammonium chloride (50 ml). The aqueous layer was extracted with diethyl ether (2 × 50 ml) and the combined organic extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure. The residue was purified by distillation (0.01 mmHg, 50 °C) to give methyl [1-^{13}C,2-^{2}H]octanoate (501 mg, 53%). This material was directly hydrolysed by treatment with 1 M KOD in D_2O (20 ml) under reflux for 12 h. After being allowed to cool, the solution was washed with diethyl ether (2 × 20 ml) and acidified with 50% aqueous HCl. The product was then extracted into diethyl ether (3 × 20 ml) and the combined ethereal extracts were then dried ($MgSO_4$), filtered and evaporated under reduced pressure to give [1-^{13}C,2-$^2H_1$]-octanoic acid (287 mg, 33%) as a clear oil; ν_{max}/cm^{-1} 3049br, 2956, 2928, 1668 and 1272; *m/z* (EI+) 146 (M^+, CHD, 0.99%) and 145 (M^+, CH_2, 0.51); $\delta_H(CDCl_3)$ 0.88 (3 H, t, *J* 6.5, CH_3), 1.28 [8 H, m, $(CH_2)_4$], 1.61 (2 H, m, CH_2) and 2.34 (1.1 H, m, CHD); $\delta_C(CDCl_3)$ 14.1 (C-8), 22.6 (C-7), 24.6 (C-6), 28.9 (C-5), 29.0 (C-4), 31.6 (C-3), 33.8 (dt, $J_{^{13}C,^{13}C}$ 54.6, $J_{^{13}C,^2H}$ 19.3, CHD, C-2), 34.0 (d, $J_{^{13}C,^{13}C}$ 54.6, C-2) and 180.3 (enriched C-1).

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