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ARTICLE

Direct biosynthetic cyclization of a distorted paracyclophane highlighted by double isotopic labelling of L-tyrosine†

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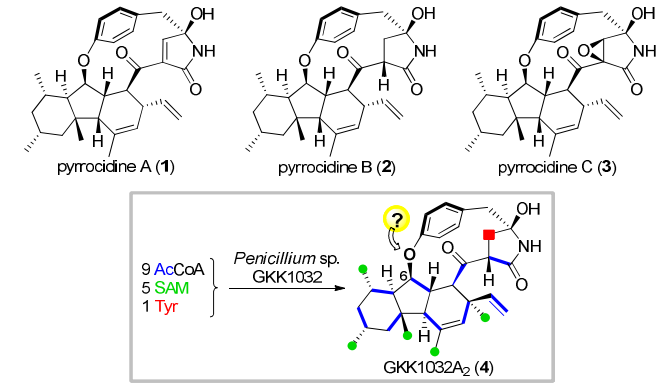
The biosynthesis of pyrrocidines, fungal PK-NRP compounds featured by a strained [9]paracyclophane, was investigated in *Acremonium zeae*. We used a synthetic L-tyrosine probe, labelled with oxygen 18 as a reporter of phenol reactivity and carbon 13 as a tracer of incorporation of this exogenous precursor. The (^{18}O)phenolic oxygen was incorporated, suggesting that the phenol behaves as a nucleophile during the formation of the bent aryl ether.

Introduction

The biosynthetic macrocyclization of bent [*n*]paracyclophanes is a challenging problem in biosynthetic studies as bending results in highly energetic benzene rings. A reasonable hypothesis suggests the transient loss of aromaticity during the cyclization, followed by rearomatization and distortion triggered by tautomery or dehydration. This mechanism has been suggested for the biosynthesis of haouamine whose bent [7]paracyclophane would be formed after oxidative phenol coupling.[1] We report the first evidence that the phenolic oxygen of tyrosine is directly involved in the formation of the bent [9]paracyclophane of pyrrocidines **1**-**3** (Scheme 1),[2-4] possibly through enzymatic templation and without breaking the aromatic character of the phenol ring.



Scheme 1 Structures of pyrrocidines A-C (**1**-**3**) and the biosynthetic building blocks of GKK1032A2 (**4**, in box).

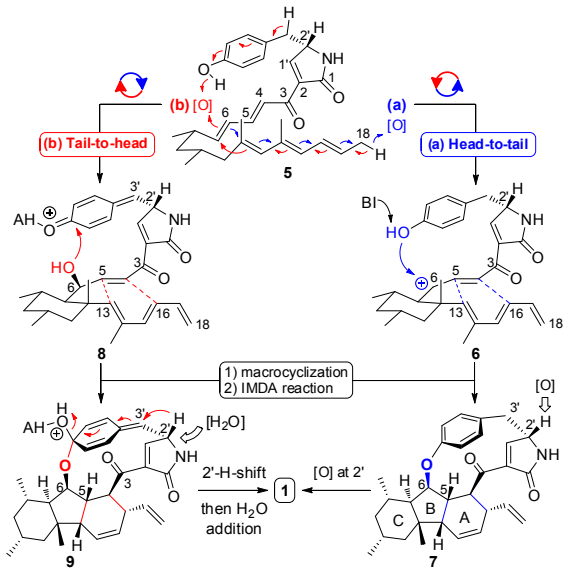
An early biosynthetic study was performed on the parent GKK1032A2 compound **4** isolated from a *Penicillium*,[5] using extensive isotopic labelling.[6] With this work, Oikawa gave clues to the mixed polyketide–non ribosomal peptide nature of **4** (Scheme 1), originated from nine acetate units (AcCoA), five methyls from *S*-adenosylmethionine (SAM) and one L-tyrosine (Tyr). However the complexity of the mechanisms[6] leading to the full carboheterocyclic core of these compounds still questions us about the cyclization of the distorted [9]paracyclophane from a linear *N*-acyl tyrosine precursor constructed by a PKS-NRPS enzymatic complex.[7,8] In particular, the role of the phenolic oxygen in the closure of the strained paracyclophane was unknown.

Results and Discussion

Biosynthetic mechanism hypotheses

With pyrrocidine A (**1**), two mechanisms can be hypothesized for the cyclization of linear precursor **5**, ultimately leading to benzene ring distortion in the [9]paracyclophane (Scheme 2), either head-to-tail through remote activation at C-18, route (a), or tail-to-head through oxidative activation of the double bond at C-6–C-7, route (b), both leading to the electrophilic cyclization of ring C.[9] In route (a), a nucleophilic quench of the allylic cation **6** by the phenol would proceed with formation of the paracyclophane, while intramolecular Diels-Alder (IMDA) reaction and C-H oxidation at C-2' in **7** would complete the biosynthesis of **1**. In route (b), oxidation of the phenol ring would result in an electrophilic quinone methide (**8**) whose attack by the hydroxyl at C-6 would allow bypassing the drawbacks of benzene distortion through a transient tetrahedral ketal carbon in **9**. In this case, the aromaticity of the paracyclophane would be recovered by the 2'→3' hydride shift before final hydration at C-2'. It is worth noting that these routes may also involve radical species. Obviously, the

phenolic oxygen is conserved in route (a) but not in route (b) where it is lost at the rearomatization step.

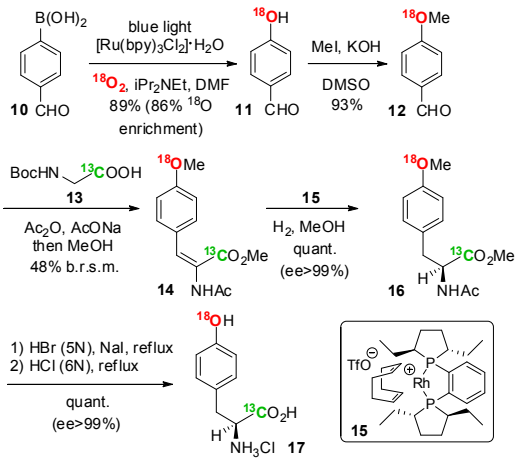


Scheme 2 Two hypothetic scenarios for the electrophilic cyclization leading to pyrrocidine A (**1**): (a) head-to-tail and (b) tail-to-head, following oxidative cyclization of linear precursor **5** into **6** or **8**, respectively.

To distinguish between these two routes, biosynthetic experiments in the presence of labelled L-tyrosine were envisaged. Our strategy relied on a double labelling to test, in a one-shot experiment, the possibility of these hypotheses. Oxygen 18 was used to report on the phenol reactivity while carbon 13 allowed tracing at the same time the incorporation of doubly labelled L-tyrosine in pyrrocidines, especially in the eventuality ^{18}O would be lost. Since such doubly labelled L-tyrosine (**17**) was not commercially available, an isotope-incorporating enantioselective total synthesis was undertaken.

Synthesis of doubly labelled L-tyrosine

A key photoredox oxidation of commercially available 4-formylphenylboronic acid **10** was performed in the presence of $[Ru(bpy)_3Cl_2]\cdot 6H_2O$ under blue led visible light irradiation at 420 nm (Scheme 3), using 97.1% enriched $^{18}O_2$ (1 atm).[10] The resulting (4-^{18}O)-4-hydroxybenzaldehyde **11** was obtained with 86% ^{18}O-enrichment due to slight air-dilution and was methylated into (4-^{18}O)-4-anisaldehyde **12** in 83% yield over two steps. Knoevenagel reaction between **12** and *N*-Boc-(1-^{13}C)glycine **13** in the presence of Ac_2O and NaOAc, followed by quenching with methanol, directly gave the *N*-acetyl-*p*-(^{18}O)methoxy-(1-^{13}C)dehydrophenylalanine methyl ester **14**.[11] The enantioselective hydrogenation of **14** was undertaken quantitatively in the presence of [(*S*,*S*)-Et-Duphos]Rh(cod)(OTf) **15**,[12] yielding *N*-acetyl-*p*-(^{18}O)methoxy-(1-^{13}C)-L-phenylalanine methyl ester **16** with >99% enantiomeric excess. At last, deprotection in acidic media provided doubly labelled L-tyrosine hydrochloride **17** in quantitative yield as a sole enantiomer (ee>99%), with 86% ^{18}O and 99% ^{13}C enrichments.



Scheme 3 Enantioselective synthesis of isotopically labelled L-tyrosine (**17**).

Culture conditions for the optimal production of pyrrocidines and crystallographic evidence of aromatic distortion

The plant associated fungus *Acremonium zeae* NRRL 13540 (syn. *A. strictum*)[3] was grown in a plant-mimicking liquid medium containing glucose and ground corn leaves. The three pyrrocidines A-C (**1-3**) were identified in the fungal extracts by LC-HRMS and LC-HRMS/MS experiments.[3a] Supplementing the medium with pulsed additions of unlabelled L-tyrosine (3 additions at 48h, 72h and 96h before stopping the culture at 168h) greatly enhanced their production by *ca.* 10-fold and allowed isolation of **3**. The separation of **1** and **2** was not initially possible in our chromatographic conditions. However, **1** was spontaneously converted into **3** upon storage,[13] allowing for separation of **2** from the epoxide **3**. X-ray crystallographic analysis of **3** could be performed after crystallization from CH_3CN (Figure 1),[14] showing two molecules per asymmetric unit, each exhibiting a [9]paracyclophane and displaying different bending angles $\Delta\theta$ of 12.26° and 12.64° ($\Delta\theta = \theta_2 - \theta_1$).

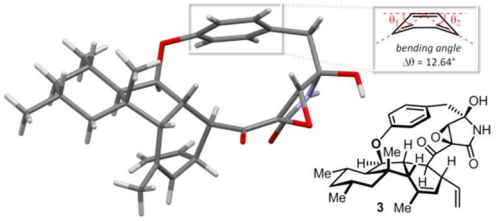


Figure 1 X-ray crystallographic drawing of **3** (only one among the two molecules per asymmetric unit is shown), showing the bent [9]paracyclophane.

In vivo isotopic labelling of pyrrocidines

The reporting amino acid **17** was added to a spore-seeded culture broth of *A. zeae*, using the pulsed protocol described before. After one week of culture, the mycelial ethyl acetate extract was analyzed by UHPLC-MS under atmospheric pressure chemical ionization (APCI). In a preliminary experiment, it was observed that exogenous L-tyrosine (*i.e.* labelled) was predominantly used in this biosynthesis. This

result was duplicated and showed incorporation of the labelled phenolic oxygen in the natural products, with *m/z* incremented by 3 amu for **1** and **2** at 491 and 493, respectively (pyrrocidine C was not observed), corresponding to the protonated adducts $[M+H]^+$ (Figure 2).

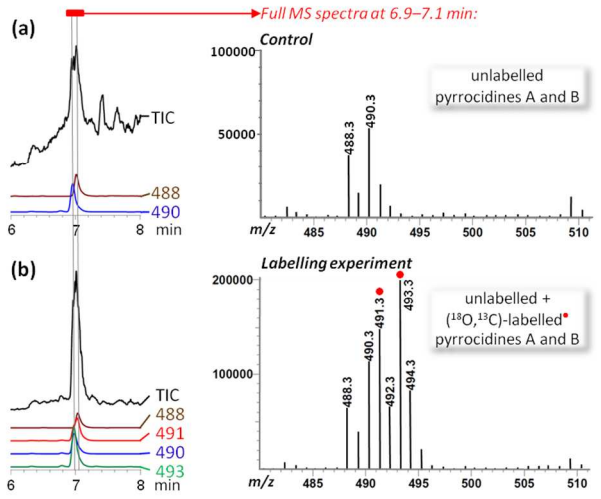
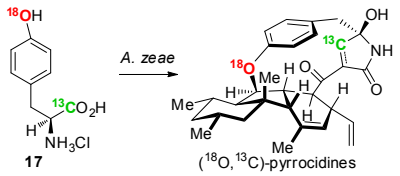


Figure 2 UHPLC-MS(APCI) of the labelling experiment performed in *A. zeae*, showing incorporation of ^{18}O and ^{13}C into pyrrocidines A and B. Left: total ion chromatogram (TIC); Right: full MS spectra. (a) Control experiment; (b) Labelling experiment showing production of both unlabelled and labelled pyrrocidines.

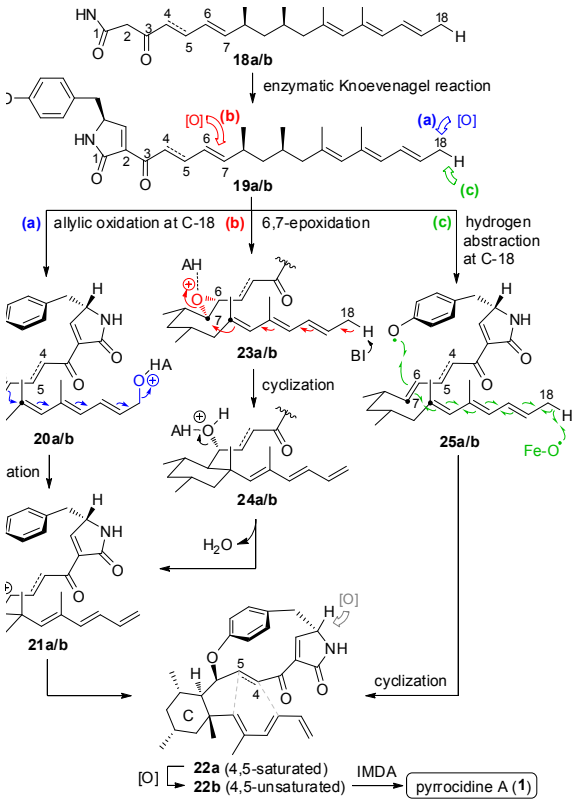
This (^{18}O,^{13}C)-labelling pattern (Figure 2b) strongly supports the hypothesis of a phenolic attack at C-6 on the acyl chain, as suggested by the head-to-tail route (a) in Scheme 2 and thus the direct formation of the paracyclophane, without the loss of aromaticity for the phenol, resulting in the conservation of ^{18}O (Scheme 4). However, a deeper analysis of the isotope distribution of products showed irregular relative intensities of peaks at *m/z* 488 (100) and 489 (relatively 62 observed for 34 expected for the isotope pattern of unlabelled natural pyrrocidine A). While it is highly improbable that both mechanisms co-occur during this biosynthesis, this increased peak at *m/z* 489 may sign the loss of oxygen 18. In fact, this situation was logically explained by the 86% ^{18}O enrichment of our doubly labelled tyrosine, containing 14% of the ^{16}O isotopomer, leading to higher peak intensity at *m/z* 489 for the labelled (^{13}C)pyrrocidine A. Indeed simulation with an isotope distribution calculator (mMass),[15] using the isotope enrichment of precursor **17** and considering that **1** is produced at a 71% level of **2** (as seen in Figure 2a), allowed reproducing and thus demonstrating the origin of relative intensities of peaks at 488 (100) and 489 (relatively 66 as simulated, Figure S3, Table S1).



Scheme 4 *In vivo* incorporation of labelled L-tyrosine **17** into pyrrocidines.

Possible biosynthetic mechanisms of the head-to-tail route

Even though paracyclophane cyclization could be easier on a flexible intermediate like **18** which is the product of the PKS-NRPS complex, it is most probable that the γ-lactam **19** is the effective substrate for the cyclization (Scheme 5). Indeed it would be formed by a specialized enzyme, as hypothesized for cytochalasins,[16] catalyzing the Knoevenagel reaction of **18**. In cytochalasins, this enzyme also catalyzes an IMDA reaction which is not possible in **19** due to the different topology and reactivity of olefins. Instead, an oxidative polycyclization is likely to occur after oxidation by a cytochrome P_{450} or a non-heme iron(II) oxygenase. An electrophilic process involving a cation like **21a** (4,5-saturated) or **21b** (4,5-unsaturated) could lead to the paracyclophane **22a** or **22b**, respectively. The cationic intermediates **21** could arise either from the terminal oxidation of **19** at C-18 into the allylic alcohol **20** (Scheme 5, pathway a), or from epoxidation of internal olefin C-6=C-7 into **23** and electrophilic cyclization into **24**, then OH elimination (Scheme 5, pathway b). At last, a radical mechanism cannot be excluded through phenolic radical **25a** or **25b**. The mechanism involving radical species would involve H-abstraction at the C-18 allylic position of the linear intermediate **25** (Scheme 5, pathway c).[17]



Scheme 5 Hypothetical biosynthetic pathways involving a head-to-tail cyclization (compounds **a**: 4,5-saturated; compounds **b**: 4,5-unsaturated).

Owing to the disfavouring π-bond polarization of the dienoyl moiety in **20b** and **25b**, it is questionable if C-6 is electrophilic toward the phenol, especially in pathways a and c. It suggests that the bond C-4–C-5 in **20a** and **25a** could be saturated up to the macrocyclization step. In such case, once the paracyclophane has been formed, an additional β-oxidation of **22a** into the *E*-enoyl derivative **22b** would release a strained and very reactive bridgehead dienophile (anti-Bredt olefin) prompt to click with the diene in the IMDA reaction.

Conclusions

We demonstrate for the first time that a distorted paracyclophane can directly be formed during a biosynthetic process, especially through the key ^{18}O-labelling of the phenol oxygen which was conserved during the experiment. Such tyrosine reporter, also provided with a ^{13}C tracer in case of oxygen loss, may be useful to study the biosynthesis of other mixed polyketides like macrocidins[18] or cyclopeptides[19] like pandamine[20] or mauritine A.[21] Pyrrocidines and structurally related compounds have been described in several fungal genus.[8] These biologically active compounds form a large family of secondary metabolites, sharing common features with cytochalasans (PKS-NRPS nature, polycyclic skeleton, linear biosynthetic precursors) and thus deserving extensive studies to understand their role and origin in nature. Our biosynthetic study shows again how Nature is capable of exploiting the molecular properties of reactive intermediates, sometimes up to an extreme degree of efficiency as observed with the formation of this distorted paracyclophane, to build an armada of biologically active compounds.

Acknowledgements

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental procedures, characterization data, and ^{1}H and ^{13}C NMR spectra of products. See DOI: 10.1039/c000000x/

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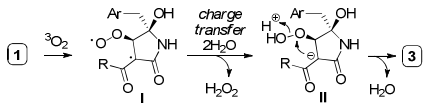

14 Crystallographic data for compound **3** were deposited with the Cambridge Crystallographic Data Centre as number CCDC 1031563. Drawings in Figure 1 were generated using the Mercury software.
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Graphical and short textual abstracts for

Direct biosynthetic cyclization of a distorted paracyclophane highlighted by double isotopic labelling of L-tyrosine

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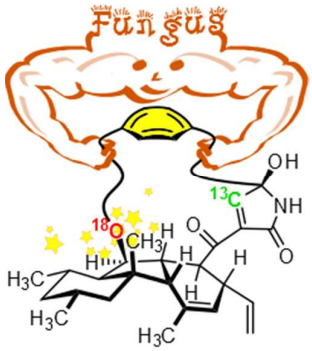

The biosynthesis of pyrrocidines was investigated using a double labelling of L-tyrosine obtained by enantioselective total synthesis. It was found that the phenolic (^{18}O)oxygen is conserved all over the cyclization process, suggesting that the bent paracyclophane core is directly installed during the biosynthesis through aryl ether bond formation.