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Biosynthesis of the mitochondrial adenine nucleotide translocase (ATPase) inhibitor bongkrekic acid in *Burkholderia gladioli*†

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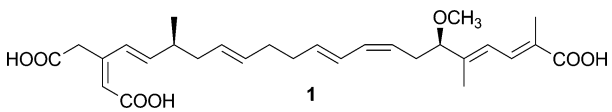
Biosynthetic studies with ^{13}C-labelled acetates and methionine revealed that the infamous, food-related toxin bongkrekic acid from *Burkholderia gladioli* is a polyketide with acetate-derived β-branches and a carboxylate terminus derived from the methyl group of an acetate.

Introduction

Tempe bongkrek is a popular, coconut-derived dish in Southeast Asia (Indonesia). However, its consumption has led to severe, often fatal intoxications.[1] From 1951–1990, over 7700 victims and almost 1000 deaths have been reported.[2] As a consequence the Indonesian government officially banished the production and distribution of tempe bongkrek with little effect.[2] It was obvious that the poisonous agent in the inexpensive meat surrogate is produced during the fermentation of coconut patties with the fungus *Rhizopus oligosporus*.[3] In 1933, Mertens and Van Veen showed that this fungal culture is often contaminated with a bacterium, *Pseudomonas cocovenenans*,[4] (later renamed to *Burkholderia gladioli* pathovar *cocovenenans*)[5]—and that this bacterium is the true producer of the deadly toxin. The causative agent for the lethal intoxications is bongkrekic acid (**1**); a polyunsaturated fatty acid substituted with three carboxylate moieties.[6] Typical symptoms, resulting from its oral uptake, are strong hyperglycemia followed by hypoglycemia and death.[7]



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Biochemical studies revealed that **1** efficiently inhibits oxidative phosphorylation in mitochondria through blockage of adenine nucleotide translocation.[8,9] Conditions for bacterial growth and toxin production have been the focus of various studies.[2,10,11] However, there has been no report on the biosynthesis of this severe toxin. Here we provide the first insights into the biogenetic origin of bongkrekic acid (**1**).

Results and discussion

The linear structure of **1** implies a biosynthetic pathway involving a polyketide synthase. Yet, due to the multiple carboxylate termini, the direction of chain assembly is enigmatic. We carried out isotope labelling studies to elucidate the biogenetic origins of **1** and to pinpoint its assembly mode. As a prerequisite, we reinvestigated the metabolic profile of *B. gladioli*. Since we were not able to produce significant amounts of **1** using published conditions,[11] we first optimised the fermentation conditions (supplemental material†). In short, **1** is only produced in detectable amounts when the media is supplemented with copious amounts of coconut fat, 2% glycerol, and amberlites XAD-16 in potato-dextrose broth. This prerequisite excluded the possibility to use minimal media for the feeding experiments.

Initially, we administered sodium [1-^{13}C]acetate, sodium [1,2-$^{13}C_2$]acetate, [1-^{13}C]propionate and [2-^{13}C]malonic acid to a growing *B. gladioli* culture in pulse- and bolus-feeding mode. Disappointingly, we could not detect any incorporation of the labelled building blocks by means of HPLC-MS and ^{13}C-NMR spectroscopy. However, supplementation of the culture with [methyl-^{13}C]methionine in both pulse- and bolus-feeding mode resulted in the formation of ^{13}C-enriched **1**, as evidenced by mass spectrometry. Next, labelled **1** was isolated from a scaled-up fermentation broth and purified using various chromatographic steps. Compared to the natural abundance spectrum, the ^{13}C NMR spectrum revealed significant signal enhancements for signals corresponding to positions C-6a, C-18a, and the methoxy group at C-17 (Fig. 1 and supplemental material†). Consequently, these carbons are derived from *S*-adenosyl-methionine (SAM) involving C- and O-methyl transfers, respectively.

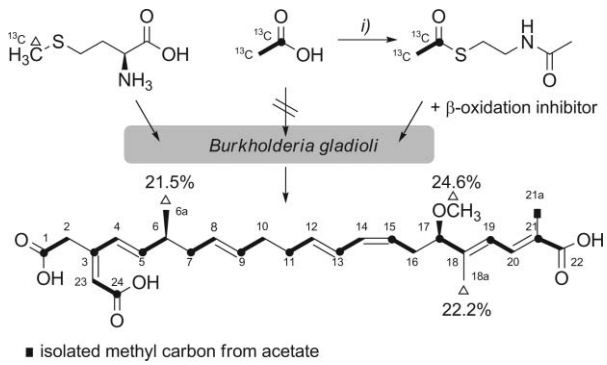

Fig. 1 ^{13}C Labelling deduced from feeding experiments with ^{13}C-Met and [1,2-$^{13}C_2$]acetyl SNAC. i) HSNAC, EDC, DMAP, DCM, 0 °C to r.t., 3 d, 68%.

The striking lack of labelled acetate and malonate incorporation may be rationalized by an impaired cellular uptake, as was observed previously.[12] Thus, to facilitate acetate delivery, we synthesized the corresponding *N*-acetylcysteamine (NAC)

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thioesters.[13] Both [1-^{13}C]acetyl and [1,2-$^{13}C_2$]acetyl SNAC were prepared using the DCC method,[14] and administered to the culture broth. However, no ^{13}C isotope enrichment of **1** could be observed. It appears that the lipids, which are essential for upregulating bongkrekic acid biosynthesis, represent the preferred carbon source and out-compete any alternative acetate building blocks. We thus aimed at limiting the lipid-derived acetyl CoA pool. This goal was achieved by adding an inhibitor of fatty acid β-oxidation, acrylic acid,[15] to the medium. According to MS analyses, this proved to be a viable approach to obtain detectable amounts of labelled **1**. Due to the very low incorporation, no labelling pattern for singly labelled acetyl-SNAC could be determined. Thus, we decided to employ doubly labelled [1,2-$^{13}C_2$]acetyl SNAC, since acetate units incorporated intact would give rise to satellite signals in the ^{13}C NMR spectrum.[16] Again, *B. gladioli* was supplemented with both a β-oxidation inhibitor and a labelled precursor, and the produced **1** was isolated and investigated by NMR. As expected, the incorporation was <2% (see supplemental material†). However, a large number of satellite signals could be observed allowing the detection of adjacent labelled carbon nuclei. Due to the usual dilution of ^{13}C labelled precursors, coupling to other vicinal ^{13}C carbons could be excluded.[17] Thus, the determination of the coupling constants permitted the identification of acetate units incorporated intact. For the isolated **1** we detected an all over labelling of the carbon backbone, which revealed that **1** is solely composed of acetate units (Fig. 1). However, it remained to be established, in which direction the chain grows and how the side chains are introduced.

As mentioned above, two alkyl branches (C-6a, C-18a) result from SAM-mediated methylation. Interestingly, according to the acetate ^{13}C-labelling experiment, C-21a is acetate-derived. Since this signal does not show any satellite signals, but is clearly enhanced in comparison to unlabelled **1**, we concluded that cleavage of the acetate has taken place. Furthermore, C-23 derived satellite signals provide clear evidence that the carboxymethine branch at C-3 results from the incorporation of an intact acetate unit. Only for C-24 no ^{13}C–^{13}C coupling satellites are visible due to the low signal intensity.

Taken together, there are two different biosynthetic modes for chain branching: C-6a and C-18a are SAM-derived methyl carbons, and thus represent α-methyl branches which result from methyl transfer onto the α-positions of former malonyl/acetyl units. In contrast, the C-23/24 and C-21a are not SAM-derived, but originate from acetate carbons. These acetate-derived side chains result from alkylation of a former β-position of an acetyl unit.[18–20] Two alternative routes have been described: a vinylogous, Michael-type addition of a malonyl unit,[21,22] and an aldol reaction of an acetyl unit with a β-ketoester, analogous to the mevalonate pathway. The formation of the C-21 β-branch may be installed in analogy to the bacillaene[23–25] and pseudomonic acid (mupirocin)[26] pathways (Fig. 2), which involves an HMG-CoA synthase(HCS)-catalysed aldol addition of a free-standing acetyl-ACP with the β-ketoacyl-ACP, followed by decarboxylation. On the other hand, the C-23/24 side chain of **1** is reminiscent of the two β-branches of the cytotoxic bryostatin. Haygood, Sherman, and coworkers suggest that the olefinic side chains of bryostatin emerge from the condensation of acetyl-CoA at the β-keto groups.[27] An analogous route would lead to the C-23/24 side chain of **1** in the bongkrekic acid pathway (Fig. 2).

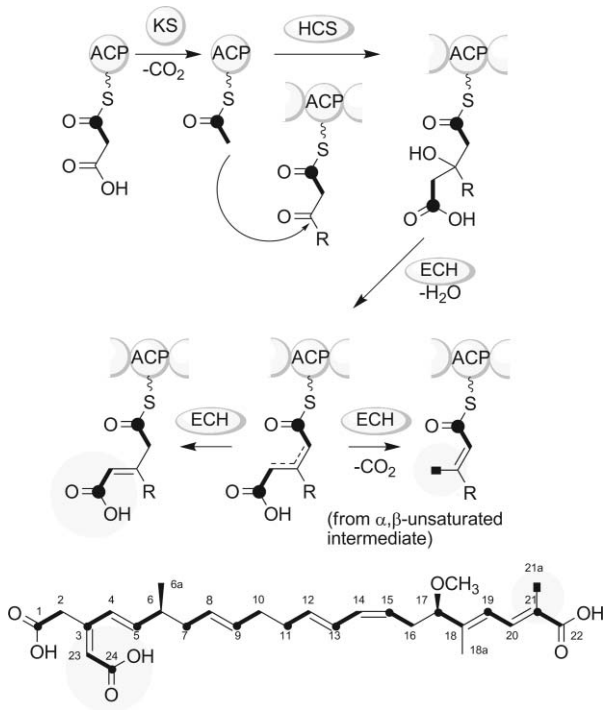

Fig. 2 Model for the incorporation of β-alkyl branches in bongkrekic acid; KS: ketosynthase, ACP: acyl carrier protein, ECH: enoyl-CoA hydratase/crotonase, HCS: HMG-CoA synthase.

Combining the results from the isotope labelling experiments allowed us to make various conclusions, and the data provide a clear picture of the building blocks and their assembly. First, it became evident that **1** is indeed a polyketide metabolite. Based on the positions and types of α- and β-branches, we could also establish the orientation of the former acetate units. Since C-6 and C-18 are former methylene carbons and C-17 represents a former carbonyl carbon, there is only room for this particular scenario. Accordingly, C-22 denotes the starting point of the carbon chain, because the reverse situation (elongation of the chain from C-1 or C-24) would result in the contrary positioning of each branch. Yet, a carboxyl group at the C-2 of the former starter acetyl unit is unusual. Obviously, this carbon has undergone complete oxygenation after chain assembly. Alternatively, it results from an oxidative cleavage of a larger precursor molecule, as has been proposed for pederin biosynthesis.[28] Ongoing studies at the molecular level will shed more light on the enzymatic assembly line involved in bongkrekic acid.

Finally, it should be noted that bongkrekic acid is a new member of the small family of polyketide metabolites from bacteria of the genus *Burkholderia*, such as thailandamide[25] and rhizoxin.[29] Interestingly, the latter is also produced by *Burkholderia* spp. growing in association with fungi, albeit in a persistent symbiosis.[29,30]

In summary, we have reevaluated bongkrekic acid production in *B. gladioli* to allow for the first insight into the biosynthesis of this infamous, food-related toxin. Only through inhibiting β-oxidation in *B. gladioli* and providing synthetic [1,2-$^{13}C_2$]acetyl SNAC were we able to succeed in stable isotope labelling of the carbon backbone of the molecule. Analysis of satellite signals revealed for the first time that **1** is a polyketide. Furthermore, through

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[methyl-^{13}C]methionine labelling and mechanistic considerations regarding α- and β-branching, we were able to deduce the direction of chain assembly. These data also led to the surprising conclusion that the C-22 carboxylate corresponds to a former methyl group of acetate. Our results not only highlight the biogenetic origin of an important food-borne toxin, but are also of importance in future molecular studies with the aim of monitoring potential toxin producers.

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