

The biosynthesis of bisorbicillinoids: evidence for a biosynthetic route from bisorbicillinol to bisorbibutenolide and bisorbicillinolide

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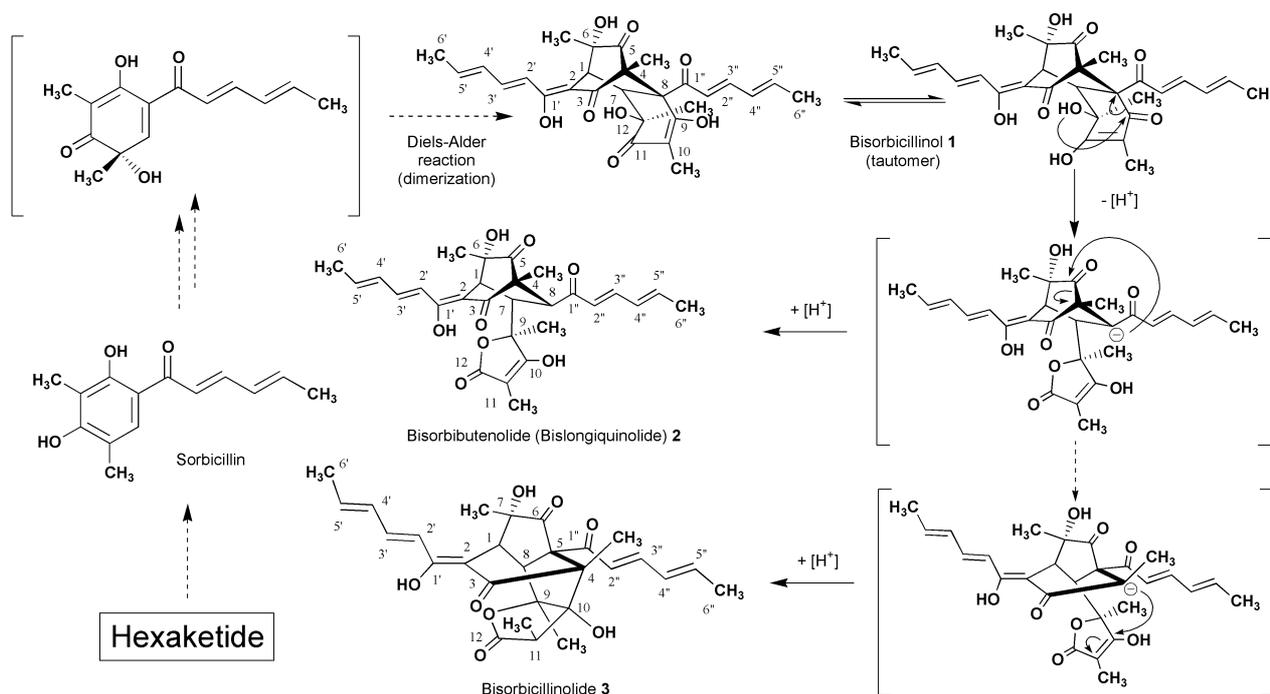
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Biosynthetic incorporation of labelled sodium acetates into bisorbicillinol in *Trichoderma* sp. USF-2690 suggests that bisorbicillinol is derived from 12 acetate units. Successful bioconversion of the labelled bisorbicillinols to bisorbibutenolides (bislongiquinolides) and bisorbicillinolides using the washed mycelium of the strain suggests that there are biosynthetic routes from bisorbicillinol to bisorbibutenolide and from bisorbicillinol to bisorbicillinolide.

The 'Bisorbicillinoids', comprised of dimeric sorbicillin-related natural products, have recently been described by Nicolaou *et al.*¹ Several compounds in the group, possessing complex structures, exhibit interesting biological activities, *e.g.*, inhibition of the production of TNF- α by macrophages and monocytes,² inhibition of β -1,6-glucan biosynthesis,³ and scavenging of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical.⁴⁻⁷ The biosynthesis of bisorbicillinoids is of interest to many investigators^{5,8-10} and, recently, biomimetic total synthesis has been investigated.¹¹⁻¹³ In particular, the biosynthetic pathway of bisorbibutenolide⁵ (bislongiquinolide, **3**)¹⁴ has been proposed to involve several distinct routes.^{5,10} We recently reported that *Trichoderma* sp. USF-2690 produced seven bisorbicillinoids, including bisorbicillinol **1**, bisorbibutenolide **2**, and bisorbicillinolide **3**,^{4,5} with presumed biogenetic routes from **1** to **2** and from **1** to **3** through a branch point as a common intermediate anion (Scheme 1).⁵ The routes were postulated based on the finding that ¹³C-labelled sodium acetate was incorporated into **1** via a polyketide pathway prior to incorpora-

tion into **2** and **3**. We wished to establish the biosynthetic relationship between **1**, **2**, and **3**, and report herein the result of a preliminary labelling experiment that provides evidence of the biosynthetic routes from **1** to **2** and from **1** to **3**.

Trichoderma sp. USF-2690 was first cultivated on a reciprocal shaker at 30 °C for 2 d.[†] The washed mycelium from the fermentation broth was then suspended in five 0.5-liter flasks each holding 150 mL of the medium (pH 7) containing 0.1% sodium [^{1-¹³C}]acetate or 0.1% sodium [^{2-¹³C}]acetate and 0.5% polypeptone, and again fermented with reciprocal shaking at 30 °C for 2 d. The filtered broth (1 L) was extracted with ethyl acetate (0.5 L \times 2) at pH 3. The organic extract, concentrated *in vacuo*, was applied to a Sephadex LH-20 column and eluted with methanol. The fraction including **1** was rechromatographed using medium-pressure liquid chromatography (MPLC) under the following conditions: support, YMC-ODS-AQ 120-S50; solvent, acetonitrile–H₂O (1:1, containing 0.1% trifluoroacetic acid); detection, UV at 370 nm. Two types of labelled bisorbicillinol **1** were obtained. The experiment using sodium [^{1-¹³C}]acetate gave **1** ([^{1-¹³C}]-**1**) with 10 ¹³C-enriched carbons (C-1, C-3, C-5, C-7, C-1', C-3', C-5', C-1'', C-3'', and C-5'') in the ¹³C-NMR spectrum. The expected incorporations at C-9 and C-11 were not observed. We previously reported that bisorbicillinol **1** had a keto–enol tautomerism between C-9 and C-11 in solution, and the ¹³C signals of C-9 and C-11 did not appear in the ¹³C-NMR spectrum.⁴ Therefore, the lack of peaks at C-9 and C-11 did not necessarily mean that the ¹³C atoms from sodium [^{1-¹³C}]acetate were not incorporated at C-9 and



Scheme 1 Biosynthetic pathway from bisorbicillinol **1** to bisorbibutenolide (bislongiquinolide **2**) and bisorbicillinolide **3**.

C-11. On the other hand, the feeding experiment using sodium [2-¹³C]acetate enhanced the peak strength of 12 carbons (C-2, C-4, C-6, C-8, C-10, C-12, C-2', C-4', C-6', C-2'', C-4'', and C-6'') in the ¹³C-NMR spectrum of **1** ([2-¹³C]-**1**). These results suggested that **1** was formed by the dimerization of six acetate units, which combined in the head-to-tail manner typical of fatty acids and polyketides, to give linkages between C-1 and C-7 and between C-4 and C-8 according to the Diels–Alder reaction.

The incorporation study employing each labelled bisorbicillinol **1** as a precursor of bioconversion was accomplished in the following manner. The fungus, inoculated in 0.5 L flasks containing 150 mL of the medium (pH 7) composed of 2.0% glucose and 0.5% polypeptone, was preincubated on a reciprocal shaker at 30 °C for 9 d. Mycelia were washed with sterilized water and then the washed mycelium was inoculated in 0.5 L flasks containing 150 mL of sterilized water with 10 mg of non-labelled and 5 mg of ¹³C-labelled bisorbicillinol **1**. The cultures for isolation of bisorbibutenolide **2** and bisorbicillinolide **3** were incubated for 72 and 10 h, respectively. The filtrate obtained from each broth was adjusted to pH 3 and extracted with ethyl acetate. LH-20 column chromatography and repetitive MPLC yielded **2** and **3**, respectively.

The ¹³C-NMR spectrum of bisorbibutenolide **2** obtained from the broth fed with [1-¹³C]-**1** had 12 ¹³C-enriched peaks at C-1, C-3, C-5, C-7, C-10, C-12, C-1', C-3', C-5', C-1'', C-3'', and C-5''. This definitive result established the existence of a biological synthetic route from **1** to **2**, as we had proposed previously.⁵ In addition, the enrichment of C-10 and C-12 of **2** meant that the two invisible carbons in the ¹³C-NMR spectrum of **1** were enriched by sodium [1-¹³C]acetate. Furthermore, the consecutive feeding study of [2-¹³C]-**1** confirmed the validity of bioconversion from **1** to **2** in the fungal strain. This finding was consistent with the expectation based on a typical polyketide pathway that the ¹³C-enrichments at C-2, C-4, C-6, C-8, C-9, C-11, C-2', C-4', C-6', C-2'', C-4'', and C-6'' were observed in the ¹³C-NMR spectrum of **2**.

Proof of biosynthesis of bisorbicillinolide **3** via bisorbicillinol **1** as a precursor, was then performed in the same manner. The bisorbicillinolide **3** obtained from the broth fed with [1-¹³C]-**1** gave the characteristic ¹³C-NMR spectrum of **3**, which had 12 peaks enhanced at C-1, C-3, C-6, C-8, C-10, C-12, C-1', C-3', C-5', C-1'', C-3'', and C-5''. The continued experiment using [2-¹³C]-**1** led to the expected **3**, into which excess ¹³C-atoms were incorporated at C-2, C-4, C-5, C-7, C-9, C-11, C-2', C-4', C-6', C-2'', C-4'', and C-6''. The results of a feeding study using [1-¹³C]-**1** and [2-¹³C]-**1** ascertained that bisorbicillinolide **3** was biosynthesized from bisorbicillinol **1** as a precursor.

To examine the possibility that the biosynthesis from bisorbicillinol **1** to bisorbibutenolide **2** occurred via bisorbicillinolide **3**, or from **1** to **3** via **2**, time course studies on bioconversion of products in the washed mycelium were performed using high performance liquid chromatography

analysis. Non-labeled bisorbibutenolide **2** or bisorbicillinolide **3** was added to the washed mycelium prepared above, and the culture broth was incubated at 30 °C for 72 h. No other products were detected in either experiment.

These observations from the ¹³C-feeding studies indicate that there are biosynthetic routes from **1** to **2** and from **1** to **3** in *Trichoderma* sp. USF-2690. The supporting experiments might rule out the possibility of biosynthesis from **1** to **2** via **3** or from **1** to **3** via **2** and the reverse biosynthesis from **2** or **3** to **1**. These results suggest the presence of the first anion as a branch point and the second anion following the C–C bond cleavage. Further incorporation studies will clarify the detailed biosynthetic mechanisms through the two precursor anions.

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Notes and references

† The fermentation broth was pooled from ten 0.5 L flasks each containing 150 mL of the following medium: 2.0% glucose, 0.05% polypeptone, 0.2% yeast extract, 0.1% KH₂PO₄, 0.1% MgSO₄·7H₂O, and 0.1% trace salt mixture at pH 7. The filtered mycelial cake was washed with 2 L of sterilized water.

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