Biosynthesis of the 2*H*-Azepine Alkaloid Chalciporone

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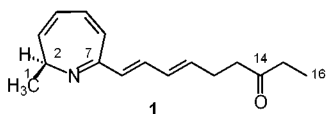
The unusual 2-*H*-azepine alkaloid chalciporone (**1**),[1] is found in the mushroom *Chalciporus piperatus* (Basidiomycetes). The pungent effect of this compound seems to protect the fruit bodies since they are rarely attacked by insects and other predators. Synthetic studies by our group established the (2*S*)-stereochemistry of **1**.[2]



Because of the unique structure of this natural product we became interested in its biosynthesis. Here we present the results of a range of experiments that now allow us to postulate a biosynthetic route for this alkaloid. It appeared reasonable to speculate that **1** could be formed by cyclization of a linear polyketide chain. For this reason, young fruit bodies of *Ch. piperatus*, while growing in their natural forest habitat, were fed with sodium [U-$^{13}C_2$]acetate.[3] These experiments were not successful, presumably due to the basicity of the precursor not being tolerated by the mushrooms. However, we were pleased to find that when a mixture of [U-^{13}C]-labeled fats[4] was applied it was readily accepted by the fungi. The experiment revealed the degradation of the fatty acids to doubly labeled acetate and the incorporation of seven acetate units into chalciporone,[5] leading to ^{13}C-enrichment of carbons 3−16 (see Table 2 and Scheme 1).

In contrast, the methyl group and the neighboring ring carbon atom C-2 exhibited no detectable ^{13}C-enrichment. The enrichment was about 10% and was derived from the ^{13}C NMR spectrum by integration of the doublet signals of labeled in relation to the integral of the corresponding singlet signal of the unlabeled alkaloid **1** (Table 2).

Concerning the origin of C-1 and C-2 we speculated that these could be derived from an α-amino acid since the H_3C-CH- unit is attached to the nitrogen atom in **1**. Indeed, when a mixture of [U-^{13}C]-L-amino acids[4] was injected into the fruit bodies, partial ^{13}C-labeling of C-1 and C-2 was observed. Both signals were split into doublets indicating their origin from one and the same amino acid. Not surprisingly, doublet signals were also detectable for all the other carbon nuclei. This is explained by degradation of the [U-^{13}C]-labeled amino acids to doubly labeled acetate, which is then incorporated into **1**. The incorporation of seven intact acetate units and the carbons of the CH_3CH- moiety was proven by an INADEQUATE experiment (Figure 1). ^{13}C-enrichments were found to be about 50% (Table 2). $^{1}J_{C,C}$ coupling constants derived from the ^{13}C NMR spectrum are listed in Table 1.

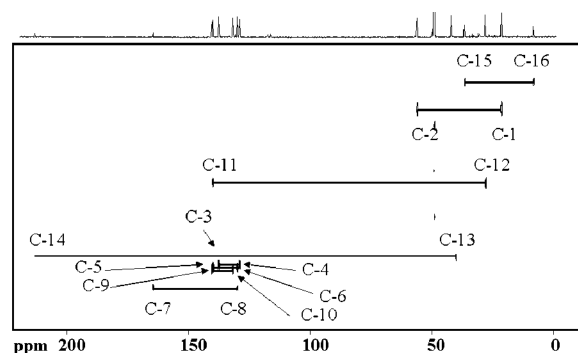


Figure 1. INADEQUATE experiment (CD_3OD, 151 MHz); labeling pattern of chalciporone (**1**) (20 mg) after applying a mixture of [U-^{13}C]-L-amino acids to young fruit bodies of *Ch. piperatus.*

Scheme 1. Incorporation of Seven Acetate Units into Chalciporone

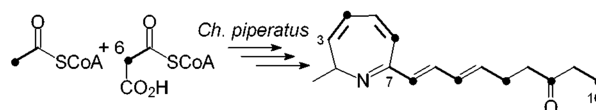


Table 1. Chemical Shifts δ_C (Assigned Due to COSY, HMQC, and HMBC Experiments) and Coupling Constants $^{1}J_{C,C}$ of **1** after Feeding *Ch. piperatus* with a Mixture of [U-^{13}C]-L-Amino Acids (151 MHz, CD_3OD)

C-atom	δ_C [ppm]	$^{1}J_{C,C}$ [Hz]	C-atom	δ_C [ppm]	$^{1}J_{C,C}$ [Hz]
1	21.37	41.4	9	141.62	55.6
2	56.00	41.4	10	131.64	55.6
3	137.45	65.2	11	140.95	43.2
4	128.80	65.2	12	28.03	43.2
5	139.78	64.2	13	41.97	38.9
6	129.80	64.2	14	213.02	38.9
7	164.59	62.2	15	36.58	35.7
8	129.88	62.2	16	8.02	35.7

Further feeding experiments were aimed to identify the amino acid being specifically incorporated into **1**. Providing the mushrooms with [U-$^{13}C_3$]-L-alanine as a potential biosynthetic precursor led to an interesting result: a significantly increased ^{13}C-enrichment was observed for C-1 and C-2 (>100%) compared to all other carbon atoms (~50%) (Table 2). This is consistent with alanine being the precursor of C-1 and C-2 in chalciporone and indicates that the carboxyl group of alanine is lost in the course of the biosynthesis.[6] Unfortunately, a feeding experiment with [U-^{13}C,^{15}N]-L-alanine aimed to prove the retention of the $^{13}C-^{15}N$ bond was inconclusive due to low incorporation.

The experiments described so far do not allow to distinguish whether the polyketide chain is generated starting from C-3 or

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(2) Hamprecht, D.; Josten J.; Steglich W. *Tetrahedron* **1996**, *52*, 10883−10902.

(3) Typically, 5−10 mg of the precursor in 50 μL of water (or diethyl ether in the feeding experiment with fats) were injected into each stalk of 20 fruit bodies in a forest near Regensburg, Bavaria. After 7 days, the mushrooms were harvested and kept frozen at −20 °C until workup.

(4) The mixtures of [U-^{13}C]-labeled fats and [U-^{13}C]-labeled amino acids (99% ^{13}C) were obtained from algae with $^{13}CO_2$ as the only carbon source. They are C,C-bond-labeled throughout and are commercially available from Cambridge Isotope Laboratories, Inc., Andover, MA.

(5) Isolation of chalciporone (**1**): Frozen fruit bodies of *Ch. piperatus* (50 g) were crushed and immediately extracted for 30 min with ethyl acetate. The extract was dried (Na_2SO_4) and the solvent removed carefully. The residue was dissolved in MeOH (3 mL) and purified by elution with MeOH through a RP-18 cartridge. Yield: 21 mg. Since **1** rearranges readily to isochalciporone[1] and decomposes on silica gel, further purification is not recommended. ^{1}H NMR (600 MHz, MeOD): δ = 1.02 (t, J = 7.3 Hz, 3 H, H-16), 1.58 (d, J = 6.7 Hz, 3 H, H-1), 2.40 (td, J = 7.2 Hz, J = 6.9 Hz, 2 H, H-12), 2.48 (q, J = 7.3 Hz, 2 H, 15-H), 2.60 (t, J = 7.2 Hz, 2 H, H-13), 2.97 (dq, J = 6.7 Hz, J = 5.3 Hz, 1 H, H-2), 5.72 (dd, J = 9.1 Hz, J = 5.3 Hz, 1 H, H-3), 6.00 (dt, J = 14.8 Hz, J = 6.9 Hz, 1 H, H-11), 6.22 (d, J = 15.8, H-8), 6.23 (dd, J = 14.8, J = 10.5 Hz, H-10), 6.34 (dd, J = 9.1 Hz, J = 5.4 Hz, H-4), 6.81 (dd, J = 15.8, J = 10.5 Hz, 1 H, H-9), 6.99 (dd, J = 11.5 Hz, J = 5.4 Hz, H-5), 7.24 (d, J = 11.5 Hz, 1 H, H-6).

(6) Since [U-$^{13}C_3$]-glycerol is readily metabolized to L-alanine via pyruvate, similar ^{13}C-enrichments were observed by feeding [U-^{13}C]-glycerol to the mushroom (^{13}C-enrichments: ~40% for C-1 and C-2 and ~20% for the other carbons).

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Table 2. ^{13}C-Enrichment in Chalciporone after Feeding *Ch. piperatus* with Labeled Precursors[a] (151 MHz, CD_3OD)

	^{13}C-enrichment [%]					^{13}C-enrichment [%]			
C	FA	AA	uAla	3Ala	C	FA	AA	uAla	3Ala
1	0	43	166	210	9	9	42	52	0
2	0	34	134	0	10	7	46	45	120
3	8	45	48	0	11	10	54	37	0
4	9	46	47	160	12	9	51	65	150
5	9	34	45	0	13	9	51	47	0
6	5	47	49	120	14	8	28	39	100
7	7	27	24	0	15	9	51	60	0
8	8	55	49	160	16	12	52	66	170

[a] FA = feeding experiment with [U-^{13}C]-fats. AA = feeding with [U-^{13}C]-L-amino acids; uAla = feeding with [U-$^{13}C_3$]-L-alanine; 3Ala = feeding with [3-^{13}C]-L-alanine.

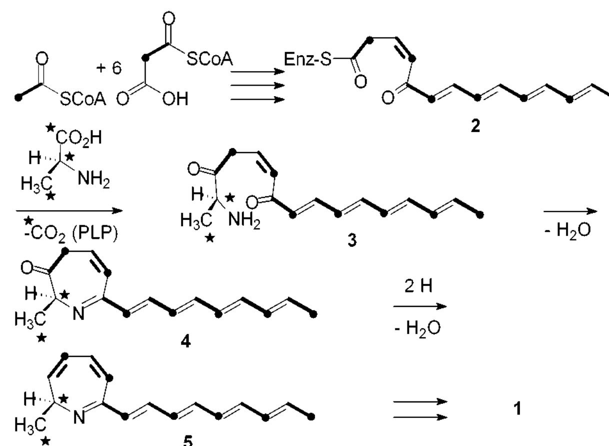
C-16. To resolve this issue, singly ^{13}C-labeled acetate precursors are required. Initial feeding experiments with [2-^{13}C]acetate or [1-^{13}C]ethanol failed, despite the ethanol being tolerated better by the mushroom than the acetate. Better results were obtained with [3-^{13}C]-L-alanine as precursor of acetate. To ensure sufficient enrichment of the labeled chalciporone, the inoculated fruit bodies were harvested after an unusually long growth period of 12 days. Quantitative ^{13}C NMR measurements revealed ^{13}C-enrichment of the carbons at positions 1, 4, 6, 8, 10, 12, 14, and 16 (Table 2, Scheme 1), indicating that assembly of the carbon chain commences at C-16.

These results are in accordance with the formation of the complete carbon chain of **1** by condensation of L-alanine with a heptaketide CoA thioester **2**[7] and concomitant decarboxylation (Scheme 2). The resulting aminoketone **3** is formed with overall retention of the absolute configuration. The same applies to reactions catalyzed by enzymes of the α-oxoamine synthase family,[8] which depend on pyridoxal phosphate (PLP) as cofactor. Thus, a reaction mechanism analogous to that proposed[9] for those conversions can be assumed for the transformation of L-alanine into aminoketone **3**.

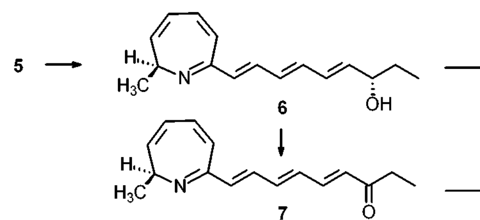
The next steps in chalciporone biosynthesis should be ring closure to **4**, reduction of the C=O-group in position 3, and elimination of water leading to 2*H*-azepine **5** (Scheme 2).[11] The side chain C=O-group is very likely introduced at the end of the biosynthesis, since compounds **5**,[12] **6**, and **7** can be detected in traces in the mushroom.[13] A proposal for their formation is depicted in Scheme 3.

Interestingly, conjugates of polyenedicarboxylic acids with amino acids have been found as pigments in *Boletus laetissimus*, a close relative of *Ch. piperatus*.[14]

Scheme 2. Hypothetical Biosynthesis of Chalciporone[10]



Scheme 3. Proposal for the Late Steps in Chalciporone Biosynthesis



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(7) The involvement of a CoA ester is proposed in analogy to other α-oxoamine synthase reactions. The functionalization of the polyketide chain is arbitrary.

(8) (a) 8-Amino-7-oxononanoate synthase (AONS): Webster, S. P.; Alexeev, D.; Campopiano, D. J.; Watt, R. M.; Alexeeva, M.; Sawyer, L.; Baxter, R. L. *Biochemistry* **2000**, *39*, 516−528. (b) 5-Aminolevulinate synthase (ALAS): Jordan, P. M. In *Biosynthesis of Tetrapyrroles*; Jordan, P. M., Ed.; Elsevier: Amsterdam, 1991; pp 1−66. (c) Serine palmitoyltransferase (SPT): Merrill, A. H., Jr.; Jones, D. D. *Biochim. Biophys. Acta* **1990**, *1044*, 1−12. (d) 2-Amino-3-ketobutyrate CoA ligase (AKB): Mukherjee, J. J.; Dekker, E. E. *J. Biol. Chem.* **1987**, *262*, 14441−14447 and references cited therein.

(9) (a) Ploux, O.; Marquet, A. *Eur. J. Biochem.* **1996**, *236*, 301−308. (b) Alexeev, D.; Alexeeva, M.; Baxter, R. L.; Campopiano, D. J.; Webster, S. P.; Sawyer, L. *J. Mol. Biol.* **1998**, *284*, 401−419. (c) Ploux, O.; Breyne, O.; Carillon, S.; Marquet, A. *Eur. J. Biochem.* **1999**, *259*, 63−70. (d) Ifuku, O.; Miyaoka, H.; Koga, N.; Kishimoto, J.; Haze, S.; Wachi, Y.; Kajiwara, M. *Eur. J. Biochem.* **1994**, *220*, 585−591.

(10) The exact structures of the intermediates and the sequence of the late biosynthetic steps are unknown.

(11) The formation of pyrrole rings from α-amino acids and 3-oxocarboxylic acid CoA thioesters has been observed in the biosynthesis of prodiginines: (a) Gerber, N. N.; McInnes, A. G.; Smith, D. G.; Walter, J. A.; Wright, J. L. C.; Vining, L. C. *Can J. Chem.* **1978**, *56*, 1155−1163. (b) Wasserman, H. H.; Sykes, R. J.; Peverada, P.; Shaw, C. K.; Cushley, R. J.; Lipsky, S. R. *J. Am. Chem. Soc.* **1973**, *95*, 6874−6875.

(12) **5** was only identified by GC−MS.

(13) Hamprecht, D., Ph.D. Thesis, University of München, 1996.

(14) Kahner, L.; Dasenbrock, J.; Spiteller, P.; Steglich, W.; Marumoto, R.; Spiteller, M. *Phytochemistry* **1998**, *49*, 1693−1697.