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Labelling studies on the biosynthesis of terpenes in *Fusarium fujikuroi*†

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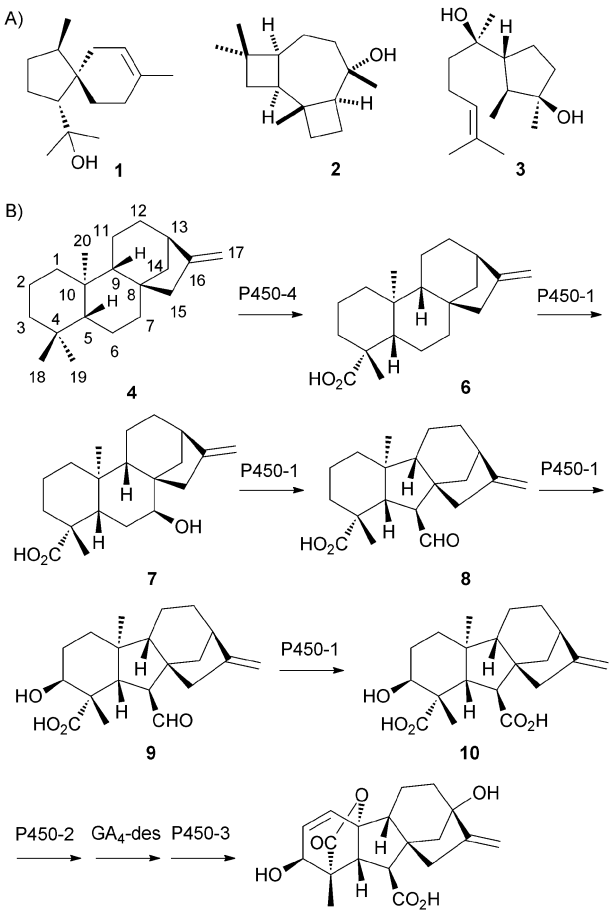
Christian A. Citron,[a] Nelson L. Brock,[a] Bettina Tudzynski[b] and Jeroen S. Dickschat*[a]

Synthetic [2-^{13}C]mevalonolactone was fed to the gibberellin producer *Fusarium fujikuroi* and its incorporation into four known terpenoids was investigated by ^{13}C NMR analysis of crude culture extracts. The experiments gave detailed insights into the mechanisms of terpene biosynthesis by this fungus.

Fusarium fujikuroi is a known producer of the sesquiterpenoids α-acorenol **1**,[1] koraiol **2**,[2] and cyclonerodiol **3**,[3] and of gibberellins,[4] a class of highly oxidised nor-diterpenoids (C_{19}, Scheme 1). Gibberellins are produced by plants, fungi, and bacteria, and exhibit significant biological activity as plant growth hormones, while the function of the sesquiterpenoids is unknown. The complex gibberellin biosynthesis starts with the cyclisation of geranylgeranyl diphosphate to *ent*-kaurene **4** (Scheme S2 of ESI†), followed by a series of oxidation steps with contraction of the B ring and loss of the C-20 methyl group to give gibberellic acid GA_3 **5**.[5] While the pathways in plants and fungi proceed *via* different intermediates,[4] bacterial gibberellin biosynthesis is unexplored.

The plant pathogenic fungus *F. fujikuroi* produces large amounts of **5** that causes the bakanae or foolish seedling disease in infected rice plants. Conversion of the terpene hydrocarbon **4** into **5** involves the activity of four monooxygenases (P450-1, P450-2, P450-3, and P450-4) and a desaturase (Scheme 1).[6] The first enzyme, P450-4, catalyses the oxidation of **4** *via ent*-kaurenal to *ent*-kaurenoic acid **6**, followed by a series of four oxidations by P450-1, starting with hydroxylation of the B ring at C-7 to *ent*-7α-hydroxykaurenoic acid **7** that is transformed into GA_{12}-aldehyde **8** by ring contraction.[7] Another hydroxylation in the A ring (C-3) to GA_{14}-aldehyde **9** and oxidation of the aldehyde moiety yield GA_{14} **10**. Consecutive oxidative conversions of **10** by P450-2, the GA_4 desaturase, and P450-3 eventually result in **5**.[8–10]



Scheme 1 (A) Structures of sesquiterpenoids from *Fusarium fujikuroi*; (B) structures of diterpenoids **4** and **5** from *F. fujikuroi* and biosynthetic steps from **4** towards **5**.

The ring contraction from **7** to **8** was investigated using semi-synthetic stereospecifically deuterated isotopologues of **6** carrying deuterium labels in the α- or β-positions of C-6 or C-7.[11] Incubation with cell free extracts of *F. fujikuroi* mutants demonstrated that the oxidative ring contraction to **8** proceeds with stereospecific loss of the 6β- and 7β-hydrogens, whereas the 6α- and 7α-hydrogens were retained,

[a] *Institut für Organische Chemie, Hagenring 30, 38106 Braunschweig, Germany. E-mail: j.dickschat@tu-braunschweig.de; Fax: +49 531 3915272; Tel: +49 531 3915264*

[b] *Institut für Biologie und Biotechnologie der Pflanzen, Schlossplatz 8, 48143 Münster, Germany*

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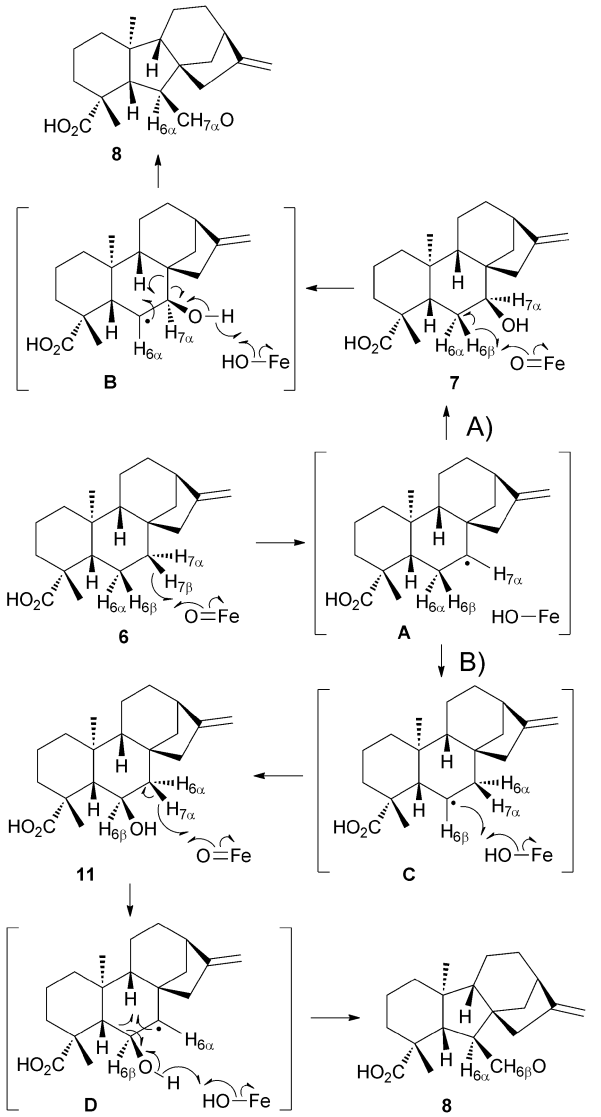
as determined by GC-MS analysis of the corresponding methyl ester. GC-MS analysis also revealed that only the 6α-hydrogen is retained in **5**. These observations are in accord with a ring contraction mechanism for the formation of **8** *via* radical intermediates as shown in Scheme 2, pathway A.[12] A problem of these experiments is the partial loss also of deuterium labelling from 6α-H that is mechanistically not explained, but may be a result of the C,H-acidity of **8** and the downstream metabolites. The mechanism is in agreement with feeding experiments by Birch and coworkers using ^{14}C-labelled precursors in which the site of incorporation was followed by degradation of the carbon backbone of **5**.[13] On the other hand, the mechanism contradicts a previous report showing that both C-6 hydrogens, if labelled with tritium, are retained in the conversion of **6** to **8**, one of which is lost in the chemical oxidation of the aldehyde function.[14]

These observations are only explainable *via* a mechanism that (a) does not involve oxidative hydrogen removal from C-6 and (b) includes a rearrangement of one of the two hydrogens at C-6 by a shift to C-7. A logical consequence of such a mechanism is the initial formation of the intermediate alcohol **11** that may undergo a similar ring contraction to **8** as suggested in Scheme 2, pathway A, albeit with extrusion of C-6 instead of C-7 (Scheme 2, pathway B).

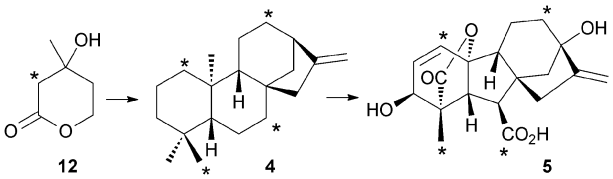
The critical point to distinguish between the two mechanisms is the extrusion of C-6 or C-7 in the ring contraction step. This question was addressed by Birch in a difficult experiment by chemical degradation of **5** after incorporation of radioactive labelling from acetate and mevalonolactone.[13] The experiment was performed with lack of knowledge about the structure of **5** that may have engendered misinterpretations. Since the results from other labelling studies are contradictory,[11,14] we decided to repeat Birch's experiment using modern NMR analytical techniques.

For this purpose racemic [2-^{13}C]-mevalonolactone **12** was synthesised using a modification of a previously reported route (ESI†).[15–17] The synthetic [2-^{13}C]-**12** was fed to the fungus *F. fujikuroi* and its incorporation into C-7 of **4**, further converted into **5** (Scheme 3), was followed by ^{13}C NMR analysis of crude culture extracts and comparison with an authentic sample of **5** and ^{13}C NMR literature data.[18,19] The analysis revealed four carbon signals in the NMR spectrum (Table 1, Fig. 1B) matching shifts of the standard (Fig. 1A). To further validate these data the sample was mixed with **5** followed by ^{13}C NMR spectroscopy (Fig. S3 and S4 of ESI†). The results unequivocally established the extrusion of C-7 in the ring contraction step of GA biosynthesis, in full agreement with Birch's earlier report[13] and the mechanism as shown in Scheme 2, pathway A, while the apparent observation by Hanson and coworkers[14] is hard to explain. A source of error in the respective study may be the mentioned extremely low incorporation rate. Alternative mechanisms to pathway A in Scheme 2, *e.g. via* oxidation of radical intermediate **B** to a cation and subsequent pinacol-type rearrangement, or formation of a diol followed by extrusion of a hydroxide to generate such a putative cationic intermediate, cannot be excluded based on this or any of the previous labelling studies.

Subsequently, the incorporation of [2-^{13}C]-**12** into the sesquiterpenes **1**, **2**, and **3** was investigated. The volatiles **1** and **2** were trapped on charcoal filters[20] from agar plate cultures of two overexpression mutants (ESI†),[2] followed by extraction with $CDCl_3$ and direct ^{13}C NMR analysis of the crude headspace extracts.[21] For **1** strongly elevated ^{13}C signals were detected at 30.2, 27.94, and 27.89 ppm,



Scheme 2 Suggested mechanisms for the ring contraction in agreement with the observations by Birch *et al.*[13] and Castellaro *et al.*[11] (pathway A), and by Hanson *et al.*[14] (pathway B). Reactive intermediates are shown in brackets.



Scheme 3 Labelling in **5** after feeding of [2-^{13}C]-**12**. Details of the conversion of **12** to **4** can be followed in Scheme S2 of ESI.†

indicating the expected incorporation into the assigned methylene groups (Scheme 4 and Fig. S5–S7 of ESI†). Due to the conformational flexibility incorporation into one of the stereochemically distinct geminal methyl groups could not be distinguished. A microreaction with the headspace extract was carried out that chemically converted **1** into the conformationally fixed α-cedrene **14** by treatment with 88% formic acid for 10 min at room temperature.[22] Analysis by ^{13}C NMR revealed enhanced signals at 119.2, 36.1, and 27.6 ppm (Scheme 4 and

Table 1 Comparison of ^{13}C-NMR data of authentic **5** to ^{13}C-labelled **5** obtained after feeding of [2-^{13}C]-**12** to *F. fujikuroi*

Carbon no.[a]	δ [b][ppm]	Carbon no.[a]	δ [b][ppm]
C-19	178.1	C-9	52.1
C-7	**173.2**	C-5	50.8
C-16	157.8	C-6	50.5
C-2	133.3	C-8	49.4
C-1	**131.5**	C-14	44.3
C-17	106.3	C-15	42.7
C-10	90.5	C-12	**38.8**
C-13	76.6	C-11	16.6
C-3	68.4	C-18	**14.5**
C-4	53.1		

[a] Carbon numbering according to Scheme 1. [b] ^{13}C NMR shifts of **5** are referenced to [$^{2}H_6$]DMSO. ^{13}C-NMR signals of labelled carbons in the feeding experiment with [2-^{13}C]-**12** are given in bold.

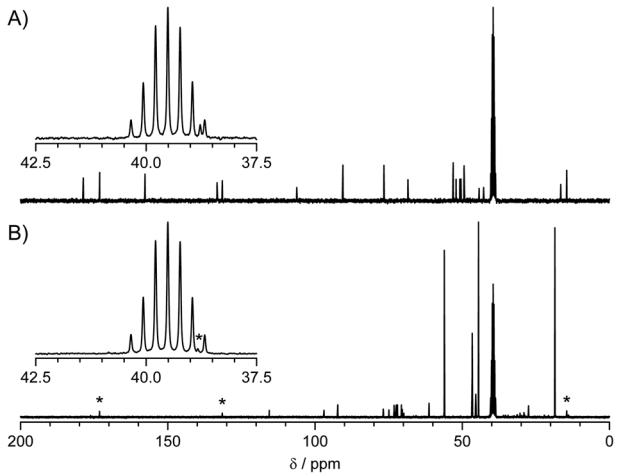
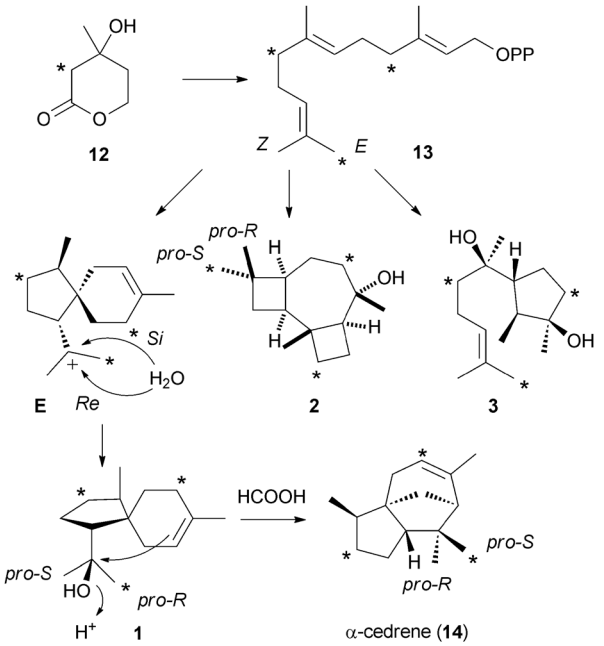


Fig. 1 (A) ^{13}C NMR spectrum of **5** in [$^{2}H_6$]DMSO; (B) ^{13}C NMR spectrum of a crude culture extract from *F. fujikuroi* after feeding of [2-^{13}C]-**12**. Asterisks indicate carbon signals originating from **5** after incorporation of [2-^{13}C]-**12**.

Fig. S8–S10 of ESI†). The signal at 27.6 ppm is due to incorporation into the geminal *pro-S* methyl group of **14**, whereas only low incorporation (*ca.* 10% by ^{13}C NMR peak integration) into the adjacent methyl group was detected (25.6 ppm).[23] This defined stereochemical course is in strong favour of a concerted S_N2 type reaction with major inversion of configuration, which in turn demonstrates incorporation of labelling mainly into the *pro-R* methyl group of **1**. This experiment also shows that attack of the acorenyl cation **E** by water must have occurred from the *Re* face of the molecule (*Re*/*Si* distinguished due to labelling, Scheme 4). In contrast, an alternative stereochemical course with elimination of water from **1** to generate a cation that is intramolecularly reattacked by the olefinic double bond from the same side, *i.e.* with retention of configuration, is difficult to understand.

For **2** the obtained spectrum showed enhanced signals at 46.3, 37.6, and 31.0 ppm (Scheme 4 and Fig. S11–S13 of ESI†). This, together with published NMR data of **2**,[2] allowed for conclusions on the stereochemical course of the FPP cyclisation that proceeds with conversion of the terminal *E* methyl group of FPP into the *pro-S* of the two geminal methyl groups in **2**. Finally, incorporation of labelling from [2-^{13}C]-**12** into **3** was observed by enhanced ^{13}C signals at 40.39, 40.36, and 25.7 ppm (Scheme 4 and Fig. S14 and S15 of ESI†), in agreement with Cane's earlier report.[3]



Scheme 4 Detected ^{13}C labelling patterns in sesquiterpenes from *Fusarium fujikuroi* after feeding of [2-^{13}C]-**12**. Asterisks indicate ^{13}C-labelled carbons.

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