Terpene Biosynthesis

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Identification of Intermediates in the Biosynthesis of PR Toxin by *Penicillium roqueforti*

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Abstract: *The sesquiterpenoid 7-epi-neopetasone was synthesized via the Wieland–Miescher ketone. The compound was identical to a previously tentatively identified headspace constituent of Penicillium roqueforti. Feeding experiments with ^{13}C-labeled mevalonolactone isotopomers demonstrated that oxidation at C12 and an isomerization of the C11=C12 to a C7=C11 double bond must occur independently and not via a C7-C11-C12 allyl radical in one step. Feeding with (11,12,13-$^{13}C_3$)-7-epi-neopetasone resulted in labelling of the PR toxin, thus establishing this compound as a newly identified pathway intermediate.*

The ascomycete *Penicillium roqueforti* can be isolated from soil, plants, or decomposing organic matter and is traditionally applied in the production of Roquefort and other blue cheese. Its spectrum of secondary metabolites not only includes flavor compounds, but also highly bioactive compounds such as the clinically used immunosuppressant mycophenolic acid (**1**, Figure 1),[1] the neurotoxin roquefortin C (**2**),[2,3] and the sesquiterpenoid mycotoxin PR toxin (**3**). PR toxin inhibits critical cellular processes including transcription[4] and protein biosynthesis[5] and it exhibits a lethal dose (LD_{50}) of approximately 5 mg kg^{-1} in mice.[6] The low concentrations of bioactive secondary metabolites in molded cheese may also explain the "French paradox", i.e. the low rates of cardiovascular mortality in the French population despite comparably high consumption of saturated fats and alcohol.[7] After the structure elucidation of **3** in 1975 by Strong and co-workers,[8] the related eremofortins B (**4**), A (**5**), and C (**6**), and the oxygenated eremophilanes **7**–**9** were discovered.[9–12]

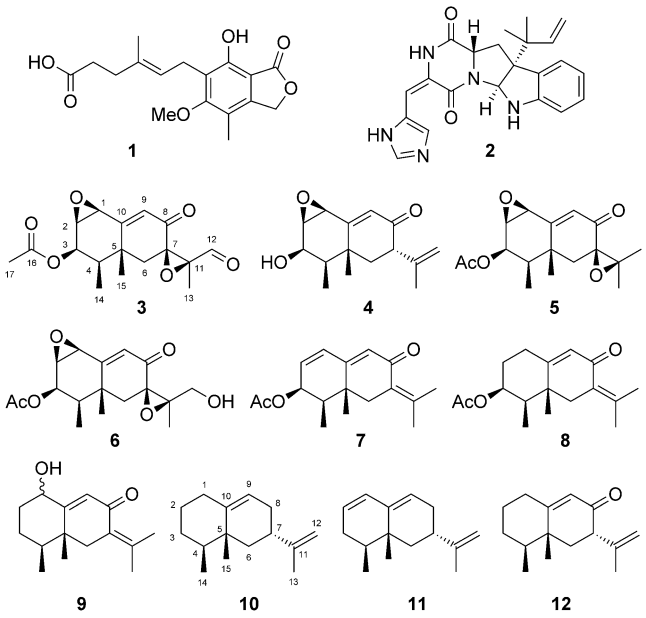
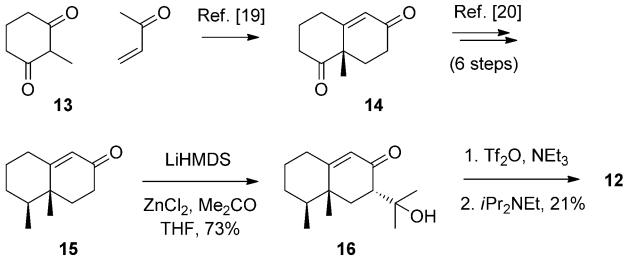


Figure 1. Secondary metabolites from *Penicillium roqueforti*.

The biosynthesis of **3** has not been investigated in detail. The first committed step is the conversion of farnesyl diphosphate (FPP) into the parent hydrocarbon aristolochene (**10**) by the aristolochene synthase.[13,14] Although partial information about the biosynthetic gene cluster has recently been obtained,[15] the nature of most pathway intermediates and the precise functions of the genes and enzymes in their interconversion is still unclear. The eremofortins A–C and **7** were suggested to be pathway intermediates,[12,16,17] but some of these metabolites could also be shunt products. We have recently reported the detection of nootkatene (**11**) and 7-*epi*-neopetasone (**12**) in headspace extracts from *P. roqueforti*.[18] While **11** was identified from its EI mass spectrum and retention index, both of which matched literature data, **12** was only tentatively identified. Herein, we present the unambiguous identification of **12** by total synthesis, as well as data from feeding experiments with isotopically labeled precursors that give insight into the biosynthetic pathway of **3**.

The synthesis of **12** was started from 2-methylcyclohexan-1,3-dione (**13**), which was converted via the Wieland–Miescher ketone (+)-**14** into the octalone **15** through known procedures (Scheme 1).[19,20] Similar to the reported procedure for the synthesis of petasin,[21] **15** was subsequently used in an aldol addition to acetone to yield ligudicin C (**16**), a known natural product from *Ligularia dictyoneura*.[22] Treatment with triflic anhydride in NEt_3 was followed by



Scheme 1. Synthesis of 7-*epi*-neopetasone (**12**).

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elimination to the Hoffmann product **12** by using Hünig's base. The synthetic 7-*epi*-neopetasone was identical in its EI mass spectrum and retention index to the volatile constituent in the *P. roqueforti* headspace extracts (Figure S1 in the Supporting Information). Compound **12** proved to be highly instable in terms of isomerization of the newly installed olefinic double bond into the conjugated α,β-position. This isomerization was even observable after storage for one week at −80 °C.

For unambiguous biosynthetic studies, the ^{1}H- and ^{13}C NMR spectroscopic data of **3** were reassigned through the application of two-dimensional NMR techniques including ^{1}H,^{1}H-COSY, HSQC, HMBC, and NOESY (Table 1). The obtained data cleared some wrongly assigned chemical shifts in the structure elucidation of **3** (confusion of C13 with C15)[8] and were in agreement with two later reports.[11,23]

Table 1: Reassigned ^{1}H- and ^{13}C NMR data of **3** in $CDCl_3$.

C[a]	^{1}H (δ, m, J)[b]	^{13}C (δ)[c]
1	3.62 (d, ^{3}J = 3.5, 1 H)	55.9 (CH)
2	3.92 (dd, ^{3}J = 4.9, ^{3}J = 3.6, 1 H)	55.5 (CH)
3	5.11 (dd, ^{3}J = 5.1, ^{3}J = 5.1, 1 H)	69.9 (CH)
4	1.77 (dq, ^{3}J = 5.4, ^{3}J = 6.9, 1 H)	42.7 (CH)
5	–	38.1 (C_q)
6a	1.80 (d, ^{2}J = 14.5, 1 H)	41.5 (CH_2)
6b	2.13 (d, ^{2}J = 14.5, 1 H)	
7	–	67.3 (C_q)
8	–	191.7 (C_q)
9	6.38 (s, 1 H)	129.9 (CH)
10	–	164.7 (C_q)
11	–	67.4 (C_q)
12	9.66 (s, 1 H)	198.6 (CH)
13	1.45 (s, 3 H)	13.6 (CH_3)
14	0.99 (d, ^{3}J = 7.1 Hz, 3 H)	10.1 (CH_3)
15	1.41 (s, 3 H)	21.9 (CH_3)
16	–	170.6 (C_q)
17	2.13 (s, 3 H)	20.7 (CH_3)

[a] Carbon numbering as shown in Figure 1. [b] Chemical shifts δ in ppm, multiplicity m: s = singlet, d = doublet, m = multiplet; coupling constants ^{n}J are via *n* bonds and given in Hertz. [c] Carbon assignments (CH_3, CH_2, CH and C_q) were delineated from a DEPT spectrum.

Subsequently, the biosynthesis of **3** was investigated by feeding with (2-^{13}C)- and (6-^{13}C)mevalonolactone (**17**).[18,24] The isotopically labeled compounds were fed to fungal cultures on hydrated rice medium,[15] and after two weeks of incubation, **3** was isolated with a yield of 50 mg from 15 g of medium. Feeding with (2-^{13}C)-**17** followed by isolation of **3** and ^{13}C NMR analysis revealed the incorporation of the label into C3, C9, and C12, while feeding with (6-^{13}C)-**17** gave incorporation into C13, C14, and C15 (Figure 2B,C). These results are in agreement with previously reported results from feeding experiments with ^{13}C-labeled precursors.[23,25,26] Together with our finding that isotopic labeling from (6-^{13}C)-**17** is specifically incorporated into the olefinic C12 of **10**,[18] these feeding experiments give detailed insight into the stereochemical course and mechanism of PR toxin biosynthesis.

A proposed biosynthetic pathway to **3** that was developed as a working hypothesis is shown in Scheme 2. Generally, this

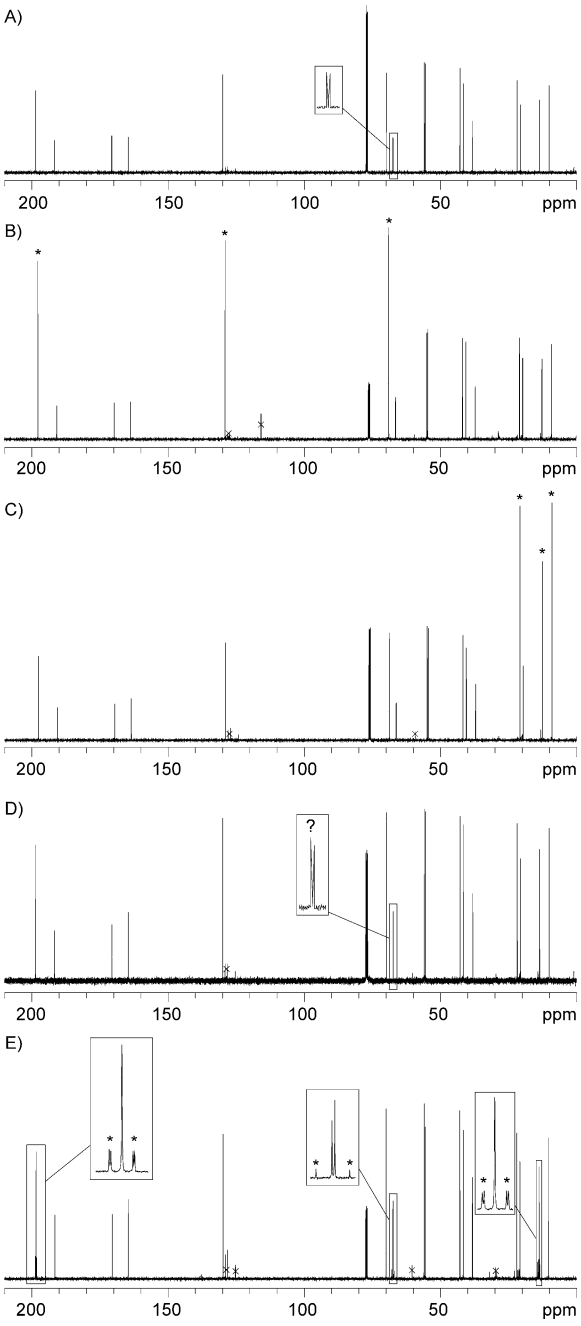
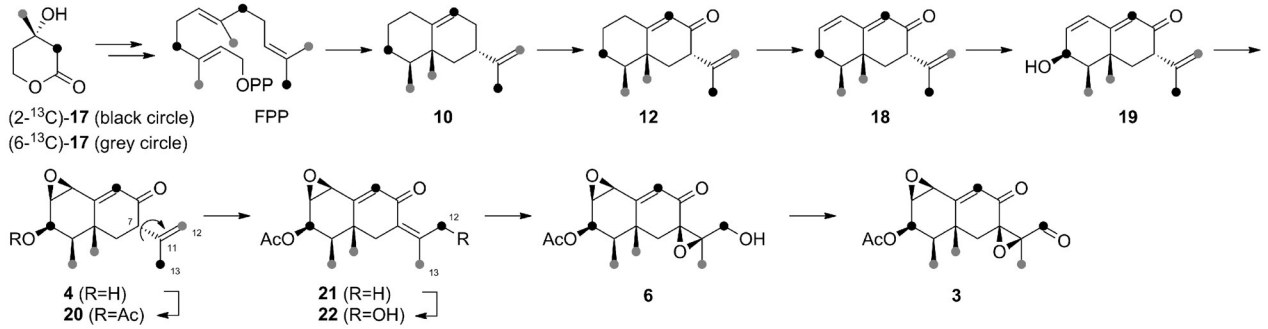


Figure 2. ^{13}C NMR spectra of PR toxin (**3**). A) Unlabeled **3**, B) [3,9,12-$^{13}C_3$]-**3** after feeding with (2-^{13}C)-**17**, C) [13,14,15-$^{13}C_3$]-**3** after feeding with (6-^{13}C)-**17**, D) [11-^{13}C]-**3** after feeding with isomerized (11-^{13}C)-**12** (see text, incorporation unclear), and E) [11,12,13-$^{13}C_3$]-**3** after feeding with (11,12,13-$^{13}C_3$)-**12**. Asterisks indicate signals of ^{13}C-enriched carbons, question marks indicate peaks corresponding to carbon atoms for which incorporation of ^{13}C-labeling was expected but unclear. Peaks from impurities are crossed through.

pathway takes into account that oxidations of the carbon skeleton of **10** and all later intermediates are most favorable in allylic positions. Following this principle, **10** may first be converted into **12** by allylic oxidation. Introduction of an additional olefinic double bond, either by a dehydrogenase or via allylic oxidation and elimination of water, thus explaining the formation of compound **9**, may result in **18**, which can be



Scheme 2. Hypothetical biosynthetic pathway for PR toxin (**3**). Black and grey circles indicate ^{13}C-labeled carbons from two feeding experiments.

oxidized to **19**. The epoxidation of **19** may yield eremofortin B (**4**), which can be acetylated to give **20**. This compound is then oxidized to **22**, either directly by oxidation via a C7-C11-C12 allyl radical with introduction of the hydroxy function at C12, or in a two-step process via double-bond isomerization to **21** and allylic oxidation at C12. As the results from the feeding experiments with (2-^{13}C)- and (6-^{13}C)-**17** with respect to the positions of incorporation into **10** and **3** demonstrate, C12 of **20** must be converted into C13 of **21**/**22**, while C13 of **20** ends up as C12 of **21**/**22** (or a similar positional exchange of C12 and C13 may occur for any corresponding alternative intermediate). This finding contradicts a mechanism by which **20** is directly converted into **22** via a C7-C11-C12 allyl radical, but is in agreement with an isomerization of **20**, reacting from a different conformation that arises by rotation around the C7–C11 bond, to yield the conjugated enone **21**. This process could be spontaneous, albeit with the requirement of a strict stereochemical course, similar to the observed isomerization of synthetic **12**. The subsequent allylic oxidation of **21** to **22**, epoxidation to give eremofortin C (**6**), and oxidation would yield **3**.

Eremofortin A (**5**), which was previously suggested as a pathway intermediate,[16,17] is more likely a shunt product that arises by premature epoxidation of **21**; its turnover into **22** would require a disfavored oxidation of a non-activated methyl group. The existence of **5** also supports the idea that the conversion of **20** into **22** is a two-step process via isomerization and allylic oxidation, and not a one-step allylic oxidation. The fact that **22**, in contrast to **5**, has never been isolated from *P. roqueforti* does not contradict its nature as a pathway intermediate, since an intermediate will be rapidly turned over, whereas a shunt product will accumulate.

The proposed biosynthetic pathway was further studied by feeding with advanced ^{13}C-labeled intermediates. For this purpose, (11-^{13}C)-**12** was synthesized via the route shown in Scheme 1, using (2-^{13}C)acetone in the conversion of **15** into (11-^{13}C)-**16**. Labeled (11-^{13}C)-**12** was fed to *P. roqueforti*, but surprisingly no incorporation could be observed (Figure 2D). Since the synthetic compound (11-^{13}C)-**12** was prepared several weeks before the feeding experiment was performed, its identity was re-checked by GC–MS, which revealed that the complete material had undergone double-bond isomerization with conversion of the isopropenyl group into a conjugated isopropylidene group, even though the compound had been stored at −80 °C. Two explanations are possible for why no incorporation into **3** was observed. First, it could be that the isomerized material or one of its downstream metabolites is not accepted by one or several enzymes of the PR toxin biosynthetic pathway. Second, the experimental design may not have been well chosen in that advanced pathway intermediates may not be efficiently taken up by the fungus. Another problem seemed to be that the experimental design would not allow the detection of incorporation of the labeling with low rates (< 0.2 %), since this would only lead to slightly enhanced and thus poorly conclusive ^{13}C-signals. However, low incorporation rates may have to be considered owing to the high production level of **3** by *P. roqueforti*. To overcome these problems, (11,12,13-$^{13}C_3$)-**12** was synthesized from ($^{13}C_3$)acetone and fed to *P. roqueforti* immediately after its preparation. Although the incorporation rates of (11,12,13-$^{13}C_3$)-**12** were low, the incorporation of labeling into the C11-C12-C13 portion of **3** was clearly evident from satellite signals that originate from ^{13}C,^{13}C-couplings and are observed around the peaks of the corresponding carbon atoms of unlabeled **3** (Figure 2E).

In summary, we have synthesized 7-*epi*-neopetasone (**12**), which was shown to be identical to a previously tentatively identified compound in headspace extracts from the PR toxin producer *Penicillium roqueforti*. The instability of **12** in terms of a double-bond isomerization may also play a role in a corresponding spontaneous double-bond migration along the PR toxin biosynthetic pathway. Feeding experiments with two ^{13}C-labeled mevalonolactone isotopomers revealed that oxidation of an appropriate intermediate with a C11=C12 double bond via a C7-C11-C12 allyl radical and with introduction of a hydroxy function at C12 cannot explain the double-bond isomerization. The results from these feeding experiments rather prove that C12 oxidation is independent of the double-bond migration. Compound **12** was demonstrated to be a pathway intermediate of PR toxin biosynthesis through feeding with (11,12,13-$^{13}C_3$)-**12**. Many pathway intermediates have been suggested so far,[12,16,17] but now we have the first experimental evidence for **12** being a true intermediate. The strategy of labeling three neighboring carbons in **12** allowed very sensitive detection of incorporation through the observation of satellite signals resulting from ^{13}C,^{13}C-coupling. The phenomenon of ^{13}C,^{13}C-coupling in ^{13}C NMR spectroscopy can also be used to follow rearrangements of carbon skeletons, for example, during terpene cyclizations, through the observation of rearrange-

ment-induced $^{13}C,^{13}C$-couplings,[27,28] or for structure elucidation by $^{13}C,^{13}C$-COSY NMR.[28–30] Further applications of isotopic labelling techniques for investigating key steps of biosynthetic pathways are currently being developed in our laboratories.

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